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IN VIVO AUGMENTATION OF NATURAL KILLER CELL ACTIVITY WITH A DEOXYRIBONUCLEIC ACID FRACTION OF BCG

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A fraction extracted from BCG and designated MY-1, which was composed of 70.0% DNA and 28.0% RNA, was previously reported to possess strong antitumor activities against various syngeneic mouse and guinea pig tumors. An intraperitoneal injection of MY-1 (100 μg) 1 day before rendered mouse peritoneal cells cytotoxic to YAC-1 cells. The effector cells were nonadherent to plastic dishes, and the activity was destroyed by treatment with anti-asialo GM1 antiserum plus complement or carrageenan *in vitro*, but not with carbonyl-iron or anti-Thy 1.2, suggesting that the cells are natural killer (NK) cells. *In vivo* augmentation of NK activity was dependent on MY-1 dose, and reached the peak 1 day after MY-1 injection. Since NK activity in lipopolysaccharide (LPS)-nonresponder mice could be augmented by MY-1, the possibility that LPS contaminated the MY-1-augmented NK was excluded. MY-1 digested preliminarily with DNase lost its NK-inducing activity, suggesting that the DNA entity of MY-1 was essential for the activity. When mice were pretreated with anti-asialo GM1 or carrageenan, MY-1 could not render the peritoneal cells cytotoxic. Antitumor activities of MY-1 were also abolished if the animals were pretreated with anti-asialo GM1 antiserum or carrageenan, suggesting that the activities can be ascribed mainly to activated NK cells.

Key words: DNA — BCG — Natural killer cell — Biological response modifier

We reported previously that a purified nucleic acid fraction extracted from *Mycobacterium bovis* BCG, which was composed of 70.0% DNA, 28.0% RNA, 1.3% protein, 0.27% hexose and 0.1% lipid with no detectable amounts of cell wall components such as α,ε-diaminopimelic acid and hexosamine,[1] showed antitumor activity against nine different syngeneic mouse tumors and one guinea pig tumor.[1, 2] This fraction, designated MY-1, showed no direct cytotoxicity *in vitro* against these tumors.[2] MY-1 after digestion with RNase contained 97.0% single-stranded DNA with a guanine-cytosine content of 69.8%, and showed stronger antitumor activities than undigested MY-1, while MY-1 digested with DNase contained 97.0% RNA, and had reduced activity.[1] These results suggest that under certain conditions the DNA from BCG possesses strong antitumor activity which acts through host-mediated mechanism(s).

In this paper, we report that MY-1 augments NK*3 cell activity *in vivo*. Evidence that this mechanism may play an important role in the antitumor activity of MY-1 is also presented.

MATERIALS AND METHODS

MY-1 and Its Nuclease-digested Fractions Methods of preparation and physicochemical characteristics of MY-1, of the RNase digest of MY-1 consisting of 97.0% DNA, and of the DNase digest of MY-1 consisting of 97.0% RNA, were described previously.[1] The lyophilized preparations were dissolved in PBS before use.

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*3 Abbreviations used: NK, natural killer; E/T ratio, the ratio of the effector cells to target cells; FCS, fetal calf serum; FCS-HBSS, HBSS containing 5% FCS; FCS-RPMI medium, RPMI 1640 medium supplemented with 10% FCS, 100 U of penicillin per ml and 100 μg of streptomycin per ml; HBSS, Hanks' balanced salt solution; ^{3}H-TdR, [methyl-^{3}H]thymidine; id, intradermal(ly); il, intralesional(ly); ip, intraperitoneal(ly); iv, intravenous(ly); LPS, lipopolysaccharide; PBS, phosphate-buffered saline; poly I:C, polyinosinic-polycytidylic acid.

Culture Medium RPMI 1640 medium (Gibco, Grand Island, N.Y.) was supplemented with 10% FCS (Gibco), 100 U of penicillin per ml and 100 μg of streptomycin per ml (FCS-RPMI medium).

