Clinical Significance of Adenosine Deaminase in Veterinary Medicine

（獣医学領域におけるアデノシンデアミナーゼの
臨床診断的意義に関する研究）

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ABBREVIATIONS

AC	Asymptomatic carrier
ACP	Acid phosphatase
Ado	Adenosine
ADA	Adenosine deaminase
AIDS	Acquired immune deficiency syndrome
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
AMV	Avian myoblastosis virus
ARC	AIDS-related complex
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
ConA	Concanavarin A
CPA	Cyclophosphamide
CRF	Chronic renal failure
dAdo	2`deoxyadenosine
DXR	Doxorubicin
D-PBS	Dulbeccos' PBS
EHNA	Erythro-9- (hydroxyl-3-nonyl) adenine
FAIDS	Feline AIDS
FBS	Fetal bovine serum
FIV	Feline immunodeficiency virus
GGT	Gamma glutamyl transferase
GLDH	Glutamate dehydrogenase
H1.077	Histopaque 1.077

H1.119	Histopaque 1.119
HIV	Human immunodeficiency virus
L-ADA	Lymphocyte ADA
L-ASP	L-Asparaginase
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
mRNA	Messenger RNA
MTT	2-[4,5-dimethylthiazol-2-y]-2,5-diphenyl tetrazolium bromide
N-ADA	Neutrophil ADA
P-ADA	Plasma ADA
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGL	Persistent generalized lymphadenopathy
PMNC	Polymorphonuclear neutrophil cells
Pred.	Prednisolone
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SCID	Severe Combined Immunodeficiency Disease
SI	Stimulation indexes
T-ADA	T cell ADA
Tm	Melting temperature
VRC	Vincristine

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Enzymes are proteins that have catalytic properties that include specific activation of their respective substrates. Much emphasis is placed on the application of plasma enzymes as markers of organ damage, with many enzymes used in toxicological studies to measure cellular injury, enzyme induction and activation or inhibition of enzymes. The distribution of enzymes in different tissues varies between tissues, and therefore influences their diagnostic value in particular species. The tissue distribution of an enzyme can be affected by age and sex and may vary in the different cell types within an organ (Braun et al, 1983).

The intracellular distribution of enzymes also varies and the proportions may be such that an enzyme can be regarded as relatively specific to a particular type of organelle. Several enzymes are cytosolic, for example LDH, whilst other enzymes are located in organelles such as GLDH in mitochondria or ACP in lysosomes. Cytoplasmic enzymes are usually soluble, easily released, and readily pass through the cell membrane (even when it appears microscopically intact). This property makes them sensitive diagnostic markers. Some enzymes occur both in mitochondria and in the cytosol, whereas other enzymes may be largely membrane bound e.g. GGT. Membrane–bound enzymes are not soluble and are firmly attached to the cell membrane and may be shed after severe damage.

When there is tissue injury there is increased release of some enzymes. Injury may range from overt cell death (damaged membrane and organelles) to metabolic

alterations without microscopically visible cell changes. Hence, cells need not die to release their enzymes. A short period of hypoxia is enough to disrupt the integrity of the cell membrane and potentially allow soluble cytosolic enzymes to escape or leak into their surrounding matrix to be drained away in lymph (Lemasters et al, 1983). Hypoxic or toxic injury results in exocytosis or formation of membrane blebs where their cytosolic enzymes are released into the surrounding plasma (Gores et al, 1990).

Plasma enzymes may be classified as (i) Plasma specific enzymes, (ii) other secreted enzymes, e.g. amylase, and (iii) intracellular enzymes. Plasma enzymes include those enzymes that are secreted by some organs and have a direct action in the plasma, for example coagulation enzymes.

For several enzyme measurements, it is preferable to use plasma rather than serum because of the release of erythrocytic enzymes during the clotting process (Korsrud and Trick, 1973). Some enzymes are present at relatively high concentrations in erythrocytes compared to plasma and therefore may interfere with the measurements (Czerwek and Bleuel, 1981).

There are a number of enzymes that are established as diagnostic enzymes in small animal medicine including, ALP which has been as an indicator of hepatic injury since the twenties. This enzyme is found primarily in intestine, kidney, liver and bone. ALT is a cytoplasmic enzyme that is of great importance in diagnosis of liver disease in small animals. LDH is contained in various tissues and is elevated during tissue damage. Despite the existence of many enzymes that are available for clinical diagnosis in small animals, investigations into new enzymes of clinical significance is justified in order to improve or compliment the diagnostic ability of current ones.

1.2. ADENOSINE DEAMINASE

ADA (EC 3.5.4.4) is an important enzyme of purine catabolism. It catalyses the deamination of Ado and dAdo to produce inosine and 2`deoxyinosine respectively. The importance of the enzyme for vertebrate organisms stems in part from the physiological impact of its substrates. ADA has in the past been thought to be purely cytosolic but has been also found on the cell surface of lymphocytes (Aran et al, 1991). There is recent evidence about a specific role of ecto-ADA, which is different from that of intracellular ADA. Apart from degrading extracellular Ado or dAdo, ecto-ADA has an extraenzymatic function via its interaction with CD26. CD26 is a sialoglycoprotein whose physiological role seems to be related to cell activation. The ADA-CD26 interaction results in co-stimulatory signals in T cells (Franco et al, 1998).

ADA is present in all cell types but the amount of enzyme differs widely amongst tissues. The highest ADA levels in humans are found in lymphoid tissues (Hirschhorn et al, 1978). In animals ADA has been shown to have higher activity in organs such as spleen, lymph nodes and thymus in most species (Tanabe, 1993). In human, the activity of ADA has been shown to be greatest in lymphocytes and higher in T than in B cells. One report has documented the levels of T-ADA activity to be ten times that of B cells. As ADA activity is increased in plasma or body fluids in diseases where cell mediated immunity is stimulated, it has been considered a marker of T cell activation (Kose et al, 2001, Hovi et al, 1976). In addition, ADA has been reported as a marker of cell-mediated immunity in human (Baganha et al, 1990).

The enzyme plays a vital role in the maturation of the immunological system because congenital deficiency of this enzyme in erythrocytes and lymphocytes in human is associated with SCID. The patients usually present in infancy with recurrent infections, lymphopenia, defective proliferative responses to mitogens,

hypoglobulinemia, and an inability to mount specific antibody responses (Bollinger et al, 1996). This condition is characterized by both T- and B- cell function impairment. Several theories exist as to how deficiency in a purine catabolic enzyme can cause lymphopenia. Most evidence suggests that accumulation of ADA substrates is detrimental to lymphocyte development and survival (Aldrich et al, 2000). Another theory is that elevated Ado levels could also trigger aberrant Ado receptor signaling. Ado transduces extracellular signals by binding to G-protein-coupled Ado receptors that can regulate intracellular cAMP and calcium levels. Ado receptor engagement can thus lead to elevation of cAMP levels that can lead to thymocyte apoptosis and developmental arrest (McConkey et al, 1990). Like Ado, elevated dAdo is thought to inhibit S-adenosylhomocysteine hydrolase, an enzyme critical to cellular transmethylation reactions, resulting cell death and apoptosis mediated by the latter (Ratter et al, 1996). Most of the clinical signs of the disease in humans, can be attributed to the existing T, B and NK cell lymphopenia, other abnormalities include hepatic pathology, costochondral junction abnormalities, increased asthma incidence and neurological abnormalities (Aldrich et al, 2000, Hirschhorn, 1995). In animals, some studies have shown that ADA deficient mice die perinatally with marked liver-cell degeneration (Migchielsen et al, 1995 and Wakamiya et al, 1995). In addition, other studies, in ADA deficient mice, have showed marked metabolic and immunological abnormalities such as lymphopenia, elevated plasma Ado, severe liver impairment, pulmonary insufficiency and elevated adenosine levels in plasma and some organs (Blackburn et al, 1996). However, a study by Tax and Veerkamp (1978) reported that ADA activity levels in horse lymphocytes were comparable to those in lymphocytes of human patients with SCID associated with ADA deficiency. Additionally, a study by McGuire et al (1976) also showed that ADA activity might

not be necessary for normal lymphocyte function in horses because low ADA activity was found in lymphocytes of both healthy adults and foals with combined immunodeficiency.

ADA has two principal isozymes, ADA1 and ADA2, which have different optimal pH, Michaelis constants and relative substrate specificity patterns (Ungerer et al, 1992). ADA 1 activity is inhibited by EHNA while ADA2 is not. ADA1 is found in most body cells, particularly lymphocytes (Shibagaki et al, 1996), where it is present not only in the cytosol but also as the ecto-form on the cell membrane attached to CD26. This isozyme is critically important in lymphocyte proliferation and development and its deficiency leads to SCID in humans (Conlon and Law, 2004). The isozyme ADA2 is the major component (73%) of the activity of total ADA in the serum of healthy persons (Merrikhi, 2001). ADA2 has been suggested to be an indicator of macrophage activation or turnover (Casal et al, 2002). ADA2 is increased in many diseases, particularly those associated with the immune system: for example rheumatoid arthritis, psoriasis and sarcoidosis. The plasma ADA2 isoform is also increased in most cancers. In animals according to a study by Tanabe (1993), there was no serum ADA2 activity in cows and rats, whereas other species such as dogs, cats and pigs showed only slight levels. However, a recent study in rats, by Conlon and Law (2004), ADA2 was shown to be in greater quantities in macrophages than monocytes and also that these cells released ADA2 into their surroundings following an inflammatory response.

1.3 GENERAL OBJECTIVES

i) To evaluate the usefulness of ADA as an enzyme of clinical significance in canine and feline disease.

ii) To investigate the relationship between ADA and the immune system with particular reference to lymphocytes in dogs and cats.

iii) Study ADA activity as a prognostic factor for disease progression in some canine and feline diseases.

CHAPTER 2

PLASMA ADENOSINE DEAMINASE ACTIVITY IN NORMAL AND DISEASED DOGS AND CATS

2.1 INTRODUCTION

ADA is widely distributed in human tissues with the highest activity found in the spleen and gastrointestinal tract. It is considered as an auxiliary diagnostic tool and a reliable marker of human tuberculosis (Orphanidou et al, 1996). ADA activity is increased in several other diseases including hepatic disease (Goldberg, 1965), cutaneous leishmaniasis (Erel et al, 1998), meningitis (Baheti et al, 2001), leukemia (Morisaki et al, 1985), lymphoma (Ganeshaguru et al, 1981), nephrotic syndrome (Misra et al, 1997), brucellosis (Cesur et al, 2004), hepatitis (Vasudha et al (2006), pneumonia (Nishikawa et al 1988) and sarcoidosis (Taylor, 1986). In addition ADA has been proven useful in differentiating causes of some diseases such as meningitis (Baheti et al, 2001), jaundice (Goldberg, 1965), peritonitis (Leksrisakul et al, 2001) and hepatic disease (Nishikawa et al, 1986). In humans, the diagnostic value of ADA activity in various body fluids has also been analyzed, such as in the sputum of patients with pulmonary tuberculosis in which the enzyme was elevated compared to that from cancer and obstructive lung diseases (Dilmac et al, 2002). Saracoglu et al (2005) analyzed ADA in patients with oral and laryngeal cancer, in which ADA was lower in the latter than the former.

2.2. OBJECTIVE

To investigate P-ADA activity in normal and in different canine and feline diseases in order to establish whether age has an effect on ADA activity and whether ADA activity is of any clinical value.

2.3. MATERIALS AND METHODS

2.31. ANIMALS

Blood was collected from normal dogs (n= 42, 20 males, 22 females) and cats (n=16, 6 males and 10 females). The age ranged from 4.6±2.9 yrs in dogs and 6.6± 4.6 years in cats. The dogs with lymphoma comprised of 7 males and 6 females with age 7.3±2.7 years. The age of dogs with hepatitis was 5.0±2.3 years of which 6 were male and 4 female. The dogs with tumors were comprised of 6 males 4 females and the age ranged from 10.0±3.0 years. The dogs with demodicosis were 2 males and 1 female with age of 5.0±6.0 years. The FIV positive cats were comprised of 4 males and 4 females and the age ranged from 9.0±2.5 years. The cats with CRF composed of 2 males and 3 females and the age ranged from 7.0±3.0 years. Heparinized blood was collected largely from the jugular vein.

2.32. MEASUREMENT OF P-ADA ACTIVITY

Heparinized blood was centrifuged at 1500 rpm for 15 minutes and the plasma analyzed for ADA activity. P-ADA activity was assayed using a commercial ADA kit (Serotec, AD-L, Sapporo, Japan.) using an autoanalyzer (Accute, Toshiba-40FR Clinical Laboratory System, Tokyo, Japan) at 37°C.

Statistical analyses were carried out using Student T test and Pearson's correlation. Probability with values $P<0.05$ were considered statistically significant. Data are summarized as mean $\pm$ SD.

2.4. RESULTS

P-ADA activity in healthy normal dogs (n=42) was 3.44 ± 2.02 (IU/L), in dogs less than 5 years (n=25) 2.9 ± 2.17 (IU/L) and those over 5 years (n=17) 4.4 ± 2.4 (IU/L, Fig. 2-1). Dogs older than 5 years had a significantly higher P-ADA than the younger dogs ($P<0.05$). There was no correlation between age and P-ADA in the normal dogs. P-ADA activity was significantly elevated in lymphoma (n=13), hepatic disease (n=10), and demodicosis (n=3) in Fig 2-2. There was no increase in P-ADA activity in dogs with other tumors (n=10).

P-ADA activity in healthy normal cats was 48.6 ± 14.6 (IU/L). Cats less than 5 years (n=8) had P-ADA activity of 32.4 ± 3.28 (IU/L) and over 5 year olds (n=8), 55.1 ± 11.9 (IU/L, Fig. 2-3). Cats older than 5 years (n=8) had a significantly higher P-ADA activity than the younger ones ($P<0.01$). There was a positive correlation between age and P-ADA activity in the normal cats ($r = 0.48$, $P<0.05$, Fig. 2-4). FIV positive cats ADA activity was as follows: 49.8 ± 16.6 (IU/L), AC 35.6 ± 4.97 (IU/L) and ARC 64.1 ± 9.1 (IU/L, Figs. 2-5 & Fig. 2-6). P-ADA activity was significantly increased in cats in the ARC stage of FIV infection when compared with the AC ($P<0.005$) and control groups ($P<0.05$). P-ADA activity in cats with CRF was $48.6 \pm$ 0.36 IU/L (Fig. 2-5). There was no significant difference between controls and cats with CRF.

2.5. DISCUSSION

Our results also show that in both dogs and cats, animals younger than 5 years had significantly lower P-ADA activity than older ones. This is further highlighted by the positive correlation seen between age and P-ADA activity in cats. Our results concur with those of Vasudha et al (2006) who also observed higher levels in serum ADA in older people compared to younger ones. In this study canine P-ADA activity was lower than that of the feline species. In addition, normal P-ADA activity in cats has been reported to be higher than other species including dogs, rabbits, cows, pigs, horses and rats (Tanabe, 1993). It is difficult to explain the complexities that are involved in the differences of P-ADA activity between the different species, however factors involved in plasma enzyme modulation may play a key role. The plasma activity of an enzyme depends on several factors including the enzyme concentrations in different tissues, the intracellular location of the enzyme, rate of synthesis of the enzyme, severity of tissue and cellular damage, the molecular size of the enzyme and the rate of clearance of the enzyme from plasma. The 'normal' serum enzyme activity probably reflects a balance between physiologic cell death and degradation/activation by the macrophage system or, less commonly excretion. It is therefore possible that any of the afore-mentioned factors may be responsible for differences seen in P-ADA activity of dogs and cats.

In animals, total serum ADA activity is reported to be elevated in bovine leucosis (Chikuma, 1997 and Yasuda et al, 1996), liver diseases (Abd Ellah et al, 2004 and Chikuma, 1997) and tuberculosis (Silva et al, 2006); canine liver disease (Altug and Agaoglu, 2000 and Tanabe, 1993) and feline infectious peritonitis (Tanabe, 1993), chronic fasciolasis in sheep (Kozat et al, 2006) and white muscle disease in lambs (Altug et al, 2006).

In dogs P-ADA activity was markedly elevated in lymphoma, hepatic disease and demodicosis. Our results are in agreement with previous studies in human that showed that serum was elevated in lymphoma (Vezzoni et al, 1984, 1985). Canine demodicosis, a common skin disease of dogs in which proliferation of *Demodex canis*, is associated with the development of cutaneous lesions. Caswell et al (1997) have recently demonstrated that this disease is characterized by lymphocytic folicullitis and peripheral blood increase of cytotoxic T lymphocytes. The increase of P-ADA activity seen in demodicosis may therefore be a reflection of activation and mobilization of these cells. The current results concurred with a study on ADA in dogs with induced liver toxicity, by Altug and Agaoglu, (2000), that also revealed high ADA activity. Abd Ellah et al (2004) reported that serum ADA activity was increased in cows with liver disease and suggested a possible link to the degree of hepatocellular damage. Furthermore, in human studies high serum ADA activity has been reported in chronic hepatitis, liver cirrhosis, chronic active hepatitis and hepatoma and the authors suggest that serum ADA isozymes may be a new marker for liver disease (Kobayashi et al, 1993). Increased ADA activity also paralleled liver damage demonstrated histopathologically in the acute group in this study. Determination of P-ADA activity may be useful in the assessment of liver disease in dogs. This may be of particular value in chronic hepatic disease where routine liver enzymes are usually unremarkable.

