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IDENTIFICATION OF A SUBPOPULATION OF IMMUNE NIGERIAN ADULT VOLUNTEERS BY ANTIBODIES TO THE CIRCUMSPOROZOITE PROTEIN OF *PLASMODIUM FALCIPARUM*

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Abstract. Collections of human sera from malaria-endemic areas would be valuable for identifying and characterizing antigens as malaria vaccine candidates if the contributing serum donors' ability to resist infection were fully characterized. We prepared such a serum collection from 26 apparently immune Nigerian adults who failed to develop patent parasitemia for at least 20 weeks following a documented increase in antibodies to the circumsporozoite protein (CSP) from *Plasmodium falciparum.* Volunteers were evaluated five times per week for malaria symptoms and bimonthly for parasites by examining thick blood smears. The incidence rate over 13 months for the cohort was 42% (47 malaria-confirmed volunteers) and the risk of infection was 1.3 infections/year. Responses to CSP did not correlate with protection. Because antibody responses to antigens other than CSP may be associated with protection, the sera from these immune individuals may be useful for identifying and characterizing other potential malaria vaccine candidates.

Malaria, particularly that caused by *Plasmodium falciparum*, remains one of the most serious diseases in the world, endangering infant and early childhood development in many tropical regions lacking the resources to implement thorough and widespread control programs. With the emergence of insecticide-resistant *Anopheles* mosquitoes and of drug-resistant *P. falciparum*, the prospect of human survival in malaria-endemic areas has become increasingly grave. Hope for successful control of malaria seems to lie in a concerted multi-pronged approach that includes environmental and continued vector control methods, combined with an appropriate use of anti-malarial drugs and continued efforts towards the development of malaria vaccines.

Antigens from several developmental stages of *P. falciparum* are being evaluated for inclusion in a malaria vaccine,[1–11] with circumsporozoite protein (CSP) from the sporozoite stage and merozoite surface protein-1 (MSP-1) from the erythrocytic stage[12–17] receiving the most attention. Antibody responses to the central repeat region of the CSP have been studied in several malaria-endemic areas. Some investigators concluded that anti-CSP antibodies were protective,[18–20] while others did not.[21–23]

Adult sera from malaria-endemic areas have been used in a number of clinical[24–26] studies to show that antibody can passively transfer protection against erythrocytic stage infections. Such sera have also found application in defining parasite antigens for inclusion in a malaria vaccine.[27–30] Sera such as these should be most valuable for the purpose of antigen discovery and characterization if the relative immune status of the donors were known.

In the absence of detectable erythrocytic stage parasites, one method for assessing if a person living in an area of endemic malaria has been exposed to *P. falciparum* sporozoites is to measure boosting of the levels of the anti-CSP antibodies. Gordon and others[31] assumed that a two-fold or greater increase in antibody level over baseline values established 2–4 weeks earlier indicates sporozoite exposure in a natural setting. This definition will underestimate the number of individuals actually exposed to sporozoites by the percentage of CSP nonresponders in the population studied.[32] In this study, we used a similar definition to identify relatively immune and nonimmune individuals in a 13-month longitudinal study of 111 adults from a rural area in Nigeria.

MATERIALS AND METHODS

Study area and subjects. Volunteers were recruited under a Human Use Protocol reviewed and approved by the Ethical Committee of the College of Medicine, University College Hospital, Ibadan, and their consent to participate in the study was obtained. Seven villages in the Igbo-Ora and Idere areas of the Ifeloju Local Government Area of Oyo State in southwestern Nigeria were chosen as the study site because they are proximal to Igbo-Ora. In Igbo-Ora, The College of Medicine of the University of Ibadan, the Local Government Council, and the Government of Oyo State of Nigeria have maintained a program for training doctors in primary health care delivery and in rural and preventive medicine for more than 20 years. This program makes the villages around Igbo-Ora attractive sites for field studies because it has produced well-documented records of the prevalent diseases in the area and because the local inhabitants are accustomed to medical research. Malaria is hyperendemic with a prevalence rate of approximately 75% among children during the rainy season from April to October and 55–60% during the dry season from November to March (Sodeinde CA, unpublished data).