Antisera, Enzymes and Other Agents Anti-asialo GM1 antiserum was prepared as an ammonium sulfate precipitate of whole antiserum of rabbits immunized with asialo GM1[3] purified from bovine brain.[4] Anti-Thy 1.2 antiserum (F7D5 monoclonal antibody, Serotec Ltd., Bicester, Oxon, England) and guinea pig complement (low-tox. complement for mouse lymphocytes, Cedarlane Laboratories Ltd., Hornby, Ontario) were purchased. DNase I (EC 3.1.4.5, 2253 U/mg) and RNase A (EC 3.1.27.5, 79 U/mg, type IA) were obtained from Worthington Biochemicals Corp., Freehold, N.J. and Sigma Chemical Co., St. Louis, Mo., respectively. LPS (*Escherichia coli*, Serotype 0111:B4, Sigma), poly I:C (Yamasa Shoyu Co., Ltd., Choshi, Chiba), carrageenan (Lambda type, Sigma) and carbonyl-iron (Wako Pure Chemical Industries, Ltd., Osaka) were also used.

Mice Female mice of BALB/c, BALB/c *nu/nu*, DBA/2, CDF1 (BALB/c♀ × DBA/2♂), C57BL/6, and A/J strains were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Shizuoka). Female C3H/HeN and C3H/HeJ mice were obtained from Charles River Japan, Inc. (Atsugi, Kanagawa) and from the Jackson Laboratory (Bar Harbor, Me.), respectively. The animals were bred under specific-pathogen-free conditions at a constant temperature and humidity, and used at 6–7 weeks of age.

Preparation of Various Effector Cells Peritoneal cells were harvested from mice by lavage with 5 ml of FCS-HBSS. The cells were resuspended in 10 ml of FCS-RPMI medium and seeded into Costar #3100 plastic dishes (Data Packaging Corp., Cambridge, Mass.). After incubation at 37° for 60 min, the dishes were swirled gently, and the nonadherent cells were recovered. The dishes were rinsed once with 10 ml of warm FCS-RPMI medium, and nonadherent cells were recovered again and pooled.

In some experiments, plastic-adherent cells were also used as the effector cells. The plastic dishes, after nonadherent cells had been removed, were rinsed once with 5 ml of warm PBS, and 10 ml of PBS was added. After incubation at 4° for 30–40 min, the plastic-adherent cells were recovered with a rubber policeman, and resuspended in FCS-RPMI medium.

These nonadherent and adherent peritoneal cells were adjusted to desired cell concentrations with FCS-RPMI medium on the basis of viability determined by trypan blue dye exclusion, and used as effector cells for the cytotoxicity test.

***In vitro* Treatment of Effector Cells** In an experiment, peritoneal nonadherent cells (1×10^6) were suspended in HBSS containing serially diluted anti-asialo GM1 antiserum or anti-Thy 1.2 antiserum and held at 4° for 30 min. The cells were washed with HBSS, ar appropriate concentration of guinea pig complement was added, and the cells were incubated at 37° for 40 min. After being washed twice with FCS-HBSS, the cells were resuspended in FCS-RPMI medium.

Nonadherent cells were also treated with carbonyl-iron.[5] One-tenth volume of 5% carbonyl-iron was added to nonadherent cells suspended in RPMI 1640 supplemented with 10% autologous mouse serum. After incubation at 37° for 60 min, the cell suspension was subjected to Ficoll-sodium metrizoate (d=1.090, Japan Immunoresearch Laboratory Co., Ltd., Takasaki, Gumma) gradient centrifugation (1,200*g*, 20 min). The mononuclear cells recovered from the interface of the gradient contained less than 1% macrophages as determined by yeast-phagocytosis[5] and morphologic examination. The cells were washed twice with FCS-HBSS and resuspended in FCS-RPMI medium.

The viability of the nonadherent cells thus treated was always more than 95% as determined by trypan blue dye exclusion.

Target Cells Cells of the cultured cell lines YAC-1, Meth A, P815, RLδ1 and EL4 were grown in FCS-RPMI medium and used as target cells in the cytotoxicity test. The origins of these cells were described previously,[2] except for RLδ1 of BALB/c mice, which was a gift from Prof. N. Ishida, Tohoku University (Sendai).

