HEMOLYTIC ACTIVITY OF *MONOCERCOMONAS* SPP.

DA SILVA A.C.*, DE CARLI G.A.*, BRASSEUR P.**, TASCA T.*, CASTILHOS D.* & WENDORFF A.*

Summary :

The hemolytic activity of an isolate of *Monocercomonas* spp. from *Tropidophis melanurus* (snake:*Boidae*) was investigated. The isolate was tested against human erythrocytes of groups A, B, AB and O and against erythrocytes of six adult animals of different species (rabbit, rat, chicken, horse, bovine, and sheep). Results show that *Monocercomonas* spp. exerted an hemolytic activity against all erythrocytes tested.

KEY WORDS : *Monocercomonas* spp., snake: *Boidae, Tropidophis melanurus,* hemolytic activity.

Résumé : ACTIVITÉ HÉMOLYTIQUE D'UN ISOLAT DE *MONOCERCOMANAS* SPP.

L'activité hémolytique d'un isolat de Monocercomonas *spp. provenant de* Tropidophis melanurus *(serpent:* Boidae*) a été étudiée. L'isolat a été testé avec les hématies appartenant aux groupes sanguins humains A, B, AB et O et les hématies de six animaux adultes d'espèces différentes (lapin, rat, poulet, cheval, bœuf et mouton). Les résultats ont montré que* Monocercomonas *spp. exerce une activité hémolytique sur toutes les hématies testées.*

MOTS CLÉS : Monocercomonas *spp., Serpent :* Boidae, Tropidophis melanurus, *hémolyse.*

INTRODUCTION

Monocercomonas Grassi, 1879 is a flagellate protozoan of the large intestine of squamate reptiles. It is considered the most primitive known member of the order Trichomonadida Kirby (Honigberg, 1963) and limited information is currently available on the pathogenicity of this parasite. The pathogenic mechanisms of several species of trichomonads: *Trichomonas vaginalis, Trichomonas gallinae, Tritrichomonas foetus* and *Tritrichomonas suis* have been widely studied in animal models especially in mice since 1934 (Bos, 1934), providing valuable informations on specific host-parasite interactions (see review by Kulda *et al.*, 1990). Interactions between *T. vaginalis* and cell culture monolayers have also been studied and have demonstrated a contact dependent cytopathic effects of the parasite. In addition, several studies have suggested that molecules released by *T. vaginalis* may exert contact independent cytopathic effects (see review of Honigberg, 1990). Cytopathogenicity induced *in vitro* by other protozoan parasites have also been demonstrated for *Entamoeba histolytica*, and *Giardia lamblia* (Lopez-Revilla & Said-Fernandez, 1980, Ravdin & Guerrant, 1981, Ortega *et al.*, 1987).

Using *in vitro* methods, the hemolytic activity of different species of Trichomonadida, such as *T. vaginalis* (Grys & Hernik, 1973; Krieger *et al.*, 1983; De Carli *et al.*, 1989, 1994; Dailey *et al.*, 1990; Potamianos *et al.*, 1992), *T. gallinae* (De Carli *et al.*, 1996*a*), *T. foetus* (Burgess *et al.*, 1990; De Carli *et al.*, 1994, 1996*b*) and *T. suis* (De Carli *et al.*, 1994) has been studied. For *T. vaginalis*, Fiori *et al.*, in 1993 showed that hemolysis was a contact and temperature dependent phenomenon and hypothetized that cytopathic effects could be related to pore-forming in the membrane of red blood cells. However the contact of *T. vaginalis* and red blood cells is not a prerequisite for hemolysis which could also be due to a pH dependent lytic protein secreted by the parasite (Fiori *et al.*, 1996). Although attempts to demonstrate a hemolytic activity in different species of *Trichomonas* have been conducted, the hemolytic activity of *Monocercomonas* spp. have never been studied.

MATERIAL AND METHODS

ORGANISMS

The *Monocercomonas* spp. isolate (R183) used in this study was isolated by Kulda in Cuba in 1965 from *Tropidophis melanurus* (snake: *Boidae*) and kindly provided by Dr Benchimol (CBB-

* Faculdade de Farmacia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, Porto Alegre 90610-000 RS, Brazil.
** Laboratoire de Parasitologie, Centre Hospitalier Universitaire, Hôpital Charles Nicolle, 76931 Rouen, France.
Correspondence : Philippe Brasseur.
Tel. : 33 2 32 88 80 15 – Fax : 33 2 32 88 80 17.

