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Oligonucleotide Sequences Required for Natural Killer Cell Activation

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Based on the previous finding that certain 30-mer single-stranded oligodeoxyribonucleotides (oligonucleotides) having particular 6-mer palindromic sequences could induce interferon-alpha and -gamma, and enhance natural killer activity, the present study was carried out to clarify the entire relationship between the activity and the sequence of 30-mer oligonucleotides. The results indicated that the activity depended critically on the presence of particular palindromic sequences including the 5′-CG-3′ motif(s). The size and the number of palindromes as well as the extra-palindromic sequences also influenced the activity. An oligonucleotide with a 10-mer palindrome and extra-palindromic oligoguanylate sequences showed the strongest activity among the oligonucleotides tested.

Key words: Oligonucleotide — Palindrome — NK cell

In a series of studies to characterize the antitumor and the immunostimulatory activity of a DNA fraction extracted from *Mycobacterium bovis* BCG,[1-5] we found that some of the 45-mer single-stranded oligonucleotides with sequences randomly chosen from the cDNAs coding for three BCG proteins could induce interferon (IFN)-alpha and -gamma, enhance natural killer (NK) cell activity of mouse spleen cells, and inhibit tumor growth.[6-8] All the active oligonucleotides included a palindromic sequence, such as GACGTC, AGCGCT and AACGTT, whereas the inactive ones did not.[6,7] When a portion of an inactive oligonucleotide was substituted with a palindromic sequence from active oligonucleotides, the oligonucleotide acquired the ability to enhance NK cell activity.[7] In contrast, a sequence substitution with ACCGGT palindrome did not give rise to the activity. Furthermore, the active oligonucleotide lost its activity after an exchange or a deletion of bases within, but not outside, the 6-mer palindromic sequence.[7] Taken together, these findings indicate that some, but not all, of the 6-mer palindromic sequences are essential for the activity of oligonucleotides. Extra-palindromic sequences have also been suggested to be necessary, because trimming an active 45-mer oligonucleotide molecule to 15-mer, while keeping the palindromic sequence intact, resulted in decreased activity.[6] This study was designed to get a comprehensive picture of the sequence requirement of oligonucleotides for enhancing the NK cell activity.

All oligonucleotides used in this study were synthesized by the standard phosphoramidite method using an automatic DNA synthesizer. The oligonucleotide concentration was determined by ultraviolet absorption measurement, assuming $A_{260} = 50$ at 1 mg/ml. Purity of oligonucleotides was confirmed by polyacrylamide gel electrophoresis. BALB/c mouse spleen cells (1×10^7/ml) were incubated at 37°C for 20 h in the presence of oligonucleotides before being tested for NK activity against ^{51}Cr-labeled YAC-1 cells at an effector-to-target ratio of 100.[6,7] NK cell activity was calculated as % specific lysis with the standard deviation using the following formula: (experimental release cpm − spontaneous release cpm)/(maximal release cpm − spontaneous release cpm) × 100.

The palindromic sequence (GACGTC) of an active oligonucleotide named BCG-A4a, the sequence of which is 5′-accgatGACGTCgccggtgacggcaccacg-3′,[6] was replaced with each of the 63 theoretically possible 6-mer palindromic sequences. The resulting BCG-A4a analogues were tested for their ability to enhance NK cell activity (Table I). The results are expressed as the % relative enhancement of NK cell activity by each analogue oligonucleotide as compared to that by BCG-A4a, which was calculated using the following formula: [% specific lysis (analogue) − % specific lysis (control)]/[% specific lysis (BCG-A4a) − % specific lysis (control)] × 100. More than 100% relative activity was observed in the 8 oligonucleotides with one of the following palindromic sequences: AACGTT, AGCGCT, ATCGAT, CGATCG, CGTACG, CGCGCG, GCGCGC and TCGCGA (indicated by asterisks in Table I). These palindromes are referred to as 'potent' palindromes henceforth. All the potent palindromes included one or more 5′-CG-3′ motif(s). In contrast, palindromes composed entirely of adenines (A) and thymines (T) and

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Table I. NK Cell-stimulating Ability of Oligonucleotides with Different 6-mer Palindromes

N_1	N_2	Relative ability to activate NK cells (%) N_3			
		A	T	G	C
A	A	−19.5	25.8	8.3	*136
	T	−16.2	−22.6	−9.4	*171
	G	3.7	−27.7	56.5	*112
	C	−4.3	31.0	53.4	−4.9
T	A	−18.4	−9.8	29.1	34.8
	T	−0.6	−4.7	43.0	2.6
	G	30.1	66.4	33.9	9.5
	C	32.6	−13.7	*169	−11.5
G	A	−3.2	45.3	2.9	(100)
	T	12.1	53.6	3.8	80.4
	G	37.9	0.4	43.4	24.2
	C	21.1	21.4	*126	13.1
C	A	28.0	73.9	33.3	46.6
	T	−12.8	32.5	−27.9	32.3
	G	*233	*119	*106	58.6
	C	26.7	−26.1	−11.6	−23.5

