J. Sci. Soc. Thailand, 10 (1984) 109 - 118

SUPPORTIVE EFFECTS OF MAGNESIUM CHLORIDE ON VIABILITY OF *PLASMODIUM VIVAX* IN VITRO

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(Received 4 November 1983)

Abstract

Peripheral blood infected with ***Plasmodium vivax*** *showed a decrease in parasite density and viability after the blood cells were washed conventionally in RPMI medium. Treatment of infected cells with a culture medium supplemented with 1000 mg/L $mgCl_2$ gave the most satisfactory yield. Marked differences in parasite viability were demonstrated in parasite schizogonic development in short-term cultures. Only the $MgCl_2$-treated parasites were able to form merozoites.*

Introduction

It is generally accepted that many current problems in malaria research can be answered adequately only by the use of cultivation techniques. So far, only one species of human malaria, *Plasmodium falciparum,* can be cultured continuously[1]. It is cultured in a number of laboratories around the world[2] . *Plasmodium vivax,* the second most medically important species, cannot be grown with equal ease. There are reports[3,4] claiming success in long-term cultivation of *P. vivax* using essentially the same technique described for *P. falciparum,* but this has not been repeated elsewhere.

P. vivax infection in peripheral blood differs from that of *P. falciparum* in two distinct ways: a low parasitemia of only 0.1 to 0.5%, and the appearance of the parasites in various stages at once - ring, amoeboid form, and schizont[5]. Erythrocytes harboring parasites which have grown beyond the ring stage become fragile and distorted easily when subjected to centrifugation and repeated washing (Brockelman, C.R. unpubl.). Our preliminary observations have revealed that handling patient's blood infected with *P. vivax* in the same manner as *P. falciparum* blood results in a decrease in parasite density as well as parasite viability. Therefore we have attempted to develop a method to prepare a substantial number of viable *P. vivax* parasites in human blood which can be used adequately to start an *in vitro* culture.

Materials and Methods

Blood samples

Infected blood samples were collected aseptically in 10 ml volumes (in heparin 10 IU/ml) from patients with *P. vivax* who were attending malaria clinics of the Malaria Control Division, Ministry of Public Health. Blood was chilled and transported on wet ice to our laboratory on the same day. For this study two *P. vivax* isolates PV 44 and PV 46 were used.

Reagents

RPMI 1640 powder (Grand Island Biological Company, N.Y.) was reconstituted as described by Trager and Jensen[1]. The complete medium contained 25 mM HEPES, 0.2% $NaHCO_3$ (w/v). Magnesium chloride (BDH) solution was prepared by dissolving 0.231 g of $MgCl_2.6H_2O$ in eight ml of doubly distilled water. The volume of the solution was adjusted to 10 ml, so that the final concentration of $MgCl_2$ in the solution was thus 100 g/L. After sterilization by filtering through a Millipore Swinnex-type filter, the solution was added 1:100 (v/v) to RPMI complete medium, giving a final concentration of 1 g/L (1000 mg/L). This medium was further diluted with RPMI complete medium 1 : 10 and 1 : 20 (v/v), so that the final concentrations of $MgCl_2$ were 1000 and 50 mg/L respectively. The control medium was RPMI complete. It was supplemented with 15% human serum group AB for *in vitro* cultivation. It was referred to as complete RPMI with serum.

