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Babesia: The protective effects of killed *Propionibacterium acnes* on the infections of two rodent *Babesia* parasites in mice

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Abstract

In the present study, we investigated the protective effects of killed *Propionibacterium acnes* on the infections of two rodent *Babesia* parasites in mice. Pre-treatment with "EqStim" (a commercially available immunostimulant containing killed *P. acnes*) showed significant resistance to both infections. To elucidate the immunological status in the mice, the concentrations of multiple cytokines were measured in serum collected from infected mice. After *B. microti* infection, the levels of interleukin (IL)-2, IL-4, IL-5, IL-10, IL-12p70, and tumor necrosis factor (TNF)-α in the treated group were significantly lower than in the control group. In contrast, after *B. rodhaini* infection, only IL-12p70 and TNF-α were detectable at significantly higher levels in the treated group than in the control group. The present findings indicated the protective effects of killed *P. acnes* on rodent babesiosis even with different immune responses between the *B. microti* and *B. rodhaini* infections. Killed *P. acnes* might be a powerful tool for the control of serious livestock babesiosis.

Index Descriptors and Abbreviations: *P. acnes*, *Propionibacterium acnes*; *B. microti*, *Babesia microti*; *B. rodhaini*, *Babesia rodhaini*; TNF-α, tumor necrosis factor; IFN-γ, interferon; IL, interleukin; Cytokine; Mice

1. Introduction

Members of the genus *Babesia* belong to a diverse group of intraerythrocytic, tick-transmitted protozoa that infect a broad variety of vertebrate hosts (Krause, 2002). *Babesia* parasites destroy host erythrocytes and induce fever, anemia, and icterus in the infected animals (Irwin, 2002). The vaccine control of severe livestock babesiosis, such as bovine and equine babesiosis, has faced increasing failures (Bock et al., 1995). For example, because their wide use may put the parasites under further selection pressure, it may result in the possible appearance of emerging strains capable of evading the host immune responses (Dalrymple, 1992). Two kinds of rodent *Babesia* parasites, *Babesia microti* and *Babesia rodhaini*, have been used as experimental models to study the immunological responses for livestock babesiosis (Chen et al., 2000; Igarashi et al., 1999; Yokoyama et al., 2003). Mice infected with *B. microti* exhibit a transiently high parasitemia, but they subsequently recover from acute infection (Pastusiak et al., 2003). On the other hand, *B. rodhaini* causes a lethal infection in mice (Kamiya et al., 2005; Shimada et al., 1991).

An extract of killed *Propionibacterium acnes* has been reported to stimulate non-specific immunity to viral (Glasgow et al., 1977; Kirchner et al., 1977), bacterial (Adlam et al., 1972; Miyata et al., 1980), and protozoan (Clark et al., 1977; Nussenzweig, 1967) diseases. The protective effect of *P. acnes* pre-treatment on an intraerythrocytic protozoan infection was first reported by Nussenzweig (1967), who found that the pre-treated mice showed complete resistance against the challenge of *Plasmodium berghei* sporozoites. Additionally, the treated mice also exhibited a significant resistance to the subsequent challenge infection of *B. microti* on day 120 after the treatment (Clark et al., 1977). In *Babesia bigemina*, *P. acnes*-treated calves

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showed consistently lower parasitemia and less marked disease (Corrier and Wagner, 1984). At present, the immunomodulation of killed *P. acnes* is being used for the prophylaxis of respiratory diseases and the chemotherapy of chronic pulmonary inflammation in horses (Davis et al., 2003). However, the correct mechanism underlying this effect is poorly understood.

The present study was conducted to confirm that killed *P. acnes* has a preventive effect on babesiosis using the mouse models. First, we studied the effect of heat-killed *P. acnes* on *B. microti* infection based on parasitemia development. Next, the effect of "EqStim," which is a commercially available immunostimulant consisting of killed *P. acnes*, was studied on *B. microti* and *B. rodhaini* infections. Finally, we discussed the protective immunity of killed *P. acnes* against rodent babesiosis using the data of multiple cytokine responses and its possible utility for livestock babesiosis.

