Cordyceps sinensis mycelium protects mice from group A streptococcal infection

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Group A streptococcus (GAS) infection can cause severe invasive diseases, including necrotizing fasciitis and streptococcal toxic shock syndrome. Cordyceps sinensis, a Chinese herbal medicine, is an immunomodulator. In this study the air-pouch bacterial inoculation model was used to investigate the protective efficacy of C. sinensis mycelium extract against GAS infection. Force-feeding mice with C. sinensis mycelium extract for 3 consecutive days before GAS infection increased the survival rate and reduced local skin-tissue injury compared with mice fed PBS. Bacterial numbers in the air pouch exudates from C. sinensis-treated mice were lower than those from PBS-treated mice. Blood and organs in PBS-treated mice showed bacterial dissemination, but those in C. sinensis-treated mice did not. Three days of pretreatment with C. sinensis extract followed by C. sinensis treatment every other day after GAS infection resulted in 100 % survival. The post-GAS-infection levels of aspartate aminotransferase, alanine aminotransferase and blood urea nitrogen in the sera of C. sinensis-treated mice were lower than those of PBS-treated mice. Taken together, these results show that C. sinensis mycelium extract protects by decreasing bacterial growth and dissemination, thereby increasing mouse survival rate. IL-12 and IFN-γ expression and macrophage phagocytic activity also increased after C. sinensis treatment.

INTRODUCTION

Cordyceps sinensis, a member of the Ascomycetes class of fungi, is a parasite on the larvae of the Lepidoptera moth. C. sinensis has been used as a Chinese herbal medicine with a variety of functions, including modulating immune responses (Shimizu, 1978). C. sinensis possesses anti-tumour properties (Kuo et al., 1994; Yoshida et al., 1989), and one of the mechanisms of inhibiting tumour growth might be activation of the host immune response (Chen et al., 1997; Yamaguchi et al., 1990). The production of cytokines such as interferon (IFN)-γ, tumour necrosis factor (TNF)-α and interleukin (IL)-12, and the upregulation of phagocytic activity by C. sinensis fractions might play a role in anti-tumour and anti-bacterial activity (Chen et al., 1997; Kuo et al., 2001; Yamaguchi et al., 1990).

Group A streptococcus (GAS) causes a wide spectrum of diseases in humans, including pharyngitis, impetigo, scarlet fever, streptococcal toxic shock syndrome and necrotizing fasciitis. A variety of factors have been implicated in their virulence, including streptococcal pyrogenic exotoxins (SPEs), various M-protein types, hyaluronic acid capsule, streptolysin O and S, and C5a peptidase (Bisno et al., 2003; Cunningham, 2000; Efstratiou, 2000). Studies in vivo have shown that genetic inactivation of SPE-B, a cysteine protease, decreased the resistance of the mutant to phagocytosis (Lukomski et al., 1998). Previous studies in our laboratory also showed that SPE-B reduced the phagocytic activity of U937 human monocytic cells (Kuo et al., 1999). It is likely that GAS employs SPE-B to resist phagocytosis by host cells.

In the present study, the protective efficacy of C. sinensis against bacterial infection was evaluated in an animal model. We pretreated mice with C. sinensis mycelium or PBS and then used air pouch inoculation to infect them with GAS (Kuo et al., 1998). We then examined and compared bacterial growth and dissemination as well as pathogenic effects in the organs of both groups of mice. GAS-infection mortality rates were lower in mice pretreated with C. sinensis mycelium than in those pretreated with PBS. The mechanisms involving...
cytokine production and phagocytic activity were investigated.

**METHODS**

**Mycelium extract of *C. sinensis***. Cultured *C. sinensis* mycelium was supplied by Simpson Biotech. Crude *C. sinensis* (100 g) was extracted with 800 ml of distilled water and shaken at 37 °C for 2 h. The solution was then centrifuged at 12 000 g for 30 min at 4 °C. The supernatant was collected and lyophilized to form a powder. Before it was used for experiments, the lyophilized *C. sinensis* powder was dissolved in PBS and passed through a 0.2 µm filter. The protein concentration of *C. sinensis* mycelium extract was measured using a Bio-Rad protein assay kit.

