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**Stimulatory factors for interleukin-12 production from murine splenic macrophages co-cultured with *Babesia microti* and *Babesia rodhaini* infected erythrocytes**

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**ABSTRACT**

Stimulatory factors for interleukin 12 (IL-12) production associated with *Babesia microti* and *Babesia rodhaini* infected erythrocytes were examined using an *in vitro* assay system established, since a remarkable increase of serum IL-12 concentration and differentiation of helper T cell (Th cell) into helper T cell type 1 (Th1 cell) was observed in early phase of infection with *Babesia spp.* in mice. To investigate direct stimulating activity of infected erythrocytes for IL-12 production, intact splenic macrophages were co-cultured with them and examined the expression of IL-12 mRNA by reverse transcription-polymerase chain reaction (RT-PCR). Both *B. microti* and *B. rodhaini* infected erythrocytes elicited IL-12 mRNA expression in co-cultured macrophages. Since only the supernatant obtained from the cultured medium of infected erythrocytes showed an activity for IL-12 production from macrophages, the supernatant was collected, concentrated, and heated, followed by the collection of soluble fraction. As the heat stable soluble supernatant showed an IL-12 production activity, it was fractionated by gel filtration. The elution profile of the heat stable soluble supernatant from *B. microti* infected erythrocytes was quite different to that from *B. rodhaini* infected and non-infected erythrocytes. The differences of stimulatory activity were also observed in the fraction of eluate, especially Fraction 3, between *B. microti* infected erythrocytes, and *B. rodhaini* infected and non-infected erythrocytes. These results suggested that *B. microti* infected erythrocytes and/or *B. microti* itself released some factors to stimulate IL-12 production from splenic macrophages, resulted in the Th1 differentiation.

**INTRODUCTION**

It has been widely accepted that *Babesia microti* infection showed a temporally hyperparasitemia and non-lethal to mice, whereas *Babesia rodhaini* infection caused acute and fatal with severe hemolytic anemia. These differences were considered to depend on the host's protective immunological response, especially cellular immunity. Our previous reports (Shimada et al., 1996) demonstrated that *B. microti* and *B. rodhaini* infected mice showed a selective differentiation to helper T cell type 1 (Th1 cell) and

helper T cell type 2 (Th2 cell) in the early phase of infection, respectively. Preimmunized mice with *B. microti* showed a complete protection against *B. rodhaini* infection, depended mainly on the Th1 cell mediated immunity with an increase of serum interleukin-12 (IL-12) concentration (Hashiguchi-Kato et al., 2004). The dominant factor for Th1 cell differentiation in response to infection was closely related to the ability of the pathogen to stimulate IL-12 production from macrophages (D'Andrea et al., 1993; Hsieh et al., 1993; Hunter et al., 1994; Yoshimoto et al., 1998). Skeiky et al. (1998) have already identified certain recombinant *Leishmania* antigen to produce IL-12 and to enhance Th1 cell differentiation, showing the protection against *Leishmania* infection. Unlikely to the *Leishmania* infection, which directly infected in immunocompetent cells (Sypek et al., 1993; Tripp et al., 1993), *Babesia spp.* usually infected and developed in host's erythrocytes. Schetters et al. (1998) suggested that *Babesia spp.* could mature in infected erythrocytes due to the escape from host's protective immune system. However, changes of the cell surface membrane in infected erythrocytes were reported to enhance host's protective immunological systems for eliminating infected cells and also *Babesia spp.* (Allred et al., 1995). This study deals with stimulatory factors associated with *Babesia* infected erythrocytes to produce IL-12 from macrophages.

## Materials and Methods

### Animals and protozoa

Male BALB/c mice, 8-week-old, were purchased from SLC Inc. (Shizuoka, Japan). *Babesia microti* (Munich strain) and *B. rodhaini* (Australian strain) have been maintained in our laboratory by serial passages of parasitized blood to mice.

### Experimental infection

Mice were inoculated by peritoneal injection with  $10^4$  parasitized erythrocytes of *B. microti* or *B. rodhaini* in 0.2 ml of sterile saline diluted per head. The percent parasitemia was monitored by Giemsa's-stained smears of peripheral blood.

