Activities of the Enzymes NTPDase, 5´-Nucleotidase and Adenosine Deaminase in Platelets of Rats Experimentally Infected with Leptospira interrogans Serovar icterohaemorrhagiae

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ABSTRACT

Background: Leptospirosis, a spirochetal zoonotic disease caused by different serovars of Leptospira interrogans, is increasingly recognized as an important cause of hemorrhagic fever. Although the haemorrhagic potential of leptospirosis was noted by Weil (1886) as early as 1886, its pathophysiology is still not clearly elucidated, particularly regarding the cause and mechanisms of bleeding. Studies with ectonucleoside triphosphate diphosphohydrolase (NTPDase; EC 3.6.1.5; CD39), 5´-nucleotidase (EC 3.1.3.5; CD73) and adenosine deaminase (ADA) have demonstrated the involvement of these enzymes in thromboregulation mechanisms, and altered enzymatic activities have been reported in many diseases. Since leptospirosis is a disease increasingly recognized as an important cause of hemorrhagic fever, the aim of this study was to evaluate these enzymes activities and parameters of platelet aggregation in platelets from rats experimentally infected with Leptospira interrogans serovar icterohaemorrhagiae during different periods of experimental infection.

Materials, Methods & Results: For this purpose, thirty-six adult male rats were divided into two groups: A, as uninfected control (subgroups A1, A2 and A3); and B, infected (subgroups B1, B2 and B3). Group B was inoculated intraperitoneally (Day 0) with $2 \times 10^8$ organisms per rat. Blood samples were collected on days 05 (A1 and B1), 10 (A2 and B2) and 20 (A3 and B3) post-inoculation (PI). In the infected group, platelet count had a decrease on day 10 PI and prothrombin time (PT) had an increase on day 5 PI. In the same group, platelet aggregation decreased ($P < 0.01$) day 10 PI. The hydrolysis of ATP in platelets was also decreased ($P < 0.05$) on day 10 PI, when compared to the control group. By the other side, ADP hydrolysis was increased ($P < 0.05$) on days 5 and 10 PI. 5´-nucleotidase activity was significantly increased on day 5 ($P < 0.01$) and 20 ($P < 0.05$) PI. Results of adenosine deamination into inosine by ADA in platelets showed a significant ($P < 0.01$) increase on days 5 and 10 PI in the infected group.

Discussion: Studies with NTPDase, 5´nucleotidase and ADA have demonstrated the involvement of these enzymes in the thromboregulation mechanisms, and altered enzymatic activities have been reported in many diseases. It has been established that extracellular adenosine nucleotides and adenosine are versatile signaling molecules known to participate in an array of platelet functions. For example, the nucleotide ADP is the main promoter of platelet aggregation, while adenosine can act as a vasodilator and an inhibitor of platelet aggregation. In addition, high concentrations of ATP have been shown to inhibit ADP-induced aggregation in vitro, while low concentrations of ATP can significantly enhance platelet aggregation. In our experimental study the coagulation cascade was activated, since when the activities of NTPDase, 5´-nucleotidase and ADA were analyzed is possible to suggest that levels of ATP were decreased, unlike of ADP and AMP levels, supposedly increased during determinate periods of our experiment. Adenosine levels were also enhanced due to the higher levels of its precursors. This cascade activation may be a mechanism of bleeding prevention front to leptospires infection, especially the ones caused by serovar icterohaemorrhagiae.

Keywords: Leptospirosis, Leptospira interrogans, ectonucleotidasis, adenosine nucleosides, thrombocytopenia.
INTRODUCTION

Leptospirosis, a spirochetal zoonotic disease, is increasingly recognized as an important cause of hemorrhagic fever [23,36]. Pathologist's findings in humans and animals underline the bleeding tendency, and show widespread haemorrhages [15,29,31,32,38,41]. Although the Weil’s disease to be well known by its haemorrhagic potential [19,21], its pathophysiology is still not clearly elucidated, particularly regarding the cause and mechanisms of bleeding.

