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A HIGHLY EFFICIENT *IN VITRO* CLONING PROCEDURE FOR ASEXUAL ERYTHROCYTIC FORMS OF THE HUMAN MALARIA PARASITE *PLASMODIUM FALCIPARUM*

by

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Summary. — A very reliable and productive technique for cloning of *Plasmodium falciparum in vitro* is proposed, as demonstrated by successive limiting dilution of suspensions of asexual erythrocytic forms of the NF 54 strain. The introduction and the study of reliable clones is of extreme importance for a better understanding of the behaviour of the parasite, also in field conditions. The method is rapid, simple and efficient. The growth of the clones was individually monitored and the culture conditions were constantly adjusted during their stay in recipients of increasing size. A yield of 18/96 (18.75 %) of provisional clones was obtained, while the supercloning phase resulted in 16/80 (20 %) positive cultures. The probability that the latter were derived from a single progenitor is very high (99 %). It was shown that three randomly selected clones (A1A9, A1B11, and A1C10) have excellent growth characteristics before and after cryopreservation, and after a longer period of culture in standard conditions.

KEYWORDS : *Plasmodium falciparum;* Human malaria; Erythrocytic forms; Cloning; *In vitro;* Limiting dilution; Probability; Cryopreservation; Growth characteristics.

Introduction

Malaria is still one of the most important tropical diseases, and *Plasmodium falciparum* is estimated to kill 2-3 million people per year (14). In many parts of the world the situation is even deteriorating, amongst other things by the rapid spread of drug resistant parasite strains (24). Therefore, a concerted effort is necessary to elucidate the mechanisms of drug action and resistance, to reverse resistance, and to prevent its development (18).

The availability of homogeneous populations of *P. falciparum* with defined characteristics provides a useful model, and can greatly facilitate the study of these and related biological and genetic aspects of the species. Studies on variation of drug response of clones *in vitro* have been recommended by the World Health Organization (21). Clones have been prepared by dilution techniques (20, 27), by microscopic selection in minute droplets (26, 5), or by micromanipulation (17, 4). They have been used for *in vitro* induction of resistance (2, 16), characterization of antigens (1), drug testing (8), enzymatic characterization (13, 19), karyotyping (6, 3), knob and gametocyte formation (26), and the study of cytoadherence (7).

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Many previously described limiting dilution techniques were not completely satisfactory. Therefore, we propose a combined cloning and supercloning procedure applied to the NF 54 strain of *P. falciparum*, wherein the risk of producing a dilution culture that is in fact a «clonal mixture» (22, 23) derived from 2 or more parasites, is reduced to a minimum. The aim was to obtain a collection of single-cell derived parasite populations, selected in identical circumstances and meant as essential and reliable basic material for several types of more profound investigations.

Materials and Methods

In vitro cultures

Erythrocytic cultures of the NF 54 (Amsterdam airport) strain of *P. falciparum* were maintained *in vitro*, essentially following the method of Trager and Jensen (25). A volume of culture medium of 115 ml contained 100 ml of RPMI 1640 stock solution, 4 ml of 5 % $NaHCO_3$ (Merck), 1 ml of 0.2 % gentamycin (Merck) and 10 ml of human serum (A Rh.+). The RPMI 1640 stock was prepared by solving 5.94 g HEPES (BDH), 0.05 g hypoxanthin (Sigma), 2.00 g glucose (Sigma) and 10.42 g RPMI 1640 powder (ICN) in 1130 ml of distilled H_2O. The cultures were kept under an atmosphere containing 5 % of CO_2, 5 % of O_2 and 90 % of N_2 (L'air Liquide). A volume of 100 ml of wash medium contained 96.4 ml of RPMI 1640 stock solution and 3.6 ml of 5 % $NaHCO_3$.

Human red blood cells (O Rh.+ or A Rh.+) were donated by volunteers, with ACD (citric acid - dextrose) as anticoagulant (3 ml ACD + 17 ml blood). Fresh erythrocytes were washed three times (two times with wash medium and once with culture medium), and centrifuged at 750 g during 5 min. The packed cell volume was adjusted to 50 %, prior to storage of the stock suspension at 4° C.