### Reverse transcription-polymerase chain reaction (RT-PCR) for IL-12 mRNA

Splenic macrophages from intact mice were prepared to examine the direct stimulatory effect of infected erythrocytes on IL-12 mRNA expression in macrophages. Briefly, to obtain macrophages as adherent cells, spleen cells were collected from 20 intact mice and adjusted to  $3 \times 10^7$  cells/ml in RPMI 1640 (pH7.2), supplemented with 10% fetal bovine serum (FBS: GIBCO, NY), 5 mM Hepes, 40 mg/l of gentamicin, 0.3g/l of glutamin, and 30 ml/l of 7% sodium bicarbonate. One ml of the cell suspension per well was incubated in 12-well culture plate at 37 °C in 5% CO<sub>2</sub> air for 90 min, followed by the rinsing of dish to remove non-adherent cells. The blood was collected by cardiac puncture from *Babesia* infected mice, showing parasitemia of approximate 15%, and adjusted to  $10^6$  erythrocytes/ml in RPMI 1640. The blood was also collected from intact mice as the control. Ten μl of the infected erythrocytes was added to the each contained macrophages. After the incubation for 24 hr, the macrophages were collected. Total RNA was extracted from the macrophages by a RNeasy total RNA kit (QIAGEN, GmbH Germany). First-strand cDNA was synthesized from 1 μg of extracted RNA for each sample by a Super Script™ preamplification system for first strand cDNA synthesis kit (GIBCO, BRL, Japan). The polymerase chain reaction (PCR) was carried out on the 0.5 μl of cDNA sample in a reaction mixture, containing PCR

## STIMULATORY FACTORS FOR IL-12 PRODUCTION in *Babesia* infected erythrocytes

Table 1. Primers used for PCR

		Sequence (5' to 3')
IL-12p3	sense	TCCTGGGAAAGTCTGCCG
	Antisense	TCCTATCTGTGTGAGGAG
IL-12p40	sense	GCACATCAGACCAG
	Antisense	CAACGTTGCATCCTAGGATCG

buffer (Perkin Elmen, CT), 2.5 mM dNTP (Takara, Tokyo, Japan), 5 U/ml Taq DNA polymerase (Perkin Elmen, CT), and 25 pM of the IL-12 specific primers (Table1). The reaction of PCR condition was as follows: 45 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 1 min, and extension at 72 °C for 2 min. The PCR products were analysed by an agarose gel electrophoresis in the presense of etidium bromied.

### Cultivation of infected erythrocytes

The blood was collected from intact and *Babesia* infected mice, showing parasitemia of approximate 60%. After the centrifugation and careful removal of the buffy coat, infected erythrocytes were washed twice with phosphate buffered saline (PBS; pH7.0) at 4 °C, adjusted to 10<sup>6</sup> infected erythrocytes/ml in RPMI 1640 without FBS, and cultured for 18 hr according to our established method (Shikano et al., 1995).

### Stimulatory factors for IL-12 production associated with infected erythrocytes

For detection of stimulatory factors to induce IL-12 production from macrophages, precipitate and supernatant were separated from the cultured medium of infected erythrocytes by the centrifugation at 1200 g for 10 min. For preparation of the heat stable soluble factors, the supernatant was concentrated 25 times by freeze dry method using a Bio Freeze Dryer (NIHON FREEZER CO., LTD., Tokyo, Japan), heated at 100 °C for 10 min, and then ultra-centrifuged at 100,000 g for 1 hr. The supernatant was collected as a sample of the heat stable solutions. Splenic macrophages obtained from intact mice mentioned above were co-cultured with the precipitate, supernatant, or heat stable solution for 18 hr in RPMI 1640 with 10% FBS. The level of IL-12 in each co-cultured medium was measured by an ELISA kit (BioSource Cytoscreen Mouse IL-12 kit; CA).

### Gel chromatographic analyses for the heat stable solution

Heat stable solutions were fractionated by gel filtration on a Superdex 200 column (Superdex<sup>TM</sup> 200 pc 3.2/30, Pharmacia Biotech, AB Uppsala, Sweden) using a micropurification system for large and medium sized biomolecules (Smart<sup>TM</sup> system, Pharmacia LKB Biotechnology, Uppasala Sweden). Briefly, 500 µl of each solution was loaded onto the superdex 200 column, eluted with PBS (ph7.2) at a flow rate of 250 µl/min, and monitored at 280 nm. Individual 1.5 ml fractions were collected. Ten µl of each fractionated samples was co-cultured with intact splenic macrophages in 1 ml of RPMI 1640 with 10% FBS per well. The culture medium was collected at 18 hr after the incubation and measured IL-12 levels by an ELISA KIT.

