ELEVATED CEREBROSPINAL FLUID AND SERUM ADENOSINE DEAMINASE ACTIVITIES IN EXPERIMENTAL Listeria monocytogenes MENINGOENCEPHALITIS

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Listeriosis is a severe infectious disease occurring both in humans and in most other animal species caused by Listeria monocytogenes (L. monocytogenes) that can lead to severe meningoencephalitis. Elevated ADA activities have been demonstrated in the cerebrospinal fluid (CSF) in meningitis with different etiologies. However, data on ADA activities in CSF and serum associated with listerial meningoencephalitis is limited. Thus, the present study was designed to investigate the changes in the CSF and serum ADA activities and whether there is a correlation between CSF and serum ADA in the experimental model of listeriosis. Twenty adult white New Zealand rabbits of both sexes were used in this study. Fourteen rabbits were infected by intracerebral inoculation of L. monocytogenes 1/2a and 6 healthy rabbits were included in the study as the control group. CSF samples were obtained by puncture of the cisterna magna. Blood was sampled from the auricular vein of rabbits. ADA activity was determined using the colorimetric method. Mean±SD ADA activities in the CSF and serum were 14.98±3.26 U/L and 17.71±3.41 U/L in infected rabbits, respectively, while 4.72±1.86 U/L and 6.89±3.00 U/L in healthy rabbits. CSF and serum ADA activities showed significant alteration in rabbits with experimental listeriosis compared to control rabbits (p<0.001). A positive correlation was found between CSF and serum ADA activities in infected rabbits (r=0.65, p<0.05), but not in healthy rabbits. These findings imply that ADA plays a role in this disease process. In conclusion, CSF and serum ADA measurements may be used as a supplementary biochemical test in combination with clinical and laboratory findings of listerial meningoencephalitis.

Key words: adenosine deaminase, cerebrospinal fluid, Listeria monocytogenes, rabbit, serum

INTRODUCTION

Listeriosis is a disease caused by eating food contaminated with the bacterium Listeria monocytogenes (L. monocytogenes). L. monocytogenes is a ubiquitous gram-positive bacterium that can cause life-threatening infections including meningitis, meningoencephalitis, septicemia, pneumonia, abortion,
perinatal infections and gastroenteritis, as a severe, generalized blood-borne infection in humans, domestic and wild animals (Lorber, 1997). This bacterium has tropism for the brain, particularly, meninges. Meningitis, is the most common form of listerial infection (Lorber, 2000). In the adult population, *Listeria* is the third most common pathogen in community-acquired infective meningitis (Durand et al., 1993). The mortality rate is high despite antibiotic treatment (Lorber, 1997). Thirty-seven mammals and 20 bird species including cold blooded animals and ticks have been documented as carriers (Gray and Killinger, 1966). *L. monocytogenes* is isolated as the etiological agent of a septicemic disease affecting rabbits and guinea pigs (Murray et al., 1926). The main line of defense against *Listeria* infection is cell-mediated immunity and T-cell response plays a major role in controlling the multiplication of *L. monocytogenes* and suppression of listeriosis (Pamer, 1997). *L. monocytogenes* has been used for decades as a model for the study of cell-mediated immunity (Tvinnereim et al., 2002).

Adenosine deaminase (Adenosine aminohydrolase, EC 3.5.4.4, ADA) is an important enzyme which participates in purine metabolism, which catalyses the irreversible conversion of either adenosine and/or deoxyadenosine to inosine and deoxyinosine, respectively. This enzyme is widely distributed in vertebrates with the highest activity in lymphoid tissue (Kurata, 1995). ADA is mainly produced by immature T cells (Ungerer et al., 1992). It has been demonstrated that this enzyme plays a putative role in lymphocyte differentiation and is reported to be essential for normal growth and proliferation of T lymphoid cells (Kurata, 1995). ADA is present in lymphocytes in high levels and the levels of the enzyme in T-lymphocytes varies according to cellular differentiation (Galanti et al., 1981). There is a relation between ADA activity and cellular immune response including lymphoreticular cell activity (Collazos et al., 1998).

Elevated ADA activities have been documented in pathologic conditions such as systemic lupus erythematosus, brucellosis, tuberculosis, rheumatoid arthritis and toxoplasmosis (Choi et al., 2002; Jaqueti et al., 1991; Stancikova et al., 1998; Viciana et al., 1991; Yuksel and Akoglu, 1988). In several studies, ADA activities were found increased in meningitis with different etiologies (Casado et al., 1997; Martinez et al., 1992; Ribera et al., 1987). To the best of our knowledge, there is limited data regarding CSF and serum activities of ADA, and correlation between ADA activities of both compartments in listerial meningoencephalitis. Nishida et al. (2007) reported that ADA activity in CSF was increased in the listeria meningitis case. In addition, recently, Nakae and Kuroiwa (2009) demonstrated high levels of ADA associated with elevated T-lymphocytes in the cerebrospinal fluid in a case of listeria meningitis. Thus, the aim of this study was to investigate the changes in the CSF and serum ADA activities and whether a relationship between CSF and serum ADA in listerial meningoencephalitis.

