

Phagocytosis of Alveolar Macrophages after Conagenin Injection to Rats

MASAFUMI HAMADA^{*,a}, SHIGERU YAMAMOTO^a, SATORU MORIGUCHI^b and YASUO KISHINO^c

^a Department of Nutrition, School of Medicine, The University of Tokushima,
Tokushima 770-8503, Japan

^b Department of Nutrition, Faculty of Human Life Science, Yamaguchi Prefectural University,
Yamaguchi 753-8502, Japan

^c School of Home Economics, Tokushima Bunri University,
Tokushima 770-8514, Japan

(Received for publication October 25, 2000)

Phagocytic functions of rat alveolar macrophages (AM) following intraperitoneal injection of conagenin (CNG) and of AM sub-populations fractionated by Percoll discontinuous gradient centrifugation were investigated. Phagocytosis of opsonized-sheep red blood cells (SRBC) following *in vitro* incubation with CNG showed a significant increase in a higher density of AM (fraction IV). In addition, phagocytosis was also increased in lower density ones (fractions I and II) by macrophage-activating factor (MAF) co-cultivation. CNG-injected rats for 5 consecutive days showed a dose-dependent increase in phagocytosis of AM compared to the control rats. Although the distribution of AM sub-population in rats injected CNG was not significantly different compared to the control rats, phagocytosis was significantly increased in AM of a lower density fraction (fraction II). These results suggest that CNG directly increases phagocytosis of AM in a higher density fraction, and indirectly enhances phagocytosis in AM of a lower density fraction *via* increasing MAF-like material production.

Conagenin (CNG; C₁₀H₁₉NO₆, M.W. 249.16), a low molecular weight nitrogen containing chemical, is produced by *Streptomyces roseosporus* which enhances immune functions such as lymphocyte proliferation and cytokine secretion *in vivo* and *in vitro*¹⁾. In tumor bearing mice, CNG administration inhibited the tumor growth, and NK activity maintained at normal level²⁾. In addition, CNG prevented the myelosuppression induced by antitumor agents such as 5-fluorouracil, mitomycin C and cyclophosphamide³⁾. Other immunoenhancers, MDP⁴⁾ and forphenicicol⁵⁾, enhanced macrophage functions such as phagocytosis or cytotoxicity *in vivo* and *in vitro*, and forphenicicol increased the production of superoxide anion and changes the activities of some enzymes in macrophages. In *in vitro* experiment, we have previously reported that phagocytic function of alveolar macrophages (AM) was significantly enhanced by CNG incubation⁶⁾. In this study, we have further investigated the effect of CNG on phagocytic function of AM in rats in *in vivo* experiment.

Materials and Methods

Animals

Specific pathogen-free, inbred F344 male rats, weighing 180 to 200 g, were obtained from Japan SLC (Shizuoka, Japan). They were maintained under specific pathogen-free conditions at 21±2 °C and 55±5% humidity. They were fed a Purina chow diet and water *ad libitum* during the experiment.

CNG and Treatment

CNG, gifted from KANEKA Co, Ltd (Osaka, Japan), was dissolved in sterilized saline or RPMI-1640 culture medium. The solution was then filtered through a Millipore membrane (0.22 μm; Millipore Corp., Bedford, MA). CNG solution (1.0 to 10.0 mg/kg body weight) or saline were administered by i.p. injection for 5 consecutive days.

Cell Preparation

Rats were anesthetized with sodium pentobarbital and

* Corresponding: hamada@nutr.med.tokushima-u.ac.jp

exanguinated by cutting off the arteries of both kidneys on the day after the final injection. AM were obtained from bronchoalveolar lavage. Briefly, the trachea was cannulated and the lungs were washed with physiological saline at 37°C. This procedure was repeated to obtain a total of 50 ml of lavage fluid. Then the AM were collected by centrifugation. Collected AM were resuspended in culture medium including 10% fetal bovine serum (FBS) and antibiotics such as penicillin (100 units/ml) and streptomycin (100 µg/ml).

