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Synthetic Oligonucleotides with Certain Palindromes Stimulate Interferon Production of Human Peripheral Blood Lymphocytes *in vitro*

Toshiko Yamamoto,[1] Saburo Yamamoto,[1] Tetsuro Kataoka,[1] Katsutoshi Komuro,[1] Masayoshi Kohase[2] and Tohru Tokunaga[3]

[1]*Department of Bacterial and Blood Products,* [2]*Department of Viral Disease and Vaccine Control, National Institute of Health, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208 and* [3]*National Institute of Health, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162*

We studied the ability of synthetic single-stranded 30-mer oligodeoxyribonucleotides (oligoDNAs) with three different kinds of hexamer palindromic sequence to induce interferon (IFN) production of human peripheral blood lymphocytes (PBL). When PBL was cultured with oligoDNA having a palindrome of AACGTT or GACGTC, IFN activity was detected by bioassay in the culture fluid after 8 h, and the amount of IFN reached the maximum after 18 h. IFN-α was predominantly produced, and small amounts of IFN-β and IFN-γ were also found. OligoDNA with the palindrome ACCGGT had no effect.

Key words: Synthetic oligoDNA — IFN production — Human PBL — Palindrome

In preceding studies, we have found that an oligonucleotide fraction extracted and purified from *Mycobacterium bovis* BCG, designated MY-1, augmented *in vitro* natural killer (NK) cell activity of human peripheral blood lymphocytes (PBL).[1] Since MY-1 showed little toxicity to humans, immunotherapeutic trials of MY-1 have been carried out against human malignant melanoma and stomach cancer.[2, 3]

MY-1 also showed strong antitumor activity against various murine and guinea-pig syngeneic tumors.[4, 5] In murine systems, MY-1 augmented NK cell activity and induced production of interferon (IFN)-α/β and -γ *in vitro* or *in vivo*.[6–8] We have already shown that the major DNA component in MY-1, which is responsible for the biological activities[7] of MY-1, consists of a mixture of single-stranded oligodeoxyribonucleotides (oligoDNAs) with various base lengths, mostly 45 bases.[9] To clarify the mode of action of the DNA molecules contained in MY-1, we synthesized a variety of 45-mer oligoDNAs, the sequence of which were randomly selected from the known cDNA sequences of 64 kDa heat shock protein (Antigen A),[10] MPB-70,[11] or α-Antigen,[12] and assessed their biological activities. The results showed that some of the oligoDNAs with a palindromic sequence stimulated murine spleen cells to produce IFN-α/β and -γ, and augmented NK activity, while others without a palindromic sequence were ineffective.[9] Subsequently, it was demonstrated in murine tumor systems that antitumor activity of the oligoDNAs was closely correlated with their biological activities detected *in vitro*.[13] It appeared that the presence of certain palindromic sequences, such as GACGTC, AGCGCT, and AACGTT, but not ACCGGT, is required for the immunostimulatory activity of the oligoDNAs[14]; oligoDNAs with base lenghts of 18 or more were active and the activity was proportional to the base length with a maximum at 22–30-mer.[15]

For clinical application of MY-1, it is important to confirm the biological significance of the oligoDNA molecules in humans, rather than in the murine system. It is also essential to know whether oligoDNAs with certain palindromic sequences are required for stimulation of human PBL to secrete IFN.

Human PBL from healthy donors was prepared by gradient centrifugation. Human amnion-derived epithelial FL cells[16] for potency assay of IFN were maintained in Roux bottles using Eagle's minimum essential medium (MEM) supplemented with 5% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Preparation and physicochemical characterization of MY-1 were described previously.[4] OligoDNAs were synthesized on an automated DNA synthesizer (Gene Assembler Plus: Pharmacia Inc., Uppsala, Sweden), purified by ethanol precipitation followed by gel-filtration chromatography, and then lyophilized.[14] Purity of the oligoDNAs was confirmed by high-pressure liquid chromatography and polyacrylamide gel electrophoresis. The names and the sequences of the oligoDNAs employed are shown in Table I. Polyinosinic-polycytidylic acid (poly I:C) was purchased from Sigma Chemical Company (St. Louis, Mo., USA). Rabbit anti-human IFN-α Ly antiserum (1×10^4 neutralizing units/ml) and anti-recombinant human IFN-γ antiserum (3×10^3 neutralizing units/ml) used were standard sera in Japan. Goat anti-human IFN-β antiserum (1×10^4

Table I. OligoDNA Sequences

Name	Base sequence (5′–3′)[a]
GAC-30	ACCGAT GACGTC GCCGGT GACGGC ACCACG
AAC-30	ACCGAT AACGTT GCCGGT GACGGC ACCACG
ACC-30	ACCGAT ACCGGT GCCGGT GACGGC ACCACG

a) Underlines indicate palindromic sequence.

