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GAMETOCYTE PRODUCTION IN CLONED LINES OF *PLASMODIUM FALCIPARUM*

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Abstract. Gametocyte production by cloned lines of *Plasmodium falciparum* and their parental isolates has been studied in culture over periods of several months. Many isolates differed significantly from each other in their capacity for gametocyte production. Clones derived from an individual isolate were also widely different in capacity for gametocyte production. Consistent differences in gametocyte production were observed between clones which had always been grown concurrently and thus had identical culture histories. Levels of gametocytogenesis characteristic of individual clones, although subject to transient fluctuations under environmental influence, were stable over several months.

During each growth cycle of *Plasmodium falciparum,* merozoites released from rupturing schizonts invade and become intracellular parasites of erythrocytes. A proportion of the young intracellular parasites (rings) continue the asexual cycle while others develop into sexual stages (gametocytes). The proportion forming gametocytes is known to vary during the growth of *P. falciparum* in culture, being lowest in an exponentially growing culture and increasing to a maximum when the culture reaches the plateau stage.[1] The change in rate of conversion of the parasites to gametocytes between cultures in the exponential and plateau phases of growth represents an underlying change in responsiveness of the parasites to other stimuli (such as cyclic AMP in certain strains of parasite[2, 3]) which trigger the formation of gametocytes. Thus, two distinct components of the environment affecting gametocyte production have been recognized: 1) those which determine the rate of asexual growth, and 2) those stimuli which directly initiate gametocytogenesis. Accumulation of asexual debris[4] and other adverse conditions appear to represent at least the first of these components.

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In addition to such fluctuations in gametocyte production under the influence of the immediate conditions of growth, different isolates of *P. falciparum* manifest intrinsic differences in capacity for gametocyte production.[3, 5] Moreover, capacity for gametocyte production by parasites grown in culture is unstable, and variable according to isolate. Duration of capacity for gametocyte production has been found to range from little more than 1 month for some isolates to more than 1 year of continuous cultivation.[6] Thus, among isolates of *P. falciparum* two components of variation in capacity for gametocyte production have been identified: 1) absolute rate of gametocyte production at the time of first measurement, and 2) duration of capacity for gametocyte production during growth in continuous culture.

In view of the established genetic heterogeneity of the parasites in natural infections and isolates of *P. falciparum,*[7–12] several questions arise concerning the diversity and stability of capacity for gametocytogenesis in *P. falciparum.* Do individual infections and isolates comprise mixed parasite populations of different capacities for gametocytogenesis; do changes in such capacity during continuous cultivation in vitro reflect changing proportions of lines of parasites of different capacities or do all parasites in a population undergo changes in capacity for gametocytogenesis? We have attempted to answer these questions by studying cloned lines derived in culture from different isolates of *P. falciparum.* Characterization of the clones by drug sensitivity and isoenzyme type has already been reported.[12]

MATERIALS AND METHODS

The histories of the Ituxi 084 (Brazil), KZ1 (Kenya or Zaire) and L (Liberia) isolates have been reported previously.[12] The histories of the Z (Zaire) and G1 (Gambia) isolates have been given by Carter and Miller,[1] and that of the IMTM 22 (Brazil) isolate by Burkot et al.[11] Cultures were maintained by the method of Trager and Jensen[13] with slight modifications as described by Graves et al.[12] Slow growing cultures for formation of gametocytes were initiated by dilution of parasitized blood from a culture established 7 days previously. The initial parasitemia was adjusted to about 0.1% in erythrocytes at 5% hematocrit. These were subsequently grown for 3 weeks in 5-ml volumes in 25-cm^2 tissue culture flasks (Corning). High levels of gametocytocytogenesis in these cultures usually began when the number of asexual parasites had reached a plateau at about 5% parasitemia (after 5–7 days in culture).[1] Blood smears were made from the cultures once a week for 3 weeks, and gametocytes, categorized according to each of the five conveniently defined stages of development,[1] were counted per 10,000 erythrocytes. Gametocytes are long-lived stages, which accumulate in culture and take approximately 10 days to reach morphological maturity. Once achieved, therefore, maximum densities of gametocytes do not rapidly decline; previous experience has shown that maximum densities are achieved during the 2nd and 3rd week after subculture.