The parasites were kept in static cultures (5 ml of 1 % hematocrit in sterile Nunc flasks) or rotating cultures (10 ml of 5 % hematocrit in erlenmeyers) in a New Brunswick G25 Incubator Shaker (100 rpm) at 37° C. They were subinoculated twice a week, after adding fresh red blood cells and renewing the medium. The initial parasitaemias were 1 %.

General cloning procedure

The *P. falciparum* NF 54 strain was cloned *in vitro*, generally in agreement with formerly described procedures (20, 12), but including successive cloning and supercloning phases, and continuous monitoring of the parasite growth and adjustment of the culture conditions. In order to minimize the potential errors inherent in limiting dilution techniques (16), suspensions were consecutively diluted to precalculated probabilities of 1.0 and 0.3 parasites/100 μl, and inoculated in the wells of flat bottom microtitre plates (Falcon Microtest III).

Cloning

At the beginning of the cloning procedure, a static stock culture of *P. falciparum* NF 54 (7.70 % parasitaemia, 1 % haematocrit) was diluted with complete RPMI medium (10 % human serum) to a density of 1 parasite/100 µl. The haematocrit was adjusted to 2.5 % with fresh human red blood cells. The suspension was subsequently seeded in the 96 wells of a microtitre plate (100 µl/well) and the plate was kept under standard culture conditions. The medium was renewed once a day (100 µl/well) throughout the stay of the parasites in microtitre plates, and at days 5 and 11, 100 µl of fresh erythrocytes (2.5 % haematocrit) were added per well.

At day 14, the parasitaemias of 96 Giemsa stained thin smears, each of them corresponding with a well, were evaluated. Dependent on their growth rate, the parasite cultures were successively transferred to a second microtitre plate, to a 24 wells multidish (Nunc), and to the rotating culture system. Red blood cells were added regularly. At the end of this phase, all positive cultures (provisional clones) were temporarily stored in liquid nitrogen. They were named after their location on the first microtitre plate.

Supercloning

Two different diluted suspensions of a selected provisional clone were prepared, containing 1 and 0.3 parasites per 100 µl, respectively, each with a haematocrit of 2.5 %. They were seeded in the wells of a microtitre plate (100 µl/well), as follows : columns 1-2 (16 control wells) received 1 parasite/well, and columns 3-12 (80 wells) received 0.3 parasites/well. Each well was named after the provisional clone, and after its location on this microtitre plate. The medium was renewed once a day, throughout the experiment. A fresh human red blood cell suspension (100 µl/well, 2.5 % haematocrit) was added after 6 and 10 days. After 15 days, 96 thin smears were made, Giemsa stained and evaluated.

This time, the positive cultures were successively transferred to another microtitre plate, to a 24 wells multidish, and to sterile 25 cm^2 culture flasks (Nunc). Again, red blood cells were added regularly.

Calculations

The numbers of parasites per well are assumed to follow a Poisson distribution. The probability that the parasites in a positive well are derived from one single cell is given by $\mu e^{-\mu}/(1 - e^{-\mu})$, where μ (the average parasite density of the inoculum) is estimated by $\mu = \ln [1 + (\text{no. positive wells})/(\text{no. negative wells})]$. The probability that a population obtained after supercloning is derived from one progenitor, is calculated by multiplying the probabilities (in each phase of the cloning) that a line arose from more than one parasite and subtracting the result from 1 (11).

Cryopreservation

The method of Christofinis and Miller (9) was applied for the storage of *P. falciparum* strains, provisional clones and superclones in liquid nitrogen, in

2 ml ampoules containing 1 ml of the preservation medium. For the preparation of 125 ml of this medium, 4 ml of 5 % $NaHCO_3$, 1 ml of 0.2 % gentamycin, 10 ml of human serum (A Rh.+), and 10 ml of dimethyl sulfoxide (UCB) were added to 100 ml of RPMI 1640 stock solution.

Viability of stabilates

After a stay of 2-3 min in a water bath at 37° C, the content of an ampoule was transferred to a sterile 10 ml tube and 5 ml of the wash medium was added. After centrifugation (750 g during 5 min), the pellet was resuspended in 5 ml of culture medium containing 0.1 ml of washed erythrocytes (50 % haematocrit). The culture was kept in a sterile 25 cm^2 flask (Nunc) under a $CO_2/O_2/N_2$ (5 %/5 %/90 %) atmosphere.

