Arch Pharm Res Vol 26, No 11, 960-966, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Antiviral and Tumoricidal Activities of Alginate-Stimulated Macrophages are Mediated by Different Mechanisms

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(Received August 26, 2003)

Macrophages play an important role in host defenses by killing tumors and virus infections and producing secretory products. High mannuronic acid (HMA) containing alginate was examined to determine the mechanisms by which HMA-activated macrophages resist infection with HSV-1 and inhibit the growth of tumor cells. The ability of macrophages to resist infection with HSV-1 or to inhibit the growth of tumor cells was assessed following treatment with HMA alginate in the presence of either antibodies to various cytokines or inhibitors/scavengers of toxic macrophage products. Only antibodies to IFN-α/β were able to abrogate the protective effects of HMA alginate in macrophages infected with HSV-1, suggesting that the antiviral activity induced by this immunomodulator was mediated by the production of IFN-β. In contrast, anti-TNF-α, anti-IFN and inhibitors of nitric oxide and reactive oxygen species were all able to partially abrogate HMA-induced cytostatic activity, suggesting that multiple mechanisms are involved in macrophage cytostasis. These results indicate that the HMA-induced intrinsic antiviral and extrinsic cytotoxic activites are mediated by different mechanisms.

Key words: Alginate, Macrophage, Antiviral, Tumoricidal

INTRODUCTION

Macrophages have been shown to be an important component of host defenses against virus infection by inhibiting intracellular replication of virus (intrinsic resistance) and by killing virus infected cells (extrinsic cytotoxicity) (Morahan, 1984; Morahan *et al.*, 1985). The cytotoxic (cytostatic) activity of macrophages is also involved in resistance to tumors (Cohn *et al.*, 1978). Although the mechanisms by which macrophages kill tumor cells have been studied in some detail, the way in which they inhibit replication of viruses within themselves or in other cells are not well understood. However, the role of interferon (IFN) in the antiviral activity of macrophages has been implicated in some cases (Proietti *et al.*, 1986) but not in others (Sit *et al.*, 1988).

Macrophages can be stimulated by a variety of agents such as IFN-γ, lipopolysaccharide, or other microbial products (Dullens *et al.*, 1989; Gautam and Deodhar., 1989; Paulnock and Lambert., 1985) and some of which have also been shown to trigger the release of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and nitrite and to induce tumoricidal activity in macrophages (Arden *et al.*, 1985; Arenzana-Seisdedos and Virelizier, 1983; Choriki *et al.*, 1989; Stuehr and Marletta, 1987; Keller *et al.*, 1990).

Alginate, extract of seaweeds, is a linear polymer of polysaccharides with 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 immobilization of Langerhans islets for treatment of experimental diabetes mellitus in rats (Tze and Tai, 1982; Soon-Shiong *et al.*, 1994). Microencapsulation of hormone-producing cells into calcium alginate gel was useful for the treatment of diabetes mellitus and parathyroid disease (Daquy and Sun, 1987; Fan *et al.*, 1990). It has been known that M- and MG-blocks have active polysaccharide structures to stimulate cytokine production such as IL-1, IL-6 and TNF-α, but not G-blocks (Seljelid *et al.*, 1989). Otterle *et al.* (1991) have reported that β-1,4-linked D-mannuronic acid and β-1,3-glucan which are mannose-containing poly-

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saccharide increase antitumor activities and cytokine production of macrophages or monocytes. In addition, mannoglucan showed antitumor and TNF-α-like activities against tumor tissues (Takahashi *et al.*, 1988). Alginate also increased the survival rate of sarcoma 180-bearing animals (Iizima-Mizui *et al.*, 1985).

