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Influence of experimental *Anaplasma marginale* infection and splenectomy on NTPDase and 5'nucleotidase activities in platelets of cattle

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ABSTRACT

The objective of this paper was to evaluate NTPDase and 5'-nucleotidase activities in platelets of bovine with and without spleen and infected by *Anaplasma marginale*. Our results demonstrate that infection along with splenectomy is able of inducing a profile of cellular protection, which showed an increase in the degradation of the nucleotides ATP and ADP by NTPDase, in addition to AMP by 5'nucleotidase to form the nucleoside adenosine in platelets, i.e., the enzymatic activities of platelets were increased in splenectomized animals when compared to non-splenectomized group. It notes that adenosine is a molecule with anti-inflammatory function. But this profile is related to a deficiency in immune signaling triggered by nucleotide ATP, which may be related to the increase in bacteremia and disability in combating the parasite in splenectomized host.

1. Introduction

Anaplasma marginale (*A. marginale*) is a pathogen that belongs to the Order Rickettsiales with a worldwide distribution mainly in tropical and subtropical regions [1]. This hemoparasite is transmitted to cattle by approximately 20 species of ticks, and mechanically by flies and infected fomites. After infection, the parasite settles into the bloodstream trough the invasion of the erythrocytes, the only known site of replication in cattle [2].

The acute phase of the disease is characterized by infection of more than 70% of the erythrocytes in the bloodstream, weight loss, fever, lowered milk production and often animal death. However, cattle can recover from acute infection, becoming healthy carriers while maintaining low levels of bacteremia [3]. This fact is related to the removal capacity of infected cells by the reticuloendothelial system in the spleen, which is a lymphatic organ directly connected to the blood-circulatory system and it is the largest reservoir and disposal site for blood platelets. Even though the spleen has important physiological functions, it is not considered essential for life and in some medical cases it is advised its removal, and such surgery is called splenectomy [4]. Splenectomy may cause a deficiency in removing damaged infected erythrocytes. In addition, some studies have shown that splenectomy can result on vascular endothelium disturbances, altered lipid profiles, hypercoagulability, and platelet activation [5].

Platelets are enucleate cells present in high levels in the bloodstream and are essential for the physiological process of blood coagulation, and repair of damaged endothelium [6]. Moreover, studies have demonstrated that these cells have important intercellular interactions, being a key cell in triggering innate and adaptive immune responses. This is because platelets have immune

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receptors that interact with pathogens and leukocytes, releasing cytokines and other factors that act as mediators of inflammation and immune activity [7].

Among the factors that can activate and modulate platelets function are adenine nucleotides, which can interact with purinoreceptors present in the plasma membrane of these cells [8]. Adenosine triphosphate (ATP), diphosphate (ADP) and monophosphate (AMP) can interact with the P2 type receptors, and adenosine (Ado) interacts with A2 type receptors [9]. ATP can inhibit platelet activation and also the release of pro-inflammatory cytokines, since ADP induce vasodilation and platelet aggregation, while Ado acts as a potent endogenous anti-inflammatory agent [10]. The effect of these nucleotides on the extracellular face is modulated by enzymes in the plasma membrane of platelets capable of degrading nucleotides [11]. NTPDase is the enzyme responsible for hydrolyzing ATP to ADP, and also ADP to AMP, which finally is degraded to adenosine by the 5'-nucleotidase enzyme. Authors have demonstrated the important role of ectonucleotidases in numerous physiological functions and diseases [12,13], as well as the role of purine molecules and ectonucleotidases in others parasites infections [14]. Therefore, this study was designed to evaluate NTPDase and 5'-nucleotidase activities in platelets of cattle (splenectomized and non-splenectomized) experimentally infected by *A. marginale*.

2. Materials and methods

2.1. Animals

Female calves (n = 8) with 6–8 months of age, Angus breed were selected from a farm located in a naturally tick-free zone. All animals tested were seronegative for *A. marginale*, *Babesia bigemina* and *Babesia bovis*, infectious bovine rhinotracheitis (IBR), bovine viral diarrhea (BVD), and *Leptospira* spp. three weeks before arrival at the *Instituto de Pesquisa Veterinária Desidério Finamor*, where the experiment was performed. The animals were kept in individual units protected from insects, fed with lucerne (*Medicago sativa*), hay, and water *ad libitum*. Four animals were splenectomized (Group B) seven days before *A. marginale* inoculation and four remained non-splenectomized (Group A).

