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Immunomodulatory Function of Murine NK Cell Activity by Alginate

Eun-Wha Son, Kwang-Hee Yang[1], Dong-Kwon Rhee[1], and Suhkneung Pyo[1]

Department of Pharmacognosy and Material development, Samcheok National University, Samcheok 245-711, Korea and [1]Division of Immunopharmacology, College of Pharmacy, Sungkyunkwan University, Suwon 440-746, Korea

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The *in vivo* immunomodulatory function of the activity of murine natural killer (NK) cells induced by high mannuronic acid-containing alginate (HMA) was examined. HMA was injected i.p at doses of 25 and 100 mg/kg. The NK activity was 3 times higher with 100 mg/kg HMA than the baseline. In addition, *in vitro* studies of splenocytes cultured with HMA for 20 h showed a significant increase in NK activity at E:T ratio of 100:1; a 160% and 210% increase at 10 and l00 μg/mL, respectively. There was a six fold increase in interferon-γ production in a postculture of splenocytes with 100 μg/mL HMA. HMA had no suppressive effects on the lymphocyte function in the presence or absence of mitogens. This suggests that HMA is useful in cancer immunotherapy.

Key words: Alginate, NK cell, Cytotoxicity, IFN-γ

INTRODUCTION

Natural killer (NK) cells are an important component of the innate immune system and mediate the cytolytic activities against tumor and virus-infected targets. In addition to the cytotoxic activity, there are increasing indications that NK cells can secrete a variety of cytokines and cytotoxic factors (Wayner and Brooks, 1985). Several studies have shown that the NK system is usually impaired in cancer patients (Hersey *et al.*, 1980; Wilmer *et al.*, 1984; Ghoneum *et al.*, 1996). As a result, recent attention has focused on determining new mmunotherapeutic approaches for treating cancer by increasing the host anti-tumor response through the augmentation of the NK cell activity.

Alginate is an extract of seaweed and is linear polymer of polysaccharides exhibiting gel-forming properties composed of β-(1→4)-D-mannosyluronic acid (M), α-(1→4)-L-glucosyluronic acid (G), and alternating (MG) blocks (Davidson *et al.*, 1976). Alginate has been used for the immobilization of Langerhans islets in the treatment of experimental diabetes mellitus in rats (Tze *et al.*, 1982; Soon-Shiong *et al.*, 1994). Microencapsulation of hormone-producing cells into calcium alginate gel is useful for treating diabetes mellitus and parathyroid disease (Darquy *et al.*, 1987; Fan *et al.*, 1990). The M- and MG-blocks contain active polysaccharide structures that stimulate cytokine production such as IL-1, IL-6, and TNF-α but they do not contain G-blocks (Otterle *et al.*, 1991). Seljelid *et al.* reported that β-1, 4-linked D-mannuronic acid and β-1, 3-glucan, which are mannose-containing polysaccharides, increase the antitumor activity and cytokine production of macrophages or monocytes (1989). In addition, mannoglucan showed antitumor and TNF-α-like activities against tumor tissues (Takahashi *et al.*, 1988). Moreover, alginate increased the survival rate of sarcoma 180-bearing animals (Iizima-Mizui *et al.*, 1985). From these reports, it was thought that mannuronic acid is a major component affecting the immune system. In addition, our previous reports demonstrated that high M-alginate (HMA) levels stimulate various functions of the murine peritoneal macrophages (Son *et al.*, 2001). Therefore, this study examined the effect of HMA on the NK cell activity.

Correspondence to: Suhkneung Pyo, Sungkyunkwan University, College of Pharmacy, Suwon, Kyunggi-do 440-746, South Korea
Tel: 82-31-290-7713, Fax: 82-31-292-8800
E-mail: snpyo@skku.ac.kr

MATERIALS AND METHODS

Animals and Chemicals

Male C57BL/6 mice (7 weeks old) were obtained from

the Charles River Breeding Laboratories (Japan). The animals were randomly distributed into five per group. During the experimental period, the animals were maintained at 23±1°C, 55±5% humidity, 10-18 circulation/hour and 12 h cycle of light/dark. The animals were given access to food and water *ad libitum*.

Unless stated otherwise, all chemicals were purchased from the Sigma Chemical Co. (St Louis, MO). The RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). All the tissue culture reagents and HMA were assayed for any endotoxin contamination using the Limulus lysate test (E-Toxate, Sigma), and the endotoxin levels were found to be <10 pg/mL.

