Pharmacological action of tick saliva upon haemostasis and the neutralization ability of sera from repeatedly infested hosts

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SUMMARY

Ticks are blood-feeding arthropods widely distributed in the world and vectors of several diseases. As haematophagy demands evasion strategies and repeatedly infested hosts develop protective immune responses, we investigated the mechanisms of the *Rhipicephalus* (*Boophilus*) *microplus* saliva anti-haemostatic activity and the possible relationship between the acquired natural anti-tick host resistance and anti-haemostatic action. For this purpose, we studied the effects of *R. microplus* saliva on different pathways of haemostasis and tested whether repeated infested bovine sera (RIBS) are able to abolish salivary anti-haemostatic activities. *R. microplus* saliva (i) displays inhibitory activity upon collagen-induced platelet aggregation; (ii) inhibits the induction of endothelial pro-coagulant state; and (iii) reduces thrombogenesis *in vivo*. RIBS were shown to be able to partially block the delay of coagulation and the anti-thrombotic effect of saliva, and to totally abolish the modulation of endothelium activation. Conversely, RIBS has no effect on the inhibition of platelet aggregation. These results show, for the first time, the neutralization ability of sera from acquired resistance hosts against tick anti-haemostatics. Moreover, this is the first report of a haematophagous parasite able to modulate endothelial cell pro-coagulant state, and addresses the presence of anti-platelet and anti-thrombotic activity in *R. microplus* saliva.

Key words: *Rhipicephalus (Boophilus) microplus*, tick, haematophagous, haemostasis, coagulation, platelet, endothelium, thrombosis, bovine resistance.

INTRODUCTION

Ticks are blood-feeding arthropods widely distributed in the world, which are able to parasitize humans as well as almost any domestic and wildlife vertebrate animals. In several countries, ticks are important public health hazards, since these arthropods are vectors of several bacterial, viral and protozoan tick-borne diseases (Walker, 1998).

Once a tick has found a host and reaches the attachment site, it introduces its mouthparts into the skin and induces vessel damage, which in turn triggers mechanisms to avoid blood loss and infection such as vasoconstriction, platelet aggregation, coagulation, inflammation and migration of immune cells to the lesion site (Simmonds and Lane, 1998). Therefore, it could be stated that a successful bloodmeal depends on a fine balance between the pharmacological action of tick saliva and host responses. Thus, tick salivary secretion plays a major role in the modulation of host haemostatic and immunological responses (Ribeiro, 1989, 1995; Maritz-Olivier *et al*. 2007).

Several molecules with a large range of pharmacological properties have been characterized in tick saliva (for comprehensive reviews see Champagne and Valenzuela, 1996; Ribeiro and Francischetti, 2003; Francischetti *et al*. 2009). These molecules may act as vasodilators (Dickinson *et al*. 1976; Kemp *et al*. 1983), anti-platelets (Mans *et al*. 1998, 2002; Mans and Ribeiro, 2008), anti-coagulants (Horn *et al*. 2000; Francischetti *et al*. 2002, 2004; Ciprandi *et al*. 2006), immunosuppressants (Juncadella *et al*. 2007; Konnai *et al*. 2009) and anti-inflammatories (Kotsyfakis *et al*. 2006; Déruaz *et al*. 2008). Recently,

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we conducted an *in vivo* study reporting the systemic modulation of haemostasis from tick-naïve bovines infested with *Rhipicephalus* (*Boophilus*) *microplus* (Reck Jr *et al*. 2009). The study has shown that the consequences of the biological action of tick saliva to the host may be much more far-reaching than initially suspected, and that little is known about the full impact of these compounds to the tick-host relationship.

On the other hand, a well-known phenomenon occurs after repeated tick infestations: host acquisition of resistance (Wikel and Whelen, 1986), which seems to be mediated mainly by immune responses (Allen, 1994). Although this resistance status was widely studied and is mostly related to cellular immune responses, namely mediated by basophils and eosinophils, (Wikel *et al*. 1996), it also seems to depend on serum neutralization ability against several tick molecules, and the role of these tick compounds targeted by host serum requires further investigation.

The cattle tick *R. microplus*, formerly *Boophilus microplus* (Murrel and Barker, 2003), is the most harmful bovine parasite in Latin America and Australia (Jongejan and Uilenberg, 2004). *R. microplus* is also a consistent model to study the tick-host relationship, since it remains attached to the same host throughout the whole parasitic stage, which lasts around 3 weeks (Guimarães *et al*. 2001). Until now, thrombin inhibition was the only identified pathway of anti-haemostatic activity in *R. microplus*, since the anti-haemostatics reported for *R. microplus* were 2 thrombin inhibitors in saliva (Horn *et al*. 2000; Ciprandi *et al*. 2006), and 1 thrombin inhibitor in the gut (Ricci *et al*. 2007). Yet, no information has been made available about the pharmacological activity of *R. microplus* salivary compounds upon platelets, endothelium and *in vivo* models of thrombogenesis.

The aims of this work were to identify and to explore the pharmacological mechanisms of *R. microplus* saliva anti-haemostatic activity and to investigate a possible relationship between the acquired natural tick resistance and the anti-haemostatic action of this tick. For this purpose, we have studied the effects of *R. microplus* saliva on platelet aggregation, endothelial activation and thrombogenesis. We have also investigated whether sera from acquired natural tick resistance cattle were able to counteract the anti-haemostatic action of tick saliva.

