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Full Length Research Paper

Cultivation and multiplication of viable axenic *Trypanosoma vivax in vitro* and *in vivo*

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Trypanosoma vivax **was isolated from the blood of an infected laboratory mouse, washed and introduced into the prepared culture media, ME-99 and minimum essential medium (MEM), both containing laboratory prepared (commercial) horse serum and antibiotics (streptomycin and penicillin). The cultures were monitored *in vitro* for 12 days. There was an initial decline in parasitaemia in the first 48 h in both media, which later picked up to reach the peak of about 1.10 x 10^2 parasites/ml in ME-99 on day 5 and 6.2 x 10 parasites/ml in MEM on day 4. Thereafter, the parasites number tapered off to reach zero on day 9 in ME-99 and day 10 in MEM. No growth was recorded in the control, which contained normal saline (pH 7), horse serum and antibiotics. The result of the *in vivo* culture showed a different trend when compared to the *in vitro*. Multiplication was tremendous with a peak of about 3 x 10 parasites/ml of blood on day 22 (high inoculum) and day 24 (low inoculum) post infection. The slender trypomastigote parasites recovered in the *in vitro* culture was short and had a long, free flagellum and measured 23-25 um while that of the *in vivo* culture was long, slender, clongated, torpedo shaped body measuring between 30 and 32 um. During the course of the *in vivo* culture congenital transmission of trypanosomes was observed. The *in vitro* attenuated parasite conferred a degree of protection to 25% of the mice that were later infected with viable parasites indicating possible prophylactic effect of *in vitro* attenuated parasites. The study showed that *T. vivax* could not be cultured in large numbers on their own axenically in MEM and ME-99. However, ME-99 can be said to be more suitable compared to MEM for axenic cultivation of *T. vivax* as a result of the additional nutrients supplied by the tissue culture medium 199 present in medium ME-99. Also, the parasites multiplied better *in vivo* compared to the *in vitro* study, which could mean that the best means of culturing trypanosomes still remains the *in vivo* method.**

Key words: *Trypanosoma vivax,* parasite cultivation, *in vitro* and *in vivo* cultures.

INTRODUCTION

The necessity for culturing both African and American trypanosomes *in vitro* has received much attention for a very long time because of the advantage of allowing for the maintenance of parasites without using an animal model (Balber, 1983). Although for many years the salivarian trypanosomes have proved to be among the most difficult members to cultivate *in vitro*, the works of Isoun and Isoun (1974) on *Trypanosoma vivax*, Yabu et al. (1989) on *T. Brucei*, Hirumi et al. (1977) on *T. brucei* and Otigbuo and Woo (1987) has shown that trypanosomes can be cultured axenically *in vitro*. The advancement in *in vitro* cultivation of trypanosomes has further enhanced the understanding of the biochemical make-up, nutritional requirements and transmission of the parasites (Wirth and Kierszenbaum, 1985). This work is

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focused on culturing of *T. vivax*, a salivarian species, axenically in *vitro* using two different media and to determine the viability of the cultivated forms. Also, the trypanosome will be cultured *in vivo* using mice.

MATERIALS AND METHODS

Parasite source and maintenance

T. vivax stock (identified from source) used in this study was obtained from the Department of Veterinary Pathology, University of Ibadan, Nigeria. The parasites were maintained in the laboratory by serial passages from infected to uninfected mice. The blood containing the trypanosomes from infected mice were collected in saline (pH 7) and then injected intraperitoneally into new set of mice.

Animal source and maintenance

Mice (*Mus musculus*) used in these studies were obtained from the Animal Centre of the College of Medicine, University of Lagos, Lagos State, Nigeria. They were kept in cages and fed with animal feeds produced by Ladokun Feeds Limited, Ibadan, Oyo State, Nigeria. (Feed composition: protein 21% (min), fat 3.5% (min), fibre 6.0% (max), calcium 0.8% and phosphorus 0.8%).

In vivo culture

In assessing virulence through injection of parasites into uninfected mice to determine the rates of multiplication, 0.1 ml of infected blood mixed with 2.4 ml of saline solution (low inoculum) and 0.8 ml of infected blood mixed with 1.7 ml of saline solution (high inoculum) were prepared. From the high and low inocular, 0.5 ml was inoculated into the experimental mice. Thin films were prepared and slides were then allowed to air dry before observation under the microscope using an oil immersion objective (x100). Parasite count was done using a haemocytometer.

In vitro culture

A modified medium of tissue culture 199 (ME-99) and minimum essential medium (MEM) were both prepared from the power stock (Otigbuo and Woo, 1987). To prepare MEM, 5 g of MEM and 1 g of $NaHCO_3$ were dissolved in 400 ml of distilled water, stirred gently and made up to 500 ml with more distilled water. All reagents used were obtained from the Department of Medical Microbiology and Parasitology, University of Lagos, Nigeria. The liquid (monophasic) medium was dispensed in 10 ml into half ounce bottles and then sterilised by autoclaving at 121°C (151 lbs) for 15 min. They were later stored in the refrigerator.

