

Phagocytic Activity of Ethyl Alcohol Fraction of Deer Antler in Murine Peritoneal Macrophage

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The mechanism of phagocytic activity of the ethyl alcohol fraction of *Cervus nippon* (CN-E) was investigated *in vivo*. The administration of CN-E (100 mg/kg, *p.o.*) enhanced lucigenin chemiluminescence and the engulfment of fluorescein-conjugated *E. coli* particles in murine peritoneal macrophages. Phagocytic activity was suppressed by the treatment of S-nitrosoglutathione (GSNO) which is an exogenous nitric oxide donor depending on the concentration of dose. CN-E suppressed the production of nitric oxide and enhanced the concentration in $[Ca^{2+}]_i$. The enhancement in $[Ca^{2+}]_i$ was diminished by the treatment of EGTA. These results indicate that CN-E enhances the phagocytic activity of murine peritoneal macrophage *via* a suppression of nitric oxide production and an increase in $[Ca^{2+}]_i$.

Key words *Cervus nippon*; phagocytic activity; macrophage; nitric oxide; $[Ca^{2+}]_i$

Deer antler is a widely prescribed and expensive substance in Chinese and Korean pharmacology. Antlers are used in the orient primarily as an aphrodisiac, and are also considered useful to treat a wide variety of medical problems.¹⁾ There have been several reports concerning the chemical structure of gangliosides in *Cervus nippon*²⁾ and the effects of deer antler, such as anti-aging action,³⁾ androgenic/gonadotrophic effects,⁴⁾ prostaglandin-like activity,⁵⁾ hematopoietic action,^{6,7)} immuno-regulative action,^{8,9)} radiation protective effect¹⁰⁾ and regulation of the level of glucose.¹¹⁾ The immunological activity of deer antler has been reported to promote cellular and humoral mediated immunity, such as enhancement of the production of antibodies¹²⁾ and suppression of hyphal growth in *Candida albicans*.¹³⁾ Although efforts have been made to confirm the immune response mechanism, it remains unclear. We investigated the phagocytic activity of the ethyl alcohol fraction of *Cervus nippon* (CN-E) in murine peritoneal macrophages.

RESULTS AND DISCUSSION

Chemiluminescence provides a simple method of assessing phagocyte function *in vitro*. The maximum peak of the chemiluminescence response of macrophages to opsonized zymosan was seen at 25—35 min. The chemiluminescence response of macrophages obtained from CN-E administered mice was enhanced, compared to control mice (Fig. 1), indicating that CN-E enhances the phagocytic activity in murine peritoneal macrophages. We observed the phagocytic activity using fluorescein-conjugated *Escherichia coli* K-12 bio-particles. Engulfment of the particles in peritoneal macrophages obtained from CN-E administered mice was enhanced (Fig. 2), and thus it is apparent that CN-E enhances the phagocytic activity in murine peritoneal macrophages. Lipopolysaccharide (LPS) and gamma-interferon (γ -IFN) treatments enhanced the production of nitric oxide in murine peritoneal macrophages, but CN-E suppressed nitric oxide production (Table 1). To determine the effect of nitric oxide, we observed the phagocytic activity as soon as S-nitrosoglu-

tathione (GSNO) which is an exogenous nitric oxide donor was added *in vitro*, and found that GSNO suppressed the phagocytic activity depending on the concentration of dose (Fig. 3). These findings suggest that CN-E regulates the phagocytic activity of murine peritoneal macrophages by reducing nitric oxide production. The administration of CN-E enhanced the resting concentration of $[Ca^{2+}]_i$ in a single macrophage (Table 2, Fig. 4). The increase of $[Ca^{2+}]_i$ in macrophages obtained from CN-E administered mice was diminished by EGTA treatment and the removal of $CaCl_2$ from the medium, but $[Ca^{2+}]_i$ in normal macrophages (control) was not affected (Fig. 5-a). The variation was very remarkable in RPMI1640 medium in the absence of FBS (Fig. 5-b). The intracellular pH was not changed in macrophages obtained from CN-E administered mice (data not shown). These findings indicate that CN-E regulates the phagocytic activity of murine peritoneal macrophages by promoting extracellular Ca^{2+} influx into the cytosol without damaging the cells.

