Adenosine deaminase activity as a biochemical marker of inflammatory response in goats infected by caprine arthritis–encephalitis virus

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\textbf{A B S T R A C T}

Caprine arthritis–encephalitis virus (CAEV) is a lentivirus that causes a chronic and degenerative arthritis and is associated with important economic losses. Adenosine deaminase (ADA) levels increase in some human infectious diseases, such as tuberculosis and acquired immune deficiency syndrome. Due to the similarity between CAEV and human immunodeficiency virus, we hypothesized that ADA activity in goats with clinical signs of CAEV infection is also altered and could serve as a valuable biochemical marker. Adenosine deaminase was assayed using adenosine (Ado) or 2’-deoxyadenosine (dAdo) as substrates, and ADA activity was calculated using the amount of ammonium produced. No significant difference was detected in the activity of the serum enzyme when assayed with Ado ($K_{\text{m}} = 49.19 \pm 5.28 \mu \text{mol/L}$) or dAdo ($K_{\text{m}} = 41.28 \pm 4.58 \mu \text{mol/L}$). Caprine serum ADA is a thermo-stable enzyme and can be stored at cool temperatures for at least 30 days with no loss of activity. An increase in ADA activity (approximately 2.6-fold) was found in serum and synovial fluid in CAEV infected animals with clinical signs of arthritis compared with uninfected animals. In serum, the ADA cutoff value for CAEV disease using Ado was $>34.9 \mu \text{U/L}$. Adenosine deaminase activity may be used as an important biochemical marker of the inflammatory response induced by CAEV, and its determination in serum using adenosine as a simple and inexpensive method is sufficient to assess the pathological condition of the animal.

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1. Introduction

Man domesticated goats 10,000 years ago mainly because they present great adaptability to several environmental conditions and different nutritional regimens (Aziz, 2010). Especially in poor rural areas, goats are considered an important source of milk, meat, and leather. Official data estimate that Brazil has approximately 9 million goats, and 90% of the entire herd is located in the northeast region.
(IBGE, 2009). Despite ancestral domestication, the rearing of goats also presents numerous problems, including infectious diseases, that hinder the economic production of these animals. Caprine arthritis–encephalitis virus (CAEV) is a retrovirus that belongs to the lentivirus subfamily. It is a common infection of goats causing arthritis and encephalitis (Narayan, 1990).

Originally, the caprine arthritis–encephalitis virus (CAEV) was thought to only infect goats. However, recent molecular epidemiological studies have shown that this virus and visna/maedi virus represent a spectrum of variants that infect both sheep and goats in the field and it is now referred to as a small ruminant lentivirus (SRLV) (Blacklaws, 2012).

The major CAEV tropism is for monocyte/macrophages and dendritic cells. However, other cell types may also be infected and act as virus reservoirs. These additional targets include epithelial cells in the mammary gland, which are an important source of virus for transmission of the disease from dams to their offspring (Blacklaws, 2012).

CAEV induced arthritis is characterized by a chronic and degenerative process that mainly involves the tarsus and metatarsus joints and progressive cachexia. Other clinical signs of infection include encephalitis, mastitis and pneumonitis. In the advanced phase of the arthritic form, which is the most frequent manifestation of the disease, the pelage becomes dry, and the animal loses weight with no changes in appetite and walks on its knees (Cullen, 1992; Frank, 1997; Perk, 1990).

A diagnosis of CAEV infection is often made by means of the serologic detection of anti-viral antibodies. Other tests for CAEV have been proposed using polymerase chain reaction. However, these methods may not be feasible or financially affordable for routine testing, especially in poor areas or for large herds.

Adenosine is considered an important endogenous inhibitor of the immune system for its potent suppressor action in virtually all cells in the immune system (Hasko and Cronstein, 2004). Its levels are modulated by specialized enzymes. The enzyme adenosine deaminase (ADA, E.C. 3.5.5.4.4) catalyzes the deamination of adenosine (or 2′-deoxyadenosine [dAdo]) to produce inosine (or 2′-deoxyinosine) and ammonium. Adenosine deaminase is liberated from the mononcytic-macrophage stage during the course of diseases caused by intracellular microorganisms, and the monitoring of ADA activity is useful in the diagnosis of human tuberculosis. However, ADA levels can also increase in viral infections, such as acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV) (Gakis et al., 1989).

Because of the similarity between CAEV and HIV, we hypothesized that ADA activity is altered in animals with clinical signs of CAEV infection and could serve as a valuable biochemical marker.

2. Materials and methods

2.1. Chemicals

Adenosine (Ado) and dAdo were purchased from Sigma-Aldrich (USA). Sodium nitroprusside was obtained from Merck (Germany). Disodium monohydrogen phosphate (Na₂HPO₄) and sodium dihydrogen phosphate (NaH₂PO₄) were obtained from Labsynth (Brazil). Ammonium sulfate, phenol, and sodium hydroxide were obtained from Reagen (Brazil). Sodium hypochlorite was obtained from Vetec (Brazil).