### Statistical analyses

Paired *Student's t*-test was performed to determine the statistical significance of IL-12 level in cultured medium.

**RESULTS**

**Expression of IL-12 mRNA in intact splenic macrophages co-cultured with infected erythrocytes**

The expression of IL-12 mRNA was detected in intact splenic macrophages co-cultured with both *B. microti* and *B. rodhaini* infected erythrocytes (Fig. 1). In the macrophages co-cultured with *B. microti* infected erythrocytes, the expression was observed at 12 hr after cultivation, whereas at 48 hr with

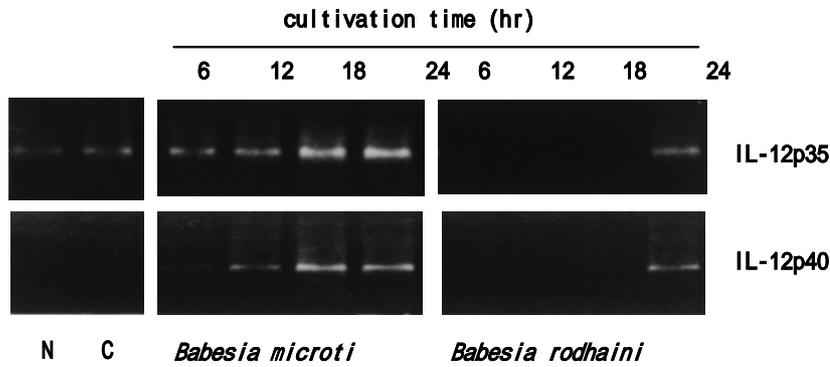


Fig. 1. The expression of IL-12 mRNA in intact splenic macrophages co-cultured with *Babesia microti* and *Babesia rodhaini* infected erythrocytes.

N: macrophages cultured alone, C: macrophages co-cultured with non-infected erythrocytes

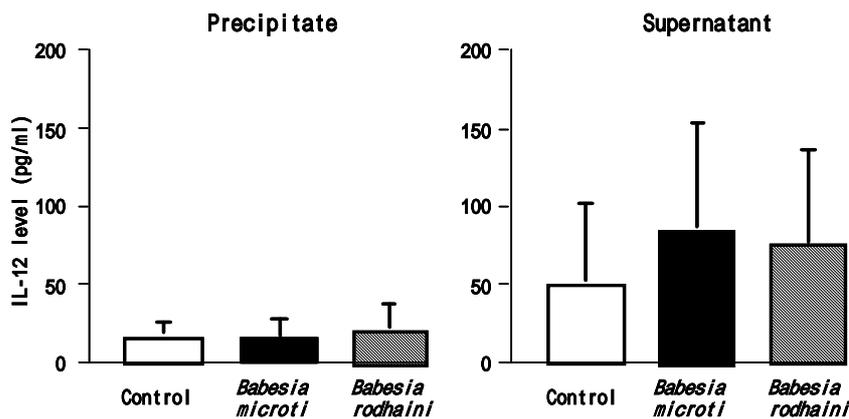


Fig. 2. Levels of IL-12 in cultured medium of intact splenic macrophages co-cultured with the precipitate and supernatant of *Babesia microti* and *Babesia rodhaini* infected erythrocytes cultured.

Control: non-infected erythrocytes cultured, \* : P<0.02

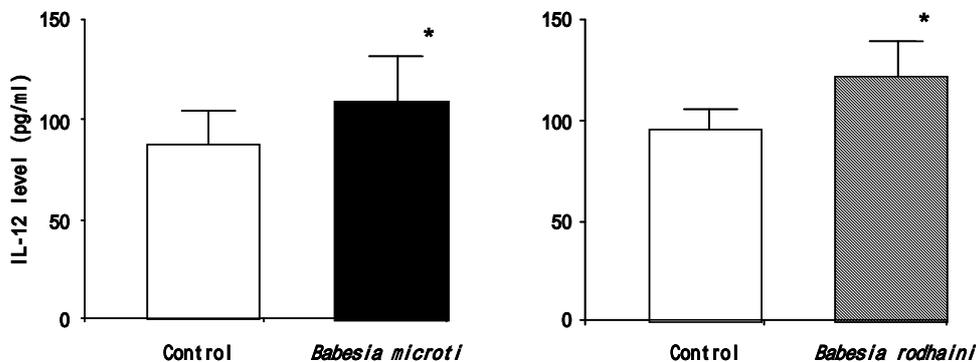


Fig. 3. Levels of IL-12 in cultured medium of intact splenic macrophages co-cultured with the heat stable solution from *Babesia microti* and *Babesia rodhaini* infected erythrocytes cultured.

