In vitro culture of plasmodium berghei using glucose and reticulocytes enriched blood cells.

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الملخص

ان زراعة كريات الدم الحمر الحاوية على اطوار طفيلي الملاريا مختبريا قد يساعد في فهم بايولوجية هذا الطفيلي وتحديد المواقع الهدف لغرض تطوير بعض اللقاحات والادوية المضادة له . في الدراسة الحالية تم قياس بعض الظروف المختبرية التي تحسن المحيط لغرض النمو المتواصل لطفيلي الملاريا في القوارض(Plasmodium berghei) درست ثمانية انماط لظروف مختلفة (A-H)واختبر النمط G لكونه الاكثر تأثيرا في المحافظة على المدى الطويل للنمو مختبريا ،اذ يحتوي النمط Gعلى الوسط RPMI-1640 مضافا اليه HEPS وبيكاربونات الصوديوم والكلوكوز ومصل الفأر الطبيعي و PHC وتم قياس حيوية الطفيلي في المزرعة ذات المدى الطويل (Long-term) بواسطة الحقن داخل الغشاء البريتوني وبمعدل (10x1^5)كرية دم حمراء مصابة بالطفيلي بعد كل 24 ساعة ولمدة 7 ايام في الفأر الطبيعي.

Abstract

***In vitro* culture of erythrocytic stages of malarial parasite can prove helpful in understanding the biology of the parasite to locate new targets for drug designing and vaccine development. In the present study an attempt has been made to assess certain *in vitro* conditions which can provide the amiable environment for continuous growth of rodent malaria parasite, *Plasmodium berghei.* A total of eight different conditions were studied out of which G type proved to be the most-effective in maintaining long-term *in vitro* culture of *P. berghei. G* type contained medium RPMI-1640 supplemented with HEPES, sodium bicarbonate and glucose, normal mouse serum and phenylhydrazine hydrochloride (PHC) - induced reticulocyte rich blood. The viability of the parasite in long-term culture was assessed by i.p. injections of 1 x 10^5 *P. berghei*- infected erythrocytes from terminated culture pellets after every 24h till 7 days in normal mice.**

Introduction

Several attempts have been made to grow intraerythrocytic stages of the human as well as other malarial parasites *in vitro.* Continuous culture of *P.falciparum* was established by Trager and Jensen (1976). Reticulocytes and young red blood cells are more susceptible to invade by *P. falciparum* as compared to older cell populations (Pasvol *et al.*, 1980). Several long term *in vitro* cultures of P. *berghei* were established (Janse *et al,*. 1984). In these cultures, ranging from 17-90 days, peak parasitaemia over 20% was observed. Using Trager and Jensen Methods (1976), long term culture of P. *berghei* was established for 45

weeks where RPMI-1640 was modified by addition glucose and bactopeptone (Ramaiya *et al.*, 1987). 10% normal mouse serum was used to prepare complete medium. Preferential invasion of reticulocytes by *P. vivax* was demonstrated both *in vitro* and *in vivo* (Mons *et al.*, 1988). Using both static and candle jar and shaker Methods , long-term culture of P. *vivax* was maintained successfully for 10-14 days (Golenda *et al.* 1997). Since P. *berghei* acts as a simulated model of P. *vivax,* the present study was conducted by combining various condtions to assess the viability in continuous cultures of P. *berghei* maintained by static candle jar Methods .

Materials and Methods

NK-65 strain of *Plasmodium berghei* was maintained in white Swiss mice, *Mus musculus* (Balb/C). 1 x 10^5 P. *berghei* infected erythrocytes were injected i. p. into naive mice and parasitaemia was monitored daily by preparing thin Giemsa-stained blood smears.Reticulocytosis was induced by i.p. injection of 0.4% (w/v) phenylhydrazine hydrochloride (PHC) in PBS, pH 7.2 to each mouse on alternate days.

