VOL. 52, NO. 6, JUNE 1999 THE JOURNAL OF ANTIBIOTICS pp. 548 — 551

Conagenin Derived from *Streptomyces roseosporus* Enhances Macrophage Functions

MASAFUMI HAMADA*,[a], EIKO SONOTAKE[a],
SHIGERU YAMAMOTO[a] and SATORU MORIGUCHI[b]

[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 Preferectural University, Yamaguchi 753-8502, Japan

(Received for publication March 18, 1999)

In contrast to the studies that describes the effects of conagenin (CNG) on the cellular immunity of lymphocytes (references), we investigated the *in vitro* effect of CNG on macrophage function. Phagocytosis of alveolar macrophages (AM) against opsonized-sheep red blood cells (SRBC) was significantly enhanced following *in vitro* incubation with CNG for 12 hours at 37°C, which was closely associated with increased expression of Fc-receptor in AM membranes. Macrophage-activating factor (MAF), prepared from splenic lymphocytes *in vitro* stimulated with concanavarin A (Con A) for 48 hours at 37°C, had also the enhancing effect on phagocytosis of AM against opsonized-SRBC. Preincubation with CNG (0.1 μg/ml) and MAF (1/100 dilution) induced the additional effect on phagocytosis of AM, which was associated with the increased expression of Fc-receptor in AM membranes. These results suggest that CNG enhances AM phagocytosis by increasing the expression of Fc-receptor on their membranes *via* either effecting different sub-populations of AM cells or by activating independent mechanism on the same AM cell population.

Conagenin (CNG; $C_{10}H_{19}NO_6$, MW 249.16) extracted from *Streptomyces roseosporus* is known to be immunoenhancer. CNG enhances the incorporation of [^{3}H]-thymidine by splenic lymphocytes following *in vitro* stimulation with Con A[1]. In addition, CNG enhances production of cytokines such as interleukin-2, 3 and 6[2,3]. Although natural killer cell (NK) activity is known to decrease in tumor-bearing mice, the administration of CNG did not induce the decrease of NK activity and maintained NK activity at the normal level[3]. Until now, most of studies on the effect of CNG on cellular immunity has focused on lymphocyte functions. In this study the *in vitro* effect of CNG on macrophage function is discussed.

Materials and Methods

Animals

Male F344 rats, 8 weeks old, were obtained from Japan SLC (Shizuoka, Japan) and maintained under specific pathogen-free conditions at 21±2°C and 55±5% humidity.

CNG

CNG was gifted from KANEKA Co., Ltd. (Osaka, Japan), and dissolved in culture medium and filtered through a 0.22 μm Millipore membrane (Millipore Corp, Bedford, MA).

Cell Preparation

Rats were anesthetized with pentobarbital sodium (0.1 ml/100 g BW) and exanguinated by cutting off the arteries of both kidneys. Then, AM were collected by

bronchoalveolar lavage with saline. AM were collected by centrifugation. Collected AM were resuspended with RPMI 1640 medium including 10% fetal bovine serum (FBS) and antibiotics such as penicillin (100 units/ml) and streptomycin (100 μg/ml).

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 through a 0.22 μm Millipore membrane and stored at −80°C until the experiment. This supernatant was diluted with RPMI 1640 culture medium and used as MAF.