Cytotoxicity Assay Approximately 2×10^6 target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, Mass.) in 0.2 ml of FCS-RPMI medium for 50 min. The labeled cells were washed three times with FCS-HBSS and suspended at a final concentration of 5×10^4 to 1×10^5 cells per ml in FCS-RPMI medium. The cells (0.1 ml) were placed in wells of round-bottomed microtiter plates (Nunc, Roskilde, Denmark), and 0.1-ml quantities of the various effector cells were added. Unless otherwise stated, the E/T ratio was 50. The plates were centrifuged at 200*g* for 2 min and incubated at 37° in a humidified atmosphere of 5% CO_2. Unless otherwise stated, the incubation time was 4 hr. At the end of the incubation period, radioactivity released from triplicate or quadruplicate cultures was assayed with an auto-gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Results were expressed as percent lysis of the target cells according to the following formula, with the standard deviation (SD):

% Lysis = [cpm (experimental release) − cpm (spontaneous release)]/[cpm (maximum release) − cpm (spontaneous release)] × 100.

Values for spontaneous and maximum release were obtained by incubating the target cells in medium alone and in 0.5*N* HCl, respectively.

To test the peritoneal adherent cells for cytostatic activity, 0.1-ml quantities of a suspension of 2.5×10^4 P815 cells were seeded into wells of flat-bottomed tissue culture plates (Falcon #3072, Falcon Products, Oxnard, Calif.) with 0.1 ml of adherent cells. The concentrations of the adherent cells were adjusted to E/T ratios of 5 and 10. The plates were incubated for 24 hr at 37° and pulsed with 0.03 μCi of ^{3}H-TdR (5.0 Ci/mmol; Amersham International plc, Buckinghamshire, England) followed by incubation for an additional 16 hr. The cells in triplicate cultures were then harvested on glass filter papers, and the radioactivity incorporated into the tumor cells was counted with a liquid scintillation counter (Packard Instrument Co.).

Tumors IMC carcinoma of CDF1 mice, L1210 leukemia of DBA/2 mice and EL4 lymphoma of C57BL/6 mice were used. The origins of these tumors and experimental protocols of tumor suppression and tumor regression were described previously.[2]

Results

Cytotoxicity of Peritoneal Nonadherent Cells from MY-1-treated Mice against YAC-1 Cells Peritoneal cells harvested from mice given MY-1 1 day earlier were tested for cytotoxic activity. The results are shown in Table I. The nonadherent peritoneal cells from mice given MY-1 showed strong cytotoxicity, while the adherent cells showed no cytotoxicity.

Peritoneal cells were harvested from BALB/c mice injected ip with or without MY-1 (100 μg) 3 hr, 1 day, 2 days, 4 days or 7 days earlier. These cells and cells of their nonadherent and adherent fractions were tested for cytotoxicity against YAC-1 cells. The nonadherent cells always showed stronger cytotoxicity than the whole cells, and no cytotoxicity of the adherent cell fractions was observed. The strongest cytotoxicity (64.8±4.0%) was exhibited by the nonadherent cells obtained 1 day after MY-1 injection. Those obtained 2 days after MY-1 injection showed strong cytotoxicity (34.2±2.4%), but those obtained 3 hr and 4 days after MY-1 showed only weak cytotoxicity (15.7±1.0 and 19.4±1.9, respectively).

BALB/c mice were injected ip with MY-1 (300 μg). Peritoneal cells were harvested 1, 2, 3, 4, 7 and 14 days after MY-1 injection, and adherent and nonadherent cells were tested for cytostatic activity against P815 cells by 40-hr ^{3}H-TdR incorporation-inhibition assay at E/T ratios of 5

Table I. Lysis of YAC-1 Cells by Peritoneal Cells from Mice Injected Ip with MY-1

Mice	Peritoneal cells	% Lysis of YAC-1 cells
Untreated	Whole cells	3.2±1.8
	Nonadherent cells	7.8±2.5
	Adherent cells	0.3±1.1
Treated	Whole cells	27.3±3.2
	Nonadherent cells	65.3±4.3
	Adherent cells	3.8±1.4

BALB/c mice were treated with single ip injections of MY-1 (100 μg/mouse) or were untreated. Peritoneal cells were harvested 24 hr later, suspended in FCS-RPMI medium, and incubated at 37° for 2 hr in plastic dishes. Then cells adherent and nonadherent to the plastic surface were separated, and these separated peritoneal cells, in addition to unseparated whole cells, were tested for lysis (%) of YAC-1 cells by a 4-hr ^{51}Cr release assay at an E/T ratio of 50.

