Jpn. J. Med. Sci. Biol., 43, 171 - 182, 1990.

**IMMUNOTHERAPEUTIC POTENTIAL IN GUINEA-PIG TUMOR
MODEL OF DEOXYRIBONUCLEIC ACID FROM *MYCOBACTERIUM
BOVIS* BCG COMPLEXED WITH POLY-L-LYSINE AND CARBOXY-
METHYLCELLULOSE**

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(Received August 16, 1990. Accepted November 1, 1990)

SUMMARY: In vivo antitumor activity of a deoxyribonucleic acid fraction obtained from *Mycobacterium bovis* BCG (named MY-1) increased when it was complexed with poly-L-lysine (poly LL) solubilized by addition of carboxymethylcellulose (CMC). The complex of MY-1 and poly LL/CMC induced interferon in vivo at a low dose of MY-1 which alone exerted no IFN induction. With Line 10 hepatoma (L10) which is syngeneic with strain 2 guinea pigs, it was demonstrated that repeated intralesional injections of the complex resulted in delay of tumor growth and complete cure of animals from L10 tumor inoculated. Similar treatment of the animals with the same amount of MY-1 or poly LL/CMC alone had little therapeutic effect on the tumor growth.

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INTRODUCTION

It was reported previously that a purified nucleic acid fraction extracted from *Mycobacterium bovis* BCG, which was composed of 70% DNA, 28% RNA, 1.3% protein, 0.27% hexose and 0.1% lipid with no detectable amount of cell wall components such as α , ϵ -diaminopimelic acid and hexosamine (1), showed antitumor activity against various murine tumors and a guinea-pig tumor (1,2). This fraction, named MY-1, showed no direct cytotoxicity in vitro against these tumors (2). Antitumor activity of MY-1 was ascribed to its DNA component, because DNase-digest of MY-1, which was composed of 97% RNA, showed no antitumor activity, whereas RNase-digest of MY-1, which contained 97% DNA, was more effective than undigested MY-1 (1,2).

Recently it has been found that id injection of MY-1 induced in situ infiltration of mononuclear cells that resembled natural killer (NK) cells (3). The incubation of mouse spleen cells with MY-1 resulted in augmentation of NK activity and induction of interferon (IFN)-alpha/beta and -gamma as macrophage-activating factors in the culture supernatant (4,5). These activities are also due to DNA component of MY-1 (4,5).

Phase I study and a pilot therapeutic study of MY-1 on skin cancer have already been completed (6,7) and phase II clinical study has been conducted since 1987. In these studies, in most of the protocol, more than 5 mg/dose of MY-1 was administrated repeatedly, i.e., 3-5 times/week in average. Although MY-1 has shown very low toxicity and no significant side-effect (6-9), decrease in frequency of administration and dose of MY-1 may be advantageous to clinical application. Levy et al. (10-12) demonstrated that the complex of polyinosinic-polycytidylic acid (poly IC) and poly-L-lysine (poly LL) which was solublized by the addition of carboxymethylcellulose (CMC) was found to be four to 10 times more resistant to hydrolysis and have higher therapeutic potential than the parent compound. We attempted to prepare a complex of MY-1 with poly LL/CMC and tested it for in vivo antitumor activity. It was demonstrated that the complex has a much stronger in vivo activity in the guinea-pig tumor model.

MATERIALS AND METHODS

Animals: Inbred Sewall Wright strain 2 guinea pigs were purchased from Nisseiken Co., Ohme, Tokyo. Animals of both sexes weighing more than 400 g were used. Inbred BALB/c female mice (>7 weeks old) were obtained from the Animal Facilities of the National Institute of Health, Tokyo. They were kept in accordance with the guide line for experimental animals of the National Institute of Health.

Tumor: An ascites variant of diethylnitrosamine-induced hepatocarcinoma line 10 (L10), which is syngeneic with strain 2 guinea pigs, was obtained from Dr. B. Zbar, National Cancer Institute, Bethesda, MD, USA, and maintained by ip passages in strain 2 guinea pigs (13,14).

