FULL PAPER *Internal Medicine*

Serum from Dogs Infected with *Babesia gibsoni* Inhibits Maturation of Reticulocytes and Erythrocyte 5'-Nucleotidase Activity *in Vitro*

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(Received 6 February 2003/Accepted 6 August 2003)

ABSTRACT. Erythrocyte 5'-nucleotidase is thought to be involved in the maturation of erythrocytes. In the present study, *in vitro* incubation of canine erythrocytes demonstrated that significant inhibition of 5'-nucleotidase activity occurred in the presence of serum from dogs infected with *Babesia gibsoni*, when the enzyme was assayed with cytidine 5'-monophosphate (5'-CMP) and inosine 5'-monophosphate (5'-IMP) as substrates. The multiplication of *B. gibsoni* in *in vitro* culture also resulted in a significant decrease in the enzyme activity of erythrocytes in the culture. Furthermore, the infected serum and 5'-CMP retarded the maturation of canine reticulocytes *in vitro*. These results suggested that nucleotides such as 5'-CMP and 5'-IMP might accumulate in young erythrocytes and/or serum in dogs infected with *B. gibsoni* as a result of decreased activity of erythrocyte 5'-nucleotidase, resulting in the delayed maturation of reticulocytes.

KEY WORDS: *Babesia gibsoni*, canine erythrocyte, 5'-nucleotidase, pyrimidine 5'-monophosphate, reticulocyte.

J. Vet. Med. Sci. 65(12): 1281–1286, 2003

During the final maturation process from reticulocytes to the mature state, erythrocytes undergo a series of biochemical and physiological transformations. The maturation of reticulocytes is known to be heavily dependent upon the function of erythrocyte 5'-nucleotidase, since its hereditary deficiency is associated with premature hemolysis in humans [21]. The enzyme mediates the hydrolysis of monophosphate ester linkages with the fifth carbon of ribose or deoxyribose in various 5'(3')-nucleotides, producing inorganic phosphate (Pi) and the corresponding nucleoside. The substrates of this enzyme seem to be derived from degradation of reticulocyte RNA in ribosomes during the maturation of reticulocytes [21]. Therefore, erythrocyte 5'-nucleotidase aids in the removal of useless ribosomal RNA and contributes to the maturation of reticulocytes into mature erythrocytes.

The activities of 5'-nucleotidase-catalyzing pyrimidine and purine substrates are also present in canine erythrocytes [7, 10]. Our previous study [10] demonstrated that canine erythrocytes also have activities equivalent to human pyrimidine 5'-nucleotidase isozymes (P5N-I and P5N-II) and purine 5'-nucleotidase, and suggested that a P5N-I-like enzyme may be involved in the removal of reticulum in reticulocytes, resulting in their morphologic change to mature erythrocytes.

Babesia gibsoni is a well-known causative pathogen of canine babesiosis and causes severe hemolytic anemia in infected dogs [4, 6, 8]. The anemia is regenerative, as characterized by polychromasia, reticulocytosis and occasionally increased numbers of nucleated erythrocytes *in vivo* [6, 8]. We previously showed that *B. gibsoni* parasites preferentially invade and multiply in reticulocytes rather than in mature erythrocytes when cultured *in vitro* [14]. Nevertheless, although anemic dogs infected with *B. gibsoni* have many young erythrocytes including reticulocytes in their peripheral blood [6, 8], the parasitemia is often very low [5, 12]. This phenomenon seems not to be consistent with the preferential multiplication of *B. gibsoni* in reticulocytes *in vitro*. The properties of reticulocytes might be changed by infection with *B. gibsoni*.

In the course of the study, we found that the serum from dogs infected with *B. gibsoni* had a suppressive effect on the maturation of canine reticulocytes *in vitro*. The purposes of the present study were to elucidate this phenomenon, and to clarify the role of 5'-nucleotidase in the maturation of reticulocytes in canine babesiosis.