Compounds such as luminol or lucigenin can enhance

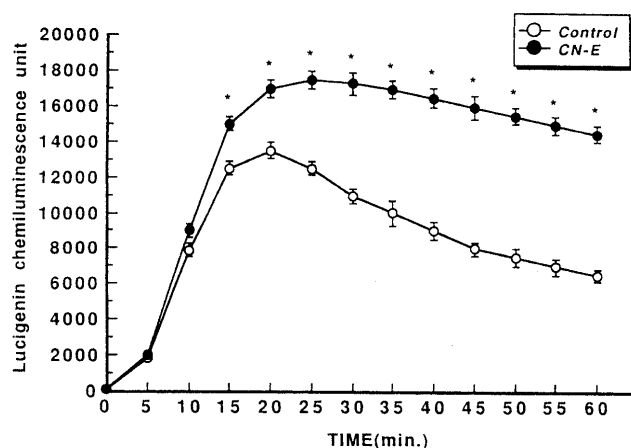


Fig. 1. Lucigenin Chemiluminescence of Peritoneal Macrophages from CN-E Administered Mice

Each point represents the mean \pm S.E. ($n=5$). *; Significantly different from control group ($p<0.001$).

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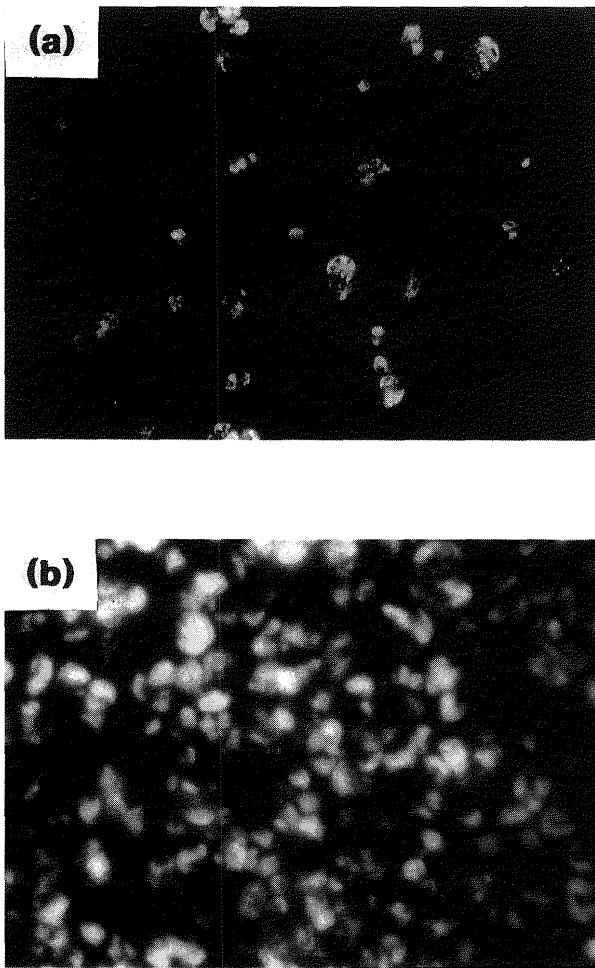


Fig. 2. Photomicrographs of Engulfment of Fluorescein Conjugated *E. coli* Particles by Peritoneal Macrophages from CN-E Administered Mice

Inverted fluoromicroscope photomicrographs (200×) showing uptake of fluorescein conjugated *E. coli* particles in control (a), and peritoneal macrophages obtained from CN-E administered mice (b).

Table 1. Nitric Oxide Production of Peritoneal Macrophages from CN-E Administered Mice

Samples	Nitric oxide (μM)	
	Without LPS and IFN- γ	With LPS and IFN- γ
Control	1.3±0.2	9.8±0.3
CN-E	0.8±0.1	1.5±0.1*

CN-E (100 mg/kg) was administered *p.o.* once a day for 7 d. Peritoneal macrophages obtained after a 2 h adherence period were cultured in RPMI1640 medium in the presence and absence of LPS and IFN- γ . Other procedures were described in detail in the Experimental. Data are presented as the mean±S.E. (n=5). *, Significantly different from control group ($p<0.001$).

chemiluminescence response. Luminol dependent chemiluminescence is based on detection of oxygen radicals produced by phagocytes during the respiratory burst following phagocytic activity of particles or by stimulation with various humoral factors.¹⁴⁾ Luminol-dependent chemiluminescence is linked to the myeloperoxidase-H₂O₂ system¹⁵⁾ whereas lucigenin-dependent chemiluminescence is independent of myeloperoxidase and is thought to reflect superoxide production by phagocytes.¹⁶⁾ We found that lucigenin-dependent chemiluminescence and engulfment of fluorescein conjugated *E. coli* particles were both enhanced in peritoneal

