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Clones of the malaria parasite *Plasmodium falciparum* obtained by microscopic selection: Their characterization with regard to knobs, chloroquine sensitivity, and formation of gametocytes

(cultivation *in vitro*)

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ABSTRACT **A culture line of *Plasmodium falciparum* (FCR-3/Gambia) was used to select and place in culture cells containing a single parasite. The method depends on examination of minute droplets of dilute cell suspensions with oil immersion phase-contrast microscopy. Droplets found to contain a single parasite were maintained under appropriate culture conditions until detectable numbers of parasites were present (generally by day 21). Of nine clones that grew up, seven were knobless and two were knobby. The clones differed somewhat in chloroquine sensitivity. Their 50% inhibition point under one set of conditions *in vitro* ranged from 0.02 to 0.06 μg of base per ml, compared with only 0.003 μg for the highly sensitive line FCR-8/West Africa. All three tested clones formed gametocytes under appropriate *in vitro* conditions.**

Since the first cultivation of *Plasmodium falciparum* 5 yr ago (1), many culture isolates of this parasite have been obtained from different geographical regions (2–5). These differ in drug sensitivity and other properties; thus, it becomes important to establish clones to permit more detailed investigation. Clones are also of special interest in relation to the knobs, structures that appear on the membrane of erythrocytes containing trophozoites or later stages of the asexual cycle (6). After prolonged cultivation, it was observed that many erythrocytes with trophozoites or schizonts lacked knobs. It was assumed that this knobless condition might represent a mutant of the parasite well suited to life in culture but perhaps not to life *in vivo* within a host. Clones are essential to test this hypothesis.

Recently clones of knobless (K−) and knobby (K+) parasites have been established by the method of limiting dilution (T. Green, personal communication). A similar dilution method has been used to initiate clones with two different isozymes of glucose phosphate isomerase (7). We report here the establishment of nine clones by a method based on microscopic selection.

MATERIALS AND METHODS

A subline of FCR-3/Gambia (2) served as the source of parasites. This subline has been in continuous culture most of the time since its isolation in August 1976 from the blood of a patient from Gambia. For the past year, it has been maintained by F. Gyang in our laboratory in flow vessels (8) with subculture every third day. When examined by electron microscopy, the subline shows under these conditions that about 40–50% of the cells with late-stage parasites have knobs.

Material from such a culture having a parasitemia of about 10% and a hematocrit of 7% was diluted in two steps, first 1:100 and then again 1:100 or 1:200 to give 1:10,000 or 1:20,000. The dilutions were made in the standard RPS medium (9) but with 15% (vol/vol) human serum and with gentamycin at 40 μg/ml, designated RP-G-15S medium. The final dilution would give about 10 erythrocytes in a droplet of 1- to 2-mm diameter held between two coverslips.

The droplet was prepared in the following way. A rectangular coverslip (30 × 22 mm) was placed over the well of a depression slide and held in place with a ring of vaseline around the well. The coverslip was kept covered with a 35-mm plastic Petri dish cover between operations. A fine circle, about 5 mm in diameter, of sterile silicone grease was placed in the center of the coverslip by using a piece of sterile glass tubing of 5- to 6-mm diameter with one end ground flat. This was touched to silicone grease that had been autoclaved in a Petri dish and then to the coverslip. A minute droplet of the 1:10,000 (or 1:20,000) cell dilution was deposited in the center of the ring of grease with a small pipette drawn to a very fine tip and controlled with a rubber bulb. This droplet was then covered with a 5 × 5 mm sterile coverslip (cut from larger no. 1 coverslips). This was set on in such a way that the droplet formed a disc but did not quite reach to the circle of grease. The latter served as a support for the small coverslip and prevented drying of the droplet. The large coverslip bearing the preparation was then inverted over the well slide and examined with oil immersion phase-contrast microscopy by using a long-working-distance condenser. The entire droplet was scanned, each cell being carefully examined. Total erythrocytes and any crenated cells and parasites were counted.

If a preparation showed a single parasite, this was put in culture as follows. The large coverslip was removed from the depression slide and placed with the preparation side up in a sterile 35-mm Petri dish. A well was constructed around the preparation by means of a glass cylinder 1 cm in diameter and 5 mm high. One end of the cylinder was touched to the sterile silicone grease and by this was attached to the large coverslip to surround the preparation. In the well of a 24-well Linbro plate was put 0.5 ml of a 1% suspension of human type A+ erythrocytes in RP-G-15S medium. A drop of this suspension was then placed over the 5 × 5 mm coverslip in the well that had been constructed around it. With two sterile needles, the small coverslip was lifted, allowing the parasite to enter the drop of 1% erythrocyte suspension. The coverslip was then held with a sterile forcep while it was rinsed with additional drops of 1% erythrocyte suspension from the 0.5 ml in the Linbro plate. The material in the constructed well then was transferred to a clean well in the Linbro plate and rinsed with additional 1% erythrocyte suspension until all of the 0.5 ml had been used and transferred. This gave a microculture consisting of 0.5 ml of 1% erythrocyte suspension that should have contained the one infected cell with one parasite.

