The Age Dependent Levels of Serum ALP Isoenzymes and the Diagnostic Significance of Corticosteroid-Induced ALP during Long-Term Glucocorticoid Treatment

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(Received 25 December 1996/Accepted 29 May 1997)

ABSTRACT. Three isoenzymes of total alkaline phosphatase (TALP) are known in canine serum: Bone alkaline phosphatase (BALP), liver alkaline phosphatase (LALP) and corticosteroid-induced alkaline phosphatase (CALP). Using an assay developed by combining selective precipitation of BALP by wheat germ lectin (WGA) and an automated levamisole inhibition method for quantifying CALP, age-related reference ranges of the isoenzymes in 75 canine serum samples were investigated. BALP comprised 96, 38 and 26% of TALP in young, middle aged and old dogs, respectively, and CALP was respectively 12, 11 and 27% of TALP. LALP was less than 10% in the young but represented more than 50% of TALP in middle aged and old dogs. Furthermore, the significance of monitoring LALP and CALP and their relationship to hepatopathy in dogs receiving long term prednisolone therapy was assessed. In this study, TALP increased in all dogs receiving prednisolone. But only LALP was responsible in dogs with minor vacuolization of the liver, while in severely degenerated cases both LALP and CALP increased. It is concluded that a high TALP due solely to LALP, rather than LALP and CALP represents lesser liver pathologic involvement. Monitoring the 2 isoenzymes has greater significance in assessing the level of liver damage than relying on an increased TALP value alone. Quantifying the individual isoenzymes may further be useful in assessing the clinical significance of these isoenzymes in various conditions that result in elevated TALP values. — **KEY WORDS:** BALP, CALP, canine, LALP, steroid.

J. Vet. Med. Sci. 59(10): 905–909, 1997

Alkaline phosphatase (ALP) is a membrane-associated enzyme present in many tissues; however, only the bone (BALP), liver (LALP) and corticosteroid-induced (CALP) isoenzymes contribute to serum ALP activity in dogs [10]. CALP is present at low levels, but high levels can be induced by abnormal exposure to high concentrations of endogenous or exogenous glucocorticoids [1, 3, 4, 7, 11]. Both LALP and CALP originate from that portion of the hepatocyte membrane that comprises the bile canaliculi [17] whereas BALP is of osteoblastic origin although its precise function is unknown [5, 9].

Quantifying the serum levels of each of the isoenzymes would provide an opportunity to evaluate the diagnostic significance of each isoenzyme in disease and/or in research. Previously, methods for selective quantification of the ALP isoenzymes using a combination of levamisole inhibition [12] and wheat germ lectin (WGA) precipitation were described [14, 16]. By these methods quantification of CALP, an isoenzyme unique to dogs, is also possible.

The purpose of this study is to establish age-dependent reference values for each of the main ALP isoenzymes in canine serum; and to apply the above reference values to assess the significance of LALP and CALP isoenzyme changes in dogs receiving long term steroid therapy.

MATERIALS AND METHODS

Experimental animals: Seventy-five normal dogs of ages ranging from 6 days to 17 years brought into the Veterinary Teaching Hospital of Hokkaido University from April, 1993 to October, 1996 mainly for vaccination and without history of any major illness or steroid therapy were used. They

were grouped as young (24 dogs from 6 days to 1 year old), middle aged (26 dogs between 1 and 7 yrs) and old dogs (25 of them above 7yrs old).

Ten normal dogs were used in the second part of the experiment. Two (Nos. 1 & 2) were untreated controls injected with normal saline for 30 days, another 2 (Nos. 3 & 4) were given an anti-inflammatory dose of 1.1 mg/kg prednisolone every other day for 30 days, while the rest (Nos. 5–10) received 4.4 mg/kg prednisolone everyday for 30 days. The dose of 4.4 mg/kg has been reported to induce CALP by several authors [1, 2, 6, 7]. The duration of medication in each of these studies has however been variable. We used the 30 days' duration as an average of the period reported by Badylak and Van Fleet [1, 2] and that by Sanecki *et al.* [17] with the prime objective of inducing CALP. Changes associated with the induction of either LALP and CALP or LALP alone biochemically and histologically from liver biopsies during the 30 days of treatment were compared. Dogs were kept in steel cages and fed a commercial dry feed (EUKANUBA®) twice a day. They were adapted to the new environment for 2 weeks before the experiment started.