The mechanisms by which high mannuronic acid (HMA) exerts its tumoricidal and antiviral effects on macrophages are not fully understood. We have recently shown that HMA alginate has an immunostimulating effect on macrophages after *in vivo* exposure (Son *et al.*, 2001). Once activated, macrophages produce various substances, some of which have both cytotoxic and antiviral activities. The role that these substances play in cytostasis and in virucidal activity has not been fully elucidated. Since macrophages may employ common mechanisms for their cytotoxic and antiviral activities, we investigated the role of HMA-induced cytokines and cytotoxic factors in the tumoricidal and intrinsic antiviral activities of macrophage. This study demonstrated that HMA-induced antiviral activity is due solely to the production of IFN-β and not to other cytotoxic compounds. In contrast, HMA-induced cytostatic activity is mediated by the production of cytokines and cytotoxic factors. Thus, HMA-induced intrinsic antiviral resistance and extrinsic cytotoxic activities are mediated by different mechanisms.

MATERIALS AND METHODS

Mouse, Herpes simplex virus strains and tumor cell

Male C57BL/6 mice (7 weeks old) were obtained from Charles River Breeding Laboratories (Japan). Five animals were randomly distributed into each group. During the experimental period, animals were maintained in an environment of 23±1°C, 55±5% humidity, 10-18 circulation/h and a 12 h light/dark cycle. Food and water was supplied ad libitum. The KOS strain of HSV-1 was kindly provided by Dr M. Nachtigal (University of South Carolina, USA). Foruse, B16 tumor cells (epidermoid carcinoma) (ATCC, Rockville, MD) were grown in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 μg/mL)(RPMI-FBS). The tumor cell line was passaged three times per week.

HMA exposure

HMA [polyuronic acid of hydrophilic colloid having anhydro-D-mannuronic acid residue (1→4)] from *Macrocystis pyrifera* (Kelp)] was used as sodium salt (Sigma) in this study. HMA was dissolved in Dulbecco's Phosphate Buffered Saline (D-PBS) and filtered with a 0.22 μm filter, and mice were intraperitoneally injected with either D-PBS or HMA (100 mg/kg). The dose selection for the present experiments was based on that of our previous report (Son *et al.*, 2001). Peritoneal macrophages were collected from mice at 20 h after the administration of HMA.

Chemicals and antibodies

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Polyclonal anti-mouse IFN-α/β (10,900 IU/mL) antibody and polyclonal anti-mouse IFN-β antibodies (10,000 IU/mL) were purchased from Lee Biomolecular Research Laboratories, Inc (San Diego, CA). Polyclonal anti-mouse IFN-α antibodies(20,000 IU/mL) were purchased from R&D system Inc (Minneapolis, MN). Polyclonal anti-mouse TNF-α antibody (1×10^6 neutralizing units/mL) was purchased from BD Biosciences (San Jose, CA). $Na_2{}^{51}CrO_4$ was purchased from Amersham Life Science LTD (England). Culture media and test reagents were assayed for endotoxin contamination by the limulus lysate test (E-Toxate; Sigma) and found to be less than 10 pg/mL.

Collection and countings of peritoneal macrophages

Pooled peritoneal exudate cells were harvested from HMA- and PBS-treated mice (n=5, each experiment). Following lavage of the peritoneal cavity with 6 mL of RPMI 1640 (GIBCO, Grand Island, NY), the cells were washed twice and resuspended in RPMI 1640 containing 10% heat-inactivated FBS, 100 IU/mL penicillin and 100 g/mL streptomycin (RPMI-FBS) (Pyo, 1994).

Total cell counts were determined with a hemocytometer. In addition, 100 μL of cell suspension was spun for 10 min at 120×g onto a microscope slide with a cytospin 3 centrifuge (Shandon, Inc., Pittsburg, PA), and cytocentrifuged cells were fixed and stained using ACUSTAIN Wright-Giemsa Stain (Sigma) to allow the calculation of percentages of macrophages. Total numbers of peritoneal macrophages were obtained by multiplying these percentages by the total number of peritoneal exudate cells. Based on this differential staining, macrophages were seeded at densities of 1×10^6 cells/well on 96-well plate (Falcon Plastics, Oxnard, CA) and allowed to adhere for 2 h in a humidified incubator at 37°C in 5% CO_2. After a 2 h incubation, nonadherent cells were removed by washing with medium three times. More than 98% of the adherent cell populations were macrophages according to morphology and phagocytic criteria (Saiki and Fidler, 1984).