P-ADA activity was significantly higher in the ARC group than in the AC and control groups. There was no significant difference between the ages of cats in the AC and those in the ARC stage of FIV infection. These observations imply that P-ADA activity is up-regulated in advanced clinical disease. These results were consistent with those in HIV infection where ADA activity was reported to vary significantly

between the infection stages (Goto et al, 1992). Inigo et al (1992) also showed that serum ADA activity progressively and significantly increases in symptomatic HIV-infected.

The increase seen in hepatic disease, lymphoma and demodicosis suggests that ADA activity may be of value in the diagnosis of these conditions. Demodicosis and the ARC stage of FIV infection are both characterized by chronic inflammation. Therefore P-ADA activity may be high in other conditions associated with chronic inflammation. Monitoring of P-ADA activity has potential in FIV infection as increases of the enzyme in cats in the AC group may suggest progression of the disease to the ARC phase. Our results also suggest the importance of taking age into consideration when interpreting P-ADA activity results in dogs and cats. Based on these results, lymphoma and FIV were highlighted for further investigations.

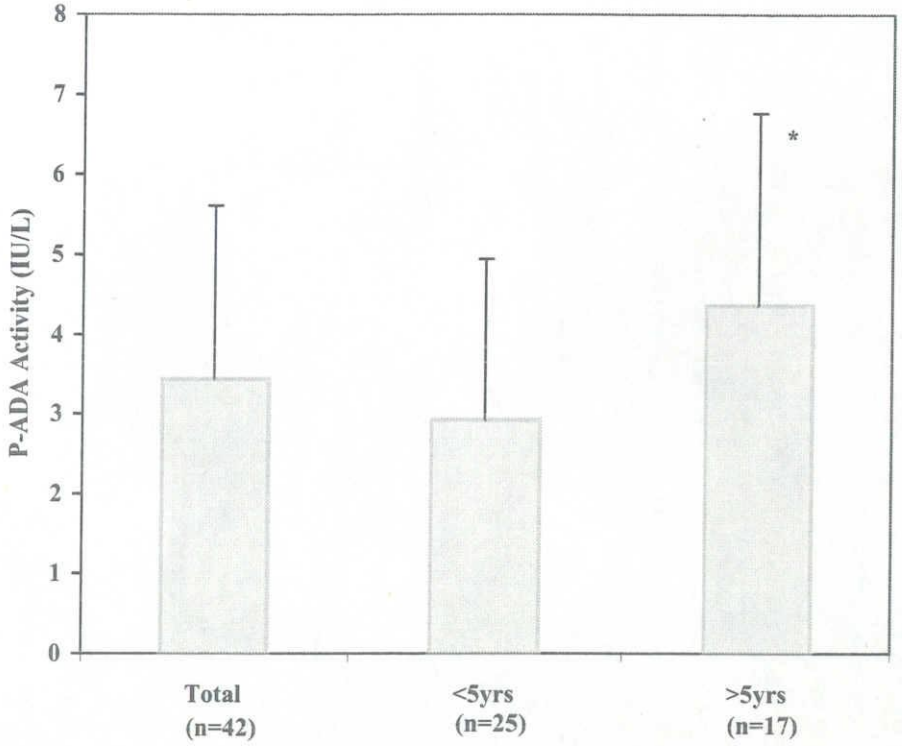

Fig. 2-1. Graph showing the normal canine P-ADA activity. P-ADA of dogs above 5 years was significantly higher than those under 5 years (*P<0.05). Total represents the whole population.

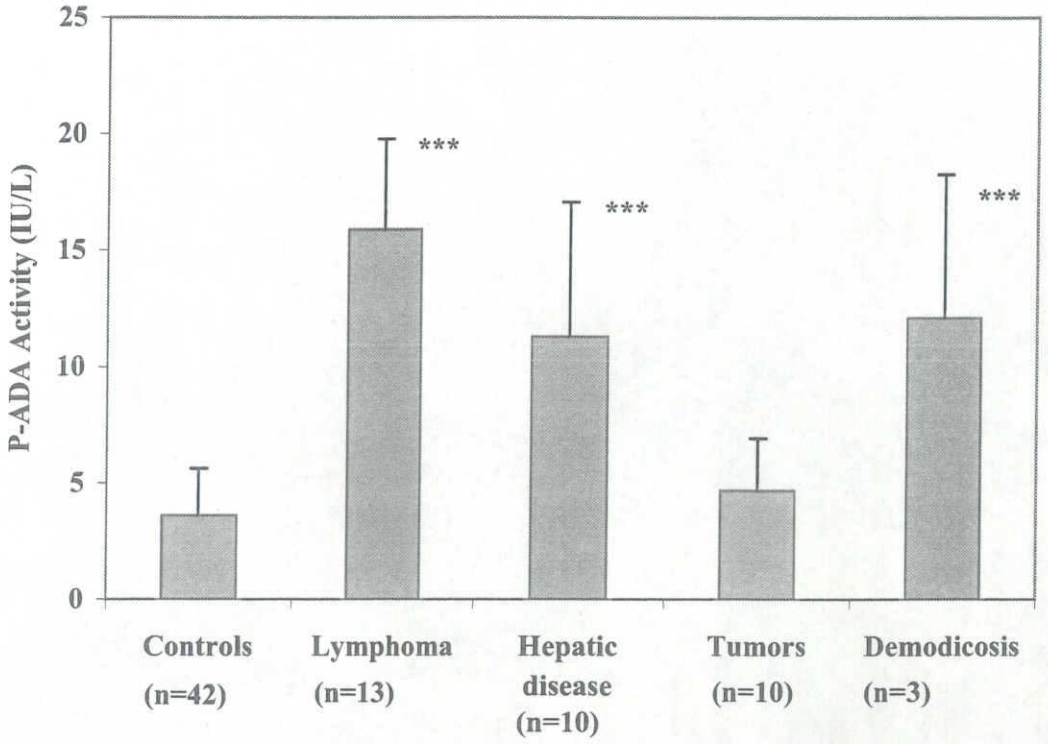

Fig. 2-2. P-ADA activity in normal and dogs with disease. There were significant increases in P-ADA activity in dogs with lymphoma, hepatic disease and demodicosis when compared with the controls (***P<0.005).

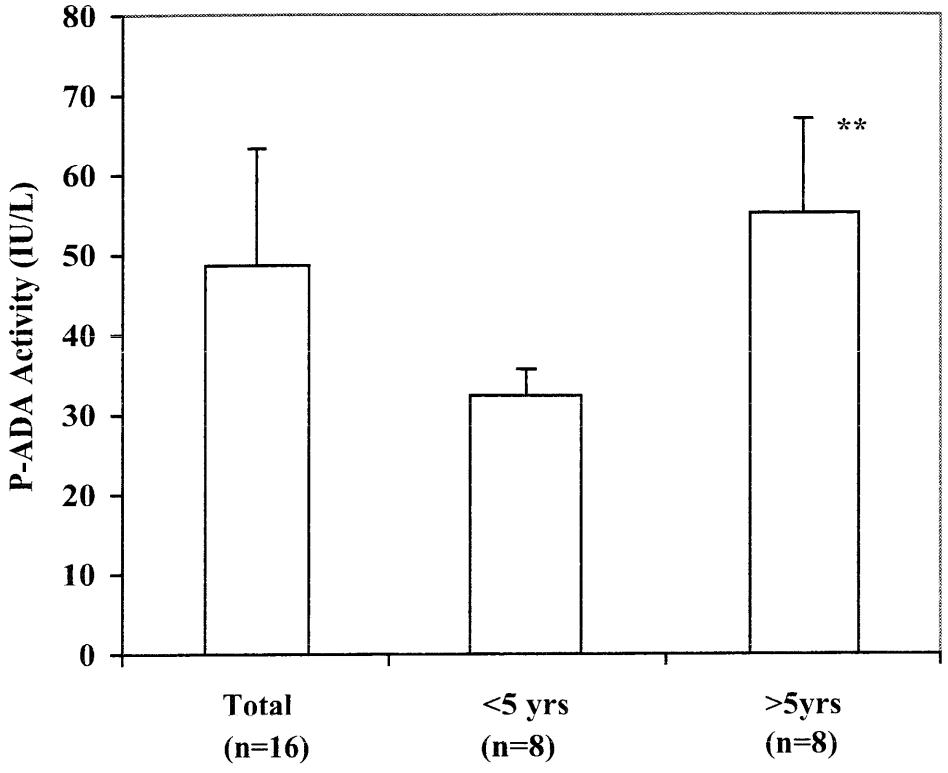

Fig. 2-3. Graph showing P-ADA activity in healthy feline controls. P-ADA activity of cats above 5 years was significantly higher than those under 5 years (**P<0.01). Young cats appear to have lower P-ADA activity levels than older ones.

Fig. 2-4. Relationship between age and P-ADA activity in feline controls (r=0.48, P<0.05).

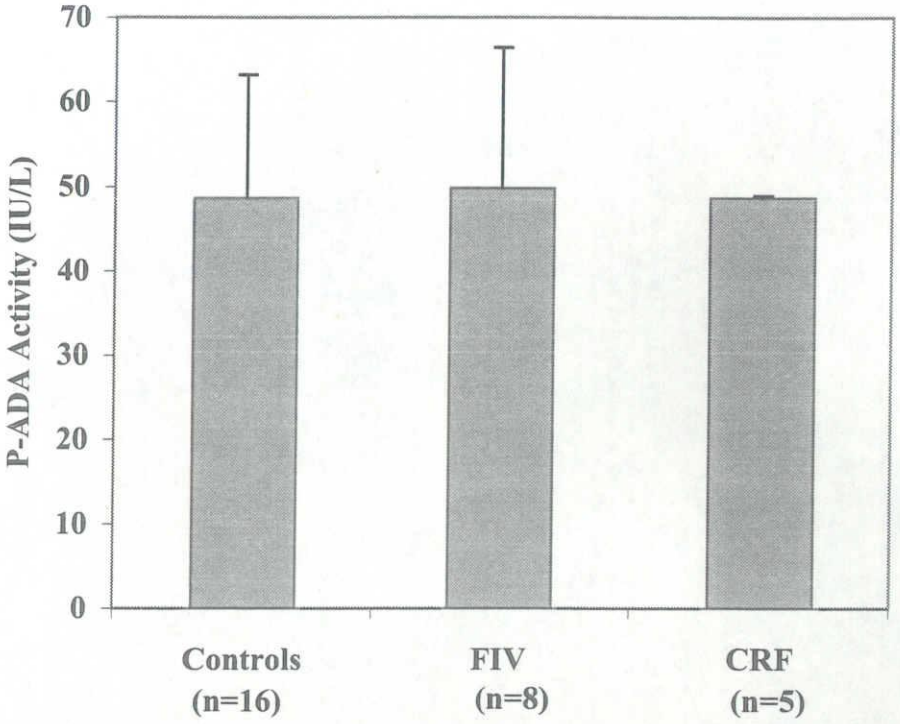

Fig. 2-5. P-ADA activity in healthy controls and cats with FIV infection and CRF.

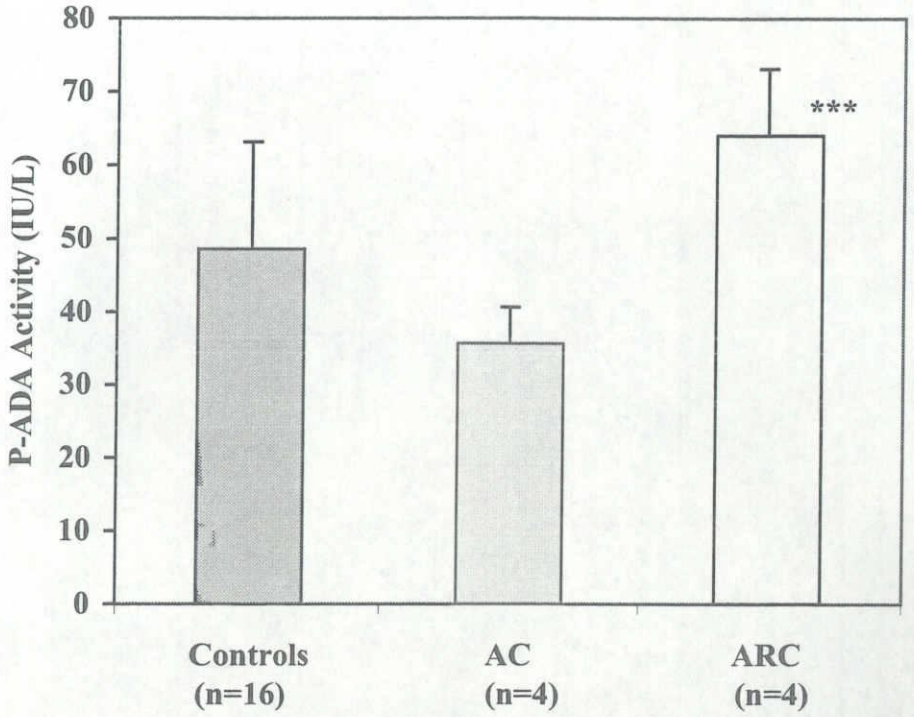

Fig.2-6.Diagram showing P-ADA activity in FIV-positive cats. A significant increase (***P<0.005) was seen in the ARC group compared to the AC and the controls (P<0.05). P-ADA activity was markedly increased in cats in the ARC stage of FIV infection.

CHAPTER 3

INVESTIGATION OF CLINICAL VALUE OF ADA IN CANINE LYMPHOMA

3.1 INTRODUCTION

Results of the previous study showed that P-ADA activity is elevated in canine lymphoma, in this study we investigated whether P-ADA has a role in monitoring of dogs undergoing chemotherapy.

Canine lymphoma is a progressive fatal disease caused by the malignant clonal expansion of lymphoid cells. Lymphoma most commonly arises from organized lymphoid tissues including bone marrow, thymus, lymph nodes and spleen. In addition to these primary and secondary lymphoid organs, common extra-nodal sites include the skin, eye, central nervous system, testis and bone. Lymphoma is a neoplasm that affects dogs of all ages and gender. The etiology of canine lymphoma is not known. A genetic component is suspected as there appears to be a breed-predisposition to this neoplasm such as Boxer, Basset Hound, Rottweiler, Cocker Spaniel, St. Bernard, Scottish Terrier, Airedale Terrier, English Bulldog and Golden Retriever (Lurie et al, 2004). Clinical features include 4 basic anatomic forms of presentation: multicentric which is characterized by generalized lymphadenopathy; splenic, hepatic and bone marrow involvement; mediastinal mainly characterized by mediastinal lymphadenopathy; alimentary, characterized by gastrointestinal infiltration and extranodal which may affect any organ or tissue. The definitive diagnosis of lymphoma can be obtained easily by either a cytological or

histopathological evaluation of the affected organ system. Factors used in evaluating affected dogs include clinical cancer stage, serum calcium levels, histologic grade and immuno-phenotype.

3.2. OBJECTIVE

To investigate whether ADA is of any clinical or prognostic value in dogs with lymphoma undergoing chemotherapy.

3.3. MATERIALS AND METHODS

Six dogs with lymphoma presented to Iwate University Veterinary Teaching Hospital were included in this study. The dogs' signalments are shown in Table 3-1. A definitive diagnosis was based on fine-needle aspiration biopsy cytological examination of enlarged superficial lymph nodes. Blood samples for ADA and other clinicopathological tests were collected at initial presentation and on a weekly basis, in heparin and plain tubes. Blood was also collected in heparin from 42 healthy dogs for ADA analysis. Samples for ADA analysis were centrifuged at 1500 rpm for 15 minutes and analyzed using an ADA reagent kit (Serotec, AD-L, Sapporo, Japan) in an autoanalyzer (Hitachi 7060,Tokyo, Japan) at 37°C.

The Wisconsin protocol (VCR, CPA, Pred., DXR and L-ASP) was employed over a 25-week course. Disappearance of all measurable tumor mass or lymph node size reduction was considered to be a complete response to therapy.

Lymph node volume cm^3= $\frac{\text{length x width x height x 3.14}}{6}$

Statistical analyses were carried out using Student's T test and Spearman's Correlation. Results were reported as mean $\pm$ SD and $P<0.05$ was considered to be of statistical significance.

3.4. RESULTS

Normal ADA activity in dogs was 3.44 ± 2.02 IU/L. There was a significant difference in total P-ADA activity between the controls and the dogs with lymphoma at first presentation ($P<0.005$, Fig. 3-1). A wide range of ADA activity was seen in the dogs with lymphoma both at presentation and in the course of the treatment. There was no significant difference in total P-ADA values due to sex or age. There was however no relationship found between lymphocytes, red blood cell number and ADA activity in dogs with lymphoma.

