Inhibitory Effect of Pyrimidine and Purine Nucleotides on the Multiplication of Babesia gibsoni: Possible Cause of Low Parasitemia and Simultaneous Reticulocytosis in Canine Babesiosis

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ABSTRACT. The present study was conducted to determine the cause of low parasitemia and simultaneous reticulocytosis in canine babesiosis. The parasitemia was significantly decreased in in vitro cultures of Babesia gibsoni by the pretreatment of host canine erythrocytes with lead acetate, which is a specific inhibitor of pyrimidine 5'-nucleotidase subclass I (P5N-I). The serum from dogs chronically infected with B. gibsoni did not decrease the activities of hexokinase, glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase in canine reticulocytes, although it was previously reported that this serum had inhibitory effects on both the maturation of reticulocytes and the canine P5N-I and purine-specific 5'-nucleotidase activities. Furthermore, the in vitro multiplication of B. gibsoni was significantly inhibited by pyrimidine nucleotides such as cytidine 5'-monophosphate (5'-CMP), which is preferentially catalyzed by P5N-I and also inhibits the morphological maturation of canine reticulocytes. Purine nucleotides such as inosine 5'-monophosphate (5'-IMP) also had an inhibitory effect on the multiplication of this parasite. These results suggest 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 the decreased activity of erythrocyte 5'-nucleotidase, and the accumulation of these nucleotides might inhibit the multiplication of this parasite and simultaneously retard the maturation of reticulocytes. The results obtained from the in vitro examinations in the present study may partially clarify the relationship between low parasitemia and simultaneous reticulocytosis in vivo in canine babesiosis.

KEY WORDS: Babesia gibsoni, erythrocyte 5'-nucleotidase, pyrimidine, purine nucleotide, reticulocyte.

Babesiosis is a widespread tick-borne blood protozoan infection of many domestic animals. Babesia gibsoni is a well-known causative pathogen of canine babesiosis and causes severe hemolytic anemia in infected dogs [3, 5, 6]. The anemia is regenerative, as characterized by polychromasia, reticulocytosis and occasionally increased numbers of nucleated erythrocytes in vivo [5, 6]. Severe anemia often occurs in dogs infected with this parasite in spite of a markedly low percentage of parasitized erythrocytes in their peripheral blood [4, 9]. It was previously reported that B. gibsoni parasites preferentially invade and multiply in reticulocytes rather than in mature erythrocytes when cultured in vitro [11]. In general, the reticulocytes contain ribosomes, polyribosomes and mitochondria, and have a higher concentration of adenosine triphosphate, reduced glutathione, amino acids and nucleic acids than mature erythrocytes [10]. We previously showed that the high level of multiplication of B. gibsoni in reticulocytes is partly due to the high concentrations of glutamate and reduced glutathione in reticulocytes, based on the data of in vitro examinations [25]. However, although anemic dogs infected with B. gibsoni have many young erythrocytes, including reticulocytes, in their circulation [5, 6], the parasitemia is often markedly low [4, 9]. This phenomenon observed in infected dogs seems not to be consistent with the preferential multiplication of B. gibsoni in reticulocytes in vitro.

Our recent study [7] demonstrated that the serum from dogs infected with B. gibsoni dose-dependently retarded the maturation of canine reticulocytes and decreased the activity of erythrocyte 5'-nucleotidase, which is involved in the morphological maturation of reticulocytes by contributing to the degradation of reticulocyte RNA in ribosomes [21]. The in vitro multiplication of B. gibsoni also induced a significant decrease of this enzyme activity [7]. Furthermore, cytidine 5'-monophosphate (5'-CMP) which is the most effective and specific substrate of pyrimidine 5'-nucleotidase subclass I (P5N-I) retarded the maturation of canine reticulocytes, as the serum from infected dogs did. From these observations, it was supposed that erythrocyte 5'-nucleotidase and nucleotides such as 5'-CMP might have important roles for the reticulocyte maturation and the multiplication of the parasites.

In the present study, we report the in vitro effects of pyrimidine and purine nucleotides on the multiplication of B. gibsoni compared with those of some inhibitors of erythrocyte metabolism, and discuss the relationship between the low parasitemia and simultaneous reticulocytosis observed in vivo in canine babesiosis based on the data obtained from the in vitro examinations.
MATERIALS AND METHODS

Reagents: α-Modified 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). 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), adenosine 5'-monophosphate (5'-AMP) and guanine 5'-monophosphate (5'-GMP) were used as purine substrates. All the nine 5'-monophosphate (5'-AMP) and guanine 5'-monophosphate (3'-GMP) were used as pyrimidine substrates. 5'-CMP, uridine 5'-monophosphate (5'-UMP), uridine 3'-monophosphate (3'-UMP) and thymidine 3'-monophosphate (3'-TMP) were used as purine substrates. All the nine 5'-monophosphate (5'-AMP) and guanine 5'-monophosphate (5'-GMP) were used as purine substrates. All the nine 5'-monophosphate (5'-AMP) and guanine 5'-monophosphate (5'-GMP) were used as purine substrates.