Two types of culture media were prepared for present study : (1) Normal medium-standard incomplete medium having RPMI-1640 (Himedia, India) supplemented with 5% (w/v) sodium bicarbonate, 0.6% (w/v) HEPES, 50|^g/ml gentamycin, 100 i.u./ml penicillin and 100^g/ml streptomycin. (2) Modified medium incomplete medium in (1) was modified by adding glucose (mg/ml). Both these incomplete media were made complete either by adding 5% (v/v) fetal calf serum (FCS) or 5% (v/v) normal mouse serum (NMS).Short-term *in vitro* culture was maintained in 24-welled culture trays (Laxbro, India). 4% haematocrit was prepared and 1 ml culture was incubated in candle jar at 37°C in 8 types of wells (A-H, each type in duplicate). The types and conditions used for different cultures are explained in Table 1. Culture was terminated after 21h and thin Giemsa-stained smears were prepared. Observations were made for both Oh and 21h smears by differential count of parasite stages i.e. rings, trophozoites and schizonts. invasion Percent was calculated as :

$$\frac{\text{Number of infected erythrocytes}}{\text{Total number of erythrocytes}} \times 100$$

For maintaining the parasite for longer duration *in vitro,* the cultures were carried in 5ml petridishes and incubated at 37°C in candle jar. After every 24h, culture of one petridish was terminated and Giemsa-stained thin smears were prepared. Differential count of parasite stages were done after every 24hs till 7 days. In the remaining petridish medium was changed daily.

Results

Induction of reticulocytosis Normal mice showed reticulocyte count of 0.7% - 1.0%. However, when reticulocytes was induced by i.p. injection of PHC, 20% - 22% reticulocytes were observed on day 4. Blood for PHC treated mice was used for maintaining long term *in vitro* culture of P. *berghei. In vitro* invasion under different culture conditions

Culture smears of both oh and 21 h from all types of wells were compared as shown in Fig.1

In G type well, 2.05% parasitaemia was observed after 21 h which was 1.7 times higher than at oh. This was followed by C and A type wells where there was 1.6 and 1.0 fold increase in parasitaemia at 21 h as compared to oh.In all other wells, there was a decrease in parasitaemia after 21 h incubation of cultures under different conditions. However, an increase in number of newly invaded rings was observed in all the wells.

Continuous culture of P. *berghei* Since maximum parasitaemia was observed with the conditions of G type well, an attempt was made to maintain continuous culture of P. *berghei* using these conditions(Fig 2). The culture was maintained successfully for one week. It was observed that percent infection was maximum at 24h (almost 2 folds) while it gradually decreased thereafter to almost one folds at 168h.Four fold increase in the number of newly invaded rings was observed at 24h culture smear. Significant number of rings were observed in smears prepared upto 168h culture while the number of mature parasites reduced in 6^{th} and 7^{th} day cultures.Inoculation of mice from culture parasite1 x 10^5 P. berghei-infected erythrocytes taken from culture after 48h incubation and onwards were injected i.p. into each of three normal mice of the groups. Course of parasitaemia was monitored in Giemsa-stained thin blood smearsPrepared every alternate day till day 6 post-infection. In all cases, parasite appeared by day 2 and parasitaemia gradually increased thereafter (Fig. 3).

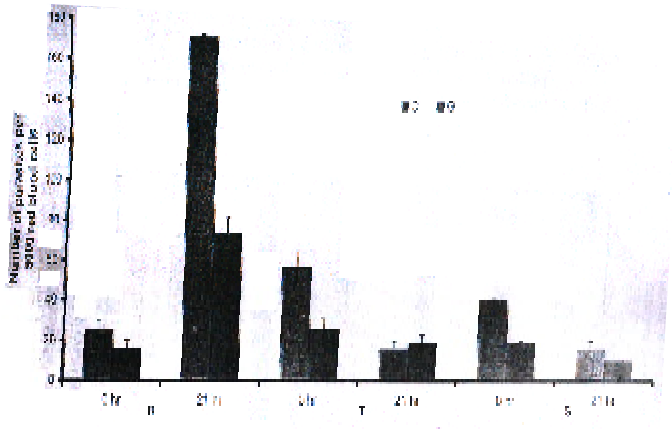

Fig. 1A: Histogram showing in vitro invasion of mouse erythrocytes by *P. berghei* under two different culture conditions (C and G)
R = rings, T = trophozoites, S = schizonts