Serum and liver biopsy collection: Blood samples were collected by vein puncture. The serum was either immediately analyzed or kept at - 20°C and analyzed within a week after collection.

Biopsies from all the 10 treated and control dogs were collected twice per week along with blood/serum samples. They were collected by an ultrasound-guided technique described by Hager *et al.* and Hoppe *et al.* [8, 13] using an automated biopsy gun (Biopty®, Radiplast, Karlsrogatan 46, 752 39 Uppsala, Sweden) with a 16 gauge 10 cm biopsy

Table 1. Grading criteria for scoring the severity of vacuolar change of hepatocytes

Scoring of severity of hepatocellular vacuolation
None: vacuoles present in less than 10% of hepatocytes. Scored as 0
Mild: vacuoles present in 10-30% of hepatocytes. Scored as 1
Moderate: vacuoles present in 30-60% of hepatocytes. Scored as 2
Severe: vacuoles present in 60-100% of hepatocytes. Scored as 3

needle. The samples were immediately checked for color and texture change before being fixed in 10% buffered formalin. Each sample was routinely processed for haematoxylin-eosin (HE) staining.

A semiquantitative method to determine severity of vacuolar change by grading the number of hepatocytes with intracellular vacuoles per hepatic lobule as described by Badylak and Van Fleet. [1] was adopted as shown in Table 1. The level of vacuolar change to represent the extent of hepatic pathology was used.

Measurement of enzyme activity: Enzyme measurements were made on serum immediately after separation or within a week after storage at -20°C . An autoanalyser (COBAS: MIRA, Roche, Switzerland) and the respective ALP kit was used. The method was however adapted to measure BALP and LALP using WGA precipitation as described earlier [14, 16] and CALP, by addition of levamisole to the substrate [12, 15]. Briefly, after total ALP (TALP) and CALP were determined, a 50 μl aliquot of the serum was mixed with 50 μl of WGA (5 g/l in de-ionized water) incubated at 37°C for 30 min and centrifuged ($2,000 \times g$) for 10 min. The total supernatant ALP and CALP were again determined. The difference between whole serum TALP and that of the supernatant represented the precipitated BALP portion while the difference between serum CALP and that of the supernatant represented that portion of CALP precipitated along with BALP. With

TALP, BALP and CALP known, the LALP was mathematically calculated.

Statistical analysis: Statistical analysis using Student's *t*-test ($P < 0.05$) was done using a computerized software Systat (Systat, Inc., Evanston, U.S.A.).

RESULTS

Enzyme activity: From the 75 normal dogs a reference range was established as shown in Table 2. The predominant isoenzyme in young dogs was of bone origin. It comprised about 96% of the TALP. The levels in middle aged and old dogs fell to 38 and 26%, respectively. The CALP levels in the young dogs tended to be higher in weeks old dogs diminishing to very low levels as the age neared the 1 yr old upper limit. On average however CALP in the young and middle aged dogs was 12 and 11% of TALP respectively, values not significantly different ($P < 0.05$). In old dogs the CALP levels increased significantly to 27% of TALP, tending to be higher towards the 17 yrs upper limit. The liver isoenzyme was less than 10% of TALP and barely detectable in young dogs but comprised more than 50% of TALP and the main isoenzyme in middle aged and old dogs.

For the treatment experiment, the effect of prednisolone on TALP, LALP and CALP is shown in Figs. 1, 2 and 3. Briefly, all dogs receiving prednisolone had increased TALP. Only LALP had uniformly increased in all these dogs. But in dogs 8, 9 & 10 the LALP increase was accompanied by massive CALP increases making CALP the predominant isoenzyme. For the sake of clarity, dogs 5, 6 & 7 in which only LALP increased were put in subgroup A, whereas dogs 8, 9 & 10 with massive elevation of both LALP and CALP were put into subgroup B. Dog 4 treated with 1.1 mg/kg prednisolone which responded as shown for dogs in subgroup B was taken to represent the subgroup B-like effect of steroids at normal dosage.