Coagulopathies are alterations related to the number of platelets or to the coagulation cascade that consequently interfere in the coagulation process [20]. Platelets play important homeostatic functions through the mechanisms of adhesion, aggregation and subsequent formation of clots at sites of vascular damage [16,26,42], performing a fundamental role in normal homeostasis [25]. They are distributed into two distinct pools; the metabolic, used for maintaining cellular functions, mainly constituted of ATP; and the storage pool, which contains ATP and ADP that are released during platelet secretion [22]. The platelet’s surface contains purinergic receptors for the nucleotides ATP and ADP and their levels are controlled by the enzymes NTPDase and 5’-nucleotidase [33]. Adenine nucleotides are considered important endogenous signaling molecules in inflammatory and immunological processes and in vasomotor responses [7,18].

The study of such enzymes and their activities in platelets from icterohaemorrhagiae-infected animals is fundamental, due to their high importance to the homeostasis maintenance. Thus, the aim of this study was to evaluate the NTPDase, 5’-nucleotidase and ADA activities in platelets from Leptospira interrogans serovar icterohaemorrhagiae-infected rats during different periods of the experimental infection.

MATERIALS AND METHODS

Animals

Thirty-six adult male rats with a mean age of 60 days and weighing an average of 200 ± 13 g were used in this study. The animals were housed in cages, six per cage, in a room with controlled temperature and humidity (25°C; 70%). They were fed with a commercial ration and water ad libitum and acclimated for 15 days. On day zero, all animals were clinically normal. Serology was performed to confirm that the animals were seronegative at day zero and seropositive on day 15.

Isolation and infection

Leptospira interrogans, serovar icterohaemorrhagiae strain was maintained in the Leptospirosis Laboratory of the Department of Microbiology and Parasitology belonging to the Universidade Federal de Santa Maria (UFSM). Ellinghausen-McCullough-Johnson-Harris (EMJH) medium¹ was used for maintenance and propagation of leptospiral cultures. Semisolid EMJH medium with 0.2% agar was used for maintenance of stock cultures. Rats were divided in two groups: A, uninfected control (subgroups A1, A2 and A3); B, infected (subgroups B1, B2 and B3); groups consisted of 18 animals (6 each subgroup). The rats from group B were inoculated intraperitoneally (Day 0) with 2 x 10⁸ organisms per rats.

Estimation of bacteremia

Infection was estimated at days 5, 10 and 20 post-infection (PI) by microscopic examination of urine on a slide. One drop of urine was placed between slide and coverslip and examined under darkfield microscopy at a magnification of 400x. Blood from each animal was collected days 5, 10 and 20 PI and a drop mixed in modified EMJH medium with 5-fluorouracil² (300 mg/L)² and nalidixic acid³ (20 mg/L), using a serial dilution technique, to evaluate the percentage of recovery of the added microorganisms [28]. These were stored at 29°C.

Samples collection and platelet counts

On days 5 (A1 and B1 groups), 10 (A2 and B2 groups) and 20 (A3 and B3 groups) PI, 6 animals per group were anaesthetized with isoflurane⁴ for blood collection. The material collected was allocated in tubes with anticoagulant (sodium citrate) for quantification of platelets, clotting time evaluation and separation of platelets to carry out the enzymatic tests.

Platelets and clotting time

The platelet counts were performed by using a Neubauer chamber filled with whole blood diluted in calcium oxalate. Prothrombin time (PT) and activated prothrombin time (aPTT) were determined by commercial kits⁵.

Platelet aggregation

Platelet aggregation was assessed by turbidimetric measurement with a Chrono-log optical aggregometer, with AGGRO/LINK® Model 810-CA
software [4] for Windows version 5.1. The platelet rich plasma (PRP) was obtained by centrifugation of blood for 20 min at 1500g and the preparation of platelet poor plasma (PPP) was obtained by centrifugation of the sample at 5000g for 30 min. After calibration of the aggregometer, the animal data concerning the assays and reagents were entered on a computer coupled to the equipment and the aggregation assay was then performed. Aggregation was measured at 37°C and expressed as the maximal percent change in light transmittance from baseline at 5 min after the addition of the agonist ADP at concentrations of 10 µM, with PPP as a reference. The results were expressed as percentage of aggregation.