HMA exposure

HMA (polyuronic acid of a hydrophilic colloid containing an anhydro-β-D-mannuronic acid residue (1→4)) from *Macrocystis pyrifera* (Kelp) was used as the sodium salt (Sigma A0682). HMA was dissolved in D-PBS and filtered through a 0.22 μm filter. The mice were injected intraperitoneally with either D-PBS or HMA (25 and 100 mg/kg). The dose and treatment times were obtained from a study that reported elsewhere (Son *et al.*, 2001). The splenocytes were collected from the mice after 20 h.

Mitogen-induced cell proliferation

A modification of the method reported by Mosmann *et al.* (1983) was used. The spleens were aseptically removed and dissociated into a single-cell suspension in a culture medium. The concentration was adjusted to 2×10^6 cells/mL. The culture medium was RPMI 1640 (GIBCO, Grand Island, NY) containing 10% heat-inactivated FBS, penicillin (100 IU/mL), and streptomycin (100 μg/mL) (RPMI-FBS). Mitogenic stimulation was carried out as follows: 5×10^5 cells per well in a total volume of 50 μL were incubated in the presence or absence (control) of the mitogens. The additions (50 μL) are as follows: concanavalin A (ConA) at 4 μg/mL for T cell activation; and lipopolysacchride (LPS) at 10 μg/mL for B cell activation. After an incubation period of 48 h, the enzyme activity of the viable cells was measured by adding 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) to each well. After an additional incubation period of 4 h to allow for the dissolution of the MTT crystals, the absorption was measured at 540 nm using a microplate reader (Molecular Devices, Menlo Park, CA).

Assessment of NK cell cytotoxicity

The NK cell cytotoxicity was determined using a standard ^{51}Cr release assay (Kim *et al.*, 2003). Spleen cells from the male C57BL/6 mice were tested as effector cells and YAC-1 mouse lymphoma cells (ATCC, Rockville, MD) were used as the target cells labeled with a sodium ^{51}Cr-chromate solution (Amersham, UK). The NK cell assays were carried out in 96-well v-bottom plates at effector/target cell ratios 10:1, 50:1, and 100:1 with 1×10^4 of the target cells in a final well volume of 200 μL at 37°C for 6 h in a 5% CO_2 humidified incubator. The specific release of ^{51}Cr by the YAC-1 target cells reflected the natural cytotoxic activity of the NK cells, and was calculated as follows:

$$\% \text{ Cytotoxicity} = [(E-S)/(T-S)] \times 100$$

where E is the activity of the ^{51}Cr released from the target cells in the presence of effector cells, S is the activity of ^{51}Cr released spontaneously from the target cells alone under identical conditions. T is the maximum activity of the ^{51}Cr released when all the target cells were destroyed.

Total lymphocyte counts

The total lymphocyte counts were examined prior to and 20 h after the HMA treatment using a hemocytometer.

IFN-γ determination by ELISA

The splenocytes from the male B57BL/6 mice were cultured with or without HMA for 16 h. The culture supernatants were collected and the IFN-γ concentration in the culture supernatants was determined using an ELISA kit according to the manufacturer's instructions (Endogen, Woburn, MA).

Statistical analysis

The data is represented as a mean±S.E.M. The statistical difference between the groups was determined using a one-way analysis of variance (ANOVA) with a Dunnett's *t*-test. A P value < 0.05 was considered significant.

RESULTS AND DISCUSSION

The cytolytic capacity of the NK cells was measured using a ^{51}Cr releasing assay at different E:T ratios. The *in vivo* treatment with HMA resulted in a significant enhancement in NK activity compared with the control at all E:T ratios (Fig. 1). The *in vitro* effect of HMA on the NK activity was also examined at an E:T ratios of 50:1 and 100:1. Culturing the splenocytes with HMA for 16 h resulted in the enhancement of NK cell activity, which was dose dependent. 10 μg/mL HMA increased the NK cell activity by 128% and 160% at E:T ratio of 50:1, respectively. The NK cell activity at E:T ratio of 100:1 was further increased (148%, 210%) with increasing the HMA concentration to 100 μg/mL (Fig. 2). There were no significant changes in the total lymphocyte counts after administering the HMA administration compared with counts prior to treatment