The approximate populations of the selected villages were Sekere (350), Geke (59), Iseme (242), Babacleshin (62), Araromi (61), Tobalogbo (180), and Afefu (75). The study area lies almost entirely within the forest-savanna mosaic vegetation belt, which gradually becomes more of a savanna (Daly MT, Filani MO, Richards P, unpublished data). The area is rocky and has the lowest mean annual rainfall in western Nigeria. The mean monthly rainfall pattern in Igbo-Ora for the years 1964–1973, for which there is a complete record, shows a rainy season beginning in April and lasting into October (Figure 1). All the villages have streams that

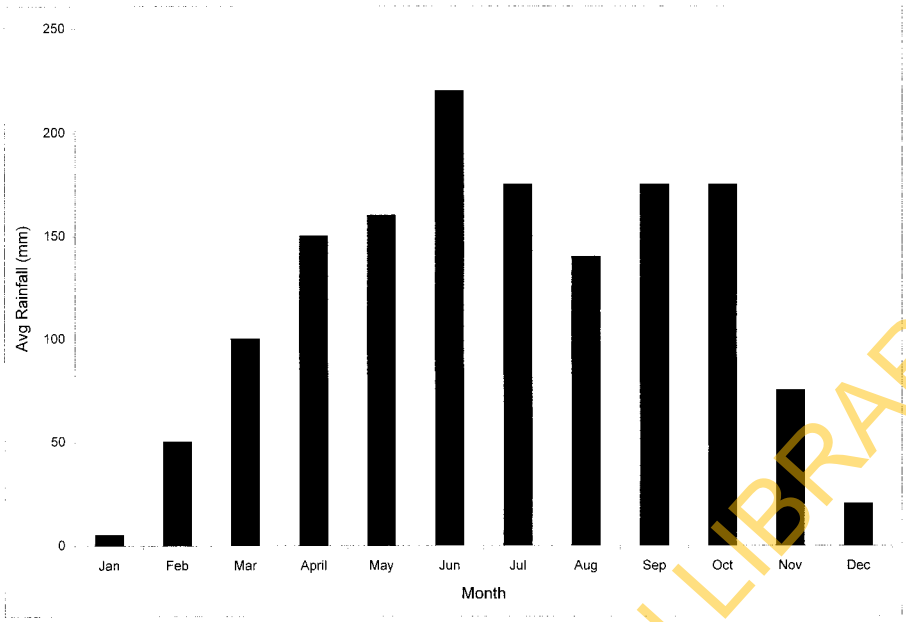

FIGURE 1. Monthly average (Avg) rainfall distribution in Igbo-Ora, Nigeria, 1964–1973 (Dally MT, Filani MO, Richards P, unpublished data). This is the only available record of the precipitation pattern at Igbo-Ora. It is not likely that the present pattern would differ markedly from this figure.

fill in the rainy season but tend to dry up from November to March. The distance from Iseme at one end of the study area to Afefu at the other end is about 20 km. The volunteers, adult males and females 25–50 years of age, were farmers who lived in the villages during the week and often went to their homes in Igbo-Ora or Idere for weekends or holiday festivals. The dwellings were huts built from mud with thatched roofs. Mosquito netting was not used and although not routinely available, the cohort was strongly discouraged from using mosquito coils in their homes.

The project commenced on April 7, 1992 with a total enrollment of 183 subjects. All of the volunteers underwent physical examinations in their respective villages and only those found medically fit were enrolled. For cultural reasons, women who were pregnant before or during the study were excluded. By the end of the program in April 1993, the cohort size had decreased to 111 because for some participation in the study was inconvenient. Thus, approximately 61% of the initial enrollment remained through the end of the study.

Measures to promote compliance with the study protocol. To encourage full participation, and to provide some benefits in the short term, the volunteers received free medical care for minor intercurrent illnesses or were referred to more definitive health care facilities for major problems. Because of the general underlying nutritional status of the population, the local ethical review committee believed that nutritional supplements (e.g., iron and vitamin supplements) provided appropriate benefit to the population. It was strongly emphasized to the volunteers that the success of the study also depended on their compliance with the protocol.

