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A SYNTHETIC SINGLE-STRANDED DNA, POLY(dG,dC), INDUCES INTERFERON-α/β AND -γ, AUGMENTS NATURAL KILLER ACTIVITY, AND SUPPRESSES TUMOR GROWTH

Tohru TOKUNAGA, Saburo YAMAMOTO and Kenji NAMBA
Department of Cellular Immunology, National Institute of Health, 2-10-35 Kamiosaki, Shinagawa-ku, Tokyo 141

When various synthetic double- or single-stranded DNAs were incubated with spleen cells from mice (BALB/c or CDF_1) at 37° for 20 hr, it was found that some of the DNAs augmented NK activity and produced factors in the culture supernatants which showed antiviral activity and activity to render mouse macrophages cytotoxic toward tumor cells. Poly(dG,dC) showed the strongest activities, when incubated with spleen cells from lipopolysaccharide-nonresponsive mice, C3H/HeJ. The activity of the culture supernatant to activate macrophages was completely abolished by a small amount of anti-IFNγ antibody. On the other hand, the virus-inhibitory activity of the supernatant was mostly neutralized by anti-IFNα/β. When IMC tumor cells (5×10^5 cells) were mixed with poly(dG,dC) (100 μg) and then inoculated intradermally into CDF_1 mice, the tumor did not take, while tumors grew progressively and killed the mice in a control group inoculated with tumor cells alone. Direct cytotoxicity of poly(dG,dC) at a concentration of 1,000 μg/ml against IMC cells was not observed *in vitro*.

Key words: Interferon — Macrophage activating factor — Natural killer cells — Synthetic single-stranded DNA — Polydeoxyriboguanylic, deoxyribocytidylic acid

Abbreviations: DNA, deoxyribonucleic acid; IFN, interferon; NK, natural killer; poly(rI)·poly(rC), polyriboinosinicpolycytidylic acid; RNA, ribonucleic acid; LPS, lipopolysaccharide; MAF, macrophage activating factor.

Synthetic polyanionic agents, such as double-stranded poly(rI)·poly(rC) and maleic vinyl ether (pyran copolymer), are known to be inducers of IFN and of NK activity either *in vivo* or *in vitro*.[1–3] In contrast, little is known about such activities of synthetic DNA, though calf-thymus DNA and polydeoxyribonucleotides have been reported to induce little or no IFN.[3–5] Colby[4] speculated that double-stranded RNA formed in cells infected with DNA-containing viruses might be responsible for the induction of IFN.

Recently, we have demonstrated that a fraction (designated MY-1) which was isolated and purified from *Mycobacterium bovis* BCG, and composed of 70.0% DNA, 28.0% RNA, 1.3% protein, 0.27% hexose and 0.1% lipid, showed strong antitumor activity against many syngeneic mouse and guinea pig tumors.[6,7] NK activity was augmented remarkably by a single injection of MY-1 (100 μg) in mouse[8] and human (unpublished data). Incubation of mouse spleen cells with MY-1 (100 μg/ml) augmented NK activity *in vitro* and produced IFN-α/β and -γ in the culture supernatant.[9] All of these activities were ascribed to DNA contained in MY-1, because MY-1 digested with DNase lost its activities, whereas MY-1 digested with RNase was more effective than the undigested MY-1. In this study, we tried to determine whether or not synthetic DNAs possess such biologic activities.

As listed in Table I, 4 single-stranded homopolymers, 2 single-stranded copolymers, 2 double-stranded homopolymers and 2 double-stranded copolymers of DNA, and 1 double-stranded homopolymer of RNA were employed. Poly(dA)·poly(dT), poly(dG)·poly(dC) and poly(dA, dT)·poly(dA, dT) were purchased from Sigma Chemicals Company (Saint Louis, Mo.), poly(rI)·poly(rC) from Yamasa Shoyu (Choshi, Chiba), and the other polynucleotides from P-L Biochemicals (Milwaukee, Wis.). Rabbit antimouse IFN-α/β antibody (1.0×10^4 IRU/ml) was purchased from Lee BioMolecular Research

Table I. NK Activity and MAF and IFN Production of Spleen Cells Stimulated with Various Synthetic DNAs and RNA