The GACGTC palindromic sequence of an oligonucleotide named BCG-A4a, the sequence of which is 5′-accgatGACGTCgccggtgacggcaccacg-3′, was replaced with 63 other theoretically possible 6-mer palindromic sequences. The resulting BCG-A4a-analogue oligonucleotides were incubated with mouse spleen cells at a concentration of 5 μM to be tested for NK cell-stimulating activity.[6,7] The results are expressed as the % relative increase or decrease in the NK cell activity caused by incubation with BCG-A4a-analogues as compared to that caused by incubation with BCG-A4a. N_1, N_2, N_3 represents the first, the second, and the third base from the 5′-end of the pelindrome, respectively. Asterisks indicate more than 100% relative increase in the activity.

those with a sequence of either Pu(purine)-Pu-Pu-Py-(pyrimidine)-Py-Py or Py-Py-Py-Pu-Pu-Pu were generally unfavorable for the activity.

To examine the role of the extra-palindromic sequence, the NK-augmenting activity was compared among 4 types of 30-mer homooligomers with one of the potent palindromic sequences, AACGTT, CGATCG or ATCGAT, at the center position (Table II). No activity was found in the homooligomers without a palindrome. Oligo-guanylate (oligo-G) showed the highest activity irrespective of the palindromic sequence included, but oligo-adenylate and oligo-cytidylate gave only marginal activity. This was also true of the homooligomers including the GACGTC palindrome (data not shown). In contrast, no activity was found in the 30-mer oligo-G containing an ACCGGT palindrome, which is impotent (data not shown). These results indicate the independent and cooperative effects of the palindromic sequence and the extra-palindromic sequence on the activity of oligonucleotides.

Next, the effect of palindrome size on the activity was investigated. The CGATCG palindrome in the oligonucleotides having extra-palindromic oligo-G sequences was expanded or truncated as shown in Table III (experiment 1). Among these oligonucleotides, one containing a 10-mer palindrome (GACGATCGTC) showed the highest activity, and those with a palindrome smaller than CGATCG had no activity. Essentially the same results were obtained with the AACGTT palindrome (Table III, experiment 2).

Finally, the effects of the number and the location of the palindromic sequence(s) on the activity were investigated. Among the oligonucleotides containing a different number of AACGTT palindromes and extra-palindromic oligo-G sequences, an oligonucleotide with one palindrome showed the strongest activity (Table IV, experiment 1). The oligonucleotides with AACGTT palindrome at the 5′-end or at the 3′-end showed slightly stronger activity than that with it in the center (Table IV, experiment 2), although the activity was influenced more drastically by the number of palindromes than by their location.

The relationships between the sequence and the activity of the palindrome are not very clear at present. The stacking stability between guanine and cytosine is higher than that between adenine and thymine.[9,10] Stacking between pyrimidines is less stable than that between purines or that between purine and pyrimidine.[11] Thus the stable helical structure formed by the palindromes including the 5′-CG-3′ but without Py-Py-Py sequence may be favorable for the activity. However, this does not quite explain the inactivity of some of the oligonucleotides with a palindromic sequence including the 5′-CG-3′. Studies are necessary on the target molecule(s) of the palindromic sequences.

The above results suggest distinct and cooperative roles of the palindromic and the extra-palindromic sequences in the mechanism of NK cell activation by the oligonucleotides. The reduced activity of the oligonucleotides with shorter oligo-G sequence (Tables III and IV) supports this assumption. Since the mixture of a palindrome-including fragment of BCG-A4a and another fragment without it had a reduced activity as compared to BCG-A4a,[6] the palindromic and the extra-palindromic sequence should be present in the same molecule in order to act cooperatively. It is unlikely that the cooperativity between the palindromic and the extra-palindromic sequences is due to some secondary structure(s), such as bulges and hairpin-loops possibly composed from these sequences, for the following reasons. First, the thermostability of secondary structures was not correlated with the activity of oligonucleotides as judged

Table II. NK Cell-stimulating Ability of Oligonucleotides Containing the Same Palindromic Sequence and Different Extra-palindromic Sequences

Experiment 1		Experiment 2	
Sequence (5′-3′)	% Lysis±SD (EI)	Sequence (5′-3′)	% Lysis±SD (EI)
gggggggggggAACGTTgggggggggggg	63.4±3.4 (7.8)	gggggggggggATCGATgggggggggggg	37.5±3.7 (2.5)
ttttttttttttAACGTTtttttttttttt	49.2±3.4 (6.1)	ttttttttttttATCGATtttttttttttt	27.2±1.3 (1.8)
aaaaaaaaaaaaAACGTTaaaaaaaaaaaa	8.5±1.9 (1.0)	aaaaaaaaaaaaATCGATaaaaaaaaaaaa	24.0±1.9 (1.6)
ccccccccccccAACGTTcccccccccccc	16.6±2.5 (2.0)	ccccccccccccATCGATcccccccccccc	14.4±0.9 (1.0)
gggggggggggggggggggggggggggg	7.4±1.7 (0.9)	gggggggggggCGATCGgggggggggggg	55.4±2.9 (3.7)
ttttttttttttttttttttttttttttt	7.4±1.8 (0.9)	ttttttttttttCGATCGtttttttttttt	34.7±1.1 (3.0)
aaaaaaaaaaaaaaaaaaaaaaaaaaaaa	7.3±1.6 (0.9)	aaaaaaaaaaaaCGATCGaaaaaaaaaaaa	41.5±1.4 (2.7)
ccccccccccccccccccccccccccccc	8.5±2.9 (1.0)	ccccccccccccCGATCGcccccccccccc	14.8±1.3 (1.0)
None	8.1±1.9 (1.0)	None	15.1±0.9 (1.0)

The experimental procedures were the same as described in Table I except that the oligonucleotide concentration was 0.5 μM. The palindromic sequences are represented by capital letters. Enhancement index (EI) indicates the relative NK cell activity as compared to that of unstimulated spleen cells.