Results

Cloning

Fourteen days after initiation of the cloning procedure, 18 out of 96 cultures were proven to be positive and well growing, after successive transfer to a second microtitre plate, a 24 wells multidish and the rotating culture system. This corresponds to 18.75 % of the initial number. These provisional clones were named as follows: A1, A4, B7, C3, C12, D10, E1, E5, E9, F2, F5, F7, F12, G1, G3, G8, H8, and H10.

At day 18, they were transferred to another microtitre plate. Their parasitaemias were allowed to increase to about 5 %, and 100 µl of a fresh erythrocyte suspension (2.5 % haematocrit) were added per well. This procedure was repeated two more times, the last time adding 200 µl of the erythrocyte suspension. Finally, the parasites were allowed to multiply until a parasitaemia of 1-2 % was reached.

They were subsequently transferred to a 24 wells multidish, where their parasitaemias were allowed to rise again to about 5 %, and this time 500 µl of an erythrocyte suspension (2.5 % haematocrit) were added to each well. This procedure was repeated once. Then, the parasitaemias were permitted to increase to 1-2%.

Next, each parasite culture was moved to the erlenmeyers of the rotating culture system, with initial conditions of 1 % parasitaemia and 5 % haematocrit. After a growth period of 4 days, all provisional clones were prepared for cryopreservation (5 ampoules/clone).

Supercloning

Two ampoules, each containing the provisional clone A1, were recovered from storage in liquid nitrogen. Their content was pooled and transferred to the rotating culture system, after adjustment of the haematocrit to 2.5 %. After a growth period of 14 days under standard conditions, evaluation of a series of Giemsa stained thin smears revealed an average parasitaemia of 12.10 %.

The parasites were inoculated in the wells of a microtitre plate at

densities of 1 and 0.3 parasites/well, respectively. In the first 2 columns (controls, 1 parasite/well), 10/16 wells proved to be positive after 15 days, namely A1A2, A1B1, A1B2, A1C1, A1C2, A1E1, A1E2, A1F1, A1H1, and A1H2, corresponding with 62.50 % of the control wells. In columns 3-12 (0.3 parasites/well), 16/80 wells were positive at day 15 : A1A8, A1A9, A1B9, A1B11, A1C5, A1C9, A1C10, A1C11, A1D3, A1D8, A1E6, A1E12, A1F7, A1G3, A1H7, and A1H9. This corresponds with 20.00 % of the number of inoculated wells, and is, proportionally, well in agreement with the percentage calculated for the control wells.

The 16 clones were subsequently transferred to another microtitre plate, to a 24 wells multidish and to 25 cm^2 culture flasks, in a scheme similar to the one described in the first part of the cloning procedure. In each well of the microtitre plate, 100 µl of fresh erythrocytes (2.5 % haematocrit) and 100 µl of complete culture medium were added initially. The medium was renewed daily, and thin smears were made on days 4 and 6. The corresponding parasitaemias are given in Table 1. After transfer to the multidish (day 7), each well received 300 µl of fresh erythrocytes. Again, the medium was renewed every day, and thin smears were made on days 10, 14, and 17 (Table 1). The erythrocyte suspension was renewed on day 14. On day 17, all 16 clones were transferred to 25 cm^2 culture flasks, after adding 5 ml of a 1 % haematocrit red blood cell suspension. From then on, each of them was routineously subinoculated twice a week, prior to cryopreservation. Their parasitaemias on day 21 are given in Table 1.

TABLE 1

Growth pattern of sixteen superclones of *Plasmodium falciparum* (NF 54) in microtitre plates, multidishes or culture flasks, immediately after their *in vitro* selection.