Cell lines: YAC-1 and L929 cells were grown in RPMI 1640 medium with 10% fetal calf serum (FCS). They were incubated at 37 C in a 5% CO₂ incubator saturated with water vapor and were maintained in logarithmic growth phase (4,5).

Preparation of MY-1 complexed with poly-L-lysine solubilized in carboxymethylcellulose (MY-1/poly LC complex): Poly-L-lysine of different molecular sizes were obtained from Sigma Chemical Co., St. Louis, MO. Carboxymethylcellulose was a product of Wako Pure Chemical Industries, Ltd., Osaka. According to Levy et al. (10), a solution of the complex (in pyrogen-free phosphate-buffered saline, pH 7.2; PBS) was prepared as follows: poly LL (5 ml, 6 mg/ml) was slowly poured with constant stirring into 5 ml of CMC (2%), then stirring was continued for additional two days. Ten milliliters of MY-1 (4 mg/ml) was then poured into the poly LL/CMC solution. The final preparation contained 2 mg of MY-1/ml and 1.5 mg of poly LL/ml in 0.5% CMC. The complex thus prepared was refrigerated until administration.

In vitro activation of spleen cells with MY-1/poly LC complex: Spleen cells from BALB/c mice (1×10^7 /ml) were incubated with MY-1, poly LL/CMC, or MY-1/poly LC complex in 24-well tissue culture plates (Costar, Cambridge, MA, USA) at 37 C for 20 hr in a CO₂ (5%) incubator. Concentrations MY-1 and poly LL in 0.025% CMC were 100 µg/ml and 75 µg/ml, respectively. After the incubation, the cells were collected and washed three times with medium and assayed for NK activity.

Assay for NK activity: The cells collected as mentioned above, were incubated with ⁵¹Cr-labeled YAC-1 cells (1×10^4) in 96-well tissue culture plates (Costar) for 4 hr at 37 C. After the incubation, the plates were centrifuged at 800 ×g for 10 min, and the radioactivity in 0.1 ml of each supernatant was measured with a gamma-scintillation counter (Packard Instruments Co., Inc., Downers Grove, IL, USA). Every effector/target (E/T) ratio was tested in triplicates. NK

activity was expressed as percent specific lysis which was calculated by the following formula:

$$\% \text{ lysis} = (\text{test cpm} - \text{spontaneous cpm} / \text{total cpm} - \text{spontaneous cpm}) \times 100$$

where test cpm is mean cpm released in the presence of effector cells, spontaneous cpm is mean cpm released by target cells alone, and total cpm is the total amount of ^{51}Cr released from target cells upon treatment with 0.1% sodium dodecyl sulfate. The spontaneous release was 5-10% of the total cpm.

Assay for antiviral activity of serum samples from the mice treated with MY-1/poly LC complex: BALB/c mice were injected intravenously with 0.5 ml of a 1:10 dilution of MY-1/poly LC complex, MY-1 or poly LL/CMC. The amount of MY-1 and poly LL in 0.025% CMC injected were 100 μg and 75 μg , respectively. Levels of IFN in the serum samples taken from the treated mice 2 hr after the iv injection were measured for estimating their ability to inhibit cytopathic effects of vesicular stomatitis virus on L929 cells. For IFN assay, a micro-method was used as described by Yamamoto et al (5). Antiviral units were expressed as international units (IU) per ml, calculated from the reciprocal of highest dilution inhibiting 50% of the cytopathic effect in comparison with that of NIH reference.