To realize this objective, we used a system of home visiting whereby the volunteers were seen daily in their homes or on their farms (except on Saturdays and Sundays) by trained personnel (home visitors) each of whom took care of the health needs and general welfare of 10–15 subjects. All volunteers were strongly discouraged from taking any local herbal drinks or drugs since their possible anti-malarial effects had not been determined. Being local residents, the home visitors could easily survey for the use of such herbs and herbal drinks without intrusion, particularly during the beginning of the study. As best as could be assessed, none of the volunteers treated themselves for malaria without a doctor's diagnosis and prescription according to the study protocol. The home visitors kept complete daily records of their visits, which were cross-checked by a supervisor.

Blood collection, parasitology, and treatments. Upon enrollment, a blood smear was prepared and 10 ml of blood was drawn by venipuncture with citrate-dextrose as anticoagulant. The blood was stored at 4°C at the medical center at Igbo-Ora for 24–48 hr and transported on ice to Ibadan, approximately 10 km away, where the plasma and erythrocytes were recovered by centrifugation and stored at -70°C. All subjects were drug-cured of any patent parasitemia with a single dose of sulfadoxine-pyrimethamine (Fansidar®; F. Hoffmann-La Roche, Basel, Switzerland, 500 mg of sulfadoxine and 25 mg of pyrimethamine).

The incidence rates and density of *P. falciparum* in the cohort was determined from evaluating blood smears prepared bimonthly for the entire 13-month period, except for one week during the Christmas period in December 1992. For serum collection, additional blood was squeezed from the punctured finger into 2-ml cryotubes, which were delivered to the local medical center within 2–6 hr and stored at 4°C. Within 48 hr, the samples were then taken to Ibadan where the sera were recovered and stored at -70°C.

Duplicate slides were stained with Giemsa and 200 high-power microscopic fields were read independently by an experienced malaria technologist stationed at the field site at Igbo-Ora, and by one of us at Ibadan. Thus, two sets of

records were kept. There was approximately 90% correspondence between the results obtained by the two workers. When discrepancies appeared, those slides were reread blindly by both workers and confirmed as positive or negative after three separate microscopic examinations. For the positive slides, the number of parasites encountered per 200 white blood cells was recorded and used to calculate parasite density on the basis of 8,000 leukocytes/μl of blood.[33]

Unless they declined treatment, subjects who tested positive in smears (malaria-confirmed) and all those who had malaria symptoms without attendant parasitemia were treated with chloroquine. For larger serum collections from the malaria-confirmed individuals, blood was withdrawn by venipuncture during the hospital visit before treatment. Blood (10 ml) was also withdrawn at intervals from individuals who were malaria-free for six and nine successive months, respectively. At the end of the study, 10 ml of blood was withdrawn from each subject.

Measurement of anti-R32tet32 response by ELISA. The ELISA titers of anti-CSP repeat antibody in the sera were determined as previously described[34] using as capture antigen recombinant R32tet32.[35] Capture antigen (1 μg/ml) was diluted 1:5 in phosphate-buffered-saline containing boiled casein (0.8 μg/ml) and 50 μl was added to each well in the even-numbered columns of 96-well U-bottomed Immulon microtiter plates (Dynatech Laboratories, Alexandria, VA). Wells in odd-numbered columns received boiled casein (0.8 μg/ml) only. All of the serum samples collected from a single individual were analyzed in duplicate on the same plate together with a positive and negative control to eliminate variability arising from the use of different plates. Initially, North American sera from nonexposed and noninfected individuals served as negative controls. In time, negative control samples were obtained from the study population. The negative control sera included seven individual sera, and a pooled serum sample from three individuals. All negative control sera were obtained from nonparasitemic subjects. Positive control standard curves were obtained from the same population by using a two-fold serial dilution of a serum sample with the highest absorbance. Serum samples in duplicate were diluted 1:200 with blocking buffer (phosphate-buffered-saline, 0.5% boiled casein, 0.1% Tween 20), incubated for 2 hr at room temperature (28°C), and washed twice with phosphate-buffered-saline, 0.05% Tween 20 (wash buffer). Horseradish peroxidase–conjugated mouse anti-human IgG (Miles-Yeda, Naperville, IL) diluted 1:2,000 in blocking buffer was added and the plates incubated for 1 hr at room temperature. The wells were aspirated and washed three times with wash buffer. The enzyme reaction was developed by addition of peroxidase substrate (100 μl each of 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) solution and hydrogen peroxide; Kirkegaard and Perry Laboratories, Gaithersburg, MD) and the absorbance of the mixture was determined after 1 hr at room temperature. The threshold for a positive reaction was determined to be the mean absorbance of 10 negative control sera plus two standard deviations, which equaled an absorbance of 0.11 at 405 nm.

