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Effects of *Astragalus* Polysaccharides and Astragalosides on the Phagocytosis of *Mycobacterium tuberculosis* by Macrophages

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The herb *Astragalus membranaceus* is used in traditional Chinese medicine to boost immunity. This study investigated the effects of *Astragalus* polysaccharides (APS) and astragalosides (AS) on the phagocytosis of *Mycobacterium tuberculosis* by macrophages. Peritoneal macrophages were obtained by peritoneal lavage from mice stimulated by starch gravy culture medium and cultured with *M. tuberculosis* and varying concentrations of APS and AS. Phagocytotic activity was measured using a real-time polymerase chain reaction assay to detect *M. tuberculosis* DNA. Levels of interleukin-1β, interleukin-6 and tumour necrosis factor-α secreted by activated macrophages in the culture supernatant were determined using an enzyme-linked immunosorbent assay. Macrophage phagocytotic activity and secreted cytokine levels were significantly increased after treatment with APS and AS. This study provides evidence that APS and AS have strong promoting effects on the phagocytosis of *M. tuberculosis* by macrophages and the secretion of interleukin-1β, interleukin-6 and tumour necrosis factor-α by activated macrophages.

KEY WORDS: *Astragalus* polysaccharides; Astragalosides; Macrophage phagocytosis; *Mycobacterium tuberculosis*

Introduction

The incidence of tuberculosis, one of the oldest known human diseases, has continued to increase rapidly over the past two decades. It is estimated that its aetiological agent, *Mycobacterium tuberculosis,* infects almost a third of the human population and kills two million people every year.[1] *M. tuberculosis* is reputed to cause the highest annual global mortality of all known pathogens.[2] In the light of the recent human immunodeficiency virus pandemic, the selection of multidrug-resistant strains of *M. tuberculosis* and increased immigration from countries with a high incidence of tuberculosis, coupled with increasing poverty and homelessness in these countries, tuberculosis has been declared a global health emergency by the World Health Organization.[3]

Mycobacterium tuberculosis bacteria parasitize host macrophages; they are able to survive and grow there by inhibiting the fusion of the *M. tuberculosis* phagosome with a lysosome.[4] This mechanism is the main reason why tuberculosis is so difficult to prevent and treat and why recrudescence occurs. Macrophages are the main immune-regulating cells and play an important role in the clearance of invading pathogens and of dying, dead and apoptotic cells; these biological functions are brought about by the self-apoptosis of macrophages engulfing such cells.[5] It has been reported that macrophages infected by *M. tuberculosis* are able to kill the bacteria parasitizing them by self-apoptosis.[6] Thus, it is possible that parasitizing *M. tuberculosis* bacteria may be completely cleared by modulating macrophage apoptosis. Enhancing the initial phagocytosis of *M. tuberculosis* by macrophages and their subsequent apoptosis may offer a new way to prevent and treat tuberculosis.

Astragalus membranaceus, a traditional Chinese medicinal herb, is known to have a balancing effect on bodily functions and is associated with significant immunomodulation.[7] *Astragalus* extract has been shown to boost significantly macrophage phagocytosis of *Candida albicans*,[8] but it is unknown whether it can enhance phagocytosis of *M. tuberculosis* by macrophages. In this study, the effects of the two main classes of active compound – *Astragalus* polysaccharides (APS) and astragalosides (AS) – on macrophage phagocytosis of *M. tuberculosis* were investigated. The phagocytotic activity of the macrophages was evaluated by measuring the number of phagocytosed *M. tuberculosis* bacteria, and the levels of cytokines secreted by the activated macrophages were assessed.

Materials and methods

MICE

Female BALB/c mice approximately 10 weeks old were purchased from the Experimental Animal Centre of Lanzhou University, China. The mice were housed under pathogen-free conditions in the Animal Facility of Lanzhou Medical College. The study was approved by our institution's ethics committee and the mice were treated in accordance with our institution's guidelines for the ethical care and use of animals.

GENERATION AND IDENTIFICATION OF PERITONEAL MACROPHAGES

The mice were injected intraperitoneally with 1.5 ml of starch gravy culture medium (Sino-American Biotechnology Co., Luoyang, China), which has been shown to stimulate the peritoneum and induce inflammation,[9] once a day for 3 days. The mice were killed and placed in 75% ethanol for 1 min. After volatilization of the ethanol, peritoneal macrophages were recovered by peritoneal lavage.