Fraction of AM

Separation of AM sub-population was accomplished by using Percoll solution (Pharmacia, Uppsala, Sweden)⁷⁾. Briefly, a discontinuous Percoll solution was prepared by a stock of Percoll (9 parts of Percoll, 1 part of 1.5 M NaCl) with sterile saline to get specific gravity of 1.030, 1.050, 1.060, 1.070 and 1.080, and subsequently layered into centrifuge tube. The AM suspension was layered on the top of layer, and then centrifuged at 400 g for 30 minutes. After centrifugation, the separated cells were carefully harvested and washed 3 times with medium. The fractions were designated as I, II, III and IV in order of increasing density. The viability of these cells in each fraction were more than 95% by tripan blue dye exclusion.

Macrophage-activating Factor (MAF)

Spleens from rats were aseptically removed and minced. Then, splenocytes were passed through a stainless steel mesh in medium. They were adjusted at 1×10^7 /ml and then incubated with 5 µg/ml Con A-sepharose for 48 hours at 37°C. After incubation, the supernatants were filtered and stored at -80°C until the experiment. This supernatant was diluted with culture medium (1/100) and used as MAF.

Phagocytosis of AM

Phagocytosis was determined by the modified method of the MORIGUCHI *et al.*⁸⁾. Sheep red blood cells (SRBC, Nihon Biotest, Tokyo) were washed 3 times with medium. Opsonization was accomplished by incubating 10 ml of washed SRBC with 0.2 ml of rat anti-SRBC antiserum (heat-inactivated) for 1 hour at 37°C, and then they were washed 3 times. The final volume was adjusted to 0.6% suspension of SRBC. AM were plated (5×10^4 /well) in 48 well flat-bottom microplate. After 1 hour, the plate was washed to remove nonadherent cells and further incubated with opsonized-SRBC for 90 minutes at 37°C. After incubation, the cultures were rinsed once with distilled water to lyse non-phagocytosed SRBC, and then washed twice with physiological saline. All remaining adherent

cells were lysed by 0.1 N NaOH. The lysate including phagocytosed-SRBC in AM which represents red color was determined by a microplate reader at 415 nm. Data were expressed as the phagocytic index, which was calculated by assigning a value of 1 to the absorbance of AM cultured with medium and by comparing this to the absorbance of AM from CNG treated groups.

In Vitro Effect of CNG or MAF on Phagocytosis of Fractionated AM

Fractionated-AM were treated and plated as described above. CNG (0.1 µg/ml) or MAF (1/100 dilution) was added to each well and incubated for 12 hours at 37°C, which concentrations and incubation time were the most effective condition against AM phagocytosis as reported previously⁶⁾. After incubation, AM were further incubated with opsonized-SRBC for 1 hour at 37°C, and then their phagocytosis was measured.

Statistical Analysis

Data are means ± SD. Statistical significance was analyzed by Duncan's multiple range test. Differences were considered as significant at $p < 0.05$.

Results

Body Weight and Number of AM

There was no significant difference in body weight between control and CNG-injected rats. The total number of AM was also not significantly different between both groups (data not shown).

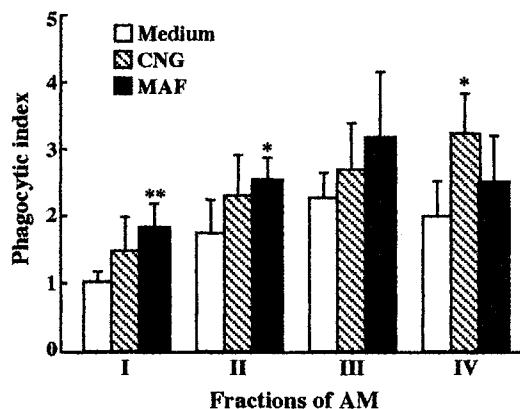
In Vitro Effect of CNG or MAF on Phagocytosis of Fractionated-AM against Opsonized-SRBC

Fractionated-AM were incubated with CNG or MAF, and then their phagocytosis was measured. Phagocytosis of AM cultured with CNG was higher in all fractions of AM compared to the culture with medium alone (Fig. 1). Especially, it was significantly increased in AM of a higher density fraction (fraction IV). Phagocytosis was also significantly increased in the lower density (fractions I and II) by MAF co-cultivation.