Initiation of culture

Sterile disposable petri dishes (Sterilin) were used for the culture. Thirteen petri dishes divided into 3 sets of 5, 5 and 3 were placed on a disinfected table. To each of the first set of 5 petri dishes was added 5 ml of ME-99 medium, to the second set was added 5 ml of MEM while the remaining 3 petri dishes, which served as the controls, contained 5 ml of normal saline solution. 0.5 ml of laboratory prepared horse serum was added to all the petri dishes, which were later placed in the incubator (37°C) to attain a uniform temperature before the parasites were introduced into the media. 0.1 ml of penicillin and streptomycin mixture (equally mixed in a syringe) was added. The antibiotics were to prevent the growth of bacteria (Otigbuo and Woo, 1987).

0.5 ml of isolated washed trypanosomes suspension in phosphate buffer was introduced into each of the 13 petri dishes. 2.5 ml of the medium was added to the culture every 48 h to avoid complete drying up of the culture media (Otigbuo and Woo, 1987). More serum was also added on the 5th day after initiation of culture to prevent nutrient depletion in the culture. The trypanosomes in the culture were also subcultured on the 5th day to monitor the life span of the parasite *in vitro*. This was done by taking about 0.1 ml of the old culture using a sterile syringe and needle and introducing it into a new culture dish with fresh medium which contain serum and antibiotics. Slides were prepared daily from each culture dish.

Parasites recovered from the two different culture media were inoculated daily into two sets of three experimental mice. The tail bloods of these mice were observed daily for viable parasites. The mice were later infected with trypanosomes from infected mice to ascertain whether the *in vitro* cultured parasites conferred any kind of immunity to the mice.

RESULTS

In vitro culture

There was an initial sharp decline in the number of parasites from day 0 to day 1 after initiation of the culture from 1.76 x 10^3 parasites per ml of culture medium to 1.1 x 10^2 parasites/ml in MEM and 8.9 x 10 parasites/ml in ME-99 (Table 1). More decline was observed on day 2 in both culture media to 3.3 x 10 parasites/ml in MEM and 2.2 x 10 parasites/ml in ME-99. By day 3, a gradual increase to 3.3 x 10 parasites/ml in ME-99 was observed while the parasites number remained the same in the MEM culture. On day 4, the concentration of parasites in MEM increased to 6.2 x 10 per ml which also marked the peak of parasitaemia for the culture and that of ME-99 rose to 5.6 x 10, however, a decline in the number of parasites cultured in MEM to 5.6 x 10 parasites/ml was observed on the 5th day after culture initiation, whereas the ME-99 culture recorded an increase to 1.1 x 10^2 parasites/ml of culture medium on the same day (Table 1). The 1.1 x 10^2 parasites/ml in culture ME-99 marked the peak of parasitaemia (Table 1). There was a leveling out in the number of parasites in MEM culture between days 5 and 7 in which the parasite number remained at 5.6 x 10 parasites/ml (Table 1). On day 6, the number of parasites in ME-99 culture declined to 7.8 x 10 parasites/ml. A form of levelling out of parasite number was also observed between days 6 and 9 in the culture medium ME-99 after which all the parasites in culture ME-99 died on day 9 post-initiation of culture (Table 1). In MEM, another decrease was observed on day 8 to 3.3 x 10 parasites/ml of culture medium. This number was maintained till day 9 after which a total decline to zero was recorded on day 10 as a result of the death of the parasites in culture. In the control experiment with normal saline, all the parasites were found dead after 24 hours of culture initiation.

Table 1. Daily mean number of parasites per ml of *in vitro* culture media (MEM and ME-99).

Days	Number of parasites/ml	
	MEM	ME-99
0	1.76×10^3	1.76×10^3
1	1.1×10^2	8.9 x 10
1	3.3 x 10	2.2 x 10
3	3.3 x 10	3.3 x 10
4	6.2 x 10	5.6 x 10
5	5.6 x 10	1.1×10^2
6	5.6 x 10	7.8 x 10
7	5.6 x 10	7.8 x 10
8	3.3 x 10	7.8 x 10
9	3.3 x 10	0
10	0	0

The typical bloodstream form of trypanosome, known as the slender trypomastigote form, was recovered from the two media used for culture and measured between 23 and 25 μm. The parasites recovered from the culture was inoculated into mice and found not to be viable in the mice in that they could not initiate an infection in the mice. However, these attenuated parasites conferred a degree of immunity to 25% of the mice that were later infected with viable parasites from an infected mouse.

In vivo culture

There was a continuous increase in the number of parasites throughout the period of the *in vivo* study (Table 2). There were no parasites in the peripheral blood of the experimental mice on days 1 and 2 in the low inoculum study. By days 2 and 3 in the low and high inoculums, respectively, few parasites were seen in the blood. During the duration of the experiment, some mice were found dead in cages containing mice injected with low dose on days 9, 10 and 11. In each of these days, 20% mortality was recorded. Post-mortem analysis showed that the dead mice were pregnant. More animals died on days 19 and 24 with about 20% mortality rate on each day. The peak of parasitaemia of mice injected with low dose was recorded on the 24th day after infection after which the animals remaining were observed dead in the cages (Table 2).