**Bacterial strain.** *Streptococcus pyogenes* NZ131 (type M49, T14) was a gift from Dr D. R. Martin, New Zealand Communicable Disease Center, Poriuia. A fresh colony was inoculated overnight into tryptic soy broth containing 0.5% yeast extract (TSBY) (Difco Laboratories), then diluted to 1:50 and cultured for 8 h at 37 °C. The bacteria were harvested using centrifugation and resuspended in sterile PBS. Bacterial density was determined by measuring the absorbance at 600 nm (A600). The bacterial suspension was then diluted with PBS to 10^4 c.f.u. ml^-1, using a standard growth curve to relate measured A600 to the bacterial concentration.

**Air pouch infection model.** The air pouch infection model was described previously (Kuo et al., 1998). We used 8–10-week-old male progeny of BALB/c mice from Jackson Laboratories in this study. Mice were anaesthetized using ether inhalation and then injected subcutaneously with 1 ml air into the air pouch. Mouse mortality rates were monitored every day during the experimental periods. In one experimental group (*C. sinensis* group), mice were force-fed *C. sinensis* mycelium extract (0.2 ml of 50 µg protein ml^-1) in PBS daily for 3 successive days (days −2, −1 and 0) before GAS inoculation (day 1). In another experimental group (*CON group*), mice were force-fed *C. sinensis* mycelium extract (0.2 ml of 50 µg protein ml^-1) in PBS on days −2, −1 and 0 before GAS inoculation (day 1), and also on days 1, 3, 5, 7 and 9 after GAS infection. Mice in one control group (PBS group) were given an equal volume of PBS control at 48 h and 72 h after GAS infection (Fig. 1). Local skin-tissue sections showed severe damage in the epidermis, subcutaneous fat and muscle fibres in the PBS-treated group at both 48 h and 72 h after GAS infection. Mouse sera were collected and the concentrations of AST, ALT and BUN were determined using a Vitros 950 chemistry system (Johnson & Johnson Clinical Diagnostics).

**Detecting cytokine mRNA expression using RT-PCR.** The expression of IL-12 p35, IL-12 p40 and IFN-γ mRNA was determined using RT-PCR analysis. Total cellular RNA of mouse spleenocytes in 10^7 ml^-1 was extracted using TRizol reagent (Gibco-BRL). The concentration of RNA was quantified by spectrophotometry at 260 nm (U-2000; Hitachi). The cDNA was prepared using reverse transcription, as previously described (Lin et al., 2002), and PCR was performed with a PCR controller (GeneAmp PCR System 2400; PerkinElmer) using 35 cycles for cytokines and 30 cycles for β-actin. Temperature cycling for the PCR run consisted of denaturation at 94 °C for 5 min and an extension at 72 °C for 2 min. The oligonucleotide primers used for mouse IL-12 p35 were 5′-GCCAAGGACACAGTCTCAGGG-3′ (sense) and 5′-TGCATCGACTCATCTCAGGG-3′ (antisense), for IL-12 p40 were 5′-GAGGTGGAAGTCCAGCTCGAG-3′ (sense) and 5′-CAATTCTCAGGCTCCTGG-3′ (antisense), for IFN-γ were 5′-TGAAGGCT AACACTGTCATCTTGG-3′ (sense) and 5′-CGACTTCCTTTCGCCGTTCTCGAG-3′ (antisense), and for β-actin 5′-TGGAAAATCGTCGTTGCCACATCATGAAAAC-3′ (sense) and 5′-TAAACGCGACTGTAAGTATGCTG-3′ (antisense). The PCR products were separated using 1.5% agarose gel electrophoresis, stained with ethidium bromide and viewed with UV light.

**Detecting phagocytic activity.** The procedure described previously (Kuo et al., 1999) was followed. Mouse peritoneal macrophages or U937 cells (0.5 ml suspension containing 2×10^6 cells ml^-1) were mixed with 1 µl FITC-conjugated beads (5×10^10 beads ml^-1 with Fluoresbrite carboxylate 0.75 µm diameter microspheres; Polysciences) and incubated for 2 h with shaking at 37 °C. The unbound beads were separated from cells using density-gradient centrifugation in 2% BSA. The binding of fluorescein beads were determined using flow cytometric analysis (Becton Dickinson). For the co-treatment of U937 cells with SPE-B and *C. sinensis* mycelium extract, U937 cells were cultured at a density of 2×10^5 ml^-1 in a volume of 0.5 ml in a 24-well plate at 37 °C. Various doses of *C. sinensis* mycelium extract, with or without purified SPE-B (Chen et al., 2003), were added to the cultures and incubated for 24 h, after which phagocytosis was determined.