Phagocytosis of AM

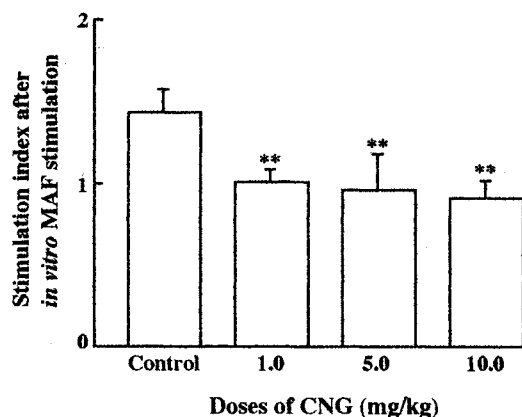
AM from CNG-injected rats showed a significant increase in phagocytosis dose-dependently (Fig. 2). In the highest concentration of CNG (10.0 mg/kg), phagocytic

Fig. 1. *In vitro* effect of CNG or MAF on phagocytosis of fractionated-AM against opsonized-SRBC.



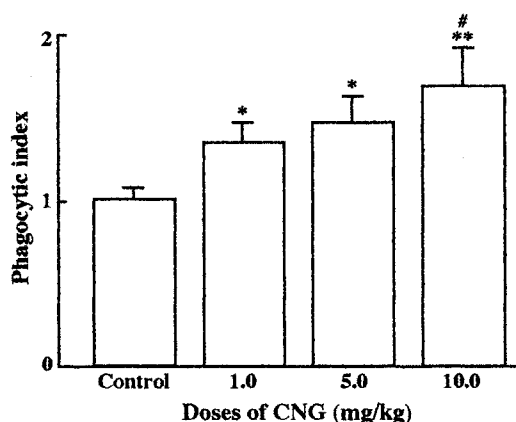
* $p < 0.05$, ** $p < 0.01$ (vs. Medium).

Fig. 3. Changes of phagocytosis of AM following *in vitro* incubation with MAF.



** $p < 0.01$ (vs. Control).

Fig. 2. Changes of phagocytosis of AM in rats following i.p. injection of CNG.



* $p < 0.05$, ** $p < 0.01$ (vs. Control); # $p < 0.05$ (vs. CNG 1.0 mg/kg).

activity of AM showed a 1.5-fold higher than that of the control rats. In addition, CNG (10.0 mg/kg) induced a significant increase in phagocytosis compared to the lowest level of CNG (1.0 mg/kg).

Effect of MAF Treatment on Phagocytosis of AM of Rats Injected with CNG

Phagocytosis of AM from control rats were largely enhanced following *in vitro* treatment with MAF, which was 1.5-times higher compared to the medium alone (Fig. 3). In contrast, phagocytosis of AM from CNG-treated rats did not show any increase to MAF treatment.

Distribution of Fractionated AM from Rats Injected CNG

There was no significant difference in the distribution of AM sub-populations between control and CNG-injected rats (Fig. 4).

Phagocytosis of Fractionated AM of Rats Injected CNG

In CNG-injected rats, phagocytosis of AM was higher in all fractions compared to control rats (Fig. 5). Phagocytosis was significantly higher in lower density of AM (fraction II), which was almost 2-times higher than that of control rats.

Discussion

Macrophages were divided into four sub-populations, which were different morphologically and functionally^{7,9,10}. In our previous study, it is reported that phagocytosis of

Fig. 4. Distribution of AM fractionated from rats i.p. injected CNG (10.0 mg/kg) for 5 consecutive days.