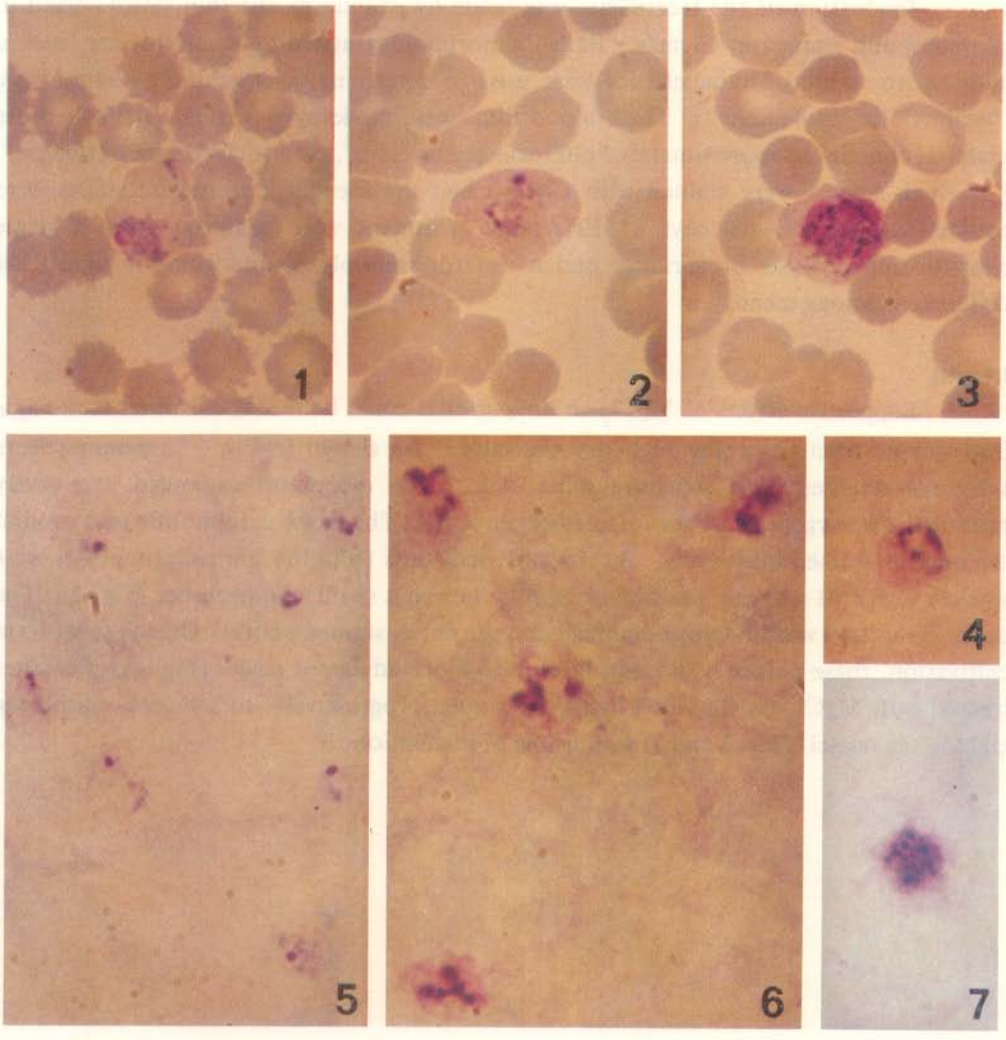
Procedure

Upon arrival of a blood specimen at the laboratory, blood cells and the buffy coat were gently mixed by passing the specimen a few times through a pipette. One drop of blood was taken to make two thick smears so that parasite density of the sample could be estimated. The sample was then divided into eight 1-ml volumes in eight tubes thus allowing us to carry out two experiments, each with four treatments. The samples were then centrifuged at 400 xg at 4°C for 8 min. After the plasma had been removed, the packed cells were resuspended in 2 ml of complete RPMI and in complete RPMI with 50, 100, or 1000 mg/L magnesium chloride. The blood cell suspensions were centrifuged again and twice rewashed in the corresponding medium. After the last wash and removal of the supernatant fluids, an equal volume of human serum group AB was mixed with the blood cells in each tube. Two blood samples were taken from each tube and smeared as thick and thin films on microscope slides. After this step was completed there was generally one ml of blood cell suspension in each tube available to start *in vitro* cultures. Six ml of RPMI complete medium containing 15% human serum were added to each tube and the culture material was dispensed into culture dishes (35 mm diam.), with four replicates for each treatment. The culture dishes were then incubated in a candle jar atmosphere[1] at 38°C. Thick and thin blood films were made after either 12 or 24 h and 46 h of incubation.