MATERIALS AND METHODS

Animals

Three male Hereford calves (*Bos taurus taurus*) of about 6 months of age from a tick-free area were utilized in this work. All procedures involving bovines were carried out in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999). Male Wistar rats (weighing 350–400 g) and male New Zealand rabbits (3·5–4 kg) were also used in this work, and were housed in temperature-controlled (21–24 °C, in 12-h light/dark cycles) rooms. All animals had access to water and food *ad libitum*. All experiments performed in this work were carried out in accordance with local institutional ethical guidelines about animal experimentation, and all procedures were also in accordance with the instructions of the Colégio Brasileiro de Experimentação Animal (COBEA) and NIH Animal Care Guidelines. Every effort was made to minimize the number of animals used and their suffering.

Drugs and reagents

Human plasma was obtained from 6 healthy male medication-free volunteers; bovine plasma was obtained from 3 tick-naïve male Hereford calves; all plasma samples were collected in 3·2% sodium citrate (1 : 10, v/v). Human thrombin was purified in our laboratory from plasma of healthy donors according to the method of Ngai and Chang (1991). Arachidonic acid was obtained and prepared following the instructions of manufacturer (Chrono-Log Co., Havertown, USA). Bovine collagen type I; adenosine diphosphate (ADP); bovine fibrinogen, lipopolysaccharide (LPS, *Escherichia coli* 0111:B4, cell culture tested, γ-irradiated); and HAT media supplement (Hybri-Max^{TM}, lyophilized powder, γ-irradiated) were purchased from Sigma-Aldrich (St Louis, MO, USA). All other culture media and additives were from Gibco Life Technologies (Gaithersburg, USA). Calcium thromboplastin (Soluplastin) was obtained from Wiener Lab (Rosario, Argentina). Sodium thiopental was purchased from Cristália Produtos Químicos Farmacêuticos (São Paulo, Brazil).

Infestation procedure and serum preparation

The tick-naïve bovines were infested with *R. microplus* larvae as described by Cruz and co-workers (2008). Briefly, 6 male *Bos taurus taurus* Hereford calves of about 6 months of age were purchased from an area naturally free of *R. microplus*. Infestations were performed with each calf being infested once a month for 12 months with *R. microplus* larvae of the Bagé strain along the back. Serum samples collected before the 1st infestation were called tick-naïve bovine sera (TNBS), and the serum samples collected after the 10th infestation were named as repeated infested bovine serum (RIBS). After 10 infestations, the bovines utilized to obtain RIBS displayed a significant decrease in the observed number of ticks that completed the parasitic cycle (Cruz *et al*. 2008).

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Tick saliva

Fully engorged *R. microplus* females that had spontaneously detached from the bovines were collected, rinsed, and induced to salivate with injection of 5 μl pilocarpine (2%). Ticks were maintained in a humid chamber and saliva was collected for 2 h (approximately 1 μl per tick). Saliva was stored at $-80\,^{\circ}$C until use. Saliva protein concentration was determined using the bicinchoninic acid method (BCA™ Protein Assay, Pierce, Rockford, USA) as previously described (Brown, 1989). Saliva quantities were expressed as μg or mg of protein. The saliva utilized in this work was collected only from engorged females which fed on bovines never exposed to ticks before.

Coagulation assay

Plasma samples from tick-naïve bovines (100 μl) were incubated for 15 min with phosphate-buffered saline (PBS) or tick saliva (5 μl; 80 μg/ml) previously incubated for 20 min with different volumes (15, 30, 60 and 90 μl) of TNBS, RIBS, or PBS. Coagulation was measured by Recalcification Time (RT), performed as previously described (Ribeiro *et al*. 1995; Berger *et al*. 2008). The assay was conducted using a 96-well microplate spectrophotometer (SpectraMax, Molecular Devices Co., Sunnyvale, USA) equipped with temperature and shaking controls. Results are the mean of 12 independent experiments.

Platelets

The platelet function was measured by *in vitro* photometric method (Born and Cross, 1963), in an optical aggregometer (Chrono-Log Co., Havertown, USA). Blood samples were collected by punction of marginal ear artery from male New Zealand rabbits using ACD solution as anticoagulant (2·5% trisodium citrate, 1·37% citric acid, 2% D-glucose; 1 : 5 v/v). Washed rabbit platelets (WRP) were obtained as follows. Blood was centrifuged 3 times at 200 ***g*** for 5 min to obtain platelet rich plasma (PRP). PRP was centrifuged at 650 ***g*** for 10 min. The supernatant was discarded and the platelet pellet was suspended in 300 μl of ACD solution. Then, platelets were purified/washed following a protocol developed by Timmons and Hawinger (1989) consisting of gel filtration in a Sepharose-2B column equilibrated and eluted with Tyrode-albumin buffer, pH 7·4. Washed platelet suspensions were supplemented with 2 mM $CaCl_2$ and 500 μg/ml of fibrinogen. The platelet count was performed manually in a Neubauer chamber (haemocytometer) using optical microscopy and the platelet concentration was adjusted to 350 000 cells/μl. Aggregation was measured using the decrease rate in WRP (300 μl) optical density (absorbance) in response to the addition of one of the following agonist agents: adenosine diphosphate (ADP) (10 μM), bovine collagen type I (40 μg/ml), arachidonic acid (10 μM), or human thrombin (2·5 U/ml). In order to verify whether *R. microplus* saliva was able to inhibit platelet aggregation, different concentrations of saliva were pre-incubated with platelets (for 15 min) before adding the agonist. For the experiments with serum, TNBS or RIBS were previously incubated (for 20 min) with *R. microplus* saliva before adding it to the platelet preparation. Results are the mean of 6 independent experiments.