2.2. Solutions

Adenosine and 2′-deoxyadenosine (22 mmol/L each) were dissolved in assay buffer that consisted of sodium phosphate buffer (50 mmol/L, pH 7.2). The phenol reagent consisted of phenol (106 mmol/L) and sodium nitroprusside (0.17 mmol/L). Alkaline hypochlorite consisted of sodium hypochlorite (11 mmol/L) in sodium hydroxide (125 mmol/L). The standard solution was ammonium sulfate (75 μmol/L).

2.3. Experimental animals and sample collection

Crossbred goats of both sexes, aged 15–53 months with an average weight of 50 kg, were obtained from farms near the city of Fortaleza, State of Ceará, Brazil. The selection of the experimental animals was based on the analysis of the clinical index for CAEV (Monicat, 1987) and ante-mortem inspection according to Brazilian law that prohibits the trade of animals suspected of carrying zoonotic diseases such as CAEV, babesiosis, brucellosis, anthrax, blackleg, foot and mouth disease, glanders, paratuberculosis, babesiosis, and others diseases.

Clinical identification of suspected cases of CAEV infection is based on an interpretation of the “clinical index,” which is calculated as the difference between the circumference of the carpus and metacarpus (approximately 3 cm below the knee). The following parameters were used as the “clinical index”: ≤5.5 (absent), 6.0–6.5 (suspected), >7.0 (positive). After animal selection, blood samples were collected for laboratory confirmation of disease by serological test (agar-gel immunodiffusion – AGID).

Blood was collected from the jugular vein. The synovial fluid was collected only from animals slaughtered, immediately after the death of the animal. The material for ADA activity determination was transported to the laboratory in an ice bath. For the evaluation of ADA levels in CAEV induced arthritis, 64 animals were used for serum analysis, 48 with an “absent” clinical index + negative antibody responses to CAEV (S−) and 16 with a “positive” clinical index + positive antibody responses to CAEV (S+). For the analysis of synovial fluid, a total of 51 animals (38 S− and 13 S+) were used.

After clot retraction, the material was centrifuged at 7840 × g at 4–6 °C for 15 min. Joint fluid was treated similarly. The levels of ADA from the supernatants were determined according to method described below.

The experimental protocols (protocol # 65/2011) were approved by the Institutional Committee on the Care and Use of Animals for Experimentation of Federal University of Ceará, in accordance with the guidelines of the Brazilian Committee of Animal Experimentation (COBEA).

2.4. ADA assay

The ADA assay was performed according to the method described by Giusti (1974), with slight modifications. This assay is based on the quantification of ammonium ions produced by the deamination of Ado and dAdo by ADA. Ado and dAdo (22 mmol/L – 200 μL) in assay buffer were incubated with 20 μL of serum or synovial fluid for 60 min at 37 °C (first incubation period). Afterward, ammonia release was measured using the Berthelot reaction (Berthelot, 1859; 600 μL of phenol reagent plus 600 μL of alkaline hypochlorite). The tubes were incubated again (second incubation period) at 37 °C for 30 min and then read with a spectrophotometer at 628 nm.

Control tubes that contained Ado or dAdo (200 μL), standard tubes that contained standard solution (200 μL), and blank tubes that contained only assay buffer (200 μL) were also produced and subjected to the same conditions as the experimental assays above, with the exception of the absence of serum or synovial liquid during the first incubation period. Only after the addition of phenol reagent (600 μL), the biological samples (20 μL) were added followed by alkaline hypochlorite (600 μL).

Optimum conditions for the enzyme assay were then established. Optimum pH, apparent Km, apparent Vmax, storage stability, thermal effects, and thermal stability were determined using serum ADA.
2.5. Optimum pH

Twenty microliters of sample (n = 8) were incubated with 200 µL Ado or dAdo (22 mmol/L) in assay buffer with pH ranging from 6.0 to 8.0. The other procedures were the same as those described for the ADA assay.

2.6. Kinetics parameters

Twenty microliters of sample (n = 9) were incubated with 200 µL Ado or dAdo in assay buffer, with final concentrations from 0 to 100 µmol/L. The other procedures were the same as those described for the ADA assay.

2.7. Storage stability

Serum ADA activity was evaluated from 1 to 30 days under different storage conditions to establish how long it could be stored without loss. The samples (n = 8) were stored at either 4 °C or −20 °C, frozen in liquid nitrogen and stored at −20 °C, or frozen and stored in liquid nitrogen at −176 °C. The analyses were performed 0, 1, 5, 15, and 30 days after collection. On the specified days, the samples were analyzed for ADA activity according to the procedures described for the ADA assay.