Phagocytosis of AM

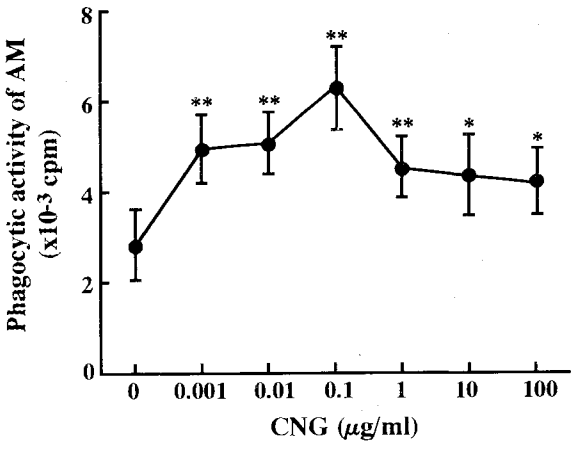
Sheep red blood cells (SRBC) (Nihon Biotest, Tokyo) were washed 3 times with RPMI-1640. Opsonization was accomplished by incubating 10 ml of washed SRBC with 0.2 ml of rat anti-SRBC antiserum (heat-inactivated) for 1 hour at 37°C, and then they were washed 3 times with medium. Radioactive labeling of opsonized-SRBC was accomplished by incubating with 0.2 ml of $Na_2{}^{51}CrO_4$ (Amersham) for 1 hour at 37°C. Labeled opsonized-SRBC were washed 3 times to remove excess ^{51}Cr, and the final volume was adjusted to 0.6% suspension of SRBC. AM were adjusted (6×10^5/ml) and plated (3×10^5/well) in 24 well flat-bottom microplate. After 1 hour, the plate was washed with medium to remove nonadherent cells, and then various concentrations of CNG (0 ~ 100 μg/ml) and/or diluted-MAF were added to each well and incubated for 12 hours at 37°C. After incubation, plates were washed with medium, added ^{51}Cr-labeled opsonized-SRBC, and incubated for 1 hour at 37°C. The cultures were rinsed once with distilled water to lyse nonphagocytosed SRBC, and then washed twice with physiological saline. All remaining adherent cells were lysed by 0.1 N NaOH, and the radioactivity of lysate was measured by gamma counter.

Fc-receptor Expression in AM Membrane

AM were incubated with ^{51}Cr-labeled opsonized-SRBC for 1 hour at 4°C. Then, the culture plate was washed 4 times with physiological saline at 4°C. All remaining cells were lysed by 0.1 N NaOH, and the radioactivity of lysate was measured by gamma counter.

Fig. 1. Phagocytosis of ^{51}Cr-opsonized SRBC by AM following *in vitro* incubation with various concentrations of CNG (0 ~ 100 μg/ml).

$^*p<0.05$, $^{**}p<0.01$ (*vs.* without CNG).



Statistical Analysis

Statistical significance was analyzed by Student's *t*-test.

Results

Phagocytosis of Opsonized-SRBC by AM

AM were preincubated with various concentrations of CNG (0 ~ 100 μg/ml) for 12 hours at 37°C, and then phagocytic activity of AM were determined by using ^{51}Cr-labeled opsonized SRBC. Phagocytic activity of AM was significantly increased by *in vitro* incubation with all concentrations of CNG (Fig. 1). The most effective concentration of CNG to enhance AM phagocytosis was 0.1 μg/ml, which showed 2 times higher phagocytosis compared to AM cultured with medium alone.

Fc-receptor Expression in AM Membrane

AM were preincubated with CNG for 12 hours at 37°C, and then Fc-receptor expression in AM was examined after incubation with opsonized-SRBC for 1 hour at 4°C. Fc-receptor expression in AM was significantly increased by *in vitro* incubation with CNG. Both 0.01 and 0.1 μg/ml of CNG induced 2.5-fold increase compared to that incubated with medium alone (Fig. 2). However, the concentration of CNG did not induce a further increase of Fc-receptor expression of AM.

Fig. 2. Fc-receptor expression in AM membrane following *in vitro* incubation with various concentrations of CNG (0～100 μg/ml).

* $p<0.05$ (*vs.* without CNG).

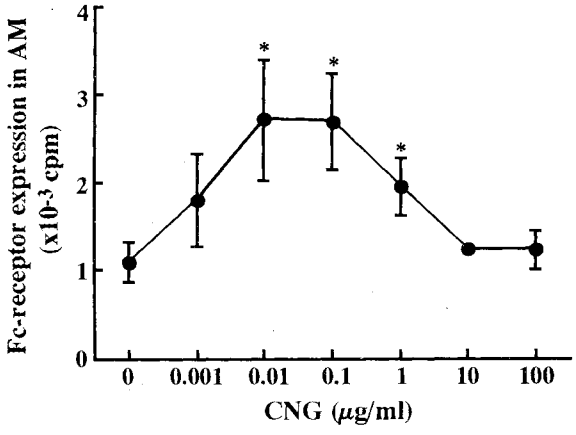


Fig. 3. *In vitro* effect of MAF on phagocytic activity of AM.

* $p<0.05$, ** $p<0.01$ (*vs.* medium alone).