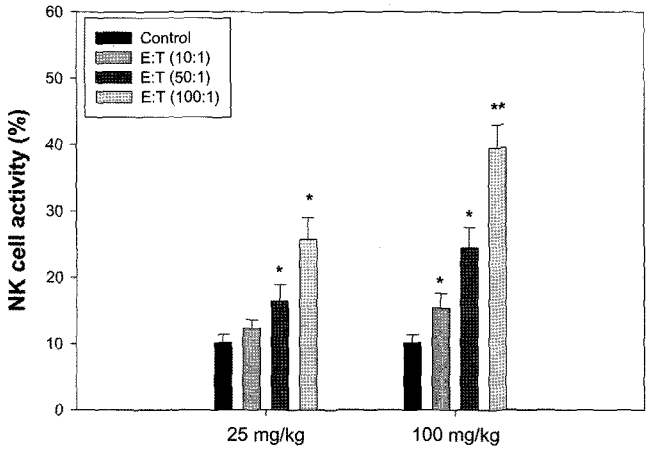

Fig. 1. *In vivo* effect of HMA on the NK cell cytotoxicity. The mice were injected intraperitoneally with HMA. The NK cell cytotoxicity was measured as described in the Experimental procedures and is expressed as the percentage cytolysis of the target cells. The results are presented as a mean ± SE, and there were 5 mice per group. *p<0.05, **p<0.01; significantly different from the vehicle control group. The experiments were repeated 3 times.

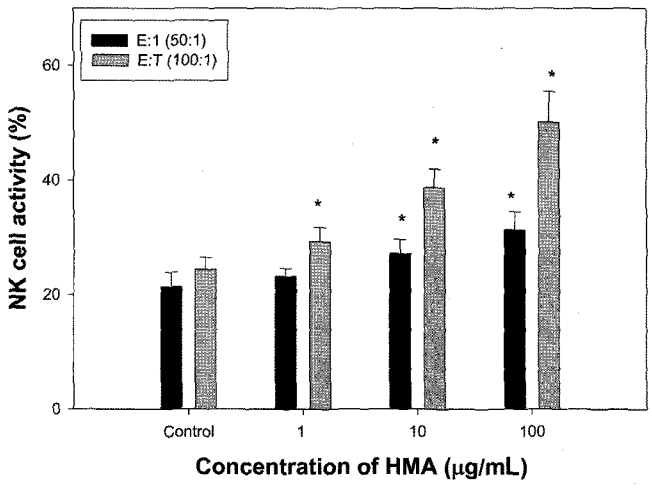

Fig. 2. *In vitro* effect of HMA on the NK cell cytotoxicity. Splenocytes were cultured with various HMA doses (1-100 μg/mL) for 20 h. The NK cell cytotoxicity was examined at E:T ratio of 50:1 and 100:1. The data represents the mean± SE of quadruplicate experiments. *: Significantly different from control (no treatment); *p<0.05.

(data not shown). This indicates that HMA can activate the cytolytic activity of NK cells without affecting the cell viability.

Fig. 3A shows the results of the MTT assays performed by stimulating the spleen cells with the mitogens, ConA or LPS, after the *in vivo* exposure to HMA for 20 h. *In vivo* exposure to HMA showed a significant increased effect on lymphocyte proliferation in the presence of ConA and LPS. This result is consistent with *in vitro* study in which HMA exposure for 3 days produced an increase of B cell and T cell proliferation in response to mitogens (Fig. 3B).

