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FUCOIDAN INHIBITS THE *IN VITRO* GROWTH OF *BABESIA BOVIS*

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SUMMARY

The present study demonstrated the inhibitory effect of fucoidan, a class of polysaccharides mainly constituted of sulfated L-fucose, on the *in vitro* growth of *Babesia bovis* in a dose-dependent manner, as determined by light microscopy. The 50% inhibitory concentration of fucoidan was determined to be 123 μg/ml. The results obtained in this study suggested that fucoidan is a potential chemotherapeutic against babesiosis, although the exact mechanisms of its inhibition require further studies.
Keywords: *Babesia bovis*; fucoidan

INTRODUCTION

Babesia bovis, a hemoprotozoan parasite, causes a virulent disease characterized by fever, anemia, a hypotensive shock syndrome and, in severe cases, a fatal cerebral disease with high mortality rate, thereby causing great economic losses throughout the world (Brown and Palmer, 1999). While much effort has so far been directed to the development of effective control strategies against *B. bovis* infection in cows (Bork et al., 2003a, b; Brown and Palmer, 1999; Combrink et al., 2002; Dalrymple, 1993; Jenkins, 2001; Palmer and McElwain, 1995; Wright et al., 1992), no satisfactory vaccines or chemotherapeutics have been developed to date.

Fucoidans are sulfated, negatively charged polysaccharides that are rich in L-fucose (fucans) extracted from the common brown algae, *Fucus vesiculosus* (Black, 1954; Killing, 1913; Percival and Ross, 1950) and other marine invertebrates (Vasseur, 1948). Recently, the search for new drugs has resulted in increased interest in fucoidans. Fucoidans are known to act as anticoagulants (Chargaff et al., 1936), thereby being proposed as alternatives to heparin (Mourao and Pereira, 1999). There are also some other potent effects of fucoidans on physiological systems: anti-inflammatory activity, which reduces the leucocyte migration into the sites of inflammation (Granert et al., 1999; Shimaoka et al., 1996; Teixeira and Hellewell, 1997); anti-proliferative effects in a mode distinct from heparin on vascular smooth muscle cells (Logeart et al., 1997; Patel et al., 2002) and fibroblasts (Haroun-Bouhedja et al., 2000); inhibitory effect on sperm binding to oviductal monolayers (Talevi and Gualtieri, 2001); and protection of cells from viral infections (Hoshino et al., 1998; Iqbal et al., 2000). Furthermore, the fucoidans have been shown to affect erythrocyte invasion by pathogenic protozoa such as *Plasmodium falciparum* (McCormick et al., 1999) and *P. berghei* (Ying et al., 1997). Although the exact mechanisms have not been clarified yet, the sulfated, negatively charged nature of fucoidan is proposed to be a major property of its inhibitory effect against these parasites.

The present study examined the inhibitory effect of a fucoidan preparation on the *in vitro* growth of *B. bovis* and characterized its inhibitory properties by light microscopy. Finally, the mechanisms by which fucoidan interferes with the asexual growth stage of *B. bovis* are discussed.

MATERIALS AND METHODS

Parasite: A Texas strain of *B. bovis* (Hines et al., 1992) was cultured *in vitro* according to the microaerophilus stationary-phase culture method (Igarashi et al., 1998; Levy and Ristic, 1980). Briefly, the parasite was cultured with bovine erythrocytes at a 10% packed cell volume in M199 (Sigma, St. Louis, MO, USA) with 40% bovine serum and an antibiotic-antimycotic (100x) supplement (Invitrogen Corp., Carlsbad, CA, USA) in 5% CO_2 and 5% O_2 at 37 °C. Cultures were routinely maintained with daily replacement of the medium and dilution at parasitemia (the percentage of parasitized erythrocytes to a total of at least 1,000 erythrocytes) between 1-8%.