MATERIALS AND METHODS

Reagents: Cellulose microcrystalline was obtained from Merck (Darmstadt, Germany). Percoll gradient solution was from Amersham Pharmacia Biotech (Uppsala, Sweden). α-Modification of Eagle medium (α-MEM) was from Life Technologies (Grand Island, NY, U.S.A.). Potassium benzylpenicillin (Penicillin G Meiji) and streptomycin sulfate (Streptomycin Sulfate Meiji) were from Meiji Seika Kaisha (Tokyo, Japan). Cytidine 5'-monophosphate (5'-CMP), uridine 5'-monophosphate (5'-UMP), uridine 3'-monophosphate (3'-UMP) and thymidine 3'-monophosphate (3'-TMP) were used as pyrimidine substrates, and inosine 5'-monophosphate (5'-IMP) was used as a purine substrate. All the substrates were obtained from Sigma

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Chemical (St. Louis, MO, U.S.A.). Lead acetate and all other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of canine serum: Sera were collected from 6 clinically healthy dogs and 3 dogs chronically infected with *B. gibsoni*. The reticulocyte percentage was 0.6 ± 0.3% (mean ± standard deviation, range 0.1–1.0%) in the clinically healthy dogs but 5.5 ± 3.7% (range 3.2–9.8%) in the infected dogs. The strain of *B. gibsoni* used in this study was originally obtained from a dog infected naturally with *B. gibsoni* in the city of Nagasaki in 1973 and has been maintained in dogs at Hokkaido University since then.

Preparation of canine reticulocytes: Canine reticulocytes were prepared following the method of Maede and Inaba [13] with some modifications. Four clinically healthy dogs weighing about 10–12 kg were used. Each dog was bled of about 200–240 m*l* blood once daily from the cervical vein for 3 days. On the third day after bleeding, 130 m*l* of whole blood was collected into heparinized syringes. At that time, the reticulocyte count in the peripheral blood from each dog was 6.0–8.5%, as determined by microscopic examination of a blood smear stained with new methylene blue staining solution [11]. The collected blood was washed twice with 10 mM phosphate-buffered saline (PBS, pH 7.4) and resuspended in PBS having a packed cell volume (PCV) of about 25–30%. Reticulocytes were separated from the washed cell suspension by Percoll discontinuous gradient centrifugation. 45% (v/v) and 64.5% (v/v) Percoll solutions containing 150 mM NaCl, 0.1% (w/v) bovine serum albumin, and 20 mM HEPES/Tris buffer (pH 7.5) were then used for preparation of the discontinuous Percoll gradients. The solutions had specific densities of 1.070 and 1.096 g/m*l*, respectively. The erythrocyte suspension was carefully layered over the Percoll gradient and centrifuged at 1,800 × g for 15 min at room temperature. The reticulocyte-rich fraction (reticulocyte count 70–95%) was located at the interface of the two Percoll solutions.

The separated reticulocytes were washed twice with PBS and then three times with a-MEM supplemented with sodium pyruvate (0.11 mg/m*l*), glutamine (0.3 mg/m*l*), sodium bicarbonate (2 mg/m*l*), potassium benzylpenicillin (100 units/m*l*) and streptomycin sulfate (100 μg/m*l*). The reticulocytes with a final PCV of 2% were incubated at 37°C under a humidified atmosphere containing 5% CO_2 and 95% air in an incubator (Model 6100–100, Napco, Tualatin, OR, U.S.A.). In these examinations, sera from normal dogs and dogs chronically infected with *B. gibsoni*, lead acetate, 5'-CMP, 3'-TMP and 5'-IMP were added to the incubation media to examine their effects on the maturation of canine reticulocytes. Every 24 hr, 60% of the incubation medium was removed without disturbing the sediment and replaced with an equal volume of fresh medium. The final concentration of each additive and the time of incubation are shown in each figure legend.

Preparation of intact canine erythrocytes: Intact canine erythrocytes were prepared to examine the effect of serum from dogs chronically infected with *B. gibsoni* on erythrocyte 5'-nucleotidase. Heparinized venous blood was collected from clinically healthy dogs, and leukocytes and platelets were removed from whole blood by filtration through a column of a 1:1 mixture of α-cellulose and cellulose microcrystalline [2]. The separated erythrocytes were washed three times with physiological saline, and then centrifuged at 1,250 × g for 10 min. The packed erythrocytes with a PCV of approximately 90% were mixed with an equal volume of serum from normal dogs or dogs chronically infected with *B. gibsoni*, and incubated at 37°C for 24 hr. After incubation, the activity of erythrocyte 5'-nucleotidase was measured according to the method reported by Hossain *et al.* [10].