Animals: Six clinically healthy dogs and 3 dogs chronically infected with B. gibsoni were used in the present study. Sera were collected from these dogs. The percentage of reticulocytes in the blood was 0.6 ± 0.3% (mean ± standard deviation, range 0.1–1.0%) in the clinically healthy dogs while it was 5.5 ± 3.7% (range 3.2–9.8%) in the infected dogs when the sera were collected. 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. All experimental procedures were in accordance with the guidelines for animal use in the Graduate School of Veterinary Medicine, Hokkaido University.

Culture of B. gibsoni in canine erythrocytes: B. gibsoni was cultivated according to the method reported by Hossain et al. [7]. Venous blood was collected from clinically healthy dogs using ethylenediaminetetraacetic acid as an anticoagulant. The buffy coat was removed after centrifugation, and the erythrocytes were washed twice with 10 mM phosphate buffered saline (PBS, pH 7.4) and then washed three times with α-MEM supplemented with sodium pyruvate (0.11 mg/ml), glutamine (0.3 mg/ml), sodium bicarbonate (2 mg/ml), potassium benzylpenicillin (100 units/ml) and streptomycin sulfate (100 µg/ml). The washed erythrocytes were resuspended in culture medium consisting of 80% α-MEM and 20% serum from normal dogs. D-Mannose and sodium fluoride as inhibitors of the glycolysis pathway [14], DHEA and 6-ANAD as inhibitors of the pentose phosphate pathway [19, 20, 23], and the pyrimidine or purine substrates were added to the culture medium to examine the effect of each additive on the multiplication of B. gibsoni. The final concentration of each additive is shown in each figure legend. For cultivation of the parasites, B. gibsoni-infected erythrocytes having high parasitemia in subculture were added to the prepared erythrocyte suspension to yield a parasitemia of 2% and a packed cell volume (PCV) of 3%. The suspension was placed in each well of a 96-well flat-bottomed microculture plate and incubated at 37°C for 3 days under a humidified atmosphere containing 5% CO₂, 5% O₂ and 90% N₂ 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 percentage of parasitemia was calculated by counting the number of parasitized cells per 2,000 cells on a Giemsa-stained smear.

Exposure of erythrocytes to lead: The canine erythrocytes were treated with lead acetate to examine the effect of the reduction of the erythrocyte 5'-nucleotidase activity induced by lead on the multiplication of B. gibsoni. The erythrocytes separated using the method described above were washed three times with PBS, and then centrifuged at 1,250 × g for 10 min. The packed erythrocytes at a cell density of approximately 90% were mixed with an equal volume of PBS containing 0 to 10 mM lead acetate, and incubated at 37°C for 24 hr. After incubation, the erythrocytes were washed twice with PBS and three times with α-MEM containing the supplements described above. The washed erythrocytes thus exposed to lead acetate were used for the cultivation of B. gibsoni.

Preparation of canine reticulocytes: Canine reticulocytes were prepared according to the method reported by Hossain et al. [7]. The separated reticulocyte-rich erythrocytes (reticulocyte percentage 70–95%) were washed twice with PBS and then three times with α-MEM containing the supplements described above. The cells were suspended in this medium at a PCV of 2% and incubated at 37°C for 4 days under a humidified atmosphere containing 5% CO₂ and 95% air using 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 were added to the incubation media at a final concentration of 20%. Every 24 hr, 60% of the incubation medium was removed without disturbing the sediment and replaced with an equal volume of fresh medium. After incubation, the cells were washed two times with ice-cold PBS, and the activities of hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) were measured according to the methods described by Beutler [1, 2] using a spectrophotometer (U-3210, Hitachi, Tokyo, Japan). The enzyme activity was expressed as IU per gram of hemoglobin. The hemoglobin concentration was measured by the cyanmethemoglobin method using a commercial kit (Hemoglobin Test Wako, Wako Pure Chemical Industries).

Measurement of erythrocyte 5'-nucleotidase activity: The activity of erythrocyte 5'-nucleotidase was measured according to the method reported by Hossain et al. [8]. The enzyme activity was expressed as micromoles of inorganic phosphorus (Pᵢ) released per hour per gram of hemoglobin.