**Platelet preparation and protein determination for enzymatic activity**

Platelets were isolated from plasma obtained from blood collected with sodium citrate as anticoagulant (0.129M, 1:9 v/v) [25,33]. Cell viability was estimated by lactate dehydrogenase release before and after incubation at 37°C. Values of the sample were compared to the enzymatic activity obtained from cells lysed with 0.1% Triton X-100 [1]. For protein determination of isolated platelets was measured by the Coomassie blue method using bovine serum albumin as standard [6].

**NTPDase and 5’-nucleotidase activity determination**

The NTPDase enzymatic assay was carried out in a reaction medium containing 5 mM CaCl2, 100 mM NaCl, 4 mM KCl, 50 mM glucose and 50 mM Tris-HCl buffer, pH 7.4, at a final volume of 200 µL [33]. For AMP hydrolysis, the ecto-5’-nucleotidase activity was carried out as previously described [25,33], except that the 5 mM of CaCl2 was replaced by 10 mM of CaCl2. Twenty microliters of platelets preparation (8-12 µg of protein) were added to the reaction mixture and the pre-incubation proceeded for 10 min at 37°C. The reaction was initiated by the addition of ATP or ADP at a final concentration of 1.0 mM, and AMP at a final concentration of 2 mM. The time of incubation was 60 minutes. Both enzymes activities were stopped by the addition of 200 µL of 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. Subsequently, the tubes were chilled on ice for 10 min. Released inorganic phosphate (Pi) was assayed using malachite green as the colorimetric reagent and KH2PO4 as standard [8]. Controls were carried out to correct for non-enzymatic hydrolyses of nucleotides by adding enzyme preparation after TCA addition. All samples were run in triplicates. Enzymes specific activities are reported as nmol of Pi released/min/mg of protein.

**ADA activity**

ADA activity was determined through the method [13], briefly described: 50 uL of platelets reacted with 21 mmol/L of adenosine pH 6.5 and was incubated at 37°C for 60 min. This method is based on the direct production of ammonia when ADA acts in excess of adenosine. The protein content used for the platelet experiment was adjusted to 0.7-0.9 mg/mL. Results were expressed in units per liter (U/L). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

**Statistical analysis**

Data were submitted to analysis of Student’s t-test (P < 0.05). The effect of enzymes NTPDase, 5’-nucleotidase and ADA in the number of platelets was analyzed by correlation. The analyses were performed using SAS statistical package (SAS Institute, Cary, NC, USA) with a significance level of 5% (P < 0.05).

**RESULTS**

**Course of infection and clinical changes**

The animals were inspected daily for clinical signs. In the first five days PI behavior was normal, but on the third day a decrease in feed intake and increase in water consumption was noticed. Weight loss, apathy and lethargy were also observed, especially in the first week PI. From about the seventh day after infection, the animals returned to normal food and water intake, weight gain and behavior. The control group did not display any of these signs. All rats were seronegative at day 0. On days 5, 10 and 15 PI, all exposed animals had titers ranging from 1:200 to 1:800.

After the fifth day, leptospires were seen in the urine of rats in the infected group and this continued until the day 20 PI. Spirochetes were not seen in the urine of any rat in the control group. Blood samples, collected on day 5 PI from the infected group, showed growth of leptospires in EMJH after the sixth week of culture. Growth was not obtained neither in the blood obtained at days 10 and 20 from the infected group, nor from the control group.
Platelet count, clotting time and platelet aggregation

Platelet count had no differences on days 5 and 20 PI, unlike of day 10 PI in which a decrease of platelets number was observed in the infected group (Figure 1-A). Prothrombin time (PT) had an increase on day 5 PI, however on days 10 and 20 it did not show any statistical differences (Figure 1-B). Activated prothrombin time (aPTT) had no statistical difference throughout the experiment between infected and non infected groups (Figure 1-C), showing only an increase tendency in all groups.