***In vitro* growth assay using fucoidan:** *B. bovis*-infected erythrocytes were adjusted to 1% parasitemia with the uninfected erythrocytes, and fucoidan (Sigma) was added at concentrations of 250, 125 and 62.5 μg/ml in 24-well tissue culture plates. The assay was carried out in 3 wells per treatment. The range of the fucoidan concentrations selected for use in this study was based on the results of earlier preliminary experiments (1,000, 500, and 62.5 μg/ml). After initiation of the culture, parasitemia was daily determined in Giemsa-stained thin smears for 3 days. Subsequently, viability tests were conducted for 3 days in a row following the method described by Bork et al. (2003b). Statistical analysis was performed by the Student's *t*-test for the comparison of parasitemia. Differences were considered significant when *P* values of less than 0.05 were obtained. The 50% inhibition concentration (IC_{50}) was determined by non-linear regression analysis.

Results

Growth inhibition effects of fucoidan on the *in vitro* growth of *B. bovis*

The concentration of 250 μg/ml of fucoidan entirely arrested the growth of the parasites, and subsequent viability tests proved the complete destruction of the parasite (Fig. 1). Although 125 μg/ml of fucoidan significantly suppressed parasite proliferation, it could not clear the parasites from the host red blood cells. In contrast, 62.5 μg/ml did not exert any inhibitory effects on the course of parasitemia.

In a light-microscopic examination, parasites treated with 125 or 250 μg/ml of fucoidan showed severe morphological changes (Fig. 2B and C) compared to the controls (Fig. 2A). Especially, in the treatment with 250 μg/ml, the entire parasite population (*i.e.* intraerythrocytic- as well as extraerythrocytic-"free" merozoites) lost its typical shape, and the parasites appeared pycnotic, indicating their complete destruction (Fig. 2C). Similarly, in cultures treated with 125 μg/ml of fucoidan, the final destructive effects solely occurred in approximately 60% of the parasites while the remaining 40% were unaffected by the drug (Fig. 2B).

Discussion

It has been reported that sulfated polysaccharides, including fucoidan, heparin and dextran sulfate, exerted anti-malarial activities *in vitro* in terms of the inhibition of erythrocyte invasion by *P. falciparum* (McCormick et al., 1999; Xiao et al., 1996), *P. berghei* (Ying et al., 1997) and *P. knowlesi* (Dalton et al., 1991). These studies suggested that sulfated polysaccharides may sterically interfere with the interaction between specific ligands and receptors. However, this concept is still controversial to date. Because of their negatively charged feature, the anti-malarial effect of sulfated glycoconjugates can also be simply considered as a result of nonspecific electrostatic interaction (Xiao et al., 1996). This is emphasized by the fact that

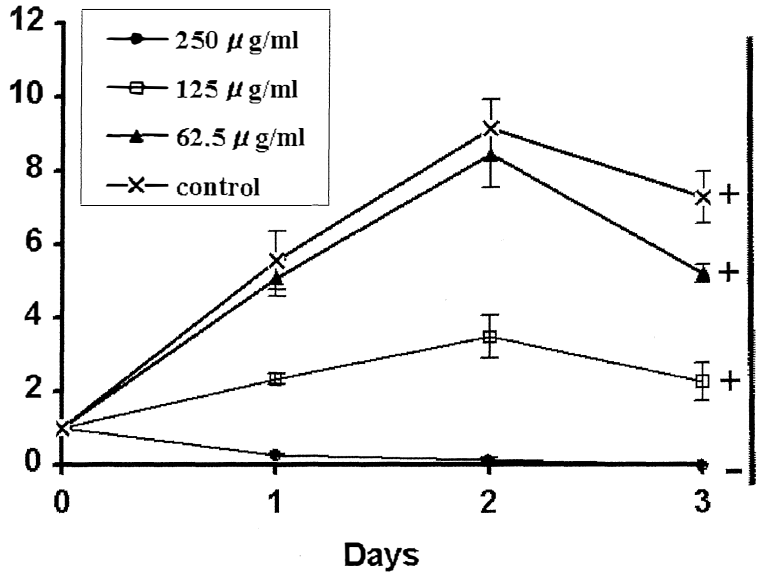

Fig. 1. *In vitro* growth of *B. bovis* in the presence of different concentrations of fucoidan. Parasitic viability was monitored for additional 3 days without fucoidan. Viable (+), Not viable (-). Asterisks indicate a significant difference ($P < 0.05$) between the fucoidan-treated and the control groups. Each value represents the mean ± standard deviation (SD) in triplicates in two separate trials.