Statistical analysis: Statistical analysis was performed using Student’s t-test. Values of P<0.05 were considered significant. The analyses were carried out on a computer using a statistical software package, Fastat 2.0 (SYSTAT Inc., Evanston, IL, U.S.A.).
RESULTS

Effects of lead and inhibitors of erythrocyte metabolism on the multiplication of B. gibsoni: The parasitemia was decreased significantly in in vitro cultures using erythrocytes exposed to lead acetate compared with cultures of intact erythrocytes (Fig. 1). The inhibitory effect of lead on the multiplication of B. gibsoni was dose-dependent at 0.1–1 mM lead acetate. Vertical bars indicate the mean ± standard deviation (n=3). † P<0.05 and †† P<0.001, compared with the value obtained using intact erythrocytes using Student’s t-test.

Fig. 1. The in vitro multiplication of Babesia gibsoni in canine erythrocytes exposed to lead acetate. B. gibsoni was cultivated at 37°C for 3 days under a humidified atmosphere containing 5% CO₂, 5% O₂ and 90% N₂ in intact erythrocytes (open column; C) and erythrocytes exposed before culturing to lead acetate at concentrations of 0.1, 0.5, 1 and 5 mM (closed columns). Vertical bars indicate the mean ± standard deviation (n=3). † P<0.05 and †† P<0.001, compared with the value obtained using intact erythrocytes using Student’s t-test.

Effect of D-mannose and sodium fluoride, inhibitors of hexokinase (HK), on the multiplication of Babesia gibsoni (Fig. 2). B. gibsoni was cultivated at 37°C for 3 days under a humidified atmosphere containing 5% CO₂, 5% O₂ and 90% N₂ in culture media without HK inhibitors (open column; C) and with D-mannose or sodium fluoride at final concentrations of 0.05, 0.1, 0.5, 1 and 5 mM (closed columns). Vertical bars indicate the mean ± standard deviation (n=3). * P<0.05, † P<0.05 and †† P<0.001, compared with the value obtained without HK inhibitors using Student’s t-test.

Fig. 2. The in vitro effects of D-mannose and sodium fluoride, inhibitors of hexokinase (HK), on the multiplication of Babesia gibsoni. B. gibsoni was cultivated at 37°C for 3 days under a humidified atmosphere containing 5% CO₂, 5% O₂ and 90% N₂ in culture media without HK inhibitors (open column; C) and with D-mannose or sodium fluoride at final concentrations of 0.05, 0.1, 0.5, 1 and 5 mM (closed columns). Vertical bars indicate the mean ± standard deviation (n=3). * P<0.05, † P<0.05 and †† P<0.001, compared with the value obtained without HK inhibitors using Student’s t-test.

Erythrocyte 5'-nucleotidase activity in dogs chronically infected with B. gibsoni: The activity measured using 5'-CMP was 7.99 ± 2.28 μmol Pi/hr/g of hemoglobin (range 6.30–11.04 μmol Pi/hr/g of hemoglobin; n=3) in normal dogs. The activity measured using 5'-CMP was 11.36 ± 5.70 μmol Pi/hr/g of hemoglobin (range 6.76–17.74 μmol Pi/hr/g of hemoglobin; n=3) in normal dogs.
infected dogs, which was approximately proportional to the percentage of reticulocytes in each individual (0.5–10.7%). There was no significant difference in the erythrocyte 5′-nucleotidase activity between normal and infected dogs when measured using 5′-CMP or any of the substrates used in the present study (data not shown).

Effect of nucleotides on the multiplication of *B. gibsoni*: *B. gibsoni* was cultivated with canine erythrocytes in culture media with or without 5 mM pyrimidine and purine nucleotides (Fig. 5). The level of parasitemia was significantly inhibited when 5′-CMP, 5′-UMP, 5′-IMP, 5′-AMP or 5′-GMP was added to the culture medium compared with that of the control culture without nucleotides, but 3′-UMP and 3′-TMP showed no significant effect on the multiplication of *B. gibsoni*. Furthermore, the dose-dependence of the effect on the multiplication of *B. gibsoni* was examined using 5′-CMP, 3′-UMP, 3′-TMP and 5′-IMP (Fig. 6). 5′-CMP and 5′-IMP inhibited the multiplication of *B. gibsoni* dose-dependently while 3′-UMP and 3′-TMP showed no effect at concentrations of up to 10 mM. The inhibitory effect of 5′-CMP (IC₅₀: 0.71 mM) seemed to be stronger than that of 5′-IMP (IC₅₀: 3.72 mM).

