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Platelet-aggregating activity of released factor(s) from *Trypanosoma brucei brucei*

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Summary

The effect of factors derived from *Trypanosoma brucei brucei* on rat platelets was studied. *T. brucei* at a concentration of 4 × 10^9 trypanosomes/ml phosphate saline glucose (PSG) was stored at −20°C for 18 h, thawed, and a supernatant fraction, trypanosome-derived supernatant (TDS) was obtained by spinning the sample at 3000 *g* for 10 min at 20°C. Normal rat platelets, prepared as platelet-rich plasma (PRP), were then incubated with TDS in the absence or presence of ADP (0.05–0.1 μM). The results showed that approximately 83% platelet aggregation was induced by addition of TDS (50 μl; 113 μg protein) to 100 μl PRP with a platelet count of 10^6. Simultaneous addition of ADP and TDS to PRP produced a synergistic effect. It was also shown that a supernatant fraction, obtained by incubating live *T. brucei* (4 × 10^9/μl PSG) at 0°C for 1 h and spinning down the trypanosomes (3000 *g* for 10 min), also induced platelet aggregation. The nature of the factor(s) derived from, or released by, *T. brucei* inducing platelet aggregation is being investigated but it has been shown not to be ADP.

Résumé

L'effet qui est produit sur des plaquettes du rat par des facteurs dérivés de *Trypanosoma brucei brucei* fut recherché. Du *T. brucei* dans une concentration de 4 × 10^9 trypanosomes/ml phosphate saline glucose (PSG) fut conservé à −20°C pendant 18 h, fut dégelé et une fraction supernatante, un supernatant dérivé de trypanosome (SDT) fut obtenue en tournoyant l'échantillon à 3000 *g* pendant 10 min à 20°C. Des plaquettes normales du rat préparés comme du plasma plaquette-riche (PPR) furent ensuite couvies avec du SDT sans ou dans la présence du ADP (0.05–0.1 μM). Les résultats montrèrent que approximativement 83% de l'agrégation des plaquettes furent provoqués par l'addition du SDT (50 μl; 113 μg protéine) aux 100 μl du PPR avec un compte plaquette de 10^6. Des additions simultanées du ADP et du SDT ont produit un effet synergistique. Il fut montré aussi qu'une fraction supernatante obtenue en couvant du *T. brucei* vivant (4 × 10^9/μl PSG) à 0°C pendant 1 h et en tournoyant les trypanosomes (3000 *g*/10 min), provoqua aussi l'agrégation des plaquettes. La nature du facteur (ou des facteurs) dérivé de, ou bien, déchargé par *T. brucei* provoquant l'agrégation des plaquettes est sous examination mais celle fut déjà montré de ne soit pas du ADP.

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Introduction

Thrombocytopenia has been shown to be a common complication in both experimental and clinical trypanosomiasis [1]. The mechanisms which have been invoked to explain this complication include consumption of platelets as part of disseminated intravascular coagulation and increased removal by the reticulo-endothelial system, from the circulation of normal or immunologically damaged platelets [2–4]. In-vivo platelet aggregation, detected by counting platelet aggregates [5] and believed to be caused by trypanosomal products or intact organisms, has also been suggested as a primary mechanism of the thrombocytopenia. The aggregation has been shown to be independent of ADP, complement, kinin, antibody and fatty

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acids [5]. Interaction between platelets and anti-trypanosomal antibody has also been shown to cause aggregation during infection [6]. In this communication, we report our findings on studies of platelet aggregation induced by trypanosomes or trypanosome fractions.

The results of the present investigation show that a factor(s) inducing platelet aggregation is released by *Trypanosoma brucei brucei*. The nature and characteristics of the putative factor(s) are yet to be investigated fully.

Materials and methods

T. brucei stock NITR 8/18 was used in this study. Wistar rats weighing 200–250 g were inoculated with the trypanosomes (approximately 10^6 per rat). The trypanosomes were subsequently isolated as described previously [7] and freed of blood elements by chromatography on DEAE-cellulose [8]. Freshly separated trypanosomes, at a concentration of 1×10^9/ml phosphate saline glucose (PSG), or 4×10^9/ml PSG, pH 8.0 (containing 285 ml of 0.2 M Na_2HPO_4; 15 ml of 0.2 M NaH_2PO_4; 300 ml of 0.85% (w/v) NaCl; and 400 ml of 2.5% (w/v) glucose) were incubated in an ice bath for 1 h. Preliminary experiments had shown that within this concentration range and time, trypanosomes were actively motile. The trypanosomes were then pelleted by centrifugation at 4°C for 10 min at 3000 *g*. The resulting supernatant was designated trypanosome-derived supernatant (TDS): that from the low trypanosome concentration (1×10^9/ml) TDS-1, and that from the high trypanosome concentration (4×10^9/ml) TDS-2.