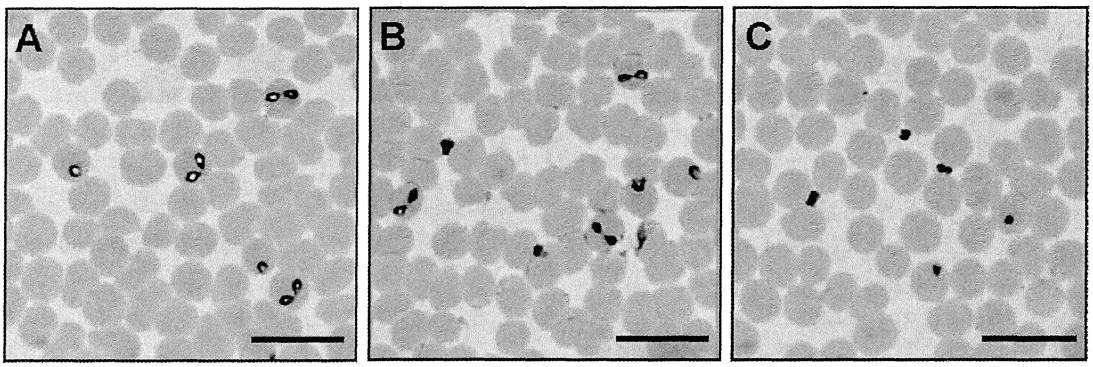

Fig. 2. Light microscopic observation of *in vitro* cultures of *B. bovis* treated with 250 and 125 μg/ml of fucoidan. Photographs were taken on the first day of culture. (A) Control; (B) 125 μg/ml; (C) 250 μg/ml. Note the increase in the number of parasites appearing the pycnotic degradation in (C). Bars = 20 μm.

merozoites and erythrocytes are negatively charged due to surface phospholipids and sialic acids, respectively (Seed and Kreier, 1976; Van Damme et al., 1994).

For *B. bovis*, the IC_{50} value of fucoidan (molecular weight of 18,000) was 123 μg/ml in the present study, while that of a heparin preparation (molecular weight of 17,000 to 19,000) was 410 μg/ml, shown in our previous study using the same experimental system (Bork et al., 2004). With regard to its charge density on sulfated polysaccharides, fucoidan has a negative charge density of 0.3/monomer, which is lower than that of heparin (1.9/monomer) (Brennan et al., 1995; DeAngelis and Glabe, 1987). Therefore, the anti-babesial effect of fucoidan might involve a specific steric configuration rather than electrostatic interaction.

Interestingly, we recently proposed the presence of a molecule(s) that has a strong affinity for heparin and is located on the surface of free babesial merozoites, thereby facilitating erythrocyte invasion (Bork et al., 2004). In that study, heparin was shown to cover the merozoite surface and inhibit the growth of *Babesia* parasites. This finding may give rise to another possibility, namely, that the fucoidan attaches to the merozoite surface, like heparin, thereby inhibiting the erythrocyte invasion by *B. bovis*. Alternatively, the *B. bigemina*-infected erythrocytes were found to preferentially bind to dextran sulfate, possibly through the parasite-derived antigen of 35 kDa (Goodger et al., 1989). In addition, the vaccination of cattle with this dextran sulfate-binding fraction resulted in decreased parasitemia after the *B. bigemina* challenge infection (Kung'u et al., 1992). Therefore, it is also possible that soluble fucoidan binds to the *B. bovis*-infected erythrocytes through a parasite-derived antigen that has affinity for fucoidan, resulting in the inhibition of the *in vitro* growth of the parasites.

In conclusion, the present study is the first to demonstrate the inhibitory effect of fucoidan on the growth of *B. bovis in vitro*. Fucoidan can be used as a potential chemotherapeutic as well as a tool for future elucidation of the mechanism(s) of *B. bovis* infection of host erythrocytes. This, in turn, would contribute to the development of vaccine candidates, although the structural nature of the compound should be further clarified.

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