There was no correlation between clinical stage and total P-ADA activity ($r=0.28$, $P>0.05$). There was no association between total P-ADA and lymph node size during the course of therapy, however one case (No. 4) of multicentric lymphoma (Fig. 3-2) showed a strong relationship between the two parameters. There was no relationship found between P-ADA activity and chemotherapy.

Of the three dogs that died, two cases (Nos. 4 & 5) showed marked elevation in total P-ADA activity upon relapse after having relatively stabilized, however the dog with mediastinal lymphoma (case No. 3) did not show such a pattern (Fig. 3-3).

3.5. DISCUSSION

In this study total P-ADA activities were higher in the dogs with lymphoma than the over 5 years-old controls. ADA is found in most tissues, its activity is greatest in the lymphoid tissues, and more specifically the T lymphocytes (Adams and Harkness, 1976). These higher levels of P-ADA activity could be a reflection of the proliferative activity of the tumors and the stage of differentiation reached by both the normal and the neoplastic lymphoid cells.

Our results did not show any significant difference in total P-ADA between dogs with mediastinal and those with multicentric lymphoma at presentation. According to a study done in human lymph node samples by Ganeshaguru et al (1981), the highest levels of ADA were found in T-cell tumors of lymphoblastic and diffuse undifferentiated types while in the B-cell type tumors, the level of ADA varied with the proportions of T-cells in the tumor. Therefore, tumor characteristics including phenotype may be responsible for the differences seen between P-ADA activities in the two groups.

According to Vezzoni et al (1985), a relationship was found between ADA activity of various histotypes of non-Hodgkin's lymphoma in humans, and their grade of malignancy. Our results did not show any association between the cancer stage and total plasma activity at presentation.

Total P-ADA activity was seen to decrease drastically upon the commencement of treatment but then was seen to fluctuate throughout the course and appeared to be very variable. There was also great variability in the P-ADA activity at presentation. A study in humans, by Ponce et al (2004), reported that significant differences seen between the lymphoma subtypes are suggestive of inherent biological

features and clinical behavior of these tumors. These findings may account for the variability seen in total P-ADA activity prior and during the chemotherapeutic regime.

The dramatic elevation of total P-ADA activity upon relapse in patients (Case Nos. 4 & 5) and the strong relationship noted between lymph node size and P-ADA activity (case No. 4) reiterates the potential use of this enzyme in patient monitoring. ADA activity of cells from human neoplastic lymph nodes is related to the proliferative activity of lymphomas (Ungerer et al, 1992). This probably in turn affects the plasma and a similar assumption could be made in dogs.

ADA may be as useful in the diagnosis of canine lymphoma like it is in human medicine. However the tumor histological type may be a major determining factor, because ADA has been reported as a marker of human lymphoblastic lymphoma that showed unusually high enzyme levels in a study by Vezzoni et al (1984). Since there is no information in the literature on ADA activity in canine lymphoma or its response to chemotherapeutic treatment, this preliminary study provides valuable insight on the potential of this enzyme in the diagnosis and assessment of the canine patient with lymphoma. Our study is, however, limited by the lack of tumor characterization. Based on these results, further studies were carried out to investigate the role of lymphocytes and neutrophils in P-ADA activity in canine lymphoma.

Table 3-1. Profile of dogs with lymphoma at first presentation

CASE NO.	BREED	AGE (yrs)	SEX	LYMPHOMA	CANCER STAGE	Initial ADA (IU/L)
1	Corgi	4	F	Multicentric	5	13.8
2	Golden Retriever	8	F	Mediastinal	1	7.8
3	Mixed breed dog	11	M	Mediastinal	5	5.4
4	Maltese Poodle	12	M	Multicentric	5	15.5
5	Shi tzu	7	M	Multicentric	4	3
6	Yorkshire terrier	9	M	Multicentric	5	7.4

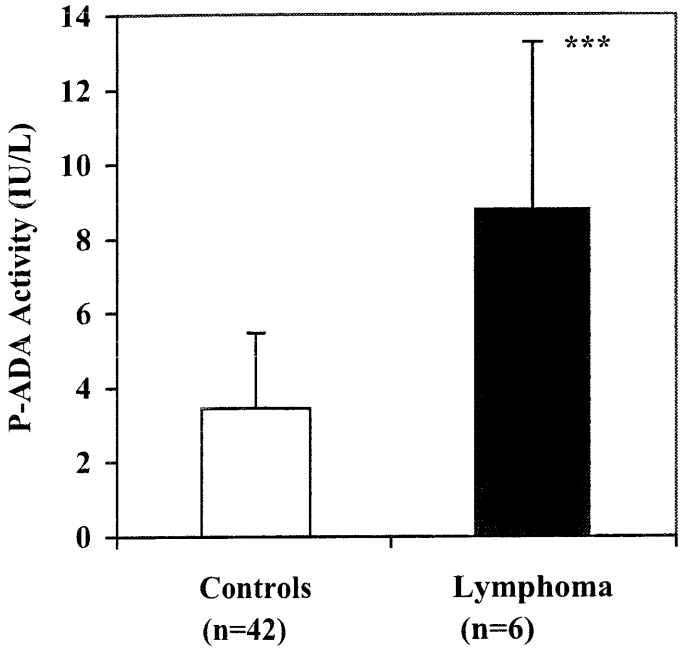

Fig. 3-1. P-ADA activity of controls and dogs with lymphoma. Dogs with lymphoma had higher P-ADA activity than controls (***P<0.005). This result showed that P-ADA activity is increased in lymphoma.

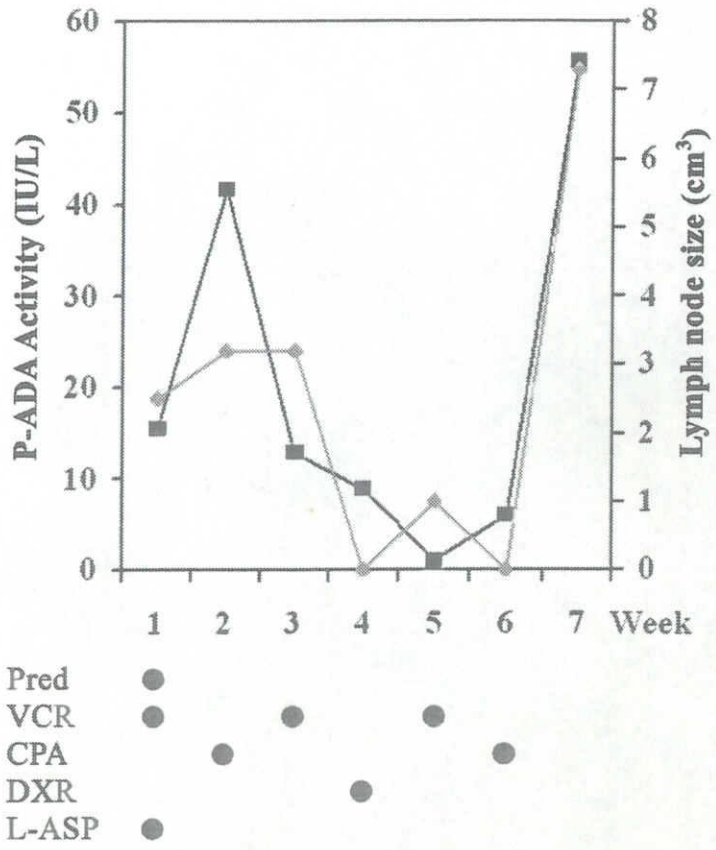

Fig. 3-2. Strong relationship between P-ADA activity and lymph node size in case No. 4 with multicentric lymphoma. The corresponding course of chemotherapeutic treatment undertaken is also shown. Pred. was administered per os every other day.

■ ; P-ADA activity, ◆ ;lymph node size.

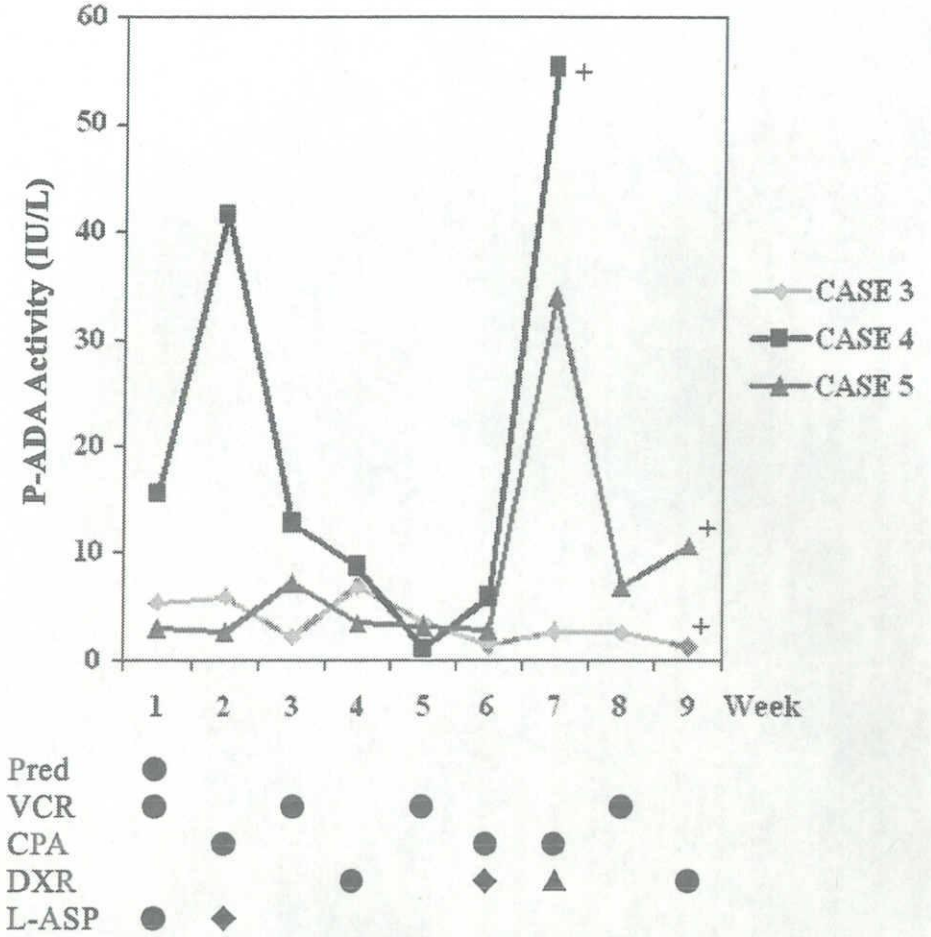

Fig. 3-3. P-ADA activity of the 3 dogs that died during course of therapy. There was a sudden increase in P-ADA activity at week 7, following relapse of the disease a prior to death in case Nos. 4 and 5, respectively. +; Point of death. ●; normal course of therapy. ◆,▲ shows points where there was a variation in therapy from normal in case Nos. 3 & 5.

CHAPTER 4

INVESTIGATION OF ADA ACTIVITY IN LYMPHOCYTES AND NEUTROPHILS IN NORMAL AND DISEASED ANIMALS

4.1. INTRODUCTION

In view of the fact that P-ADA activity has some potential in canine lymphoma, we further investigated its source in peripheral blood cells. Numerous reports suggest that spontaneous tumors in humans are recognized as antigenic by the host. In many instances, however, the malignant tissue fails to evoke an immune response capable of destroying the neoplastic cells. It has been suggested in a study by Green and Chan (1973), that increased levels of Ado in lymphocytes may result in an inability of the cells to divide. The level of ADA activity might influence the capability of the immune system to respond (Uberti et al, 1976).

FIV is a typical lentivirus that replicates preferentially in T cells and is structurally similar to HIV (Bendinelli et al, 1995). Therefore in both human beings and cats, the disease is characterized by severe impairment of T cell functions and cellular immune response due to infection of $CD4^+$ cells (Ackley et al, 1990, Joshi et al, 2004). Gradual reduction in both the percentage and the absolute number of $CD4^+$ T cells is one of the most striking immunological consequences of FIV infection resulting in the reduction of the CD4/CD8 ratio (Hoffman-Fezer et al, 1992). Consequently, an immunodeficiency syndrome develops that is characterized by wasting, neurological manifestations, chronic stomatitis and gingivitis and an

increased incidence of lymphoma. Unlike HIV infection where the primary receptor for HIV is CD4, Shimojima et al (2004) identified that the primary receptor that promotes viral binding and renders $CD4^+$ cells permissive to infection, as CD134 in FIV. Despite the progressive deterioration of T cell function, the ability of B cells to recognize and respond to T-independent antigenic stimulus was not affected (Torten et al, 1991). FIV also replicates in macrophages and astrocytes. Primary infection of cats by FIV is associated with a protracted asymptomatic phase of several months or years that in some cats culminates in the development of immunosuppression. Ishida and Tomoda (1990) have proposed classification of FIV stages as follows: Primary infection, AC, PGL characterized by generalized lymphadenopathy, ARC characterized by weight loss, bacterial and viral infections, and FAIDS characterized by severe secondary and opportunistic chronic infections, tumors and wasting. Hematologic manifestations of FIV infection include anemia, lymphopenia, neutropenia and thrombocytopenia.

4.2. OBJECTIVE

To investigate ADA activity in blood peripheral cells in canine lymphoma and FIV. The diseases chosen were based on the results from the survey obtained in the previous chapter.

4.3. MATERIALS AND METHODS

4.31. ANIMALS

Heparinized blood was collected from 11 healthy dogs (5 males and 6 females) aged 5.7±3.0 years. Eleven dogs with lymphoma (7 males and 4 females) aged 7.3±2.3 years. Lymphoma diagnosis was made by fine-needle aspiration biopsy, which was then stained and examined.

Heparinized blood was also collected from 13 healthy controls and 11 FIV positive cats. The control group was made up of 6 males and 7 females and the age ranged from 6.7±4.5 years. The FIV positive cats were comprised of 5 males and 6 females and the age ranged from 9.0±2.5 years. Of the positive cats, 7 were in the AC stage of infection while 4 were in the ARC stage. All the cats included in this work were seronegative for Feline leukemia virus antigen and the FIV-positive group was seropositive for FIV antibody (IDEXX Laboratories, Portland, Maine).

4.32. CELL SEPARATION

PBMC and PMNC were separated using the double density Histopaque® (Sigma-Aldrich, St. Louis, MO, USA) separation according to the method described by Strasser et al (1998). H1.077 was carefully layered onto 4 ml H1.119 and stored at 4°C until use. The columns were kept on ice in separate conical 12 ml centrifugal polypropylene tubes. 4 ml of blood was layered on the low gradient solution using a 21G hypodermic needle on a syringe. The tubes were centrifuged at 350 x g for 30 min at room temperature with a swing-out rotor and the process terminated without applying brakes.

In the case of cats, 3 ml of H1.119 was added to a 12 ml conical tube with a 21G needle attached to a syringe. With a separate syringe and needle, 3 ml of H1.077

was layered over the H1.119. Six milliliters of whole blood was layered over the H1.077 and. centrifuge at 700 x g for 20 minutes with no brake at room temperature.

After centrifugation the top inter-phase layer consisting of PBMC and the second layer of PMNC were collected and transferred to separate 50 ml centrifuge tubes for washing. Washing was done with D-PBS (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 200 x g for 10 minutes. Red blood cells were lysed using ammonium chloride buffer solution and the cells washed twice. The PBMCs were cultured for 1 hour in RPMI-1640 media containing 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) at 37°C and 5% CO_2 in a humidified incubator to remove monocytes. Lymphocytes were then harvested for ADA analysis.

4.33. MEASUREMENT OF LYMPHOCYTE and NEUTROPHIL ADA ACTIVITY

The cells were counted using a hemocytometer and lysed by ultrasound. The suspension was then centrifuged and the cell lysate-supernatant was analyzed for ADA activity content. P-ADA activity was measured as earlier described in chapter 2.

Statistical analyses were carried out using Student's T test and Pearson's correlation. Results were presented as mean $\pm$ SD and $P<0.05$ was considered to be of statistical significance.

4.4. RESULTS

4.41. CANINE LYMPHOCYTE/NEUTROPHIL ADA ACTVITY

Control group L-ADA activity was 2.6 ± 1.6 (IU/10^6 cells) and that of dogs with lymphoma was 7.4 ± 9.09 (IU/10^6 cells). There was a significant elevation of L-

ADA activity of the dogs with lymphoma when compared with the control group ($P<0.05$, Fig. 4-1).

N-ADA activity was 1.2 ± 0.52 (IU/10^6 cells) in the controls and 0.4 ± 0.23 (IU/10^6 cells), in the dogs with lymphoma. Healthy controls had higher N-ADA activity than dogs with lymphoma ($P<0.0001$, Fig. 4-2). L-ADA was significantly higher than N-ADA in both groups ($P<0.05$). There was, however, no significant correlation between L-ADA and P-ADA activities in neither group.