Exp.	Sample	Type[a]	NK activity	MAF activity	IFN activity
1	Poly(dA)	ss-h	12.3±0.3	6.2±2.2	<4
	Poly(dT)	ss-h	13.5±2.7	5.6±2.1	<4
	Poly(dG)	ss-h	7.2±1.6	0.4±1.1	<4
	Poly(dC)	ss-h	7.0±1.9	5.2±2.7	<4
	Poly(dA,dT)	ss-c	69.1±5.5	24.8±4.7	192
	Poly(dG,dC)	ss-c	69.0±4.7	26.7±3.4	192
	Poly(dA)·poly(dT)	ds-h	9.6±1.5	6.8±3.3	<4
	Poly(dG)·poly(dC)	ds-h	39.9±4.8	28.5±0.8	16
	Poly(dA,dT)·poly(dA,dT)	ds-c	9.1±3.2	6.4±1.7	8
	Poly(dG,dC)·poly(dG,dC)	ds-c	36.9±4.7	32.1±5.0	32
	Poly(rI)·poly(rC)	ds-h	82.8±5.1	38.5±4.5	192
	None		5.5±1.2	5.2±2.1	<4
2	Poly(dA,dT)	ss-c	1.9±2.2	2.8±3.0	<4
	Poly(dG,dC)	ss-c	28.2±3.1	30.4±0.2	46
	Poly(dA)·poly(dT)	ds-h	2.2±0.6	0.2±0.3	<4
	Poly(dG)·poly(dC)	ds-h	2.1±1.0	1.9±0.5	<4
	Poly(dA,dT)·poly(dA,dT)	ds-c	1.7±0.8	1.3±2.2	<4
	Poly(dG,dC)·poly(dG,dC)	ds-c	12.3±0.7	0.5±0.3	<4
	Poly(rI)·poly(rC)	ds-h	65.9±4.7	42.3±3.4	64
	None		3.8±3.2	2.8±1.7	<4

Spleen cells (1×10^7/ml) from BALB/c (in Exp 1) or C3H/HeJ (in Exp 2) were incubated with 100 μg/ml each of the synthetic DNA or RNA at 37° for 20 hr. Then, the cells were assayed for NK activity against RL 1 cells, and the culture supernatants were tested for MAF and IFN activity. The NK activity was expressed as % lysis of ^{51}Cr-labeled RL♂1 cells after a 4-hr incubation. The MAF activity was expressed as % lysis of ^{51}Cr-labeled EL4 cells after a 20-hr incubation. The IFN activity was expressed as international units per milliliter for the 50% inhibition of the cytopathic effect of vesicular stomatitis virus against L929 cells.
a) ss, single-stranded; ds, double-stranded; h, homopolymer; c, copolymer.

Laboratories, Inc. (San Diego, Calif.). Rabbit antimouse IFN-γ antibody (1.2×10^3 IRU/ml) and monoclonal antimouse IFN-γ antibody (6.8×10^3 IRU/ml) were gifts from Dr. Y. Watabe and Prof. Y. Kawade (Institute for Virology, Kyoto University, Kyoto), and Dr. R. D. Schreiber (Research Institute of Scripps Clinic, La Jolla, Calif.), respectively. Female BALB/c or BALB/c×DBA/2, F_1 (CDF_1) mice were purchased from Shizuoka Experimental Animal Farm (Hamamatsu, Shizuoka). C3H/HeJ mice were raised in our laboratory. All mice were used at the age of 6–8 weeks. After incubation of 1×10^7 spleen cells with 100 μg/ml of each polynucleotide for 20 hr, NK activity was evaluated by measuring the cytolysis of RL♂1 cells in a 4-hr ^{51}Cr release assay.[8] At the same time, a part of the culture was centrifuged and the activities of MAF and IFN in the supernatants were assayed. Namely, mouse peritoneal exudate macrophages induced by proteose-peptone were incubated with the supernatants supplemented with a small amount of LPS, and MAF activity was measured by a 20-hr ^{51}Cr release assay using labeled EL4 cells as target cells[8]; IFN titer was expressed as the reciprocal of the highest dilution inhibiting 50% of the cytopathic effect of vesicular stomatitis virus against L929 cells.[8]