DISCUSSION

A deficiency of P5N-I results in nonspherocytic hemolytic anemia in humans, which is characterized by marked basophilic stippling in Wright-stained blood films and an accumulation of pyrimidine nucleotides [24]. 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 [17, 18, 22]. Treatment of erythrocytes with lead also inhibits canine P5N-I [8], resulting in retardation of the morphological maturation of reticulocytes [7]. In the present study, the *in vitro* multiplication of *B. gibsoni* was inhibited by the pretreatment of host canine erythrocytes with lead acetate (Fig. 1). Certain biochemical modifications induced by a decreased activity of erythrocyte 5'-nucleotidase as a result of the lead exposure seemed to inhibit the multiplication of *B. gibsoni*, although the residual lead acetate in erythrocytes after washing with PBS might also have an inhibitory effect on the metabolism of this parasite. One of these biochemical modifications may be the accumulation of pyrimidine substrates such as 5'-CMP and 5'-UMP resulting from the decreased enzyme activity.

D-Mannose and sodium fluoride are known to inhibit the utilization of glucose in glycolysis pathway [14], in which HK is one of the rate-limiting enzymes. DHEA and 6-ANAD are also known inhibitors of G6PD [19, 20, 23], which is the rate-limiting enzyme of the pentose phosphate pathway and, together with 6PGD, catalyzes the reduction of NADP to NADPH. In the present study, these inhibitors of the energy metabolic pathways significantly inhibited the multiplication of *B. gibsoni* (Figs. 2 and 3). This may be due to the direct effect of the inhibitors on the energy metabolism of *B. gibsoni*. However, the activities of HK, G6PD and 6PGD in canine reticulocytes were not affected by the addition of serum from dogs infected with *B. gibsoni*, while the morphological maturation of reticulocytes was retarded by this serum (Fig. 4). In addition, our previous study showed that serum from dogs infected with *B. gibsoni* decreased the activities of canine P5N-I and purine-specific 5'-nucleotidase [7]. Serum which has been biochemically modified by *B. gibsoni* infection may specifically inhibit canine P5N-I and purine 5'-nucleotidase but not other enzymes, leading to the retardation of reticulocyte maturation as a result of the delayed removal of intracellular ribosomal RNA.

In the present study, 5'-CMP, 5'-UMP and purine nucleotides, which are specific substrates of P5N-I and purine 5'-nucleotidase, significantly inhibited the multiplication of *B. gibsoni* *in vitro* culture while 3'-monophosphates specific for another 5'-nucleotidase, P5N-II, had no effect (Fig. 5). The inhibitory effect of 5'-CMP and 5'-IMP was dose dependent (Fig. 6). As mentioned above, the low parasitemia *in vivo* may be the result of some immunological and biochemical activities, i.e., the protective effect of parasite-specific antibodies, toxicity of free radicals [13, 15, 16], increased phagocytic activity of macrophages [12], and so on. The decreased activity of erythrocyte 5'-nucleotidase and subsequent accumulation of nucleotides might also contribute in part to the low parasitemia *in vivo*.

In the present study, however, the erythrocyte 5'-nucleotidase activity in dogs chronically infected with *B. gibsoni* did not differ significantly from that in normal dogs. This result obtained from the *in vitro* investigation does not seem to be consistent with the results from *in vitro* examinations in the present and previous studies [7]. It is known that many enzyme activities in erythrocytes of dogs infected with *B. gibsoni* are significantly higher than those of non-infected dogs because of the high percentage of reticulocytes in infected dogs [16]. The erythrocyte 5'-nucleotidase activity in infected dogs was approximately proportional to the percentage of reticulocytes in each individual in the present study. Therefore, intact canine reticulocytes are thought to have higher activity of erythrocyte 5'-nucleotidase than mature erythrocytes. In *B. gibsoni* infection, circulating reticulocytes may lose this enzyme activity to remove ribosomal RNA and hydrolyze pyrimidine and purine nucleotides. The slight decrease of the enzyme activity may induce a slight accumulation of nucleotides which is sufficient to inhibit the multiplication of the parasite.

The results obtained from *in vitro* examinations in the present study may elucidate in part the relationship between...
the low parasitemia and reticulocytosis in vivo in *B. gibsoni* infection. These possibilities, however, remain to be proven. It is our hope that the ideas presented in this report will stimulate further work aimed at determining the cause of the reduction of erythrocyte 5’-nucleotidase activity and the inhibition of the *B. gibsoni* multiplication by pyrimidine and purine nucleotides.

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REFERENCES