Cell culture

EAhy926 is a human derived endothelial cell line originated by fusing human umbilical vein endothelial cells with the permanent cell line of human lung carcinoma, A549 (Edgell *et al*. 1983). EAhy926 has been used as a model to study the endothelium, since it displays conserved functional characteristics of an endothelial cell (Edgell *et al*. 1983). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), penicillin/streptomycin (200 units/ml and 200 μg/ml, respectively), and supplemented with 100 μM hypoxanthine, 0·4 μM aminopterin, and 16 μM thymidine (HAT media supplement), at 37 °C in an incubator with humidified atmosphere of air/CO_2 (95%/5%). As previously described, this cell line no requires additional growth factors (Edgell *et al*. 1983).

Cell surface-pro-coagulant assay

Pro-coagulant activity was measured on the surface of endothelial cell monolayers (EAhy926) using a 96-well microplate spectrophotometer (SpectraMax®, Molecular Devices Co., Sunnyvale, USA), based on the modified one-stage clotting assay (Visseren *et al*. 2000), as follows. EAhy926 cells cultured in DMEM (3% FCS) were seeded on 96-well microplates at a density of 40 000 cells/well and, after 24 h, different treatments were performed ($\approx$80% confluence). Cells were divided into 4 groups (6 wells per group) and received the following treatments (a final volume of 150 μl was completed with DMEM): (i) 30 μl of PBS; (ii) saliva (5 μl; 40 μg/ml) + 25 μl of PBS; (iii) saliva (40 μg/ml) + 25 μl of RIBS; or (iv) saliva (40 μg/ml) + 25 μl of TNBS. After 48 h all groups received 150 μg/ml of LPS to induce endothelial activation. After 24 h, culture medium was totally removed and cells were washed 3 times with PBS. Monolayers were incubated with 100 μl of Michaelis buffer (sodium-acetate 28·5 mM, sodium-barbital 28·5 mM, NaCl 50 mM, $CaCl_2$ 33 mM, pH 7·35) at 37 °C for 60 s, after which 100 μl of human pooled citrated plasma was added. The endothelial

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induced-plasma clotting was measured in a spectrophotometer. The value obtained for PBS-treated group (control) was considered as having 100% procoagulant activity. Results are the mean of 8 independent experiments. Additional experiments ($n=3$), using neutral red based cytotoxicity assay (TOX-4® *in vitro* toxicology assay kit, Sigma-Aldrich, St Louis, MO, USA), were performed to evaluate the cell viability after the different treatments.

In vivo *model of deep venous thrombosis*

For the determination of anti-thrombotic saliva activity, a rat thrombosis model was performed. The model is a combination of stasis and hypercoagulability induced by the injection of an exogenous tissue factor-rich component, as previously described (Vogel *et al*. 1989; Nazareth *et al*. 2006) with minor modifications. Wistar rats were anaesthetized with sodium thiopental (85 mg/kg, i.p.). Body temperature was monitored by a rectal thermometer and maintained at 36 °C ± 1 °C using a thermal surgical table, as previously established (Bohrer *et al*. 2007). A laparotomy was performed, and the caudal vena cava was carefully dissected from surrounding tissues. Rats received the following treatments *via* the left femoral vein (final volume of 0·7 ml was completed with PBS): (i) PBS; (ii) saliva (0·7 mg/kg of rat body weight); (iii) saliva (0·7 mg/kg of rat body weight) and 500 µl of TNBS; or (iv) saliva (0·7 mg/kg of rat body weight) and 500 µl of RIBS. After 5 min, calcium thromboplastin (3 mg/kg body weight) was injected in the vena cava (near to the right renal vein) and stasis was immediately established by the ligation of caudal vena cava (above the insertion point of the right renal vein). The distal ligations of the vena cava (above the common iliac veins confluence), left renal vein and other major tributaries were conducted exactly 20 min after thromboplastin administration. The isolated segment of the caudal vena cava was removed and carefully opened. The thrombus was separated from the isolated segment, rinsed with 0·9% NaCl (at 37 °C) and dried on a filter paper at 60 °C (1 h), and weighed. The ratio value of thrombus : rat weight was used in the comparisons. Results are the mean of 8 independent experiments.

Statistical analysis

Data are expressed as mean ± S.E.M. of n animals. Statistical significance was analysed by one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test correction. A p value of less than 0·.05 was considered significant. Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, USA) software.