Table II. Characteristics of the Killer Cells Contained in Peritoneal Nonadherent Cells from MY-1-treated Mice

Nonadherent effector cells pretreated with	% Lysis of YAC-1 cells
HBSS	60.0±2.8
Carbonyl-iron	63.3±3.6
Complement (C) alone	58.6±3.3
Anti-Thy 1.2 antiserum+C	43.2±2.2
Anti-asialo GM1 antiserum+C	1.7±0.9
Normal rabbit serum+C	56.0±2.8
Nonadherent cells from PBS-injected mice (control)	9.6±0.4

BALB/c mice were injected with MY-1 (100 μg) ip, and peritoneal cells were harvested 24 hr later. Plastic-nonadherent cells were separated and treated with either carbonyl-iron or various types of antiserum plus complement by the methods described in the text. Adequate controls were included. These cells, in addition to the unseparated whole cells from MY-1-treated mice, were then tested for lytic activity against YAC-1 cells.

and 10. Although the data are not shown, no cytostatic activity of the adherent or non-adherent cells was observed.

Characterization of Effector Cells To characterize the effector cells contained in the peritoneal nonadherent cells from mice given MY-1 ip 1 day earlier, the cells were pretreated with carbonyl-iron or various antisera plus complement. The results are shown in Table II. Only the treatment with anti-asialo GM1 antiserum plus complement abolished the lytic activity against YAC-1 cells. Treatment with carbonyl-iron had almost no influence on the effector activity, and that with anti-Thy 1.2 plus complement reduced but did not abolish the activity.

BALB/c mice injected iv with anti-asialo GM1 antiserum or normal rabbit serum, or with carrageenan, were injected ip with MY-1. Peritoneal cells were harvested 1 day after MY-1 injection, and the cytotoxicity of the nonadherent fractions was assayed. As shown in Table III, cytolytic activity was markedly reduced in the nonadherent cells from mice pretreated with either anti-asialo GM1 antiserum or carrageenan.

Table III. Effect of Pretreatment of Mice with Anti-asialo GM1 Antiserum or Carrageenan on the NK-inducing Activity of MY-1

Pretreatment	% Lysis of YAC-1 cells	
	MY-1 (−)	MY-1 (+)
None	12.3±1.4	68.7±3.9
Anti-asialo GM1 antiserum	−0.3±0.5	6.3±1.8
Normal rabbit serum	14.1±1.2	66.1±4.1
Carrageenan	4.5±1.0	19.9±1.2

MY-1 (100 μg) was injected ip into BALB/c mice that had been treated iv with 0.1 ml of anti-asialo GM1 antiserum (1:5 dilution) or normal rabbit serum (1:5 dilution) 16 hr previously, or with carrageenan (0.1 mg) 2 hr previously. Peritoneal cells were harvested 24 hr later, and plastic-nonadherent cells were tested for NK activity.

Augmentation of NK Cell Activity with MY-1 in Various Mouse Strains To determine whether the activation of NK cells by MY-1 is general in different strains of mice, MY-1 (100 μg) was given ip to various mouse strains, A/J, C57BL/6, BALB/c, BALB/c *nu/nu*, DBA/2, CDF1 (BALB/c×DBA/2), C3H/HeN and C3H/HeJ. On the next day, nonadherent peritoneal cells were harvested and tested for NK activity. Although different degrees of activity were observed among the mouse strains tested, it was clear that MY-1 augmented the NK activity of peritoneal cells from all the strains, including athymic nude mice and LPS-nonresponsive C3H/HeJ mice.[6]

Lytic Activities of NK Cells against Various Target Cells The cytolytic activity of plastic-nonadherent cells from mice given MY-1 (100 μg) ip 1 day earlier was tested in various cultured tumor cells. As shown in Table IV, MY-1 augmented the cytolytic activity to a greater or lesser extent for all of the cells except P815. Against P815 cells, nonadherent cells activated by MY-1 were not cytotoxic even in a 16-hr ^{51}Cr assay. Although the data are not shown here, when the nonadherent cells activated by MY-1 were pretreated with anti-asialo GM1 antiserum plus complement, the cyto-