Table III. Effect of the Palindrome Size on the Ability of Oligonucleotides to Enhance NK Cell Activity

Experiment 1		Experiment 2	
Sequence (5′-3′)	% Lysis±SD (EI)	Sequence (5′-3′)	% Lysis±SD (EI)
gggggggggggggggggggggggggggg	4.9±4.7 (0.9)	gggggggggggggggggggggggggggg	24.8±1.2 (1.4)
gggggggggggggATgggggggggggggg	4.1±4.7 (0.7)	gggggggggggggCGgggggggggggggg	18.9±2.1 (1.1)
gggggggggggggGATCgggggggggggggg	3.0±4.7 (0.5)	gggggggggggggACGTgggggggggggggg	22.2±2.7 (1.3)
gggggggggggCGATCGgggggggggggg	19.9±5.0 (3.6)	gggggggggggAACGTTgggggggggggg	36.7±3.2 (2.1)
gggggggggACGATCGTgggggggggg	49.9±5.7 (9.1)	gggggggggCAACGTTGgggggggggg	34.8±1.7 (2.0)
ggggggggGACGATCGTCgggggggggg	69.0±6.3 (12.5)	ggggggggACAACGTTGTgggggggggg	50.8±4.5 (2.9)
gggggggCGACGATCGTCGgggggggg	56.6±6.1 (10.3)	gggggggAACAACGTTGTTgggggggg	49.6±3.6 (2.9)
gggggCGACGACGATCGTCGTCGggggg	52.9±5.7 (9.6)	ggggggCAACAACGTTGTTGgggggg	48.6±1.9 (2.8)
None	5.5±4.8 (1.0)	None	17.4±1.7 (1.0)

The experimental procedures were the same as described in Table I except that the oligonucleotide concentration was 0.1 μM. The palindromic sequences are represented by capital letters. EI: Enhancement index.

Table IV. Effects of the Number and/or the Location of the Palindrome on the Ability of Oligonucleotides to Enhance NK Cell Activity

Experiment 1		Experiment 2	
Sequence (5′-3′)	% Lysis±SD (EI)	Sequence (5′-3′)	% Lysis±SD (EI)
gggggggggggAACGTTgggggggggggg	70.5±3.3 (3.9)	AACGTTgggggggggggggggggggggggg	65.1±4.3 (6.6)
gggggAACGTTgggggAACGAAggggg	64.5±4.0 (3.5)	gggggAACGTTgggggggggggggggg	41.1±3.5 (4.2)
AACGTTgggggggggggggggggAACGTT	36.4±2.9 (2.0)	gggggggggggAACGTTgggggggggg	44.1±5.6 (4.5)
AACGTTgggggAACGTTgggggAACGTT	43.4±1.5 (2.4)	gggggggggggggggggAACGTTgggggg	41.4±3.5 (4.2)
AACGTTAACGTTAACGTTAACGTTAACGTT	18.4±1.7 (1.0)	gggggggggggggggggggggggAACGTT	54.1±1.8 (5.5)
ggggggggggggggggggggggggggggg	12.7±1.2 (0.7)		
None	18.2±1.6 (1.0)	None	9.9±1.2 (1.0)

The experimental procedures were the same as described in Table I except that the oligonucleotide concentration was 0.5 μM. The palindromic sequences are represented by capital letters. EI: Enhancement index.

by temperature gradient gel electrophoresis (data not shown). Second, there was no correlation between the activity and the preferred secondary structure of the oligonucleotide as predicted by thermodynamic calculation.[12] And third, the activity of the single-stranded BCG-A4a was comparable to that of the double-stranded counterpart, although the latter is less likely to form secondary structure than the former (data not shown).

Our recent results show that DNAs extracted from various bacteria, viruses and invertebrate animals, but not those from a variety of vertebrates and plants, strongly enhance the mouse NK cell activity.[13] Most interestingly, the occurrence of the 8 potent palindromes shown in Table I is more frequent in bacterial cDNA sequences than in those of vertebrates or plants. Thus, the strong immunostimulatory activity of certain bacterial DNAs may be due to the potent palindromes included therein, although the influence of the framing extra-palindromic sequences may also be important. Recent reports suggest diverse biological functions for DNA/oligonucleotides bearing various sequences.[14,15] The oligonucleotides with palindromic sequences may belong to a new category of DNA/oligonucleotides having unique biological functions.

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