Histopathological changes: Control dogs and all other dogs at day 0 had only a fraction of cells (less than 10%) showing vacuolization mostly in the periportal area. Dog 3 and those in subgroup A had 10–30% vacuolization by day 3, reaching 30–60% by day 9 and remaining slightly above

Table 2. Reference ranges and means ($M \pm SD$) of the total alkaline phosphatase and its isoenzymes in young, middle-aged and old normal dogs

Age	No.	TALP (U/L)	BALP (U/L)	LALP (U/L)	CALP (U/L)
Young (6 days-1 yr.)	24	25.0–321.0 (175.4 $\pm$ 84.5)	17.0–307.0 (167.9 $\pm$ 79.6)	0.0–06.0 (2.1 $\pm$ 2.3)	0.0–89.0 (20.4 $\pm$ 25.2)
Middle age (1–7 yrs.)	26	18.0–85.0 (35.9 $\pm$ 18.1)	3.2–32.0 (13.5 $\pm$ 6.3)	2.6–57.0 (19.2 $\pm$ 15.8)	0.0–65.0 (3.9 $\pm$ 11.8)
Old age (> 7 yrs.)	25	18.0–231.0 (74.2 $\pm$ 64.3)	3.2–6.2 (19.1 $\pm$ 13.8)	8.4–158.0 (38.8 $\pm$ 44.9)	0.0–140.0 (19.9 $\pm$ 31.8)

No.- Number of dogs in the group. TALP- Total alkaline phosphatase. BALP- Bone alkaline phosphatase. LALP- Liver alkaline phosphatase. CALP- Corticosteroid-induced alkaline phosphatase.

Fig. 1. TALP levels in control and prednisolone treated dogs.

Fig. 2. Changes in LALP levels in control and prednisolone treated dogs with treatment.

Fig. 3. CALP levels in control and prednisolone treated dogs. Note that only dogs in subgroup B had elevated levels of the isoenzyme even though the same dose as in subgroup A was administered.

this range at the end of the experiment. Vacuolization in these dogs was predominantly midzonal while the

centralobular area became only slightly but gradually affected towards the end of the experiment. For dog 4 and

Table 3. Sequential vacuolation scores in control and treated dogs

Day	Controls	1.1 mg/kg Prednisolone	4.4 mg/kg Prednisolone	
			A	B
0	0	0	0	0
3	0	1	1	2
9	0	1	2	3
12	0	1	2	3
18	0	2	2	3
22	0	2	2	3
29	0	2	2	3

Significant differences were observed between the control and 1.1 mg/kg groups, control and 4.4 mg/kg groups and subgroups A and B but there was no difference between 1.1 mg/kg and subgroup A (T-test, $P < 0.05$).

those in subgroup B, about 30–60% vacuolization was observed as early as day 3 progressing to above 60% by day 9 and reaching maximum severity of about 100% before day 18. Vacuolization progressed from the midzone between days 3 and 9 to involve the entire centralobule by day 18. The vacuolization scores are shown in Table 3. Macroscopically, biopsy specimens in subgroup B became friable with a yellowish brown cast by day 9 whereas those in subgroup A and the 1.1 mg/kg group remained reddish brown almost until the end of study.