Table IV. Lysis of Various Target Cells by Plastic-nonadherent Peritoneal Cells from Mice Injected Ip with MY-1

Target cells	% Lysis			
	4-hr assay		16-hr assay	
	Control	MY-1-injected	Control	MY-1-injected
YAC-1	12.8±2.1	68.1±3.7	25.1±1.8	80.9±5.6
RLδ1	13.6±1.1	66.0±4.8	22.6±1.5	77.9±6.0
Meth A	1.1±0.6	20.1±1.6	4.4±2.3	36.0±2.4
P815	−0.4±1.2	0.3±2.0	0.3±2.1	0.4±1.1
EL4	6.7±1.2	34.8±2.0	15.9±1.3	49.3±2.5

BALB/c mice were injected ip with 100 μg of MY-1, or were not injected, and peritoneal cells were harvested 24 hr later. Plastic-nonadherent peritoneal cells were tested for cytolytic activity against various target cells by 4-hr and 16-hr ^{51}Cr release assay at an E/T ratio of 50.

Table V. Lysis of YAC-1 Cells by Plastic-nonadherent Peritoneal Cells from Mice Given Various Doses of MY-1, LPS or Poly I:C

Dose (μg)	% Lysis of YAC-1 cells		
	MY-1	LPS	Poly I:C
0	9.7±1.0		
0.1	8.9±0.9	16.4±1.8	27.9±1.1
1	10.3±1.1	23.6±2.2	54.8±0.7
3	14.7±0.8	NT	NT
10	19.4±1.2	60.9±3.7	59.3±1.2
30	51.6±2.3	NT	NT
100	61.7±3.8	58.5±2.9	82.2±2.2
300	81.6±5.2	NT	NT

BALB/c mice were injected ip with various doses of MY-1, LPS or poly I:C, and peritoneal cells were harvested 24 hr later. Plastic-nonadherent cells were obtained and tested for cytolytic activity against YAC-1 cells by a 4-hr ^{51}Cr release assay at an E/T ratio of 50. NT: Not tested.

lytic activity against YAC-1, RLδ1, Meth A and EL4 was completely abolished.

Comparison of NK-augmenting Ability of MY-1, LPS and Poly I:C Peritoneal nonadherent cells harvested from BALB/c mice injected ip with various doses of MY-1, LPS or poly I:C 1 day earlier were tested for cytotoxicity against YAC-1 cells. As shown in Table V, all of MY-1, LPS and poly I:C strongly augmented the cytotoxicity of the cells. The effect of MY-1 was somewhat inferior to that of the others; to obtain more than 50% cytolysis, 30 μg of MY-1 was necessary, while 10 μg or less of LPS or 1 μg or less of poly I:C was sufficient.

NK-inducing Activity of MY-1, RNase Digest of MY-1 and DNase Digest of MY-1 The NK-inducing activities of 100 μg of MY-1, the RNase digest of MY-1 and the DNase digest of MY-1 were compared. In addition, these substances were further treated with DNase or RNase, and tested for NK-inducing ability. The results are shown in Table VI. NK activity induced by MY-1 was a little weaker than that induced by the RNase digest of MY-1, but much stronger than that induced by the DNase digest of MY-1. Treatment of MY-1 with DNase destroyed the NK-inducing ability but treatment with RNase did not influence it. The NK-inducing activity of the RNase digest of MY-1 was abolished by DNase treatment. That of the DNase digest of MY-1 was reduced by RNase treatment to the level of that of RNase alone.

Table VI. NK-inducing Activity of MY-1, the RNase Digest of MY-1 and the DNase Digest of MY-1, with or without Further Nuclease Treatment

	Material injected	% Lysis of YAC-1 cells
MY-1	Untreated	67.4±2.0
	Treated with DNase	6.4±0.3
	Treated with RNase	63.6±1.9
RNase digest of MY-1	Untreated	71.6±2.1
	Treated with DNase	13.0±2.0
DNase digest of MY-1	Untreated	32.3±2.5
	Treated with RNase	20.3±1.5
PBS (control)		6.8±1.2
DNase alone (control)		10.1±0.7
RNase alone (control)		20.8±1.5

