TRYPANOSOMA VIVAX: A SIMPLIFIED PROTOCOL FOR *IN VIVO* GROWTH, ISOLATION AND CRYOPRESERVATION

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Summary:

A rodent adapted clone of *Trypanosoma vivax* was used to infect cyclophosphamide treated mice and rats. Fresh blood containing trypanosomes, was centrifuged in a density gradient of three Percoll solutions, 1.07, 1.06, 1.05 g/ml, respectively, carefully layered on top of each other. The yields of this simple procedure for trypanosome purification were about six times higher than those obtained with the conventional anion-exchange columns. Cryopreservation of trypanosomes using glycerol yielded 90 % viable parasites, whereas using dimethylsulfoxide, a more commonly used cryoprotectant, the viability was only 35 %.

KEY WORDS : *Trypanosoma vivax*, cyclophosphamide, isolation, cryopreservation, Percoll, rodents.

Résumé : *TRYPANOSOMA VIVAX* : UN PROTOCOLE SIMPLE POUR LA CULTURE *IN VIVO*, L'ISOLEMENT ET LA CRYOCONSERVATION

Un clone de Trypanosoma vivax adapté aux rongeurs a été utilisé pour infecter des souris et des rats traités au cyclophosphamide. Du sang frais contenant des trypanosomes a été centrifugé dans un gradient de densité de trois solutions de Percoll respectivement 1,07, 1,06, 1,05 g/ml et soigneusement déposées l'une sur l'autre. Les rendements de ce procédé simple pour la purification des trypanosomes étaient environ six fois plus élevés que ceux obtenus avec les colonnes conventionnelles d'échange anionique. Après cryoconservation des trypanosomes en utilisant le glycérol, le pourcentage de parasites viables était de 90 %, tandis qu'en utilisant le diméthylsulfoxyde, la viabilité était seulement de 35 %.

MOTS CLÉS : Trypanosoma vivax, *cyclophosphamide, isolation, cryopreservation, Percoll, rongeur.*

Research on *Trypanosoma vivax* is hampered by a number of difficulties when trying to isolate this parasite and to grow it in laboratory animals such as mice, rats and rabbits. Splenectomy or the addition of ruminant serum to the inoculum has been used to induce parasitaemia in mice and rats (Hull, 1971; Gathuo *et al.*, 1987). Even with the aid of such manipulations of the host, however, only some *T. vivax* stocks can be induced to maintain infectivity for rodents (Gathuo *et al.*, 1987). It has also been shown that the administration of cyclophosphamide, which suppresses antibody response, enhances parasite growth (De Gee *et al.*, 1982; Gould *et al.*, 1986; Jones, 1986) especially for trypanosomes (Smith *et al.*, 1982). Problems are also observed with the cryopreservation of *T. vivax* bloodstream forms resulting in low infectivity of cryopreserved stabilates (Dar *et al.*, 1972). Furthermore, separation of *T. vivax* from host blood by anion-exchange with DEAE-cellulose (Lanham, 1968; Lanham & Godfrey, 1970) is far from optimal especially in ruminants. Yields are often low and the viability of the purified parasites varies substantially (Grab & Bwayo, 1982). This separation depends on the surface charge of many species of trypanosomes being less negative than that of the blood cells of the hosts. Grab & Bwayo (1982) have introduced an alternative way of trypanosome purification by centrifugation of infected blood on a continuous Percoll density gradient. The isolation procedure described here combines the low-viscosity properties of Percoll with the short pathlenght of sedimentation of a swinging bucket rotor. This leads to a drastic reduction of centrifugation time: eight minutes versus 20 min, and a reduction in rotor speed: 700 g versus 17,500 g (Grab & Bwayo (1982). Consequently, the separation of subcellular components by gradient centrifugation can be obtained without the use of expensive ultracentrifuges.

In this report, modified protocols are described for the production of large amounts of *T. vivax* in rodents and for the subsequent isolation and cryopreservation of the bloodstream forms, suitable for a variety of applications, including antigen preparation and DNA based studies.

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MATERIALS AND METHODS

Experimental infection

Trypanosoma vivax ILRAD 700 is a derivative of Zaria Y486 which was isolated from a zebu cow in Nigeria (Leeflang *et al.*, 1976). Mice were first injected with 0.2 ml of a cryostabilate of *T. vivax* ILRAD 700, containing antilog 5.7 viable parasites/ml. When the parasitaemia reached antilog 8.7 viable parasites/ml, their blood was taken by cardiac puncture and used for the infection. Twenty four hours before infection, five outbred female OF1 mice and three Wistar rats (IFFA-CREDO), 10 to 15 weeks old, were injected intraperitonially with 200 mg/kg (Luckins, 1969; Smith *et al.*, 1982; Jones, 1986) and 80 mg/kg cyclophosphamide (Endoxan, Asta Medica AG, Germany) (Luebke *et al.*, 1992) respectively. A control group consisting of five mice and three rats did not receive cyclophosphamide before infection. The rats were intraperitoneally injected with 1 ml of mouse blood containing antilog 8.5 viable trypanosomes/ml. The mice were intraperitoneally injected with 0.5 ml of mouse blood containing 7.2 viable trypanosomes/ml. Parasitaemia was estimated daily by microscopic examination of tail blood according to the matching method as described by Herbert & Lumsden, 1976.