**MATERIAL AND METHODS**

**Rabbits**

Twenty adult white New Zealand rabbits of both sexes, weighing approximately 2.6-3.0 kg each, were used in this study. Fourteen rabbits were
infected by intracerebral inoculation of *L. monocytogenes* 1/2a and 6 healthy rabbits were included in the study as the control group. The study was approved by the Kirikkale University Ethics Committee.

**Bacteria**

*L. monocytogenes* 1/2a test strain (Dr. Weise, Bundesgesundheitsamt, Berlin) was used as the infecting bacteria. It was inoculated into tryptic soya broth (Oxoid, Basingstoke, England) with 5% yeast extract (TSB-YE) (Oxoid) and incubated at 30°C during 24 hr. Serial dilutions in peptone water of TSB-YE were dropped on modified Oxford Agar (MOX, Difco, Detroit, USA) and this was incubated at 35°C during 48 hr. This solution was further diluted in saline to a concentration of 10⁷ colony-forming units (CFU)/mL.

**Experimental Listeria monocytogenes infection**

Rabbits were anaesthetised by intramuscular acepromazine (3 mg/kg), ketamine (30 mg/kg) and xylazine (15 mg/kg) injection (Täuber *et al.*, 1993). The duration of anaesthesia was 10-15 min. Infection was induced by direct inoculation of 0.3 mL physiological serum containing 10⁷ CFU/mL *L. monocytogenes* 1/2a by intracerebral route via the atlanto-occipital region of rabbits using a 22 G syringe (Abbott, Abbott Park, IL) (Marco *et al.*, 1991). Rabbits in the control group were given only sterile saline solution. CSF samples (0.5 mL) were obtained by puncture of the cisterna magna using a 24 G needle (Mediflon, Eastern Medikit Ltd., India). Blood (2 mL) was sampled from the auricular vein with a 24 G intracat (Mediflon, Eastern Medikit Ltd., India) and serum was obtained after centrifugation at 1550 x g for 10 min at 4°C. Serum samples were stored at −80°C and −20°C, respectively until assayed.

Nine rabbits died on the 3rd day and five rabbits were euthanasized on the 4th day after bacterial inoculation. Following necropsy, the entire brain was removed and tissue samples were fixated in Bouin’s fixative overnight at 4°C. Tissues were embedded in paraffin wax after dehydration in ethanol and xylene and sectioned on a microtome at 5 µm in thickness. Tissue sections were stained by Haematoxylin and Eosin and examined with an Olympus BX-50 microscope.

**Immunohistochemical methods**

**Production of primary antisera**

*L. monocytogenes* 1/2a strain antibody was produced in two rabbits. The *L. monocytogenes* 1/2a test strain was inoculated for 18 h at 37°C in tryptic soya broth (Oxoid). Broth culture inoculated with *L. monocytogenes* 1/2a test strain was incubated into Kolle flasks of trypticase soy agar at 37°C. Rabbits were injected 0.1, 0.2, 0.4, 0.8 and 1.0 mL of antigen intravenously. One week after the final inoculation serum samples were collected from both rabbits and were prepared for immunohistochemistry.

**Immunohistochemistry**

Following dehydration, formalin fixed, paraffin-embedded tissue sections were boiled in a microwave oven (600 W) four times for 5 min in citrate buffer (2.1 g
sodium citrate/l, pH 6.0). Endogenous peroxidase was inhibited with 1% H$_2$O$_2$ in methanol (1:10; 15 min) and 10% normal goat serum (Dako, Glostrup Denmark) was applied to block non-specific binding of immunoglobulins. The immunohistochemistry for *L. monocytogenes* 1/2a antigen was performed using a polyclonal antibody. A universal LSAB2 horseradish peroxidase (HRP) kit (Dako) was used for the demonstration of *L. monocytogenes* 1/2a antigen. All steps were performed at room temperature and in a humid chamber. The sections were then incubated with a 1:1024 dilution in phosphate-buffered saline (PBS; pH 7.4) of rabbit anti-*L. monocytogenes* 1/2a antigen for 60 min at room temperature. Antibody binding was visualised with an anti-rabbit biotinylated polyvalent secondary antibody (Dako) for 10 min, streptavidin-peroxidase enzyme (Dako) for 10 min, 3, 3'-diaminobenzidine (DAB) chromogen (Dako) for 10-15 min (controlled by visual observation with a microscope) and rinsed with distilled water. Sections were counterstained with Mayer’s haematoxylin for 1-2 min, rinsed with distilled water, and mounted with aqueous mounting medium. Negative control sections of the brain were used in all immunolabelling procedures. The distribution of immunoreactive cells was examined with an Olympus BX-50 microscope.