Preparations of MY-1, the RNase digest of MY-1 and the DNase digest of MY-1 were as described.[1] These substances were further treated with DNase or RNase as follows: 10 mg of each substance was dissolved in 10 ml of PBS and incubated at 37° for 4 hr either with 200 U of DNase I in the presence of 5m*M* $MgCl_2$, or with 30 U of RNase A. Incubation mixtures consisting of substrate alone were used as controls. After incubation, an appropriate volume of PBS was added to the incubation mixtures to give a substrate concentration of 0.5 mg/ml. All of these substances, in amounts corresponding to 100 μg of each substrate, were injected ip into BALB/c mice. Nonadherent cells were derived from peritoneal cells harvested 24 hr after the injection of MY-1 and its various derivatives and tested for NK activity.

***In vivo* Effect of Anti-asialo GM1 Antiserum, Anti-Thy 1.2 Antiserum, or Carrageenan on Antitumor Activity of MY-1** CDF1 mice treated with anti-asialo GM1 antiserum, normal rabbit serum, or carrageenan were inoculated 30 min later with a mixture containing IMC tumor cells and MY-1, or with tumor cells alone. The mice were killed 28 days later, and the tumors were weighed (Table VII). Treatment with either anti-asialo GM1 or carrageenan reduced the tumor-suppressive effect of MY-1, while anti-Thy 1.2 did not influence the effect.

Table VII. Effect of Pretreatment of Mice with Anti-asialo GM1 Antiserum, Anti-Thy 1.2 Antiserum, or Carrageenan on the Suppression of the Growth of IMC Carcinoma Inoculated Along with MY-1

Pretreatment	MY-1	No. of mice	Tumor weight	
			Mean, g±SD	Inhibition (%)
PBS	−	10	4.14±1.27	
	+	10	0.41±0.38	90
Normal rabbit serum	−	10	3.51±1.00	
	+	10	0.65±0.38	81
Anti-asialo GM1	−	10	5.76±1.10	
	+	10	3.64±1.67	37
Anti-Thy 1.2	−	10	2.92±1.58	
	+	10	0.18±0.24	94
Carrageenan	−	10	2.74±1.10	
	+	10	2.15±1.16	22

CDF1 mice were injected iv with 0.1 ml of anti-asialo GM1 antiserum (1:5 dilution), anti-Thy 1.2 antiserum (1:20 dilution), carrageenan (5 mg/ml), PBS or normal rabbit serum (1:5 dilution). Thirty minutes later, they were inoculated id with a mixture of IMC carcinoma cells (5×10^5) and MY-1 (100 μg) or IMC cells only. All the mice were killed 28 days later, and the tumors were weighed. The degree of inhibition (%) was expressed by the following fourmula: $[1-(\text{mean weight of tumors from mice given MY-1/mean weight of tumors from mice given no MY-1})]\times100$ (%).

Next, CDF1 mice were inoculated with IMC cells, and given a single il injection of MY-1 12 days later. Anti-asialo GM1, normal rabbit serum, anti-Thy 1.2 and carrageenan were injected 30 min before the MY-1 injection. The tumors were weighed 35 days later (Table VIII). Anti-asialo GM1 caused some reduction in the tumor-regressive effect of MY-1. Carrageenan reduced the effect a little more. Anti-Thy 1.2 had no influence on the antitumor activity of MY-1.

Third, C57BL/6 and DBA/2 mice were injected iv with anti-asialo GM1 antiserum. Thirty minutes later, the C57BL/6 mice were inoculated id with a mixture containing EL4 cells and MY-1 or with EL4 cells alone, and DBA/2 mice with a mixture containing L1210 cells and MY-1 or with L1210 cells alone. Survival of these mice is shown in Fig. 1. The treatment with anti-asialo GM1 antiserum completely abolished the tumor-suppressive effect against both tumors.