**Statistical analysis.** Statistical analysis was performed using one-way analysis of variance (ANOVA) with SigmaStat software (Jandel Scientific). Differences were considered significant at *P < 0.05*. **RESULTS**

**Mortality of GAS-infected mice is reduced by force-feeding with *C. sinensis* mycelium extract**

In this study, an animal model was used to test whether the mycelium extract of the Chinese herbal medicine *C. sinensis* would provide the host with protection against bacterial infection. Susceptibility to GAS infection in mice is significantly different between the sexes: males are more susceptible than females (Medina et al., 2001). Male BALB/c mice were used throughout our study. Mice in the PBS control group started to die on day 6 after inoculation with 3×10^8 bacteria, and the survival rate remained at 40% after day 8. In the *C. sinensis*-pretreated group, mice started to die on day 9; the survival rate after day 10 was approximately 70% (Fig. 1). Local skin-tissue sections showed severe damage in the PBS control group at 48 h and 72 h after GAS infection (Fig. 2); the epidermis, subcutaneous fat and muscle fibres were severely damaged or destroyed in skin tissues as previously shown (Kuo et al., 1998). However, the mice force-fed *C. sinensis* and then infected with GAS did not suffer skin damage (Fig. 2). The frequency of skin-tissue damage was five of five in PBS-treated mice and none of five in *C. sinensis*-treated mice at both 48 h and 72 h after GAS infection. Therefore, *C. sinensis* extract provided the mice with protection against GAS infection. When *C. sinensis* mycelium...
extract (50 μg ml⁻¹ and 100 μg ml⁻¹) was added to the bacteria culture, no bactericidal effect was observed (data not shown), which suggests that the protection provided was not due to a direct antibacterial effect of C. sinensis mycelium.

**Bacterial counts in the air pouch, blood and organs after bacterial inoculation with or without force-feeding of C. sinensis mycelium extract**

After inoculation with GAS, mice that were not pretreated with C. sinensis extract had bacterial numbers that were low at 24 h, increased at 48 h and remained high at least up to 96 h. In contrast, the bacterial counts in mice pretreated with C. sinensis extract continuously declined (Fig. 3). Bacterial dissemination was next determined by the presence of bacteria in the blood. The number of positive blood cultures in the untreated mice was none of 10 (i.e. they were all < 10 c.f.u. ml⁻¹) at 24 h, five of 10 at 48 h and six of nine (one died before sacrifice) at 72 h after bacterial inoculation, compared with none of 10 C. sinensis-pretreated mice during the entire test period (Table 1). Bacterial numbers in various organs were then determined. At 48 h after bacterial inoculation and with PBS pretreatment, five of 10 mice had detectable bacteria, and at 72 h, five or six of nine mice had detectable bacteria present in their organs.

The presence of bacteria in the blood and organs showed a positive correlation with the levels of bacterial counts in the air pouch. At 48 h, mice with bacterial numbers in the air pouch ranging between 1.5 × 10⁸ and 6.3 × 10⁸ c.f.u. ml⁻¹.

**Fig. 1.** C. sinensis mycelium extract increases the survival rate in GAS-infected mice. BALB/c mice were force-fed C. sinensis mycelium extract (●, n = 14) or PBS (■, n = 10) for 3 consecutive days and inoculated with 3 × 10⁹ c.f.u. of GAS in the air pouch 1 day later. Mortality was monitored for 14 days.

**Fig. 2.** Local skin-tissue damage after GAS infection with (b) or without (a) C. sinensis pretreatment. BALB/c mice were force-fed C. sinensis mycelium extract or PBS and inoculated with 3 × 10⁸ c.f.u. of GAS in the air pouch 1 day later. Skin-tissue morphology at the inoculation site was observed at 48 h and 72 h using haematoxylin-eosin staining of skin slices (n = 5 per group). Magnification × 100.
Protection of mice from GAS infection by continually force-feeding them C. sinensis mycelium extract

Pretreatment with C. sinensis for 3 days protected mice from GAS infection (Fig. 1). However, the protection was not complete. We therefore tested whether continual feeding of C. sinensis would provide greater protection to mice. Mice were fed with C. sinensis extract for 3 consecutive days, then given a GAS inoculation and then fed with C. sinensis every other day (CS+ group). The survival rate was 100% in the CS+ group, 80% in the C. sinensis group, and 50% in the PBS group (Fig. 4).