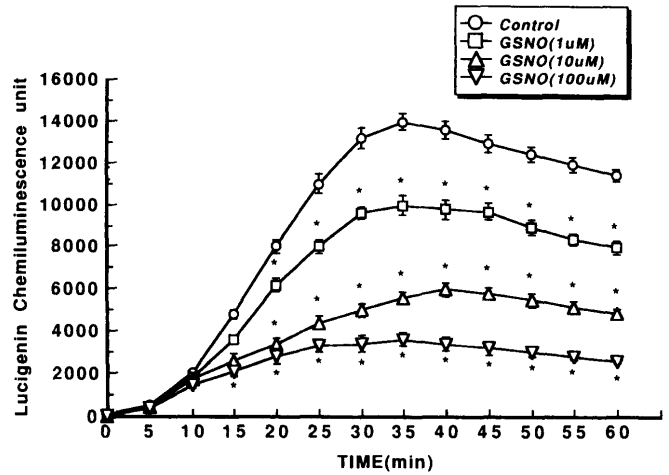


Fig. 3. Lucigenin Chemiluminescence of Peritoneal Macrophages following Treatment of S-Nitrosoglutathione *in Vitro*

Each point represents the mean±S.E. (n=5). *, Significantly different from control group ($p<0.001$).

Table 2. Concentration of [Ca²⁺]_i in Peritoneal Macrophage from CN-E Administered Mice

Samples	[Ca ²⁺] _i (nM)
Control	40.0±6.2
CN-E	164.5±8.5*

CN-E (100 mg/kg) was administered *p.o.* once a day for 7 days. [Ca²⁺]_i data were derived from cells within an intact fura-2-loaded peritoneal macrophage in CN-E administered mice. Data are presented as a mean±S.E. (n=5). Control: CN-E not administered. CN-E: CN-E administered. *, Significantly different from control group ($p<0.001$).

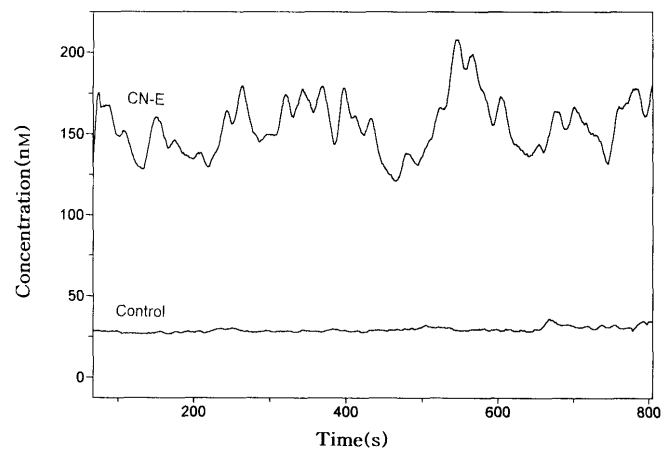


Fig. 4. The [Ca²⁺]_i Response of Peritoneal Macrophage from CN-E Administered Mice

[Ca²⁺]_i changes in cells within an intact fura-2-loaded macrophage from CN-E administered mice. CN-E (100 mg/kg) was administered *p.o.* once a day for 7 d, and then 3% thioglycollate was injected *i.p.* on the 4th day. Representative of five experiments.

macrophages obtained from CN-E administered mice. During pseudopodia formation in mammalian neutrophils and macrophages, the cytoskeleton of the phagocytosing molecule undergoes structural changes involving polymerization of F-actin from cytosolic G-actin monomers.¹⁷⁾ Nitric oxide decreases the F-actin content of human neutrophils.¹⁸⁾ Such an impairment of the cytoskeleton presumably also affects the phagocytic activity. Nitric oxide directly inhibits produc-

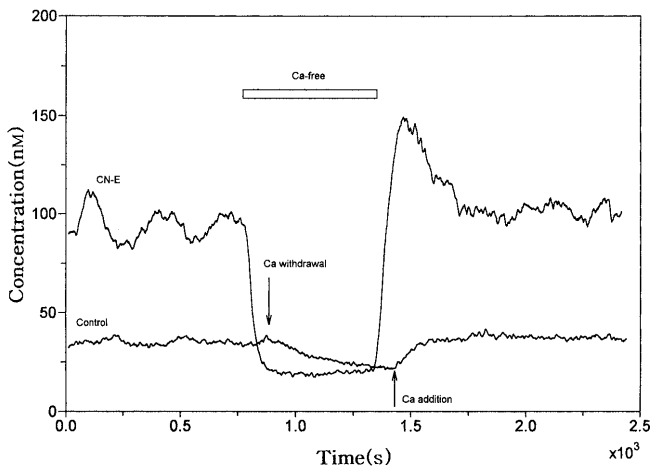


Fig. 5-a. Effect of EGTA on the $[Ca^{2+}]_i$ Response of Peritoneal Macrophage from CN-E Administered Mice

Peritoneal macrophages obtained after a 2 h adherence period were cultured in RPMI1640 medium in the presence of FBS. $[Ca^{2+}]_i$ changes in cells within an intact fura-2-loaded macrophage from CN-E administered mice. EGTA (1 mM) was added to a single cell. Representative of five experiments.