Table II. Effect of Stimulation Dose of Nucleotide on IFN Production by Human PBL

Nucleotide	μg/ml	IFN (U/ml)					
		50	10	2	0.4	0.08	0 (medium)
Donor 1							
MY-1		>12800	>12800	1600	50	25	ND[a]
AAC-30		>12800	100	ND	ND	ND	
ACC-30		ND	ND	ND	ND	ND	
Poly I:C		400	200	100	50	25	
Donor 2							
MY-1		>12800	>12800	400	ND	ND	ND
AAC-30		>12800	400	12	ND	ND	
ACC-30		ND	ND	ND	ND	ND	
Poly I:C		25	12	ND	ND	ND	
Donor 3							
MY-1		8000	250	32	NT[b]	NT	ND
GAC-30		500	63	ND	NT	NT	
AAC-30		500	63	ND	NT	NT	
ACC-30		ND	ND	ND	NT	NT	
Poly I:C		125	125	32	NT	NT	
Donor 4							
MY-1		32000	8000	125	NT	NT	ND
AAC-30		16000	250	31	NT	NT	
Donor 5							
MY-1		>32000	32000	500	NT	NT	ND
AAC-30		8000	31	ND	NT	NT	

a) Not detected.
a) Not tested.

neutralizing units/ml) was presented by Toray Industries, Inc. (Tokyo).

Human PBL at a concentration of 1×10^7 cells/ml was incubated with or without various doses of each oligoDNA in a CO_2 incubator at 37°C for 20 h. The culture fluid from each mixture of human PBL and the oligoDNA was collected and kept at −20°C until use. The levels of IFN in the culture fluids were determined in terms of the ability to inhibit the cytopathic effects of Sindbis virus on human FL cells according to the method described by Kohase *et al.*[16] with slight modifications. Briefly, 5×10^4 FL cells in a volume of 50 μl were seeded into a 96-well flat-bottomed microtiter plate. When the cells had become confluent (in general 24 h later), the wells were filled with further 50 μl aliquots of two-fold serial dilutions of the culture fluid or the National Reference IFN-α Ly (J-501, 6500 U/ml), of which the titer is equivalent to the WHO reference IFN. After incubation for a further 16 h, supernatants were discarded and the wells were washed once with warmed medium. The cultured FL cells were infected with 10^5 PFU/well of Sindbis virus in 100 μl of MEM. After 48 h of incubation, the cytopathic effect was examined by the dye (Naphthol blue black) uptake method. Antiviral units were expressed as U/ml, calculated from the highest dilution giving 50% inhibition of the cytopathic effect. IFN-γ was assayed by using an RIA kit (Medgenix, Fleurus, Belgium) and IFN-β was determined with an ELISA kit (Toray, Tokyo).

Human PBL from five donors was incubated with either AAC-30, ACC-30, GAC-30, MY-1 or poly I:C at various concentrations of nucleotide for 20 h. An ex-

Table III. Effect of Incubation Time on IFN Production by Human PBL Stimulated with oligoDNA

Incubation time (h)	IFN (U/ml)											
	0	2	4	6	8	10	12	15	18	24	48	72
Donor 6												
MY-1	ND[a]	ND	ND	ND	62	500	4000	32000	32000	32000	>32000	>32000
AAC-30	ND	ND	ND	ND	16	500	4000	32000	32000	32000	>32000	>32000
Medium	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	16
Donor 7												
MY-1	ND	ND	ND	16	500	8000	32000	32000	>32000	>32000	>32000	>32000
AAC-30	ND	ND	ND	16	125	2000	8000	32000	>32000	>32000	>32000	>32000
Medium	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	31	62

a) Not detected.