**Determination of ADA activity**

ADA activities in CSF and serum were determined using the colorimetric method described by Giusti (1974). ADA assay based on indirect measurement of formation ammonia produced when ADA acts in excess of adenosine. The production of ammonia was determined colorimetrically at 630 nm after development of blue colour with hypochloride and phenol in alkaline solution. All samples were analyzed twice. Enzyme activity was expressed U/L.

**Statistical evaluation**

Parametric values were defined as mean ± S.D. We used one-way ANOVA test for the comparison between two groups. Correlation analysis between the parameters was performed by Pearson’s correlation test, and correlative results were shown on a linear regression curve (Rao, 1973). Results were considered as significant when p values were less than 0.05.

**RESULTS**

**Microscopic findings**

In the parenchyma were prominent microabscesses and necrotic foci. Perivascular mononuclear cell layers were seen in the same areas. The meninges of all infected animals were inflamed especially in the brain stem and showed signs of meningitis. Thick perivascular inflammatory cell layers were seen in the meninges (Figure 1).

**Immunohistochemical findings**

The *L. monocytogenes* antigen immunoreactivity was characterized by prominent intracellular brown stained punctate foci by chromogen in neutrophils and macrophages. Bacterial antigen was detected in perivascular cuffs, glial foci,
microabscesses, necrotic foci and meninges (Figure 2). No antigen was detected in the brain samples collected from the control rabbits.

![Figure 1. Section of brain from an infected rabbit with *Listeria monocytogenes*, thick perivascular inflammatory cell layers were seen in meninges. Haematoxylin-Eosin. Bar: 50 μm](image1)

![Figure 2. Section of brain from an infected rabbit with *Listeria monocytogenes*, the bacterial antigen immunoreactivity with DAB chromogen is prominent in inflammatory cells in meninges. Streptavidin-peroxidase. Bar: 60 μm](image2)

**Adenosine deaminase activities**

The mean ± SD values of CSF and serum ADA activities were 14.98±3.26 U/L and 17.71±3.41 U/L in infected rabbits, respectively, compared with 4.72±1.86 U/L and 6.89±3.00 U/L in healthy rabbits (Figure 3). Compared to healthy rabbits, CSF and serum ADA activities were significantly higher (p<0.001) in infected rabbits. Besides, a positive correlation was found between CSF and serum ADA activities in infected rabbits (r²=0.65, p<0.05, Figure 4), but not in healthy rabbits.
DISCUSSION

Listeriosis is a severe infectious disease caused by a gram-positive bacterium, *Listeria monocytogenes* which is widespread in the environment, particularly in the soil, in decaying vegetation and fecal flora of many animals which causes serious infections in humans and animals (Lorber, 1997). Listeriosis characterized by high fever, severe gastroenteritis, movement disorders and seizures (Lorber, 2000). In high risk populations, including an immuno-compromised host, pregnant and newborn, infection can lead to severe infections such as septicemia and meningitis (Lorber, 1997). These features can be easily reproduced during experimental infection (Marco et al., 1991). *L. monocytogenes* has the ability to invade endothelial cells including brain microvascular endothelial cells and infects the CNS. Meningitis is the common form of CNS listeriosis (Lorber, 2000; Wilson and Drevets, 1998). Although the mechanism for entry of *L. monocytogenes* into the central nervous system (CNS) is not clarified fully, invasion of epithelial cells of the choroid plexus and microvascular
endothelial cells has been hypothesized (Zhang and Toumanen, 1999). Drevets and Leenen (2000) have also shown that bacteria can enter CNS via leukocyte-facilitated infection with adhesion of infected phagocytes to endothelial cells. Additionally, *L. monocytogenes* has the ability to invade neurons by spreading cell-to-cell (Dramsi et al., 1998). Innate and adaptive immunologic mechanisms play a crucial role in the protection against *L. monocytogenes* infection (Tvinnereim et al., 2002). Protective immunity to *L. monocytogenes* is exclusively mediated by T cells, mainly of the CD8+ type (Pamer, 1997), although CD4+ T cells have been also demonstrated to be protective (Hess et al., 1996).