Clones were isolated by limit dilution from the Ituxi 084, L and KZ1 isolates after they had been in culture for 173, 16, and 20 days, respectively. First generation clones were cryopreserved as soon as possible and thawed at a later date for further study. Subclones were isolated from two Ituxi 084 clones (It.D12 and It.G2) and from one L clone (L.F4). For full details of derivation of clones see Graves et al.[12] All references to time are given in terms of time in culture since isolation of the parasites from the natural human infection.

RESULTS

Differences in gametocyte production between isolates of P. falciparum

Gametocyte production rates by five isolates of *P. falciparum* (two Brazilian and three African) grown in continuous culture are presented in Table 1. For each isolate the individual subcultures represented are spread over periods of 10–40 weeks in culture; initial measurements were made following 2–12 weeks of continuous culture since isolation of the parasites, with the exception of the Z isolate, for which the observations represented were made following 20–40 weeks of continuous culture since isolation. Each point represents the total number of gametocytes of all stages present during a particular subculture at the time when the maximum number of gametocytes was present, usually between 14 and 20 days after initiation of a culture.

TABLE 1

Gametocyte production by isolates of Plasmodium falciparum

Isolate	No. of subcultures assayed	Gametocytes per 10^4 erythrocytes: Geometric mean + 1	Mean log	SD of mean log*	
Ituxi 084	13	162.2	2.21	0.23	
IMTM 22	6	59.5	1.78	0.16	]
L	8	32.9	1.52	0.24	]]
Z	7	19.7	1.29	0.47	]]
G1	12	10.7	1.03	0.32	]

* Isolates within the same bracket do not differ significantly at the 0.05 level by the Newman-Keuls test.

One-way analysis of variance on the log number of gametocytes produced per subculture indicates that the isolates differed in their intrinsic rates of gametocyte production during the time that each was observed ($F_{4,41} = 27.11$, $P < 0.005$). The Newman-Keuls procedure for calculating the Studentized range statistic was used to compare the mean logs of each pair of isolates. Isolates not distinguishable from one another are indicated by a shared bracket in Table 1.

Ituxi 084 was significantly different from the other four isolates; the means for these four overlaps so that neither is significantly different from its neighbor; however, each can be distinguished from other isolates more than one step away on an ordered scale of means.

Differences in gametocyte production between cloned lines from individual isolates of P. falciparum

In order to study the basis of variation in gametocyte production, cloned lines of parasite were derived from certain isolates. During the period

TABLE 2

Gametocyte production by cloned lines of Plasmodium falciparum

Clone	No. of subcultures assayed	Gametocytes per 10^4 erythrocytes: Geometric mean + 1	Mean log	SD of mean log*
It.E1	6	426.6	2.63	0.26
It.F10	6	281.8	2.45	0.25
It.D12	9	263.0	2.42	0.28
It.D12.E7	8	58.9	1.77	1.19
It.D12.G9	6	49.0	1.69	0.46
It.G10	5	16.2	1.21	0.67
It.G2.F6	3	3.1	0.49	0.43
It.G2.G4	7	1.7	0.24	0.28
L.F5	7	151.4	2.18	0.37
L.G2	7	75.9	1.88	0.46
L.E5	9	70.8	1.85	0.38
L.D1	6	5.6	0.75	0.47
L.F4.F1	4	1.0	0	—
L.F4.G5	4	1.0	0	—

* Clones within the same brackets do not differ significantly at the 0.05 level by the Newman-Keuls test.

TABLE 3

Gametocyte production in P. falciparum *KZ1 clones with identical histories in culture and grown concurrently*

Time in culture (weeks)	Gametocytes/10^4 erythrocytes: K.C7	K.D7	K.C7	K.D7
14	9	60	—	—
16	1	50	—	—
18	0	32	—	—
20	0	36	9	41
22	0	19	10	69
24	0	19	0	19
26	—	—	0	2
28	—	—	0	8
30	—	—	0	4
Geometric mean + 1	1.7	33.9	2.2	14.1
Mean log	0.22	1.53	0.34	1.15
SD of mean log	0.40	0.20	0.53	0.53
	$t_{10} = 7.15$, $P < 0.05$		$t_{10} = 2.64$, $P < 0.05$	

of measurement of gametocyte production the ages (weeks in culture since isolation of parental parasites from human infection) of the first generation clones of Ituxi 084 were as follows: It.D12, 40–51 weeks; It.E1, It.F10 and It.G10, 37–46 weeks. Most first generation clones manifested gametocyte production rates at this time as high as or higher than the rate typical of the parental isolate while one clone, It.G10, produced markedly fewer gametocytes (Table 2).