AST, ALT and BUN levels in mouse sera

AST and ALT levels were higher in the sera of GAS-infected mice with PBS pretreatment than in healthy control mice (Fig. 5a, b), indicating liver impairment in the PBS-treated mice. The AST and ALT levels in the C. sinensis and CS+ groups were similar to those of the healthy control mice. BUN levels were higher in the sera of GAS-inoculated PBS-treated mice (Fig. 5c). The CS+ group showed BUN levels similar to those of healthy control mice. While the C. sinensis group mice showed lower BUN levels than the PBS-treated GAS-infected mice, the differences were not statistically significant. These data implied that liver and kidney impairments induced by GAS infection could be reduced or prevented by C. sinensis.

Cytokine expression in C. sinensis-treated mice

IL-12-induced IFN-γ expression provided protection against lethal GAS skin infection in a mouse model (Metzger et al., 1995; Raeder et al., 2000). We therefore examined the cytokine expression induced by C. sinensis. A group of six BALB/c mice were force-fed C. sinensis mycelium extract once daily for 3 consecutive days, and controls were force-fed PBS. Results showed an increase in IL-12 p35, IL-12 p40 and IFN-γ mRNA expression in mouse splenocytes after C. sinensis treatment compared with the PBS controls (Fig. 6).

Effect of C. sinensis on phagocytic activity

Mouse peritoneal macrophages from the C. sinensis-treated group showed increased phagocytosis compared with the PBS-treated group (Fig. 7a). Our previous study showed an inhibitory effect of SPE-B on the phagocytic activity of U937 cells (Kuo et al., 1999). Additional tests indicated that C. sinensis extract blocked SPE-B-mediated suppression of phagocytosis (Fig. 7b). This was not due to a direct effect of C. sinensis extract on SPE-B activity; co-incubation of C. sinensis extract with SPE-B did not exert any effect on SPE-B protease activity (data not shown).

DISCUSSION

Clinical complications of invasive GAS infection include shock and organ failure (Cunningham, 2000). GAS is
### Table 1. Bacterial counts in the air pouch, blood and organs after GAS infection with or without force-feeding *C. sinensis*

<table>
<thead>
<tr>
<th>Treatment and time post-infection</th>
<th>Sample site</th>
<th>Bacterial number for each mouse [c.f.u. ml(^{-1}) or c.f.u. (g tissue(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>PBS-treated, 48 h</td>
<td>Air pouch</td>
<td>6·3 \times 10^8</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>2·0 \times 10^4</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>1·2 \times 10^3</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>3·4 \times 10^3</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>9·6 \times 10^2</td>
</tr>
<tr>
<td>CS-treated, 48 h</td>
<td>Air pouch</td>
<td>8·0 \times 10^7</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>&lt; 10</td>
</tr>
<tr>
<td></td>
<td>Kidney/liver/spleen</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>PBS-treated, 72 h</td>
<td>Air pouch</td>
<td>5·6 \times 10^8</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>2·5 \times 10^3</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>1·2 \times 10^4</td>
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<tr>
<td></td>
<td>Liver</td>
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<tr>
<td></td>
<td>Spleen</td>
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<tr>
<td>CS-treated, 72 h</td>
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<td></td>
<td>Blood</td>
<td>&lt; 10</td>
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<tr>
<td></td>
<td>Kidney/liver/spleen</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

*NA*, Not applicable; mouse died.
disseminated to various organs, including the kidney, liver, spleen and lung, after intraperitoneal injection (Lukomski et al., 1998). We also showed that liver and kidney damage resulted from local NZ131 skin infection in mice (Fig. 5; Kuo et al., 2004). As shown in the present study, *C. sinensis* mycelium extract protected GAS-infected mice from bacterial growth and dissemination as well as from organ damage. The mechanisms of this protective effect provided by *C. sinensis* remain to be investigated. Previous studies have shown that SPE-B, a cysteine protease produced by GAS (Kagawa et al., 2000; Rasmussen & Bjorck, 2002), plays a role in resistance of phagocytic activity (Kuo et al., 1999; Lukomski et al., 1998). *C. sinensis* mycelium extract could abrogate SPE-B-mediated suppression of phagocytic activity in U937 cells (Fig. 7). Augmentation of phagocytic activity by *C. sinensis* might help the host eliminate bacteria at the infection site. This may provide at least one explanation for the protective effect provided by *C. sinensis*.