Evaluation of the treatments was made by examining the thin smears fixed in methanol and stained in Giemsa. Parasite morphology at the light microscope level in addition to staining characteristics were used to determine whether the parasites were "healthy" or "unhealthy". Thick blood films were made by smearing 10 µl of culture material containing approximately 5×10^6 cells per cu. mm^6, on a surface area of one cm^2. The thick smears were stained with Giemsa and the asexual erythrocytic stages were counted against 200 leucocytes. Effects of treatment were analyzed using a single classification analysis of variance and a test for multiple comparison for significant differences among means[7].

Results

Supportive effects of $MgCl_2$ on the parasites could be readily observed at the light microscopic level using the high dry objective. As shown in Fig. 1, mononucleate schizonts retained their structure quite well. The cytoplasm expanded extensively resulting in enlargement of the infected erythrocyte. The dense cytoplasmic part around the nucleus stained intensively. By contrast, schizonts from the control group that were washed in RPMI without addition of $MgCl_2$ showed a swollen appearance (Fig. 2). The dense cytoplasm was no longer compact and the nuclei stained poorly. During short-term cultivation, these parasites did not develop to more advanced stages (Fig. 4). Parasites treated with $MgCl_2$ on the other hand, developed progressively to schizonts harboring eight to ten nuclei (Figs. 3 and 7) within 46 h of incubation.



Figures 1-3 Giemsa-stained thin blood smears showing a healthy mono-nucleate schizont of *P. vivax* (Fig. 1) after the infected blood had been washed in RPMI supplemented with $MgCl_2$. This can be compared with an unhealthy schizont (Fig. 2) where $MgCl_2$ was omitted in the washing medium. Fig. 3: a segmenter after 46 h of cultivation x1000.

Figure 4 A typical ill-looking schizont of *P. vivax* after 46 h of cultivation in medium without $MgCl_2$. x1000.

Figures 5-7 Giemsa-stained thick blood smears made from $MgCl_2$-treated culture material of *P. vivax* showing 7 amoeboid forms per 3000 μm^2 after wash (Fig. 5) whereas the same infected blood sample showed only two parasites/ 3000 μm^2 when $MgCl_2$ was omitted from the washing medium. Fig. 6 shows young schizonts after 24 h of cultivation. A mature schizont from a 46-h culture (Fig. 7) x1000.

Differential counts of the asexual erythrocytic stages of *P. vivax* isolate PV 46 in two experiments (Exps. I and II) showed a significant favorable effect of $MgCl_2$. As shown in Fig. 5 parasite density was 7/3000 μm^2 when the infected blood was washed in medium containing 1000 mg/L $MgCl_2$. In the blood sample where $MgCl_2$ was omitted, parasite density on thick smears dropped to an average of 2/3000 μm^2. Total counts of the parasites per 200 leucocytes further confirmed high parasite density (Group D, Fig. 8). Arithmatic means obtained from differential counts of four replicate smears were 15 rings, 384 amoeboid forms, 170 mono- and binucleate schizonts, and 115 mutinucleate schizonts.