tremely high titer of IFN was released by the human PBL incubated with AAC-30, GAC-30 or MY-1 as shown in Table II. Although the value fluctuated depending on the donors, it is clear that AAC-30 and GAC-30, can induce IFN production of human PBL in a dose-dependent manner. The activities of AAC-30 and GAC-30 are nearly comparable to that of MY-1. Another oligoDNA, ACC-30, which showed no antitumor action in the murine system, failed to induce IFN production of human PBL. Subsequently, the kinetics of the IFN production of human PBL was studied. As shown in Table III, IFN was released from human PBL stimulated with AAC-30 after 8 h and reached maximum at 18 h after the start of incubation. The type of IFN produced by human PBL was determined by using a commercial ELISA kit and an RIA kit. As shown in Table IV, IFN-β and a little IFN-γ were found in culture fluid of human PBL stimulated with MY-1 and AAC-30. In contrast, poly I:C induced a lower titer of IFN and a relatively high titer of IFN-γ production as compared with the oligoDNAs. Furthermore, when the culture fluid of human PBL stimulated with MY-1 or AAC-30 was co-cultured with anti-IFN-α antibody, antiviral activity was completely neutralized. Anti-IFN-β antibody showed partial neutralization and anti-IFN-γ showed no effect. On the other hand, anti IFN-α, -β, and -γ all showed partial neutralization of the culture fluid of human PBL stimulated with poly I:C (data not shown). These results showed that human PBL mainly produced IFN-α in addition to IFN-β and a little IFN-γ when stimulated with oligoDNA but human PBL produced a relatively high titer of IFN-γ when stimulated with poly I:C.

We have reported that not only MY-1 but also synthetic oligoDNAs having certain palindromic sequences possess strong antitumor activity and their antitumor activity is closely correlated with their immunostimulatory activities, such as augmentation of NK cell activity, induction of IFN production and macrophage-activating factor production using murine systems.[9, 13, 14]

Table IV. Quantitation of IFN, IFN-β, and IFN-γ in Culture Fluids of Human PBL[a] Stimulated with Nucleotides

Nucleotide	IFN[b] (U/ml)	IFN-β[c] (U/ml)	IFN-γ[d] (U/ml)
MY-1 (50 μg/ml)	48000	685	4.5
AAC-30 (50 μg/ml)	6000	104	2.7
Poly I:C (50 μg/ml)	300	12	19.1
Medium	ND[e]	0	0

a) Donor 8.
b) IFN was determined by using a biological assay.
c) IFN-β was assayed with an ELISA kit (Toray).
d) IFN-γ was assayed with an RIA kit (Medgenix).
e) Not detected.

MY-1 is known to augment NK cell activity of human PBL.[1] The present results clearly showed that MY-1 and synthetic oligoDNAs with particular palindromic structures such as GACGTC and AACGTT could stimulate human PBL to produce a high titer of IFN, and that IFN-α was dominantly produced. Taken together with our previous data, it is suggested that the immunostimulatory activity of the oligoDNAs with certain palindromic structures is responsible for the antitumor activity of MY-1 and oligoDNAs. This is the first report showing that MY-1 and synthetic oligoDNAs with certain palindromes can induce extremely high titers of IFN production of huamn PBL, although further investigations are required to clarify the mechanism. As clinical immunotherapeutic trials of MY-1 have been carried out,[2, 3] the results presented here are not only of scientific interest, but also should aid the therapeutic application of MY-1 against cancers and viral infections.

It is well known that DNAs bind to the mammalian cell surface in a manner consistent with a ligand-receptor relationship.[17-19] However, it is not known whether the nucleotide-receptor complexes on the cell surface mediate the signals for IFN induction, or whether the oligoDNAs are simply trapped on the receptor and then

taken up by endocytosis to react with cytoplasmic or nuclear apparatus. Recently we stimulated murine spleen cells with liposome-encapsulated oligoDNAs. Remarkably enhanced IFN secretion was found after stimulation with liposome-encapsulated oligoDNA having an active palindromic sequence, whereas only a slight secretion was detected after stimulation with liposome-encapsulated oligoDNA having an inactive palindromic sequence (T. Yamamoto *et al.*, submitted for publication). These results suggest that the IFN production is initiated by the penetration of oligoDNAs into the cells, although their intracellular distribution remains unknown.

The cells which produce IFN in response to oligoDNAs have not yet been well characterized. We have already suggested that more than two types of cells in the murine spleen are necessary for IFN production in response to MY-1.[7, 14] We have also examined the susceptibility of many kinds of cultured cell lines to the oligonucleotides, but among those so far tested, we could not find any suitable target cell lines (data not shown). Recently we found that the immunostimulatory activity of some oligoDNAs was inhibited by the presence of antagonists for scavenger receptor (Y. Kimura *et al.*, submitted for publication). Attempts to find cells which produce IFN in response to oligoDNAs are still in progress.

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