Antitumor effect of intralesional injection of MY-1/poly LC complex: Viable L10 tumor cells (1×10^6 / 0.1 ml / animal) were inoculated intradermally into the shaved flanks of the strain 2 guinea pigs. One day after the tumor inoculation, 0.1 ml of the solution of MY-1, poly LL/CMC, or MY-1/poly LC complex was injected intralesionally. The amounts of MY-1 and poly LL in 0.05% CMC included in 0.1 ml were 200 μg and 150 μg , respectively. The same intralesional injections were repeated three times a week for a total of seven times. Control animals received seven intralesional injections of 0.1 ml of PBS alone. The primary tumors at the inoculation sites and metastatic tumors in the regional lymph nodes were measured weekly until animals died. The size of tumors was expressed as mean of two perpendicular diameters (mm).

RESULTS

In vitro Augmentation of NK Activity by MY-1/poly LC Complex

The complexes MY-1 and poly LL of different molecular size were tested for their ability to augment in vitro NK activity in murine spleen cells. As shown in Fig. 1, MY-1/poly LC exerted augmenting of NK activities comparable with the parent substance, MY-1, when poly LL of a smaller size (mol. wt. <39,000) was complexed with MY-1. This result indicated that MY-1 retained the original

Fig. 1. In vitro augmentation of NK activity by MY-1/poly LC complex with poly LL of different molecular sizes.

Spleen cells of BALB/c mice were incubated with either MY-1, poly LL/CMC or complex of MY-1 and poly LL with different molecular weights in a CMC solution for 20 hr at 37 C in a CO₂ incubator. After the incubation, the cells were assayed for NK activity as described in MATERIALS AND METHODS.

activity after complexed with poly LL/CMC. Therefore, poly LL with a molecular weight of 4,000 was employed for further experiments to avoid undesirable modulation of antigenicity of the complex.

In vivo Induction of IFN by MY-1/poly LC Complex

A significantly high level of IFN titer was detected in the serum samples of mice treated by iv injection of the MY-1/poly LC complex; whereas treatment of mice with the same amount of MY-1 or poly LL/CMC alone showed no effect on the IFN induction (see Table I). It was suggested that the ability of MY-1 to activate the host immune function such as IFN induction, was strengthened

Fig. 2. Antitumor activity of MY-1/poly LC complex.

Guinea pigs were inoculated intradermally with 1×10^6 L10 cells and treated by intraslesional injection of MY-1 or MY-1/poly LC complex. The solid lines indicate the growth of primary tumor at the injection site of individual animals which was treated with MY-1 alone (●—●) or MY-1/poly LC complex (○—○). The broken lines indicate the growth of metastatic tumor at the regional lymphnode of individual animals. The bold solid line indicates the mean size of primary tumors and the bold broken line metastatic tumors of the non-treated control animals ($n=5$). The arrows indicate the days when animals received intraslesional injection of the reagent. “+” indicates the day of animal’s death.

Table I. In vivo induction of interferon by MY-1/poly LC complex

Stimulant	Interferon titer (IU)
Non-treated	< 8
MY-1	< 8
Poly LL/CMC	< 8
MY-1/poly LC complex	96

BALB/c mice were injected intravenously with MY-1 100 µg, poly LL 75 µg, or MY-1/poly LC complex (MY-1 100 µg + poly LL 75 µg) 2 hr prior to bleeding of them. The serum samples were assayed for interferon levels as described in MATERIALS AND METHODS.

markedly when it was complexed with poly LL/CMC. Accordingly, antitumor activity of the complex was investigated.

In vivo Antitumor Activity of MY-1/poly LC Complex

Growth of L10 at the inoculated sites regressed completely in four out of five animals tested by repeated intralesional injection of MY-1/poly LC complex. Complete suppression of metastasis was also demonstrated in these four animals. In the rest one, delayed tumor growth and prolongation of survival time were observed, whereas the same treatment with MY-1 alone was slightly effective on inhibition of the tumor growth (Fig. 2). In another experiment, as shown in Fig. 3, treatment of tumor-bearing animals by intralesional injections with MY-1/poly LC complex brought again complete regression of tumors in 70% of the animals. Injection of poly LL/CMC alone had no effect on the tumor growth. Injection of 200 µg MY-1 alone was also less effective.