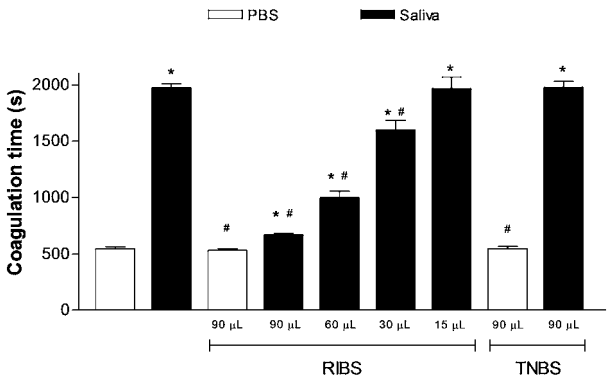

Fig. 1. Effects of repeatedly infested bovine sera (RIBS) on anti-coagulant activity of *Rhipicephalus microplus* saliva. The anti-coagulant action of saliva was measured by Recalcification Time (RT). The neutralization ability of sera from repeatedly infested cattle upon salivary anti-coagulation was also analysed. Saliva (80 µg/ml) was previously incubated with PBS, tick-naïve bovine sera (TNBS), or RIBS, as indicated, and then added to plasma samples to determine coagulation time. The volume (µl) of incubated PBS, TNBS or RIBS is also indicated. (*Statistical difference compared with group PBS + plasma, #statistical difference compared with group PBS + plasma + saliva, $P<0·05$).

RESULTS

Coagulation

R. microplus saliva significantly increased Recalcification Time (RT). When saliva (80 µg/ml) was pre-incubated with 100 µl of bovine plasma for 15 min, RT increased by almost 4 times, as compared with the control (Fig. 1). This anti-coagulant effect was almost totally neutralized when saliva was previously incubated (for 20 min) with 90 µl of repeated infested bovine sera (RIBS) before addition to the plasma in the RT assay (Fig. 1). This phenomenon seems to be dose-dependent, since the ability to block the anti-coagulant effect was directly related to serum quantity in the assay (Fig. 1). Experiments performed ($n=3$) with higher amounts of RIBS (200 µl) led to the same result observed for 90 µl (data not shown). In contrast, the tick-naïve bovine sera (TNBS) were not able to block the anti-coagulant effects of saliva (Fig. 1).

Endothelial cell modulation

In order to investigate whether *R. microplus* could impair haemostasis not only by inhibition of humoral factors but also by acting *via* endothelium, the ability of *R. microplus* saliva to negatively modulate endothelial activation was evaluated. For this purpose, a model of endothelial activation induced by LPS was employed. Cell activation was detected as a change in cell surface pro-coagulant activity. Endothelial cells (40 000 cells) previously incubated for 48 h with

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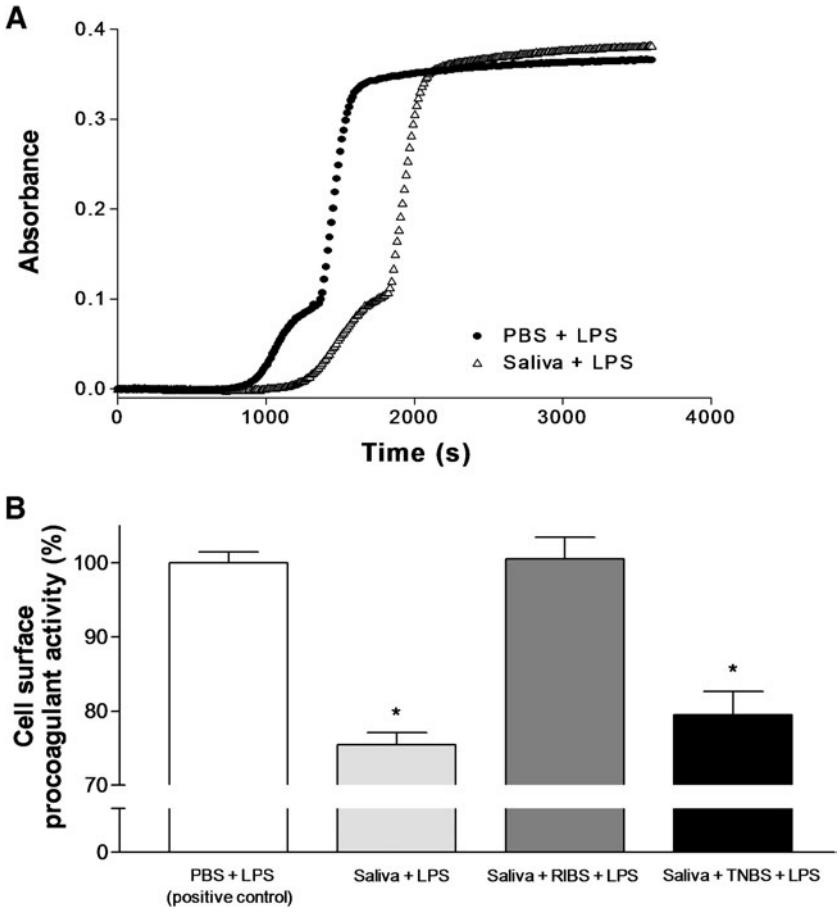

Fig. 2. Effects of *Rhipicephalus microplus* saliva and repeatedly infested bovine sera (RIBS)-treated saliva on the modulation of endothelial cell activation. The action of saliva on endothelial activation was analysed by cell surface-procoagulant assay. The neutralization ability of sera from repeated infested cattle upon salivary activities on endothelium was also analysed. (A) Representative register of the decrease in cellular pro-coagulant activity of EAhy926 monolayer pre-treated with saliva (40 μg/ml, △) compared with the control (cells pre-treated PBS, ●) after endothelial activation induced by LPS (150 μg/ml). (B) Pro-coagulant activity of LPS-activated EAhy926 cells previously treated with PBS (positive control), saliva (40 μg/ml) + PBS (25 μl), saliva (40 μg/ml) + RIBS (25 μl), or saliva (40 μg/ml) + TNBS (25 μl). (*Statistical difference compared with positive control group, $P<0{\cdot}05$).