At day 4 or 5 post-infection, when parasitaemia reached about antilog 2×9.0 (2×10^9) organisms/ml, rat were anaesthesized with chloroform and exsanguinated by cardiac puncture using a sterile heparinized syringe.

Cryopreservation of trypanosomes

Two different cryoprotectants were used: either a 1:1 (v/v) mixture of glycerol (Merck 87 %) and phosphate saline glucose (PSG, 4:6), containing 1.0 % w/v glucose; pH 8 , I = 0.145) (Lanham & Godfrey, 1970) or a 1:4 (v/v) mixture of dimethylsulfoxide (DMSO) (Merck, Darmstadt) and PSG. Polypropylene tubes with screwcaps (500 µl, Sarstedt) were filled with 100 or 200 µl of a 3:1 (v/v) blood-cryoprotectant mixture, resulting in a final concentration of 10.9 % glycerol or 5 % DMSO, respectively. Cooling was done at – 2° C/min down to – 40° C in the vapor phase of liquid nitrogen and consequently down to – 100° C at – 5° C/min before being transferred to canisters containing liquid nitrogen.

In order to evaluate the viability of the cryopreserved trypanosomes 5 µl from each vial was observed under a phase contrast microscope (10 × 40). Live and dead trypanosomes in 10 microscopic fields were counted. A total of ten cryostabilates were thawed and tested.

Isolation procedures

Trypanosome purification was carried out using Percoll (Pharmacia Biotech AB, Uppsala, Sweden) mixed with PSG in order to obtain three solutions of densities 1.07, 1.06 and 1.05 g/ml, respectively. By carefully pipetting 3 ml of each of the three solutions on top of each other, in a conical 15 ml polypropylene Falcon tube, a discrete density gradient was obtained. Three milliliters of fresh heparinized blood, withdrawn from anaesthetized animals by means of heart puncture, was then carefully pipetted on top of the density gradient and centrifuged at 700 g for eight minutes using a swinging bucket rotor at 4° C. After centrifugation, the translucent trypanosome-containing band was located between densities 1.06 and 1.05 g/ml, and was transferred to a fresh tube. Erythrocytes concentrated on the bottom of the tube.

This Percoll-based method was compared with the commonly used DEAE-cellulose column procedure, as described by Lanham & Godfrey (1970). One volume of infected blood was diluted with three volume of PSG 4:6 or PSG 6:4 buffer to a final concentration of antilog 8.7 (5×10^8) trypanosomes/ml and kept cool in ice. One volume of diluted blood was applied to eight volume of equilibrated gel.

Statistical analysis

Experiments were repeated three times. Data from experiments were evaluated using Student's *t*-test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

As shown in figures 1 and 2, parasitaemia started rising almost immediately after infection in the mice and rats treated with cyclophosphamide. In the control mice, parasitaemia started increasing only one week post infection (Fig. 1), whereas the parasitaemia in the control rats always remained under 35 trypanosomes per microscopic field (Fig. 2). Furthermore, beyond day six post infection, no parasites could be detected in the control rats.

A significant difference ($P < 0.05$) between the parasitemia of control and cyclophosphamide treated groups was observed in rats and mice from day 2 post infection.

Rat blood was diluted to a final concentration of 128 trypanosomes per microscopic field (antilog 8.7 viable trypanosomes/ml). Twenty milliliter of this dilution was passed through a DEAE columns yielding 20 ml of 20-25 trypanosomes per microscopic field suspension. Another 20 ml was centrifuged on a Percoll gradient, yielding 20 ml of 115-120 trypanosomes per microscopic field suspension with some contaminating leucocytes. The results of the cryopreservation with glycerol or

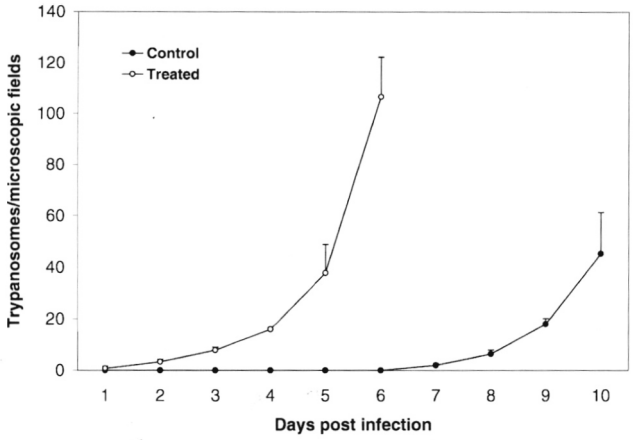

Fig. 1. – Evolution of *T. vivax* parasitaemia in control mice and mice treated with cyclophosphamide. Parasite burdens were determined from mouse blood and are represented as mean + SD for five mice per group. Statistically significant differences ($p < 0.001$) between mouse groups were observed from day 2 post infection onward. These results are representative for three individual experiments performed.

