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Anti-erythrocyte IgG in hamsters with acute experimental infection by *Leptospira interrogans* serovar Canicola

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ABSTRACT

The aim of this study was to evaluate the behavior of erythrocyte and platelet, as immunological markers, as well as evaluate the involvement of these factors in hemolytic and hemorrhagic reactions in hamsters experimentally infected by *Leptospira interrogans* Serovar Canicola. Our experimental design was composed by two randomized groups: Infected Group (IG) (n = 12) and control group (CG) (n = 6). Ninety-six hours after the inoculation, the presence of immunoglobulins (IgG and IgM) and complement C3 levels, related to erythrocytes and platelets, was assessed. Platelet's microparticles marked by CD61, reticulocytes and reticulated platelets were also quantified. Additionally, fibrinogen, prothrombin time, partially activated thromboplastin time and sera levels of IgG and IgM were assessed. Our results showed that levels of platelet decreased in IG ($P < 0.001$); as well as, there was presence of IgG and C3 associated with erythrocyte surface in the infected animals ($P < 0.01$, $P < 0.05$, respectively). Levels of prothrombin time and Activated Partial Thromboplastin Time were increased, while fibrinogen level was decreased ($P < 0.01$) in IG. CD61 microparticles were higher ($P < 0.05$) in IG due to platelet activation. Thus, it was established a positive correlation ($P < 0.01$) between platelets count and fibrinogen (Figure 3, $R = 0.84$, $P < 0.001$). Therefore, the platelet consumption component was preponderant in relation to autoimmune causes. Finally, regarding the erythrocytes, the autoimmune component played an important role, did not causing hemolytic reaction in this acute experimental time.

1. Introduction

Leptospirosis is a worldwide public health problem with great relevance in several countries, especially in Latin America and Southeast Asia. Epidemiologic studies indicate the occurrence of more than one million cases of severe human leptospirosis per year throughout the world, with a fatality rate of approximately 10% [1]. Pathophysiologically it is characterized by vasculitis, and the damage to the capillary endothelial cells is the main cause of clinical manifestations, such as renal tubular dysfunction, liver damage and pulmonary hemorrhage [2]. The injury is most likely due to deposits of immune complexes in the small vessels of the organs affected. Activation of immuno-inflammatory response determines the release of various humoral factors, which cause the inflammatory process [2]. Several different studies [3,4] have shown an important correlation of Serovar canicola and infection in dogs, since it is more adapted to dogs as hosts [5], with special significance in urban areas and, consequently, leading to human infections.

The increase of microparticles (MPs) has been the subject of studies in sepsis and in diseases with activation of the coagulation cascade, such as acute leptospirosis. Microparticles are defined as a population of vesicles originated from different cell types after

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activation or apoptosis. They measure between 50 nm and 1000 nm and contain cellular material, such as proteins, mRNA and lipoproteins, which are keys to their identification by flow cytometry [6]. All blood cells produce MPs, however platelets release the highest number of circulating MPs, corresponding to 70%–90% of total MP in the plasma of healthy individuals [7,8]. When appropriate technology and techniques are used to mark MPs, by CD 61, along with anexin as an apoptosis marker, they are an important tool and can be used as explanatory factor in studies of pathogenesis, since the attention is focused on the activation and platelet consumption phenomena [9].

Hemostatic disorders and clinical presentations with loss of erythroid mass, by either hemorrhages or hemolytic events, have been described in severe studies of infection by *L. interrogans* [10–12]. Such studies, however, have not been focused on the phenomena of platelet and/or erythrocyte opsonization. Therefore, this study aimed to: i) evaluate IgG, IgM and complement system activation by C3 in the erythrocyte and platelet membrane of hamsters experimentally infected by *Leptospira interrogans* sorovar Canicola; ii) analyze the medullary response against infection leading hematic and platelet loss (through the percentage of reticulocytes and reticulated platelets, respectively); iii) evaluate the micro expression, by CD61 binding, in platelet-poor plasma (PPP); and iv) perform a hemogram, quantification of serum IgG and IgM, assess prothrombin time (PT) and activated partial thromboplastin time (APTT), as well as plasma fibrinogen levels.

2. Material and methods

2.1. Experimental model

This study was approved by the Animal Welfare Committee of Federal University de Santa Maria (UFSM), under number 4923201015 and it is in accordance to Brazilian laws and ethical principles published by the *Colégio Brasileiro de Experimentacão Animal* (COBEA). As experimental models, we used hamsters (*Mesocricetus auratus*), since it is the ideal model for virulence and pathogenicity investigations in leptospirosis infection [13,14], providing a similar pattern when compared with to severe human leptospirosis. Hamsters underwent to a 15-day adaptation period in which they received commercial feed and water *ad libitum*. After adaptation, 2 animals were used for bacterial activation, and 18 for the establishment of the experimental groups (total of 20 hamsters). All of them were adults, males, with an average age of 60 days and weighing 80–96 g. Hamsters were randomly divided into 2 groups [CG = control (n = 6) and IG = infected (n = 12)], housed in cages (3/each cage) in an experimental room (under controlled temperature and humidity, 25 °C and 70%, respectively).