R. microplus saliva (40 μg/ml) and activated with LPS (150 μg/ml for 24 h) exhibited reduced pro-coagulant surface activity as compared to the control (incubated with PBS) (Fig. 2A). Under the conditions used, this effect does not seem to be dose dependent, since a higher saliva concentration (80 μg/ml) induced the same effect as that caused by 40 μg/ml of saliva (data not shown). Similarly, lower saliva concentrations (10 and 20 μg/ml) were not able to induce significant changes in pro-coagulant activity (data not shown). A previous incubation of *R. microplus* saliva (40 μg/ml) with RIBS (50 μl) led to a complete blockade of endothelial modulation by saliva, since the cells displayed the same pro-coagulant profile of the control (previously incubated only with PBS) (Fig. 2B). Results from saliva previously incubated with the TNBS showed the same endothelial activation profile as those treated only with saliva (Fig. 2B). None of the treatments performed induced any significant differences in endothelial cell viability (data not shown).

In vivo *thrombosis model*

Considering that *R. microplus* saliva was able to inhibit coagulation and endothelial cell activation, these anti-haemostatic mechanisms were investigated *in vivo* using the rat model of deep venous thrombosis, which includes all haemostatic pathways. It was shown that *R. microplus* saliva (0·7 mg/kg of rat body weight) is able to significantly reduce the ratio thrombus : rat weight to about 40% of the control value (Fig. 3). Moreover, pre-incubation of saliva (0·7 mg/kg) with 500 μl of RIBS led to a partial reversion of anti-thrombotic activity, since the ratio thrombus/rat weight was recovered to about 80% of the control value (Fig. 3). Experiments performed ($n=3$) with higher amounts of RIBS

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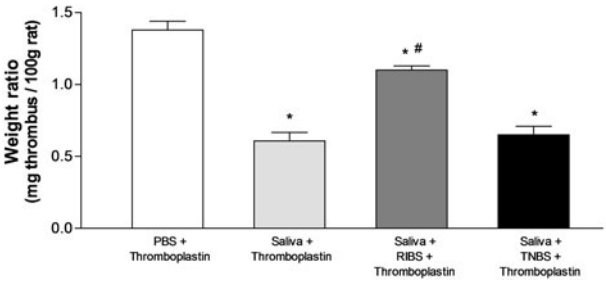

Fig. 3. Effects of *Rhipicephalus microplus* saliva and repeatedly infested bovine sera (RIBS)-treated saliva on thrombogenesis. The anti-thrombotic activity of saliva was measured by use of an *in vivo* model of rat deep venous thrombosis. The neutralization ability of sera from repeatedly infested cattle upon salivary anti-thrombotic action was also analysed. The treatments given to the rats were (i) PBS; (ii) saliva (0·7 mg/kg of rat body weight) and PBS; (iii) saliva (0·7 mg/kg) and 500 μl of TNBS; or (iv) saliva (0·7 mg/kg) and 500 μl of RIBS. The different treatments were administered (i.v.) 5 min before induction of thrombosis by thromboplastin (3 mg/kg rat body weight) and stasis, as described in the Materials and Methods section. (*Statistical difference compared with group PBS + thromboplastin, #statistical difference compared with group saliva + thromboplastin, $P < 0{\cdot}05$).

(700 and 1000 μl) led to the same result as observed for 500 μl (data not shown). TNBS did not reverse the anti-thrombotic action of *R. microplus* saliva (Fig. 3).

Platelet aggregation

Platelet aggregation studies were performed in an optical aggregometer using washed rabbit platelets (WRP) and thrombin, collagen, arachidonic acid and ADP as agonists. Firstly, experiments were performed in order to investigate whether *R. microplus* salivary secretion was able to inhibit platelet aggregation. For this purpose, different concentrations of saliva were incubated with WRP for 15 min (at 37 °C). Then, agonist was added and the change in absorbance was immediately recorded. No inhibition was observed for platelet aggregation induced by thrombin, arachidonic acid or ADP after pre-incubation of WRP with *R. microplus* saliva, even at a high saliva concentration (420 μg/ml) (data not shown). However, *R. microplus* saliva displayed inhibitory activity upon collagen-induced platelet aggregation, which was totally inhibited at the concentration of 200 μg/ml of saliva (Fig. 4A). This inhibition occurred in a dose-dependent fashion, with an IC_{50} of 53 μg/ml of saliva (Fig. 4B). It is interesting to note that, although RIBS induced a significant blockade of anti-coagulant, endothelium modulation and anti-thrombotic properties of *R. microplus* saliva, no interference in platelet aggregation inhibition induced by saliva was observed, even after incubation in the presence of high quantities of RIBS (150 μl/150 μl of WRP) (data not shown).