RESULTS

Parasitology. During this 13-month study, beginning with radical treatment with Fansidar® in April 1992 and ending in May 1993, the infecting malaria species was exclusively *P. falciparum.* The first confirmed malaria infection occurred in June 1992 more than eight weeks after treatment. Among the cohort, there were 47 confirmed cases of malaria, 23 individuals presented with clinical symptoms of malaria without attendant detectable parasitemia, and 41 individuals remained symptom and parasite free. The 23 clinical cases were treated with chloroquine and removed from the study because of the possibility that their symptoms were a consequence of intercurrent infections. Based on smear diagnosis for the rest of the cohort (88 volunteers), the risk of infection was 1.3 infections/year. Peaks in incidence rates were observed in November (13.6%), followed by August (11.7%), and lower rates of 7.2% were observed in July, September, and October (Figure 2). No parasites were detected in smears during the dry months of January and February 1993 or in April or May 1992 as described above. Forty percent of all positive smears had counts less than 101 parasites/μl of blood and 27% had counts of 101–200 parasites/μl (Figure 3). Approximately 33% of the smears had counts greater than 201 parasites/μl, of which only 4.3% had counts of 6,401 parasites/μl and higher (Figure 3). The highest densities were recorded in the months of August (> 2,000 parasites/μl) and November (1,500 parasites/μl), which were also the months with the highest incidence rates.

Of the 47 malaria-confirmed subjects, 35 (75%) received chloroquine treatment whether or not they were clinically ill. The rest of the volunteers declined to see a doctor, since they felt well, chose to decline treatment, and remained in the study group for a follow-up of their malaria status. Of the 12 subjects in this latter group, nine cleared their parasitemia without medication and were nonparasitemic for the rest of the study. One subject was first parasitemic in June, three in August, two in November, and three in December. Thus, the period when they were malaria-free after the first infection varied from four to 10 months. Follow-up blood smears prepared from the remaining three individuals 14 days following the initial positive smear showed that they were still infected, and they accepted chloroquine treatment at this time. These same three individuals (BL 11, SK 35, and SK 32) were parasitemic a total of three times during the study (Table 1).

Anti-CSP response. The serologic response of the cohort to CSP is shown in Figure 4. There was a gradual increase in absorbance from a value of 0.15 at commencement to a peak of 0.45 30 weeks later following the time of peak malaria prevalence. This peak was followed by a gradual decrease in absorbance during the period of low malaria prevalence.

Ninety-three percent of the cohort responded to the antigen (a responder is defined as someone who for the period of the entire study had a mean ELISA absorbance > 0.11 and a nonresponder is a subject whose mean ELISA absorbance was < 0.11). Among all responders, 33% were low responders with a mean absorbance of 0.11–0.20, 59% were moderate responders with a mean absorbance of 0.21–0.49, and 8% were high responders with a mean absorbance of 0.5 and greater.

Among the 47 malaria-confirmed cases, 38 showed at some time during the study a significant increase in CSP-specific antibodies that was two-fold or greater than a base-