2.8. Thermal effect on serum ADA activity

The influence of incubation temperature (first incubation period) on the activity of ADA (n = 7) was evaluated by incubating the assay mixture at temperatures that varied from 23 to 60 °C for 60 min. The other procedures were the same as those described for the ADA assay.

2.9. Thermostability of serum ADA

Initially, serum samples alone (n = 6) were incubated in a water bath at 53, 55, or 60 °C for 10, 20, 30, 40, 50, or 60 min. Afterward, the serum samples were analyzed according to the procedures described for the ADA assay.

2.10. Statistical analysis

The statistical analysis was performed using GraphPad Prism 5.0 software. The experimental data are expressed as the mean ± standard error of the mean (SEM) and were accepted as statistically significant with P < 0.05 using an unpaired t-test or the one way analysis of variance (ANOVA) followed by Tukey’s post hoc test (for multiple comparisons).

The ROC curve was used to establish cutoff values (i.e., the cutoff point between normal and abnormal), sensitivity (i.e., the fraction of sick animals correctly identified as positive), specificity (i.e., the fraction of CAE-free animals correctly identified as negative), positive predictive value (PPV; i.e., the fraction of animals with positive tests that actually presented that condition), negative predictive value (NPV; i.e., the fraction of animals with negative tests that actually presented that condition), and positive likelihood ratio (LR; i.e., how many times more likely the animal is to have the disease).

3. Results

Caprine serum ADA presented maximum activity at pH 7.2 for both substrates (Ado and dAdo). The apparent $K_m$ values for Ado and dAdo were 49.19 ± 5.28 and 41.28 ± 4.58 µmol/L, respectively (data not shown). The $V_{max}$ values for Ado and dAdo were 0.815 ± 0.08 and 0.775 ± 0.08 µmol NH$_3$/h/ml, respectively (data not shown).

A loss of enzymatic activity was not detected under the established storage conditions (i.e., 4, −20, and −176 °C) for up to 30 days. However, the samples that were stored at 4 °C presented a high ammonia content after 15 days of storage at the above temperatures in the blank and control assays, possibly attributable to protein degradation or bacteria growth.

Adenosine deaminase activity presented increasing values when incubated for 60 min (first incubation period) at temperatures from 23 °C to 55 °C. At 60 °C, a tendency toward decreased activity was observed. This effect was observed for both substrates (Fig. 1a).

The preincubation of serum alone at 53 °C for 0–60 min showed no loss of enzyme activity. Preincubation at 55 °C showed a small loss of activity at 60 min. However, when serum was preincubated at 60 °C, a decrease in activity of approximately 32% was observed beginning at 20 min, reaching a decrease in activity of approximately 70% with 60 min preincubation (Fig. 1b).

The use of Ado or dAdo did not significantly change the activity of the enzyme, either in serum or synovial fluid. In serum (Fig. 2a) and synovial fluid (Fig. 2b), enzyme activity determined with both substrates increased significantly (about two-fold) in animals with CAEV induced arthritis (S+) compared with uninfected animals (S−).

The cutoff values (Table 1) for ADA activity in clinical CAEV disease calculated using ROC curves in serum were 34.90 U/L (Ado) and 33.64 U/L (dAdo) and in synovial fluid were 15.65 U/L (Ado) and 15.50 U/L (dAdo). The areas under the ROC curves for serum ADA using Ado and dAdo were 0.9896 (Fig. 3a; standard error (SE) = 0.00924; 95% confidence interval [CI]: 0.9715–1.008) and 0.9870 (Fig. 3b; SE = 0.01039; 95% CI: 0.9666–1.007), respectively. In synovial fluid, the areas under the ROC curves using Ado and dAdo were 0.9919 (Fig. 3c; SE = 0.009419; 95% CI: 0.9734–1.010) and 1.000 (Fig. 3d; SE = 0.0; 95% CI: 1.000–1.000), respectively.

4. Discussion

The first step of this study was dedicated to investigating some of the characteristics of caprine ADA to establish the best conditions for the determination of the enzyme, especially storage conditions, thermostability, optimum pH, and some kinetics parameters. This step was necessary because of the absence of such data in the literature (Harem et al., 2009).

The optimum pH and apparent $K_m$ for caprine serum ADA suggest some similarities with the human ADA1 isoenzyme. According to Gakis (1996), human ADA1 presents a $K_m$ of approximately 52 µmol/L and an optimum pH of 7.0–7.5, whereas caprine ADA presented a $K_m$ of approximately 49 µmol/L and an optimum pH of 7.2.

Although the samples could be stored for up to 30 days with no loss of enzymatic activity, high ammonia content can be detected after 15 days, at 4 °C, possibly due to protein degradation or bacteria growth.