In the high inoculum, 40% of the experimental mice died on the 19th day post infection. More animals died on the 21st day (20% mortality) and 22nd day (40% mortality) after infection with about 2.7×10^6 and 3.0×10^6 parasites/ml of blood on the days, respectively. The multiplication rate between days 9 and 18 was with a constant increase of about 100,000 parasites per day, and the highest increase in the rate of multiplication was recorded between days 20 and 21post infection (Table 2). The peak of parasitaemia was observed on days 22 with about 3.0×10^6 parasites/ml of blood (Table 2).

The parasites recovered from the *in vivo* experiment with low and high doses of parasite were typically the trypomastigote forms with long, slender, elongated torpedo shaped body measuring between 30 and 32 μm. During the experiment, embryos from the infected pregnant mice were found to be with trypanosomes. The parasites recovered from the embryo measured between 25 – 28 μm and they were considerably few in number.

DISCUSSION

T. vivax introduced into the culture media ME-99 and minimum essential medium (MEM) both had the same pattern of multiplication *in vitro.* The initial decline in the *in vitro* pattern of multiplication of *T. vivax* cultured axenically in ME-99 and MEM could be due to the inability of most of the parasites to adapt to the media environment which is known to play an important role in the continuity of the developmental cycle as shown by the work of Ribero et al. (1990). The trypanosomes that were adapted to the culture in MEM did not start multiplying until after 24 hours whereas the ones in ME-99 started multiplying immediately (Table 1). On the other hand, the decline in the number of parasites observed in ME-99 between day 1 and day 3 and more than that recorded in MEM. The control did not have growth nutrient as supplied by the culture media to sustain the parasites *in vitro* indicating the probable reasons why all the parasites in the control died after 24 hours.

The peak of parasitaemia in the two media 1.1×10^2 parasites/ml in ME-99 and 6.2 x 10 parasites/ml in MEM) could not yield sufficient parasites for scientific research, unlike the yield obtained in the study conducted by Balber (1983), Hirumi (1984) and by Kamisky et al. (1987) which successfully cultured trypanosomes *in vitro.* The success of their work could be linked to the cultivation of the parasites together with some supportive cells of mammalian origin as earlier observed by Hirumi et al. (1977). The present work also differs from the axenic cultivation of *T. vivax* by Isoun and Isoun (1974) that was with an initial 3-fold rise in parasite count in 24 hours. This increase, unlike the decrease observed in this study, could be attributed to the fact that the parasites used in their culture were collected directly from infected cattle. Evans (1978) believed that the history of the parasites stock used in culture dictates the success of such culture and that freshly isolated parasite that had not been passed through laboratory animals by syringe are more successfully cultivated *in vitro.*

The *in vivo* multiplication rate is much better than the *in vitro* rate. The peak of parasitaemia in the *in vitro* culture was 1.10×10^2 parasites/ml in ME-99 and 6.2x10 parasites/ml in MEM (Table 1), compared to about 3 million parasites/ml of blood in the *in vivo* culture (Table 2). The death of 60% of the mice during the *in vivo* cour-

Table 2. Mean number of parasites per ml of blood for the low and high inocula *in vivo* study.

Days	Low		High	
	Number per ml of blood	% Mortality	Number per ml of blood	% Mortality
1	0		0	
2	0		6.7×10^4	
3	4.6×10^4		1.7×10^5	
4	5.9×10^4		3.2×10^5	
5	1.1×10^5		4.9×10^5	
6	1.4×10^5		6.1×10^5	
7	1.7×10^5		7.6×10^5	
8	1.8×10^5		9.2×10^5	
9	3.1×10^5	20	1.0×10^6	
10	4.2×10^5	20	1.1×10^6	
11	4.6×10^5	20	1.3×10^6	
12	5.3×10^5		1.4×10^6	
13	6.4×10^5		1.5×10^6	
14	9.3×10^5		1.6×10^6	
15	1.3×10^6		1.7×10^6	
16	1.5×10^6		1.8×10^6	
17	1.6×10^5		1.9×10^6	
18	1.85×10^5		2.0×10^6	
19	1.9×10^6	20	2.1×10^6	40
20	2.1×10^6		2.2×10^6	
21	2.3×10^6		2.7×10^6	20
22	2.4×10^6		3.0×10^6	40
23	2.7×10^6		0	
24	2.9	20	0	
25	-	-	0	

se of study may be due to the inability of the mice to cope with the parasitic infection probably as a result of pregnancy stress. The congenitally transmitted parasites were smaller than the parasites recovered from adult mice, which probably indicates that the newly divided parasites were the ones that were able to pass through the placenta to infect the embryo. Harold and Franklin (1983) had earlier observed the congenital transmission of trypanosomes in mice.

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