Cholinesterases as markers of the inflammatory process in rats infected with *Leptospira interrogans* serovar Icterohaemorrhagiae

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The aim of this study was evaluate changes in the cholinesterase activity in blood, lymphocytes and serum of rats infected with *Leptospira interrogans* serovar Icterohaemorrhagiae ('*L. icterohaemorrhagiae*'). Sixty adult Wistar rats were divided into six groups of 10 animals: three control groups and three test groups. The animals from the test groups were intraperitoneally inoculated with 1 ml medium containing 1×10^8 leptospire. The activity of acetylcholinesterase in blood and butyrylcholinesterase in serum increased on days 5 ($P < 0.05$) and 30 ($P < 0.021$) post-infection, respectively. A decrease in lymphocyte count was observed on days 15 ($P < 0.01$) and 30 post-infection ($P < 0.05$). On day 15 post-infection, acetylcholinesterase activity ($P < 0.001$) in lymphocytes decreased in infected rats. However, on day 30 post-infection there was an increase in acetylcholinesterase activity in lymphocytes. In conclusion, our results showed that the activity of enzymes of the cholinergic system in the total blood, lymphocytes and serum is altered as a result of inflammation caused by infection with *L. icterohaemorrhagiae*. The possible causes of these alterations will be discussed in this paper.

Received 20 June 2011

Accepted 14 September 2011

INTRODUCTION

Leptospirosis is a bacterial zoonotic disease prevalent worldwide, caused by spirochaetes of the species *Leptospira interrogans* (Faine *et al.*, 1999). This agent can affect animals and humans, and represents an important public health problem. Infection occurs through contact with urine, contaminated soil and water; the spirochaetes enter the mucosa or injured skin, and spread via the bloodstream to all organs (Bolin & Prescott, 1999). The course of human leptospirosis varies from mild to severe and fatal, the latter being principally caused by *L. interrogans* serovar Icterohaemorrhagiae ('*L. icterohaemorrhagiae*') (Faine *et al.*, 1999). This disease is characterized by a broad spectrum of clinical manifestations, from subclinical infection to Weil's

syndrome, a serious and potentially fatal disease characterized by haemorrhage, acute renal failure and jaundice (Vinetz, 2001). Leptospirosis is typically a biphasic illness, with a first phase of leptospiraemia or septicaemia, which lasts from 4 to 9 days, and a second phase in which immune antibodies appear, such as immunoglobulin M (IgM), which determine the formation of circulating immune complexes that can cause uveitis, circulatory collapse and other disorders. The duration and clinical manifestations of this phase are variable (O'Neil *et al.*, 1991).

Although major progress has been made in basic research on leptospirosis, the pathogenesis remains to be well elucidated (He *et al.*, 2004; McBride *et al.*, 2005). The existence of the cholinergic anti-inflammatory pathway is well documented (Tayebati *et al.*, 2002; Kawashima & Fujii, 2003; Kimura *et al.*, 2003), but it has not yet been investigated in leptospirosis. Cholinesterases are enzymes present in cholinergic and non-cholinergic tissues like blood and other body fluids,

Abbreviations: ACh, acetylcholine; AchE, acetylcholinesterase; AcSch, acetylthiocholine; BchE, butyrylcholinesterase; BcSch, butyrylthiocholine; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); mAChR, muscarinic Ach receptor; nAChR, nicotinic Ach receptor; p.i, post-infection.

and are divided into two classes (acetylcholinesterase, AChE; and butyrylcholinesterase, BChE) according to their catalytic properties and specificity for substrates, sensitivity to inhibitors and tissue distribution (Kawashima & Fujii, 2003; Kimura *et al.*, 2003). AChE and BChE both catalyse the hydrolysis of the neurotransmitter acetylcholine (ACh), a fundamental process in regulating the cholinergic system (Li *et al.*, 2000; Kimura *et al.*, 2003). These enzymes perform functions related to the transmission of nerve impulses in cholinergic synapses, haematopoiesis, inflammatory markers, production and coordination of movement, and memory (Kawashima & Fujii, 2003). The two enzymes have similar molecular formula but different tissue distribution. Recently, a new property of AChE and BChE was identified: as markers of inflammation (Das, 2007). The cholinergic anti-inflammatory pathway is mediated by ACh, which acts by inhibiting the production of tumour necrosis factor, interleukin-1 and macrophage migration inhibitory factor (Borovikova *et al.*, 2000). ACh regulates the levels of serotonin, dopamine and other neuropeptides and thus modulates the immune response as well as neurotransmission (Ramírez *et al.*, 1997). Signs of the cholinergic anti-inflammatory pathway occur through the efferent vagus nerve and are mediated initially by nicotinic ACh receptors on tissue macrophages. Activation of afferent vagus nerve fibres by endotoxin or proinflammatory cytokines stimulates the hypothalamic-adrenal-pituitary anti-inflammatory response, leading to anti-inflammatory signals through the efferent vagus nerve (Pavlov & Tracey, 2006; Das, 2007).

When the inflammatory process is mild and localized in internal organs it is difficult to detect and confirm the presence of inflammation; this is especially true when there is mild systemic inflammation (Das, 2007). During leptospirosis a mild inflammatory process occurs characterized by the development of vasculitis, endothelial damage, and inflammatory infiltrates composed of monocyte cells, plasma cells, histiocytes and neutrophils (Arean, 1962). Due to the high zoonotic potential of leptospirosis and the enormous economic losses generated by this disease, further research is necessary. The combined analysis of haematological parameters and enzymic markers of inflammation may lead to a better understanding of leptospirosis and may be useful to elucidate the pathogenesis of the disease. The aim of this study was to evaluate the activity of cholinesterase in blood, lymphocytes and serum of rats experimentally infected with *L. icterohaemorrhagiae*.

METHODS

Experimental animals. Sixty adult male Wistar rats (*Rattus norvegicus*), average 60 days old and 200 g in weight, from the central vivarium of the Universidade Federal de Santa Maria (UFSM) were used. The animals were kept in an experimental room with controlled temperature and humidity (25 °C; 70 % RH). They were fed a commercial ration, with water *ad libitum*, and submitted to a period of 7 days of adaptation.

Experimental groups and *Leptospira* infection. The rats were divided into six groups of 10 animals each: three infected groups 'T' (T5, T15 and T30) with *L. icterohaemorrhagiae* and three control groups 'C' (C5, C15 and C30) consisting of healthy and non-infected rodents. Group T were inoculated intraperitoneally with 1 ml Ellinghausen–McCullough–Johnson–Harris (EMJH) medium containing 1×10^8 leptospores, while group C received 1 ml EMJH medium alone, also intraperitoneally.

The density of leptospiral organisms used for inoculation was determined by direct counting using a dark-field microscope (Axioplan 2, Zeiss). The reference serovar used was provided by the Laboratory of Leptospirosis, Department of Microbiology and Parasitology, UFSM. Semisolid EMJH medium with 0.2 % agar was used for maintenance of stock cultures. Throughout the experiment clinical monitoring of animals was done.

Ethical approval. The procedure was approved by the Animal Welfare Committee of UFSM, number 47/2009, in accordance with Brazilian laws and ethical principles published by the Colégio Brasileiro de Experimentação Animal (COBEA).

Estimation of bacteraemia. Infection was estimated on days 5, 15 and 30 post-infection (p.i.) by microscopic examination of urine. A drop of urine was collected and placed between slide and coverslip and examined at a magnification of $\times 400$. Blood from each animal was collected (from the tail vein) on the same days (5, 15 and 30) and a drop was mixed in modified EMJH medium with 5-fluorouracil (300 mg l^{-1} ; Sigma) and nalidixic acid (20 mg l^{-1}), termed 'selective medium' (Miraglia *et al.*, 2009), using the serial dilution technique, in order to evaluate the percentage recovery of the added micro-organism. These cultures were incubated at an average temperature of 29 °C.

Sample collection. On days 5 (C5, T5), 15 (C15, T15) and 30 (C30, T30) p.i. the animals were anaesthetized with isoflurane in a gas chamber and blood was collected by cardiac puncture. Blood samples for haematology (1 ml) were collected in tubes containing EDTA. For serum BChE assays 2 ml blood was conditioned in tubes without anticoagulant and serum was obtained by centrifugation at 5000 r.p.m. for 8 min. For determination of the blood AChE activity the whole blood was haemolysed in 0.1 mmol l^{-1} potassium/sodium phosphate buffer, pH 7.4, containing 0.03 % Triton X-100, 1:100 (v/v). The samples for enzymic assay were frozen at -20 °C until analysis.

Samples (4 ml) of blood were stored in tubes with anticoagulant (10 % EDTA) for separation of lymphocytes. The peripheral lymphocytes were isolated using Ficoll Hypaque density gradient as described by Böyum (1968). After separation, only samples with at least 95 % of lymphocytes, as verified in a Coulter STKS haematology analyser, were used. Lymphocyte viability and integrity were confirmed by determining the percentage of cells excluding 0.1 % Trypan blue and measuring lactate dehydrogenase activity (Strober, 2001).

Haematological evaluation. Complete blood count and haemoglobin determination were performed using an automated cell counter (Vet Auto Haematology Analyser, model BC 2800). The packed cell volume (haematocrit) was obtained by centrifugation using a microcentrifuge (Sigma) at 14 000 r.p.m. for 5 min. For morphological evaluation of the blood and differential count of white blood cells, the blood smears were stained using a Diff-Quick commercial kit and visualized under the microscope. Mean corpuscular volume and mean corpuscular haemoglobin concentration were calculated according to Feldman *et al.* (2000).

AChE activity in total blood. The AChE enzymic assay in total blood was done by the method of Ellman *et al.* (1961) as modified by Worek *et al.* (1999). The incubation system was composed of 0.1 mol l^{-1} sodium phosphate buffer pH 7.4, 5,5'-dithiobis-(2-nitrobenzoic acid)

(DTNB) 10 mmol l^{-1} for haemolysis of blood. The increase in absorbance was registered over 2 min at 436 nm. The specific activity of whole-blood AChE was calculated from the quotient between AChE activity and haemoglobin (Hb) concentration and the results were expressed as $\text{mU l}^{-1} (\text{mol Hb})^{-1}$.

AChE activity in lymphocytes. After isolation of the lymphocytes, AChE activity was determined according to the method described by Ellman *et al.* (1961) as modified by Fitzgerald & Costa (1993). Briefly, proteins of all samples were adjusted to $0.1\text{--}0.2 \text{ mg ml}^{-1}$, and 0.2 ml of intact cells was added to a solution containing, 1.0 mmol acetylthiocholine (AcSCh), 0.1 mmol DTNB and 0.1 mmol phosphate buffer (pH 8.0). Immediately before and after incubation for 30 min at 27°C the absorbance was read on a spectrophotometer at 412 nm. AChE activity was calculated from the quotient between lymphocyte AChE activity and protein content and the results were expressed as $\mu\text{mol AcSCh consumed (mg protein)}^{-1}$.

BChE activity in serum. The enzymic activity of BChE in serum was determined by the method of Ellman *et al.* (1961). The system, containing 0.1 mol l^{-1} potassium phosphate buffer, pH 7.4, DTNB 0.30 mmol l^{-1} and 50 ml plasma, was incubated for 2 min at 30°C and the reaction was started by the addition of 1 mmol butyrylthiocholine (BcSCh). The reading was performed by spectrophotometer for 2 min at 412 nm. The enzyme activity was expressed as $\mu\text{mol BcSCh consumed h}^{-1} (\text{mg protein})^{-1}$.

Statistical analysis. The data were evaluated by ANOVA for means comparison with subsequent application of Student's *t*-test to verify the accuracy of results. The analyses were performed using SAS statistical package (SAS Institute) with a significance level of 5% ($P < 0.05$). The values were represented as mean $\pm$ SD. All samples were processed in triplicate.

Fig. 1. Haematocrit (a) and AChE activity in blood (b) of rats infected with *L. icterohaemorrhagiae* (white bars) as compared with non-infected rats (black bars), on days 5, 15 and 30 p.i. *, $P < 0.05$, Student's *t*-test.

RESULTS

Bacteraemia and clinical course of infection

The animals were inspected daily for clinical signs. Apathy and presence of animals with fur erect was observed, especially in the first week p.i. On days 5, 15 and 30 p.i., all infected animals had serological titres ranging from 1:200 to 1:800. The control group did not display any of these signs and were serologically negative.

From day 5 p.i., leptospire were seen in the urine of rats in the infected group, and this continued to days 15 and 30 p.i. Spirochaetes were not seen in the urine of any rat in the control group. Blood samples, collected on day 5 p.i. from the infected group, showed growth of leptospire in EMJH medium after the sixth week of culture. Growth was not obtained from samples taken on days 15 and 30 p.i. in the infected group, or from the control group.

Haematological findings

The haematocrit (Fig. 1a), the number of erythrocytes and the haemoglobin concentration did not differ significantly between infected and control groups, nor did total leukocyte count. However, the number of lymphocytes in the infected group decreased significantly on days 15 and 30 p.i. (Fig. 2a).

Acetylcholinesterase activity in total blood

On day 5 p.i., the activity of AChE in blood increased significantly in rats of group T5 ($P < 0.05$), when compared to the control group C5 (Fig. 1b). However, on days 15 and

Fig. 2. Lymphocyte count (a) and AChE activity in lymphocytes (b) of rats infected with *L. icterohaemorrhagiae* (white bars) as compared with non-infected rats (black bars), on days 5, 15 and 30 p.i. *, $P < 0.05$; **, $P < 0.001$ (Student's *t*-test).

Fig. 3. BChE activity in serum of rats infected with *L. icterohaemorrhagiae* (white bars) as compared with non-infected rats (black bars), on days 5, 15 and 30 p.i.). †, $P < 0.021$ (Student's *t*-test).

30 p.i. there was no significant difference between the infected and control groups.

AChE activity in lymphocytes

On day 5 p.i., there was no significant difference in AChE activity in lymphocytes between groups T and C. However, on day 15 p.i. there was a significant ($P < 0.001$) decrease in AChE activity in lymphocytes of infected rats when compared to non-infected rats (Fig. 2b). On day 30 p.i. there was a significant increase in AChE activity in lymphocytes ($P < 0.001$) of infected rats (Fig. 2b).

BChE activity in serum

On days 5 and 15 p.i. no significant difference in BChE activity in serum was observed between groups C and T. However, on day 30 p.i. there was an increase of BChE activity ($P < 0.021$) in the serum of infected rats when compared to the control group (Fig. 3).

DISCUSSION

Rat models have been used to investigate leptospirosis (Thiermann, 1981; Nally *et al.*, 2005; Viriyakosol *et al.*, 2006; Tonin *et al.*, 2011). In Wistar rats the leptospiraemic phase leads to rapid dissemination, followed by the accumulation of leptospires in the lumen of proximal tubules and the clearance of leptospires from all other tissues 9 days after infection. Thus, the experimental rat model is suitable for the investigation of leptospirosis (Athanasio *et al.*, 2008). Similar behaviour was observed in monkeys, mice, guinea pigs, hamsters, dogs and skunks (Faine, 1957; Tabel & Karstad, 1967; Miller *et al.*, 1974; Marshall, 1976; Palmer *et al.*, 1987; Alves *et al.*, 1991; Branger *et al.*, 2005).

This study investigated the alterations in enzymes of the cholinergic system in total blood, lymphocytes and serum as a result of inflammation caused by experimental infection with *L. icterohaemorrhagiae* in rats. The activity of the enzymes AChE and BChE in plasma and tissues is

commonly elevated in patients with Alzheimer's disease and diabetes mellitus (Das, 2007). In infectious diseases caused by *Staphylococcus aureus* (Kawashima *et al.*, 1980) and trypanosomosis (Wolkmer *et al.*, 2010) changes were observed in the enzymic activity of cholinesterase related to the immune response and clinical signs. In post-stroke patients the activity of cholinesterase (AChE and BChE) was directly and prominently correlated with inflammatory markers such as fibrinogen, interleukin 6 and C-reactive protein; these findings support the idea that AChE and BChE promote the systemic inflammatory response (Ben Assayag *et al.*, 2010). In a recent study of our research group, we observed changes in the enzyme adenosine deaminase in rats experimentally infected with *L. icterohaemorrhagiae* (Tonin *et al.*, 2011).

In the present study, the AChE activity in blood was increased on day 5 p.i., a phase in which the leptospirosis is characterized by generalized leptospiraemia (Maneewatch *et al.*, 2009), causing lesions due to the action of undefined leptospiral toxin(s) or toxic cellular components (Adler & de la Peña Moctezuma, 2010). Leptospiral haemolysins, also known as phospholipases, are one of the potential virulence factors which act on erythrocytes and possibly other cell membranes containing substrate phospholipids, leading to cytolysis (Thompson, 1986; Faine *et al.*, 1999). However, we did not observe a reduction in red blood cells, unlike that observed by Tonin *et al.* (2011). The activation of AChE is related to a stimulus to the host's inflammatory response. Recently it was demonstrated that rats exposed to bacterial lipopolysaccharides show an initial increase in AChE activity followed by a reduction of this activity. An increase in levels of interleukin-1b, interleukin-6 and miR-132 was also observed, suggesting that systemic reduction of AChE activity is an integral part of the post-inflammatory response (Shaked *et al.*, 2009).

This increase in blood AChE at the beginning of leptospirosis inflammation probably occurs through the cholinergic anti-inflammatory pathway. This mechanism is regulated by neural signals transmitted via the vagus nerve, which suppress pro-inflammatory cytokine release, specifically via the $\alpha 7$ subunit-containing nicotinic ACh receptor ($\alpha 7nAChR$) (Kawashima & Fujii, 2003). ACh acts on these receptors to open a channel in the cell membrane. Binding of ACh results in reduced nuclear factor (NF- κ B) activation and preservation of HMGB1 nuclear localization, thereby preventing its release into the extracellular compartment and thus preventing mortality due to lethal sepsis (Wang *et al.*, 2004); it also leads to reduced production of inflammatory cytokines (Das, 2007). Thus ACh regulates, partially through autonomic nervous system pathways, the release of tumour necrosis factor (TNF), interleukin-1 (IL-1) and other pro- and anti-inflammatory cytokines from immune cells (Elenkov *et al.*, 2000; Tracey, 2002; Pavlov *et al.*, 2003; Pavlov & Tracey, 2004). Whereas ACh is an anti-inflammatory molecule (Das, 2007), the increase of total blood activities of AChE enzymes reflects reduced levels of ACh, that will enhance local and systemic inflammatory events caused by leptospirosis infection.

Similarly to AChE, BChE could serve as a marker of low-grade systemic inflammation (Das, 2007) since it is able to hydrolyse choline esters (Li *et al.*, 2000). In the infection by *L. icterohaemorrhagiae* we observed increased BChE activity in serum on day 30 p.i. This late increase may reflect the fact that BChE is less efficient in ACh hydrolysis at low concentrations but highly efficient at high concentrations (Silver, 1974). BChE can increase in activity and also take the place of AChE in ACh degradation when AChE is inhibited (Li *et al.*, 2000). Another important role of BChE activity is as a marker to predict the prognosis of diseases. When the activity of the enzyme is low, subjects are at high risk of death (Whittaker, 1980). This idea is reinforced by the fact that in our study no rat died due to infection up to day 30 p.i.

In this study, infected rats showed a reduction in the number of circulating lymphocytes, concomitantly with alterations in AChE activity in these cells. Lymphopenia is common in an inflammatory response during acute inflammation due to the fact that inflammatory mediators stimulate the movement of lymphocytes from blood to inflamed tissue and lymphoid tissue (Imhof & Dunon, 1995). This relation between AChE and lymphocytes also was demonstrated in mice engineered to overexpress the synaptic variant of acetylcholinesterase (AChE-S). This enzyme is actively involved in controlling haematopoiesis and the potential for modulating immune functions; thus alterations in AChE can suppress T lymphopoiesis (Perry *et al.*, 2007). There was decreased AChE activity in lymphocytes on day 15 p.i., a time at which the bacterium was not found in the blood, but leptospiuria was observed. With the decrease of AChE more ACh is free to bind to lymphocytes and inhibit inflammation (Descarries *et al.*, 1997). It is known that ACh is produced within the lymphocytes, and ACh by binding to muscarinic ACh receptors (mAChRs) and nAChRs enhances lymphocyte cytotoxicity, increases their content of cGMP and inositol 1,4,5-triphosphate (IP₃), and modulates DNA synthesis and cell proliferation, supporting the idea that the lymphocytic cholinergic system is involved in the regulation of immune function (Kawashima & Fujii, 2003). Furthermore, findings from Fujii & Kawashima (2000) suggest that ACh released from T cells induces intracellular Ca²⁺ signalling in T and B cells via mAChRs. Additionally, the influx of extracellular Ca²⁺, promoted by nAChRs, and the release of intracellular Ca²⁺ into the cytoplasm promotes inhibition of pro-inflammatory cytokines. Thus, inhibition of AChE activity can be explained as a compensatory effect of the organism to attenuate inflammation and tissue damage caused by *L. icterohaemorrhagiae* in the acute phase of illness.

On day 30 p.i., with the chronicity of infection, the AChE activity in lymphocytes significantly increased, but the lymphopenia remained in infected animals. These results demonstrate the sensitivity of the enzyme activity in lymphocytes as a marker of low-grade systemic inflammation. According to the literature, when there is an increase in the AChE activity, a rapid degradation of ACh occurs.

ACh is considered as a molecule with anti-inflammatory action, because it binds to nicotinic receptors on lymphocyte surfaces, and thus inhibits the proliferation of cytokines, serotonin, histamine, nitric oxide, lysosomal enzymes, prostaglandins and leukotrienes, which are among the mediators of the inflammatory process (Kawashima & Fujii, 2003; Czura & Tracey, 2005; Nizri *et al.*, 2006; Das, 2007). The observed increase in AChE activity may be related to its expression on the surface of lymphocytes.

When the inflammatory process is low-grade and localized to the internal organs it is difficult to detect and confirm the presence of inflammation. This is especially true when there is low-grade systemic inflammation (Das, 2007). In this study, despite the infection, the animals showed no changes in blood count, findings consistent with inflammation, or clinical signs like those observed by Tonin *et al.* (2011), but although the number of lymphocytes had decreased on days 30 p.i., activity of both AChE and BChE increased in lymphocytes, demonstrating the usefulness of these enzymes as markers of inflammation in infection with *L. icterohaemorrhagiae*.

In conclusion, the results obtained in the present study demonstrate alterations in the activity of AChE in total blood and lymphocytes, and BChE in serum, in rats experimentally infected with *L. icterohaemorrhagiae*, indicating that the cholinergic system is altered in leptospirosis. Thus, we suggest that cholinesterases can be used as markers of the inflammatory process caused by experimental infection with *L. icterohaemorrhagiae* in rats.

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