UENF, Campos, RJ, Brazil). The flagellates were cultured axenically in a Trypticase-Yeast extract-Maltose (TYM) medium (Diamond, 1957) without agar, pH 7.0, supplemented with 10 % (v/v) heat inactivated cold horse serum, in air, at a temperature of 28 °C. Isolates were subcultured every 48 hours in TYM medium. Monocercomonads in the logarithmic phase of growth and subcultured every 48 hours exhibited more than 95 % of mobility and a normal morphology. The protozoans were counted with a hemocytometer and adjusted to a concentration of 1×10^6 living organisms per ml in TYM medium. Isolates were stored in liquid nitrogen (– 196 °C) with 5 % dimethyl sulfoxide (DMSO).

Erythrocytes

Fresh human blood of groups A, B, AB and O was obtained at the City Emergency Hospital (HPS) blood center of Porto Alegre (Brazil) from volunteer donors in an equal volume of Alsever's solution (dextrose 10.5 g, sodium citrate 8.0 g, citric acid 0.55 g, sodium chloride 4.2 g, distilled water to one liter), and from six different adult animal species: rabbit, rat, chicken, horse, ovine, sheep. All erythrocytes were harvested and washed three times using centrifugation (250 × g for 10 min) in an equal volume of Hank's balanced salt solution (HBSS) (Bio-Mérieux, France). The supernatants were discarded. Each experiment was performed using fresh erythrocytes from human blood donors and adult animals. Erythrocytes were stored in HBSS at 4 °C.

Hemolysis assay

Parasites were harvested from a 24 hours culture (viability > 95 %) in TYM medium, in air, at 28 °C, and washed three times in HBSS by centrifugation (750 × g for 20 min). A volume of 50 ml of washed fresh undiluted erythrocytes was mixed with 2.5 ml of HBSS containing a total number of 1×10^6 trophozoites of *Monocercomonas* spp. (Krieger *et al.*, 1983). After 18 hours of incubation at 28 °C, in air, without shaking, the mixture was centrifuged (250 × g for ten min). Absorbance of the supernatants and controls were measured at 540 nm (De Carli *et al.*, 1989) with a spectrometer and was compared with a standard curve obtained by osmotic lysis of erythrocytes of each species. Control tubes without parasites were included in all assays to measure the spontaneous hemolysis. A kinetic study of *Monocercomonas* spp. hemolysis was conducted in the standard conditions described above, with human group O erythrocytes. Hemolysis was evaluated hourly from 1 to 10 hours, and at 14 and 18 hours. This study was performed three times in triplicate. Results were expressed as percentages of total hemolysis (100 %). The mean and standard error of the hemolytic activity of monocercomonads with the different erythrocytes were calculated after performing the assay at least 12 times and each sample was done in triplicate.

Cell lysates

Parasites harvested in late exponential phase were washed three times in PBS pH 7.2. The suspension adjusted at 1×10^6 was sonicated (five cycles of ten sec at 50 watts in ice bath), centrifuged and supernatants filtered through a 0,22 µm filter (Millipore).
Statistical analysis was performed using the Student's t-test.

RESULTS

Results show that *Monocercomonas* spp. isolate tested had a hemolytic activity ranging between 33 and 71 % with human erytrocytes and between 17 to 56 % with animal erythrocytes (Table 1). The hemolytic activities observed with human erythrocytes of group O or group AB were significantly higher than thoses of group A or group B ($p < 0.001$). Among animal erythrocytes, the lowest activities were observed with bovine or sheep erythrocytes (19 and 17 % respectively). Neither adhesion nor agglutination was microscopically observed between *Monocercomonas* spp. and erythrocytes at any phase of hemolysis assay.
Monocercomonads isolated at the end of hemolysis assays were alive and were successfully cultured in

No of assays	Percentages of hemolysis*									
	Human erythrocytes groups				Animal erythrocytes					
	A	B	AB	O	rabbit	rat	chicken	horse	bovine	sheep
12	33 ± 2.3	36 ± 2.7	71 ± 1.9	63 ± 1.4	28 ± 0.3	56 ± 1.5	43 ± 2.9	32 ± 1.5	19 ± 0.8	17 ± 0.1

* Mean values ± one standard deviation of triplicate experiments

Table I. — Hemolytic activity of R183 strain of *Monocercomonas* spp. on human erythrocytes of groups A, B, AB, O and six adult animal erythrocytes.

