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Evidence for a 6.5-Day Minimum Exoerythrocytic Cycle for *Plasmodium falciparum* in Humans and Confirmation that Immunization with a Synthetic Peptide Representative of a Region of the Circumsporozoite Protein Retards Infection

J. R. MURPHY,* S. BAQAR, J. R. DAVIS, D. A. HERRINGTON, AND D. F. CLYDE

Center for Vaccine Development, Division of Geographic Medicine, Department of Medicine, University of Maryland School of Medicine, 10 South Pine Street, Baltimore, Maryland 21201

Received 27 July 1988/Accepted 31 March 1989

Immunization with a synthetic peptide which is representative of part of the repeating region of *Plasmodium falciparum* circumsporozoite protein resulted in an immunity which allowed vaccinees to retard the development of patent malaria as compared to nonimmunized controls. Analysis of infection dynamics showed that immunity could be attributed to either neutralization of about 92% of inoculated sporozoites, delayed development of the majority of parasites, or a combination of neutralization and delayed development. In spite of this impressive antiplasmodial capacity, all volunteers after being bitten by infected mosquitoes developed malaria, and seven of eight developed parasitemia between 6.5 and 7.0 days after infective mosquito bites.

In a previous report (J. Davis, S. Baqar, J. R. Murphy, D. F. Clyde, D. Herrington, R. S. Nussenzweig, V. Nussenzweig, and M. Levine, Abstr. Annu. Meet. Am. Soc. Trop. Med. Hyg. 1987, abstr. no. 285, p. 203), a mathematical model of the course of sporozoite-induced human *Plasmodium falciparum* infection was presented which incorporated data on volunteer parasitemia at interval after infection, hematologic and anthropometric data, and published values for aspects of plasmodial life cycle to equate initial patency with the minimum number of sporozoites which could have caused the infection. To improve the precision of those calculations, we have determined the minimum duration of exoerythrocytic infection by applying a sensitive blood culture procedure; our results support the conclusion that the minimum duration of exoerythrocytic *P. falciparum* NF54 infection is about 6.5 days.

MATERIALS AND METHODS

Volunteers. *P. falciparum* infections were induced in four vaccinated and four nonvaccinated volunteers (Table 1). The vaccine was a synthetic peptide [$(NANP)_3$] conjugated to a tetanus toxoid carrier and absorbed to alum (14). Vaccinees received two doses of 320 μg each 87 and 31 days before initiation of infections; details of the vaccination study are presented elsewhere (D. A. Herrington, manuscript in preparation). Challenge occurred during an approximate 4-h interval and was through bites of approximately five *Anopheles stephensi* mosquitoes.

Model. The minimum number of sporozoites causing initial erythrocytic patency was estimated using the following relationship:

$$S = [e^{(\log_e\{(K)(C)[(P/500)(W)]\}) - [(\log_e M)/T](D-E)}]/H \qquad (1)$$

where S is the number of sporozoites initiating infection; e is the base of the natural logarithm; K is the mass of the volunteer in kilograms; C is the average number of milliliters of whole blood per kilogram of body mass (a value of 62.4 is accepted [24]); P is the number of parasitized erythrocytes per 500 leukocytes, determined from examination of Giemsa-stained thick blood films; W is the number of leukocytes per milliliter of whole blood, determined using an electronic cell counter; M is the number of merozoites per schizont (a value of 16 is accepted [3]); T is the time, in days, required for completion of asexual erythrocytic schizony (a value of 2 days is accepted [3]); D is the day after infection on which the first positive thick smear was found; E is the minimum duration of exoerythrocytic schizony; and H is the number of merozoites per hepatic schizont (a value of 30,000 is accepted [3]).

Plasmodium culture. Cultures were maintained in medium RPMI 1640 containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–2 mM glutamine (Whittaker M.A. Bioproducts, Walkersville, Md.) and supplemented to 50 μg/ml each with gentamicin and hypoxanthine (referred to hereafter as an incomplete medium). Incomplete medium further supplemented by addition of 10% heat-inactivated human serum is referred to as complete medium (sera for medium supplementation were obtained from blood group A individuals and exhaustively absorbed with type B erythrocytes). Cultures were maintained at 37°C in an atmosphere of 4.5% CO_2–4.5% O_2–91% N_2.

Microcultures were maintained in 96-well plates. Each well contained 4.5×10^7 erythrocytes and 0.20 ml of complete medium.

