Infectivity to mosquitoes of *Plasmodium falciparum* clones grown *in vitro* from the same isolate

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Summary

In an attempt to produce a line of cultured *Plasmodium falciparum* parasites consistently infective to mosquites, a Brazilian isolate, IMTM 22, was cloned by the limiting dilution method. Five of the resulting clones were examined in detail. The clones were found to differ in their ability to produce micro- and macrogametocytes, to exflagellate and to infect *Anopheles freeborni* mosquitoes. The stability of one clone in producing microgametocytes and in its ability to produce oocysts and sporozoites in mosquitoes has been documented through 15 subcultures. This clone should provide a reliable source of infectious gametocytes for genetic studies and vaccine development.

Introduction

Sexual reproduction in the malaria parasite *Plasmodium falciparum* takes place in the midgut of the mosquito vector where gamete formation and fertilization occur. Parasite development in the mosquito culminates in the formation of sporozoites which transmit the infection back to man. Areas of investigation such as antigenic analysis, genetics and vector competence would be greatly facilitated by the isolation of culture-adapted falciparum clones infective to mosquitoes. In addition, such clones should provide a reliable source of sporozoites for studies leading towards vaccine development.

Previous falciparum clones have been isolated by limiting dilution or micromanipulation. These clones were characterized as to electrophoretic forms of glucose phosphate isomerase, presence of knobs on the erythrocyte surface, chloroquine sensitivity and gametocyte production (TRAGER *et al.*, 1981; ROSARIO, 1981). Such studies have limited value unless the clones can infect mosquitoes since the inability to reproduce sexually would preclude determining the genetic basis of those characteristics. The recent development of methods to produce gametocytes in culture that are infective to mosquitoes (IFEDIBA & VANDERBERG, 1981) has made the production of large numbers of falciparum sporozoites possible. However, a drop in gametocyte production has been reported with continuous subculturing (PONNUDURAI *et al.*, 1982) and a drop in infectivity with time has been observed as well (K. Campbell, personal communication). P. Graves and R. Carter (personal communication) have shown that the rate of gametocyte production may differ between clones obtained from a single isolate of *P. falciparum*. Thus loss of gametocyte production in culture may be due to clones with a lower rate of gametocyte production overgrowing other parasite subpopulations. Cultures of a cloned parasite would be more likely to maintain their infectiousness through subculturing. Described in this paper are the first isolations of cultured falciparum clones which are characterized with respect to their ability to form macro- and microgametocytes, to exflagellate and to infect mosquitoes.

Materials and Methods

The parental stock of falciparum parasites to be cloned was isolated from a 12-year-old male near Manaus, Brazil on 12 March 1980 and cryopreserved. The isolate, IMTM 22, was later put into a static culture system slightly modified from that described by IFEDIBA & VANDERBERG (1981). Cultures were initiated with a parasitaemia of 0·3% or less in a 6% haematocrit of culture media (RPMI 1640 with 32 nM sodium bicarbonate, 25 nM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), 10 μg/ml gentamicin, 50 μg/ml hypoxanthine and 10% heat-inactivated type A positive human serum added). The culture medium was changed daily and the flasks flushed with a gas mixture of 5% oxygen, 5% carbon dioxide and 90% nitrogen. When the parasitaemia exceeded 1%, the haematocrit was reduced to 3% by doubling the volume of culture medium added.

After more than 19 weeks in culture, a sample of IMTM 22 was cloned by the limiting dilution method. 96-well tissue culture plates were seeded with 100 μl of either a precalculated 0·5, 0·1 or 0·01 dilution of parasitized cells per well with a 1·5% haematocrit. To minimize the possibility of cross contamination of clones, every second well was left empty. Four plates per dilution were made with the parasites grown under candle jar culture conditions (JENSEN & TRAGER, 1977) and the media changed daily. Five of the clones isolated were examined for their ability to produce infectious gametocytes. These clones were grown simultaneously under identical culture conditions in a double blind manner, the individual examining the cultures being unaware of the identity of the clones.