4.42. FELINE LYMPHOCYTE/NEUTROPHIL ADA ACTIVITY

The control cats' L-ADA activity was 0.78 ± 0.67 (IU/10^6 cells) and of FIV-positive cats was 1.4 ± 2.06 (IU/10^6 cells) and is shown in Fig. 4-3. There was no significant difference between FIV positive and FIV negative L-ADA activity. The AC group L-ADA activity was 0.68 ± 0.28 (IU/10^6 cells) and ARC group was 3.55 ± 3.096 (IU/10^6 cells). There was a significant difference between the ARC group and the AC and control groups ($P<0.05$, Fig. 4-3).

N-ADA activity in the normal cats was 0.19 ± 0.22 (IU/10^6 cells) and 0.22 ± 0.36 (IU/10^6 cells) in the FIV positive cats. In AC and ARC groups, N-ADA was 0.09 ± 0.15 (IU/10^6 cells) and 0.98 ± 0.54 (IU/10^6 cells), respectively. N-ADA activity was significantly higher in cats in the ARC stage of FIV infection than the other groups ($P<0.05$, Fig. 4-4). L-ADA activity was significantly higher than N-ADA in all groups ($P<0.05$). There was a negative correlation between P-ADA activity and L-ADA activity in the control group ($r= -0.55$, $P<0.05$, Fig. 4-5), but there was none found in the FIV positive cats. Similarly there was no relationship found between P-ADA and N-ADA activities.

4.5. DISCUSSION

This study reported for the first time, L-ADA activity, in both the canine and feline species. In case of the dogs, L-ADA was significantly elevated in the dogs with lymphoma compared with the healthy controls ($P<0.05$). The result concurred with that of Meier et al (1976) who reported high L-ADA activity values in human patients with lymphoma. Muller et al (1982), on the other hand, reported reduced levels of ADA activity in Hodgkin lymphoma. Carter et al (1986) have shown that there are strong similarities of morphology and behavior between human non-Hodgkin's lymphomas and canine lymphomas. Uberti et al (1976) have suggested that variations in purine or pyrimidine concentrations within the lymphocytes may result in reduced immune response directed against tumor antigens and neoplastic cells. In addition, previous studies in human have suggested that L-ADA activity may offer insight into molecular aspects of the immune mechanism and host-tumor interactions (Sufrin et al, 1977 and 1978). The increase in L-ADA activity in the dogs with lymphoma may be largely attributed to tumor cells present in the peripheral blood at presentation.

The results showed a significant difference in L-ADA activity between ARC group and the other groups ($P<0.05$). Our results concur with those of Christensen et al (1988) who found that L-ADA activity was increased in HIV patients and that this increase was only significant in the AIDS and ARC patients. Previous studies have reported that mitogen-stimulated lymphocytes produce increased ADA (Hovi et al, 1976). Therefore, the results seen in cats in the ARC stage of infection may be a reflection of the on-going immune activation and virus multiplication that has been reported in this phase of the disease.

Increased L-ADA activity has been reported in leprosy (Sehgal et al, 1992), typhoid fever (Galanti et al, 1981), and HIV infection (Christensen et al, 1988). Decreased L-ADA activity has been found in diseases causing an impairment of the immune response, such as acute lymphocytic leukemia in children (Zimmer et al, 1975), tumor patients (Uberti et al, 1976), glomerulonephritis (Klinger et al, 1983), chronic active liver diseases (Nardiello et al, 1983) and renal adenocarcinoma (Sufrin et al, 1978).

The results show that canine and feline neutrophils also produce ADA. However, in both cases L-ADA activity was significantly higher than that of neutrophils. This finding concurs with the findings of other authors who have suggested that lymphocytes are an important component of ADA activity. Erel et al (1998) made similar observations where L-ADA activity was significantly higher than N-ADA activity in both controls and patients with leishmaniasis. In dogs with lymphoma, N-ADA activity was seen to significantly decrease compared to the controls. Studies in humans with lymphoma, have shown some degree of neutrophil dysfunction exists including reduced chemotaxis and adherence to nylon wool in some patients which improved following commencing therapy (Fliedner et al, 1979 and McCormack et al, 1978). In addition, one study showed a marked increase in cell count, enzyme release, phagocytosis and killing, following stimulation (Fossat et al, 1994). These findings suggest that, like in the case in humans, neutrophils in canine lymphoma may also exist in a depressed state resulting in a reduction in enzyme synthesis including ADA. On the other hand, cats in the ARC stage of FIV infection had significantly elevated N-ADA activity levels. In HIV infection, patients had activated neutrophils that showed increased apoptosis, decreased viability and dysfunction characterized by impaired chemotaxis, phagocytosis and bacterial killing

(Pitrak, 1999 and Kubes et al, 2003). In cats therefore the increased N-ADA activity may be due activation status of the cells in FIV infection resulting in increased enzyme release.

Despite high normal P-ADA activity in cats, L-ADA activity was relatively low, contrary to the case in dogs. Tanabe (1993) also observed that tissue ADA activity was much lower than P-ADA activity. In dogs with lymphoma, elevated L-ADA activity may largely be a reflection of tumor cell presence in the peripheral blood. In this regard L-ADA activity may be useful in distinguishing lymphoma from mere lymphocytosis due to other causes. In cats L-ADA activity may be an indicator of the immune system status and phase of FIV infection. Further experiments were carried out to investigate the role of T or B-lymphocytes in the selected canine and feline disease.

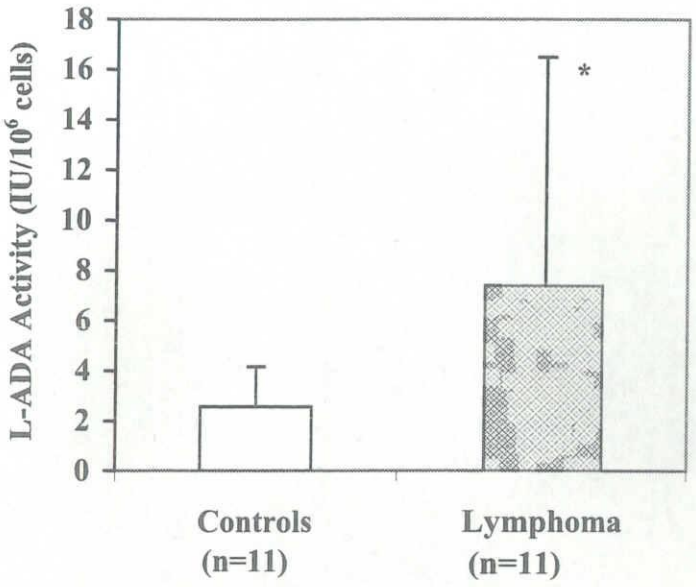

Fig. 4-1. Significant increase in L-ADA activity in dogs with lymphoma compared to the healthy controls (*P<0.05). Dogs with lymphoma have higher L-ADA activity than healthy controls.

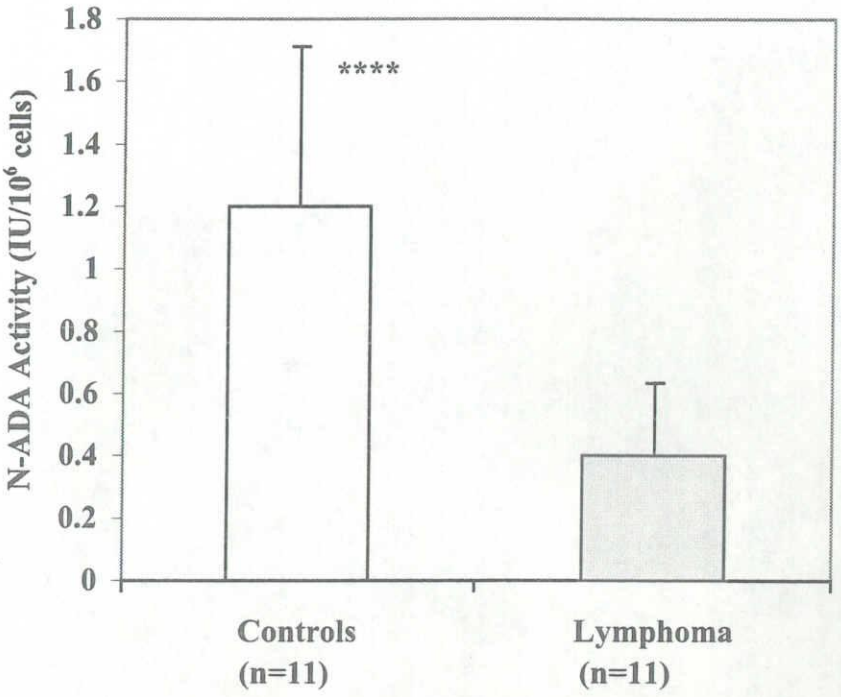

Fig. 4-2. N-ADA activities in healthy controls and dogs with lymphoma. Dogs with lymphoma had a significantly lower N-ADA than the control group (****P<0.0001).

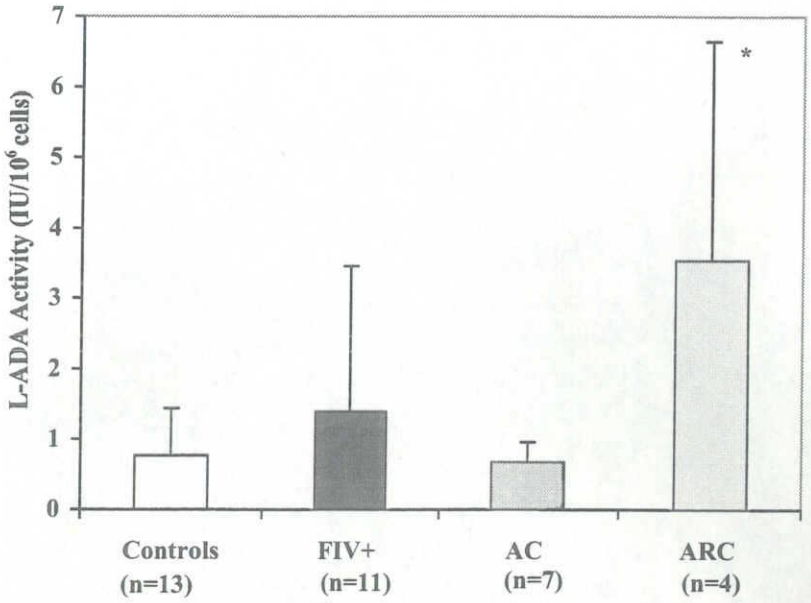

Fig. 4-3. L-ADA activity of controls, FIV+, AC and ARC stages of FIV infection. The L-ADA of the ARC group was significantly elevated compared to the other two groups (*P<0.05). Increase in L-ADA occurs in cats in the ARC stage of FIV infection. FIV+; FIV-positive.

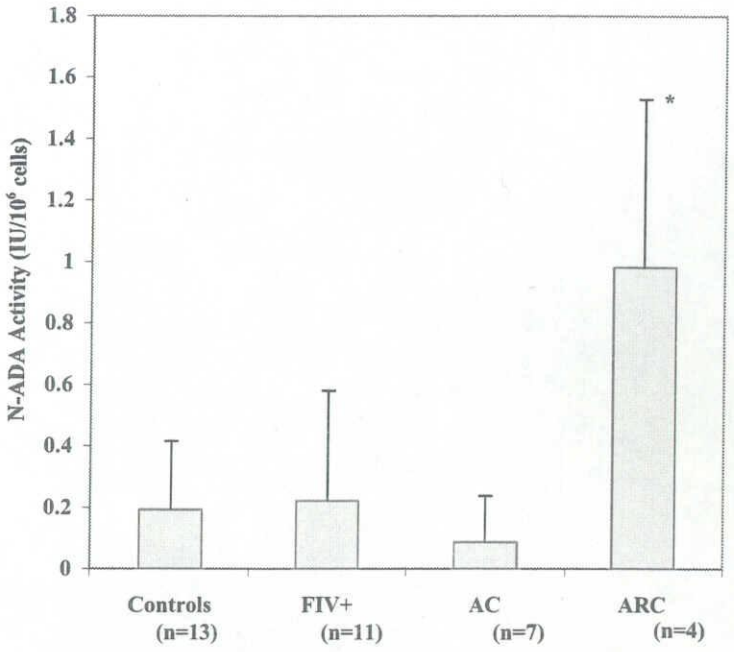

Fig. 4-4. N-ADA activities of controls, FIV-positive, AC and ARC stages of FIV infection. N-ADA activity in the ARC group was significantly higher than the other groups (*$P<0.05$).

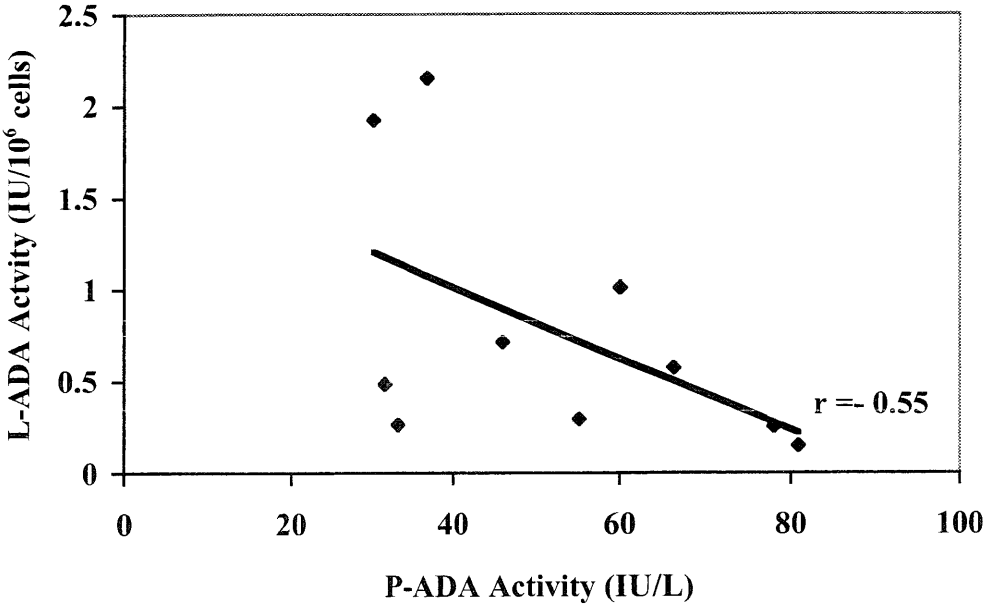

Fig. 4-5. Negative correlation between P-ADA activity and L-ADA activity in normal cats ($r = -0.55$, $P<0.05$).

CHAPTER 5

INVESTIGATION OF ADENOSINE DEAMINASE IN T AND B LYMPHOCYTES

5.1. INTRODUCTION

In the previous chapter L-ADA activity was shown to be the major component of ADA activity, we further investigated the relationship between T and B-lymphocytes and ADA activity.

ADA is an enzyme capable of catalyzing the catabolism of purine bases and whose principal biologic activity is detected in T lymphocytes. The role of this enzyme in cellular immune function was highlighted following the discovery of reduced levels in patients with SCID. In humans, ADA activity is 5-20-fold higher in T lymphocytes than B lymphocytes (Sullivan and Osbourne, 1977). In humans, ADA has been considered a marker of cell-mediated immunity (Baganha et al, 1990). Low T-ADA activity has also been reported in patients with multiple sclerosis (Vivekanandhan et al, 2005). A negative correlation was observed between serum ADA and T lymphocytes percentage in human patients with nephrotic syndrome (Misra et al, 1997). ADA activity has been identified as a marker of T cell activation (Kose et al, 2001). Furthermore, several reports have observed that ADA interacts with CD 26 on the surface of T cells surface resulting in a co-stimulatory effect in the activation of T cells (Blazquez et al, 1992, Morimoto and Schlossman, 1998, Cordero et al, 2001 and Pacheco et al, 2005).

Most of the lymphomas described in canines originate from B lymphomas (Teske, 1994). These lymphomas react much better to chemotherapy than do T cell lymphomas, which make up 10-38% of the cases and have a much worse prognosis (Hahn et al, 1992).

It is generally accepted that FIV may be useful as a model for AIDS. Several authors have reported that serum ADA activity is elevated in HIV infection (Gakis et al, 1989, Goto et al, 1992 and Inigo et al, 1992). Furthermore, a relationship has been described in which serum ADA correlated with retroviral infection in HIV infection (Mastrioanni et al, 1987). ADA is also reported to be a useful marker of progression of HIV infection to AIDS in humans (Inigo et al and 1992, Planella et al, 1998).

EXPERIMENT A: INVESTIGATION OF T AND B LYMPHOCYTE ADA ACTIVITY IN DOGS AND CATS

5.2. OBJECTIVE

The present study was undertaken to investigate the role of T and B lymphocyte in ADA activity in dogs and cats.

5.3. MATERIALS AND METHODS

5.31. ANIMALS

Heparinized blood was collected from 10 healthy dogs of various breeds, and 8 dogs with lymphoma. The control group composed of 6 males, 4 females, with an average age of 3.7 ± 1.8 years. The group of dogs with lymphoma composed 5 males, 3 females and an average age of 7.5 ± 2.1 years. None of the dogs were receiving immunosuppressive medication at the time of sampling.