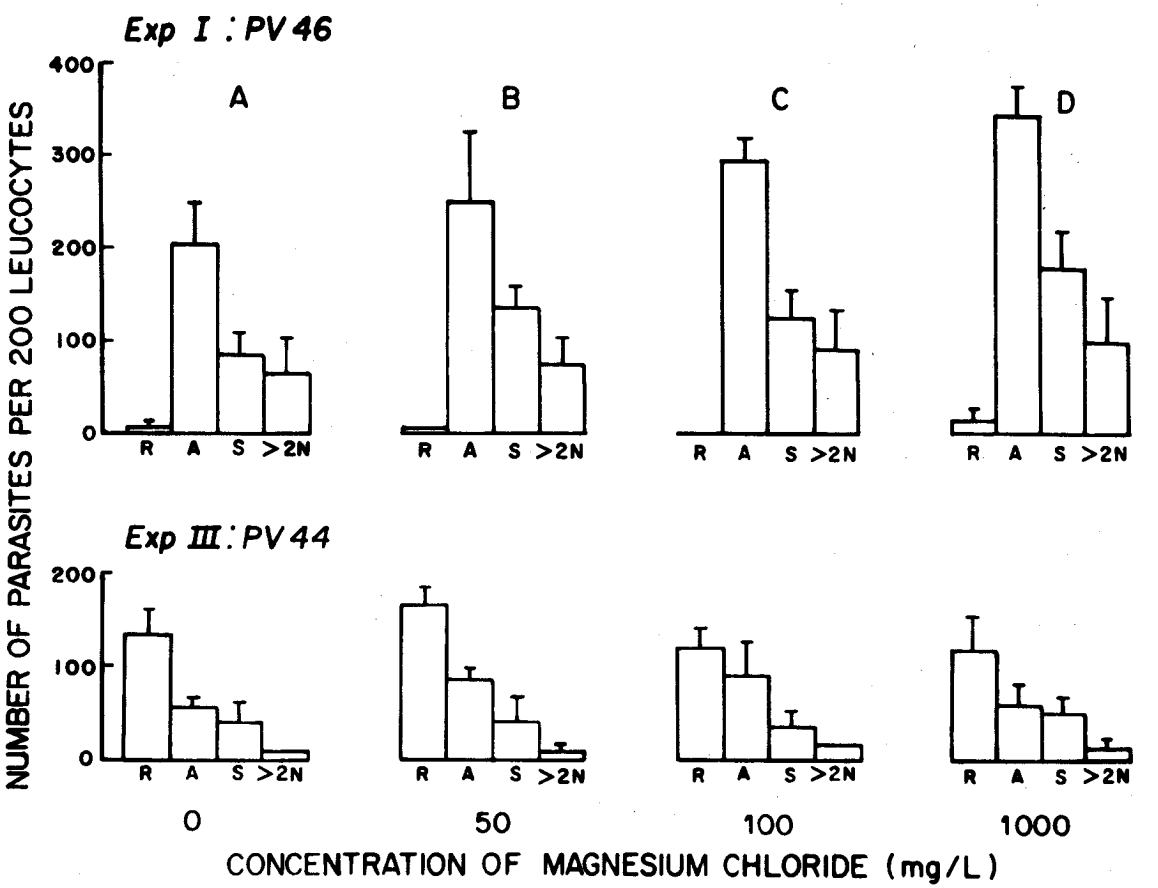


Figure 8 Distribution of various stages of *P. vivax* after washing with RPMI 1640 supplemented with $MgCl_2$ at varying concentrations. Vertical bars represent standard deviations of the mean. Abbreviation: R, ring; A, amoeboid; S, young schizont; 2N, multinucleate schizont.

When four groups were compared, the $MgCl_2$ treated groups B through C yielded parasite densities higher than that of the control (group A). The one-way analysis of variance revealed significant differences among means of parasite densities from each treatment. In the multiple comparison test, the $MgCl_2$ treated groups showed higher parasite densities than the control group A ($P < 0.05$). Among the treated groups, D (1000 $MgCl_2$/L) had a higher parasite density than B and C ($F_{3,15} = 4.145 : P < 0.005$. Results from Experiment 2 did not differ much from those obtained from Experiment 1. Total parasite counts of the culture treated with 1 g/L $MgCl_2$ were higher than the control group, particularly for young schizonts (Fig. 9, at time 0).

To determine whether or not treatment by $MgCl_2$ was also necessary for *P. vivax* isolates whose populations consisted primarily of rings, the same experimental protocol was conducted in two more experiments using isolate PV 44 (Exps. III and IV). Results of Experiment 3 which are summarized in Fig. 8 seemed to indicate a favorable effect of $MgCl_2$ at 50 mg/L. Statistical analysis, however, did not show any significant differences between means of the total parasite counts. However, number of schizonts in $MgCl_2$ treated samples were slightly higher. These results were consistent with those for Experiment 4 (Exp. IV) using PV 44 again (Fig. 9, time 0).