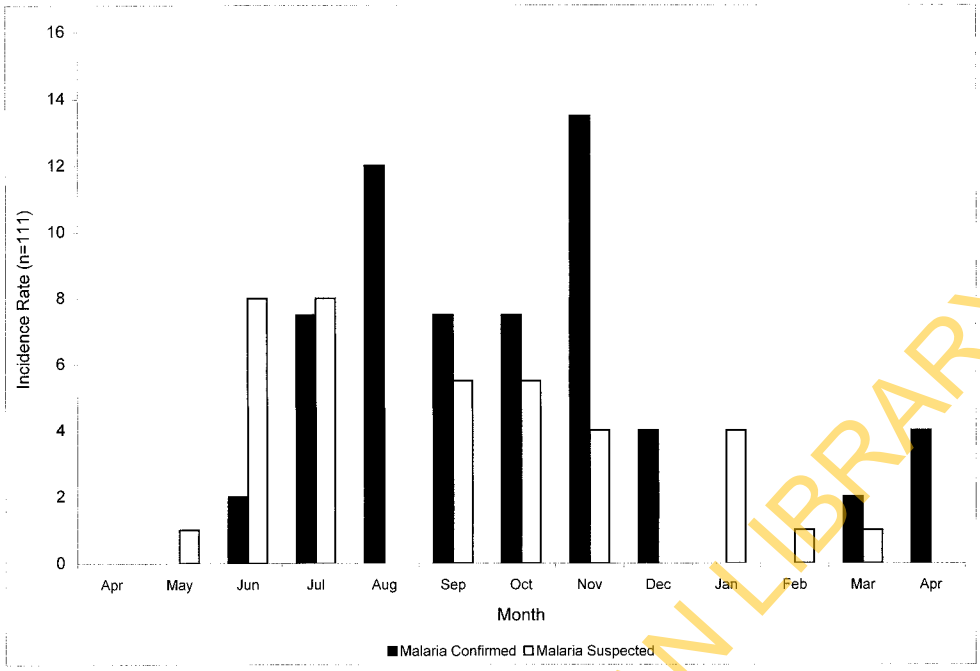

FIGURE 2. Incidence rates of malaria cases in the study area. Blood smears were prepared from fingerpricks at two-week intervals and the incidence rate was calculated as the total number of malaria-positive smears for any given month **(solid bars)** expressed as a percentage of the total number of subjects (111) in the study. Subjects who were ill for any reason were brought to a medical doctor who made a clinical diagnosis of malaria if the symptoms so indicated **(open bars)**.

line value recorded 2–4 weeks weeks earlier. Of these, 32 had ELISA-positive results that coincided with or preceded confirmation of malaria infection by blood smear (Figure 5B and A, respectively). Among the 41 individuals who remained parasite and symptom free throughout the study, 26 showed a significant CSP-specific boosting (Figure 5C). All of these latter individuals were categorized as immune because they continued to remain symptom and parasite free for the duration of the study, which lasted 20 weeks beyond their boosting responses. The mean time of boosting for the nonimmune and immune groups were the same (24 weeks, SD = 21, and 20 weeks, SD = 9, respectively; $P < 0.05$). When the analysis was restricted to individuals showing significant boosting prior to or concurrently with a positive