The results are shown in experiment 1 of Table I. Four deoxyribonucleotides, poly(dA,dT), poly(dG,dC), poly(dG)·poly(dC) and poly(dG,dC)·poly(dG,dC) augmented NK activity and induced both MAF and IFN, as did an RNA, poly(rI)·poly(rC), which was used as a positive control. Some of the other synthetic DNA showed these activities, but at much lower levels. We wondered if the activities of the polynucleotides were caused by

possible LPS contamination of the commercial preparations, because some preparations showed positive reactions in the *Limulus* test. To rule out this possibility, spleen cells of an LPS non-responder mouse strain, C3H/HeJ, were employed for examining the activity of the 7 selected polynucleotides. The experiment was repeated 3 times, and similar results were obtained. The results of one experiment are shown in Exp. 1 of Table I. It was indicated that only poly(dG,dC) and poly(rI)·poly(rC) augmented NK activity, and induced MAF and IFN. This does not necessarily mean that the activities of the other polynucleotides in experiment I were all caused by contaminating LPS; it is possible that such polynucleotides were not effective on the spleen cells from this particular strain of mouse for some reason. In any case, we decided to use poly(dG,dC) in the subsequent experiments. Poly(dG,dC) purchased from P-L Biochemicals was a random copolymer prepared from deoxynucleoside triphosphates and terminal deoxy-nucleotidyl transferase, and contains approximately equal amounts of the two bases arranged in a random sequence. However, the DNA in solution seemed to form a conformational intermediate with hydrogen-bonded double helixes and single strands, because the hyperchromicity measured was 15.4%. The values of λ_{max}, A250nm/A260nm and A280nm/A260nm at pH 7.0 were 253 nm, 1.06 and 0.55, respectively. The molecular size of the poly(dG,dC) measured by alkaline agarose gel electrophoresis was 0.3–1.0 kilobases.

A nonadherent cell fraction of C3H/HeJ mice was obtained by passage through a Sephadex G-10 column. Residual macrophages in this fraction were less than 0.7% when measured by latex bead ingestion. Adherent cells were obtained by incubating the spleen cells in plastic dishes for 2 hr; more than 70% of these cells ingested latex beads. The fractionated cells (1×10^7) were incubated with poly(dG,dC) or poly(rI)·poly(rC) for 20 hr. As shown in Table II, nonadherent cells incubated with these agents showed no NK activity and produced neither MAF nor IFN. In contrast, adherent cells incubated with either of the agents could produce MAF strongly and IFN weakly. When a mixture of the nonadherent and adherent spleen cells was incubated with the agents, strong activities were observed.

C3H/HeJ spleen cells were incubated with poly(dG,dC) (100 μg/ml) for 20 hr, and then the culture supernatant was treated with various anti-IFN antisera. As shown in Table III, the treatment with a small amount of either

Table II. Effects of Poly(dG,dC) and Poly(rI)·poly(rC) on the Adherent, Nonadherent and Reconstituted Fraction of Spleen Cells

Spleen cells	Sample	NK activity	MAF activity	IFN activity
Whole	None	5.6±1.2	8.3±2.2	<4
	Poly(dG,dC)	29.9±2.4	39.9±5.4	64
	Poly(rI)·poly(rC)	42.9±0.9	35.5±5.6	128
Nonadherent	None	2.5±1.5	3.7±1.8	<4
	Poly(dG,dC)	4.5±3.3	4.8±3.0	<4
	Poly(rI)·poly(rC)	7.8±3.7	3.2±2.9	<4
Adherent	None	2.9±0.2	9.8±3.2	<4
	Poly(dG,dC)	4.4±1.2	37.7±4.6	8
	Poly(rI)·poly(rC)	7.8±0.4	27.1±5.5	4
Reconstituted	None	6.9±0.8	7.3±4.4	<4
	Poly(dG,dC)	22.4±1.1	32.2±3.8	64
	Poly(rI)·poly(rC)	50.9±0.6	31.0±2.9	256