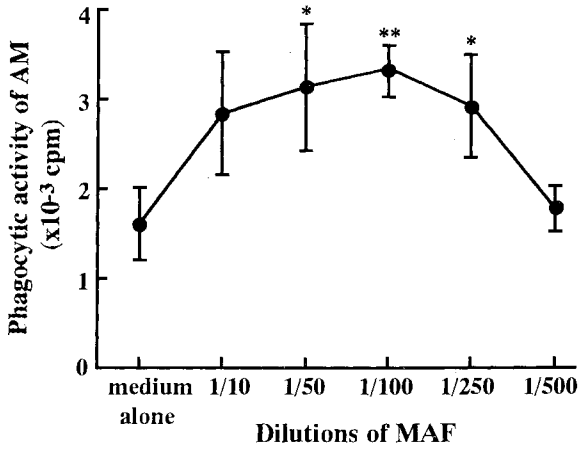


Fig. 4. Phagocytosis of ^{51}Cr-opsonized SRBC by AM following *in vitro* incubation with CNG (0.1 μg/ml) and/or MAF (1/100 dilution).

* $p<0.05$ (*vs.* medium alone); # $p<0.05$ (*vs.* MAF).

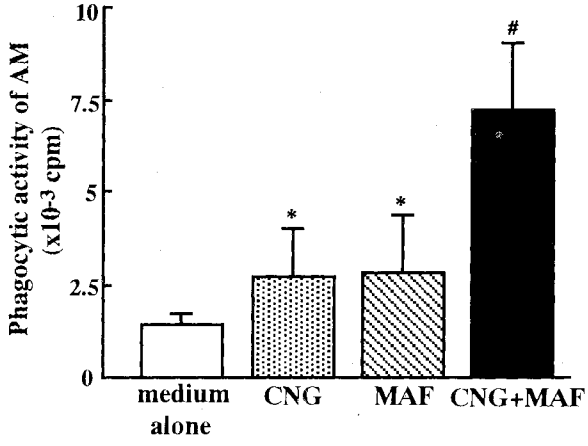
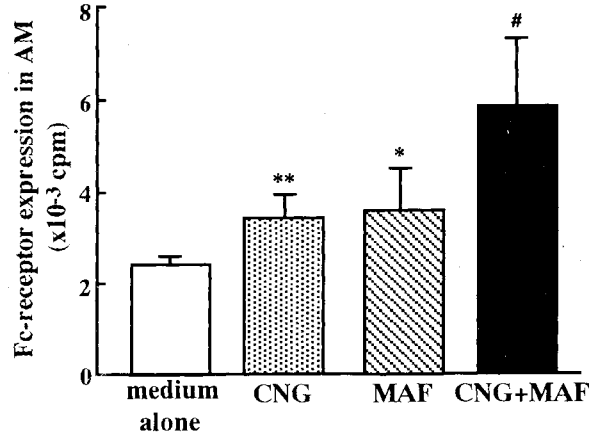


Fig. 5. Fc-receptor expression in AM following *in vitro* incubation with CNG and/or MAF.

* $p<0.05$, ** $p<0.01$ (*vs.* medium alone); # $p<0.05$ (*vs.* MAF).



Phagocytic Activity of AM after MAF Treatment

Phagocytic activity of AM against opsonized-SRBC was also significantly enhanced by *in vitro* incubation with MAF (Fig. 3). The highest enhancement of AM phagocytosis was shown in the dilution of MAF at the ratio of 1:100.

Phagocytosis and Fc-receptor Expression of AM Following the Coculture with CNG and MAF

Phagocytic activity and Fc-receptor expression of AM against opsonized-SRBC was measured after *in vitro* incubation with both MAF (1/100 dilution) and CNG (0.1 μg/ml) for 12 hours at 37°C. The coculture with CNG and MAF induced the additive increases in both phagocytic activity and Fc-receptor expression in AM

(Fig. 4 and 5).

Discussion

CNG prepared from *Streptomyces roseosporus* enhances the phagocytic activity of AM against opsonized-SRBC. Other investigations have reported that microbial extract such as muramyldipeptides (MDP)[4], ubenimex[5] and forphenicinol[6,7] also activate macrophage function. CNG and other microbial extracts may activate macrophage function by a similar mechanisms. As shown in Fig. 2, the enhancement of phagocytic activity of AM against opsonized-SRBC following *in vitro* incubation with CNG was closely associated with increased expression of Fc-receptor in AM.