Table VIII. Effect of Pretreatment of Mice with Anti-asialo GM1 Antiserum, Anti-Thy 1.2 Antiserum, or Carrageenan on the Regression of IMC Carcinoma Caused by Single Intralesional Injection of MY-1

Pretreatment	MY-1	No. of mice	Tumor weight	
			Mean, g±SD	Inhibition (%)
PBS	−	9	3.10±1.14	
	+	9	0.70±0.82	77
Normal rabbit serum	−	9	3.29±1.53	
	+	9	0.95±0.67	71
Anti-asialo GM1	−	9	3.42±1.87	
	+	9	1.79±1.31	48
Anti-Thy 1.2	−	9	3.35±1.82	
	+	9	1.05±0.81	69
Carrageenan	−	9	2.05±1.35	
	+	9	1.44±0.91	30

CDF1 mice were inoculated id with 5×10^5 cells of IMC carcinoma, and 12 days later they were injected iv with the same amounts of the same agents as described in Table III. These mice were given single il injections of MY-1 (100 μg/0.1 ml of PBS) or PBS (0.1 ml) 30 min after the iv treatment. All the mice were killed 35 days later, and the tumors were weighed. Inhibition (%) was expressed as described in the footnote to Table VII.

Discussion

A nucleic acid fraction composed of 70.0% DNA and 28.0% RNA, which was extracted from BCG and designated MY-1, exhibited marked antitumor activity against various syngeneic mouse and guinea pig tumors by a host-mediated mechanism(s).[1,2] In the present study, we showed that an ip injection of MY-1 into BALB/c mice rendered peritoneal cells cytolytic for YAC-1 cells in a dose-dependent manner; the activity was ascribed exclusively to the plastic-non-adherent fraction of the peritoneal cells. The cytolytic activity was detectable 3 hr after MY-1 injection and reached the maximum 1 day later.

The cytotoxic activity was not influenced by treatment with carbonyl-iron or anti-Thy 1.2 antiserum plus complement, but was abolished by anti-asialo GM1 antiserum plus complement. These results suggest that the killer cells in MY-1-activated peritoneal cells are NK cells[8,9] which are asialo GM1-positive.[3,10] We reported that activated peritoneal macrophages are also asialo GM1-

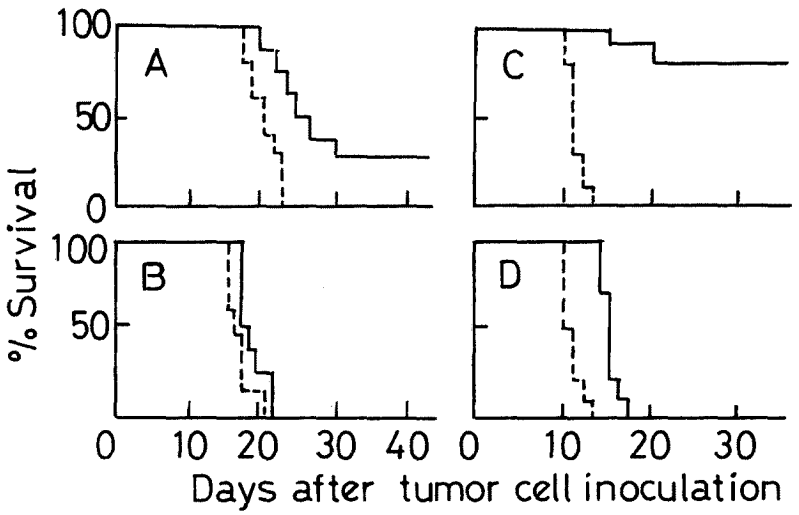

Fig. 1. Effect of pretreatment of mice with anti-asialo GM1 antiserum on the suppression of the growth of EL4 and L1210 leukemia cells inoculated as a mixture with MY-1. C57BL/6 mice (groups A and B) and DBA/2 mice (groups C and D) were injected iv with 0.1 ml of anti-asialo GM1 antiserum (1:5 dilution) (groups B and D) or normal rabbit serum (1:5 dilution) (groups A and C). Thirty minutes later, the C57BL/6 mice were inoculated id with a mixture (0.1 ml) containing EL4 cells (5×10^4) and MY-1 (400 μg) or with EL4 cells alone, and the DBA/2 mice were given id a mixture (0.1 ml) containing L1210 cells (1×10^4) and MY-1 (800 μg) or L1210 cells alone. Observation for survival was terminated on day 45 for EL4 and day 35 for L1210. Solid lines indicate the survival rates for mice inoculated with a mixture of tumor cells and MY-1, and dotted lines indicate the rates for those given tumor cells alone.

positive.[11] However, they are adherent and are removed by carbonyl-iron. In addition, peritoneal adherent cells from mice given MY-1 ip 1, 2, 3, 7 or 14 days earlier were not cytostatic against P815 cells, though adherent cells obtained from mice given MY-1 4 days previously showed a weak cytostatic activity. Therefore, the role of activated macrophages seems to be less important than that of NK cells. Anti-Thy 1.2 antiserum plus complement slightly reduced the cytotoxic activity of the peritoneal cells. These findings seem to be in good agreement with the observation by Durdik *et al.*[12] that "activated" NK cells display a higher density of Thy 1 antigen on their surface than unstimulated NK cells.