Platelet aggregation was decreased ($P < 0.01$) in the infected group on day 10 PI and showed a tendency to reduction day 5 when it was compared to the control group (Figure 2).

Enzymatic activities

Results of ATP, ADP and AMP hydrolysis by NTPDase and 5′-nucleotidase activities are presented
in Figures 3 and 4. The hydrolysis of ATP by NTPDase was decreased \( (P < 0.05) \) on day 10 PI, when compared to the control group (Figure 3-A). By the other side ADP hydrolysis was increased \( (P < 0.05) \) on days 5 and 10 PI compared to control group, while day 20 PI the hydrolysis was normal and equal to both groups (Figure 3-B). Hydrolysis of 5’-nucleotidase was significantly increased on day 5 \( (P < 0.01) \) and day 20 \( (P < 0.05) \) PI, while on day 10 PI it had only a tendency of increase (Figure 4). Results of adenosine deamination by ADA activity in platelets showed a significant increase \( (P < 0.01) \) on day 5 platelets showed a significant increase on day 5 \( (P < 0.01) \) and day 10 \( (P < 0.05) \) in the infected group (Figure 5).

A positive and significant correlation \( (P < 0.05) \) between platelet count versus ATP \( (r = 0.63) \) hydrolysis was observed on day 10 PI. By the other side, negative and significant correlations \( (P < 0.05) \) between platelet count versus ADP \( (r = 0.59) \) hydrolysis and platelet count versus adenosine \( (r = 0.68) \) deamination were observed in the infected group on day 10 PI. No significant correlations \( (P < 0.05) \) were observed between platelet count versus enzyme activities on days 5 and 20 PI.

**DISCUSSION**

The clinical signs that usually accompany bacterial infections include a decrease in food intake and disturbances of carbohydrate, protein, and fat metabolism [10,24]. This might explain the observed decrease in food and water consumption, and also the apathetic behavior of infected animals in the first days PI. Researchers observed a slight elevation in body temperature in guinea pigs following the injection of a leptospiral infected tissue suspension. Marked hyperthermia was observed at the end of the first day, and it continued to rise for the next two days [17]. Other signs observed were anorexia and prostration, also seen in our study.

Thrombocytopenia is frequently found in leptospirosis [12,30], and has been found also in dogs with experimental infection with serovar icterohaemorrhagiae [14], however the mechanism is not well defined. A prospective study showed that low platelet count was not a risk factor associated with mortality in patients with leptospirosis [27]. A previous study considered thrombocytopenia as the only hemostasis-related factor independently associated with clinical bleeding [9]. Thrombocytopenia was observed on day 10 PI, while on days 5 and 20 PI the platelets count was normal. Severe leptospirosis may result in an acute endothelial injury. *Leptospira* lipopolysaccharide stimulates neutrophil adherence and platelet activation, which may precipitate inflammatory and coagulation abnormalities [14]. During this period of leptospiremia (lasting anywhere from 3 to 20 days), the organism also invades and colonizes the liver, kidney, spleen, eye, and reproductive tract. Circulating antibodies are generated early in the course of infection and usually remove leptospires from the bloodstream and most tissues [40]. This fact may contribute to explain our findings, since during the leptospiremia the organism is reproducing in the bloodstream of the host, generating micro vascular injuries which lead to platelet activation and subsequent thrombocytopenia. The first evaluation (day 5 PI) may be too early to observe the reduction of platelets number, since at this time it may be occurred the leptospiremia onset. By the other hand, on day 10 PI, the animals were already exposed to the vascular effects of the leptospiral multiplication phase and the platelets number showed a decrease resulting of the organism response to the vascular injury. Appearance of circulating antibodies coincides with the clearance of leptospires from blood and most organs [3], minimizing the vascular injuries, neutrophil adherence and platelet activation, leading the platelet return to normal levels. This phenomenon may explain the normal count of platelets number day 20 PI.