Subclones were isolated from two Ituxi clones, It.D12 and It.G2, both of which were producing gametocytes at the time of recloning. Two subclones of the It.D12 line (It.D12.E7 and It.D12.G9) continued to produce gametocytes. Subclones of It.G2 (It.G2.F6 and It.G2.G4) were all very low producers from the earliest time of measurement after recloning (Table 2). The few gametocytes produced by these clones never progressed to maturity. It.G2, itself a producer, was lost before its gametocyte production could be accurately quantified. The ages of the subclones during the period of measurement represented in Table 2 were: It.D12.E7, 46–56 weeks; It.D12.G9, 54–63 weeks; It.G2.F6, 53–64 weeks; It.G2.G4, 47–55 weeks. Analysis of variance of clones of Ituxi 084 revealed significant differences in the mean log gametocytes produced by the clones ($F_{7,42} = 12.36$, $P < 0.005$). Mean logs which are not significantly different from one another are encompassed by a single bracket in Table 2.

As was observed for Ituxi 084, clones of L differed in their gametocyte production. The L.F5 produced large members of gametocytes and L.D1 significantly fewer (Table 2). Second generation clones of L.F4 (a gametocyte-producing line lost before its production could be accurately quantified) produced no gametocytes at all at the time of first measurements. The ages of the clones during the measurements in Table 2 were: L.F5 and L.G2, 13–24 weeks; L.E5 and L.D1, 15–41 weeks; L.F4.F1 and L.F4.G5 27–39 weeks. Analysis of variance of mean logs between clones showed them to be significantly different ($F_{7,42} = 12.36$, $P < 0.005$). When pairs of means were compared, the clones fell into three distinct groups comprising L.F5, L.G2, and L.E5 in one, L.D1 alone in the second group, and the two L.F4 subclones in the third.

Two clones of a third isolate, KZ1, also differed significantly in their gametocyte production. The clones K.C7 and K.D7 were grown together on two separate occasions and each time K.D7 produced more gametocytes than did K.C7 (Table 3).

Evidence for innate differences in gametocyte production between cloned lines of P. falciparum

The information in Table 2 was collected over a period of several months, not all clones being grown concurrently. Thus, the effects of different cumulative times in culture and environmental

TABLE 4

Gametocyte production in P. falciparum *L clones with identical histories in culture and grown concurrently*

Time in culture (weeks)	Gametocytes/10^4 rbc	
	L.D1	L.E5
15	3	17
16	1	21
19	5	137
24	8	49
29	35	125
41	1	102

differences are possible causes of variation in gametocyte production between parasite lines. To control for such effects it was necessary to look at groups of clones which had been grown in parallel under similar culture conditions since isolation of the parental parasites. Differences measured between such clones are likely to be due to intrinsic differences between the clones themselves established prior to isolation. The cloned lines of the KZ1 isolate were grown according to these criteria (Table 3), and showed consistent differences in gametocyte production on both occasions when they were grown together. Cloned lines of L and Ituxi 084 which had always been grown concurrently also showed consistent differences (Tables 4 and 5).

Loss of gametocyte production by cloned lines of P. falciparum

Cloned lines of *P. falciparum* were observed to undergo loss of gametocyte production during the course of successive blood subcultures. This

TABLE 5

Gametocyte production in P. falciparum *Ituxi 084 clones with identical histories in culture and grown concurrently*

Time in culture (weeks)	Gametocytes/10^4 rbc		
	It.E1	It.F10	It.G10
37	750	228	9
39	180	162	94
41	720	540	51
43	460	640	11
44	282	184	1
46	480	208	ND*

* ND, not done.

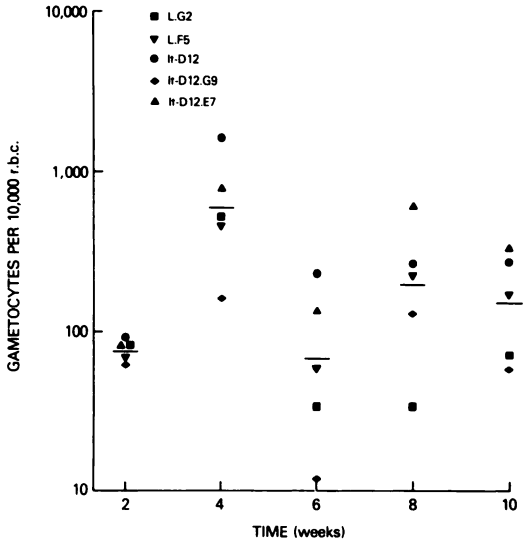

FIGURE 1. Gametocyte production by five different clones of *P. falciparum* during concurrent culture under identical conditions.

trend is shown for clones K.C7 and K.D7 in Table 3. Similar observations have been made with other cloned lines including some lines that passed from consistent maximum production of several hundred gametocytes per 10^4 erythrocytes to total non production in the course of a few weeks (data not shown).