Culture of B. gibsoni in canine erythrocytes: *B. gibsoni* was cultivated according to the method reported by Yamasaki *et al.* [24] with some modifications to examine the effect of *B. gibsoni* parasites on erythrocyte 5'-nucleotidase. Venous blood was collected from clinically healthy dogs with EDTA as an anticoagulant. The buffy coat was removed after centrifugation, and the erythrocytes were washed twice with PBS and then washed three times with α-MEM containing the supplements described above. The washed erythrocytes were resuspended in a culture medium consisting of 80% α-MEM and 20% serum from normal dogs. For cultivation of the parasites, *B. gibsoni*-infected erythrocytes with high parasitemia in the subculture were added to the prepared erythrocyte suspension to yield a parasitemia of 1% and a PCV of 3%. The suspension was placed in each well of a 96-well flat-bottomed microculture plate and incubated at 37°C under a humidified atmosphere containing 5% CO_2, 5% O_2 and 90% N_2 using an incubator (APMW-36, Astec, Fukuoka, Japan). Every 24 hr, 60% of the culture supernatant was removed without disturbing the sedimented erythrocytes and replaced with an equal volume of fresh culture medium. The activity of 5'-nucleotidase in *in vitro* cultured *B. gibsoni*-infected and not-infected erythrocytes was also measured as described above with 5'-CMP, 5'-UMP, 3'-UMP, 3'-TMP and 5'-IMP as artificial substrates after the cultivation, and the enzyme activity was expressed as a percentage of the initial value obtained before cultivation.

Statistical analysis: Statistical analysis was performed by means of Student's *t*-test. Values of $P<0.05$ were considered as significant. These analyses were carried out on a computer with a statistical software package, Fastat 2.0 (SYSTAT Inc., Evanston, IL, U.S.A.).

All experimental procedures were in accordance with the guidelines for animal use of the Graduate School of Veterinary Medicine, Hokkaido University.

RESULTS

Effect of serum from dogs infected with B. gibsoni on the maturation of canine reticulocytes: Canine reticulocytes collected by gradient centrifugation rapidly lost their cytoplasmic reticulum, and the percentage of reticulocytes in the culture decreased during the 6-day incubation period *in vitro*