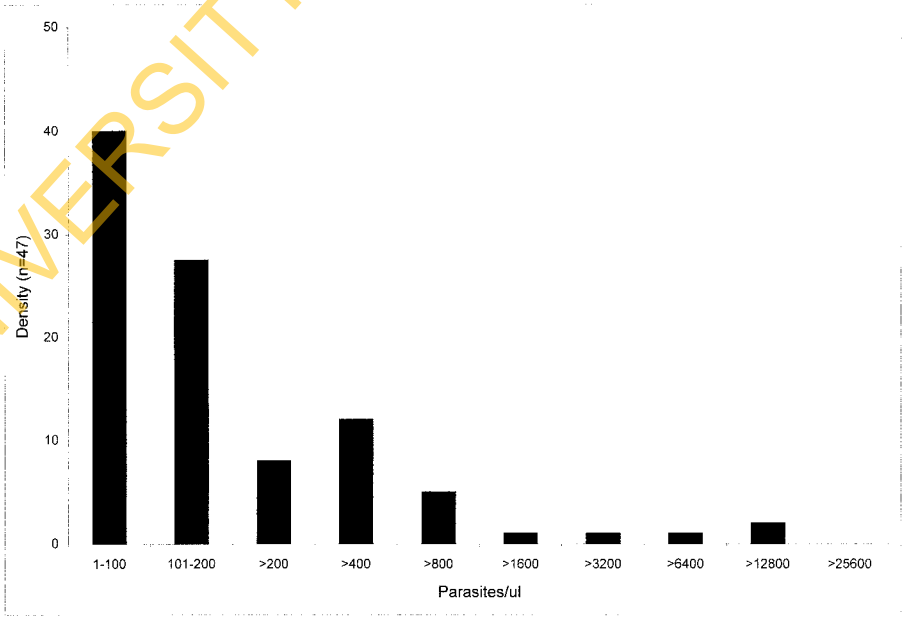

FIGURE 3. Parasite density classes of malaria-positive smears. The number of parasites/200 leukocytes was counted and expressed as the number of parasites/μl of blood. The different density classes (after Bruce-Chwatt[46]) were identified and the number of subjects in each class was determined and expressed as percentage of the total number of parasitemic subjects.

TABLE 1
Reappearance of parasitemia among malaria-confirmed volunteers*

Second infection	First infection: Apr–Jul (Group A, n = 10)	Aug–Oct (Group B, n = 20)	Nov–Jan (Group C, n = 16)	Feb–Apr (Group D, n = 4)
Apr–Jul	–	–	–	–
Aug–Oct	TB 07 [120; 80] **SK 32** [1,200; 120; 440] **BL 11** [80; 80]	AR 03 [200; 160] **SK 35** [80; 800; 40] TB 31 [420; 80]	–	–
Nov–Jan	SK 18 [180; 160] FA 07 [80; 40]	TB 13 [120; 120]	GG11 [16,800; 80]	–
Feb–Apr	**BL 11** [80; 280]	–	BL 16 [80; 80]	–

* The group identification and n (number) for each group is indicated in parentheses. Parasite density/μl of blood in the initial and subsequent surveys are shown in brackets. Subjects who were parasitemic on more than two separate occasions are in **bold.**

smear, the mean time of boosting for the malaria-confirmed group decreased to 14 weeks (SD = 7). This result was significantly less than the mean for the malaria-immune group ($P < 0.05$, by Student's *t*-test). In evaluating the malaria-confirmed group, only nine volunteers (19%) failed to show any boosting of the CSP-specific antibody response (either before or after malaria was confirmed by smear). Fifteen volunteers who remained symptom and parasite free throughout the study failed to generate such a response.

Serum bank. Sera collected from the individuals of this cohort have been categorized according to immunologic and parasitologic parameters as shown in Table 2. This bank includes sera from individuals who remained parasite and symptom free throughout the study (23 samples) and those who were infected but resolved their parasitemias after chloroquine treatment (37 samples) or without treatment (nine samples).

DISCUSSION

Some adults living in malaria-endemic areas are able to control their parasitemias to low or undetectable levels, at least in part, through immune mechanisms. The objective of this work was to prepare a collection of sera from immune and nonimmune inhabitants of a malaria-endemic area by identifying individuals that showed a significant increase in CSP-specific antibodies but were not infected throughout the study.

Forty-one individuals from a cohort of 111 remained malaria free throughout the 13-month study. The risk of infection among the cohort, excluding those individuals who presented malaria symptoms but were smear negative, was 1.3 episodes/year in this study. This rate underestimates the exposure rate because malaria-immune individuals may be challenged but not infected.[33] Although mean entomologic inoculation rates can be used for predicting the probability that an individual was challenged, the transmission within a geographic area defined by this rate is not necessarily homogeneous. Thus, a direct measurement of challenge would be most useful. We determined that 68% of malaria-confirmed volunteers showed a significant boosting of CSP-specific antibodies indexed to a baseline set 2–4 weeks earlier, in which this boosting occurred coincident with or prior to confirmation of infection by smear. Similarly, 63% of the volunteers who remained parasite and symptom free throughout the study (the immune group) showed significant boosting of the antibody response at some time during the