Serum ADA showed no loss of enzyme activity when pre-incubated until 55 °C, but a significant loss was found at 60 °C. Similar results were found for bovine ADA, which suffers thermal unfolding at temperatures above 60 °C, while the enzyme undergoes detectable conformational changes during pre-unfolding heating (Tavirani et al., 2008).

The thermostability of ADA facilitates collection, transport, and laboratory analysis with no need for specialized conditions for those procedures or storage.

ADA activity increases in clinical disease caused by CAEV, at least in serum and in synovial fluid. The increased
levels of the enzyme in animals infected by CAEV and with clinical disease suggest its involvement in the mechanisms of the disease, possibly reflecting the activation of cell-mediated immunity. Similarly high values for serum ADA were found in bovine leukemia (Yasuda et al., 1996) and HIV-positive patients (Gakis et al., 1989).

One of the first clinical signs of CAEV infection frequently observed in animals of at least 1 year of age is arthritis characterized by the chronic degenerative inflammation of carpometacarpal articulation, proliferation of synovial membrane cells, deposits of fibrinogen on synovial surfaces, and sub-synovial infiltration of inflammatory mononuclear cells (Crawford et al., 1980).

The natural immune response to CAEV is inadequate to clear the virus, thus permitting the establishment of lifelong persistence in the infected host. However, the immune response is sufficient to keep virus replication at a low level, slowing the progression of the disease (Fluri et al., 2006).

These characteristics suggest that increased ADA activity, both in serum and synovial fluid in animals with positive serology, should be a consequence of the involvement of that system.

Little is known about human ADA2, but it does represent the main component of enzyme activity in human serum when monocytes and macrophages are activated (Shibagaki et al., 1996). Caprine tissues present at least

Table 1

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Substrate</th>
<th>Cutoff (U/L)</th>
<th>Sensitivity (%) (95% CI)</th>
<th>Specificity (%) (95% CI)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>LR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Ado</td>
<td>34.90</td>
<td>93.75 (69.77–99.84%)</td>
<td>97.92 (88.93–99.95%)</td>
<td>93.75</td>
<td>97.92</td>
<td>45.00</td>
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<tr>
<td></td>
<td>dAdo</td>
<td>33.64</td>
<td>93.75 (69.77–99.84%)</td>
<td>95.83 (85.75–99.49%)</td>
<td>88.24</td>
<td>97.92</td>
<td>22.50</td>
</tr>
<tr>
<td>Synovial</td>
<td>Ado</td>
<td>15.65</td>
<td>92.31 (63.97–99.81%)</td>
<td>100.00 (90.75–100.00%)</td>
<td>100.00</td>
<td>97.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dAdo</td>
<td>15.50</td>
<td>100.00 (75.29–100.0%)</td>
<td>100.00 (90.75–100.00%)</td>
<td>100.00</td>
<td>100.00</td>
<td></td>
</tr>
</tbody>
</table>

Ado, adenosine; dAdo, 2′-deoxyadenosine; PPV, positive predictive value; NPV, negative predictive value; LR, positive likelihood ratio.
three ADA isoforms (Rodrigues et al., 2000), so investigating which isoform is responsible for increased ADA activity in serum and synovial fluid after CAEV infection is important. This information may reveal the role of ADA in the caprine immune system.

The study of the molecular weight and distribution of ADA isoforms in caprine tissue (Rodrigues et al., 2000) showed similarities with other species (Lupidi et al., 1992), including humans (Gakis, 1996). However, serum caprine isoenzymes, in contrast to human ADA, did not exhibit significantly different affinity for Ado and dAdo in the present study.

Lymphocytes, macrophages, and other blood cells are predominant in joint infiltration in CAEV induced arthritis (Norman and Smith, 1983). Increased ADA activity in synovial fluid may result from the involvement of these cells. Similar results were found in human patients with rheumatoid arthritis, osteoarthritis, and reactive arthritis (Yuksel and Akoglu, 1998).

The present study suggests the use of ADA activity as possible biochemical marker for the inflammatory response induced by CAEV. However, it is important to extend the study to infected animals with CAEV and without clinical signs. Also, it should be interesting determining which isoform is pivotal for increased ADA levels in the disease.

Our results demonstrated similarities in ADA kinetics parameters and cutoff values for Ado and dAdo. Serum ADA determination using only Ado (an inexpensive reagent) may be a useful diagnostic tool, making the collection
of synovial fluid for diagnostic analysis unnecessary, thus avoiding additional animal suffering.

5. Conclusion

Adenosine deaminase activity may be used as an important biochemical marker of the inflammatory response induced by CAEV, and the determination of its activity in serum using adenosine is a simple and inexpensive method to assess the pathological condition of the animal.

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