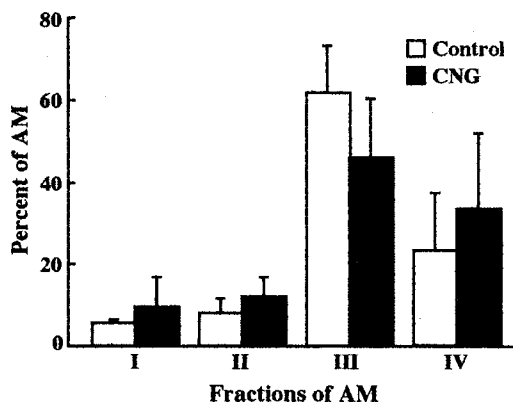
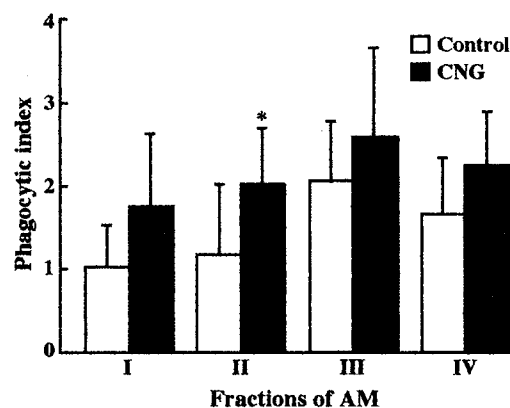


Fig. 5. Phagocytosis of AM fractionated from rats i.p. injected CNG (10.0 mg/kg) for 5 consecutive days.



* $p < 0.05$ (vs. Control).

AM was increased following *in vitro* incubation with CNG or MAF. However, it is unclear whether they act *via* independent mechanism on the same AM or the same mechanism on different sub-populations of AM. In the present study, we have further investigated the activating mechanism of CNG on phagocytic function in each sub-population of AM. Phagocytosis of AM following *in vitro* incubation with CNG induced a significant increase in higher density AM (fraction IV) (Fig. 1). In contrast to this result, phagocytosis of AM cultured with MAF induced in lower density ones (fractions I and II). These results indicate that CNG and MAF directly enhance phagocytic activity on different sub-population of AM, respectively. ZWILLING *et al.* have reported that AM from higher density (fractions III and IV) have higher phagocytic activity than lower density ones (fractions I and II)¹¹. High density AM are rich in microvilli on their membrane compared to lower density ones⁷. In contrast, lower density AM are considered as mature cells having more lysosomes and lysosomal enzymes such as cathepsin B, L, and acid phosphatase than higher density ones. In this study, acid phosphatase activity of fractionated AM was not affected by CNG (data not shown). It is known that phagocytic process of macrophage was largely divided into four stages, *i.e.*; migration, attachment, ingestion and digestion. The antioxidants such as vitamin C, E and *N*-acetyl cysteine enhance these phagocytic process of murine peritoneal macrophages^{12,13}. The above result may indicate that CNG enhances phagocytosis in ingestion stage but not in digestion stage.

It is known that interferon (IFN) influences the functions of M ϕ and acts as MAF¹⁵. The MAF effect of IFN results in clear enhancement of both differentiation markers such as MHC class II antigens and Fc-receptor expression, and functions typically associated with M ϕ activation such as phagocytosis, intracellular killing, non specific lysis of tumor cells and secretion of monokines. As shown in Fig. 3, although phagocytosis of AM after *in vitro* incubation with MAF showed a further enhancement in control rats, CNG-injected rats did not have any response. Similar results have been reported in vitamin E-supplemented rats¹⁴. Phagocytic response to MAF gradually decreased in accordance with increasing amount of vitamin E in the diet. In contrast to this results, AM from CNG-injected rats did not show any response to MAF regardless of concentrations of CNG. This suggests that AM from CNG-injected rats have already been activated state by a MAF-like materials secreted from activated T-lymphocytes. This possibility is supported by the reports that cytokine secretion from splenic lymphocytes is increased following administration of CNG¹. However, since phagocytosis of AM from rats injected CNG was increasing dose-dependently, CNG may have other mechanism to enhance phagocytic activity of AM.