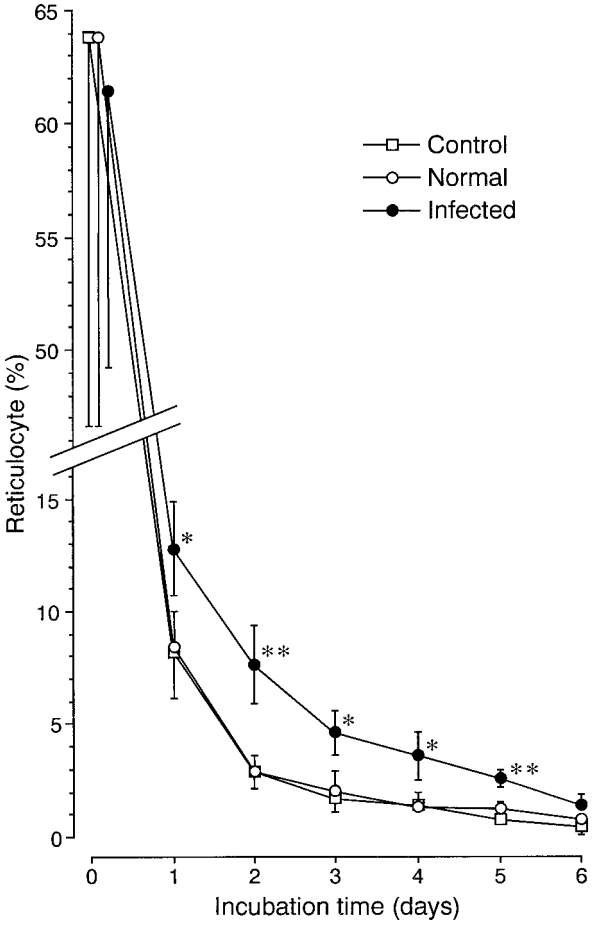

Fig. 1. The *in vitro* effect of serum from dogs infected with *Babesia gibsoni* on the maturation of canine reticulocytes. Reticulocyte-rich erythrocytes were incubated at 37°C under a humidified atmosphere containing 5% CO_2 and 95% air in incubation media without serum (□, Control); with 20% serum from normal dogs (○, Normal); and with 20% serum from dogs infected with *B. gibsoni* (●, Infected). Vertical bars indicate the mean ± standard deviation (n=3). * $P<0.05$ and ** $P<0.01$, compared with the value obtained in the incubation medium with normal canine serum (○, Normal) by Student's *t*-test.

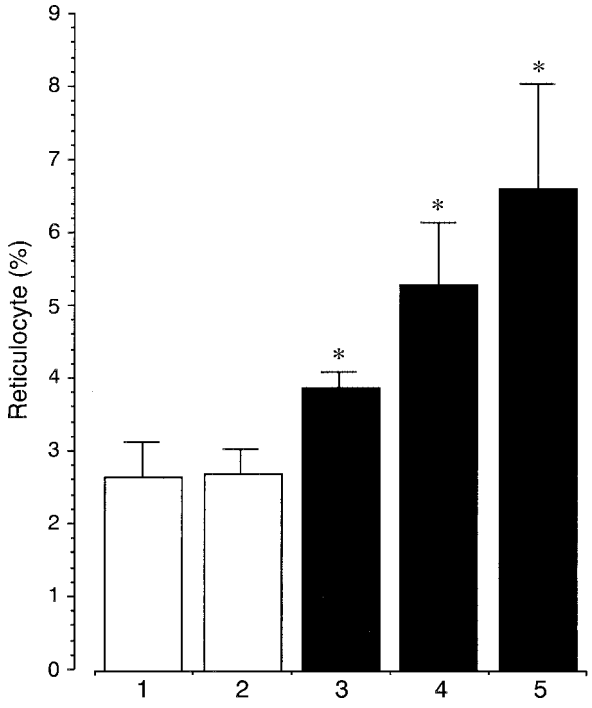

Fig. 2. The dose-dependent effect of serum from dogs infected with *Babesia gibsoni* on the maturation of reticulocytes. Reticulocyte-rich erythrocytes were incubated at 37°C for 3 days under a humidified atmosphere containing 5% CO_2 and 95% air in incubation media without serum (1); with 20% serum from normal dogs (2); with 10% normal canine serum and 10% serum from dogs infected with *B. gibsoni* (3); with 20% serum from dogs infected with *B. gibsoni* (4); and with 40% serum from dogs infected with *B. gibsoni* (5). Vertical bars indicate the mean ± standard deviation (n=3). * $P<0.05$, compared with the value obtained in the incubation medium with 20% normal canine serum (2) by Student's *t*-test.

(Fig. 1). The rate of disappearance of reticulum was significantly slowed by the addition of serum from dogs infected with *B. gibsoni*, whereas normal dog serum had no effect on the morphological maturation of reticulocytes. Furthermore, serum from dogs infected with *B. gibsoni* showed a dose-dependent effect on the maturation of reticulocytes (Fig. 2).

Changes in erythrocyte 5'-nucleotidase activity associated with B. gibsoni infection: The effect of serum from dogs infected with *B. gibsoni* on erythrocyte 5'-nucleotidase activity is shown in Fig. 3. The enzyme activity of canine erythrocytes incubated with the serum from infected dogs was significantly lower than that of the cells incubated with normal dog serum when 5'-CMP and 5'-IMP were used as substrates. There was no significant difference in the enzyme activity between erythrocytes incubated with infected serum and normal serum when using 5'-UMP, 3'-UMP and 3'-TMP. The change in erythrocyte 5'-nucleotidase activity induced by the multiplication of *B. gibsoni in vitro* culture is shown in Fig. 4. The parasitemia was approximately 5% on day 7 in this cultivation. The enzyme activities measured by using 5'-CMP, 5'-UMP and 5'-IMP were significantly lower in erythrocytes in *B. gibsoni*-infected culture than in non-infected culture. There was no significant difference in the activity of the enzyme between erythrocytes in the infected and non-infected cultures when 3'-UMP and 3'-TMP were used.