In another set of experiments, the trypanosomes (4×10^9/ml PSG buffer) were stored at −20°C for 18 h immediately after separation. Before use the samples were thawed, centrifuged at 3000 *g* for 10 min at 20°C and the supernatant, TDS-3 used to induce platelet aggregation.

Blood was collected under ether anaesthesia from the exposed heart of normal rats and dispensed into a tube containing heparin (5 units/ml of blood); at this concentration heparin does not alter platelet function [9]. The heparinized blood was centrifuged at 120 *g* for 10 min at 20°C and the platelet-rich supernatant (PRP) was collected. The remaining plasma supernatant was re-centrifuged at 3000 *g* for 10 min at 20°C and the resultant platelet-poor plasma (PPP) was collected.

All the TDS samples (TDS-1, TDS-2, TDS-3) were used to induce platelet aggregation. Appropriate blanks containing PSG and PRP were included in all experiments.

Platelet aggregation was tested in PRP (0.5–1 $\times 10^6$) at 37°C by a modification of Born's method [10] using a Payton aggregometer module (8008) and recorder (Payton Associates, Scarborough, Canada) running at 2.45 cm/min. Each PRP aliquot was stirred at 1000 r.p.m. and stimulated with TDS (3–120 μg protein). The points of minimum and maximum light transmission for each sample were preset using the rat PRP and the corresponding PPP respectively.

Aggregation was allowed to run for 4 min in each sample. The total volume in the cuvette including PRP and aggregating agent was 500 μl. Aggregation was expressed as a percentage of the preset range. Where peak aggregation was followed by rapid de-aggregation, the point of maximum light transmission was used to determine percentage aggregation.

Platelets were counted by the standard method of Bretcher and Cronkite [11] using light microscopy.

Results

The effects on platelet aggregation of different supernatant fractions of *T. brucei* and ADP were studied. As shown in Table 1, ADP (0.1 μM) caused 62.1% aggregation of platelets. The TDS-3 supernatant induced high levels of platelet aggregation in proportion to the volume of supernatant added, the highest aggregation (82.5%) was achieved with 50 μl (113 μg protein) of supernatant. A relatively high volume of TDS-2 (40–100 μl; 48–119 μg protein) induced a lower level of platelet aggregation (10.2–32.5%). TDS-1 did not induce platelet aggregation at the concentration tested (2.5–25 μg protein).

A probable synergism between the supernatant fractions and ADP was also investigated. The mean percentage aggregation induced by addition of ADP (0.05 μM) alone was 40.2%. A synergistic response of 68.3% was obtained by addition of 40 μl of TDS-1 (10 μg protein) which did not cause platelet aggregation alone

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Table 1. Effect on platelet aggregation of supernatant fractions obtained by spinning live, or frozen and thawed, *T. brucei* incubated in phosphate saline glucose buffer

Volume of supernatant fraction added (μl)	Percentage aggregation induced by		
	TDS-1	TDS-2	TDS-3
10	—	—	—
20	—	—	18.1 ± 3.4
30	—	n.d.	20.4 ± 5.2
40	—	10.2 ± 2.6	57.5 ± 4.8
50	—	n.d.	82.5 ± 6.4
50*	n.d.	n.d.	85.1 ± 5.9
80	n.d.	19.4 ± 4.0	n.d.
100	n.d.	32.5 ± 1.4	n.d.

*ADP (0.1 μM) caused 62.1% platelet aggregation.

The protein concentrations of TDS fractions were as follows: TDS-1, 0.25 mg/ml; TDS-2, 1.19 mg/ml; TDS-3, 2.25 mg/ml. All reactions contained 0.5–1 × 10^6 platelets. The results are means ± s.d. of four separate experiments.

(Table 1) and 0.05 μM ADP together with TDS-1 caused 68.3% platelet aggregation (Table 2). Similarly ADP (0.1 μM) and TDS-3 together induced a 90% platelet aggregation (Figs 1 & 2).

Discussion

The results obtained in this investigation showed that a factor(s) released by *T. brucei* caused platelet aggregation. As far as can be determined this is the first quantitative and sensitive assay of trypanosome-induced platelet aggregation and extends earlier observations [1,5] that studied a similar phenomenon by the less accurate method of counting aggregated platelets under phase-contrast microscopy. It should also be noted that Davis *et al.* [5] exposed platelets to trypanosomes and observed platelet aggregation in their presence.