Infection of macrophages with HSV-1

Macrophages were infected with the KOS strain of HSV-1 at multiplicity of infection (MOI) of 7 in a volume of 25 μL. After 1 h at 37°C, the nonadsorbed virus was removed by washing with RPMI-FBS and the cells were

incubated for 48 h at 37°C in 5% CO_2. Cytopathic effects (CPE) were determined using a neutral red dye uptake assay (Pyo, 1994). Assessment of CPE by this method correlated well with visual observation of the cells.

Neutral red dye uptake assay

The neutral red dye uptake assay to measure virus (CPE) was performed as described below. After incubation of the HSV-1 infected macrophages, the monolayers were washed twice with medium and stained for 2 h with 0.02% neutral red in medium. The stained monolayers were washed with medium by aspiration and the dye extracted with 0.2 mL of Sorensen's citrate buffer (pH 4.1) containing 50% ethanol for 15 min on a rotary shaker. The optical density was read at 540 nm in microplate reader (Molecular Devices, Menlo Park, CA). Virus CPE was evaluated by calculation of a viability index, which is expressed as the ratio of dye uptake by infected cells to dye uptake by uninfected cells, according to the following formula:

$$\text{Viability index (\%)} = \frac{\text{O.D. of virus infected cells}}{\text{O.D. of uninfected cells}} \times 100$$

In cultures treated with HMA alginate or inhibitors, controls to assess possible toxic effects were also employed and the viability index was calculated using the values obtained from HMA-treated infected and uninfected cells. No toxicity was observed at the dosages employed.

Nitrite determination

Macrophages were cultured for 20 h in the presence or absence of antibody to cytokine and the accumulation of nitrite in the culture supernatants was measured using the assay system described by Ding *et al.* (1988). Briefly, 100 μL of supernatant was removed from each well into an empty 96-well microtiter plate. After the addition of 100 μL of Griess reagent to each well, absorbance at 540 nm was measured using a Molecular Device microplate reader. Nitrite concentration was calculated from a $NaNO_2$ standard curve. The levels of nitrite are indicative of nitric oxide (NO) production. Griess reagent was prepared by mixing 1 part of 0.1% naphthylethylene diamine dihydrochloride in distilled water with 1 part of 1% sulfanilamide in 5% phosphoric acid.

Macrophage-mediated cytotoxicity

The assay for macrophage cytotoxicity was based on an assay described by Verstovsek *et al.* (1992). Briefly, macrophages (1×10^5 cells/well) were plated into 96-well microtiter plates for 2 h in an incubator at 37°C in 5% CO_2. After two washings with warm media, ^{51}Cr-labeled tumor target cells were added (1×10^4 cells/well: an initial effector:target cell ratio of 10:1) to the macrophages for an additional 20 h incubation. In some experiments, the antibody to cytokine, the isotype-matched control antibody or inhibitor of metabolic pathway was included. The plates were then centrifuged at 300×g for 5 min and the aliquots (0.1 mL) of the cell-free supernatant solutions were harvested for counting in a Packard Cobra series gamma counter (Meriden, CT). The percentage of specific ^{51}Cr release was calculated as follows: % of specific ^{51}Cr release=(cpm experimental release–cpm spontaneous release)/(cpm maximum release–cpm spontaneous release) ×100. All experiments were repeated at least two times.

Statistical analysis

All data are presented as means±S.E.M. Statistical difference between groups was determined by one-way analysis of variance (ANOVA) and significant values were represented by an asterisk (*$p<0.05$).

RESULTS

Mediators of HMA-induced macrophage cytotoxicity/cytostasis

A number of compounds have been implicated in macrophage cytotoxicity, including H_2O_2, TNF, NO and IFN (Decker *et al.*, 1987; Hibbs *et al.*, 1987a; Mavier and Edgington, 1984; Remels *et al.*, 1990). To determine whether these compounds were involved in HMA-mediated cytostasis, we attempted to abrogate cytostasis by inhibiting their production or by neutralizing their activity. The general design of these experiments was to culture macrophages, challenge them with tumor cells in the presence of inhibitors or antibodies, and assess their cytostatic activity. At the concentrations employed none of the inhibitors or antibodies affected the growth of the tumor cells (data not shown). The role of H_2O_2 was evaluated by inactivating it with catalase, that of TNF and IFN by neutralizing with antibodies, and that of NO by inhibiting its production with NMMA.