In this study, the total number of AM was not significantly different between control and CNG-injected groups (data not shown). The distribution of AM sub-populations (fractions I to IV) was also not significant (Fig. 4). However, phagocytosis of AM from CNG-injected rats showed a significant enhance in lower density of AM

(fraction II) (Fig. 5). These results suggest that enhanced phagocytosis of AM by CNG administration was not due to change in sub-population of AM, but increase in phagocytic ability in AM of lower density fraction. Recently, it is especially noteworthy that lung cancer and infectious diseases such as pneumonia or tuberculosis are increasing in elderly people with the reduced immunity. AM are the important immune cells to protect these respiratory disorders in the lungs. Thus, CNG having the ability to enhance AM function may be an effective agent for these treatment.

References

- 1) KAWATSU, M.; T. YAMASHITA, M. OSONO, M. ISHIZUKA & T. TAKEUCHI: T cell activation by conagenin in mice. *J. Antibiotics* 46: 1687~1691, 1993
- 2) KAWATSU, M.; T. YAMASHITA, M. OSONO, T. MASUDA, M. ISHIZUKA & T. TAKEUCHI: Effect of conagenin in tumor bearing mice. Antitumor activity, generation of effector cells and cytokine production. *J. Antibiotics* 46: 1692~1698, 1993
- 3) KAWATSU, M. & T. YAMASHITA: Effect of conagenin on thrombocytopenia induced by antitumor agents in mice. *J. Antibiotics* 47: 1123~1129, 1994
- 4) MATTER, A.: The effect of muramyl dipeptide (MDP) in cell-mediated immunity. *Cancer Immunol. Immunother.* 6: 201~210, 1979
- 5) ISHIZUKA, M.; S. ISHIZEKI, T. MATSUDA, A. MOMOSE, T. AOYAGI, T. TAKEUCHI & H. UMEZAWA: Studies on effects of forphenicol on immune responses. *J. Antibiotics* 35: 1042~1048, 1982
- 6) HAMADA, M.; E. SONOTAKE, S. YAMAMOTO & S. MORIGUCHI: Conagenin derived from *Streptomyces roseosporus* enhances macrophage functions. *J. Antibiotics* 52: 548~551, 1999
- 7) SAKAI, K.; H. MORIYA, A. UHEYAMA & Y. KASHINO: Heterogeneity of fractionated alveolar macrophages in starved rats. *Nutr. Res.* 10: 1141~1149, 1990
- 8) MORIGUCHI, S.; N. KOBAYASHI & Y. KISHINO: Effects of vitamin E deficiency on the functions of splenic lymphocytes and alveolar macrophages. *J. Nutr. Sci. Vitaminol.* 35: 419~430, 1989
- 9) NII, Y.; S. MORIGUCHI & Y. KISHINO: Enhanced phagocytosis of rat alveolar macrophages by intravenous infusion of an arginine-enriched solution. *J. Nutr. Sci. Vitaminol.* 38: 565~578, 1992
- 10) SAKAI, K.; Y. NII, A. UHEYAMA & Y. KISHINO: Fluorescence demonstration of cathepsin B activity in fractionated alveolar macrophages. *Cell. Mol. Biol.* 37: 353~358, 1991
- 11) XWILLING, B. S.; L. B. CAMPOLITO & N. A. REICHES: Alveolar macrophage subpopulations identified by differential centrifugation on a discontinuous albumin density gradient. *Am. Rev. Respir. Dis.* 125: 448~452, 1982
- 12) DEL RIO, M.; G. RUEDAS, S. MEDINA, V. M. VICTOR & M. DE LA FUENTE: Improvement by several antioxidants of macrophage function *in vitro*. *Life Sci.* 63: 871~881, 1998
- 13) MULERO, V.; M. A. ESTEBAN & J. MESAGUER: Effects of *in vitro* addition of exogenous vitamins C and E on gilthead seabream (*Sparus aurata* L.) phagocytes. *Vet. Immunol. Immunopathol.* 66: 185~199, 1998
- 14) MORIGUCHI, S.; N. KOBAYASHI & Y. KISHINO: High dietary intakes of vitamin E and cellular immune functions in rats. *J. Nutr.* 120: 1096~1102, 1990
- 15) VIRELIZIER, J. L. & F. ARENZANA-SEISDEDOS: Immunological functions of macrophages and their regulation by interferons. *Med. Biol.* 63: 149~159, 1985