It was reported from our laboratory[14] that iv injection of a small amount of anti-asialo GM1 antiserum abolishes NK activity in the spleen cells from athymic nude mice and enhances the local growth of syngeneic lymphoma as well as the incidence of tumor take, which indicates an important role of NK cells in resistance to transplanted tumors. In the present study, iv injection of anti-asialo GM1 16 hr before MY-1 injection abolished the augmentation of NK cell activity by MY-1.

Furthermore, pretreatment of mice with carrageenan also abolished the augmentation of NK activity with MY-1. Carrageenan is known to suppress macrophages[15] and NK cells,[7, 16] and the present results can be explained by the fact that macrophages are required for augmentation of NK cell activity.[16] Although the data will be published separately, the NK activity of normal mouse spleen cells was augmented by *in vitro* incubation with MY-1, and the augmentation was abrogated by removal of macrophages from the spleen cells before adding MY-1. This may support the above hypothesis.

MY-1 is composed of 70.0% DNA, 28.0% RNA, 1.3% protein, 0.27% hexose, and

0.1% lipid, and DNA is essential for the antitumor activity of MY-1, because the RNase digest of MY-1 containing mostly DNA (97%) showed stronger antitumor activity than MY-1, while the DNase digest of MY-1, composed mostly of RNA (97%) showed weaker activity.[1] In the present study, we showed that the NK-augmenting activity of MY-1 is also due to DNA. The RNase digest of MY-1 activated NK cells as strongly as MY-1, and the activity was almost completely lost after digestion with DNase (Table VI). The DNase digest of MY-1, on the other hand, showed only a weak activity.

Several reports have presented evidence that NK cells play an important role in host resistance to malignant cells *in vivo*,[14, 17–21] and, therefore, we investigated whether or not the antitumor activities of MY-1[1, 2] can be ascribed to its NK-augmenting activity. Mice were pretreated with either anti-asialo GM1 antiserum, anti-Thy 1.2 antiserum or carrageenan, and 30 min later, various kinds of syngeneic tumor cells were inoculated. When a mixture of MY-1 and the cells of IMC carcinoma, EL4 leukemia or L1210 leukemia was inoculated, the antitumor activity of MY-1 was decreased markedly in mice pretreated with either anti-asialo GM1 antiserum or carrageenan, but not anti-Thy 1.2 (Table VII and Fig. 1). When established IMC tumor were injected il with MY-1, similar results were obtained; mice pretreated with either anti-asialo GM1 or carrageenan showed reduced antitumor activity, compared with mice treated with either PBS, normal rabbit serum or anti-Thy 1.2 antiserum (Table VIII). These results suggest an important role of NK cells in the antitumor activity of MY-1.

Mouse NK cells are reported to be activated by a variety of immunomodulating agents including BCG,[22] LPS,[13] and synthetic double-stranded RNA, especially poly I:C.[13] The possibility that the NK-augmenting activity of MY-1 is caused by contamination with LPS can be excluded, because MY-1 augmented NK activity in LPS-nonresponsive C3H/HeJ mice,[6, 7] and the activity was DNase-sensitive. This paper reports for the first time the activation of NK cells by DNA. Although the activity of MY-1 seemed a little inferior to that of poly I:C or LPS (Table V), the toxicity of MY-1 was much less than that of poly I:C or LPS (unpublished). Recently, we found that incubation of normal mouse spleen cells with MY-1 resulted in not only augmentation of NK activity but also induction of interferon (E. Kuramoto *et al.*, manuscript in preparation). We found also that synthetic DNA with certain structures showed such activities.[23] Details will be published elsewhere.

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