IN VITRO CULTIVATION OF MALARIA PARASITES

Bulletin of the World Health Organization, 55 (2-3): 363-365 (1977)

Cultivation of erythrocytic stages *

W. TRAGER [1] & J. B. JENSEN [2]

Two methods are described for the continuous cultivation of Plasmodium falciparum. *One method provides for a continuous slow flow of medium over a settled layer of blood cells and does not require daily attention. With the other method, carried out in Petri dishes, the medium has to be changed manually every day. The problems and possibilities of continuous cultivation are discussed.*

The older work on cultivation of erythrocytic stages has been sufficiently reviewed. Certainly further studies on cultivation of the various malarial parasites of experimental animals remain a desirable objective. So, too, do continued attempts at axenic cultivation, that is extracellular culture in a non-living medium, of an obligate intracellular parasite such as one of the erythrocytic malarial parasites.

In the context of this workshop, however, it seems far more important to report on the present status of our continuous cultures of *Plasmodium falciparum*.

We have two practical methods for continuous *in vitro* growth of this parasite. In both we use human erythrocytes in a thin settled layer in RPMI 1640 medium [a] with 10% human serum and an atmosphere of raised carbon dioxide and lowered oxygen concentrations. The proper ratio of cells to medium is obtained by using a 12% red cell suspension. The layer of medium over the cells is kept at a depth of 2–4 mm.

In one method, the flow-vial method, we provide a continuous slow flow of medium over the settled cells. This has the advantage of not requiring daily attention. In this method, an atmosphere of 7% CO_2, 5% O_2, and 88% N_2, is kept flowing constantly over the culture.

In the second method the cultures are held in plastic Petri dishes and the medium has to be changed manually at least once a day. An atmosphere with 3% CO_2 and about 17% O_2 is provided in a candle jar. This method has the advantages of simplicity and flexibility.

In both methods, subculture is effected by mixing an appropriate quantity of infected cells from a culture with a larger quantity of freshly washed uninfected cells. We have used type AB serum throughout and, for most of the work, type AB cells. The convenience of type AB serum is that any type of cells can be put in it.

The first strain obtained in culture was a South-East Asian strain, FVO, originally isolated into *Aotus* monkeys by Siddiqui & Geiman. This strain, started in culture in February 1976, is still going. It was kept in flow-vials until the end of June, then in Petri dishes during the summer, and since the autumn it has been maintained in a new type of flow-vial. Its morphology is entirely normal by both light and electron microscopy. It was infective to an *Aotus* monkey after nearly a year of continuous growth *in vitro*.

The new type of flow vessel holds 6 ml of 12% red cell suspension. It has a flat surface area of 18 cm^2, and hence the depth of liquid is slightly over 3 mm. The medium flows in slowly (2 ml/h) at one end and is withdrawn at the opposite end through a withdrawal tube the tip of which is in contact with the surface of the medium. The vessel is equipped with a tube for delivery of the gas mixture. It also has a vaccine port through which samples can be withdrawn and fresh cells introduced.

Ordinarily, fresh cells in complete medium are added three times a week. First, 4-5 ml of infected suspension are withdrawn from the vessel. This harvest can be used to start other cultures or for other purposes. Then 5 ml of fresh cell suspension are injected and a sample is again taken for a smear. Films prepared before addition of fresh cells give the extent of multiplication. By keeping the parasite level just after addition of fresh cells at 1–1.5%, a proportion of infected cells of 6–10% was obtained

* From the Laboratory of Parasitology, The Rockefeller University, New York, NY 10021, USA.

[1] Professor.

[2] Assistant Professor.

[a] Roswell Park Memorial Institute 1640 medium.

2 days later. Hence one such vessel provides a harvest at each subculture of 4–5 ml of 12% red-cell suspension, equivalent to about 0.5 ml of packed cells or 5×10^9 cells, giving, if the level of infection is 10%, 500×10^6 parasites.

