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INHIBITORY EFFECT OF NUCLEOTIDES ON THE MULTIPLICATION OF *BABESIA GIBSONI* IN CANINE ERYTHROCYTES

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ABSTRACT

The inhibitory effects of nucleotides on the multiplication of *Babesia gibsoni* was studied on the *in vitro* culture of canine erythrocytes. *B. gibsoni* was cultivated at 37°C for 3 days under a humidified atmosphere containing 5% $C0_2$, 5% O_2, and 90% N_2 in culture media with no nucleotides (Control), 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), adenine 5'-monophosphate (5'-AMP) and guanine 5'-monophosphate (5'-GMP), in which a final concentration of nucleotides was 5 mM. The adding of 5'-CMP, 5'-UMP, 5'-IMP, 5'-AMP and 5'-GMP as artificial nucleotides significantly ($p < 0.01$) inhibited the multiplication of *B. gibsoni* in canine erythrocytes. Further more, the 5'-CMP and 5'-IMP showed the dose-dependently significant inhibitory effect on the multiplication *B. gibsoni*. It may be concluded from these results that the accumulation of specific nucleotides might have inhibited the multiplication of *B. gibsoni* in canine erythrocytes.

Key words : *Babesia gibsoni,* multiplication inhibition, canine erythrocytes, nucleotides

INTRODUCTION

Babesiosis is a wide spread tick-borne blood protozoan disease of domestic animals. *Babesia gibsoni* is well-known causative pathogen of canine babesiosis and causes chronic disease associated with hemolytic anemia in infected dogs (Botros *et al.,* 1975; Farwell *et al.,* 1982). Although severe anemia often occurs in dogs infected with this protozoa inspite of markedly low percentage of parasitized erythrocytes in their peripheral blood (Carson and Phillips, 1981; Kawamura *et al.,* 1987). It was postulated that the nucleotides such as cytidine 5'-monophosphate (5'-CMP) and inosine 5'-monophosphate (5'-IMP) might be accumulated in the young erythrocytes and / or serum in dogs infected with *B. gibsoni* as a result of decreased activity of pyrimidine 5'-nucleotidase (P5N-I) and purine 5'-nucleotidase, resulting the delayed maturation of reticulocytes and participate in partly in the mechanism of hemolysis (Hossain *et al.,* 2003b). Deficiency of the erythrocyte enzyme pyrimidine 5'-nucleotidase (5'-ribonucleotide phosphohydrolase EC 3.1.3.5, PyrNase), first described by Valentine *et al.* (1974) gives rise to a haemolytic syndrome in which the affected cells accumulate high levels of cytidine and uridine nucleotides, affected individuals retain 10% or less of normal enzyme activity and are mildly anemic. The purpose of the present study to investigate the effects of nucleotides on multiplication of *B. gibsoni* and discuss the relationship between accumulation of nucleotides and low parasitemia in canine babesiosis *in vivo*.

MATERIALS AND METHODS

Babesia gibsoni was cultivated according to the method reported by Yamasaki *et al.* (2000) with some modifications. 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-Modification of Eagle medium (α-MEM, Life Technologies, Grand Island, NY, U.S.A.) containing with sodium pyruvate (0.11 mg / ml), glutamine (0.3 mg / ml), sodium bicarbonate (2 mg / ml), potassium benzylpenicillin (Penicillin G Meiji, Meiji Seika Kaisha, Tokyo, Japan, 100 units / ml) and streptomycin sulfate (Streptomycin Sulfate Meiji, Meiji Seika Kaisha, 100 mg / ml). The washed erythrocytes were resuspended in a culture medium consisting of 80% α-MEM, 20% serum from normal dogs and the pyrimidine or purine substrates (0 - 10 mM). For cultivation of the parasites, the *B. gibsoni*-infected erythrocytes having a high parasitemia (8%) subculture were added to the prepared erythrocyte suspension to yield a parasitemia of 2% and a PCV of 3%. The suspension was placed in each well of a 96-well flat-bottomed microculture plate and incubated using a incubator (APMW-36, Astec, Fukuoka, Japan) at 37°C for 3 days under a humidified atmosphere containing 5% CO_2, 5% O_2 and 90% N_2 in culture media with no nucleotides (Control; open column), 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), adenine 5'-monophosphate (5'-AMP) and guanine 5'-monophosphate (5'-GMP), in which a final concentration of nucleotides was 5 mM. The dose-dependent effect of nucleotides on the *in vitro* multiplication of *B. gibsoni* was studied at 37°C for 6 days under a humidified atmosphere containing 5% CO_2, 5% O_2 and 90% N_2 in culture media with no nucleotides (open column, C), 5'-CMP, 3'-TMP and 5'-IMP at a final concentration of 0.1 mM, 1 mM, 5 mM and 10 mM using a incubator (APMW-36, Astec, Fukuoka, Japan). Every 24 h, 60% of culture supernatant was removed without disturbing the sedimented erythrocytes and replaced with an equal volume of the fresh culture medium. Percentage of parasitemia was calculated by counting the number of parasitized cells per 2000 cells on a Giemsa stained blood smear. Statistical analysis was performed using Student's t-test. These analyses were carried out on a computer using a statistical software package, Fastat 2.0 (SYSTAT Inc., Evanston, IL, U.S.A).