The addition of catalase inhibited the cytostatic activity of activated macrophages against B16 tumor cells (Table I). Moreover, we observed that HMA alginate increased the generation of superoxide anion and hydrogen peroxide, as compared to that in untreated cells (data not shown). Thus, anti-TNF-α, anti-IFN antibodies and the NO inhibitor, NMMA, were all able to abrogate, in part, HMA-induced cytostasis against the target cell (Table I). Taken together these results suggest that TNF-α, IFN-α/β, IFN-β and NO are all partially involved in the tumoricidal activity of HMA-stimulated macrophages.

Since HMA alginate is able to induce the production of IFN and TNF-α which in turn may stimulate NO production (Gao *et al.*, 1998; Son *et al.*, 2001), we also addressed the question of whether the induction of NO by HMA

Table I. Inhibition of tumoricidal activity of HMA-exposed macrophages by antibody or inhibitors

Treatment	% Cytotoxicity of target cell (B16)
None	6.2 ± 1.8
HMA (100 mg/kg)	40.7 ± 4.3†
HMA + catalase (400 units/mL)	29.3 ± 2.4*
HMA + NMMA (0.5 mM)	19.9 ± 3.6*
HMA + anti-TNF-α (500 units/mL)	26.7 ± 2.9*
HMA + anti-IFN-α/β (500 units/mL)	25.4 ± 2.2*
HMA + anti-IFN-β (500 units/mL)	27.1 ± 1.1*

HMA-exposed macrophages were cocultured for 20 h with the target of an initial effector/target cell ratio of 10:1. Macrophage tumoricidal activity was determined as described in the Materials and Methods. The results shown are the mean ± SEM of quintuplicates from a representative experiment.
†Significantly different from control (no treatment); $p<0.05$
*Significantly different from HMA-treated; $p<0.05$

Table II. Effects of anti-IFN-α/β, anti-IFN-β or anti-TNF-α antibodies on the production of NO_2^- by HMA-exposed macrophages

Treatment	NO_2^- production (μM)
None	5.7 ± 0.8
HMA	23.3 ± 2.2†
HMA + anti-IFN-α/β (500 units/mL)	12.4 ± 1.2*
HMA + anti-IFN-β (500 units/mL)	13.9 ± 2.3*
HMA + anti-TNF-α (500 units/mL)	22.1 ± 3.3

HMA-exposed macrophages were cultured for 20 h in the presence or absence of anti-IFN-α/β, anti-IFN-β or anti-TNF-α antibodies. The supernatants were collected for NO_2^- assay. The results shown are the mean ± SEM of quintuplicates from a representative experiment.
†Significantly different from control (no treatment); $p<0.05$
*Significantly different from HMA-treated; $p<0.05$

alginate was mediated via IFN or TNF-α. This was done by measuring NO_2^- production in cells treated with HMA alginate for 24 h in the presence of anti-IFN-α/β, anti-IFN-β or anti-TNF-α antibodies. As shown in Table II, anti-IFN antibodies partially inhibited the production of NO_2^-. These results suggest that IFN-β was at least partly involved in the HMA-induced NO_2^- production, whereas TNF-α was not.

Mechanism of HMA-induced intrinsic anti-HSV-1 activity

Following our demonstration that HMA-induced tumoricidal activity was mediated by a number of factors, we next determined whether any of these factors were also involved in macrophage anti-HSV-1 activity, particularly since most of these factors have been implicated to play a role in antiviral activity in other cells (Rager-Zisman *et al.*, 1989; Wong and Goeddel, 1986). This was done by attempting to abrogate the HMA-induced antiviral activity using inhibitors or antibodies, as described above. In addition to the inhibitors and antibodies used in the cytostasis studies, we also employed superoxide dismutase as a superoxide anion scavenger. We have shown previously that the neutral red dye uptake assay to assess CPE is correlated with virus yields in HSV-1 infected macrophages, and that treatment of macrophages with HMA alginate results in a 2~3 log reduction in virus yield (Pyo *et al.*, 1991).