DISCUSSION

Ticks are blood-feeding animals that require mechanisms to modulate host defence against parasitism, such as vasoconstriction, haemostasis, inflammation, pain, and innate and adaptive immune responses (Maritz-Olivier *et al.* 2007; Francischetti *et al.* 2009). Despite the powerful tick evasion mechanisms, after repeated tick infestations vertebrate hosts may develop some degree of resistance to the parasite (Wikel, 1996*a*; Brossard and Wikel, 2004). Nevertheless, it occurs mainly in situations of repeated high parasite load. Acquired resistance to tick infestation is expressed as a decrease in engorgement weight, increased time of feeding, impaired egg production and viability, inhibition of moult as well as death of ticks (Wikel, 1982, 1996*b*). Although the host-tick relationship is one of the most extensively studied host-parasite associations, the knowledge about host reactivity, tick resistance and saliva modulators is not enough to afford a complete understanding of this process.

In this work, anti-haemostatic mechanisms of *R. microplus* saliva and the humoral response within anti-tick natural acquired resistance were investigated. According to a previous report (Horn *et al.* 2000), it was shown that *R. microplus* saliva was able to induce a significant increase (about 4 times) in plasma coagulation time measured in the RT assay. This saliva activity was imputed to the action of the thrombin inhibitors, BmAP (Horn *et al.* 2000) and microphilin (Ciprandi *et al.* 2006). Figure 1 shows that repeatedly infested bovine sera (RIBS) were able to significantly decrease the anti-coagulant effects of saliva in a dose-dependent fashion. Although there was a marked reduction in the saliva effect, a complete reversion of anti-coagulant effect was not observed. The blockade of salivary anti-coagulant activity clearly indicates that there are neutralizing molecules in RIBS, not present in TNBS, acting upon the salivary anti-coagulants, probably antibodies, since these anti-coagulants are secreted in the saliva and inoculated into the host. Analogously, a study by Prevot and co-workers (2007) reported the ability of repeatedly infested rabbit serum to neutralize the enzymatic inhibitory activity of *Ixodes ricinus* salivary serpin. This kind of serum neutralization assay, although frequently reported against biological action of microbial diseases (Rutter *et al.* 1975; Johnson *et al.* 1995), was poorly reported for ectoparasites.

Although the effects of *R. microplus* saliva upon coagulation are well known (Horn *et al.* 2000; Ciprandi *et al.* 2006), other possible anti-haemostatic mechanisms have not yet been fully characterized,

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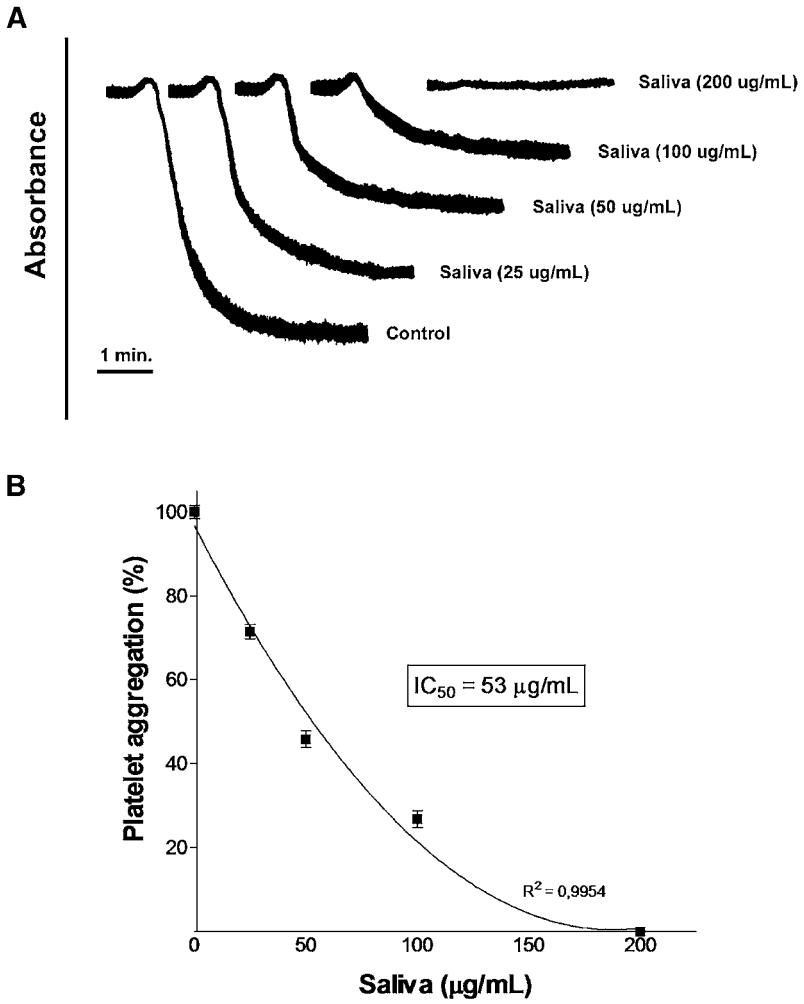