Cultures to detect *P. falciparum* in volunteer blood were made in 25-cm^2 tissue culture flasks. Each blood sample was centrifuged (400 × *g* for 10 min) and the plasma fraction was removed. The cells were then washed twice by suspension in incomplete medium followed by centrifugation. After the last wash, cells were suspended in complete medium and a total of 4.05×10^9 cells in 6 ml of medium was placed into one flask. Medium was changed two times per week through culture day 38 and daily thereafter; fresh erythrocytes (10^9 per culture) were added at culture days 18, 33, and 51. Cultures were examined weekly for presence of parasites; those cultures which did not show parasites by culture day 70 were defined as negative for *P. falciparum*.

It is estimated that this flask culture system can detect

* Corresponding author.

TABLE 1. Characteristics of *P. falciparum*-infected volunteers[a]

Group	Volunteer	Mass (kg)	Leukocytes/ml (10^6)	Patency (days)	Parasitemia (parasites per 500 leukocytes)
Control	1	67.5	4.7	8.5	2
	3	63.0	4.9	7.0	1
	5	83.3	6.7	7.0	1
	7	99.0	5.8	7.0	1
Vaccine	2	72.0	4.8	9.0	1
	4	97.2	3.2	9.0	1
	6	74.3	5.1	8.5	1
	8	85.5	7.3	9.0	1

[a] Patency, Day after mosquito bites that parasites were found in thick blood films; parasitemia, number of parasites found during a count of 500 leukocytes.

TABLE 2. Sensitivity of blood culture as compared with thick smear in detection of erythrocytic *P. falciparum* infection

Group	Volunteer	Culture result[a] at day of infection:										
		0.0	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5
Control	1	–	–	–	–	+	+	+	+	[+]	+	+
	3	–	–	–	–	+	[+]	+	+	+	+	+
	5	–	–	–	–	+	[+]	+	T	T	T	T
	7	–	–	–	–	+	[+]	+	T	T	T	T
Vaccine	2	–	–	–	–	+	+	+	+	+	[+]	+
	4	–	–	–	–	–	+	+	+	+	[+]	+
	6	–	–	–	–	C	+	+	+	[+]	+	+
	8	–	–	–	–	C	–	–	+	–	[–]	+

[a] –, Culture negative for *P. falciparum*; +, culture positive for *P. falciparum*; T, culture not made because volunteer was previously treated with chloroquine; C, culture contaminated. Symbols enclosed with brackets denote the day on which the first parasite-positive thick smears were found.

parasitemia comprising the hepatic merozoite progeny of less than one sporozoite; there is no requirement for multiplication in vivo of blood forms before their potential detection by culture. Our estimate of sensitivity is based on unpublished studies directed to isolation of cloned lines of *P. falciparum* NF54 which show that about one parasitized erythrocyte is sufficient to establish infection of a culture. Therefore, for a 75-kg volunteer with an erythrocyte count of 5×10^9/ml, 1 parasitized erythrocyte in a sample of 4.05×10^9 erythrocytes (or a parasitemia of about 1.2 parasitized cells per ml) would correspond (under this model) to a total body parasitemia of 5,777 parasitized erythrocytes, which in turn corresponds to the hepatic merozoite progeny of about 0.2 sporozoite.

RESULTS

Volunteer serum and erythrocytes, singularly or in combination, were capable of supporting in vitro growth of *P. falciparum*. Cells and serum were collected from all volunteers before challenge. Immediately after blood collection, separate microcultures were established containing either (i) volunteer erythrocytes and medium containing 10% O-absorbed serum, (ii) volunteer serum (10%) and group O erythrocytes, (iii) volunteer erythrocytes and volunteer serum (10%), or (iv) O-absorbed serum (10%) and O erythrocytes. Triplicate cultures were made for each condition for each volunteer. A sample of 450 erythrocytes containing log-phase *P. falciparum* was then added to each culture (resulting in an initial parasitemia of 0.001%), and after 7 days blood films were made from each culture. All combinations of culture components supported at least a 1,000-fold increase in parasitemia, demonstrating that the components derived from all volunteers before challenge were capable of supporting rapid growth of *P. falciparum*.

Course of *P. falciparum* infections in volunteers. Blood samples and thick blood smears were obtained before infection and at 12-h intervals from day 5 through day 9.5 after infection. First isolation of *P. falciparum* occurred from blood samples collected at day 6.5 from all nonvaccinated volunteers (Table 2). Onset of blood infection was not as uniform for vaccinees. One vaccinated individual developed infection on day 6.5, two were positive at day 7, and the remaining subject had parasites isolated from the day 8 blood sample.