The peritoneal cell suspension was centrifuged (1200 ***g*** for 10 min at 4 °C) and the supernatant was removed. The cells were suspended in RPMI-1640 medium (Invitrogen Corporation, Carlsbad, California, USA) supplemented with 5% fetal calf serum (Hangzhou Sijiqing Bio-engineering Material Co. Ltd, Hangzhou, China), 1% L-glutamine and antibiotics (100 kU/l penicillin and 100 mg/l streptomycin) (RPMI-FCS), and plated in 33 mm plastic dishes. The adherent cell monolayer was prepared by incubation for 3 h at 37 °C in the presence of 5% CO_2 and washed twice with RPMI-FCS. The viability of the macrophages isolated by this procedure was evaluated by staining with trypan blue (Sino-American Biotechnology Co.). Their purity was measured by staining with non-specific

esterase (Sino-American Biotechnology Co.). The concentration of macrophages was then adjusted to 1×10^9 cells/l.

CELL CULTURE

The cells were plated into 48-well cell-culture plastic plates in 500 µl medium and cultured at 37 °C in the presence of 5% CO_2. The supernatant and free cells were removed after 3 h and the cultures washed twice with RPMI-FCS, so the adherent cell monolayer in the bottom of each well consisted of highly purified macrophages. Then 500 µl of RPMI-FCS, 500 µl of attenuated *M. tuberculosis* (1×10^{12} cells/l) (Tiantan Biotechnology Co., Beijing, China) and 100 µl of APS or AS (Shanxi Jingxin Shuanghe Pharmaceutical Co. Ltd, Taiyuan, Shanxi, China) at concentrations of 0.05, 0.2, 0.6, 1.5, 4 and 8 mg/ml were added and the cultures were incubated under the same conditions for 5 h. Wells treated in the same way except for the addition of APS or AS were used as controls. Wells without macrophages were also prepared, to indicate background levels. Six samples were used for each of the experimental conditions.

MEASUREMENT OF CYTOKINE SECRETION

The cultures were centrifuged (4000 ***g*** for 10 min at 4 °C) and an ELISA (enzyme-linked immunosorbent assay; Jingmei Biotech Co. Ltd, Shenzhen, China) was performed on the supernatant to measure the levels of the cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), according to the manufacturer's instructions.

MEASUREMENT OF PHAGOCYTOSED *M. TUBERCULOSIS* BACTERIA

The culture plates were washed with RPMI-FCS three times in order to remove any free *M. tuberculosis*. Samples were extracted by strict adherence to the protocol of the real-time polymerase chain reaction (PCR) assay kit (PG Biotech Co. Ltd, Shenzhen, China) for *M. tuberculosis* DNA. First, 30 µl of extraction solution was added to the wells, and the culture plates were incubated at 37 °C for 30 min. After the cells had been completely lysed, the lysate was removed into 0.5 ml microcentrifuge tubes and placed in a dry heater at 100 °C for 10 min. The tubes were then centrifuged (13 000 ***g*** for 10 min) and 0.2 µl of the supernatant was removed and added to Lightcycler® capillaries (Roche, Basel, Switzerland) together with 17.8 µl reaction solution, 0.2 µl *Taq* enzyme and 0.03 µl uracil-*N*-glycosylase, and the capillaries were covered.

Real-time PCR was performed using the Lightcycler® system (Roche). The upstream primer was 5′-AAGGAGTTCTTCGGCACCAG-3′ and the downstream primer was 5′-GGTTTC-GATCGGGCACATCC-3′. The fluorescent probe used had a special sequence matched with *M. tuberculosis* DNA. Amplification was performed according to the manufacturer's instructions and included an initial denaturation step at 95 °C for 240 s, followed by 40 cycles at 95 °C for 15 s and 68 °C for 30 s.

All manipulations of the specimens were performed in a biological safety cabinet with unidirectional workflow. The levels of phagocytosed *M. tuberculosis* were expressed as the $\log_{10}$ of the number of copies of *M. tuberculosis* DNA detected.

STATISTICAL ANALYSIS

Results obtained from the blank wells were subtracted from the experimental data to eliminate background interference. All values were expressed as the mean ± SD. Data were analysed using SPSS® for Windows® version 10.0 (SPSS Inc., Chicago, Illinois, USA). Parameters were evaluated using one-way

analysis of variance. A *P*-value < 0.05 was considered statistically significant.

Results

The viability of the macrophages generated was 96.3 ± 2.2% and the level of purity was > 90%.