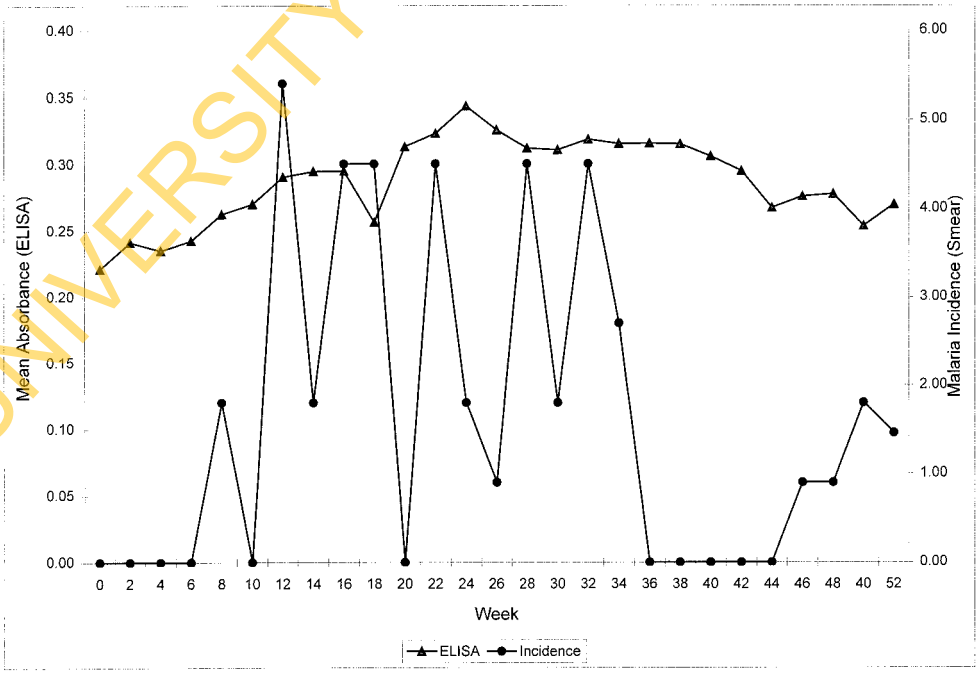

FIGURE 4. Mean anti–circumsporozoite protein (CSP) and malaria incidence rates. The anti-CSP response was measured by ELISA (Δ) using R32tet32 as capture antigen as previously described. Malaria incidence rates (O) were determined as described in Figure 2.

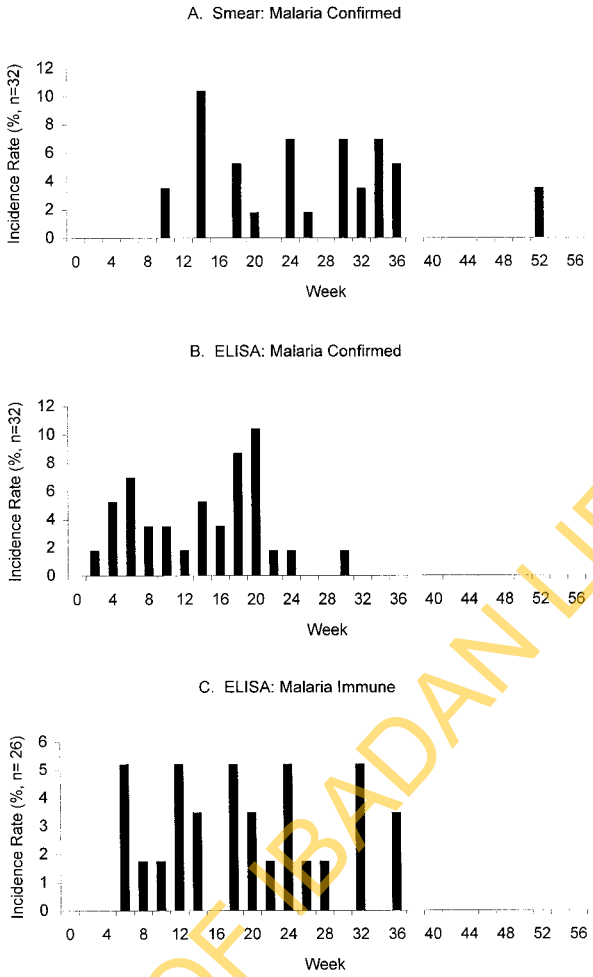

FIGURE 5. Kinetics of appearance of malaria-positive smears and significant boosting of ELISA responses among nonimmune and immune volunteers. Malaria incidence rates as determined by blood smear **(A)** or ELISA **(B)** for nonimmune subjects who showed significant boosting before or concomitantly with a positive smear. **C,** malaria incidence rates as determined by ELISA for immune subjects who were nonparasitic and asymptomatic throughout the study.

study. For this subset of the nonimmune and the immune groups, the mean time to boosting was significantly different (14 weeks, SD = 7 and 20 weeks, SD = 9, respectively, $P < 0.05$). Within the immune group, all 26 cases were monitored for patent parasitemia for at least 20 weeks after CSP boosting. Assuming that significant boosting implies that challenge has occurred, one can calculate the exposure risk during this period as 2.8 episodes per year.