Transient environmentally induced fluctuations in gametocyte production by clones of P. falciparum

It was noted that gametocyte production by cloned parasites was transiently affected by undefined conditions associated with successive subcultures. In Figure 1 data from five cloned lines (L.G2, L.F5, It.D12, It.D12.G9 and It.D12.E7) are presented. These data represent five successive subcultures over a period of 10 weeks during which the cloned parasites were cultured in parallel under identical conditions using the same batches of erythrocytes and medium. It is evident that marked differences in gametocyte production affecting all clones occurred between successive subcultures. This is especially striking between first, second and third subcultures. Such variation appears to represent transitory environmental effects on gametocyte production associated with an individual sub-

TABLE 6
*Asexual parasitemia and gametocyte production**

Subpass	K.C7	K.D7	It.E1	It.G10	It.D12	It.G2.G4
2	4.6/0	5.2/11	3.0/180	3.6/94	6.7/200	1.3/1
3	3.3/0	4.0/36	2.0/720	3.7/39	3.2/234	19.8/1
4	5.1/0	7.9/19	5.6/460	9.6/11	12.3/279	ND†

* Each entry is the asexual parasitemia (%) at 14 days of culture/total no. of gametocytes/10^4 rbc at 21 days of culture.
† ND, not done.

culture and affecting all clones in a similar fashion.

Relationship between gametocyte production and asexual growth

The differences in gametocyte production rate among the different parasite clones were not due to differences in asexual growth. This is illustrated by Table 6 which gives examples of the asexual parasitemia 14 days after subculture compared to gametocyte densities on day 21.

Asexual parasitemia on day 14 is related to gametocytemia on day 21 for the following reason. By about day 14 the rate of production of gametocytes from asexual parasites is generally at a maximum. However, gametocytes require at least 2 days of growth before they are readily identifiable and about 8–10 to reach morphological maturity; therefore, it is convenient to approximately relate the asexual parasites present on day 14 to the gametocyte population present about 1 week later, i.e., on day 21.

Although the asexual parasitemias varied there was no correlation between the number of asexual parasites and number of gametocytes subsequently produced. Moreover such differences as occurred in asexual parasitemia were relatively slight and not strongly related to the clone involved. Differences in gametocyte production were, on the other hand, generally very great and characteristic of the line of parasites involved.

DISCUSSION

In the present study, and in those of others,[3, 5] considerable variation has been found among isolates of *P. falciparum* in their capacity to produce gametocytes. Although characteristic levels of gametocyte production may be stable for extended periods, capacity for gametocyte production by individual isolates of *P. falciparum* tends eventually to be lost during in vitro culture, the time of loss, varying from a few weeks to more than 1 year, being characteristic of an isolate.[6] Such loss of gametocyte production also occurs during in vivo blood passage of *P. falciparum* in humans and other primates.[14–16] We have attempted here to study the diversity and stability of gametocyte production among the parasites represented in individual isolates of *P. falciparum* by deriving several cloned lines of parasite from each isolate and following their gametocyte production during in vitro cultivation.

Our results indicate that individual isolates of *P. falciparum* contain mixtures of parasites of innately different capacities for gametocytogenesis under in vitro conditions. This was shown by the demonstration that cloned parasites maintained under identical conditions of culture since isolation from an individual human infection manifested significant, and frequently large, differences in gametocyte production. Thus, not only do different isolates differ in capacity for gametocytogenesis but the parasites within an isolate may be represented by different lines, themselves heterogenous with respect to gametocyte production.

Capacity for gametocytogenesis of individual clones of *P. falciparum* as measured by rate of gametocyte production was generally stable within a characteristic range for several weeks to months depending upon the clone involved. Eventually, however, many clones, including previously high gametocyte producers, declined to very low levels of production or ceased gametocytogenesis completely. In view of such eventual instability of gametocyte production by cloned lines during culture it cannot be concluded that the diversity of levels of gametocytogenesis found amongst such lines as measured in vitro is representative of the diversity of capacities for gametocyte production among the parasites as they existed in the original human infection.

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