The control group values for the mean $\log_{10}$ of copies of *M. tuberculosis* DNA were 4.33 ± 0.40 and 4.58 ± 0.69 for APS and AS, respectively. Phagocytotic activity initially increased gradually with increasing concentrations of APS and AS. It was highest at a concentration of 0.6 mg/ml for both APS and AS (Fig. 1), with maximum values of 7.04 ± 0.67 and 7.40 ± 0.87 respectively ($P < 0.01$ versus control group). The increase in phagocytotic activity was greater after treatment with AS compared with APS, but this difference was not statistically significant. After peaking at 0.6 mg/ml, phagocytotic activity began to decrease with further increases in the concentration of APS and AS, falling to levels comparable with those of the control group.

The levels of IL-6, IL-1β and TNF-α were 6.28 ± 1.85, 4.68 ± 1.29 and 9.90 ± 1.24 pg/ml respectively for the APS control group, and 9.41 ± 1.63, 7.44 ± 1.75 and 8.62 ± 1.57 pg/ml respectively for the AS control group. Changes in the levels of cytokines followed a similar pattern to the changes in macrophage phagocytotic activity (Figs 2 and 3). The maximum levels of IL-6, IL-1β and TNF-α secreted by the activated macrophages were 30.7 ± 2.95, 20.5 ± 1.55 and 68.8 ± 5.73 pg/ml respectively after treatment with APS, and 46.8 ± 4.12, 29.7 ± 5.46 and 76.2 ± 6.66 pg/ml respectively after treatment with AS ($P < 0.01$ compared with controls for both experimental groups). The maximum effect on each cytokine was seen with different concentrations of APS and AS: APS produced the greatest secretion

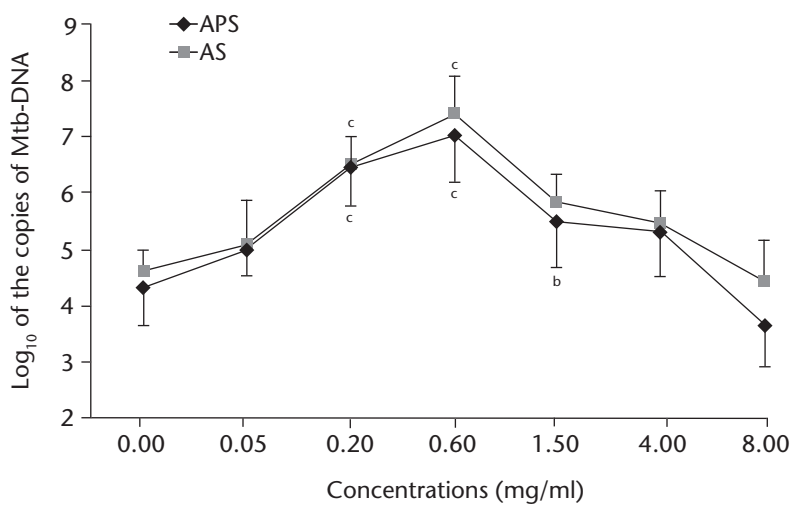

FIGURE 1: Effect of *Astragalus* polysaccharides (APS) and astragalosides (AS) on the phagocytosis of *Mycobacterium tuberculosis* by mouse macrophages ($n = 6$ samples). The levels of phagocytosed *M. tuberculosis* were expressed as the $\log_{10}$ of the number of copies of *M. tuberculosis* DNA (Mtb-DNA) detected. Data are mean ± SD. A concentration of 0 mg/ml indicates the control group. $^{b}P < 0.05$ and $^{c}P < 0.01$ versus control group

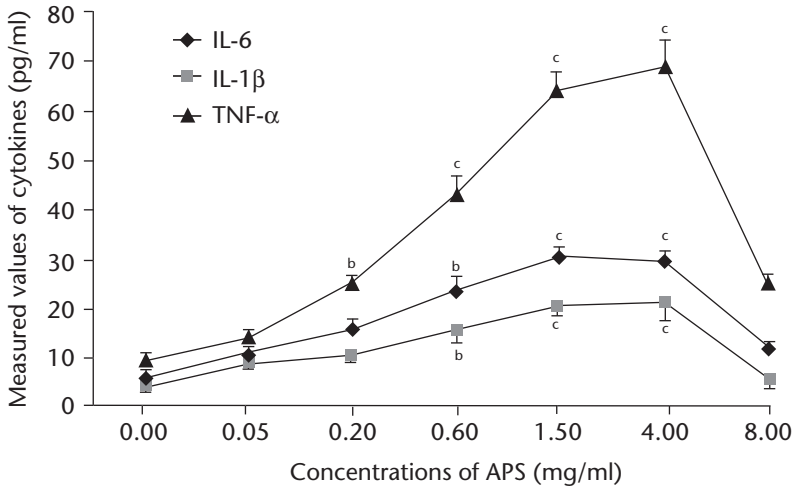

FIGURE 2: Levels of interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) secreted by activated macrophages after treatment with *Astragalus* polysaccharides (APS) (n = 6 samples). Data are mean ± SD. A concentration of 0 mg/ml indicates the control group. $^{b}P < 0.05$, $^{c}P < 0.01$ versus control group