Control: heat stable solution from non-infected erythrocytes

*Brodhaini* infected erythrocytes. No expression was observed in the macrophages co-cultured with non-infected erythrocytes as the control.

### IL-12 production from intact splenic macrophages

Levels of IL-12 in cultured medium of macrophages co-cultured with the precipitate, supernatant, and heat stable solution, all of which were obtained from cultured medium of infected and non-infected erythrocytes, are shown in Fig. 2. No production of IL-12 from macrophages was observed in the precipitate, whereas both supernatant from *B. microti* and *B. rodhaini* infected erythrocytes showed a high IL-12 production activity compared to that from non-infected erythrocytes. In addition, both heat stable solution from *B. microti* and *B. rodhaini* infected erythrocytes significantly elicited macrophages to produce IL-12 (Fig. 3).

### Gel permeation chromatographic analyses for the heat stable solution

Since both heat stable slution from *B. microti* and *B. rodhaini* infected erythrocytes induced IL-12 production from macrophages, gel permeation chromatographic analyses were performed. The solution was fractionated from fraction 1 (approximate 2,000,000 MW) to fraction 4 (approximate 400 MW). The elution profile of the solution from *B. microti* infected erythrocytes was quite different from that from *B. rodhaini* infected and non-infected erythrocytes (Fig. 4).

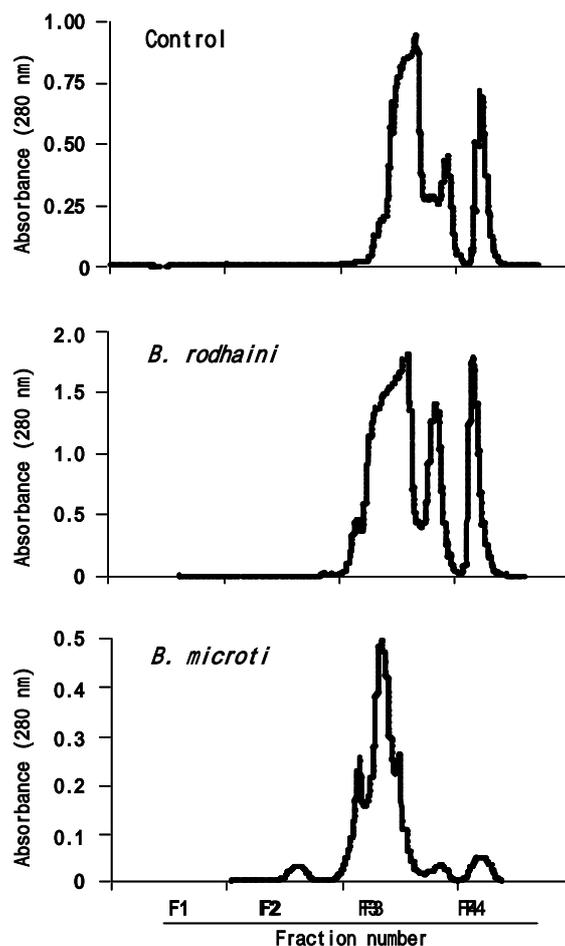


Fig. 4. Elution profiles in gel permeatin chromatography of the heat stable solution from *Babesia microti* and *Babesia rodhaini* infected erythrocytes, and non-infected erythrocytes cultured. To determine the relative molecular size, Blue Dextran 2000 (2,000,000 MW) and Phenol Red (354 MW) were used.