Serum collected after infection did not inhibit in vitro growth of *P. falciparum*. To determine whether volunteers developed serum factors capable of inhibiting in vitro growth of *P. falciparum*, sera collected from each volunteer before infection and 7 and 120 days after infection and stored at −20°C were used separately to prepare a series of complete media (final serum concentration, 10%). The microculture system as described above was used, and cultures were maintained for 7 days. All sera supported at least a 1,000-fold increase in parasitemia.

Analysis. With the exception of volunteer 8, erythrocytic infection was documented by culture between 6.5 and 7.0 days after mosquito bites. Because the minimum reported duration of exoerythrocytic infection is 5.5 days (21) and because the erythrocytic cycle of *P. falciparum* is 48 h (3), isolation of parasites at 6.5 to 7.0 days is consistent with the proposition that the culture system is capable of detecting erythrocytes infected with liver-produced merozoites; e.g., there is no requirement for in vivo multiplication of erythrocytic forms before parasitemia can be detected by culture.

For three volunteers (no. 3, 5, and 7), 12 h elapsed between collection of blood samples in which *P. falciparum* was detectable by culture and samples wherein parasites were detectable by thick smear. Because thick smears provide quantitative data (e.g., parasitemias of between 9,800 and 13,400 parasitized erythrocytes per ml [average, 11,600] for these volunteers at day 7), estimates can be made of the number of sporozoites causing the infection. Two conditions demand evaluation: (i) application of equation 1 with duration of exoerythrocytic infection set to 6.5 days and (ii) application of equation 1 with duration of exoerythrocytic infection set to the day of first positive thick smear. The first condition allows for increases in number of blood forms between the assumed end of the exoerythrocytic infection and detection of parasites in blood and shows an average of 997 sporozoites for the cited volunteers (Table 3). The second condition assumes that all parasites detected in thick smears exited the liver immediately before the preparation of the smear and yields a result of 1,997 sporozoites.

Four of the remaining five volunteers (no. 1, 2, 4, and 6) had infections which were detected by cultures initiated on day 6.5 or 7.0 and by thick smears on day 8.5 or 9.0. Therefore, parasitemias as seen in thick smears (averaging 11,250 parasites per ml) could represent (i) second-generation erythrocytic progeny of liver merozoites which had entered circulation 48 h or more before detection or (ii) a summation of second-generation parasites plus additional first-generation parasites. One extreme possibility is that one hepatic schizont may have released its content of parasites about 48 h before thick smears became positive (thereby

TABLE 3. Extreme-case analysis of number of sporozoites corresponding to parasitemia at first positive thick smear[a]

Group	Volunteer	No. of infecting sporozoites on day of completion of liver cycle:	
		6.5	Thick smear +
Control	1	165	2,640
	3	642	1,280
	5	1,160	2,320
	7	1,190	2,390
Mean		789	2,158
Vaccine	2	45	1,440
	4	40	1,290
	6	99	1,580
	8	81	2,600
Mean		66	1,728

[a] Number of infecting sporozoites which correspond to parasitemia at the first positive thick smear, under the extreme conditions where equation 1 was applied with duration of exoerythrocytic infection set to (i) 6.5 days for all volunteers or (ii) the day on which the thick smear was found positive. Mean percent protection, determined as unity minus the ratio of mean sporozoite number of vaccinated and control groups, was 91.6% for the 6.5-day duration and 19.9% when calculated from the first positive thick smear. Upper and lower limits of percent control were determined with the duration of the exoerythrocytic cycle set to 6.5 days. The lower limit on percent control was 40%, calculated as unity minus the ratio of the highest calculated sporozoite number among vaccinees for a given condition (i.e., 99) to the lowest parasitemia among controls (i.e., 165). The upper limit on percent control, derived from the lowest vaccinee sporozoite number and the highest control sporozoite number, was 97%.

accounting for the positive cultures) and the remainder of the parasitemia resulted from hepatic schizonts which ruptured immediately before the thick smear was made. Another possibility is that all detected parasites may have exited the liver about 48 h before detection by thick smear. The average numbers of sporozoites that would account for these two extreme possibilities are 1,738 or 109, respectively.