Innate immunity is important in the elimination of bacteria at the infection site. IL-12-induced IFN-γ expression provided protection against lethal GAS skin infection in a mouse model by enhancing innate immunity (Metzger et al., 1995; Raeder et al., 2000). Our studies also showed an increase in

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**Fig. 5.** Levels of AST (a), ALT (b) and BUN (c) in mouse sera after GAS inoculation in the air pouch with or without force-feeding *C. sinensis* extract. BALB/c mice were force-fed *C. sinensis* mycelium extract for 3 consecutive days before GAS infection only (●, n = 10), or for 3 consecutive days before GAS infection and every other day after GAS infection (■, n = 10), or were force-fed PBS for 3 consecutive days before GAS infection (□, n = 10 for AST/ALT and n = 8 for BUN). Mouse sera were collected on day 4 to determine AST (a) and ALT (b) levels, and on day 6 to determine BUN levels (c). Mouse sera without any treatment (○, n = 4) were used for the basal levels. Horizontal bars represent means. *P < 0.05; **P < 0.01.
was assessed using flow cytometric analysis after 2 h. (b) U937 cells cells with FITC-conjugated beads. The binding of fluorescent beads were prepared and the phagocytic activity was determined by mixing PBS (n = 6) for 24 h with or without 10

Fig. 6. Levels of cytokine mRNA in mouse splenocytes with (b) or without (a) C. sinensis-extract treatment. BALB/c mice were force-fed C. sinensis mycelium extract (n = 6) or PBS (n = 6) for 3 consecutive days. Mouse splenocytes were prepared as a single-cell suspension and cultured for 24 h, then mRNA was extracted and IL-12 p35, IL-12 p40, and IFN-γ expression was detected using RT-PCR, with β-actin as the internal control.

Fig. 7. C. sinensis mycelium extract increased phagocytic activity. (a) BALB/c mice were force-fed C. sinensis mycelium extract (n = 6) or PBS (n = 6) for 3 consecutive days. Mouse peritoneal macrophages were prepared and the phagocytic activity was determined by mixing cells with FITC-conjugated beads. The binding of fluorescent beads was assessed using flow cytometric analysis after 2 h. (b) U937 cells were incubated with 50 or 100 μg ml⁻¹ C. sinensis mycelium extract for 24 h with or without 10 μg ml⁻¹ SPE-B. Phagocytic activity was determined as described in (a) after 2 h. The data are shown as mean ± SD for duplicate cultures of one representative experiment.

IL-12 and IFN-γ production caused by C. sinensis stimulation (Fig. 6). Phagocytic activity was enhanced in both mouse peritoneal macrophages and U937 cells (Fig. 7). Taking all these results together, we speculate that C. sinensis-induced cytokine production augments phagocytic activity that in turn causes bacterial elimination. This hypothesis needs further testing. An examination of the efficacy of C. sinensis extract for protective activity against systemic infection by Salmonella enteritidis has shown that mice receiving C. sinensis live longer (Yamaguchi et al., 1990). Whether a similar effect will occur in other bacterial infections warrants further investigation.

One group of mice, the CS+ group, was fed with C. sinensis extract for 3 consecutive days, inoculated with GAS and, additionally, then continually fed with C. sinensis every other day. The survival rate of these mice was 100 %, but the survival rates in the C. sinensis groups were 70 % or 80 %. Whether this was because the C. sinensis given after GAS infection provided greater protection or because the accumulated dose in the CS+ group was higher needs to be clarified. In addition, whether C. sinensis would provide protection if given only after bacterial infection remains to be evaluated. Nevertheless, C. sinensis is deemed a healthy food product rather than a medicine. In other words, C. sinensis should be considered a preventive rather than a therapeutic remedy.

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REFERENCES