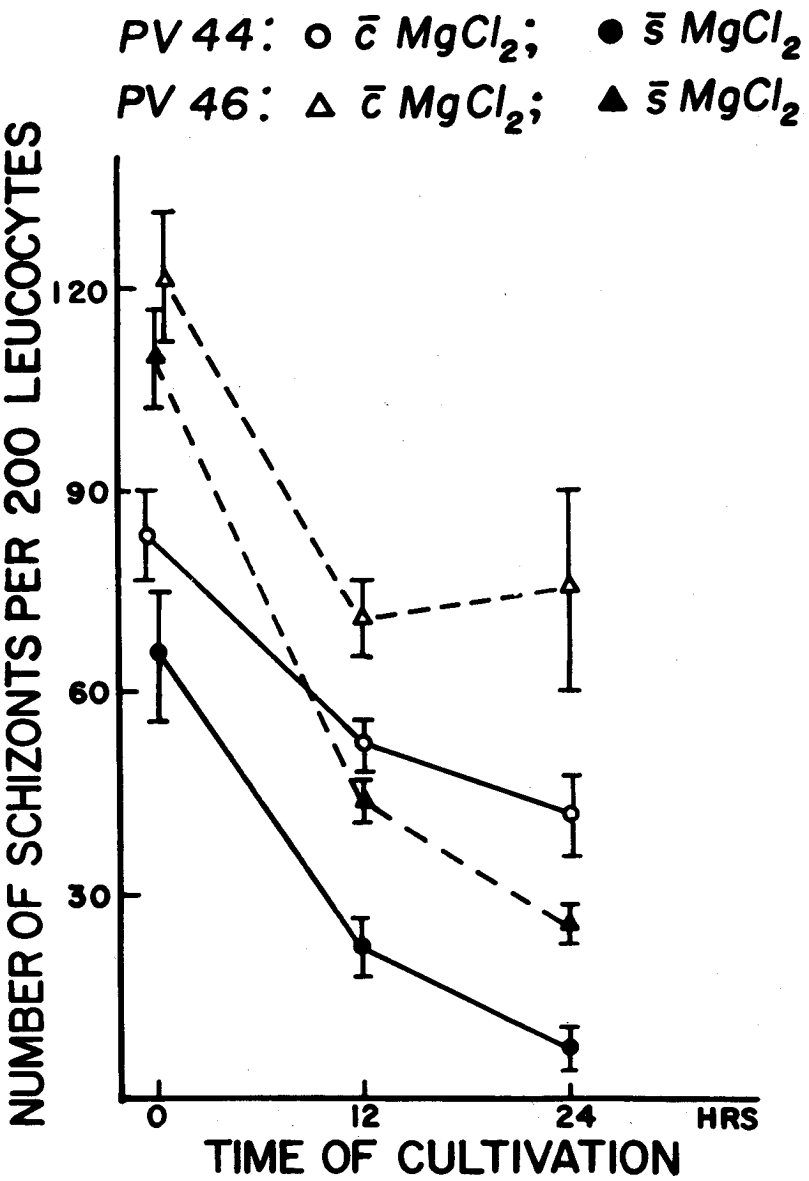


Figure 9 Growth of *P. vivax* mononucleate schizonts to multinucleate schizonts within 24 h of cultivation. Isolate PV 44: o—o, washed with RPMI supplemented with 1000 mg/L $MgCl_2$. ●—●, washed in RPMI without $MgCl_2$. Isolate PV 46: △—△, washed with RPMI supplemented with 1000 mg/L $MgCl_2$; ▲—▲, washed in RPMI without $MgCl_2$.

Short-term cultivation

Viability of *P. vivax* after washing was examined using the ability of the mononucleate schizonts (N) to grow to multinucleate ($\geq$2N) schizonts in RPMI complete medium with human serum as a criterion. PV 46 from Exp. II and PV 44 from Exp. IV were cultured. After 24 h of cultivation for both PV 44 and PV 46, there were more binucleate schizonts in cultures which were previously washed in medium containing 1000 mg/L $MgCl_2$ than in those washed in RPMI alone (Fig. 9). The differences in numbers of multinucleate schizonts were also marked in two other experiments using isolates PV 40 and PV 48, when the cultivation time was extended to 46 h (Table 1). A number of young, mononucleate schizonts in $MgCl_2$-treated cultures had developed to the multinucleate stage with a morphological characteristics resembling those of mature schizonts, whereas those washed in RPMI failed to develop beyond four-nucleate schizonts.