Heparinized blood was collected from 10 healthy FIV-negative and 8 FIV-infected cats. All the cats included in this work were seronegative for Feline leukemia virus antigen and the FIV-positive group was seropositive for FIV antibody (IDEXX Laboratories, Portland, Maine). The cats were of mixed-breed with the FIV-negative group composed of 6 males, 4 females, with an average age of 4.7 ± 4.2 years. The FIV positive group composed 5 males, 3 females and an average age of 7.5 ± 2.1 years. The FIV-positive group composed of cats in the AC (n=4) and ARC (n=4)

stages. None of the cats were receiving immunosuppressive medication at the time of sampling.

5.32. P-ADA ACTIVITY MEASUREMENT

P-ADA was assayed as described earlier using a commercial ADA kit (Serotec, AD-L, Sapporo, Japan.) using an autoanalyzer (Accute, Toshiba-40FR Clinical Laboratory System, Tokyo, Japan.) at 37°C.

5.33. T AND B LYMPHOCYTE SEPARATION USING NYLON WOOL

PBMCs were separated using single density gradient separation by overlaying blood diluted in D-PBS, on H1.077. The tubes were spun at 1500 rpm at 20°C for 30 minutes. The cells were then washed twice with D-PBS and any red blood cells were lysed using ammonium chloride lysing solution. The PBMCs were then depleted for monocytes by culturing in RPMI-1640 medium (Invitrogen, U.S.A.) supplemented with 10% FBS, streptomycin 100μg/m*l* and penicillin (100U/m*l*), at 37°C, 5% CO_2, in a humidified incubator for 1 hour. The non-adherent cells were then harvested by gentle washing. Nylon wool fractionation has been shown to be an efficient procedure for canine and feline T and B cell separation (Tham and Studdert, 1985). Nylon wool fiber columns (Polysciences Inc., Warrington, U.S.A.) were prepared according to the manufacturer's instructions, first by washing with RPMI-1640 medium and then equilibrating the wool fiber with the media by culturing at 37°C in an incubator for an hour. The lymphocytes were then cultured for an hour in RPMI-1640 media in the prepared column to separate T- from B cells. The T cells were collected by passive washing and counted using a hemocytometer. To check the rate of B cell contamination of the T cell suspension, aliquots of the cell suspensions were collected

for flow cytometry and labeled with a pan B cell immunoglobulin monoclonal antibody (MCA 1781PE, Serotec, Oxford, UK). Mouse anti-feline and rat anti-canine $CD4^+$ monoclonal antibody (MCA 1346F, MCA 1038F, Serotec, Oxford, UK, respectively) was used to check the amount of $CD4^+$ cell in the suspension. Labeling of the lymphocytes was analyzed by FACScan® flow cytometer (Nippon Becton Dickinson, Tokyo, Japan.) using Cellquest program. The remaining cell suspension was lysed by sonification for 60 seconds at 40 watts (Vibra Cell, Sonics and Materials Inc. Newtown. U.S.A.). The lysate was then centrifuged and the resulting supernatant measured for ADA content.

The number of $CD4^+$ cells isolated in the cell suspension was calculated as follows:

$CD4^+$ cell number = Conc. of T lymphocytes isolated × Percentage of $CD4^+$ cells in the cell suspension,

where Conc. is concentration in $\times 10^6$ cells/ml.

Statistical analyses were carried out by Pearson's correlation and Students' T test. Probability values of less or equal to 0.05 were considered to be significant. Data were presented as mean $\pm$ SD.

5.4. RESULTS

T-ADA activity of healthy controls was 1.24 ± 0.5 IU/10^6 cells and dogs with lymphoma 0.97 ± 0.61 IU/10^6 cells, with no significant difference between the two populations ($P>0.05$). There was no significant difference between $CD4^+$ cell numbers in the controls and in the dogs with lymphoma ($P>0.05$).

A positive correlation was found between P-ADA and T-ADA activity ($r = 0.58$, $P<0.05$, Fig. 5-1) in the control but there was no relationship found between the two parameters in the dogs with lymphoma ($r=-0.18$, $P=0.36$). There was no correlation between P-ADA activity and $CD4^+$ number in the healthy controls ($r=0.61$, $P=0.13$) or the dogs with lymphoma ($r=0.23$, $P=0.32$). T-ADA activity and $CD4^+$ cell number showed a strong significant correlation ($r=0.96$, $P<0.005$) in control subjects (Fig. 5-2), whereas the dogs with lymphoma showed a negative correlation ($r = -0.78$, $P<0.05$, Fig. 5-3).

T-ADA activity of FIV-negative cats was 1.014 ± 0.746 IU/10^6 cells and infected cats, 3.5 ± 3.16 IU/10^6 cells, showing a significant difference between the two populations ($P<0.05$, Fig. 5-4). A strong significant difference was found between T-ADA activity of FIV-negative group and that of the ARC group (5.29 ± 3.09 IU/10^6 cells, $P<0.01$). There was, however, no significant difference between T-ADA activity of the FIV-negative group and that of the AC group (1.7 ± 0.3 IU/10^6 cells).

A positive correlation was found between P-ADA and T-ADA ($r = 0.71$, $P<0.05$, Fig. 5-5) in the FIV negative cats. Conversely, in the FIV-positive cats, no correlations were found between P-ADA and T-ADA activities in the AC and ARC groups.

T-ADA and $CD4^+$ cell number showed a significant negative correlation ($r = -0.93$, $P<0.0005$) in FIV infected cats (Fig. 5-6), whereas FIV-negative cats showed an insignificant positive correlation ($r = 0.39$). The ARC group showed a significant negative correlation ($r = -0.94$, $P<0.05$) while the AC group did not show a statistically significant correlation.

There were no statistically significant correlations between P-ADA and $CD4^+$ cell number in the disease stages.

$CD4^+$ cell numbers in the cell suspensions were significantly lower in the FIV-positive cats when compared to those of the negative group ($P<0.05$). Whereas $CD4^+$ cell numbers in the ARC group were significantly lower than the controls ($P<0.005$), those of the AC group did not differ with the controls (Fig. 5-7).

Diagrams of both canine and feline B cell and $CD4^+$ expression are shown in Fig. 5-8.

Due to low B cell yields, the B cell ADA could not be measured. The percentages of B cells in the suspensions following check using flow cytometric analysis are shown in Table 5-1.

5.5. DISCUSSION

This study has reported for the first time T-ADA activity in controls and dogs with lymphoma. There was no significant difference between T-ADA activity of controls and dogs with lymphoma, with the latter being slightly down regulated. This result concurs with Murray et al (1986) and Muller et al (1983) who reported low T-ADA activity values in patients with Hodgkin lymphoma. These authors suggested that the findings were a reflection of the persistent abnormalities seen in T-cell function throughout the clinical course of Hodgkin lymphoma. Similarly, Walter et al (2006) and Winnicka et al (2002) showed that dogs with lymphoma were relatively T-cell deficient prior to treatment; $CD4^+$ and $CD8^+$ T cells were fewer in dogs with lymphoma, than in healthy dogs. However in this study there was no significant difference between the two groups with regard to $CD4^+$ cell numbers. This result may be a reflection of the tumor phenotype, Miniscalco et al (2003) suggested that blood

immunophenotyping might be a sensitive diagnostic and prognostic tool in canine lymphoma

Baghana et al (1990) observed a significant correlation between P-ADA activity and $CD4^+$ cells in patients with pleural effusion. However, in this study there was no correlation between P-ADA and $CD4^+$ number in the two populations. There was a positive correlation found between P-ADA and T-ADA activities in the controls but not in the dogs with lymphoma. This result suggests that P-ADA activity varies with T-ADA activity. T-ADA activity and $CD4^+$ cell number showed a strong positive correlation in control subjects, whereas the dogs with lymphoma showed a negative correlation.

These results suggest that in healthy controls T-ADA activity may be an indicator of T lymphocyte function in dogs because of the correlation seen with $CD4^+$ cell number. The negative correlation seen in the dogs with lymphoma may be suggestive of T cell dysfunctions that occur in dogs with lymphoma. Therefore, P-ADA and T-ADA activity may provide useful information on T cell function or activation status in dogs.

There was a significant increase in T-ADA of FIV-positive cats compared to the FIV-negative group. This increase was mainly attributed to the ARC group that showed a significant five–fold increase when compared with the control population. Excessive antigen-specific T cell activation is thought to contribute to chronic immune activation and progression to immunodeficiency during HIV disease (Xu et al, 2006). Similarly, FIV disease progression is characterized by aberrant and chronic immune activation that not only drives viral replication but may also lead to T cell apoptosis (Joshi et al, 2004). In addition, high plasma viral loads have been associated with the ARC and AIDS stages of infection in cats (Goto et al, 2002). It is possible

that increased immune activation during this stage results in elevation of T-ADA activity. In this context, previous reports in humans have also shown that T cells produce increased ADA when activated by various stimuli (Hovi et al, 1976 and Kose et al, 2001). Interestingly, studies in humans have suggested that ADA may be involved in the mediation of T cell activation through its interaction with the CD26 cell surface marker (Kameoka et al, 1993).

$CD4^+$ cell count has been known for a long time now to be a marker for the progression of both HIV and FIV infection (Hofmann-Lehmann et al, 1992, Pascale et al, 1997 and Planella et al, 1998). However it has recently been shown that $CD4^+$ is not the primary receptor for FIV, and that proliferation and infection of activated $CD4^+$ cells is instead facilitated via CD134 as the binding receptor (Shimojima et al, 2004). $CD4^+$ cell numbers have been reported to decrease as the FIV disease progresses (Hoffmann-Fezer et al, 1992 and Walker et al, 1994). Our study concurred with these reports in that the $CD4^+$ cell numbers were significantly lower in the ARC group cell suspensions when compared with those of the FIV-negative groups. In this study the $CD4^+$ cell number was inversely related to T-ADA activity in the ARC group of cats. FIV infection in $CD4^+$ cells has recently been reported to regulate T cell activation that in turn leads to increased viral replication and disease progression (Joshi et al, 2005). Consequently, the $CD4^+$ cells and other immune cells exist in a state of perpetual activation. Therefore in spite of declining $CD4^+$ cell numbers, it is possible that their hyper-activated state results in increased T-ADA.

In this study, P-ADA increases with T-ADA in FIV-negative cats. However no correlation was found in the FIV-positive cats. This may be suggestive of other factors' involvement in the regulation of the enzyme during the course of the disease.

Furthermore, the mechanism by which ADA is released from cells still remains unclear.

Our study was limited because the sample size was small and did not include cats in all the stages of FIV infection. However, these tentative results show that T-ADA increases in FIV-positive cats particularly during the ARC stage of infection. Our results also suggest that ADA may be an indicator of T cell activation in the ARC stage of FIV infection. However, understanding the complex underlying immunodynamics and immunomodulation could be important for elucidating the mechanism of increase of T-ADA in FIV infection. Unlike other tests currently used in the assessment of FIV infected cats, ADA activity assay has advantages in that it is cheap and can be measured easily using the automated method as is the case with other routine biochemical enzymes. However, further studies are required to investigate the clinical relevance and application of ADA activity in the immune system of FIV-positive cats.

Table 5-1: Amount of B cell % in the suspensions obtained following separation using nylon wool fractionation.

	Canine Controls	**Dogs with Lymphoma**	**Feline Controls**	**FIV+ Cats**
B cell % in T cell suspension	19.3±9	9.4±6.8	7.38±6.1	6.8±2.7
B cell % in B cell suspension	36.4±17.6	41.7±9.1	23.8±22.6	39.2±13.8

As seen there was low B cell contamination in the T cell suspensions. However the results showed very low B cell yields in both canine and feline cell suspensions.

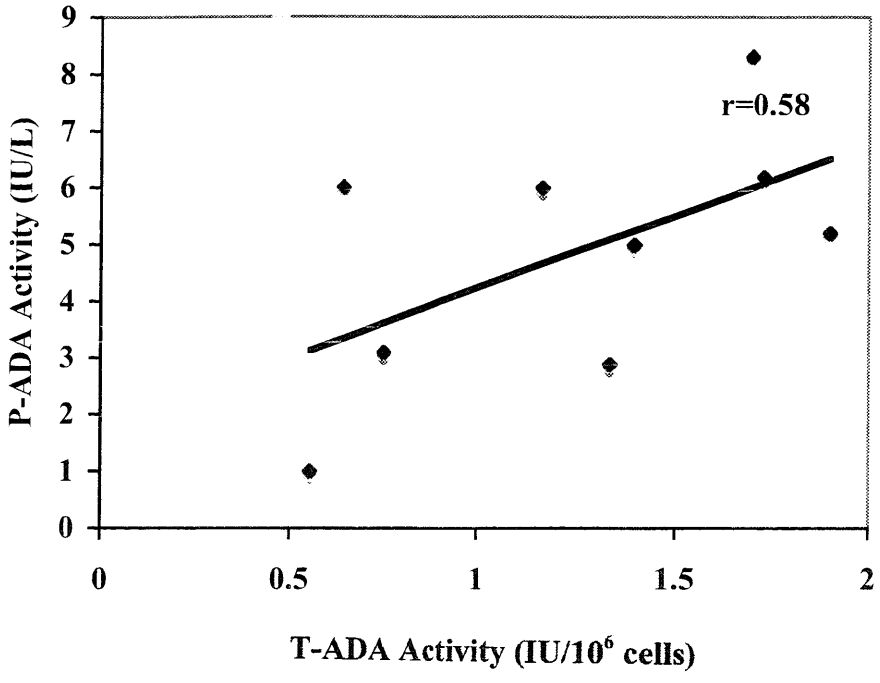

Fig. 5-1. Positive correlation between P-ADA activity and T-ADA in healthy dogs ($r=0.58$, $P<0.05$). P-ADA and T-ADA activities appear to vary together in healthy dogs.

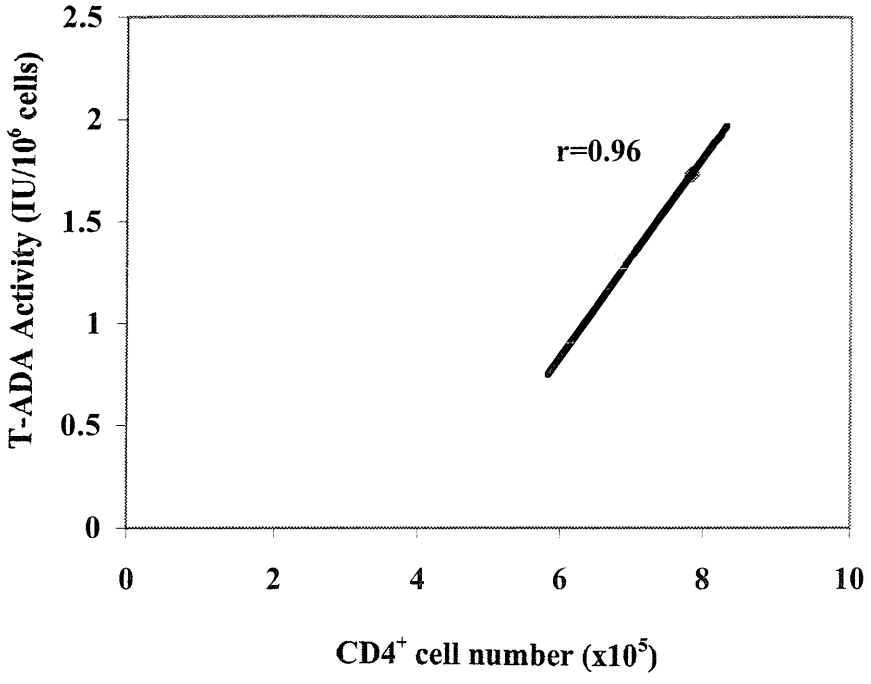

Fig. 5-2. Positive correlation between $CD4^+$ cell number and T-ADA in T cell suspensions of healthy dogs ($r=0.96$, $P<0.005$).