Fig. 4. Effects of *Rhipicephalus microplus* saliva on collagen-induced platelet aggregation. The inhibitory activity of crescent concentrations of *R. microplus* saliva (25, 50, 100 or 200 μg/ml) upon WRP aggregation induced by collagen (3 μM) was measured. (A) Representative profile of collagen induced-platelet aggregation of WRP previously incubated with *R. microplus* saliva. (B) Each point represents the mean from 6 independent experiments, and vertical lines indicate the S.E.M.

leaving significant gaps concerning the role saliva molecules play in other haemostatic events, such as platelet aggregation, endothelial response, and thrombogenesis. The data presented here clearly show that *R. microplus* saliva exerts a significant effect upon the endothelium. Figure 2A demonstrates that saliva is able to reduce the endothelial procoagulant activity induced by LPS due to a direct effect of saliva upon endothelial cells, probably *via* an alteration in protein surface composition and/or induced gene expression (Makrides and Ryan, 1998). Endothelial activation is a complex process which includes a reversion in the cell phenotype, changing from an anti-thrombotic state to a pro-thrombotic state in response to several stimuli, like cellular lesion, bacterial and viral infection, peptide signalling, or chemical damage (Gibbons, 1998). Modulation of endothelial cells by tick saliva seems to be an alternative pathway to control host defences independently of humoral mechanisms, which could allow the parasite to regulate haemostasis, inflammation, immunity and lesion cicatrization. Maxwell and co-workers (2005) have demonstrated that salivary gland extracts of *Dermacentor andersoni* significantly down-regulated the expression of inflammatory molecules, such as ICAM-1. They have also reported that salivary gland extracts of *I. scapularis* reduced the expression of different adhesion-molecules, such as selectins and VCAM-1. Francischetti and co-workers (2005) have reported the potent anti-angiogenic and anti-proliferative activities of *I. scapularis* saliva upon endothelial cells, introducing the novel notion that tick saliva is a negative modulator of wound healing and tissue repair. Despite these two very elegant reports about tick saliva and salivary gland action on the biology of endothelial cells, the modulation of the haemostatic properties of the endothelium by ticks has never been reported before. A lingering doubt surrounds the ability of other ticks, specially two and three host-ticks, to modulate the haemostatic properties of the endothelium in a similar fashion.

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It is interesting to note that when saliva was pre-incubated with RIBS, the salivary modulatory effects upon endothelium were totally blocked, differently from that which occurs with coagulation, where the serum blocking effect was only partial. Thus, host neutralization ability seems to be more powerful for the salivary molecules responsible for the modulation of endothelium than for coagulation. Despite the fact that cell modulation by ticks is possibly triggered by several molecules with synergistic action – or by a cocktail of compounds (Wikel *et al*. 1996) – it seems that infestation induces the production of different neutralizing antibodies able to block this seemingly multifactorial effect.

The occurrence of haemostatic disturbances in tick-infested calves indicates that *R. microplus* saliva is able to modulate haemostasis *in vivo* (Reck Jr *et al*. 2009), and it strongly suggests the presence of *in vivo* anti-thrombotic activity. In order to evaluate the anti-haemostatic properties of saliva in a model that reproduces all pathways involved in haemostasis, we investigated the anti-thrombotic ability of tick saliva using the *in vivo* model of rat deep venous thrombosis. Although other ticks display salivary anti-thrombotic properties (Fioravanti *et al*. 1993; Nazareth *et al*. 2006) and despite the fact that the *in vitro* anti-coagulant effects of *R. microplus* saliva are well known, the anti-thrombotic activity of *R. microplus* saliva has never been reported before. Here we have shown that *R. microplus* saliva clearly modulates thrombogenesis, and that RIBS were able to induce a significant decrease in saliva anti-thrombotic activity. This reduction, however, does not promote a complete recovery of the thrombotic activity, since the group treated with RIBS significantly differs in comparison with both the control group (treated only with thromboplastin) and the saliva-treated group. The residual anti-thrombotic activity of saliva was probably due to the fact that RIBS neutralization activity seems to be different in the three haemostatic pathways studied here, since RIBS are able to totally neutralize the action of tick saliva upon endothelium and to nearly block the effect of salivary anti-coagulant, although it has no neutralizing activity upon platelet aggregation inhibition. Taking into account the variety of compounds that participate in the thrombogenesis *in vivo*, the anti-thrombotic effect exerted by *R. microplus* saliva probably implies a wide arsenal of molecules that, as regards immune evasion mechanisms, tend to avoid the recognition of the epitopes necessary to completely neutralize the overall effect. In fact, a full blockage of all activities involved in anti-thrombosis would probably result in suppression of blood feeding. But in fact this does not occur, since some ticks remain attached to the resistant host and succeed to complete their parasitic cycle, though with a marked reduction in number and viability of parasites (Wikel, 1982; Cruz *et al*. 2008).