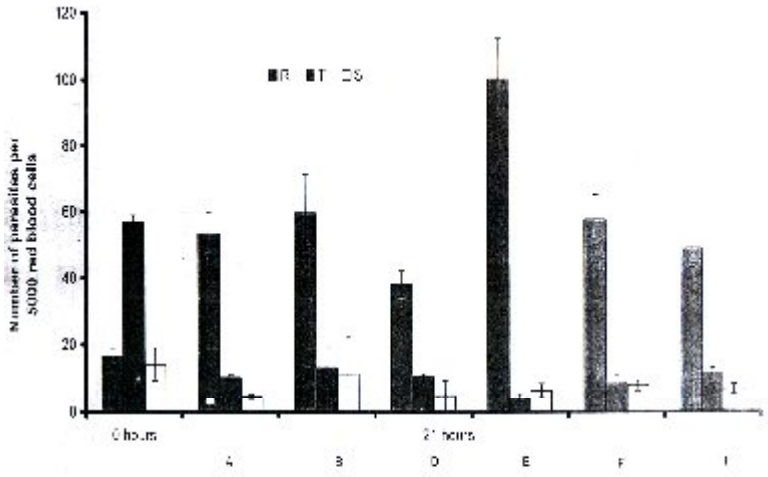

Fig. 1B: Histogram showing in vitro invasion of mouse erythrocytes by P. berghei under six different culture conditions (A, B, D, E, F and H)
R = rings, T = trophozoites, S = schizonts

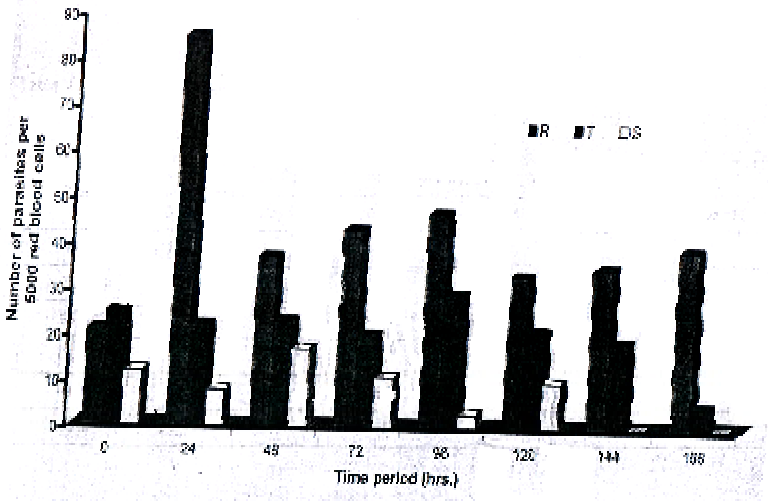

Fig. 2 : Histogram showing in vitro invasion of mouse erythrocytes by Plasmodium berghei in continuous culture
R = rings, T = trophozoites, S = schizonts

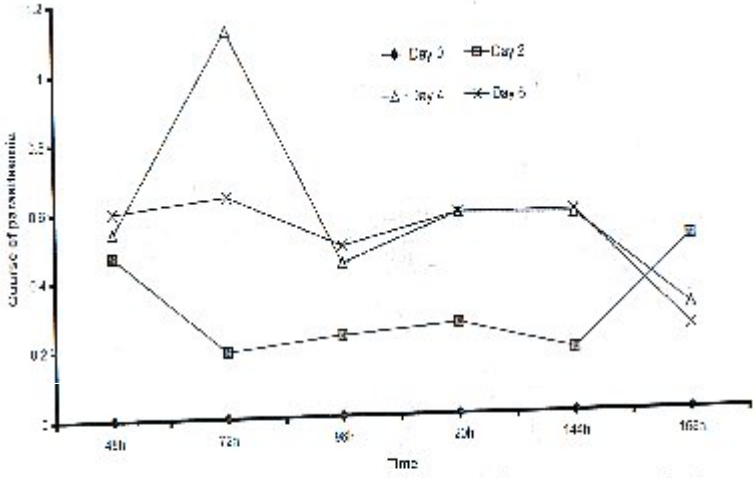

Fig. 3 : Parasitaemia in mice infected with 1×10^5 *P. berghei*-infected red blood cells taken from In vitro culture at 48h, 72h, 96h, 120h, 144h and 168h.