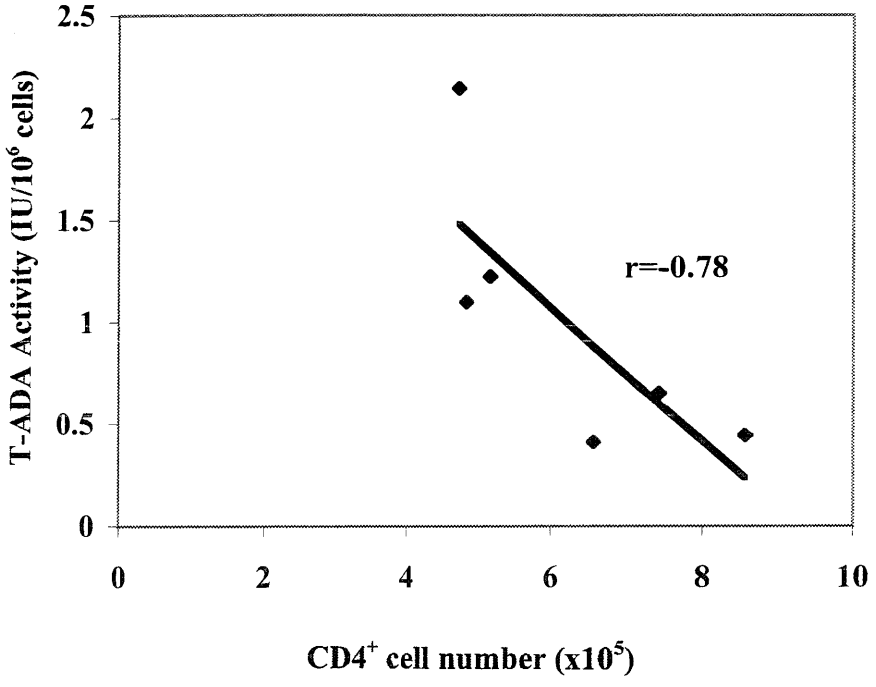

Fig. 5-3. Diagram showing the significant negative correlation between T-ADA activity and $CD4^+$ cell number in the suspensions of dogs with lymphoma ($r = -0.78$, $P<0.05$).

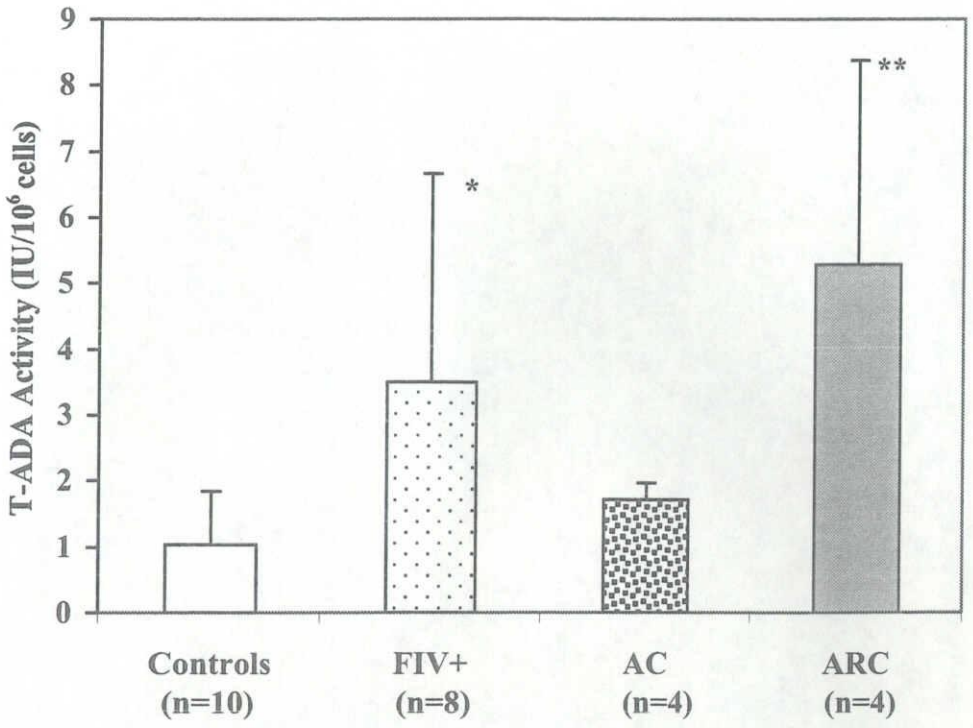

Fig. 5-4. Diagram showing T-ADA activity in FIV-negative controls, AC and ARC FIV-positive (FIV+) groups following cell separation using nylon wool fiber column. There was a significant difference between control and FIV-positive group (*P<0.05) T-ADA activity, which was mainly attributed to the ARC group (**P<0.01).

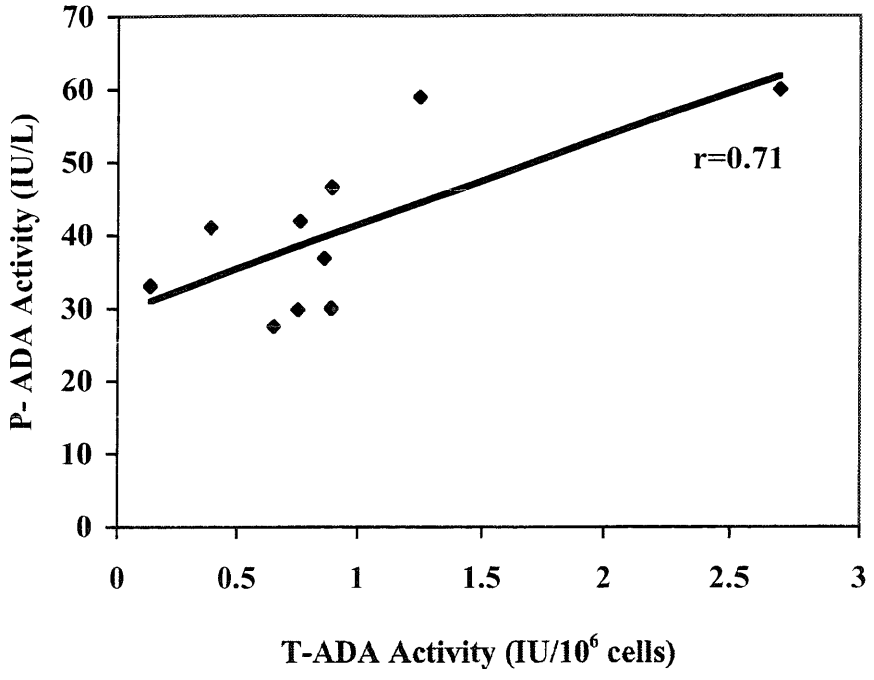

Fig. 5-5. Graph showing the positive relationship between P-ADA and T-ADA activities in FIV-negative cats ($r = 0.71$, $P<0.05$). P-ADA and T-ADA activities appear to vary together in healthy controls.

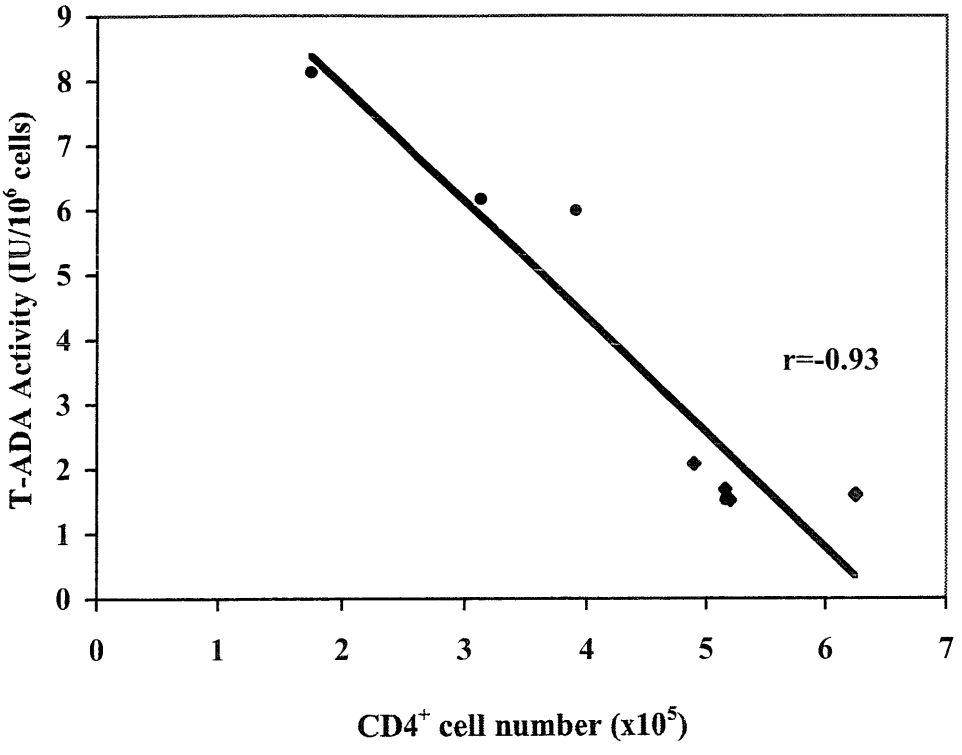

Fig. 5-6. Diagram showing the significant negative correlation between T-ADA activity and $CD4^+$ cell number in the suspensions of FIV-positive cats ($r = -0.93$, $P<0.0005$). The solid circle (●) represents the ARC group and the solid diamond (◆), the AC group. FIV-positive cats showed that as $CD4^+$ cell numbers reduced, T-ADA activity tended to increase.

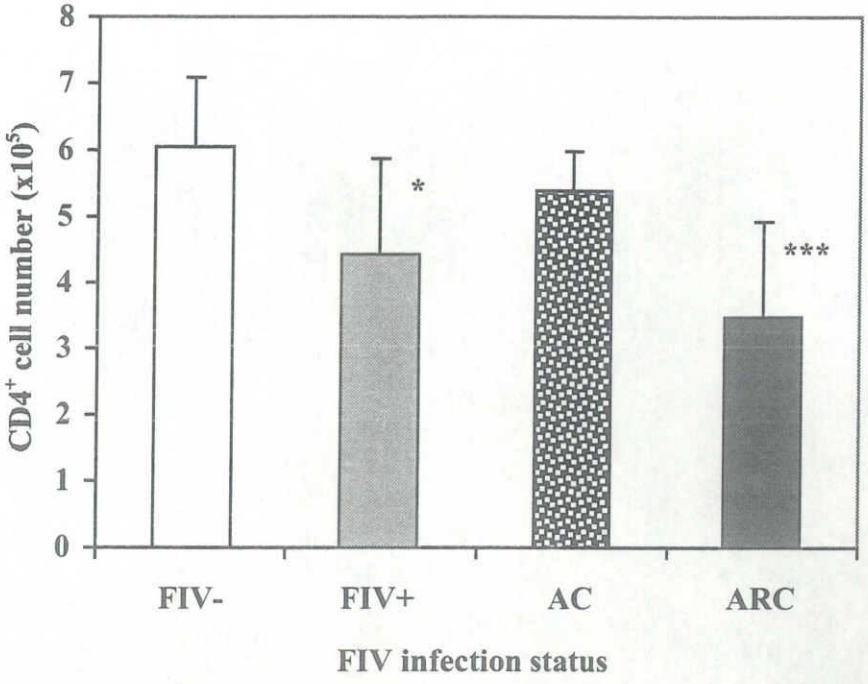

Fig 5-7. $CD4^+$ cell number in the T cell suspensions following separation. FIV-positive and ARC group $CD4^+$ cell numbers were significantly reduced (*P<0.05 and ***P<0.005, respectively). CD 4^+ cell numbers are reduced in cats in the ARC stage of FIV infection. FIV+; FIV-positive, FIV-; FIV-negative.

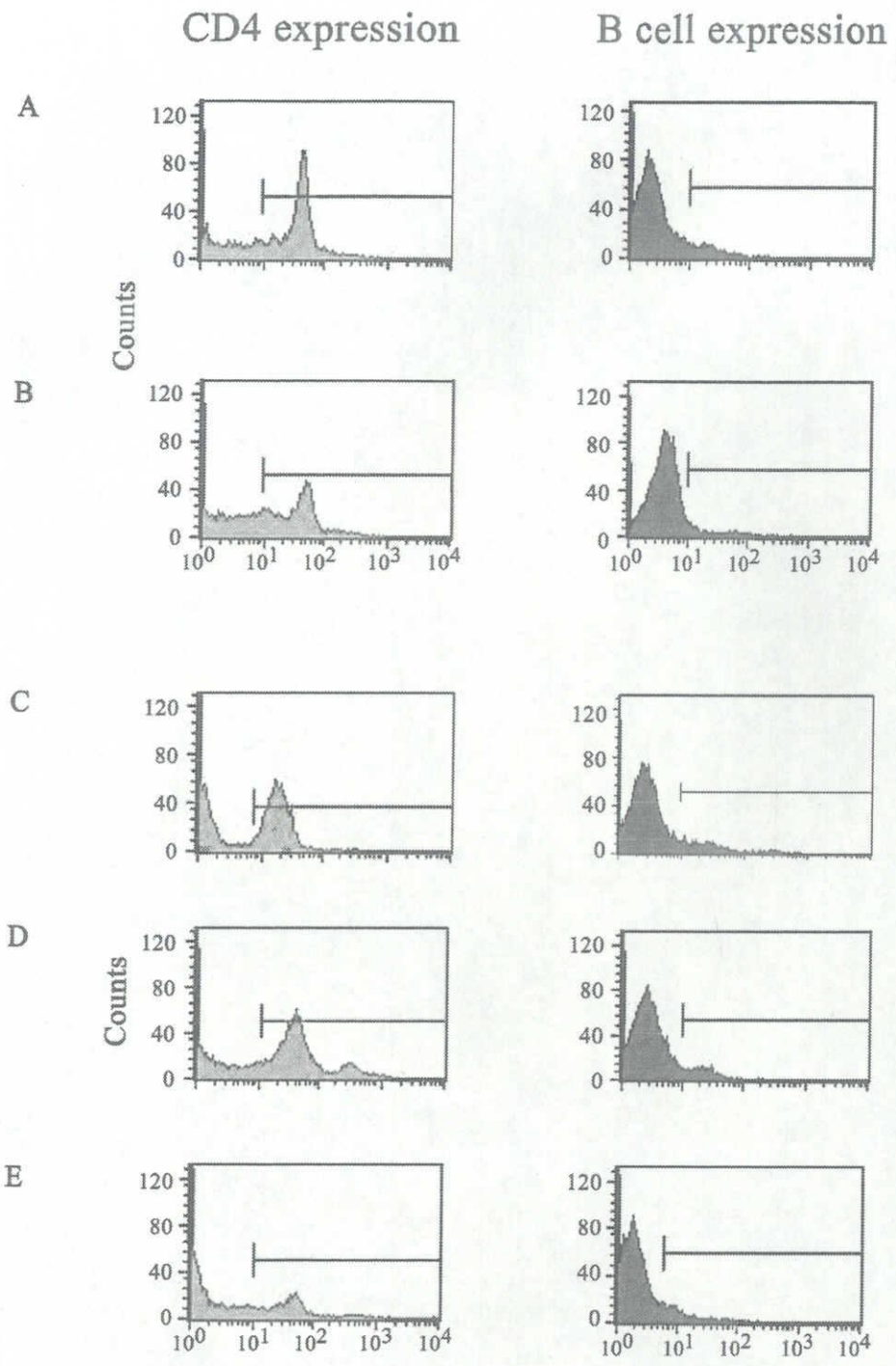

Fig. 5-8. Flow cytometric analysis results of B cell and $CD4^+$ expression in the cell suspensions. A; control (dog), B; lymphoma, C; control (cat), D; AC stage, E; ARC stage.

EXPERIMENT B: PROLIFERATION ASSAY AND ADA mRNA MEASUREMENT IN CANINE LYMPHOCYTES AND TUMOR CELLS

5.6. OBJECTIVE

In the previous section it was shown that T cells are related to ADA activity in dogs. We carried out further studies to clarify whether activation of T and B cells affects ADA activity and the expression of this enzyme in established tumor cell lines.

5.7. MATERIALS AND METHODS

5.71. ANIMALS

Blood was collected in heparin from 4 healthy dogs of various breeds. There were 3 males and one female the age ranged from 6.8 ± 1.0 years.

5.72. TUMOR CELLS

T and B tumor cells (CL-1 and GL-1, respectively) were obtained from a previously established cell lines at the Departments of Veterinary Internal Medicine, University of Tokyo and of Veterinary Surgery, Yamaguchi University, respectively. CL-1 cells were cultured in RPMI-1640 supplemented with 20% FBS, streptomycin (100μg/m*l*) and penicillin (100U/m*l*), and were maintained in a humidified atmosphere of 5% CO_2 in air at 37℃. GL-1 was cultured in similar conditions in RPMI-1640 containing 10% FBS.

5.73. CELL PROLIFERATION

LYMPHOCYTES

Lymphocytes were separated from heparinized blood as previously described and then depleted for monocytes by culturing for an hour in RPMI media containing 10% FBS in a incubator at 5% CO_2 at 37°C. Lymphocytes were cultured in RPMI media containing 10% FBS, at 2 x 10^5 cells/well in triplicate wells in 96-well plates. The lymphocytes were cultured with ConA (Sigma-Aldrich, St. Louis, MO, USA) at 5 μg/ml and bacterial (*E. coli* 055:B5) LPS (Sigma-Aldrich, St. Louis, MO, USA), at 0.001μg/ml in media (the dose of LPS was determined after culture at different concentrations). Upon determination of mitogen concentration, the cells were cultured for 72 hours as the maximum stimulation time. Separate wells were cultured for ADA activity determination.