Ordinarily three to four such cultures could be prepared in approximately 3 hr (from about twice as many preparations ex-

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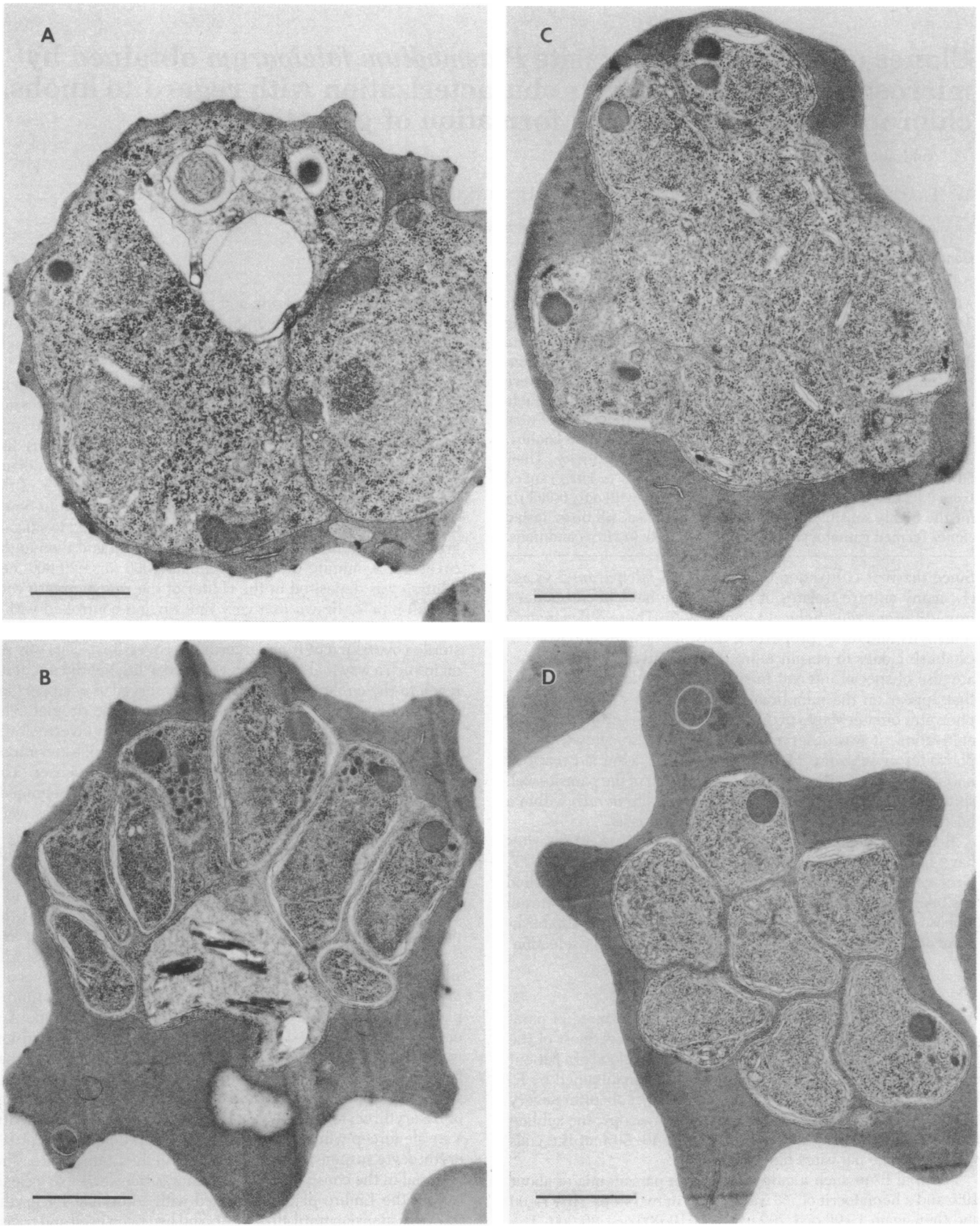