2.2. Virulence recovery, inoculum, inoculation process and success of experimental infection

Leptospira interrogans sorovar Canicola used in our study was a virulent strain (LO4). In order to obtain a fully virulent pattern, 0.5 mL of culture, containing approximately 2×10^8 leptospires (quantified through Petroff-Hausser counting chamber in darkfield microscope), were inoculated intraperitoneally in one adult hamster (H1). When H1 showed severe clinical signs of leptospirosis (5 days post-infection), we performed its euthanasia, recovering samples of liver and kidney for further re-inoculation. A pool of these organs were obtained (composing a macerate) and, then, it was reinoculated in a second animal (H2) following a protocol of 1/10 proportion (g tissue/mL of distilled water at 28°c). The onset of clinical signs of disease in H2 was 4 days post-infection, period that was carried out the euthanasia.

Thus, after the second passage (virulence recovery), it was performed the liver and kidney collection for *Leptospira recovery*, carrying out the inoculation of our infected group (IG). Inoculation was performed subcutaneously according to a consolidated methodology [15]. The animals in IG received, as inoculum, 0.5 mL of supernatant (pool macerated of kidney/liver) at 28 °C and containing 20 to 30 leptospiras per microscopic darkfield (quantified through Petroff-Hausser counting chamber) at 400× magnification. Animals from CG received, also subcutaneously, 0.5 mL of sterile saline solution at 28 °C. Prostration, food and water ingestion and presence of blood in the cages were evaluated every 12 h after inoculation.

For confirmation of infection success, it was performed two protocols: 1st: leptospire culture of urine aspirated from bladder. One drop of urine was placed between slide and coverslip and examined under darkfield microscopy at a magnification of 400×. 2nd: inoculation of renal and hepatic macerated tissue in EMJH media. Briefly, samples of kidney and liver (01 g of pool) were mixed in modified EMJH medium with 5-fluorouracil (300 mg/L – Sigma USA) and nalidixic acid (20 mg/L), using a serial dilution technique, to evaluate the percentage of recovery of the added microorganisms. Other characteristics findings of severe leptospirosis were observed during necropsies and were also taken into account [free liquid (ascites) into the abdominal cavity, jaundice, blood in the intestinal lumen, hepatosplenomegaly and macroscopic renal changes].

2.3. Sampling and auxiliary analysis

Blood samples collection were carried out (by cardiac puncture with hamsters under anesthesia) on the fourth day post-infection (PI) in both groups. In average 5 mL of blood was obtained and divided into tubes containing ethylenediaminetetraacetic acid (EDTA), sodium citrate and tubes without anticoagulant. Euthanasia was conducted by cervical dislocation after a deep anesthesia. Complete blood count and platelet count was carried out through a blood cell counter (BC 2800 Vet). Erythrocyte and platelet morphological evaluation was performed on blood smear stained with Fast Paranotico®, using optical microscopy.

Quantification of IgG and IgM in serum was performed by immunonephelometric assay, using a Behring BN II Nephelometer (Dade Behring - USA) with reagents from Dade Behring. To measure Prothrombin time (PT) and Activated Partial Thromboplastin Time (APTT), a commercial reagent containing rabbit brain extract was used, with preservative and plasma activator for PT and ellagic acid and calcium chloride for APTT. For determination of Fibrinogen, via commercial kit (Labtest, Minas Gerais, BR), a standardized amount of Thrombin was added to a sample of diluted citrated plasma and the clotting time was measured. The coagulation time of diluted citrated plasma was found to be inversely proportional to the concentration of Fibrinogen. In order to obtain the concentration of Fibrinogen, coagulation time of plasma being tested was compared with the coagulation times for a series of dilutions of plasma containing a known concentration of Fibrinogen. For PT, APTT and Fibrinogen the formation of fibrin was monitored in a semi-automated device (Quick timer®).

2.4. Antibodies and C3 detection on the surface of erythrocytes

The detection of IgG, IgM and C3 in surface of erythrocytes was performed in samples containing, at least, 1 mL of whole blood. These samples were centrifuged at 1500 g for 5 min, in order to obtain the fraction of plasma-free erythrocytes. Upon removing the proteins from the plasma, 100 μL of erythrocytes were added to 4 mL of phosphate buffered saline solution (Phosphate Buffered