Clone	Parasitaemia (%) day 4 (1)	day 6 (1)	day 10 (2)	day 14 (2)	day 17 (3)	day 21 (4)
A1A8	0.4	0.5	2.6	3.6	1.5	2.0
A1A9	0.3	1.3	1.9	2.1	2.0	3.0
A1B9	0.6	0.9	1.0	2.7	1.4	2.0
A1B11	0.8	1.1	1.6	3.0	2.4	2.1
A1C5	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
A1C9	0.6	0.4	1.4	3.0	1.9	1.9
A1C10	0.5	0.4	0.9	3.5	1.8	2.1
A1C11	1.0	1.1	2.2	2.6	1.5	1.9
A1D3	0.7	1.2	1.9	1.6	1.9	1.8
A1D8	1.3	0.8	0.9	3.1	1.3	1.7
A1E6	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
A1E12	1.4	1.9	1.9	3.6	2.1	2.0
A1F7	1.0	0.3	1.3	2.1	1.2	1.5
A1G3	1.4	1.4	1.8	3.2	2.9	2.1
A1H7	0.9	0.5	1.4	2.6	2.0	1.4
A1H9	1.7	0.8	1.6	3.5	1.5	2.7

(1) Uninterrupted growth in microtitre plate from day 0 on. (2) Uninterrupted growth after transfer to multidish on day 7. (3) Continued growth in multidish, after addition of fresh erythrocytes on day 14. (4) Growth after transfer to culture flasks on day 17.

Only two of the clones (A1C5 and A1E6) did not appear to grow at all, and had to be discarded. The vast majority however (14/16), had a considerable growth rate during their stay in the different recipients (Table 1). At day 21, they all had parasitaemias of at least 1.4 %, and many of them reached levels of at least 2.0 % (A1A8, A1A9, A1B9, A1B11, A1C10, A1E12, A1G3, and A1H9). Among the latter series, A1A9 displayed the highest growth rate, with a parasitaemia of 3.0 % at day 21.

Probabilities

In agreement with the distribution type of the numbers of parasites per well (11), the values of μ (the average parasite densities of the inocula) were estimated as follows: μ = 0.208 (cloning, 1 parasite/well), μ = 0.981 (supercloning, control), and μ = 0.223 (supercloning, 0.3 parasites/well). The probabilities that the parasites in the positive wells were derived from one single cell in each phase, were estimated as 0.899 (cloning, 1 parasite/well), 0.589 (supercloning, control), and 0.890 (supercloning, 0.3 parasites/well). The probability that the 16 produced superclones each were overall derived from one progenitor, was calculated as 1 - 0.011 = 0.989, or about 99 %. Even in the case of combining the first cloning procedure with the control part of the supercloning procedure, this probability would have been 1 - 0.042 = 0.958, or about 96 %.

Viability

The supercloned parasites were further cultured until reasonable amounts were obtained for storage in liquid nitrogen. Three well growing clones were randomly selected (A1A9, A1B11, and A1C10), and tested for their viability after cryopreservation. Two parameters were determined for each clone. The first one was the initial time required to reach a parasitaemia of 1 % *in vitro*, immediately after recovery of the parasites from liquid nitrogen. The second one was the mean parasitaemia calculated over a longer term with regular subinoculations. These results are represented in Table 2.

TABLE 2

Growth characteristics of asexual erythrocytic forms of *Plasmodium falciparum* (NF 54) superclones A1A9, A1B11, and A1C10 *in vitro*, after cryopreservation.

Clone	Time (d)*	Parasitaemia (%)**
A1A9	5	7.54 ± 2.72
A1B11	9	6.23 ± 1.96
A1C10	6	6.04 ± 2.06

* Time required to reach a parasitaemia of 1 %, immediately after cryopreservation.
** Parasitaemias after 3 or 4 days of culture, calculated after 13 subinoculations. Mean values ± standard deviations.

Discussion

The proposed method includes successive cloning and supercloning phases, and continuous monitoring of the parasite growth and adjustment of

the culture conditions. This principle, combined with the lack of complicating additional techniques (15, 10) leads to a high yield and a number of advantages.

Indeed, the described cloning procedure yielded a high number of provisional clones (18/96). Subsequent supercloning of one of them (A1) was equally successful and yielded 16/80 superclones. The latter value proves that the culture conditions were nearly optimal. It is equivalent to a yield of 20 % and not too far apart from the theoretical value of 30 %, calculated by taking the inoculum density into account.