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RESULTS AND DISCUSSION

Babesia gibsoni was cultivated with canine erythrocytes in culture media with or without 5 mM of pyrimidine and purine nucleotides (Fig. 1). The level of parasitemia was significantly ($p < 0.001$) inhibited when 5'-CMP, 5'-UMP, 5'-IMP, 5'-AMP and 5'-GMP were added to the culture medium, but 3'-TMP and 3'-UMP showed no significant ($p < 0.05$) effect on the multiplication of *B. gibsoni*. Furthermore, the dose-dependent effect on the multiplication of *B. gibsoni* was examined using 5'-CMP, 3'-TMP and 5'-IMP (Fig. 2). 5'-CMP and 5'-IMP inhibited the multiplication of *B. gibsoni* dose-dependently while 3'-TMP showed no effect at the concentration of up to 10 mM. The inhibitory effect of 5'-CMP seemed to be stronger than that of 5'-IMP.

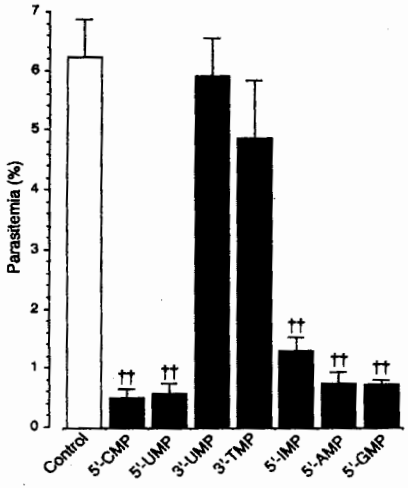

Fig. 1. The *in vitro* effect of nucleotides on the multiplication of *Babesia gibsoni*. Values are Mean ± SD (N = 4). †† $p < 0.001$.

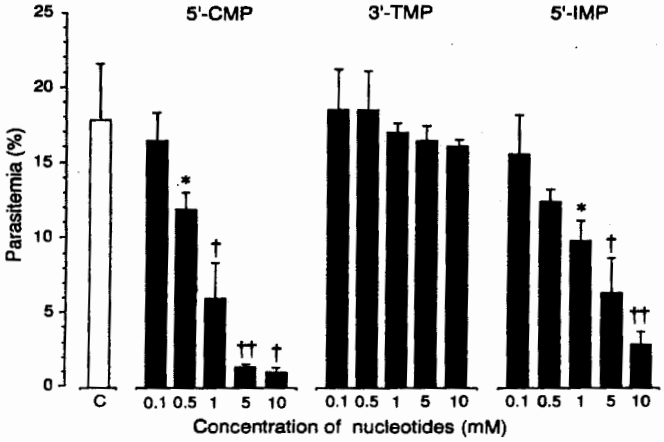

Fig. 2. The dose-dependent effect of nucleotides on the multiplication of *Babesia gibsoni*. Values are Mean ± SD (N = 4). * $p < 0.05$, † $p < 0.005$ and †† $p < 0.001$.

As described elsewhere, canine erythrocytes have two isozymes equivalent to human pyrimidine 5'-nucleotidase (P5'N-I, P5'N-II) and purine 5'-nucleotidase (Amici *et al.*, 1991; Hirono *et al.*, 1985; Hossain *et al.*, 2003a). The P5'N-I is mainly involved in the degradation of pyrimidine 5'-monophosphate, whereas P5'N-II preferentially catalyzes the breakdown of 3'-monophosphate. 5'-CMP and 5'-UMP are the most effective and specific substrates of P5'N-I.

P5'N-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 hydrolyses purine 5'-ribonucleotides and their deoxy-counterparts (Bontemps *et al.*, 1988).

We previously also reported that the serum from dogs infected with *B. gibsoni* and the parasite itself inhibit P5N-I-like activity and purine-specific 5'-nucleotidase activity in dogs, but not P5N-II-like activity (Hossain *et al.*, 2003b). Furthermore, 5'-CMP, 5'-UMP and 5'-IMP which are specific substrates of these two enzymes inhibited by the multiplication of *B. gibsoni* in *in vitro* culture while 3'-monophosphates specific for P5N-II had no effect. In humans, a hereditary deficiency of P5N-I results in nonspherocytic hemolytic anemia, which is characterized by marked basophilic stippling in Wright-stained blood films and an accumulation of pyrimidine nucleotides (Webster *et al.*, 1995). 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 (Paglia *et al.*, 1977a; Paglia *et al.*, 1977b; Valentine *et al.*,1976). In canine babesiosis, these features seen in a deficiency of P5'N-I are not observed although hemolytic anemia are induced by the infection of *B. gibsoni*. However, concerning the enzyme activity and the concentration of specific nucleotides, changes in a part of circulating reticulocytes might induce changes in the characteristic of serum resulting in the inhibition of the parasite's multiplication. The results obtained from *in vitro* examinations in the present study may elucidate partly the relationship between accumulation of nucleotides and low parasitemia *in vivo* in *B. gibsoni* infection in dogs.

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