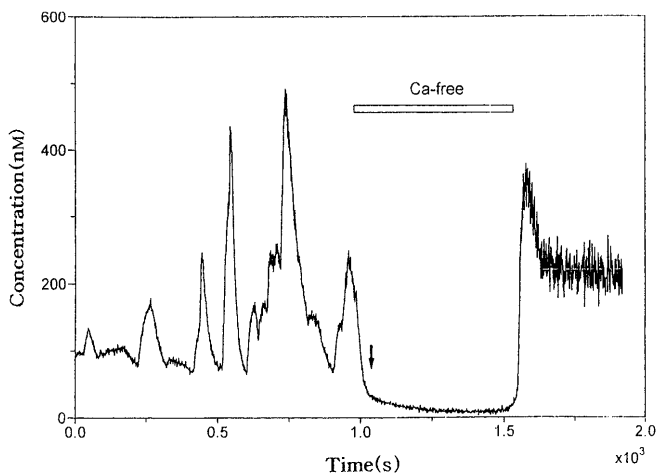


Fig. 5-b. Effect of EGTA on the $[Ca^{2+}]_i$ Response of Peritoneal Macrophage from CN-E Administered Mice

Peritoneal macrophages obtained after a 2 h adherence period were cultured in RPMI1640 medium in the absence of FBS. $[Ca^{2+}]_i$ changes in cells within an intact fura-2-loaded macrophage (without FBS) from CN-E administered mice. EGTA (1 mM) was added to a single cell. Representative of five experiments.

tion of oxygen metabolites in the phagosome and the NADPH-oxidase.¹⁹⁾ It also has a negative effect on pseudopodia formation and phagocytic activity of murine macrophages.²⁰⁾ Recently, it has been reported that deer antler extracts stimulated proliferation of hematopoietic stem cells,⁷⁾ and that nitric oxide suppressed human hematopoiesis.²¹⁾ Also, we found that exogenous nitric oxide suppressed the phagocytic activity. These results suggest that nitric oxide regulates the phagocytic activity. CN-E suppressed the production of nitric oxide and enhanced the phagocytic activity. Our results indicate that the reduction of nitric oxide is one possible factor contributing to enhancement of phagocytic activity. The activation mechanism of superoxide release has been reported to be mediated by the movement of Ca^{2+} into the cytosol.^{22–24)} We found that CN-E enhanced the concentration of $[Ca^{2+}]_i$ via an influx of extracellular Ca^{2+} into the cytosol. These findings indicate that activation of phagocytic activity in macrophages is caused by promo-

tion of superoxide release via an increase in $[Ca^{2+}]_i$. Further study in our laboratory will include investigation of the main component of CN-E which activates the phagocytic activity in murine peritoneal macrophage.

MATERIALS AND METHODS

CN-E Extraction We bought *Cervus nippon* which is produced in Heilongjiang, People's Republic of China. Slices of *Cervus nippon* (1.75 kg) were extracted two times with hexane (12 l) for 2 h at 80 °C and filtered. Chloroform (12 l) was added, and the residue was heated at 70 °C for 2 h, extracted two times and separated. The residue was then extracted three times with 70% ethyl alcohol (12 l) at a cold temperature for 2–3 d. The 70% ethyl alcohol fraction (CN-E) was concentrated in a rotary evaporator and the yield of CN-E was 91.0 g.²⁵⁾

Chemicals and Animals Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), lipopolysaccharide (026:B6), γ -interferon (Hu γ -IFN), zymosan, GSNO and lucigenin were all purchased from Sigma (St. Louis, U.S.A.). RPMI1640 media, fetal bovine sera (FBS) and thioglycollate were purchased from Gibco/BRL (Grand Island, U.S.A.). Fluorescent (FITC)-conjugated *E. coli* K-12 bio-particles, ethylene glycol bis (2-aminoethyl-ether)-tetraacetic acid (EGTA) and Fura-2/AM were purchased from Molecular Probes (Eugene, OR). The animals used were male BALB/c mice weighing about 18–20 g and obtained from Daehan Animal Center at Eumsung, Korea. The animals were maintained in a constant temperature environment with a 12 h day/night cycle before use, and were fed lab Chow (Cheil Jedang Co.) and tap water *ad lib*. CN-E (100 mg/kg) was administered orally once a day for 7 d.