FIG. 1. Electron micrographs of representative sections through cells with schizonts from four clones. (*A*) Clone I (K+). (*B*) Clone A-2 (K+). (*C*) Clone D-3 (K−). (*D*) Clone D-4 (K−). (Bar = 1 μm; ×18,150.)

amined). In order to provide better maintenance of moisture conditions, one row of six wells of the Linbro plate was filled with medium alone. The plate was incubated in a candle jar at 37°C. Each microculture was subjected to the following routine in which the day of preparation is considered day 0. Day 1: only the candle was burned to assure an appropriate atmosphere. Day 2 and daily thereafter: the overlying medium was removed, replaced with an equivalent amount of fresh RP-G-15S medium, and the cells were resuspended carefully. Day 6: the fresh medium contained 1% erythrocytes. Day 12: the fresh medium contained 2% (vol/vol) erythrocytes. Day 17: the entire contents of the well were transferred to a 3.5-cm Petri dish holding 1 ml of 4% erythrocyte suspension. Daily replacement of the medium then continued. On day 21 the first slide was made, and on day 22 the fresh medium contained 2% erythrocytes. On day 26 a slide was again made. When this was negative, the preparation was discarded.

Cultures with positive slides on day 21 or 26 were grown up and expanded as rapidly as possible, first in small Petri dishes and then in flow vessels. As soon as sufficient parasitemias were attained, samples were cryopreserved (9), and other samples were fixed and prepared for electron microscopy (10).

Chloroquine sensitivity of the clones was determined by the 48-hr method (11). To see whether the clones could form gametocytes, they were kept in flow vessels for 10–12 days without provision of fresh erythrocytes (8).

RESULTS AND DISCUSSION

Clones Obtained. In the first series attempted, only one culture (clone I) grew up out of nine prepared. Four further series were then set up for a total of 14 additional preparations of which 8 yielded cultures (Table 1). There was a considerable range in total number of erythrocytes per droplet. This depended on whether a 1:10,000 or 1:20,000 dilution was used and on the size of the droplet. Tiny droplets could be prepared with only a few cells. It is noteworthy that two parasites were sometimes present in such droplets. For example, one preparation had only two red cells, of which one contained two rings. The first barely positive slides were obtained at 21 days, the same time when the clones studied by Rosario (7) reached a detectable level. With the methods used here, calculations based on an assumed 5-fold increase with each two-day cycle predicted day 18 as the earliest at which one could expect to reach a level of one parasite per 10,000 erythrocytes.

Table 1. A typical series of preparations to show the cells seen in each droplet, and the results of cultures prepared when a single parasite was seen

Droplet no.	RBC in droplet: Total	Crenated	With parasites†	Culture well	Results*: 21 days	26 days
1	5	2	3:1-R			
2	12	1	0			
3	12	2	1:1-R	D-1‡	−	+
4	6	0	1:1-T	D-2	−	−
5	12	0	1:1-T	D-3‡	−	+
6	20	0	1:1-T	D-4‡	+	+

RBC, erythrocytes.
* −, No parasites seen on slides; +, parasites seen.
† This gives the number of infected cells and number and stage of parasites per cell. Thus, 3:1-R indicates three cells each with one ring. 1:1-T indicates one cell with one trophozoite.
‡ Established clones; the other six clones (data not shown) were designated I, A-2, A-3, B-3, B-4, C-1.

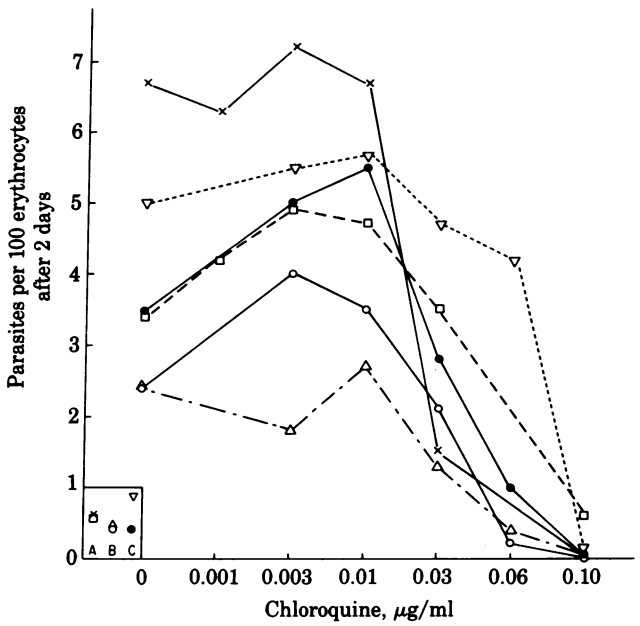

FIG. 2. Effect of chloroquine on growth of three clones and a wild-type knobby line (F-86). (*Inset*) Initial parasitemias. Parasite strains in experiments: A, clone D-3 (×—×) and clone A-3 (□– – –□); B, clone D-3 (○——○) and clone A-2 (△–·–·–△); C, clone D-3 (●——●) and a highly knobby line (▽– – –▽).