Table 2. Synergistic effect on platelet aggregation of trypanosome-derived supernatant (TDS) and low ADP concentration (0.05 μM)

Volume of TDS added (μl)	Percentage aggregation induced with ADP (0.05 μM) in the presence of	
	TDS-1	TDS-3
10	n.d.	42.5 ± 3.8
20*	n.d.	90.0 ± 4.7
40	68.3 ± 4.6	n.d.
60	87.5 ± 3.2	n.d.

*ADP (0.1 μM) was added.

The mean aggregation recorded by addition of 0.05 μM ADP alone was 40.2%.

The protein concentrations of TDS fractions were: TDS-1, 0.3 mg/ml; TDS-3, 3.75 mg/ml. All reactions contained approximately 1 × 10^6 platelets. The results represent the means of five separate experiments ± s.d.

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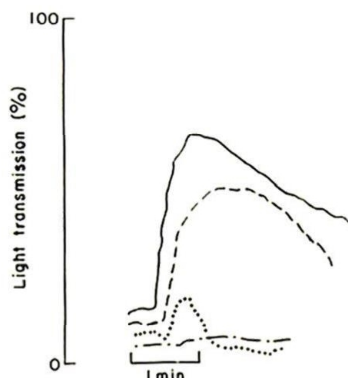

Fig. 1. Recordings of platelet aggregation induced with trypanosome-derived supernatant (TDS) fractions. The suspensions contained approximately 1×10^6 platelets, and TDS or ADP was used as agonist. (——) 40 μl of TDS-3 (90 μg protein), (– – –) 0.05 μM ADP; (–·–·–) 40 μl of TDS-1 (12 μg protein); (·····) 40 μl of TDS-2 (48 μg protein).

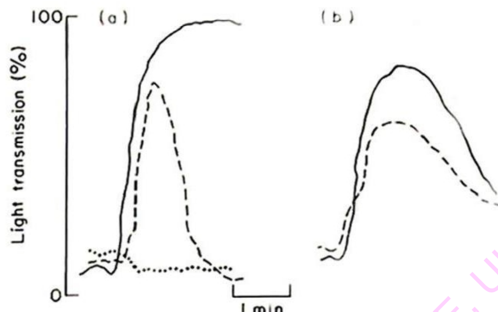

Fig. 2. Recording of platelet aggregation in the presence of both ADP and trypanosome-derived supernatant (TDS). The suspension contained approximately 1×10^6 platelets. (a) Platelet aggregation effect on addition of: 10 μl TDS-3 (25 μg protein) and 0.1 μM ADP (——); 10 μl phosphate saline glucose (PSG) and 0.1 μM ADP (– – –); and 10 μl of TDS-3 (····). (b) Platelet aggregation effect on addition of: 40 μl of TDS-1 (10 μg protein) and 0.05 μM ADP (——); and 40 μl of PSG and 0.05 μM ADP (– – –). In these control experiments aggregation induced by TDS was determined under conditions in which any free ADP was inactivated by addition of phosphoenol pyruvate (50 μM) and pyruvate kinase (2 units) [17].

In this study the platelet aggregation activity of different cell-free trypanosome-derived supernatant fractions was determined. Under these conditions any aggregating effects would be attributable directly to the factor(s) released by actively motile trypanosomes; into the medium in the case of TDS-1 and TDS-2, or extracted by freezing and thawing of trypanosomes for TDS-3.

Extensive in-vivo platelet aggregation has been suggested as a mechanism of thrombocytopenia associated with trypanosomiasis. The basis of this suggestion was that the platelet aggregation was induced by trypanosomal components or products and coating of host platelets with anti-trypanosomal antibodies [1,5]. It has been observed that the aggregation induced by cell-free supernatant fractions of disrupted *T. rhodesiense* was independent of ADP, kinins, complement and antibody [5]. The results of the present investigation suggest that the release of factor(s) by *T. brucei* could be the basic mechanism of the thrombocytopenia observed in infected animals, and may be helpful in investigating a similar feature in other infective states such as that associated with *Plasmodium falciparum* infections [12–14]. The synergism between the factor(s) and low ADP concentrations could be significant in causing the thrombocytopaenia.

Results from control experiments showed that the factor inducing aggregation was not ADP. The hypothesis that the released factor might be a protease is being tested, since proteolytic conversion of pre-proteins to active factors is a prerequisite for blood clotting and, in certain instances, in platelet aggregation [15,16]. Preliminary analysis by sodium dodecylsulphate polyacrylamide gel electrophoresis of plasma from infected rats and TDS-1 revealed a polypeptide band of molecular weight 12,000 which was absent in normal rat plasma but also present in a trypanosome extract. The possible role in platelet aggregation of this protein is being investigated.

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