ADA is the catabolic enzyme of nucleotides involved in purine metabolism that converts adenosine to inosine, which is mainly produced by immature T cells and plays a significant role in immune functions (Kurata, 1995). The significance of this enzyme in the cellular immune system was confirmed by reduced activities in severe combined immunodeficiency syndrome (Van der Weyden and Kelley, 1977). Lymphocytes are known to contain the highest ADA activity and many investigators have suggested that ADA in body fluids have originated from this cell type (Galanti et al., 1981). It has been reported that the increased ADA activity is associated with T lymphocyte proliferation and macrophage activation (Ungerer et al., 1992). ADA activity was used to evaluate cell mediated immune responses in several diseases. Its plasma activity is found to be elevated in diseases where cellular immunity is stimulated, therefore, ADA has been considered as a marker of cell mediated immunity (Galanti et al., 1981; Piras and Gakis, 1973). It has been established in a mouse model that protective acquired immunity to listeria involves T-cell related activation to protection against *L. monocytogenes* infection (D’Orazio et al., 2003).

High serum ADA activity has been recorded in various diseases, including, infection, malignancy and autoimmune disease (Stancikova et al., 1998; Viciana et al., 1991; Yuksel and Akoglu, 1988). Several studies have been carried out to evaluate the role of ADA in bacterial meningitis and it has long been known that there is an important relationship between meningitis and the activities of some enzymes in the CSF and serum. In previous studies, ADA activities of CSF were found to be higher in patients with cryptococcal (Martinez et al., 1992) and candidal (Casado et al., 1997) meningitis, and cerebral toxoplasmosis (Pedro-Botet et al., 1991). Recently, Nakae and Kuroiwa (2009) demonstrated high levels of ADA associated with elevated T-lymphocytes in the cerebrospinal fluid in a case of listeria meningitis. They suggested that high levels of CSF ADA should be considered in the differential diagnosis of listeria meningitis.

In the study described here, CSF and serum ADA activities showed significant elevations in rabbits with experimental listeriosis compared to healthy rabbits (p<0.001). Results of the present study also revealed an intense relationship between CSF and serum ADA activities in infected rabbits (r=0.651, p<0.05) (see Figure 4), but not in healthy rabbits. These results suggest that elevations in the ADA activity may involve the stimulated cellular immune response and increased T-cell activation in rabbits infected with *L. monocytogenes*. CSF ADA activities were elevated in rabbits with meningitis, which is in agreement with other studies (Nakae and Kuroiwa, 2009; Piras and
Gakis, 1973; Ribera et al., 1987). In conclusion, the findings from this study show a dramatical increase of CSF and serum ADA activity in rabbits experimentally infected with \textit{L. monocytogenes}. Based on these results, ADA measurements in CSF and serum may be used as a supplementary biochemical test in combination with clinical and laboratory findings of listerial meningoencephalitis.

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Listerioza je teško infektivno oboljenje ljudi i većine sisara izazvano bakterijom *Listeria monocytogenes*, a može dovesti do ozbiljnog meningoencefalitisa. Povećana aktivnost enzima adenozin deaminaze (ADA) u cerebrospinalnoj tečnosti (CSF) je registrovana tokom meningo-encefalitisa različite etiologije ali su podaci za nivo aktivnosti ADA u serumu i CSF za meningoencefalitis izazvan bakterijom *Listeria monocytogenes* oskudni. Cilj ovih ispitivanja je bio da se ispita aktivnost ADA u serumu i CSF tokom eksperimentalne listerioze i utvrdi eventualna korelacija izmedju ova dva parametra. U ogledu je korišćeno 20 kunića oba pola, novozelandske bele rase. Grupi od 14 jedinki je intracerebralno inokulisana kultura *L. monocytogenes* 1/2a, dok je 6 zdravih kunića predstavljalo kontrolnu grupu. Za prikupljanje uzoraka CSF punktirana je *cisterna magna* a krv je uzorkovana iz v. *auricularis*. Aktivnost AD je određivana kolorimetrijskom metodom. Srednja vrednost za aktivnost ADA ± SD u CSF i krvnom serumu inficiranih kunića je iznosila 14,98 ± 3,26 i 17,71 ± 3,41 respektivno, dok je kod zdravih jedinki ona bila 4,72 ± 1,86, odnosno 6,89 ± 3,00. Ove razlike su bile statistički značajne na nivou od 99,9 %. Utvrđena je pozitivna korelacija ova dva parametra kod obolelih (r = 0,65, p<0,05) ali ne i kod zdravih kunića. Ovi rezultati ukazuju da ADA ima ulogu u ovom patološkom procesu tako da se određivanje njene aktivnosti može koristiti kao dodatni biohemijijski test kod meningoencefalita izazvanog *L. monocytogenes* uz kliničke i druge laboratorijske nalaze.