While thrombocytopenia is especially common, a minority of patients also presented prolonged prothrombin time (PT) due to hypoprothrombinaemia [9,34], therefore our results showed a prolonged PT on day 5 PI. The prothrombin time is the time it takes plasma to clot after addition of tissue factors, measuring the quality of the extrinsic pathway of coagulation. This enhancement is directly associated to the reduction in the platelet aggregation, as observed in our results. We believe that the increased prothrombin time (day 5) may be a consequence of the high number of leptospires in the bloodstream during the leptospiremia period, which could interfere, directly or indirectly, in the coagulation factors.

Studies with NTPDase, 5’ nucleotidase and ADA have demonstrated the involvement of these enzymes in the thromboregulation mechanisms, and altered enzymatic activities have been reported in many diseases [35]. It has been established that extracellular adenosine nucleotides and adenosine are
versatile signaling molecules known to participate in an array of platelet functions [11]. For example, the nucleotide ADP is the main promoter of platelet aggregation [2], while adenosine can act as a vasodilator and an inhibitor of platelet aggregation [39]. In addition, high concentrations of ATP have been shown to inhibit ADP-induced aggregation in vitro, while low concentrations of ATP can significantly enhance platelet aggregation [37]. Our results allow suggesting that the coagulation cascade was activated, since the hydrolysis of ATP by NTPDase was decreased on day 10 PI in contrast to what occurred to the hydrolysis of ADP, which was increased on days 5 and 10 PI. Hydrolysis of AMP by the enzyme 5’-nucleotidase was also increased on days 5 and 20 PI and had a tendency on day 10 PI, both enzymatic mechanisms contributing to the platelet aggregation. The activity of ADA was also increased on days 5 and 10 PI. It is well known that ADA is responsible for regulating the extracellular concentrations of adenosine [37], which has anti-inflammatory and antiplatelet activity [5]. Higher activity of ADA may lead to a fast conversion of adenosine to inosine, contributing in the process of platelet aggregation.

These facts taken together may lead to an enhancement of platelet aggregation, especially on day 5 PI when the PT was increased and on day 10 PI when we observed thrombocytopenia. Knowing that during the leptospiremia the host is more exposed to endothelial damage, we also suggest a protective effect in an attempt to prevent possible bleeding in an acute phase (days 5 and 10 PI) of leptospirosis.

CONCLUSIONS

Analyzing the activities of NTPDase, 5’-nucleotidase and ADA it is possible to suggest that levels of ATP were decreased, unlike of ADP and AMP levels, supposedly increased during determinate periods of our experiment. Adenosine levels were also enhanced due to the higher levels of its precursors. More studies are necessary to better understand the bleeding phenomena which sometimes happened in leptospirosis. These results also suggest that a primary hemostasis was activated and may a secondary hemostasis by depletion of coagulation proteins as a consequence of the enhanced coagulation or the activated fibrinolysis could be more strongly associated to the bleedings.

SOURCES AND MANUFACTURERS
1 EMJH - Becton-Dickinson Biosciences/DIFCO, Detroit, USA.
2 5-Fluorouracil - ICN Pharmaceuticals Ltd, Basingstoke, U.K.
3 Nalidixic Acid - SIGMA, St. Louis, MO, USA.
4 Isoforine - CRISTÁLIA Produtos Químicos Farmacêuticos Ltda. Itapira, SP, Brazil.
5 PT and APTT Hemostasis - Labtest Diagnóstica AS, Lagoa Santa, MG, Brazil.

Ethical approval. The procedure was approved by the Animal Welfare Committee of Universidade Federal de Santa Maria (UFSM), number 47/2009.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES


