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ORIGINAL ARTICLE

Malignant ovine theileriosis: serum concentrations of some inflammatory components and adenosine deaminase activity

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Abstract *Theileria lestoquardi* (*hirci*) is a tick-transmitted protozoan parasite that causes a malignant disease in sheep. The parasite invades ovine leukocytes and stimulates a variety of immune responses in the host. In order to evaluate serum adenosine deaminase activity and the status of some inflammatory mediators, 34 fat-tailed Iranian sheep naturally infected with *T. lestoquardi* were selected and divided into three subgroups according to their parasitemia rates (<2, 2–4 and >4 %). Fifteen non-infected sheep were sampled as controls. Blood samples were taken from jugular vein into EDTA-containing tubes for measuring haematological parameters and without anticoagulant for serum concentrations of inflammatory cytokines (IL-1β, IL-6, IFN-γ and TNF-α) and adenosine deaminase (ADA) activity. Remarkable decreases were seen in haematological parameters including red blood cells (RBCs), packed cell volume (PCV) and haemoglobin in infected sheep. In contrast, serum levels of the measured cytokines and also ADA activity were significantly increased in infected animals. However, with increase in the parasitemia rate, no significant increase or decrease was observed in the levels of cytokines and ADA activity. The results showed that the infection with *T. lestoquardi* promotes significant changes in major inflammatory components in infected sheep. These remarkable alterations could be triggered from earlier stage of infection.

Keywords Ovine malignant theileriosis · IFN-γ · TNF-α · Adenosine deaminase · Interleukin-1β · Interleukin-6

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Introduction

Theileria spp. are tick-born protozoan parasites which predominantly infect ruminants in tropical and subtropical regions. Malignant ovine theileriosis is considered as a fatal disease of sheep and causes heavy losses and decreased productivity in infected animals (Morel and Uilenberg 1981). The disease occurs due to presence of highly pathogenic species of *Theileria* including *Theileria lestoquardi* (Morel and Uilenberg 1981) and the two newly described *Theileria* spp. in China (China 1 and China 2) (Schnittger et al. 2000).

The sporozoites of *Theileria* invade the host mononuclear cells and undergo differentiations to form macroschizont stage. This process induces the protective immune responses of the host. The mechanism of immunity is mainly cell mediated, which is directed against all parasite stages (Seitzer and Ahmed 2008), particularly the schizont stage (Preston et al. 1999).

Some investigations revealed that the increase in secretion of cytokines such as IFN-γ, IFN-α, TNF-α and interleukins 1 and 6 could inhibit transformations of trophozoite-infected cells into macroschizont-infected cell lines. Also, in vitro studies have shown that macroschizont-infected cells express messenger (m)RNA for IL-1α, IL-1β, IL-6, IL-10 and TNF-α (Brown et al. 1995). Although several studies have shown the emergence of inflammatory cytokines in bovine theileriosis, it seems that the pattern of changes in inflammatory mediators in serum of *Theileria*-infected sheep was not previously described.

Adenosine deaminase (ADA) is a cytoplasmic enzyme which is essential for lymphocytes differentiation and proliferation. Increased ADA is due mainly to increased immune cell numbers (Ungerer et al. 1992). It has been shown that serum ADA activity could increase in diseases in which cell-mediated immunity is the dominant response (Altug and Agaoglu 2007). The main biological activity of ADA is to

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protect lymphocytes from toxic effects of 2-deoxyadenosine, deoxyadenosine triphosphate and deoxyadenosine diphosphate, which depress immune functions (Senesi et al. 1990). Thus, changes in ADA activity could likely reveal some aspects of immunity to protect itself during parasitemia.

The objective of the present study was to investigate the serum concentrations of the main inflammatory cytokines (IL-1β, IL-6, IFN-γ, TNF-α) and ADA activity and their correlations in different parasitemia levels in malignant ovine theileriosis.

Materials and methods

Animals

This study was conducted in the southwest region of Iran (Fars province) where theileriosis due to *T. lestoquardi* is prevalent. Two distinct groups were established: The diseased group, comprised of 34 fat-tailed sheep, 1–2 years old, naturally infected with *T. lestoquardi*, was divided into three subgroups according to different parasitemia rates (<2, 2–4 and >4 %). Fifteen non-infected sheep were also sampled as control group.

Haematological measurements

Blood samples were drawn from jugular vein into EDTA-containing tubes for measuring haematological parameters and put into plain tubes without anticoagulant for conducting serum assays. Thin blood films were prepared, fixed with absolute methanol (5 min), stained with 10 % Giemsa solution (30 min) and examined under oil immersion (×1,000). In order to detect intraerythrocytic forms of the parasite in blood smears and confirming the disease, piroplasms of *T. hirci* which appear as a small oval, round (as a ring) bodies were searched in red blood cells. The parasitemia rate was quantified by examination of at least 1×10^4 RBC for each case, and the proportion of the infected erythrocytes to the total number of the counted cells was expressed as the percentage of parasitemia. The blood values were estimated through standard haematological techniques. Total red blood cell (RBC) and white blood cell (WBC) counts in blood were performed using a standard haemocytometer. Differential leukocyte counts were made from Giemsa-stained smears of the blood. The packed cell volume (PCV) was measured by microhaematocrit method. The concentration of haemoglobin was measured using cyanmethemoglobin method. Reticulocytes were counted in a NMB-stained blood smear and were expressed as the percentage of the total erythrocyte population (Bellwood and Andrasik-Catton 2014).

Inflammatory mediator measurements

The serum concentration of interleukin-1 beta (IL-1β) was measured by a quantitative enzyme immunoassay (sandwich ELISA) kit (Genorise Scientific Inc., Paoli, USA) and presented as nanograms per millilitre. IL-6 was assayed in serum using interleukin-6 ELISA kit (CUSABIO®, Wuhan, China) which employs the quantitative sandwich enzyme immunoassay technique and expressed as picograms per millilitre. The concentrations of IFN-γ and TNF-α were measured by a solid phase sandwich ELISA (AbC 606 and AbC 607, respectively; Votre fournisseur AbCys S.A. Paris, France) and expressed as picograms per decilitre and nanograms per millilitre, respectively.

Adenosine deaminase activity measurement

ADA activity was assessed by an enzymatic-calorimetric assay kit (Diazyme Laboratories, Gregg Court, USA). This assay is based on the enzymatic deamination of adenosine to inosine which is converted to hypoxanthine by purine nucleoside phosphorylase (PNP). Hypoxanthine is then converted to uric acid and hydrogen peroxide (H_2O_2) by xanthine oxidase (XOD). The generated quinone dye was monitored spectrophotometrically and expressed as units per litre of serum.

Statistical analysis

Student's *t* test was used for comparison of measured parameters between control and infected groups. Analysis of variance (ANOVA) and Tukey tests were used for statistical differences among subgroups and Pearson's correlation coefficients to determine relationships between parameters. All values in tables were expressed as mean and standard error of mean (SEM), and $P<0.05$ was considered as statistically significant.

Results

Haematological parameters

Examination of blood smears in all infected animals with different parasitemia rates revealed a wide range of erythrocyte abnormalities including reticulocytosis, macrocytosis, basophilic stippling and anisocytosis.

The comparison among the values of the haematological parameters in non-infected sheep and those naturally infected with *T. lestoquardi* with different parasitemia rates is presented in Table 1. The data showed that the haematological parameters including red blood cell (RBC) count, haemoglobin concentration and packed cell volume (PCV)

Table 1 Haematological parameters in non-infected sheep and those infected with *Theileria lestoquardi* (*hirci*) with different parasitemia rates (values are expressed as mean±SEM)

	Parasitemia (%)	RBC ($\times10^{12}$/L)	PCV (L/L)	Hb (g/dL)	WBC ($\times10^{9}$/L)	Neutrophil ($\times10^{9}$/L)	Lymphocyte ($\times10^{9}$/L)
Control	0 (n=15)	10.43^{a} ±0.12	0.338^{a} ±0.003	12.05^{a} ±0.07	10.5^{a} ±0.18	2.45^{a} ±0.14	7.76^{a} ±0.12
Diseased	<2 (n=11)	8.22^{b} ±0.2	0.315^{b} ±0.002	10.17^{b} ±0.09	11.69^{b} ±0.16	2.55^{a} ±0.06	8.69^{b} ±0.16
	2–4 (n=13)	6.74^{c} ±0.07	0.267^{c} ±0.006	8.45^{c} ±0.04	13.37^{c} ±0.16	2.64^{a} ±0.05	9.67^{c} ±0.22
	>4 (n=10)	4.67^{d} ±0.1	0.172^{d} ±0.003	5.95^{d} ±0.15	13.87^{c} ±0.15	2.8^{a} ±0.07	10.13^{c} ±0.15

Values in each column with different superscript letters denote significant difference (P<0.05)

were significantly decreased in the diseased subgroups compared to the healthy ones (P<0.05). In addition, in animals with higher parasitemia levels, a significant decrease was observed in RBCs, PCV and haemoglobin concentration, which confirmed the anaemia in affected animals, as higher parasitemia levels coincided with the higher degrees of anaemia.

White blood cells (WBCs) were significantly increased in infected sheep (P<0.05). According to our data, although the neutrophil count remained unchanged in infected animals, the lymphocyte count showed a marked elevation compared to control. In addition, increasing the parasitemia rate had no significant correlations with peripheral circulatory lymphocytes and neutrophils in infected animals.

Cytokines and adenosine deaminase activity

The levels of cytokines IL-1β, IL-6, IFN-γ and TNF-α and ADA activity in non-infected and infected animals with different parasitemia rates are shown in Table 2. The results revealed a significant increase (P<0.05) in the serum level of ADA activity, IL-1β, IL-6, IFN-γ and TNF-α in infected animals. However, the parasitemia rate had no significant correlation to these cytokines and also to ADA activity. In addition, no statistically significant relationship was identified between the values of measured cytokines and/or between cytokines and ADA activity.

Discussion

Remarkable decreases in the main haematological parameters (RBC count, PCV and haemoglobin) indicate the occurrence of anaemia in sheep infected with *T. lestoquardi*. This finding has been previously indicated in ovine (Nazifi et al. 2011) and bovine theileriosis (Schnittger et al 2000; Razavi et al. 2010). Some preceding researches have attributed the erythrocytes destruction to the function of macrophages in the lymph nodes, spleen and other organs capturing the infected RBCs (Singh et al. 2001). However, more recent studies indicate that anaemia is a consequence of the parasite interference with protective antioxidant mechanisms of RBCs which enhance the susceptibility of erythrocytes to be lysed (Nazifi et al. 2011).

The results obtained by the present study indicate that the infection with *T. lestoquardi* in sheep leads to a remarkable elevation in the levels of some cytokines (IL-1β, IL-6, IFN-γ and TNF-α) and ADA activity; however, the increased concentrations of these mediators did not change significantly along with the elevation in parasitemia rate.

During *Theileria* infections, T cell blasts are found surrounding foci of macroschizont-infected cells in the lymph nodes draining infection. These cells are unlikely to be stimulated by classical antigen-presentation mechanisms. The sporozoites are able to induce a non-specific activation of T cells in vivo (Campbell et al. 1995) through the production of T cell stimulatory cytokines by infected cells (Brown et al. 1995), which may be responsible for the immunopathological

Table 2 The serum concentrations of interleukin-1 Beta (IL-1β), interleukin-6 (IL-6), gamma interferon (IFN-γ), tumour necrosis factor-alpha (TNF-α) and adenosine deaminase activity (ADA) in control and infected groups (values are expressed as mean±SEM).

	Parasitemia (%)	IL-1β (ng/mL)	IL-6 (pg/mL)	IFN-γ (pg/dl)	TNF-α (ng/mL)	ADA (U/L)
Control	0 (n=15)	0.21^{a} ±0.01	58.1^{a} ±1.61	12.42^{a} ±0.22	0.74^{a} ±0.03	6.67^{a} ±0.64
Diseased	<2 (n=11)	0.36^{b} ±0.01	94.05^{b} ±2.11	19.24^{b} ±0.14	1.22^{b} ±0.06	125.64^{b} ±4.06
	2-4 (n=13)	0.37^{b} ±0.01	97.22^{b} ±2.43	19.26^{b} ±0.19	1.24^{b} ±0.06	122.85^{b} ±2.1
	>4 (n=10)	0.36^{b} ±0.01	94.67^{b} ±3.15	19.09^{b} ±0.22	1.14^{b} ±0.04	121.6^{b} ±3.58

Values in each column with different superscript letters denote significant difference (P<0.05)

symptoms observed during infection. The in vitro studies on bovine theileriosis suggested that the outcome of activation of T cells by parasitized macrophages is a skewing of their cytokine responses towards preferential expression of IFN-γ mRNA. The in vitro response may be a refractory of in vivo infection, as greatly elevated amounts of IFN-γ protein are found in infected efferent lymph node (Campbell et al. 1997). On the other hand, the production of IFN-γ stimulates macrophages to produce more TNF-α (Brown et al. 1995).

Previous in vitro studies on cattle infected with *Theileria annulata* confirmed that cells infected with *T. annulata* produce high levels of inflammatory cytokines, especially TNF-α (Sileghem et al. 1994). These investigations can corroborate our data showing TNF-α increase in ovine theileriosis.

In our work, the significant increase in the serum concentration of cytokines IL-1β, IL-6, IFN-γ and TNF-α in infected sheep is largely in line with the in vitro study conducted by Brown et al. (1995) on *Theileria*-infected cattle. They assayed *T. annulata*-infected cell lines and clones for cytokine mRNA expression and showed that cells infected with *T. annulata* constitutively produce mRNAs for a number of macrophage-associated cytokines, including IL-lα, IL-1β, IL-6, IL-10 and TNF-α. By isolating a number of *T. annulata*-infected clones, they suggested that the levels of T cell proliferation induced by infected cells correlated with the levels of expression of the T cell stimulatory cytokines, IL-lα, IL-β and IL-6.

The marked elevation in ADA activity in diseased sheep may confirm the increase in cytokines originating from leukocyte proliferation and also the significant increase in lymphocyte count in infected animals. ADA is known as an essential enzyme for lymphocytes proliferation and differentiation. Increased ADA is derived mainly from the number of increased immune cells (Ungerer et al. 1992; Altug and Agaoglu 2007), and serum ADA activity increases in diseases in which cell-mediated immunity is the dominant response of the body (Altug and Agaoglu 2007). Altug et al. (2008) revealed a significant ADA increase in cattle infected with *T. annulata* with higher packed cell volumes (PCV). They suggested that the observed increase may reflect the involvement of the cellular immune responses in infected cattle. However, they showed that ADA level significantly decreased in cattle with PCVs lower than 25, which may be due to depletion of lymphocytes in end stage animals. Kontas and Salmanoglu (2006) also showed a remarkable increase in ADA activity in cattle infected with the other haemoparasite, *Babesia bigemina*. They affirmed that the increase in serum ADA levels may result from the phagocytic activity of macrophages and/or erythrocyte damage caused by the parasites.

Taking together, malignant ovine theileriosis of sheep can significantly reduce the major haematologic indices (RBC count, PCV and haemoglobin). Thus, anaemia is a characteristic of the disease in infected animals. In addition, *T. lestoquardi* (*hirci*) promotes significant elevations in serum levels of the main inflammatory cytokines and ADA. In infected sheep, these remarkable changes could be induced even in low parasitemia levels.

Conflict of interest We declare that we have no conflict of interest.

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