DISCUSSION

TALP activity in dogs is well studied. But to our knowledge, this is the first time the isoenzyme composition is age-related. More particularly so is the inclusion of the age dependent reference range for the CALP isoenzyme. BALP is reflected to rate of growth in the young, making reference values clinically important in conditions affecting bones and growth [5, 9]. The high levels of BALP observed in young dogs and the subsequent drop with age is consistent with what is already well recognized [20]. Unexpectedly, however, even though BALP dropped it still comprised one-third to a quarter of the TALP at 38 and 26% in middle aged and old dogs, respectively. On the other hand, the LALP age distribution pattern was the opposite of that observed for BALP. It was negligible in the young, but became the prominent isoenzyme of middle aged and old dogs. CALP levels in young and middle aged dogs at 12 and 11% respectively, were not significantly different between the two age groups. However, levels tended to be higher in the younger dogs becoming lower towards the 1 yr. old upper limit of young dogs. The exact reason for this could not be deduced from this study. But stress is known to induce CALP in dogs [14, 17], so stress in the neonates due to changes in the environment, e.g., ambient temperature may have resulted in elevated glucocorticoid levels (19) with the subsequent induction of the isoenzyme. Alternatively, BALP and CALP in younger dogs may be incompletely differentiated thus having similar properties

not distinguished by this assay. In the old dogs, the CALP levels at 27% were significantly higher ($P < 0.05$) than those of the young and middle aged dogs. Old age and the accompanying geriatric problems are also known to cause a state of stress-induced hypercortisolism [14, 17]. This state may thus be responsible for inducing high CALP levels observed in this age group. Alternatively, the high CALP values confirm a state of hypercortisolism in old dogs and the well recognized susceptibility of this age group to Cushing's and related diseases that are a result of this state.

In the second part of the study, we induced LALP and CALP and monitored the accompanying liver pathology to establish a relationship to each isoenzyme. Establishing such an association would then enable the use of the 2 ALP isoenzymes to monitor long term glucocorticoid therapy regimens. Both LALP and CALP originate at the bile canalicular domain of the plasma membrane and are reported to increase considerably under prolonged glucocorticoid treatment [4, 11, 17, 18]. The dose of 4.4 mg/kg used in this study is, however, inapplicable clinically and was chosen solely as a model to induce CALP. The duration of 30 days was influenced by previous reports in which massive induction was possible within 20 days [1, 2], and that in which even after 32 days at 4.4 mg/kg the isoenzyme was still relatively insignificant [17].

All dogs on 4.4 mg/kg prednisolone had significantly elevated TALP levels. This was attributed solely to LALP in subgroup A and both LALP and CALP in subgroup B. These findings agree with those previously reported by other authors [1–4, 6, 7, 17, 18]. Unique to this study was the observation that even when the TALP in both subgroups A and B were similarly high, the extent of liver vacuolization was more severe in dogs of subgroup B. Simply stated, if the high TALP level was a result of both high LALP and CALP, severe vacuolization occurred but when LALP was the only predominant isoenzyme relatively lesser vacuolization occurred. At a significantly abnormal value of about 1,000 U/L TALP in both subgroups A and B by day 18, vacuolar hepatopathy in subgroup A, although significantly higher than before treatment, was not any more severe than that observed in dogs administered 1.1 mg/kg, whereas in subgroup B it was much more severe. These differences may depend upon each individual dog's susceptibility to steroids as speculated by Dillon *et al.* [3] and suggest that differences in severity of liver damage may be reflected in induced ALP isoenzymes. Therefore, a high TALP in itself may not be as valuable as the determination of isoenzymes involved in assessing the extent of liver damage to steroids. Generally, most clinicians stop corticosteroid treatment when TALP increases because of the consideration of adverse effects [1, 6]. This study, however, suggests that termination of treatment should be considered by an increase in CALP levels not only on the basis of a high TALP value. A high isoenzyme composition of both LALP and CALP results in severe vacuolar hepatopathy and should encourage the clinician to consider in selecting a relatively relaxed corticosteroid treatment

regimen as opposed to that when only LALP is elevated.

In conclusion, the results of this study confirm that the isoenzyme composition of TALP in dog serum is dependent on age. The study further suggested an association of high levels of both LALP and CALP with severity of liver vacuolization during long term steroid therapy, as opposed to only a high LALP increment. Further studies are however encouraged to enable more understanding of these findings and their application in the clinical laboratory.

ACKNOWLEDGMENT. This work was supported in part by Grant-in-Aids for scientific research (No. 06454127) from the Ministry of Education, Science, Culture and Sports of Japan.

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