The pattern of results for volunteer 8 was markedly different from that of the other seven individuals. Blood cultures made at days 8 or 9.5 of infection, when thick smears were negative, yielded parasites; however, cultures made at day 9.0, at which time the thick smear showed parasitemia of 14,600 parasitized erythrocytes per ml, did not yield parasites. The difficulty in establishing in culture parasites from this donor could not be attributed to serum factors which inhibited growth. A separate blood sample obtained from this individual at day 9 was inoculated into a 75-cm^2 culture flask and maintained with daily changes of medium for 21 days. Parasites were not detected. Similar cultures made from the remaining volunteers on the day of their first positive thick smear yielded *P. falciparum*. The composite results support the position that parasites from donor 8 were for some reason compromised in their ability to propagate in culture.

DISCUSSION

This study and two others have shown that vaccination of humans with recombinant (1) or synthetic (14) peptides containing the NANP sequence representative of the repeating region of *P. falciparum* circumsporozoite protein provides most recipients with a capacity to delay onset of malaria.

Our analysis is an attempt to estimate quantitatively the degree of antiparasite activity generated by the synthetic peptide vaccine. We address this question mathematically because the procedures employed in the past to obtain direct evidence, i.e., injection by syringe of sporozoites or salivary glands (6, 15) and liver biopsies (15, 20, 21), cannot be easily applied to our volunteers. The equation we present is an extension of the often observed direct relationship between the number of injected sporozoites and the duration of prepatent interval (13, 17, 22). Estimates of densities of the various parasite stages can be made by including in the relation data on the anthropometric characteristics of the volunteers.

We found onset of erythrocytic infection at about 6.5 days after infective mosquito bite. This interval is about 1 day longer than the minimum reported time (21) from mosquito bite to onset of *P. falciparum* blood stage infection. Our somewhat longer interval could be due to dissimilarity in development times of the different strains of parasite used for the respective studies or to differences in sensitivity of the procedures used to detect erythrocytic infection. For instance, Shortt et al. (21) subinoculated 130 ml of blood from a sporozoite-infected volunteer into a naive recipient volunteer.

Our nonvaccinated volunteers showed patent infections an average of 7.4 days after mosquito bites. We have found only three reports of earlier patencies: a single parasite found at day 5 (Shute as cited in reference 7) and two individuals found positive at day 6 (2). More common prepatent periods are 8 (21), 9 (5, 11, 15, 19), 11 (8, 9, 16), 12 (6), 13 (10, 16), or 15 (4) days. Because prepatent interval is often a reflection of the number of sporozoites inoculated (13, 17), our short prepatent interval suggests that our volunteers had received a massive sporozoite challenge. This view is consistent with the data of Shortt et al. (21) and Jeffery et al. (15), who report 7-day prepatent intervals for individuals challenged with greater than 350 mosquito bites (93% mosquito infection rate) or greater than 869 mosquito bites combined with intravenous injection of greater than 233 salivary glands dissected from infected mosquitoes (87% mosquito infection rate).

Because of the growth characteristics of *P. falciparum*, small delays in prepatent interval can be a reflection of large differences in the number of parasites initiating infection. In an attempt to quantitate the degree of antiparasite action which could account for the average 1.5-day delay in patency seen between the vaccinated and control groups, we applied equation 1, assuming a 6.5-day prepatent interval. Under these conditions there was an average 92% reduction in sporozoites for the vaccinated group (this estimate refers to the ratio of mean number of parasites in vaccinees and controls; see Table 3 for derivation of lower and upper limits on this estimate). Although the absolute numbers of sporozoites predicted from equation 1 are probably inaccurate, the relative numbers of parasites predicted for nonvaccinated and vaccinated groups are more precise.

An alternative interpretation for the difference in prepatent periods for vaccinees and controls is that immunity retarded the rate of development of exoerythrocytic parasites. This cannot be ruled out. However, the preponderance of evidence from studies in animals, where it is possible to get direct evidence, shows that a major action of antisporozoite antibodies, or cytokines with activity against exoerythrocytic stages, is a reduction in the number of parasites per liver. This seems due to a decrease in infected hepatocytes, not to a delay in rate of development of those parasites which successfully infect hepatocytes (12, 23).

Our results, viewed within the perspective of previous

volunteer studies (15, 21), support the position that the number of sporozoites injected by the mosquitoes used for this study was extremely large. This suggests that our challenge protocol is a most rigorous test of antisporozoite vaccines. It is encouraging to find that a first-generation synthetic vaccine has a capacity to raise an immunity which neutralized or delayed development of a majority of parasites; it has been suggested that reductions in infectious dose correspond to diminution in severity of illness (18).

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