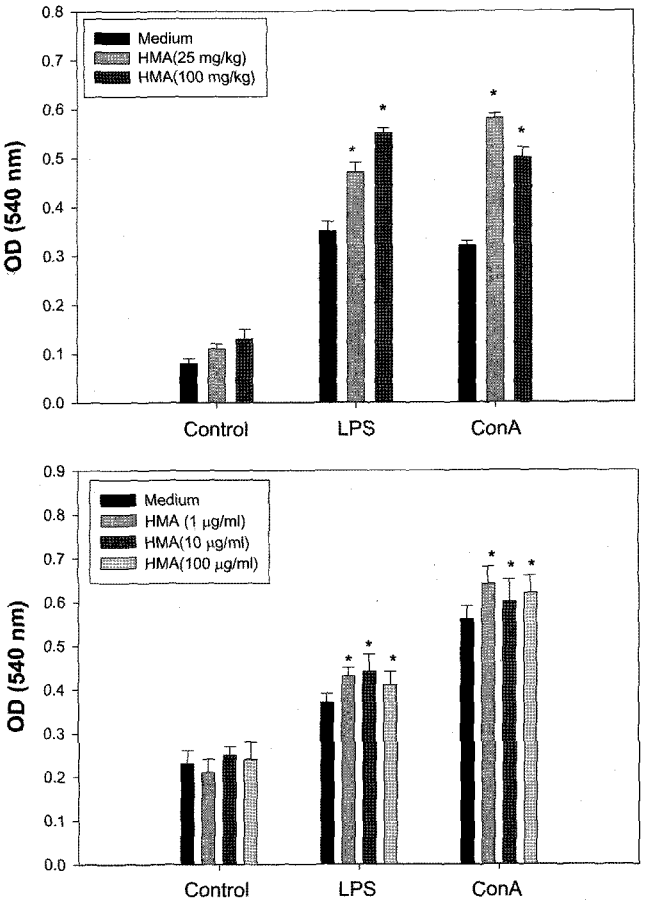

Fig. 3. *In vivo* (A) and *In vitro*(B) effect of HMA on the lymphocyte function. (A) The splenocytes were treated with or without Con A and LPS for 3 days, respectively, and the absorption was measured at 540 nm. (B) The splenocytes were incubated with various HMA concentrations (1-100 μg/mL) in the presence or absence of Con A and LPS for 3 days, and the absorption was measured at 540 nm. The data is expressed as a mean ± SE of quadruplicates of a representative experiment. *: Significantly different from control (no treatment); *p<0.05.

However, this stimulating effect was more for *in vivo* exposure than for *in vitro* exposure.

The mechanism by which HMA enhances the NK cell activity is not completely understood but might be due to an increase in IFN-γ production. Fig. 4 shows the *in vitro* results on the effect of HMA on IFN-γ production from splenocytes. The HMA treatment resulted in a significant increase in IFN-γ production in a dose dependent manner, and was maximized at a concentration of 100 μg/mL (6 fold). Many biological agents can induce rapid IFN production from the large granular lymphocytes (LGL), and it is the production of IFN that produces the self-activation of the NK activity in the LGL (Djeu, 1983). This suggests that the increased tumoricidal activity by HMA is mediated by the increased IFN-γ production.

The possibility that the alginate used to stimulate the

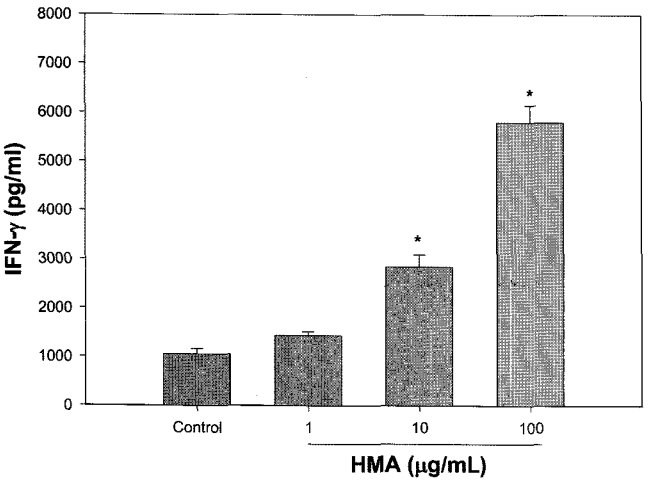

Fig. 4. *In vitro* effect of HMA on IFN-γ production. The splenocytes were incubated with HMA at different concentrations (1-100 μg/mL) for 20 h and the supernatants were analyzed for IFN-γ using an ELISA assay. The results are shown a mean ± SE of quintuplicates from a representative experiment. *: Significantly different from the control (no treatment); *p<0.05.

NK cells contained a small amount of contaminants such as polyphenols and endotoxin cannot be ruled out. Alginate isolated from alga is generally contaminated with polyphenols and endotoxins that have various immunosuppressive and immunostimulatory effects, respectively (Skjank-Brek *et al.*, 1991). Therefore, the HMA was tested for the presence of endotoxin using the Limulus amoebocyte lysate assay in order to discount the possibility of any impurity problems that might interfere with the interpretation of the results. The HMA contained the lower limit detectability (data not shown). Accordingly, the possibility of NK cell activation by contaminants was ruled out.

Overall, these findings clearly demonstrate that HMA can induce the tumoricidal and secretory activity of NK cells.

Immunological stimulation is attracting a great deal of attention as a major treatment modality in the management of cancer patients. Several studies have shown that a single administration of a biological response modifier (BRM) significantly enhanced the NK cell activity (Herberman *et al.*, 1981; Herberman *et al.*, 1983). Accordingly, the augmentation of NK activity by different BRMs has been the focus of many investigations. Recently, we reported that HMA has an immunostimulating effect on macrophages after in vivo exposure (Son *et al.*, 2001).

In conclusion, HMA is a potent immunomodulator that significantly enhances the NK cell activity both *in vivo* and *in vitro*. In addition, the dose used and the data presented is expected to expand the database on the effect of HMA on the immune system. Furthermore, these results reaffirm that NK cells are the primary cells in the immune system for destroying tumor cells and that the induced tumoricidal activities of the NK cells may be mediated by IFN-γ. The high supplementary effect of HMA and the absence of notable side effects make this material a promising immunotherapeutic agent for treating cancer patients.

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