The clones were subcultured at weekly intervals until three flasks of each clone were established. Thick film preparations were made daily, stained with Giemsa and counts were made of the asexual and sexual stages of the parasites. Microgametocyte prevalence was determined by sexing 100 stage V gametocytes as described by CARTER & MILLER (1979). The number of stage V gametocytes per 2500 rbc was recorded on the day of each feed. When equal numbers of stage IV and V gametocytes were seen, half of the culture was prepared for mosquito ingestion by centrifuging the culture material at 350 *g* for five minutes and removing the culture media. A 50% haematocrit was restored with serum and a three-fold volume of fresh defibrinated human blood was added. The mixture was then placed into a water-jacketed membrane feeder at 37°C upon which *An. freeborni* were allowed to feed. While the mosquitoes engorged, a slide was made to determine exflagellation by examination of the culture mixture diluted approximately three fold with 0·85% saline. 50 fields at 400 × magnification of single cell thick suspensions were examined under phase contrast microscopy. On the following day, the remaining culture material was similarly treated.

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Table I—Gametocye production and infectivity to *Anopheles freeborni* of IMTM 22 clones

Clone	No. Feeds	Microgametocytes ($\bar{X} \pm \sigma$)[1]	Total Exflag. (6 feeds)	Stage V Gametocytes (2500 rbc) ($\bar{X} \pm \sigma$)	Positive feeds (%)	Infected mosquitoes (%)	Average oocysts (No.)
11G8[2]	6	2·5 ± 1·9a[3]	1	17·8 ± 6·6a[4]	0	—	—
6A9[5]	6	5·2 ± 2·2b	3	42·0 ± 17·9b	0	—	—
6E7[5]	6	5·8 ± 4·0b	4	20·8 ± 13·6a	0	—	—
10G4[5]	6	8·7 ± 3·1c	12	19·3 ± 12·1a	50	20	1·3
7G8[5]	6	10·0 ± 2·0c	62	17·5 ± 4·6a	67	28	2·2

[1] Mean number microgametocytes counted per 100 stage V gametocytes at time of feed.
[2] Seeded with a parasitaemia of 0·05 parasites/well; the probability of the clone being derived from a single parasite is 0·95 as calculated from formula $\mu e^{-\mu}/(1-e^{-\mu})$ where μ = parasites/well.
[3] Means followed by the same letter do not differ significantly at the 0·05 level by the Newman-Keuls Test.
[4] Means followed by the same letter do not differ significantly at the 0·01 level by the Newman-Keuls Test.
[5] Seeded with a parasitaemia of 0·2 parasites/well. The probability of a clone being derived from a single parasite is 0·90 as calculated by the formula $\mu e^{-\mu}/(1-e^{-\mu})$ where μ = parasites/well.

In this way the three flasks of each clone provided material for six attempts at infecting mosquitoes. Engorged mosquitoes were held at 26°C, 80% relative humidity for seven to nine days before dissecting. At least 10 mosquitoes per feed were dissected to determine the presence and number of oocysts.

The stability of IMTM 22 and the clones 7G8 and 10G4 in infecting mosquitoes was followed by examination of 19, 31 and 7 cultures, respectively. Salivary gland dissections for sporozoites of oocyst-positive mosquitoes derived from IMTM 22 and 7G8 fed cultures were made at least 14 days after feeding.

Results

Parasites from the early subcultures of the parental IMTM 22 strain readily infected mosquitoes with a maximum infectivity in the ninth subculture of 87·5% and a mean of 11·9 oocysts per infected mosquito. Thereafter, infection rates for this isolate declined sharply with a mosquito infection of 5·4% and 1·2 oocysts per infected midgut in the 11th through 14th subcultures.

23 clones were isolated from the twelve 96-well plates seeded with IMTM 22 parasites, 18 from the four plates seeded at 0·5 parasites per well, four from the plates seeded at 0·1 parasites per well and one from the plates seeded at 0·01 parasites per well. The number of parasites per well was calculated from the initial parasitaemia of the stock culture and the subsequent dilutions. The actual parasitaemias were somewhat lower as determined from the proportion of negative wells and the formula $\mu = -\log_e P_o$ where P_o = proportion of negative wells; and μ = parasites per well. Using this equation the actual initial parasitaemias of the plates seeded at 0·5, 0·1 and 0·01 parasites per well were 0·2, 0·05 and 0·01 parasites per well respectively. The probability that the parasites are clones derived from single infected erythrocytes are 0·90 for the 0·2 plates, 0·95 for the 0·05 plates and 0·99 for the 0·01 plate as calculated from the formula $p = \mu e^{-\mu}/(1 - e^{-\mu})$.