Effect of nucleotides on the maturation of canine reticulocytes: The effect of nucleotides on the maturation of canine reticulocytes was examined with 5'-CMP, 3'-TMP and 5'-IMP, and compared with that of lead acetate (Fig. 5). The percentage of reticulocytes in the culture incubated with 10 mM 5'-CMP was significantly higher than that in the control culture without it on cultivation, whereas neither 10 mM

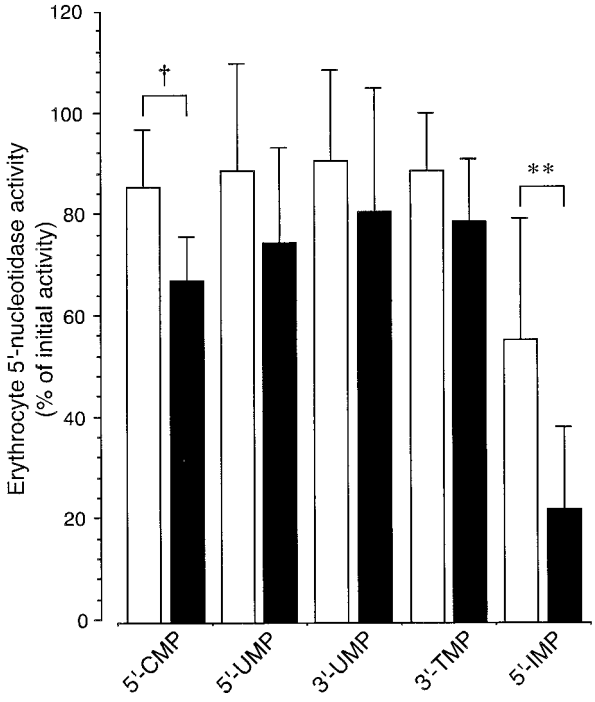

Fig. 3. The change in erythrocyte 5'-nucleotidase activity after incubation at 37°C for 24 hr with serum from normal dogs (open column) and serum from dogs infected with *Babesia gibsoni* (closed column). The erythrocyte 5'-nucleotidase activity was measured with cytidine 5'-monophosphate (5'-CMP), uridine 5'-monophosphate (5'-UMP), uridine 3'-monophosphate (3'-UMP), thymidine 3'-monophosphate (3'-TMP) and inosine 5'-monophosphate (5'-IMP) as artificial substrates. The effect of incubation is expressed as the percentage of the enzyme activity after incubation compared with that of the activity before incubation. Vertical bars indicate the mean ± standard deviation (n=8). ** $P<0.01$ and † $P<0.005$, compared with the value obtained in normal canine serum (open column) by Student's *t*-test.