2.2. Anaplasma marginale isolate and infection

The strain of *A. marginale* used as inoculum was received from UNESP-Jaboticabal/SP -Brazil (São Paulo State University), and maintained in liquid nitrogen for four months until inoculation. All animals were inoculated intravenously (jugular vein) with approximately 1×10^6 of erythrocytes parasitized by *A. marginale* (day zero). Blood smears of all animals were performed on days 0, 5, 10, 15, 20, and 25 post-infection (PI), stained with Romanowsky, and observed under optical microscope (100×). After inoculation, the animals were daily observed for clinical alterations (e.g. elevated body temperature, prostration, and food intake).

2.3. Blood samples

Blood samples were collected on days 0, 8, 15, 17, 25, and 29 PI from all animals by puncture of the jugular vein in vacuum system tubes, and these dates were set based on previous studies of our research group. Aliquots of 4 mL of blood were collected into tubes containing sodium citrate as anticoagulant for platelets separation.

2.4. Platelets

Platelet counts were performed by using a Neubauer chamber filled with whole blood diluted in ammonium oxalate 1%. Platelets were isolated from plasma obtained from blood collected with sodium citrate as anticoagulant (0.129 M, 1:9, v/v), as described by Pilla et al. [15] and modified by Lunkes et al. [16] to evaluated NTPDase and 5'-nucleotidase activities. Cell viability was estimated by lactate dehydrogenase release before and after incubation at 37 °C. The integrity of the platelet suspension was confirmed by the determination of the lactate dehydrogenase (LDH) activity using the kinetic method of the Labquest apparatus (Diagnostics Gold Analyzer).

Protein was measured by the Coomassie blue method using bovine serum albumin as standard as described previously [17].

2.5. NTPDase and 5'-nucleotidase

The NTPDase enzymatic assay was carried out in a reaction medium containing 5 mM $CaCl_2$, 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 as described by Lunkes et al. [16]. Twenty microliters of the enzyme suspension (8–12 μg of protein) were added to the reaction mixture. The pre-incubation proceeded for 10 min at 37 °C, after which the reaction was initiated by the addition of ATP or ADP at a final concentration of 1.0 mM, and incubated for 60 min. 5′-Nucleotidase activity was determined essentially as described by Pilla et al. [15] in a reaction medium containing 10 mM $MgSO_4$ and 100 mM Tris–HCl buffer, pH 7.5 at a final volume of 200 μL. Twenty microliters of enzyme preparation (8–12 μg of protein) was added to the reaction mixture. After the pre-incubation period of 10 min at 37 °C, the reaction was initiated by the addition of AMP at a final concentration of 2.0 mM and the reaction mixture was incubated for 60 min.

The enzymatic assays for NTPDase and 5'nucleotidase activities were stopped by the addition of 200 μl of 10% trichloroacetic acid (TCA). Subsequently, the tubes were chilled on ice for 10 min. Released inorganic phosphate (Pi) was assayed by the method of malachite green [18] as the colorimetric reagent and KH_2PO_4 as standard. Controls were carried out to correct non-enzymatic hydrolysis of nucleotides by adding platelets after TCA addition. All samples were run in triplicate. Specific enzyme activities were reported as nmol Pi released/min/mg of protein.

2.6. Statistical analysis

Data was analyzed primarily through descriptive statistics. Central tendency was calculated, as well as data dispersion. Furthermore, all variables were subject to Shapiro–Wilk test for normality verification. Since most variables were not normally distributed, the data was analyzed by nonparametric tests for two independent groups (Mann–Whitney U) for repeated measurements to test for statistically significant changes on parameters over time and between groups. In addition, two independence groups test Mann–Whitney U at each collection time period (0, 8, 15, 17, 21, 25, 29), as post hoc analysis by Tukey's HSD test. The *P* value considered statistically different was <0.05. All statistical process was perform according to R v.2.15.2 (R Development Core Team, 2012).

3. Results

3.1. Clinical signs and bacteremia

During daily clinical evaluation, the animals showed some characteristic signs of anaplasmosis, such as prostration and slight reduction in food consumption. However, there was no significant change between groups in body temperature (data not shown),