Of the five clones examined in detail, all produced microgametocytes and demonstrated exflagellation, but to different degrees (Table I). Although clone 6A9 produced twice as many gametocytes as the other clones, the microgametocytes did not readily exflagellate and the gametocytes were not infectious to mosquitoes. Only two clones (10G4 and 7G8) produced gametocytes that were infectious to anophelines. These produced the highest proportion of microgametocytes, resulting in the most frequent exflagellations. In the simultaneous infection trials, clone 7G8 was the most reliable infector of mosquitoes; cultures of 7G8 resulted in more frequent positive feeds with a higher oocyst level than the other clone.

In additional cultures, 7G8 and 10G4 consistently differed in their ability to infect *An. freeborni*. Cultures of 7G8 resulted in more positive feeds and higher oocyst levels than IMTM 22 cultures. 10G4 was less infectious, giving fewer positive cultures with less mosquito infections and fewer oocysts than IMTM 22 (Table II). Infections from IMTM 22 and 7G8 cultures produced sporozoite infections of the salivary glands as well.

The stability of clone 7G8 in producing microgametocytes and in its ability to infect mosquitoes has thus far been documented through 15 subcultures. The original level of microgametocyte prevalence was 9% in the second subculture. A range of microgametocyte levels from 9 to 11·2% was found in subcultures 2 through 15. There was no significant difference in microgametocyte prevalence with subculturing. Average microgametocyte levels of 9·0, 10·3 and 10·0% were found in subcultures 2, 3 and 4 as compared to microgametocyte prevalences of 10·0, 10·3, 10·2 and 10·0% in subcultures 12, 13, 14 and 15. The infectiousness of the clone has similarly held, allowing for variations in infectiousness due to differences in culture conditions (e.g., different sera used in the culture media, parasite growth inhibition due to nutrient depletion, differences in defibrinated blood added immediately before feeding, etc.). One flask in the 12th subculture infected over 94% of the engorged mosquitoes with an average of over 24 oocysts per midgut.

Discussion

Clones isolated from the IMTM 22 strain exhibited a range of infectiousness from the completely non-infective 6A9 to the mildly infective 10G4 to the highly infectious 7G8. They also differed in gametocyte production, proportion of microgametocytes produced and the ability to exflagellate. Although there appears to be an over-all correlation between the ability of a clone to demonstrate exflagellation and to produce infections, exflagellation at the time of an individual feed does not predict that mosquito infections will occur. Some feeds resulted in 100% mosquito infections but showed no exflagella-

Table II—Infectivity of the parental strain of *Plasmodium falciparum*, IMTM 22, and its clones to *Anopheles freeborni*

Culture	No. of cultures	Positive cultures[1] (%)	Mosquitoes infected from positive cultures (%)	Mean No. oocysts per infected mosquito	Oocyst range
IMTM 22	19	58	37	4·7	1-42
7G8	31	90	38	7·9	1-78
10G4	7	43	12	1·3	1-2

[1]Feeds from cultures which resulted in mosquito infections.

tion. Conversely, up to 14 exflagellations per fifty 400 × fields were seen with no subsequent mosquito infections. The demonstration of exflagellation is dependent on when and how long a microgametocyte will undergo exflagellation at the time of stimulation. It is not inconceivable to mistime the examination for exflagellation and thereby miss, in total or in part, the occurrence of exflagellations.

Evidently an isolate of falciparum malaria from a natural infection is a mixture of genetically diverse parasite subpopulations. Culture conditions may well select for subpopulations with high asexual growth rates at the expense of infectious gametocyte production. Mosquito infections with the original IMTM 22 isolate may have occurred at times when parasite subpopulations similar to clones 10G4 and 7G8 predominated. Subsequent drops in infectivity could result from a subtle selective pressure in culture against these populations of infectious parasites. Cloning of the material apparently isolated cell lines with a range of abilities to infect mosquitoes, and in one clone (7G8) infectiousness has remained high for 15 subcultures.

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