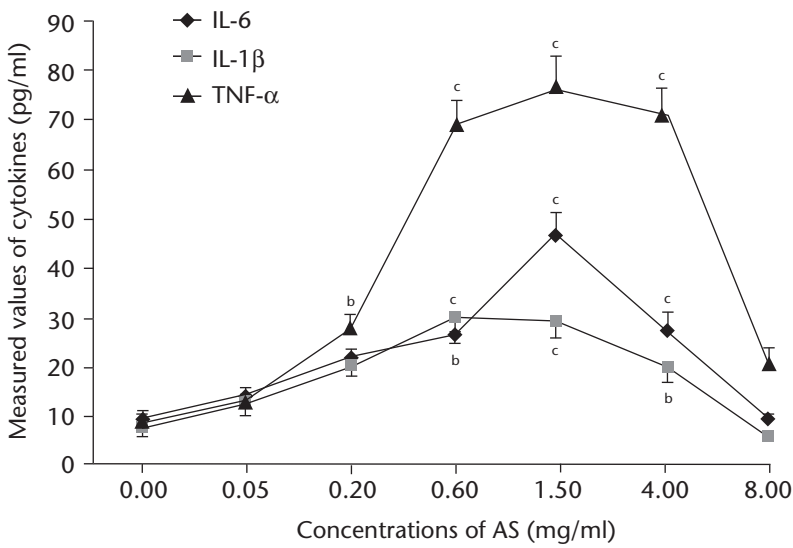

FIGURE 3: Levels of interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) secreted by activated macrophages after treatment with astragalosides (AS) (n = 6 samples). Data are mean ± SD. A concentration of 0 mg/ml indicates the control group. $^{b}P < 0.05$, $^{c}P < 0.01$ versus control group

at a concentration of 4 mg/ml for IL-1β and TNF-α (Fig. 2) and at 1.5 mg/ml for IL-6, whereas AS produced the greatest secretion at a concentration of 1.5 mg/ml for IL-6 and TNF-α and at 0.6 mg/ml for IL-1β (Fig. 3).

Discussion

Astragalus is a non-toxic natural substance that in traditional Chinese medicine is thought to 'make up energy and redress balance'.[10] Studies have confirmed that APS and AS are the two main active compounds that affect the body's immune functions; APS occur at higher concentrations and appear to have stronger immune activity than AS.[11]

The present study showed that the phagocytotic activity of macrophages on *M. tuberculosis* and the secretion of cytokines by activated macrophages were significantly increased after treatment with APS and AS. These results are consistent with published studies in which *Astragalus* was shown to boost the activity of macrophage phagocytosis on *C. albicans*[8] and carbon granules.[12] It has also been demonstrated with mouse macrophages that *Astragalus* can promote the activity of C3b and Fc receptors, which mediate the phagocytotic activity of macrophages.[13] In addition, APS-mediated macrophage activation may involve the Toll-like receptor-4 (TLR-4),[14] which plays a part in the induction of pro-inflammatory cytokine production in response to invasion by bacteria, viruses or fungi.[15]

It is not clear why higher concentrations of APS and AS suppress macrophage phagocytotic activity; the molecular basis for this double regulation requires further investigation. In the present study, APS and AS both increased the phagocytosis of *M. tuberculosis* by macrophages and induced cytokine secretion, but these changes occurred maximally at different concentrations: with increasing concentrations, macrophage phagocytotic activity was increased first and then cytokine secretion. It is well known that a very high concentration of cytokines can downregulate or even destroy cellular function. It is therefore possible that the suppression of macrophage phagocytotic activity at higher concentrations of APS and AS may be caused by the high levels of cytokines produced at these concentrations.

In summary, the present study provides evidence that APS and AS have strong promoting effects on the phagocytosis of *M. tuberculosis* by macrophages and the secretion of the cytokines IL-1β, IL-6 and TNF-α by activated macrophages. The concentration of APS or AS is a critical factor in determining the effect on macrophage function.

Acknowledgement

H-D Xu and C-G You contributed equally to this article.

Conflicts of interest

No conflicts of interest were declared in relation to this article.

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