The observed significant boosting of CSP-specific antibody in most of the malaria-free individuals in this study indicates that these individuals were probably challenged with *P. falciparum* and forms the basis for our assumption that they are immune. The mechanism responsible for this immunity, however, is not clear. These data differ from results obtained from the Kilombero Malaria Project, which showed that in this highly endemic area, the anti-sporozoite antibody level was not a good indicator of recent exposure.[36]

In a study in rural school children in Nigeria beginning in April 1988 at a time of relatively low malaria prevalence, Salako and others[37] found that all 168 nonparasitemic children who received chloroquine remained parasite negative for the next three weeks. Subsequently, 22 (13.1%) became positive at week 7, 31 (26.5%) at week 12, and 27 (16.1%) at week 26. It is not clear if parasites would have been de-

TABLE 2
Categories of sera obtained from the 111-member cohort

Category	Immune status	Parasitology	Immune response and treatment	n
A	Immune	Nonparasitemic	Responded to apparent sporozoite inoculation	25
B	Unknown	Nonparasitemic	Nonresponder to apparent sporozoite inoculation	13
C	Not immune	Parasitemic once only	Chloroquine treated	38
D	Not immune	Parasitemic more than once	Chloroquine treated each time	11
E	Partial immunity?	Parasitemic	Declined chloroquine treatment then cleared infection	9

tected before week 7 if the sampling had been more frequent. That adults in our study remained negative for a longer period may be explained by a number of factors, one of which may be induction of an age-dependent acquired immunity.[38–43]

The results of a study in Burkina Faso are similar to those presented herein. Esposito and others[20] reported that among 28-year-old adults, there was 0% *P. falciparum* prevalence at the beginning of the transmission season in June and approximately 10% at the end of the rainy season in November. In a study conducted in Cameroon, Nkuo-Akenji and others[43] found that none of the individuals more than 39 years old was positive for *P. falciparum* in the month of June, during a period of high malaria transmission. The results of our present study, however, sharply contrast with those of the Garki Project[44] conducted in northern Nigeria, which reported a malaria prevalence of approximately 20% in the dry season among subjects at least 44 years old. During the wet season, this figure increased to approximately 40%. Prevalence of infection is affected by a number of factors including vectorial capacity, entomologic inoculation rate, incidence rate, recovery rate, and immunity. We can only speculate that the epidemiologic factors prevalent in the Igbo-Ora area of our study may be markedly different from those in the Garki region and may have contributed to the differences observed.

The results of our investigation in the adult rural population of Igbo-Ora may be related to acquired immunity to malaria. The volunteers appear to clear parasites during periods of reduced transmission associated with the dry season, and become reinfected after the beginning of the next rainy season, but reinfection appears to be delayed compared with what happens in children in the same area.[37] The extent of this immune protection may be reflected in the finding that of a total of 111 subjects seen throughout the study, 41 had neither patent parasitemia nor clinical malaria symptoms for the entire 13-month period despite evidence that 63% of these individuals had been challenged. The immune status of the population may also explain the relatively low parasite densities recorded in the study. Nonimmunologic factors such as sickle cell trait, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and HLA status[33, 44, 45] are known to influence the outcome of malaria infection; however, the sickle cell trait and G6PD deficiency did not affect the outcome of our study.

Our assumption that the anti-CSP response was a useful indicator of recent sporozoite inoculation allowed us to identify a group of individuals who were parasite and symptom free, but appeared to have been challenged. Serum collections from these individuals may play an important role in further identifying and characterizing antigens for use in malaria vaccines.

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