Table 1 Schizont development of *Plasmodium vivax* after 46 h of *in vitro* cultivation.

Exp.	Isolate	time (h)	Number of parasites per 200 leukocytes						
			R[b]	A	N	2N	>2N	MS	Total
1	PV 40	0	120	42	4	0	0	0	166
		46	0	2	67	42	32	0	143
2[a]	PV 40	0	126	66	0	0	2	0	206
		46	0	0	92	58	28	16	194
3	PV 48	0	172	32	0	0	0	0	214
		46	0	0	0	8	14	0	20
4[a]	PV 48	0	152	24	4	2	0	0	174
		46	0	0	0	26	64	48	138

[a]washed in RPMI +$MgCl_2$ 1000 mg/L.

[b]R, ring; A, amoeboid form; N, mononucleate schizont; 2N, binucleate schizont; >2N, multinucleate schizont; MS, mature schizont (a segmenter).

Discussion

In order to study conditions favorable for the *in vitro* growth of *Plasmodium vivax,* a substantial amount of healthy parasite material must be obtained. It is a characteristic of *P. vivax* infection that the blood stage parasite attacks only young corpuscles, resulting in a very low parasite density[7]. A second limiting factor is that the

infected erythrocytes becoming larger and lighter[8]. As a consequence, they have a tendency to float near the upper layer of the suspension and to sediment less rapidly than do normal erythrocytes. Sedimentation of these cells can be improved by increasing the centrifugal force. This method, however, is not desirable because it damages the infected cell membrane (Brockelman, C.R., unpubl.). Healthy and higher parasite yields can be obtained, as shown in this study, by adding $MgCl_2$ to the washing medium.

In the same series of experiments we have also tried to prepare culture materials by washing blood cells in other physiological solutions. Sodium chloride and sucrose at varying concentrations and complete RPMI medium supplemented with 15 percent (v/v) fetal calf serum were used without success. Results from this study suggested that $MgCl_2$ maintained viability of the parasites not only by providing an appropriate osmolarity in the washing medium, but perhaps also by stabilizing the infected erythrocytes, which is essential for the maintenance of cell integrity.

It should be noted that erythrocytes infected only with young ring forms of *P. vivax* can be handled as easily as those infected with *P. falciparum*. This can be explained by the fact that erythrocytes harboring ring forms of *P. vivax* have not undergone changes in membrane structure. Once the parasites develop to amoeboid and later stages, the erythrocytes become enlarged and fragile[9]. Therefore, isolates of *P. vivax* that have a large population of ring forms such as isolate PV 44, show no positive response to $MgCl_2$. Effects of magnesium treatment could be recognized later when the isolate was cultured for 24 h. Unfortunately, isolates such as PV 44 are rare due to the asynchronous nature of *P. vivax* during its erythrocytic cycle[5].

Acknowledgements

This work was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. We thank Mr. Udom Haemawath, Mrs. Nualsri Nadkaew of the Malaria Control Center, Tak, and the staff of the malaria clinic Mae Sod, for kindly supplying the infected blood used in this study.

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บทคัดย่อ

เลือดจากผู้ป่วยด้วยมาลาเรียจากเชื้อชนิด พลาสโมเดียม ไวแวกซ์ นั้น เมื่อนำมาล้างด้วยน้ำเลี้ยง RPMI แล้ว ปรากฏว่าจำนวนของเชื้อลดลง และคุณสมบัติที่จะเจริญต่อไปได้ลดลงด้วย ผลของการวิจัยในบทความนี้แสดงว่า ถ้าเติมแมกนิเซียม คลอไรด์ลงไปในน้ำเลี้ยง ให้มีความเข้มข้น $MgCl_2$ 1000 mg°L แล้วจึงนำไปใช้ล้างเม็ดเลือดติดเชื้อ ผลที่ได้เป็นที่น่าพอใจ คือเชื้อมาลาเรียในเม็ดเลือดที่ล้างด้วยน้ำเลี้ยงผสมแมกนิเซียม คลอไรด์เท่านั้น ที่สามารถเจริญเติบโตและสร้างระยะเมอโรซอยท์ได้