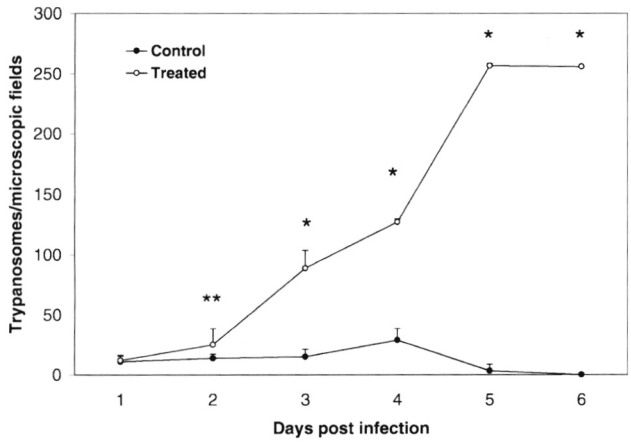

Fig. 2. – Evolution of *T. vivax* parasitaemia in control rats and rats treated with cyclophosphamide. Parasite burdens were determined from rat blood and are represented as mean + SD for three rats per group. Statistically significant differences between rats groups $p < 0.001$ (*) and $p < 0.01$ (**) are indicated. These results are representative for three individual experiments performed.

DMSO have been summarized in Table I. When glycerol was used as cryoprotectant and the total frozen volume was kept low (100 µl), about 90 % of the trypanosomes were still alive after thawing against only 35 % for DMSO (100 µl).

DISCUSSION

Administration of cyclophosphamide clearly permitted parasite growth in mice. The drug also allowed the development of high levels of parasitaemia in rats, making it possible to produce large amounts of trypanosome antigen in a relatively short period of time. For the cryopreservation of viable trypanosomes, glycerol seems to be preferable to the DMSO. Espinoza *et al.* (1997) reported satisfactory results using 10 % DMSO. In the 100 µl aliquots frozen with glycerol, 90 % of all trypanosomes did survive. On the other hand, freezing 200 µl aliquots with glycerol yielded only 57 % live trypanosomes. This phenomenon was also observed by Hertsens (1997). This difference in viability is probably due to a difference in speed of trypanosome freezing, greater volumes taking more time to reach ultra low temperatures, allowing ice crystal formation in the cells.

The recovery of trypanosomes from the DEAE-cellulose (~ 20 %) using PSG 4:6 or PSG 6:4 was poor relative to that obtained from the Percoll gradient (~ 90 %). DEAE-cellulose adsorbs the more negatively charged blood components. Lanham & Godfrey (1970) in a detailed study of the adsorption-elution characteristics of three West African strains from cattle, sheep and goats found differences between a drug-sensitive and two drug-resistant strains although routine blood separations gave 50 to 65 % over-all recoveries. This separation depends on the surface charge of the trypanosomes, and it is known that some *T. vivax* have a higher negative charge (Lanham & Godfrey, 1970). Kilgour *et al.* (1975) noted that some West African infections from cattle were difficult to separate from the DEAE cellulose and that only those infections with Set

	Cryoprotectants			
	Glycerol (10.9 %)*		DMSO (5 %)*	
Total volume (µl) of aliquot	100	200	100	200
Trypanosome survival (after thawing)	90 % ± 1.48[a]	57 % ± 3.57[a]	35 % ± 2.14[a]	29 % ± 6.04[a]

* : final concentration,

Table I. – Comparison between the results obtained with glycerol and DMSO for the cryopreservation of *T. vivax*, using ten cryostabilates of 100 µl and 200 µl aliquots (total volume). Data are presented as mean ± SD. These data are representative of three individual experiments performed. Means having the common superscript "a" are significantly different from each other ($p < 0.05$).

I aminotransferase patterns were successfully processed. In addition, this variability in separation may occur in *T. vivax* stocks from South America (Murray, 1979). Mild centrifugation of fresh heparinised blood in a discrete Percoll gradient, as described, is our method of choice for the isolation of trypanosomes. The use of Percoll for trypanosome isolation has been described earlier by Grab & Bwayo (1982). However, they used continuous gradient densities, which can only be achieved using high-speed centrifuges, which are not available in many laboratories. Furthermore, using continuous density gradients, often bad cell separation was achieved and consequently a very low trypanosome yield. Interestingly our yields from the Percoll gradient had a negligible white blood cells contamination (one cell per 100 microscopic fields), and the infectivity and antigenicity of the trypanosomes were unaffected (data not shown). Lastly, while Percoll solutions of a given density can be kept in sterile vials for months, continuous density gradients have to be freshly made before each cell separation.

The use of three Percoll solutions of different densities may be useful to obtain material for other studies involving *T. vivax* such as antigen preparation for serological tests, biochemical and DNA analyses.

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