2. Materials and methods

2.1. Parasites and mice

The Munich strain of *Babesia microti* and the Australian strain of *B. rodhaini* were maintained by blood passage in mice (Nishisaka et al., 2001). Female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). Six-week-old mice were prepared at the beginning of the mouse experiments described below.

2.2. Propionibacterium acnes and EqStim

Propionibacterium acnes was purchased from ATCC (American Type Culture Collection, No. 6919). The bacterium was cultured under an aerobic condition for 2 days at 37 °C in a GAM medium (NISSUI, Tokyo, Japan) (Ninomiya et al., 1976). The bacterial pellet was collected, washed with phosphate-buffered saline (PBS), and resuspended in PBS. The bacterial suspension was treated at 80 °C for 30 min and then stored at −80 °C until use (Kuwahara et al., 2005). A commercially available immunostimulant EqStim, consisting of killed *P. acnes*, was purchased from the NEOGEN Corporation (Lexington, KY, USA).

2.3. Mouse experiments

Approximately 6 weeks old mice were injected intraperitoneally (i.p.) with 0.4 mg of the heat-killed *P. acnes* or EqStim diluted with PBS and then challenged with 1×10^7 *B. microti* or *B. rodhaini*-infected erythrocytes at the indicated interval. In an initial experiment, a total of 40 mice were divided into 8 groups. The first to fourth groups were inoculated (i.p.) with the heat-killed *P. acnes* and challenged with *B. microti* at weekly intervals (second group; 2 weeks, third group; 3 weeks, fourth group; 4 weeks) except the first group. Fifth to seventh groups were challenged with *B. microti* as positive control at the indicated interval (fifth group; 2 weeks, sixth group; 3 weeks, seventh group; 4 weeks), and eighth group was inoculated PBS instead of the heat-killed *P. acnes*. On the other hands, a total of 48 mice were also divided into 6 groups in the second experiment. The first, second, and third group were inoculated (i.p.) with the EqStim and then challenged with *B. microti* or *B. rodhaini* at 3 weeks interval except the first group. Fourth and fifth group were challenged as positive control, and sixth group was inoculated with PBS instead of the EqStim as negative control. After the challenge infection, Giemsa-stained thin blood smears were prepared from the tail veins every 2 days (*B. microti*) or daily (*B. rodhaini*), and the parasitemia and survival rate were monitored for a total of 35 days or until the death of the mice. The serum fraction was also prepared from the collected blood. All mouse experiments were conducted in accordance with the Standard Relating to the Cage and Management of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

2.4. Cytokine analyses

Th1 and Th2 cytokines, including interleukin (IL)-2, IL-4, IL-5, IL-10, IFN-γ, and inflammatory cytokines, including IL-6, IL-12p70, and tumor necrosis factor (TNF)-α, were simultaneously quantified from the collected sera using mice Th1/ Th2 cytokine and inflammatory cytometric bead array (CBA) kits (BD Biosciences Pharmingen, San Diego, CA, USA). These kits provided a mixture of 8 microbead populations with distinct fluorescent intensities (FL-3); the populations were pre-coated with capture antibodies specific for each cytokine. Fifty microliters of each serum diluted 10 times with PBS was added into the pre-mixed microbeads in a 12 mm × 75 mm Falcon tube (BD Biosciences Pharmingen). After the addition of 50 μl of phycoerythrin-conjugated antibodies against the cytokines, the mixture was incubated for 3 h in the dark at room temperature. This mixture was washed by centrifuging at 500*g* for 5 min, and the pellet was resuspended in 300 μl of a washing buffer (BD). The FACSCalibur flow cytometer (BD Biosciences Pharmingen) was calibrated with setup beads, and 3000 events were acquired for each sample. The concentration of individual cytokines was indicated by their fluorescent intensities (FL-2) using the standard reference curve of the CBA software (BD Biosciences Pharmingen).

2.5. Statistical analysis

The survival rates of the *B. rodhaini*-infected mice were plotted according to a Kaplan–Meier method, and the statistical significance in the survival rate was analyzed among the groups by the generated Wilcoxon test. Differences in parasitemia were analyzed using an independent Student's *t*-test. In the cytokine analyses, the independent Student's