MAF prepared from splenic lymphocytes treated *in vitro* with Con A for 48 hours is known to enhance macrophage functions as reported previously[8,9]. As shown in Fig. 3, 1/100 dilution of MAF induced the maximum increase of phagocytic activity, which was similar to that observed with CNG treated AM as shown in Fig. 4. The coculture of AM preparation with CNG and MAF induced an additive effect on phagocytic activity. This additive stimulation could be due to CNG and MAF acting by either acting by independent mechanism on the same cells or by acting on different sub-populations of AM by the same mechanisms. Both MAF and CNG increased expression of Fc-receptors in AM (Fig. 5). The coculture with CNG and MAF induced a higher expression of Fc-receptor than either agent alone. Although both agents independently activate the expression of the same receptor, the additive activation again suggests that they act either by independent mechanism on the same cell or by the same mechanism on different sub-populations of cells.

KAWATSU *et al.* have reported that CNG inhibits both macrophage function and the secretion of monokine from macrophages in tumor-bearing mice[2,3]. This does not agree with the results in the study. The discrepancy between KAWATSU *et al.* and the present studies may be due to the difference in the experimental condition. They used tumor-bearing mice in their experiment. Not only macrophage function but also the secretion of inflammatory cytokines such as TNF-α and IL-1 were remarkably enhanced during the development of cancer[10]. In addition, their cytokines promote tumor growth[11]. Therefore, CNG may inhibit macrophage function in tumor-bearing mice due to suppression of tumor growth. In normal mice, CNG may enhance macrophage function. These results suggest that CNG is a potent immunomodulator and has the ability to normalize macrophage function depending on the condition of the host. Although CNG and MAF enhanced macrophage phagocytic function in this experiment, it is unclear whether they act independent mechanism on the same cell or the same mechanism on different sub-population of cells. Further work is needed in order to define the detailed mechanism of CNG stimulation of AM function.

References

1) YAMASHITA, T.; M. IIJIMA, H. NAKAMURA, K. ISSHIKI, H. NAGANAWA, S. HATTORI, M. HAMADA, M. ISHIZUKA & T. TAKEUCHI: Conagenin, a low molecular weight immunomodulator produced by *Streptomyces roseosporus*. J. Antibiotics 44: 557~559, 1991
2) KAWATSU, M.; T. YAMASHITA, M. OSONO, M. ISHIZUKA & T. TAKEUCHI: T cell activation by conagenin in mice. J. Antibiotics 46: 1687~1691, 1993
3) KAWATSU, M.; T. YAMASHITA, M. OSONO, T. MASUDA, M. ISIZUKA & T. TAKEUCHI: Effect of conagenin in tumor bearing mice antitumor activity, generation of effector cells and cytokine production. J. Antibiotics 46: 1692~1698, 1993
4) MATTER, A.: The effect of muramyldipeptide (MDP) in cell-mediated immunity. Cancer Immunol. Immunother. 6: 201~210, 1979
5) ISHIZUKA, M.; J. SATO, Y. SUGIYAMA, T. TAKEUCHI & H. UMEZAWA: Mitogenic effect of bestatin on lymphocytes. J. Antibiotics 33: 653~662, 1980
6) ISHIZUKA, M.; S. ISHIZEKI, T. MASUDA, A. MOMOSE, T. AOYAGI, T. TAKEUCHI & H. UMEZAWA: Studies on effects of forphenicinol on immune responses. J. Antibiotics 35: 1042~1048, 1982
7) OKURA, A.; H. ARAKAWA, T. YOSHINARI, K. NAITO, M. ISHIZUKA, T. TAKEUCHI & H. UMEZAWA: Effect of forphenicinol on the production of Ia-positive macrophages in mice with or without L1210 leukemia and on the growth of L1210 in immunized mice. J. Antibiotics 39: 575~580, 1986
8) MORIGUCHI, S. & Y. KISHINO: Phagocytosis of alveolar macrophages of pyridoxine-deficient rats. J. Nutr. 114: 888~893, 1984
9) 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
10) ALLEVA, D. G.; C. J. BURGER & K. D. ELGERT: Tumor-induced macrophage tumor necrosis factor-alpha production suppresses autoreactive T cell proliferation. Immunobiology 188: 430~445, 1993
11) WU, S.; C.M. BOYER, R. S. WHITAKER, A. BERCHUCK, J. R. WIENER, J. B. WEINBERG & R. C. BAST Jr: Tumor necrosis factor alpha as an autocrine and paracrine growth factor for ovarian cancer: Monokine induction of tumor cell proliferation and tumor necrosis factor alpha expression. Cancer Res. 53: 1939~1944, 1993