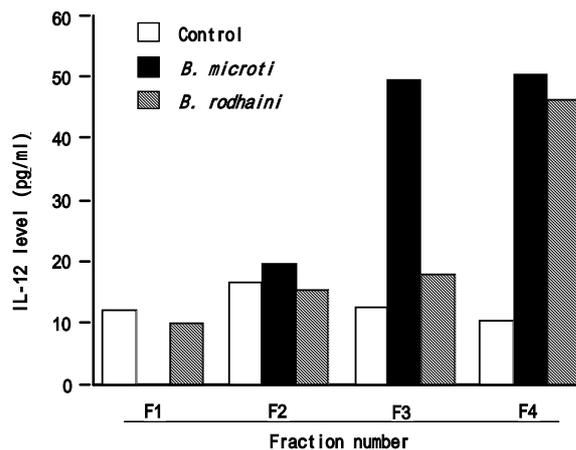


Fig. 5. Levels of IL-12 in cultured medium of intact splenic macrophages co-cultured with the fraction of the heat stable solution from *Babesia microti* and *Babesia rodhaini* infected erythrocytes cultured.

### IL-12 production activity in the fraction of heat stable solution

Levels of IL-12 in *cultured* medium from intact splenic macrophages co-cultured with each fraction of the heat stable solutions were measured by an ELISA method. Both F3 and F4 fraction of the heat stable solution from *B. microti* infected erythrocytes showed an IL-12 production activity, while only F4 fraction of it from *B. rodhaini* infected erythrocytes showed the activity (Fig. 5).

### DISCUSSION

The cytokine IL-12, which is mainly produced from macrophages, is known to be a powerful stimulator of INF-production in NK cell (Hunter et al., 1994; Scharton-Kersten et al., 1995), and also Th1 differentiation and proliferation (Hsieh et al., 1993; Macatonia et al., 1995; Hino et al., 1996). Although IL-18 derived from activated macrophages stimulates INF-production in Th1 cell, it shows no effect on the Th1 cell differentiation (Matsui et al., 1997; Robinson et al., 1997; Takeda et al., 1998). Firstly, direct stimulatory activity of *Babesia* infected erythrocytes were examined to detect the express of IL-12 mRNA in intact splenic macrophages co-cultured with them by RT-PCR. Both *B. microti* and *B. rodhaini* infected erythrocytes elicited IL-12 mRNA expression in splenic macrophages. Since protozoa lysates prepared by freezing and thawing method showed no effect (data not shown), the stimulatory factors for IL-12 production from macrophages were considered to be released from infected erythrocytes.

As shown in the results of the precipitate and supernatant obtained from the cultured medium of infected erythrocytes, only the supernatant showed an activity for IL-12 production from macrophages. For further characterization of the supernatant, it was heated and separated into precipitate and soluble solution. As the precipitate had no activity for IL-12 production from macrophages, the heat stable solution was fractionated by gel filtration. The elution profile of the solution from *B. microti* infected erythrocytes was quite different with that from *B. rodhaini* infected and non-infected erythrocytes. In the heat stable solution from *B. microti*, both F3 and F4 fractions showed an IL-12 production activity from macrophages, while only F4 fraction of it from *B. rodhaini* infected erythrocytes showed the activity. The different stimulatory activity in F3 fraction was considered to closely relate with the Th1 cell differentiation observed in the early phase of infection with *B. microti* in mice, showing a remarkable

elevation of serum IL-12 concentration.

Numerous studies were reported that stimulatory factors in soluble and membrane fractions of merozoites induced Th1 cell development in the protozoa infection (Brown et al., 1992; Wasserman et al., 1993; Schetters et al., 1995), especially in *Leishmania* infection (Skeiky et al., 1998). One of the recombinant *Leishmania* protein was characterized as the factor to stimulate IL-12 production, resulted in the Th1 cell differentiation in *L. magor* infected BALB/c mice. As for the *Babesia* infection, the recombinant *B. bovis* merozoite protein induced Th1 cell differentiation in ruminants (Brown et al. 1993, Tetzlaff et al. 1992). Carcy et al. (1995) demonstrated that a low molecular (37 kDa) glycoprotein, which exerted and/or released from the merozoites, was detected in *B. divergens* cultured medium. Considering above reports and our results, *B. microti* infected erythrocytes and/or *B. microti* itself produced and released factors to stimulate IL-12 production from splenic macrophages.

From these results, the different course of infection observed between *B. microti* and *B. rodhaini* infected mice was mainly caused by the stimulatory factors to produce IL-12, which exerted and/or released from infected erythrocytes and also from protozoa itself.

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