TYM medium. Parasite culture supernatants of hemolysis assays, tested in the presence of different species of erythrocytes did not induce any hemolytic activity. A kinetic study of hemolysis with human group O erythrocytes showed that hemolysis was over 30 % after one hour, and reached a plateau (63 %) after 18 hours (Fig. 1).

Parasite culture supernatants, extracts of sonicated parasites or heat killed organisms tested with human or animal erythrocytes did not exhibit hemolytic activity (details not shown).

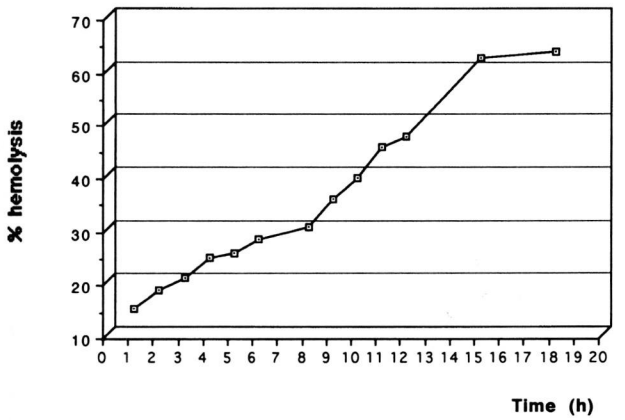

Fig. 1. — Kinetic study of hemolytic activity of *Monocercomonas* spp. on human erythrocytes of group O.

DISCUSSION

A hemolytic activity has been demonstrated in several protozoan parasites including *Trypanosoma congolense* (Tizard *et al.*, 1977) and *T. brucei* (Tizard, 1978), *E. histolytica* (Lopez-Revilla & Said-Fernandez, 1980), *T. vaginalis* (Grys & Hernik, 1973; Krieger *et al.*, 1983; De Carli *et al.*, 1989, 1994; Dailey *et al.*, 1990; Potamianos *et al.*, 1992) and *T. gallinae* (De Carli *et al.*, 1996*a*). *T. foetus* (De Carli *et al.*, 1994, 1996*b*), and *T. suis* (De Carli *et al.*, 1994) have no hemolytic activity against human erythrocytes. The mechanism of hemolysis is not yet well established and vary from one species to another. For *T. congolense*, hemolysis has been found related to release of fatty acids from endogenous phosphatidyl choline by a phosphalipase A (Tizard & Holmes, 1976). For *T. vaginalis* and *T. gallinae* no activity could be detected in culture supernatants, suggesting that hemolytic activity was not related to a hemolysin or soluble metabolites released by the parasite (De Carli *et al.*, 1996*a,b*). A relationship between adhesion and cytopathogenicity was demonstrated for *T. vaginalis* in cell cultures (Brasseur & Savel, 1982), and Fiori *et al.* in 1993, showed that *T. vaginalis* lysed human erythrocytes by pore-forming in their membrane. Proteins involved in cytoadherence and pathogenesis of *T. vaginalis* have been identified (Fiori *et al.*, 1993; Alderete *et al.*, 1995). Although adherence of parasite on the target cell surface has been considered for long as a prerequisite to cell damage, and particularly hemolysis of erythrocytes, Fiori *et al.* in 1996, showed that a contact independent hemolysis was mediated by a protein of more than 30 kDa released by *T. vaginalis* under triggering conditions. Such a cytopathic effect due to pore-forming protein has been previously postulated for *E. histolytica* (Young *et al.*, 1982; Ravdin, 1986). Although pore-forming activity was high in *E. histolytica* compared to non pathogenic *Entamoeba*, it was found irrespective of the virulence of strains (Keller *et al.*, 1988).

In our experiments, adhesion between *Monocercomonas* spp. and erythrocytes were not observed and contact independent hemolytic activity could not be exhibited using supernatants of hemolysis assays, culture medium or sonicated parasite extracts. Although a hemolytic activity of *Monocercomonas* spp. was clearly demonstrated, a relationship between hemolysis and cell pathogenicity is not yet clearly established.

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