Table III. Lack of neutralization of HMA-induced anti-HSV-1 activity by inhibitors of reactive oxygen species and nitric oxide production

Treatment	Viability index (%)
None	33.7 ± 3.5
HMA	56.2 ± 3.2†
HMA + catalase (400 units/mL)	55.4 ± 4.4
HMA + superoxide dismutase (450 units/mL)	56.7 ± 2.9
HMA + NMMA (0.5 mM)	52.9 ± 1.1*

HMA-exposed macrophages were infected with HSV-1 (KOS strain; MOI=7) and cultured for 48 h in the presence or absence of scavengers of reactive oxygen species or inhibitor of NO production. The viability index was determined by the neutral red dye uptake assay. The results shown are the mean ± SEM of quintuplicates from a representative experiment.
†Significantly different from control (no treatment); $p<0.05$
*Significantly different from HMA-treated; $p<0.05$

Table III shows that neither of the scavengers of reactive oxygen intermediates, superoxide dismutase or catalase, could abrogate the antiviral activity of HMA alginate, Similarly, the inhibitor of NO production, NMMA, was unable to abrogate HMA alginate's protective effects. Although NMMA did cause a slight reduction in the viability index compared to HMA alginate treatment alone, this difference, while it was statistically significant, is of questionable biological significance. These results suggest that oxygen radicals and NO are not involved in HMA-induced antiviral activity. In contrast, treatment with anti-IFN-α/β or anti-IFN-β antibodies partially abrogated the protective effects of HMA alginate, whereas anti-IFN-α antibodies did not exert any such effect (Fig. 1). Furthermore, antibodies to TNF-α and IL-1 also showed no effect (data not shown). Thus, although the cytostatic activity induced by HMA alginate is mediated by a variety of different mechanisms, HMA-induced anti-HSV-1 antiviral activity is mediated solely by the production of IFN-β.

DISCUSSION

HMA-stimulated macrophages have been reported to secrete cytokines and cytotoxic factors (Dinarello, 1984; Greene, 1986; Wong and Goeddel, 1986; Miner *et al.*,