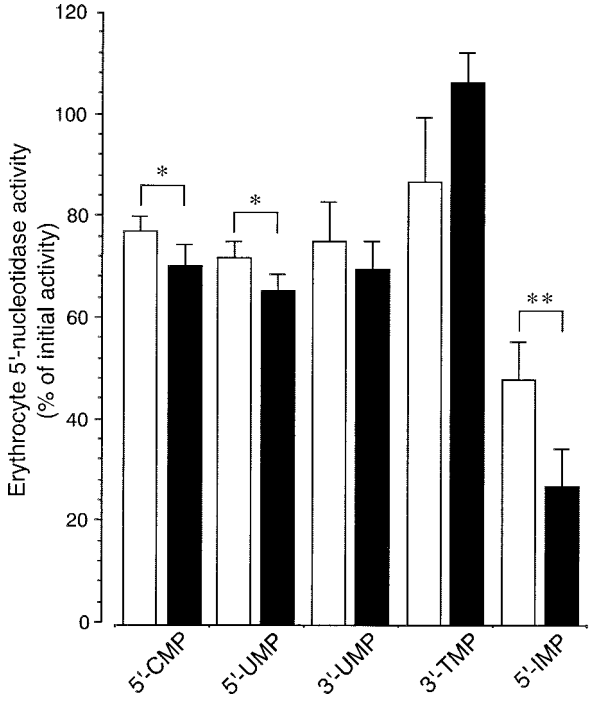

Fig. 4. The change in erythrocyte 5'-nucleotidase activity in control (open column) and *Babesia gibsoni*-infected cultures (closed column). *B. gibsoni* was cultivated at 37°C for 7 days under a humidified atmosphere containing 5% CO_2, 5% O_2 and 90% N_2. The erythrocyte 5'-nucleotidase activity was measured with cytidine 5'-monophosphate (5'-CMP), uridine 5'-monophosphate (5'-UMP), uridine 3'-monophosphate (3'-UMP), thymidine 3'-monophosphate (3'-TMP) and inosine 5'-monophosphate (5'-IMP) as artificial substrates. The effect of cultivation is expressed as the percentage of the enzyme activity after cultivation, compared with that of the activity before cultivation. Vertical bars indicate the mean ± standard deviation (n=4). * $P<0.05$ and ** $P<0.01$, compared with the value obtained in the control culture (open column) by Student's *t*-test.

3'-TMP nor 5'-IMP had any effect on the maturation of reticulocytes.

DISCUSSION

The pyrimidine 5'-nucleotidase in human erythrocytes includes two subclasses, P5N-I and P5N-II, which possess different substrate specificities, optimal pHs and thermostabilities [1, 9]. P5N-I is mainly involved in the degradation of pyrimidine 5'-monophosphate, whereas P5N-II preferentially catalyzes the breakdown of 3'-monophosphate. 5'-CMP and 5'-UMP are the most effective and specific substrates of P5N-I. P5N-II is characterized by its high Michaelis constant and maximum velocity of 3'-TMP and 3'-UMP. In addition, a third erythrocyte 5'-nucleotidase, a purine 5'-nucleotidase, is also present in human erythrocytes, and it preferentially hydrolyzes purine 5'-ribonucleotides and their deoxy counterparts [3]. As described elsewhere, canine erythrocytes have two isozymes similar to human P5N-I and P5N-II, and higher purine-specific 5'-nucleotidase activity than human erythrocytes [10]. Furthermore, it was suggested that the P5N-I-like activity may be involved in the maturation of canine erythrocytes, since the reticulocyte count was approximately proportional to the P5N-I-like activity in dogs [10].

In the present study, *in vitro* incubation of canine reticulocytes with serum from dogs infected with *B. gibsoni* resulted in delayed maturation of the cells, and the effect of the serum on the cell maturation was dose-dependent. These results suggested that the serum from dogs infected with *B. gibsoni* might contain certain factor(s) that retard the maturation of canine reticulocytes *in vitro*. In addition, the infected serum inhibited the activity of erythrocyte 5'-nucleotidase measured with 5'-CMP and 5'-IMP *in vitro*. The multiplication of *B. gibsoni* in *in vitro* culture induced a significant decrease in the 5'-nucleotidase activity measured