The advantages of the proposed method are manifold. In the first place, the time needed for achieving the whole routine is limited, and could even be reduced by omitting the intermediate cryopreservation step. Assuming a reproducible production in the supercloning phase, the 18 provisional clones could have yielded 18 x 16 = 288 genuine clones in only a few months. Secondly, all manipulations are performed on a practical laboratory scale, and only a limited bench surface and incubator space is needed. During a considerable part of the experiment, a high number of parasite cultures is kept in only one microtitre plate or multidish. Only in the final parts of both the cloning and the supercloning phase, when high quantities of parasites need to be produced, one separate recipient is required per culture. Thirdly, the technique is relatively simple. Other authors obtained in certain cases equally satisfying results, but often after introducing more complicated or time-consuming manipulations, such as co-culture of the clones on rodent hepatocyte feeder layers (15), or applying a third sequential round of limiting dilutions (10).

Last but not least, the probability that each clone is derived from only one progenitor is high and approaches one hundred percent. In other words, the risk of dealing with «clonal mixtures» (22, 23) is reduced to an absolute minimum. In most cases, the probabilities calculated by other authors for their own superclones reached only lower values (20, 11).

The high efficiency of the proposed method is also reflected in the low proportion of superclones with unsatisfactory growth rates : only two of them, or 12.5 %, had to be discarded because of this reason. This means that the vast majority of the produced superclones is immediately ready for culture, parasite production, and storage in liquid nitrogen at the end of the cloning procedure. These generally excellent characteristics are confirmed by determining the growth parameters of three randomly selected superclones (A1A9, A1B11, and A1C10), after their recovery from liquid nitrogen. In all cases, the initial time needed to reach a parasitaemia of 1 % was short, and the mean parasitaemias after 3-4 days of growth were high, as calculated over a longer period of culture and regular subinoculations.

The produced *P. falciparum* clones are to be considered as useful tools for the study of many aspects of the behaviour of the parasite, in laboratory and in field conditions.

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Een zeer efficiënte methode voor het klonen van ongeslachtelijke erythrocytaire vormen van de menselijke malariaparasiet *Plasmodium falciparum in vitro.*

Samenvatting — Een zeer betrouwbare en produktieve methode voor het klonen van *Plasmodium falciparum in vitro* wordt beschreven en wordt gedemonstreerd via opeenvolgende gelimiteerde verdunning van suspensies van ongeslachtelijke bloedvormen van de NF 54 stam. Onderzoek van betrouwbare klonen is van groot belang voor een beter begrip van het gedrag van de parasiet, ook in het veld. De methode is snel, eenvoudig en efficiënt. De groei van de provisionele klonen werd individueel gevolgd en de kultuurvoorwaarden werden kontinu aangepast tijdens hun verblijf in recipiënten van toenemende grootte. De opbrengst van het klonen bedroeg 18/96 (18,75 %) in de eerste fase en 16/80 (20 %) na het superklonen. De waarschijnlijkheid dat de superklonen afkomstig waren van een enkele cel is zeer groot (99 %). Er werd aangetoond dat drie willekeurig uitgekozen klonen (A1A9, A1B11 en A1C10) uitstekende groeikarakteristieken hebben, voor en na cryopreservatie en na een langere periode van kultuur onder standaardvoorwaarden.

Une méthode très efficace pour le clonage de formes érythrocytaires asexuées de l'agent de paludisme humain *Plasmodium falciparum in vitro.*

Résumé — Une technique très fiable et productive est proposée pour le clonage de *Plasmodium falciparum in vitro* et démontrée par la dilution limitée successive de suspensions de formes érythrocytaires asexuées de la souche NF 54. L'introduction de clones fiables est d'un intérêt capital pour l'étude de la conduite du parasite, aussi sur le terrain. La méthode est rapide, simple et efficace. La croissance des clones provisoires a été suivie soigneusement et leurs conditions de culture ont été adaptées constamment pendant leur séjour dans des récipients de tailles augmentantes. Un rendement de 18/96 (18,75 %) a été obtenu pour les clones provisoires et le superclonage a produit 16/80 (20 %) cultures positives. La probabilité que ces dernières étaient dérivées d'une cellule unique est très haute (99 %). On a montré que trois clones (A1A9, A1B11 and A1C10), sélectionnés au hasard, ont d'excellentes caractéristiques de croissance avant et après cryopréservation, et après une période plus longue de culture dans des conditions établies.

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