Table 1 : Culture conditions used for maintaining short-term *in vitro* culture of *Plasmodium berghei.*

Type of Well	Medium	Serum	Haematocrit
A	NM	NMS	RBC
B	NM	FCS	RBC
C	MM	NMS	RBC
D	MM	FCS	RBC
E	NM	NMS	RBCr
F	NM	FCS	RBCr
G	MM	NMS	RBCr
H	MM	FCS	RBCr

NM - Normal medium i.e. RPMI - 1640 supplemented with 5% (w/v) NaHCOS, 0.6% (w/v) HEPES, 50|J,g/ml gentamycin, 100 i.u./ml penicillin and 100 µ/ml streptomycin.

MM - Modified medium i.e. all ingredients of normal medium plus mg/ml glucose.

NMS - 5% (w/v) normal mouse serum

FCS - 5% (w/v) foetal calf serum

RBC - red blood cells, 4% haematocrit

RBCr - Blood cells containing 20% - 22% PHC - induced reticulocytes.

Discussion

Erythrocyte invasion is a process essential for the survival of malarial parasite. Moreover, it is because of the availability of *in vitro* invasion assays that this process can be examinedrelatively easily. This study has shown that *P. berghei* can be maintained *in vitro* using RPMI-1640 supplemented with glucose and normal mouse serum in reticulocyte enriched red blood cells in candle jar.*Plasmodium* exhibits high degree of host as well as host cell specificity for invasion. This specificity changes drastically especially under *in vitro* conditions. It has already been reported that *P. berghei can* be grown in erythrocytes of different animals *in vitro* i.e. hamster, rat, guinea pig etc. However, parasitaemia remains very low in such cases (Ramaiya and Renapurkar, 1988). *P. berghei merozo\{es* have been shown to prefer reticulocytes during invasion (Janse *et al.*, 1984).Reticulocyte enriched blood has been used as haematocrit for maintenance of continuous *in vitro* culture of *P. berghei* in present study. 20% - 22% reticulocytosis was induced in normal mice by i.p. injections of PHC on alternate days. It was observed that both C and G type wells proved to be most suitable conditions after 21 h *in vitro* culture. While 5.8% increase in newly invaded rings was observed in C type wells, an approximate 4.4% increase was observed in G type wells. However, conditions of G type wells were used to maintain long-term *in vitro* culture of *P. berghei* because reticulocytes have been reported to be preferred by *P. berghei* merozoites for invasion as compared to other cell populations and 50% heat-inactivated FCS has been reported to provide the best growth rate for P. *berghei* (McNally et al. 1992).In the present study,5% of FCS

was used and the parasite did grow in this medium but the invasion was less as compared to normal mouse serum. (Ramaiya *et al.* 1987) used 10% NMS while in the present investigation, long-term culture was maintained using 5% NMS and satisfactory growth of parasite was observed.P. *berghei* - mouse is a significant model in malaria studies. The present study shows that modified RPMI-1640 medium (with mg/ml glucose) and addition of 20% - 22% reticulocyte containing blood alongwith 5% NMS can aid in long-term cultivation of P. *berghei* as compared to 50% FCS used in earlier studies. The present continuous culture shows maximum invasion at 24h (4 fold) and thereafter the invasion follows the order: 48h (1.8 fold) < 72h (2 fold) < 96h (approx. 2.3 fold). The viability of cultures has been established with the inoculation of 1 x 10^5P. *berghei* - infected erythrocytes from culture upto 7^{th} day into normal mice. These parasitized cells have been able to infect normal mice. Slight modifications of temperature, medium and higher concentrations of reticulocytes can further refine this petridish candle jar Methods for continuously growing *of P. berghei in vitro.*

References

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