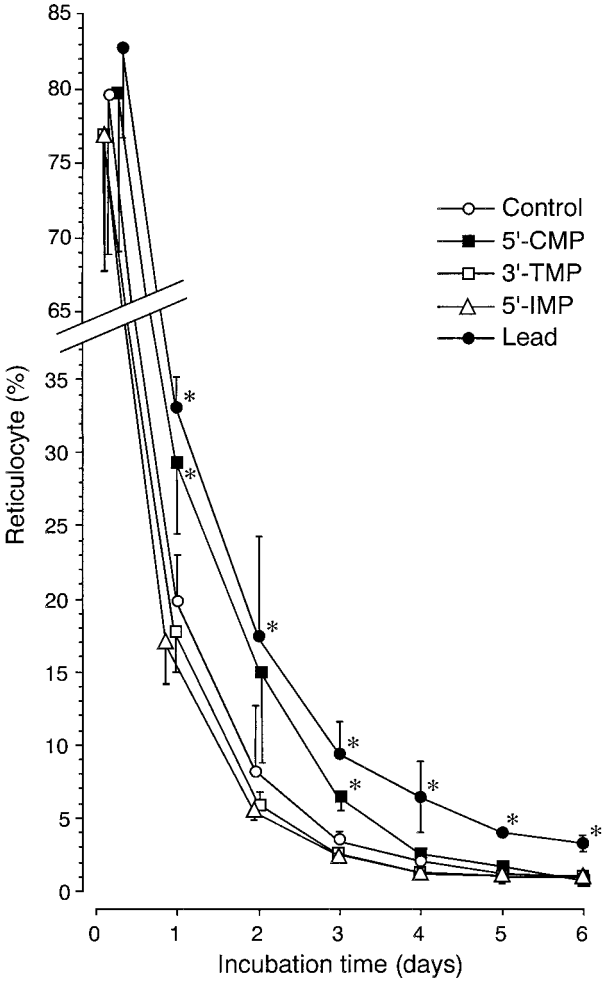

Fig. 5. The *in vitro* effects of nucleotides and lead acetate on the maturation of canine reticulocytes. Reticulocyte-rich erythrocytes were incubated at 37°C under a humidified atmosphere containing 5% CO_2 and 95% air in incubation media without additives (○, Control); with 10 mM cytidine 5'-monophosphate (■, 5'-CMP); with 10 mM thymidine 3'-monophosphate (□, 3'-TMP); with 10 mM inosine 5'-monophosphate (△, 5'-IMP); and with 100 μM lead acetate (●, Lead). Vertical bars indicate the mean ± standard deviation (n=4). * P<0.05, compared with the value obtained in the incubation medium (○, Control) without additives by Student's *t*-test.

with 5'-CMP, 5'-UMP and 5'-IMP in erythrocytes in the culture.

The results obtained in the present study indicate that both serum from dogs infected with *B. gibsoni* and probably the parasite itself or some metabolites produced by the multiplication of the parasites might inhibit P5N-I-like activity and purine-specific 5'-nucleotidase activity in dogs, but not P5N-II-like activity. Furthermore, 5'-CMP also retarded the maturation of canine reticulocytes in *in vitro* culture like lead acetate, which reduces canine P5N-I-like activity to 50% or less [10]. We found that 5'-CMP did not reduce the 5'-nucleotidase activity in canine erythrocytes (data not shown). From the results obtained, it was postulated that nucleotides such as 5'-CMP and 5'-IMP might accumulate in serum in dogs infected with *B. gibsoni*, resulting from decreased activity of erythrocyte 5'-nucleotidase, and that the accumulation of those nucleotides in reticulocytes might retard the maturation of the cells. The delayed maturation of reticulocytes in the culture seems to be very convenient for multiplication of the parasites, since *B. gibsoni* parasites preferentially multiply in reticulocytes rather than in mature erythrocytes when cultured *in vitro* [14].

In addition, the inhibitory effect of serum in infected dogs on the maturation of reticulocytes may contribute partly to reticulocytosis *in vivo* in canine babesiosis, but severe hemolytic anemia often occurs in dogs infected with this parasite in spite of a very low percentage of parasitized erythrocytes in their peripheral blood [5, 12]. Although the low parasitemia *in vivo* might be due to toxicity of free radicals [16–18], increased phagocytic activity of macrophages [15], immunological defenses against the parasites and so on, decreased activity of 5'-nucleotidase and subsequent accumulation of nucleotides might also contribute to the low parasitemia *in vivo*, but these possibilities remain to be elucidated.

In humans, a hereditary deficiency of P5N-I results in nonspherocytic hemolytic anemia, which is characterized by noticeable basophilic stippling in Wright-stained blood film and accumulation of pyrimidine nucleotides [23]. A lead-induced deficiency of P5N-I also results in the induction of basophilic stippling and premature erythrocyte hemolysis analogous to that encountered in genetically induced enzyme-deficiency syndrome [19, 20, 22]. In canine babesiosis, those hematological features seen in patients with a deficiency of P5N-I have not been observed in infected dogs, except for hemolytic anemia. The mechanism of hemolysis induced by *B. gibsoni* infection is not fully understood. The results of the present study suggest that the decreased activity of erythrocyte 5'-nucleotidase induced by *B. gibsoni* infection may participate in part in the mechanism of hemolysis in canine babesiosis.

ACKNOWLEDGMENT. This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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