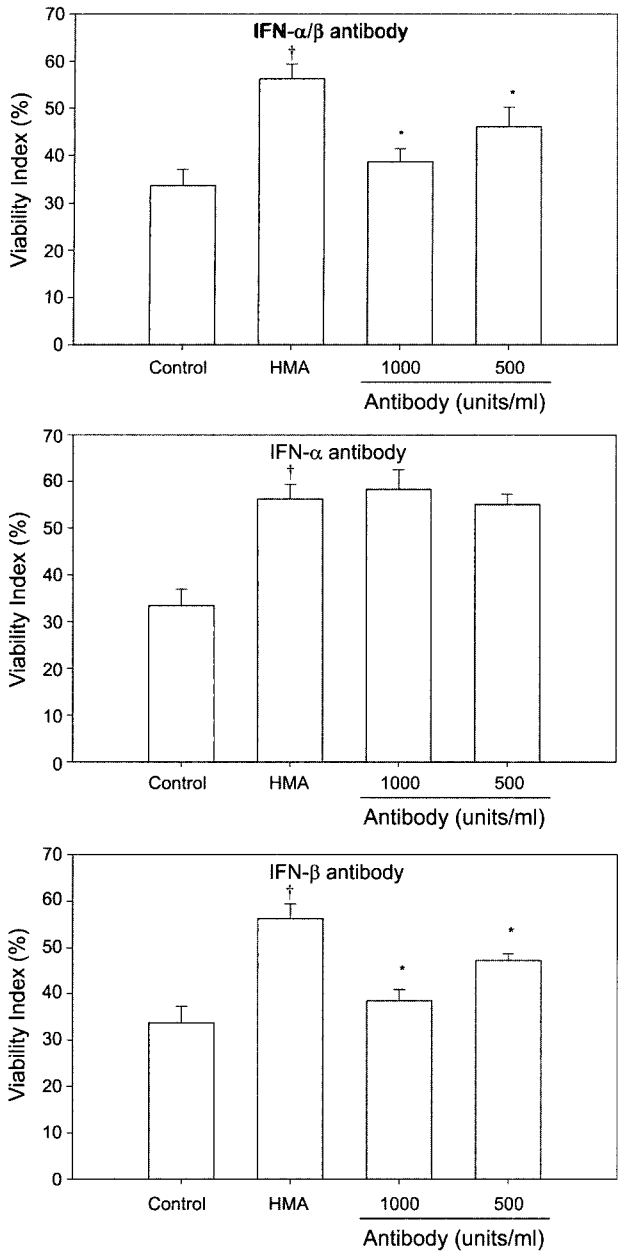

Fig. 1. Neutralization of HMA-induced anti-HSV-1 activity by anti-IFN-antibodies. HMA-exposed macrophages were infected with HSV-1 (KOS strain; MOI=7) and cultured for 48 h in the presence or absence of antibodies. CPE was determined by the neutral red dye uptake assay. The results shown are the mean ± SEM of quintuplicates from a representative experiment. †Significantly different from control (no treatment); $p<0.05$. *Significantly different from HMA-treated; $p<0.05$

1983; Miner and Nicolson, 1983). In this study we investigated the role that these and other factors play in macrophage tumoricidal and antiviral activity, in order to determine whether macrophages employ common mechanisms for their antiviral and cytotoxic activities. Reactive oxygen/nitrogen intermediates have been implicated as mediators of antiviral activity (Rager-Zisman *et al.*, 1982; Djeraba *et al.*, 2000; Messaoudi *et al.*, 2002). In the present experiments, reactive oxygen scavengers reversed HMA-induced tumoricidal but not antiviral activity, suggesting that these mediators play a role in HMA-induced tumoricidal activity.

Hibbs *et al.* (1987a, 1987b) have demonstrated that activated macrophages produce NO which inhibits mitochondrial respiration and results in cytostasis of target cells. The present studies suggest that reactive NO induced by HMA alginate is one of the effector molecules involved in macrophages-mediated tumor cytostasis. However, NO is not involved in HMA-induced anti-HSV-1 activity.

Sharon *et al.* (1989) have shown that IFN-β caused a dose-dependent increase in macrophage cytotoxicity to tumor cells. Furthermore, IFN has been reported to dramatically potentiate the lytic capacity of TNF-α (Tsujimoto *et al.*, 1986). In the present study, IFN-β's implication as a mediator in HMA-induced tumoricidal activity is substantiated by the observation that anti-IFN-β antibodies could abolish the cytotoxic action of macrophages. In addition, we have shown that IFN-β was involved in the antiviral effects of HMA alginate in macrophages.

Decker *et al.* (1987) showed that TNF acts as an effector molecule in macrophage cell-mediated cytolysis against tumor cells that are highly sensitive to TNF. The present data demonstrating that the tumoricidal effect of HMA alginate on macrophages was clearly diminished by anti-TNF antibodies are in keeping with the previous notion that TNF-α plays a central role in the macrophage-mediated killing of target cells. Higuchi *et al.* (1990) demonstrated that TNF-α and L-arginine-derived NO act synergistically in macrophage cytotoxicity. In addition, our observation that the addition of anti-IFN antibodies partially inhibited NO_2^- production by HMA-activated macrophage is in agreement with the finding that IFN induces NO_2^- production (Gao *et al.*, 1998). Based on these findings it is conceivable that HMA alginate synergizes with other cytolytic factors.

In conclusion, the data in this study show that HMA alginate is capable of stimulating the production of different substances, many of which play a role in macrophage-mediated cytostasis. However, of these substances only IFN-β is involved in macrophage intrinsic anti-HSV-1 activity, even though many of these factors may have antiviral activity in other cells.

ACKNOWLEDGEMENT

This work was supported by the Brain Korea 21 project in 2003.

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