Separation of Peritoneal Macrophages CN-E (100 mg/kg) was administered orally once a day for 7 d, and 3% thioglycollate was injected intraperitoneally on the 4th day. Macrophages were obtained from the peritoneal cavity of the mice, and the peritoneal macrophages obtained after a 2 h adherence period in RPMI1640 medium were scraped. Adherent cells were judged to be >95% macrophages by Wright and nonspecific esterase staining.²⁶⁾

Assessment of Phagocytic Activity Previous assays of phagocyte chemiluminescence have required large numbers of cells and have not been able to follow responses from a large number of samples in a single experiment. Recently, sensitive luminometers which use a 96 well microplate format have become available.²⁷⁾ We examined the application of this equipment to the measurement of phagocyte chemiluminescence using lucigenin to enhance the response for estimation of opsonic activity. The basis of the technique is the detection of oxygen radicals produced by phagocytes during the respiratory burst following phagocytic activity of particles or by stimulation with various humoral factors.¹⁴⁾ The obtained macrophages were washed twice in DPBS-A solution before being resuspended in DME medium without phenol red. The cells were diluted to 1×10^6 cells/ml. S-nitrosoglutathione was added to the cells at concentrations of 1, 10 and 100 μ M *in vitro*. Zymosan was prepared by the method of Lachmann and Hobert²⁸⁾ and diluted to 1×10^7 particles/ml in DPBS-A solution before use. Zymosan and serum were pre-

incubated at 37 °C for 30 min to opsonize the zymosan. The cells were pre-incubated with fresh lucigenin at 37 °C for 15 min in a white multi-well plate (Costar), then the opsonized zymosan was added to individual wells. The chemiluminescence (CL) assays were performed using a luminometer (Berthold 96LP) at 5 min intervals for 60 min.²⁹⁾

Another method we used for phagocytic activity was developed using fluorescein conjugated *E. coli* K12 particles.³⁰⁾ The particles were suspended in HBSS and stored at -20 °C in the dark. The suspension was then thawed and briefly sonicated just before use. Trypan blue was dissolved in citrate buffer, pH 4.4, at a concentration of 250 µg/ml.³¹⁾ The incubation was performed in a humidified 37 °C incubator with 5% CO₂. The obtained macrophages were harvested and resuspended in RPMI1640 supplemented with 10% FBS. The cell concentration was adjusted to 5 × 10⁵ cells/ml and 100 µl of cell suspension was then pipetted into each well. Cells were incubated for 1 h, then the culture medium was aspirated. The *E. coli* suspension, pre-warmed to room temperature, was then briefly sonicated to disperse any aggregates and 25 µl was added to each well. The plate was covered and incubated for 1 h, and then the buffer in the plate was removed by aspiration. Extracellular fluorescence was quenched by adding 100 µl of trypan blue (250 µg/ml, pH 4.4). After 1 min, the dye was removed, and the morphology of macrophages was observed with an inverted fluoromicroscope.

Measurement of Nitrite Concentration The macrophage monolayers prepared in 24 well tissue culture clusters (1 × 10⁶ cells/ml) were incubated for 24 h in RPMI1640 medium in the presence and absence of LPS (1 µg/ml) and γ-IFN (10 units/ml). The concentration of NO₂⁻ in culture supernatants was measured using Griess reagent.³²⁾

Measurement of [Ca²⁺]_i in Single Cells The obtained macrophages were cultured with 2 µM fura-2/AM in HEPES buffered solution for 30 min at room temperature under 100% O₂. They were washed twice and resuspended in a HCO₃⁻-buffered solution containing (in mM): NaCl 110, KCl 4.5, NaH₂PO₄ 1.0, MgSO₄ 1.0, CaCl₂ 1.5, HEPES-Na 5, HEPES free acid 5, D-glucose 10 (equilibrated with 95% O₂, 5% CO₂ to give a pH of 7.4). To make a Ca²⁺ free solution, CaCl₂ was removed and 1 mM EGTA was added. The cells were allowed to attach to a coverslip that formed the base of a cell chamber mounted on the stage of an inverted microscope. [Ca²⁺]_i was measured by spectrofluorometry (Photon Technology International, Brunswick, NJ, U.S.A.) with excitation at 340 nm and 380 nm and emission measured at 510 nm. The values of [Ca²⁺]_i were calculated from the ratio of fluorescence intensities (F₃₄₀/F₃₈₀) according to Grinkiewicz *et al.*³³⁾

Data Analysis Data were shown as the mean ± S.E. obtained from 5 experiments. Evaluation was done using Student's *t*-test.

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