Since several ticks and other haematophagous arthropods display platelet antagonists as one of their anti-haemostatic resources, the occurrence of platelet aggregation inhibitors in *R. microplus* was investigated. The present study reports, for the first time, that *R. microplus* saliva is able to inhibit the collagen-induced platelet aggregation in WRP. This inhibition could be related to the collagen-binding component reported in the salivary glands of *R. microplus* (Ferreira *et al*. 2002). Collagen-induced platelet aggregation may occur through two distinct pathways, directly *via* activation of phospholipase C or *via* arachidonic acid pathway or indirectly *via* ADP release from cell granules. The fact that *R. microplus* saliva does not inhibit arachidonic acid-induced platelet aggregation indicates this tick uses a distinct mechanism to inhibit collagen-induced platelet aggregation, as compared to *Ornithodoros moubata* (Mans and Ribeiro, 2008). Surprisingly, no saliva inhibition was observed in platelet response to thrombin, arachidonic acid or ADP (data not shown), in spite of the fact that *R. microplus* possesses two thrombin inhibitors in saliva. The lack of inhibition in thrombin-induced platelet aggregation could be due to an insufficient concentration reached by the salivary thrombin inhibitors in the saliva preparations utilized, leading to an unfeasible inhibition only because the inhibitor concentration is not high enough to suppress thrombin activity. This hypothesis is corroborated by comparing the saliva concentration used in the experiment with the Ki of microphilin, which is in the micromolar range (Ciprandi *et al*. 2006). Indeed, this result seems to contradict a previous report, since microphilin, an *R. microplus* thrombin inhibitor, inhibits fibrinocoagulation and thrombin-induced platelet aggregation (Ciprandi *et al*. 2006). However, besides microphilin Ki, this contrasting finding could be explained by analytical differences between the studies, since we performed the incubation of saliva with the platelets and added the agonist after a time interval (thrombin, collagen, arachidonic acid or ADP), while Ciprandi and co-workers (2006) performed the incubation of saliva with thrombin and added the mixture to platelets after a time interval. The approach employed by Ciprandi and co-workers (2006) was designed to characterize the effects of thrombin inhibition by microphilin binding.

It is interesting to note that among all activities investigated, the inhibition of platelet aggregation is the only one in which the RIBS used were not able to interfere (data not shown), which suggests that the salivary anti-platelet molecule(s) has (have) low immunogenicity and is probably due to the naturally acquired resistance to ticks in the bovines used. However, this does not imply that the platelet aggregation inhibitor should be dismissed as an unfeasible resource for the development of an anti-tick vaccine. In this sense, Kotsyfakis and co-workers

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(2008) conducted an efficient study that introduced a new concept in the tick-host relationship–the silent antigens–like sialostatin L2. This molecule was found in *I. scapularis* saliva and was not recognized by sera of guinea pigs repeatedly exposed to ticks; yet, when sialostatin L2 was used as a vaccine antigen, the serum of vaccinated animals recognized the protein and the immunization procedure lent protection to the hosts (Kotsyfakis *et al*. 2008).

Despite the widely accepted notion that tick saliva is largely non-immunogenic, our study suggests that anti-coagulants, endothelial modulators and anti-thrombotic molecules are immunogenic and could be used as vaccinal antigens to reproduce the resistance status of repeatedly infested bovines, which would be accomplished through a vaccination strategy, and possibly reinforced by natural infestations. However, our results were obtained with non-diluted sera, indicating that this immunogenicity is not as high as that obtained by highly immunogenic antigens, as concealed antigens. Accordingly, our data indicate that the molecules involved in anti-haemostasis may be candidates to compose a cocktail vaccine, possibly in a strategy associating concealed and silent antigens.

Studies evaluating the relative importance of an adaptative humoral response in bovine resistance against *R. microplus* presented conflicting results. The levels of anti-*R. microplus* antibodies were not positively correlated with resistance in some analyses (Johnston *et al*. 1986; Kimaro and Opdebeeck, 1994; Jackson and Opdebeeck, 1995), but were positively correlated in others, either originated from vaccination (Willadsen, 1987; Willadsen *et al*. 1995) or repeated infestations (Cruz *et al*. 2008). Here we show that sera from resistant bovines, although presenting low titres of anti-tick antibodies (Cruz *et al*. 2008), were able to impair anti-haemostatic tick saliva activities. These results suggest that global levels of anti-tick antibodies are not undoubtedly indicative of host resistance. Since levels of antibodies are modulated by tick infestation (Cruz *et al*. 2008), antibodies induced by high-density infestations could be predominantly targeted to unessential and/or a fraction of functionally redundant molecules.

Although the biochemical and immunological basis of anti-haemostatic mechanisms of ticks (Ribeiro, 1995; Ribeiro and Francischetti, 2003; Maritz-Olivier *et al*. 2007; Francischetti *et al*. 2009) and the natural acquired resistance (Wikel, 1996*a*, *b*) have been widely studied, reports concerning the possible relationship between these two phenomena are scarce. The present work shows, for the first time, the occurrence of neutralizing activity against the salivary anti-haemostatics of ticks in sera of repeatedly infested hosts that exhibit natural acquired resistance. Also, this study is the first to report a haematophagous parasite modulating the endothelial cell pro-coagulant state, and shows the presence of platelet aggregation inhibitory and anti-thrombotic action in *R. microplus* saliva.

Finally, since acquired resistance and successful parasitism seem to depend on a state of balance between host defences and tick modulators (Wikel, 1996*a*), this study allows a better understanding of the relationship between the acquired natural tick resistance and the anti-haemostatic action and gives more insights to elucidate mechanisms related with the tick-host interaction.

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