The microscopic method described here presents certain advantages over the method of limiting dilution. Although it requires more work initially, subsequent blind maintenance of cultures is much reduced. There is greater certainty that the culture is derived from a single parasite. This is especially true

Table 2. Chloroquine sensitivity *in vitro**

Parasite strain	K+ or K−†	Chloroquine IC_{50}, μg/ml
F-79 unselected	≈50% K+	0.03
Clone I	K+	0.05
Clone A-2	K+	0.05, 0.03
Clone A-3	K−	0.06
Clone B-3	K−	0.02
Clone B-4	K−	0.04
Clone C-1	K−	0.04
Clone D-1	K−	0.05
Clone D-3	K−	0.02, 0.025, 0.03, 0.03
Clone D-4	K−	0.02, 0.04
FCR-3 after 1 mo in culture	ND	0.03
F-86 selected for K+	K+	0.07
FCR-8/ West Africa	ND	0.003

ND, not determined.
* For three culture lines of FCR-3/Gambia (F-79, F-86, and material frozen after 1 mo in culture), for nine clones derived from F-79, and for another isolate FCR-8/West Africa.
† Knobby (K+) or knobless (K−).
‡ Concentration for 50% inhibition; estimated from charts (see Fig. 2).

Table 3. Gametocyte formation by clones in flow vessels

Clone no.	Parasites* per 5000 erythrocytes					Gametocytes, %	Stages of gametocytes in 100 fields				
	R	T	S	G	Total		II	III	IV	V	Total
A-2	64	18	173	31	286	11	8	13	7	6	34
D-3	31	9	99	35	174	20	9	13	7	7	36
D-4	40	4	124	17	185	9	2	3	8	5	18

The cultures were subcultured to a parasitemia of 0.2% in a 6% erythrocyte suspension. Gametocytes were seen 7 days later. Counts on day 12 are shown.

* R, rings; T, trophozoites; S, schizonts; G, gametocytes.

for an intracellular parasite where limiting dilution has to be based on the number of host cells and a single host cell may contain two or more parasites. The one source of uncertainty in the microscopic method lies in the crenated erythrocytes. It is possible to miss a ring in a crenated cell. For this reason, clones D-3 and D-4 have been chosen for more detailed study as knobless clones, and clone A-2, likewise derived from a droplet with no crenated erythrocyte, has been selected for study as a knobby one.

Presence or Absence of Knobs. Transmission electron microscopy showed that clone I was knobby; of the eight additional clones, seven were completely knobless and one (A-2) was knobby (see Fig. 1). These results were confirmed by electron microscopy of whole infected erythrocytes and by carbon replicas of the erythrocyte cell surface. Both methods revealed the knobs without the need for thin sectioning (N. Lanners, personal communication). Clone D-3 was selected for extensive cultivation in flow vessels. After 5 mo of continuous culture, it remains entirely knobless.

Chloroquine Sensitivity. Curves relating growth to chloroquine sensitivity are shown for three clones and the highly K+ wild-type line in three separate experiments (Fig. 2). An estimate of the 50% inhibition point can be made from the right-hand portion of each curve, where the extent of growth drops from a maximum. Such estimates usually but not always agreed closely. As judged in this way, chloroquine sensitivity for most of the clones was much the same as that of the parent line (Table 2). The wild-type K+ line (F-86) was less sensitive than any of the clones. All derivatives of FCR-3/Gambia tested were classed as chloroquine resistant and were about 1/10th as sensitive as another West African culture line (FCR-8/West Africa) (Table 2; ref. 11).

Gametocyte Formation. Several workers have noted that gametocytes appear *in vitro* especially under conditions not suitable for best asexual growth—e.g., when fresh erythrocytes are not provided (12). When three of the clones (one K+ and two K−) kept in flow vessels were diluted with fresh cells to a parasitemia of about 0.2% and then kept without further addition of erythrocytes, gametocytes appeared within a week and were relatively abundant by day 12 (Table 3). About 20–25% of those seen were in Stage V with equal proportions of the two sexes.

This observation, combined with the recent demonstration that mosquito infection can be produced with gametocytes formed in culture (ref. 13; T. Ifediba and J. Vanderberg, personal communication) indicates future possibilities for genetic studies with *P. falciparum*.

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