Fig. 3. Antitumor activity of MY-1/poly LC complex.

Guinea pigs (seven/group) were inoculated intradermally with 1×10^6 L10 cells and treated by intralesional injection of MY-1 (●), poly LL / CMC (×), or MY-1/poly LC complex (○). Bold line indicates control group treated with PBS alone (n=5).

DISCUSSION

In the guinea-pig model, suppression of tumor growth at the site of infection with BCG and regression of established dermal tumors after intralesional injection of BCG were first reported by Zbar and his coworkers (15,16). Such approaches with BCG to “local immunotherapy” (17) or “adjuvant contact immunotherapy” (18) have been made by many investigators and produced convincing therapeutic effects on tumors in many experimental animals (17-19) and in humans (20). Attempts to isolate the BCG fraction(s) responsible for the antitumor effects have been made by many researchers, and a variety of fractions, for example, methanol-extraction residue (21), cell-wall skeleton (22), ribosomal RNA (23) and some water-soluble fractions (24,25), have been reported to be active against tumors. No report had indicated, however, that a DNA-rich fraction from BCG has antitumor activity until our previous papers (1,2) showed that repeated injections or a single intralesional injection of MY-1 attached to

Freund's incomplete adjuvant caused regression of primary tumors of L10 in guinea pigs and prevented metastasis in most of the animals.

Although MY-1 has proved its low toxicity (6-9), if the dose and frequency of administration decreased, the above facts may be very advantageous to clinical application. The half life of radioactivity of ^{125}I -labeled MY-1 in plasma of male rats after sc administration was estimated at 11-13 hr by Lochmann et al (26). We attempted to detain MY-1 at the injection site by modifying it according to Levy et al. (10), who complexed poly-IC with poly LL/CMC and made them 4-10 times more resistant to hydrolysis than the original compound. As described in this report, we succeeded in marked improvement of MY-1 by modifying it with poly LL/CMC. Namely, in the previous experiment with unmodified MY-1 (1), guinea pigs received repeated intralesional injections of 500 or 1,000 μg of MY-1 every other day for a total of 10 times and three and four of six animals survived by the treatment, respectively. The same treatment with less than 200 μg of MY-1 showed little effect on the tumor regression (1). However, MY-1/poly LC complex exhibited the antitumor activity comparable to that of parent MY-1 with a reduced amount of MY-1 (from 500 or 1,000 to 200 μg) and with less injections (from 10 to 7 times).

About the toxicologic character of the poly LL derivatives, Levy et al. (10) stated that (a) iv injection of 200 μg of the poly ICLC complex daily for 6 days did not cause any overt toxicity in mice, (b) intrathecal injection of 3 mg/kg of the complex showed no evidence of toxicity to the central nervous system in the monkeys, (c) iv injection of 3 mg/kg of the complex did not cause any toxic effect in chimpanzees. Talmadge et al. (11,12) also indicated that (a) a single ip or iv injection of 5 mg/kg of poly ICLC, (b) iv injection of 0.5 mg/kg of the derivative daily for 2 weeks, or (c) iv injection of 1.25 mg/kg three times a week for 4 weeks caused no toxic effect in mice. In our experiments described here, guinea pigs were received intralesional injection of the complex containing MY-1 and poly LL equivalent to 0.5 mg/kg and 0.38 mg/kg, respectively, three times a week for 2 weeks. However, the animals showed no sign of overt toxic effect during the observation period. These findings suggest that the poly LL/CMC derivative is no more toxic than the parent compound, the toxicity of which is considered to be acceptable for systemic use, at least in seriously diseased cases. Augmentation of the activity of an antitumor agent by such a simple method is very useful model for potentiation of immunotherapeutic effect.

ACKNOWLEDGEMENTS

This study was partly supported by a Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10 Year Strategy for Cancer Control, and from Japan Health Sciences Foundation. The authors thank Ms. M. Takegawa for her technical assistance, and Ms. K. Takahashi for her secretarial assistance.

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