TUMOR CELLS

The cells were cultured in quadriplicate wells in 96 well plates at 10^4 cells per well. GL-1 cells were cultured with and without the B cell mitogen, LPS, while the T cell mitogen, ConA was added to the CL-1 cells. The concentrations of the mitogens, LPS and ConA in the media, were 100 ng/ml and 5 ug/ml, respectively. The concentrations of the mitogens was determined following culture at different concentrations and culture times. The cells were then cultured for 24, 48 and 72 hours. Separate wells were also cultured for ADA activity measurement.

5.74. MTT MEASUREMENT

10 μl of MTT (Sigma-Aldrich, St. Louis, MO, USA.) was added to the

cultures and further incubated for 3 hours to complete the incubation period. The cultures were removed from the incubator and MTT formazan crystals were dissolved by adding MTT solvent (0.1 N HCl in isopropanol) directly into the culture in an amount equal to the original culture volume. Gentle pipetting enabled dissolution of crystals. Absorbance was measured at 570 nm using a micro plate reader (MPR A4i, Asahi Techne Ion, Tokyo, Japan). The SI was calculated as follows:

$$SI = \frac{\text{Average culture absorbance-Average background absorbance}}{\text{Average background absorbance}}$$

5.75. RNA EXTRACTION AND cDNA SYNTHESIS

Total RNA was extracted using a kit (RNeasy Mini Kit, Qiagen GmbH, Hilden, Germany) using the method prescribed by the manufacturer. The purified RNA was eluted in a final volume of 30 µl RNase-free water and stored at -80°C until cDNA synthesis. The cDNA was synthesized from total RNA using AMV reverse transcriptase and an RNA PCR Kit Ver. 3.1 (Takara Biomedicals, Shiga, Japan) according to the manufacturer's instructions. Briefly, 10 µl containing 5 mM $MgCl_2$, 1 mM dNTPs, I U/µl Rnase inhibitor, RT buffer and 2.5 µM random 9 mers primer and 0.25 U/µl of AMV reverse transcriptase were prepared, and the reverse transcriptase carried out by subsequent incubation for 10 min at 30°C, 30 min at 42°C, 5 min at 99°C and 5 min at 5°C. The cDNA was stored at -80°C until use.

5.76. PRIMERS

The nucleotide sequences of the canine ADA-mRNA PCR primer are shown in Table 5-2. This primer set was based on the cDNA sequence deposited in the

GenBank database and designed to detect canine ADA genes and β-actin acted as an internal control.

5.77. SEMI-QUANTITATIVE RT-PCR

The amplification of ADA was performed using an RNA PCR Kit (AMV) Version 3.1 (Takara Biomedicals). One µl of cDNA was added to a reaction mixture with the final concentration of 1 x PCR buffer, 0.2 µM each primer and 0.25U/µl Taq polymerase (Takara EX Taq HS® Takara Biomedicals) in a final volume of 10µl. The PCR profile used was 2 min at 94°C for the first cycle, and 30 sec at 94°C, 30 sec at Tm and 30 sec at 72°C for 30 cycles. The PCR products were electrophoresed on 3 % agarose gel. The gel was stained with ethidium bromide and photographed through the UV light of a transilluminator using a digital camera. The density of each band of ADA and β-actin was quantified by NIH Image Ver 1.62, and calculated by the ratio between the ADA and the β-actin gene products for each sample.

5.78. CELLULAR ADA MEASUREMENT

Cellular ADA activity was measured as described earlier.

Statistical analyses were carried out using Student's T test and Pearson's correlation. Results were reported as mean $\pm$ SD and $P<0.05$ was considered statistically significant.

5.8. RESULTS

LYMPHOCYTES

Following culture of lymphocytes were maximally stimulated at 72 hr by the

mitogens. The SI (at 0, 72 hours) for the un-stimulated cells, LPS-stimulated and ConA-stimulated cells were 6.556±1.155 (0 h), 6.233±1.68 (72 h), 7.21±1.7 (LPS), 10.80±5.20 (ConA), respectively (Fig 5-9). Cell lysate ADA activities were as follows: 0 hr cells (2.0±1.73 IU/10^6 cells, 72 hr unstimulated cells (1.3±1.06 IU/10^6 cells), LPS-stimulated (3.86±1.77 IU/10^6 cells) and ConA-stimulated cells (4.50± 2.29 IU/10^6 cells). The ConA and LPS-stimulated cells produced significantly more ADA than the unstimulated cultures ($P<0.05$).

TUMOR CELLS

The SI and ADA activities of the tumor cells following culture at different times, with and without mitogens, are tabulated in Table 5-3 and shown in Figs.5-10 and 5-11. The results showed that CL-1 cell ADA activity was significantly higher than GL-1 cell activity in both unstimulated and stimulated cells for each culture period as shown in Table 5-4.

The ADA mRNA level in unstimulated and stimulated cells (expressed as a ratio to β-actin) was 0.419 and 0.433 and 0.29 and 0.296, in CL-1 and GL-1, respectively. There was a significant difference between ADA mRNA expression between CL-1 and GL-1 tumor cells ($P<0.05$, Fig. 5-12). There were also differences seen between unstimulated; 1.78±0.18 (T cell 0 hour), 2.323±0.26 (T cell 72 h), 0.46±0.066 (B cell 0 h), 0.79±0.09 (B cell 72 h) and stimulated; 4.12±0.49 (T ConA), 0.62±0.07 (B LPS 72h), CL-1 and GL-1 tumor cells (Fig. 5-13 & 5-14). There was a significant difference between unstimulated and stimulated cells in CL-1 and GL-1 cell lines ($P<0.002$ and $P<0.04$, respectively) at 72 hours. There was a

strong positive correlation between ADA mRNA level and ADA activity in both CL-1 and GL-1 tumor cells ($r = 0.92$, $P<0.005$, Fig. 5-15).

5.9. DISCUSSION

Our results show that stimulated lymphocytes produce an increased amount of ADA. The results concur with those of Hovi et al (1976) who reported considerable increase in ADA activity of human lymphocytes following exposure to T and B cell mitogens. In this study, following a 72-hour culture, there was no statistical difference between ConA- stimulated and LPS- stimulated ADA activity. Differences exist between T and B lymphocytes in relation to cellular metabolism and to cell proliferation (Hovi et al 1976). Some authors have reported that differences exist in the control of purine metabolizing enzyme activities in lymphocytes and that estimation of purine salvage enzyme activity indicates the metabolic activity of T cells (Tritsch and Minowada, 1978). A recent *in vitro* study observed that the ADA assay might be a reliable and accurate method of measuring T-lymphocyte proliferation (Kainthla et al 2006). In this case we did not see a statistical difference between T and B lymphocytes following mitogen stimulation, this may be because ADA activity peaks before maximum proliferation (72 hours) occurs.

T cell tumor ADA activity was considerably higher than that of the B cell tumor. This is consistent with the findings of other authors who have reported markedly high levels of ADA activity in T cell tumors when compared to B cell tumors (Tritsch and Monowada 1978). The ConA-stimulated T cell tumor ADA activity showed a marked increase at 24 hours and then declined at 48 and 72 hours. The unstimulated cells on the other hand remained relatively the same during the culture time. A previous study by Desrivieres et al (1997) showed that ConA inhibited

growth of a murine T cell lymphoma cell line by causing cell arrest at both G1 and G2/M phases of the cell cycle. This inhibitory effect may be responsible for the decline in ADA activity after 24 hr incubation. Moreover, ADA activity is highest in T lymphoblasts but activity has been observed to decrease in the course of T-cell maturation (Martin et al, 1995). Therefore, it is possible that this may also be true for T lymphoma cells as they age. The highest ADA activity was seen at 24 hrs before maximum cell in CL-1 in the stimulated cells. A study by Hovi et al (1976) showed that increased ADA activity in human lymphocytes preceded the initiation of DNA synthesis. This may be the case in the CL-1 cells too.

Our results showed that both T and B cell tumors express ADA-specific mRNA, with the T cells showing higher expression than the other cells ($P<0.05$). The level of ADA mRNA expression was directly related to ADA activity in both CL-1 and GL-1 cell lines. Our results are in agreement with findings in human lymphoid malignancies, where the level of ADA activity was directly related to the amount of ADA-specific mRNA present (Gan et al, 1987). In the T cell tumor line there was an obvious increase of ADA mRNA in comparison to the unstimulated cells. A study by Karbownik et al (2003) reported increase in ADA mRNA expression in leukocytes of patients with autoimmune thyroid disorders and suggested that measurement of this gene could help explain mechanism of the diseases.

Our results showed that stimulated canine lymphocytes and tumor cells produced increased amounts of ADA. T cells produce more ADA compared to B cells. This was particularly clear in the tumor cell lines. There was also a corresponding increase in ADA mRNA. These results suggest that ADA activity may be useful in differentiating T and B cell tumor-originated conditions and showing the status of the immune system the canine species.

Table 5-2: Canine ADA primer sets used for PCR amplification.

Forward primer (5`--->3`):	**CCTGGTCCAGCTACCTCAC**	
Reverse primer (5`--->3`):	**GCGGGTCATCTGTGTTGAG**	109 bp

Table 5-3: SI and corresponding ADA activities of CL-1 and GL-1 tumor cells cultured for shown period of time.

	Culture time (Hours)	**SI** (unstimulated)	**SI** (stimulated)	**ADA activity** ($IU/10^6$ cells) (unstimulated)	**ADA activity** ($IU/10^6$ cells) (stimulated)	**Probability**
CL-1	24	4.036±0.035	3.695±0.072	88.33±1.17	290	8.47E-06
	48	7.58±0.1240	6.981±0.290	88.88±1.57	131.66±1.18	0.0005
	72	10.345±0.218	11.244±0.098	83±0.7	105±1.8	0.002
GL-1	24	14.691±0.162	12.58±0.458	29.2±21	20±8.8	0.02
	48	24.294±0.638	20.415±0.579	28.75±2.65	26.66	0.46 (NS)
	72	26.186±0.364	31.626±1.141	26.66±1.17	32.22±0.7	0.03

Probability shows significance of difference between unstimulated and stimulated cells, according to culture time, for each cell type.
NS indicates that there was no significant difference. mean±SD

Table 5-4. List of the stastistical differences between CL-1 and GL-1 ADA activities for unstimulated and stimulated cells at different culture times.

Culture time (Hours)	**Probability** (Unstimulated)	**Probability** (Stimulated)
24	0.0003	8.17E-11
48	0.0006	3.10E-05
72	0.0002	0.0002

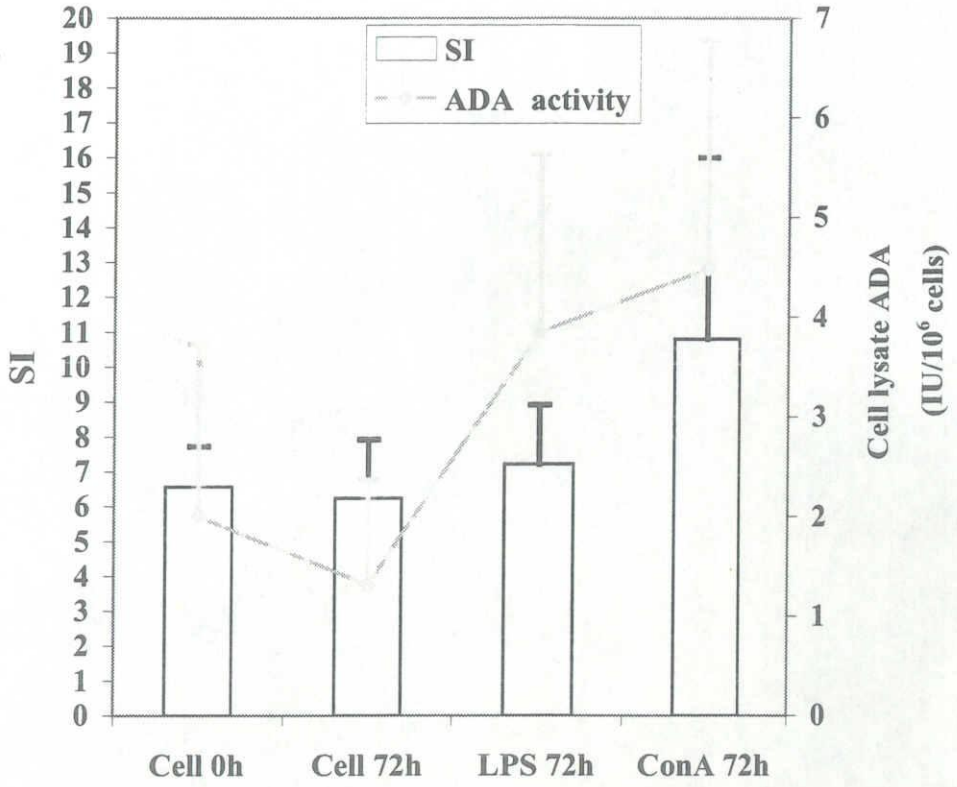

Fig. 5-9. Proliferation assay of canine lymphocytes following culture for 72 hours. The amount of ADA activity in the ConA-stimulated cells was higher than LPS-stimulated cells, however, the difference was not significant. ADA activities in LPS and ConA stimulated lymphocytes were significantly higher than ADA activity in the unstimulated cells (*$P<0.05$).

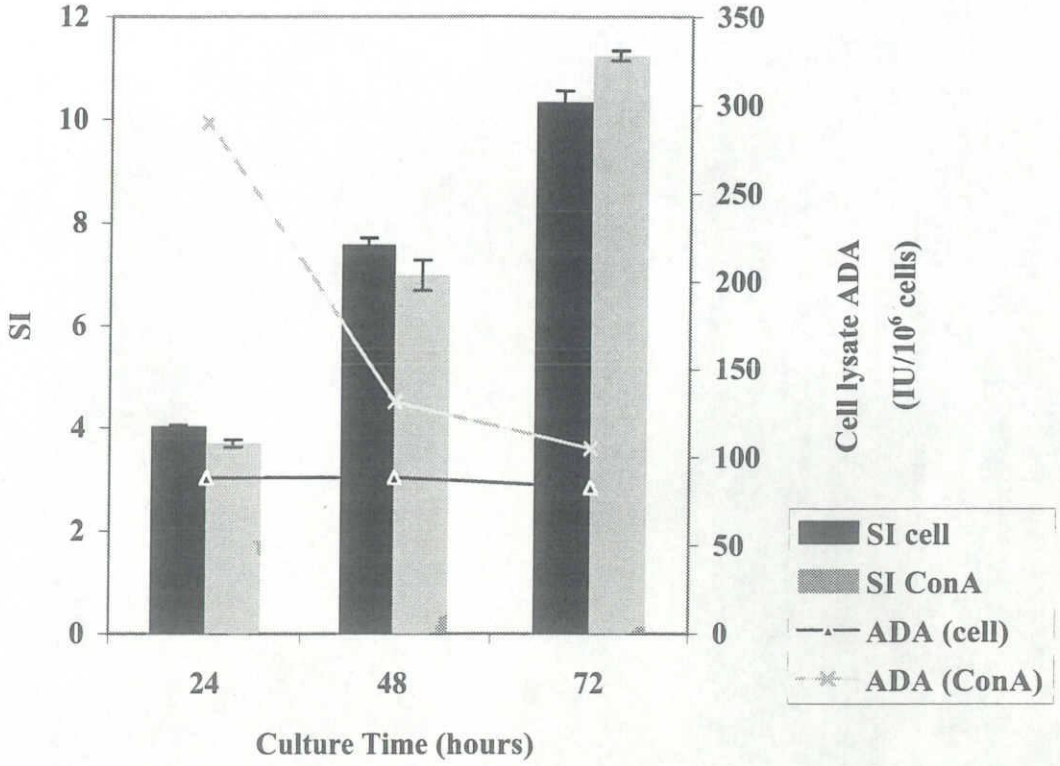

Fig. 5-10. Proliferation assay of CL-1 cells cultured with/without ConA. ADA was shown to progressively decrease in the ConA-stimulated cells whilst that of the un-stimulated cells remained relatively unchanged. Results of statistical analyses are shown in Tables 5-3 and 5-4.