Nonadherent cells were obtained from C3H/HeJ spleen cells by passage through a Sephadex G-10 column. Adherent cells were obtained by incubation in plastic dishes for 2 hr. Cells (1×10^7/ml) of each fraction or the mixed fractions were incubated with poly(dG,dC) (100 μg/ml), poly(rI)·poly(rC) (10 μg/ml) or medium alone for 20 hr. The cells were tested for NK activity, and the supernatants were assayed for MAF and IFN activities.

polyclonal or monoclonal anti-IFNγ antiserum neutralized the MAF activity, but could not destroy the antiviral activity. On the other hand, the treatment with anti-IFNα/β antiserum did not influence the MAF activity but destroyed the IFN activity. These results suggest that poly(dG,dC) stimulated the spleen cells to produce both IFN-α/β and -γ. The viral plaque formation was inhibited mainly by the IFNα/β produced. The amount of IFNγ produced was not enough to inhibit plaque formation; the IFNγ was detectable only by a more sensitive assay method, MAF assay.

Table III. Effect of Anti-IFNα/β or Anti-IFNγ Antisera on MAF and IFN Activities in Culture Supernatant of Spleen Cells Incubated with Poly (dG,dC)

Treatment	MAF activity	IFN activity
None	25.0±6.4	45
Anti-IFNα/β	26.6±2.1	<4
Anti-IFNγ (A)	9.0±4.7	32
Anti-IFNγ (B)	8.7±3.7	32

C3H/HeJ mouse spleen cells (1×10^7/ml) were incubated with poly(dG,dC) (100 μg/ml) for 20 hr. The supernatant was harvested and treated with anti-IFNα/β (1,000 U/ml), rabbit anti-IFNγ (10 U/ml) (A) or monoclonal anti-IFNγ (1 U/ml) (B) for 60 min. The treated supernatants were tested for MAF and IFN activities.

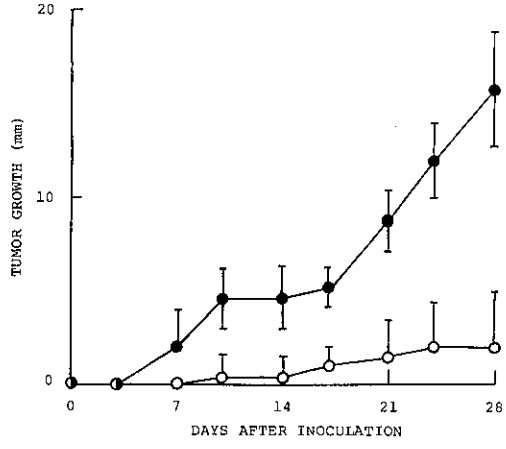

Fig. 1. Effect of poly(dG,dC) on the growth of IMC tumor cells in CDF_1 mice. IMC tumor cells (5×10^5 cells) were inoculated intradermally with poly(dG,dC) (100 μg) (○) or with saline (●) into 5 mice each. The lesions at the injection sites were measured with callipers twice a week. Results are expressed as average diameters (mm±SD).

To test the *in vivo* activity of the DNA, 10 female CDF_1 mice were inoculated intradermally with 5×10^5 IMC cells with or without poly(dG,dC) (100 μg). The mice were examined twice a week to evaluate tumor growth. Results are expressed as average tumor diameter (mm±SD) and illustrated in Fig. 1. In the group given IMC cells alone, tumors grew progressively; at day 28, the mean tumor diameter was 15.6±3.0 mm. In the group given IMC cells mixed with poly(dG,dC), small nodules were produced at the injection sites, but in 3 out of the 5 mice the nodules regressed completely. The tumors in the remaining 2 mice grew gradually but much more slowly than in the control group. The 3 animals whose nodules had regressed were challenged intradermally with 5×10^5 viable IMC cells at the contralateral sites at day 35, but all of them rejected the challenge grafts, while the tumors grew progressively in 3 control normal mice.

This paper may be the first definitive report of biologic responses to synthetic DNA. It is interesting to note that MY-1 DNA extracted from BCG, which shows strong anti-tumor activity and very low toxicity, is single-stranded and GC-rich (GC%: 69.8).[6] However, this particular base sequence may be essential for the biologic activity, because the stereostructural and physicochemical nature of the DNA in solution is unknown under the experimental conditions, and may greatly influence the biologic activities. Further investigations are required.

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