Once the FVO strain was successfully established in culture, we tried to culture a second strain. The only one available last spring had been isolated the previous autumn into an *Aotus* monkey, using blood from a patient from South America (obtained through the courtesy of Dr Tom Jones). This strain, which produced only a very light infection in *Aotus*, was nevertheless successfully cultured in a flow-vial. This strain was of great interest because it produced gametocytes. Indeed, in Petri dishes it produced only gametocytes. It was later lost by contamination.

Several strains from Africa have been started in Petri dish cultures and two are still being maintained.

Cultures have been used to study a variety of culture conditions, including the following:

(*a*) various changes in composition of the tissue culture medium—none has given improvement;

(*b*) storage of serum: this is best kept frozen—at 4°C it should not be kept more than 1 month;

(*c*) use of outdated cells: see below for details;

(*d*) use of antibiotics; these are not ordinarily used, but gentamicin at 133 μg/ml or penicillin at 100 μg/ml appear not to be injurious.

The Petri dish cultures have also been used in preliminary studies with antimalarials. Thus, with the FVO strain, exposure to chloroquine for 2 days had no effect at levels of 0.03 and 0.1 μg/ml (counts of 300 parasites/10 000 red cells as compared to 260 in controls) but did inhibit at 0.3 μg/ml (count of 100/10 000 red cells). With this strain, pyrimethamine gave complete inhibition at 0.03 μg/ml. These cultures should provide a cheap, simple way of screening possible compounds for antimalarial activity, directly with *P. falciparum*.

Failure to cultivate *Plasmodium* spp. successfully in the past has often been attributed to the apparent instability of the erythrocyte in artificial culture systems, and thus in our early experiments with continuous propagation of *P. falciparum* we tried to use erythrocytes that were as "fresh" as possible—usually cells that had been stored for less than 7 days in citrated preservatives. However, there were times when we were forced to use erythrocytes that had been stored for longer periods and these older cells appeared to support the development of *P. falciparum* even better than fresher cells. Thus a series of experiments was conducted to determine the effects of storage of erythrocytes on their ability to support the development of *P. falciparum*. Generally, the storage of erythrocytes in citrated preservatives improves their ability to support *P. falciparum* with optimum growth occurring in cells stored for 21–28 days. Since blood is no longer used for transfusion after 21 days of storage, and is considered by blood banks outdated after this period, a supply of erythrocytes can be easily obtained for large-scale cultivation.

Although variations in developmental rates of *P. falciparum* can be attributed to several factors, e.g., the length of blood storage discussed above or a particular batch of serum or medium, the principal variant appears to be an innate characteristic of any given donor's cells to support plasmodial development. For example, some erythrocytes consistently gave multiplication rates of 50–70× in 96 hours, whereas others supported growth poorly, or in one case, not at all.

Because some workers may have difficulty in obtaining regular supplies of human serum, we tested 3 different lots of commercially available fetal calf serum (FCS) against fresh human serum. There was great variation from lot to lot in the ability of FCS to support *in vitro* development of *P. falciparum*. Moreover, even the best lot gave less than half the level of development obtained with human serum—an increase of 21× compared to 50× in 96 hours. More significant perhaps was the fact that the growth rates in FCS decreased with each subculture. In view of our observation that human serum contains certain labile components, and must be stored at − 20 °C until used, the commercially available FCS may not have been suitably handled, and fresh FCS may prove to be an acceptable substitute for human serum.

Concerning the matter of gametocyte formation in culture, it should be sufficient to mention here that gametocyte formation does occur *in vitro*. It takes 8-10 days for the gametocytes to become morphologically mature, as reported recently by other workers. However, our studies are the first to demonstrate that the induction of gametocytogenesis can occur *in vitro* and that gametocytes continue to be produced in culture for a reasonable length of time.

RÉSUMÉ

CULTURE DES STADES ÉRYTHROCYTAIRES

Deux méthodes de culture continue de *Plasmodium falciparum* sont décrites ici. L'une consiste à assurer un écoulement lent et permanent du milieu sur une couche de globules sanguins décantés et n'exige pas de surveillance quotidienne. L'autre, exécutée sur des boîtes de Pétri, nécessite le renouvellement manuel quotidien du milieu. L'article examine les problèmes soulevés par la culture continue et les possibilités offertes par cette technique.