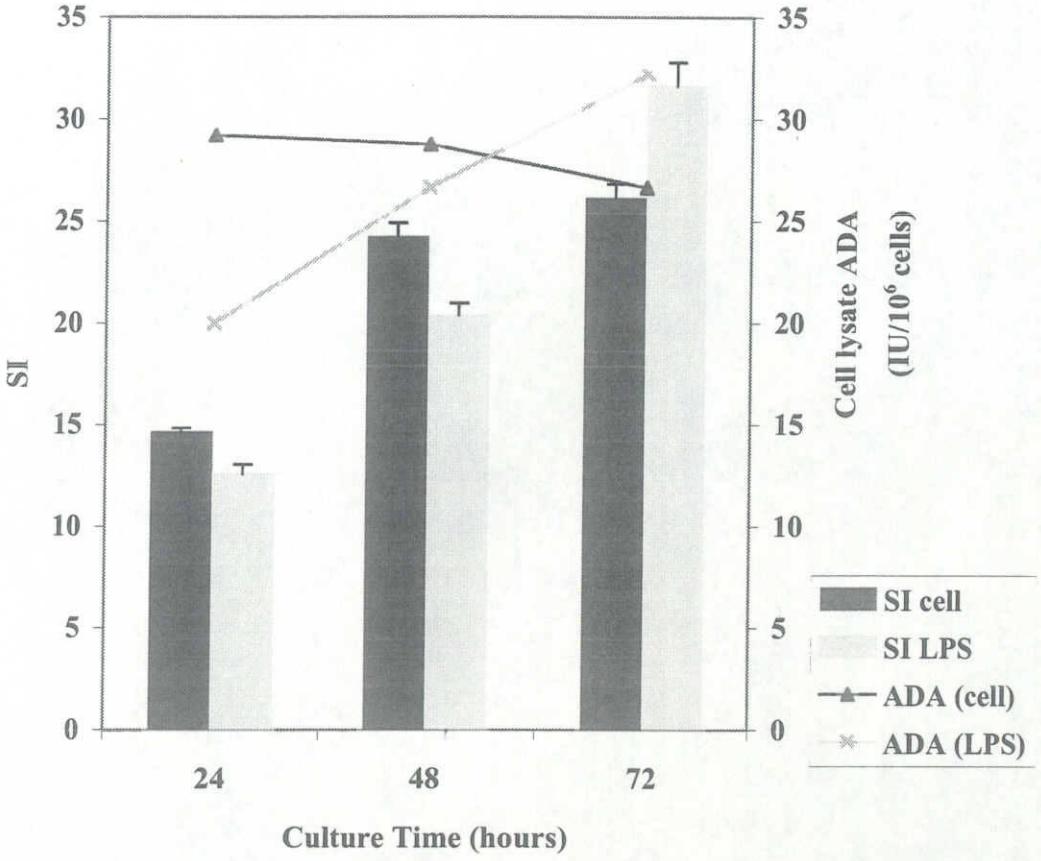

Fig. 5-11. Proliferation assay of GL-1 cells cultured with/without LPS. ADA was seen to progressively increase in the LPS-stimulated cells whilst that of the un-stimulated cells decreased. Results of statistical analyses are shown in Tables 5-3 and 5-4.

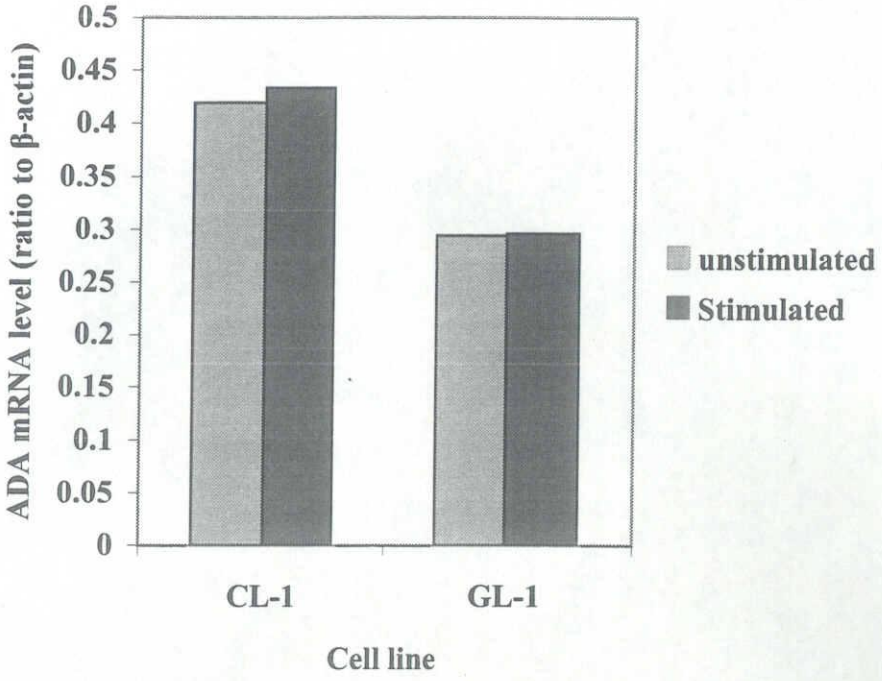

Fig. 5-12. ADA mRNA level of expression in unstimulated and stimulated CL-1 and GL-1 tumor cells at 72 hour culture showing the difference between T and B cell level of expression. The CL-1 tumor cells had higher expression than the GL-1 cells. PCR condition was as follows: 2μl cDNA, Tm 62.5°C, 30 cycles.

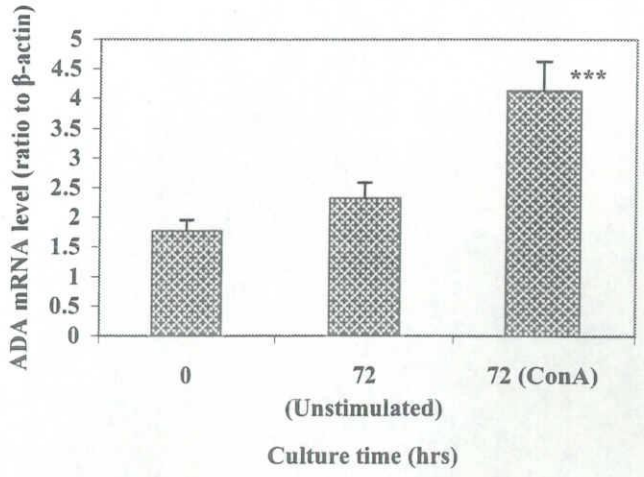

Fig. 5-13. ADA mRNA level in CL-1 showing the difference between unstimulated and stimulated cells. Lower ADA mRNA expression was seen at 0 and 72 hour culture compared to the stimulated cells (***P<0.002, n=3). PCR condition was as follows: 1 μl cDNA, Tm 65°C, 30 cycles (β-actin 20 cycles).

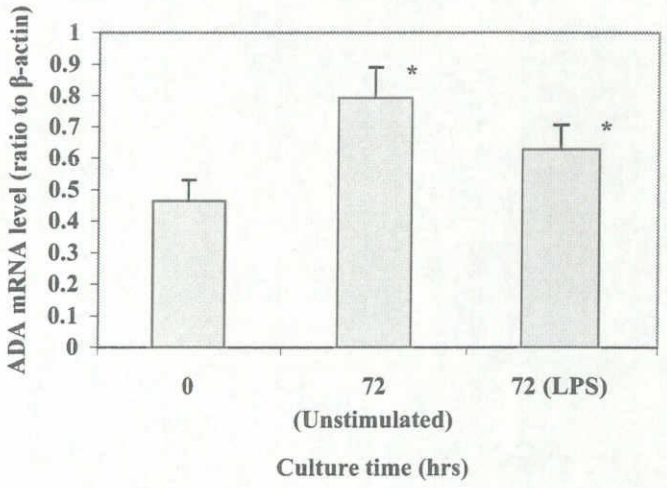

Fig. 5-14. ADA mRNA level in GL-1 showing the difference between unstimulated and stimulated cells (*P<0.05, n=3). Reduced expression was seen at 0 hr culture compared to the 72 hours. PCR condition was as follows: 1 μl cDNA, Tm 65°C, 30 cycles.

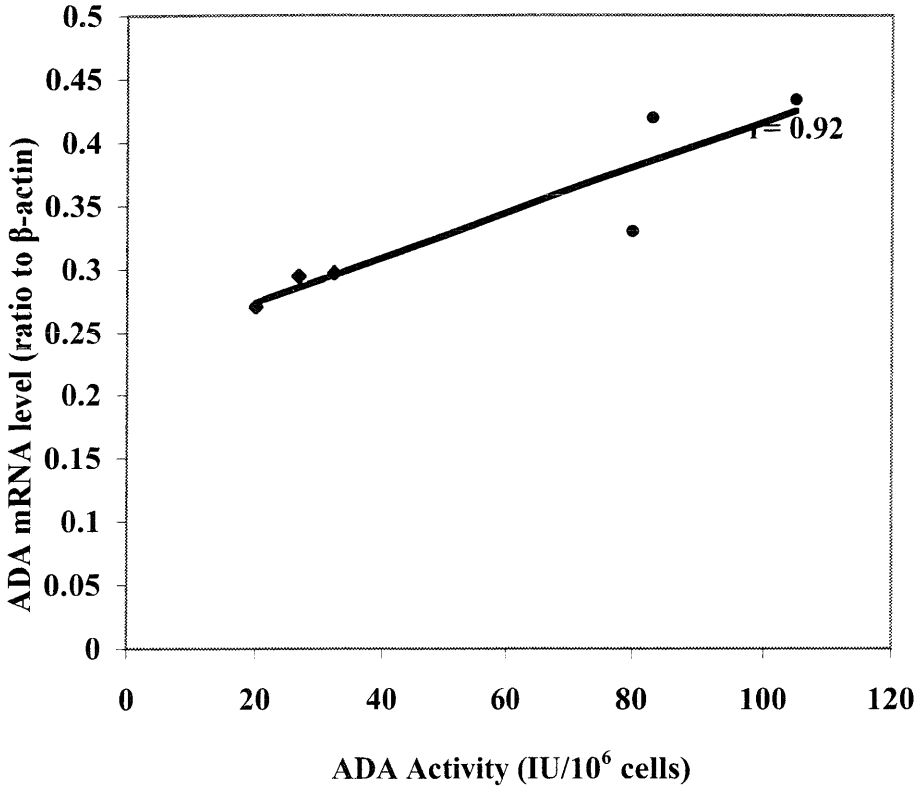

Fig. 5-15. Positive correlation between ADA mRNA level and ADA activity in GL-1 and CL-1 tumor cells ($r = 0.92$, $P<0.005$) for both unstimulated and stimulated cells at 0 and 72 hrs culture time. The solid circle (•) represents the CL-1 and the solid diamond (◆), GL-1.

CHAPTER 6

GENERAL CONCLUSION

We have investigated ADA activity in dogs and cats to find if it is of any clinical significance. Our results have shown that ADA may have some potential in the diagnosis of diseases such as lymphoma, hepatitis and demodicosis in which cases P-ADA activity was significantly elevated. Many enzymes are available for diagnosis of liver disease in dogs, however these enzymes are of limited use in chronic liver disease where levels tend to normalize. Furthermore, ADA has been shown by other authors (Kobayashi et al, 1983) to be useful in chronic hepatic disease; it is possible that it may have the same potential in dogs.

There was no distinct pattern in P-ADA activity in dogs with lymphoma undergoing chemotherapy. Canine lymphomas may have strong similarities with human non-Hodgkin's lymphoma with regard to morphology and clinical behavior (Carter et al, 1986). A study in humans, by Ponce et al (2004), reported that significant differences seen between the lymphoma subtypes are suggestive of inherent biological features and clinical behavior of these tumors. These differences may account for the variation in ADA levels seen in this study. However, two dogs with multicentric lymphoma showed a sudden increase in P-ADA activity upon relapse whereas one patient did not. Similar findings have been cited in human medicine; Morisaki et al (1985) reported that P-ADA was elevated in leukemia patients and it was noted upon serial monitoring that untreated and relapse patients had high activity which returned to normal or subnormal levels during complete remission. One case showed that P-ADA activity was varying with lymph node size. These results suggest that P-ADA activity may have a role to play in the diagnosis of

lymphoma but the variability seen in the results suggest that factors such as phenotype and histologic type should be taken into consideration.

Our results showed that both lymphocytes and neutrophils produce ADA, however the former produce more of the enzyme than the latter. The elevation in L-ADA activity in lymphoma dogs suggests that this is probably the source of elevated P-ADA activity. These results showed that lymphocytes are an important source of P-ADA activity in dogs and cats. This result concurred with reports by other authors in human medicine. Our results showed that stimulated canine lymphocytes and tumor cells produced increased amounts of ADA. T cells produce more ADA compared to B cells. This was particularly clear in the tumor cell lines. The lack of significant increase seen in T-ADA activity may be a reflection of the phenotype of the tumors of the dogs involved. This conclusion is supported by the fact that there was a positive correlation found between P-ADA and T-ADA activities in the controls but not in the dogs with lymphoma. This result suggests that P-ADA activity varies with T-ADA activity. T-ADA activity and $CD4^+$ cell number showed a strong positive correlation in control subjects, whereas the dogs with lymphoma showed a negative correlation. These results suggest that in healthy controls T-ADA activity may be an indicator of T lymphocyte function in dogs as it correlated with $CD4^+$ cell number. The negative correlation seen in the dogs with lymphoma may be suggestive of T cell dysfunctions that occur in dogs with lymphoma. Therefore P-ADA and T-ADA activity may provide useful information on T cell function or activation status in dogs.

In the case of cats, even though no significant differences seen in the diseases studied, with regard to P-ADA activity, cats with ARC showed a significant increase when compared to the other groups. In human ADA activity has been observed to be useful in the monitoring and progression in HIV (Inigo et al, 1992, Planella et al,

1998), therefore, P-ADA activity may be a clinical marker of advancement of FIV from asymptomatic to symptomatic stage and may be used in conjunction with currently offered tests for monitoring FIV infected cats.

As was the case of P-ADA activity, L-ADA and T-ADA activities were noted to be significantly elevated in the ARC stage of FIV infection. Previous reports in humans have shown that T cells produce increased ADA when activated by various stimuli (Hovi et al, 1976 and Kose et al, 2001). Interestingly, studies in humans have suggested that ADA may be involved in the mediation of T cell activation through its interaction with the CD26 cell surface marker (Kameoka et al, 1993). As has been reported by other authors (Hoffmann-Fezer et al, 1997 and Walker et al, 1994), our study showed reduced $CD4^+$ cell numbers lower in the ARC group cell suspensions when compared with those of the FIV-negative groups. In this study the $CD4^+$ cell number was inversely related to T-ADA activity in the ARC group of cats. FIV infection in $CD4^+$ cells has recently been reported to regulate T cell activation that in turn leads to increased viral replication and disease progression (Joshi et al, 2005). Consequently, the $CD4^+$ cells and other immune cells exist in a state of perpetual activation. Therefore, in spite of declining $CD4^+$ cell numbers, it is possible that their hyper-activated state results in increased T-ADA. The increased levels of L-ADA activity seen in cats with ARC may be attributable to T cell activation. Our results suggest that ADA may be an indicator of T cell activation in the ARC stage of FIV infection. However, understanding the complex underlying immunodynamics and immunomodulation could be important for elucidating the mechanism of increase of T-ADA in feline FIV infection. Unlike other tests currently used in the assessment of FIV infected cats, ADA activity assay has advantages in that it is cheap and can be

measured easily using the automated method as is the case with other routine biochemical enzymes.

The results of stimulation of canine lymphocytes and tumor cells with T and B mitogen showed increased ADA activity at varying degrees. T cell tumor ADA activity was considerably higher than that of the B cell tumor. This is consistent with the findings of other authors who have reported markedly high levels of ADA activity in T cell tumors when compared to B cell tumors (Tritsch and Monowada, 1978). ADA activity is highest in T lymphoblasts but activity has been observed to decrease in the course of T-cell maturation (Martin et al, 1995). Therefore, it is possible that this may also be true for T lymphoma cells as they age.

Our results showed that both T and B cell tumors express ADA-specific mRNA, with the T cells showing higher expression than the other cells. The level of ADA mRNA expression was directly related to ADA activity in both CL-1 and GL-1 cell lines. Our results are in agreement with findings in human lymphoid malignancies, where the level of ADA activity was directly related to the amount of ADA-specific mRNA present (Gan et al, 1987). In the T cell tumor line there was an obvious increase of ADA mRNA in comparison to the unstimulated cells. A study by Karbownik et al (2003) reported increase in ADA mRNA expression in leukocytes of patients with autoimmune thyroid disorders and suggested that measurement of this gene could help explain the mechanism of the diseases. This finding suggests that phenotype may play a pertinent role in P-ADA activity as a distinct difference was seen between the tumor cell lines ADA activity.

ADA is of clinical significance in dogs and cats because it is an indicator of immune system activation as was shown in cats with ARC and stimulated cells that produced increased amounts of ADA. Therefore, the relationships seen between ADA

activity and T and $CD4^+$ cells also strongly suggests that it is related to the immune system in dogs and cats. T cells produce more ADA compared to B cells; this was particularly clear in the tumor cell lines and reiterates the fact that phenotype is an important factor in ADA interpretation in canine lymphoma. There was also a corresponding increase in ADA mRNA. These results suggest that ADA activity may be useful in differentiating T and B cell tumor-originated conditions and showing the status of the immune system.

In conclusion ADA is related to the feline and canine immune system. It is useful in clinical conditions of dogs and cats and appears to be of particular importance in diseases characterized by immune system activation such as is the case in FIV. In lymphoma ADA may have some limited in the monitoring of patients however, its usefulness may be limited by tumor phenotype and histological type. ADA also has potential of monitoring progression of disease such as elevations were prominent in only the ARC stage. The results suggest that ADA activity may be useful in differentiating T and B cell originated conditions and showing the status of the immune system.

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