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Oligodeoxynucleotides Containing Palindrome Sequences with Internal 5'-CpG-3' Act Directly on Human NK and Activated T Cells to Induce IFN-γ Production In Vitro

Sumiko Iho, Toshiko Yamamoto, Takayuki Takahashi, and Saburo Yamamoto

Previous studies have shown that the action of bacterial or synthetic oligodeoxynucleotide (oligo-DNA) on mouse NK cells to produce IFN-γ is mediated mostly by monocytes/macrophages activated by oligo-DNA. However, its action on human IFN-γ-producing cells has not been well investigated. In the present study, we examined the effect of oligo-DNAs on highly purified human NK and T cells. Bacillus Calmette-Guérin-derived or synthetic oligo-DNAs induced NK cells to produce IFN-γ with an increased CD69 expression, and the autocrine IFN-γ enhanced their cytotoxicity. The response of NK cells to oligo-DNAs was enhanced when the cells were activated with IL-2, IL-12, or anti-CD16 Ab. T cells did not produce IFN-γ in response to oligo-DNAs but did respond independently of IL-2 when they were stimulated with anti-CD3 Ab. In the action of oligo-DNAs, the palindrome sequence containing unmethylated 5'-CpG-3' motif(s) appeared to play an important role in the IFN-γ-producing ability of NK cells. The changes of base composition inside or outside the palindrome sequence altered its activity: The homooligo-G-flanked GAC appeared to be an inducer for NK cells. The CG palindrome was also important for activated NK and T cells in their IFN-γ production, although certain nonpalindromes acted on them. Among the sequences tested, cell activation- or cell lineage-specific sequences were likely; i.e., palindrome ACCGGT and nonpalindrome AACG were favored by activated NK cells but not by unactivated NK cells or activated T cells. These results indicate that oligo-DNAs containing CG palindrome act directly on human NK and activated T cells to induce IFN-γ production.


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whether the CG palindromes and other CG-oligo-DNAs are truly immunogenic in human NK and T cells.

Materials and Methods

Culture medium, cytokines, Abs, and reagents

RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated FCS (Equitech-Bio, Ingram, TX; endotoxin <0.05 ng/ml), 100 μM penicillin G potassium (Banyu Pharmaceutical, Tokyo, Japan), and 100 μg/ml streptomycin sulfate (Meiji Seika, Tokyo, Japan) was used as a complete medium for cell culture. Human rIL-2, TNF-α, and IFN-γ were generously provided by Shionogi Pharmaceutical (Osaka, Japan), Dainippon Pharmaceutical (Osaka, Japan), and Hayashibara Biochemical Laboratories (Okayama, Japan), respectively. Human rIL-12 was purchased from R&D Systems (Minneapolis, MN), and human rIL-15 and rIL-18 were commercially obtained from BioSource International (Camarillo, CA). mAbs against human IFN-γ (IgG2a), TNF-α (IgG1), IL-12 (IgG, clone Ch6.8), and IL-15 (IgG1) were purchased from Genzyme (Boston, MA). Anti-IL-18 (IgG2a) and anti-IFN-γ mAbs were purchased from R&D Systems and Pestecke Biomedical Laboratories (New Brunswick, NJ), respectively. Polyclonal rabbit anti-IL-2 Ab was commercially obtained from Collaborative Research (Bedford, MA). Based on our preliminary experiments, 1 μg of the anti-IFN-γ mAb neutralizes 4 ng of human rIFN-γ, 100 ng of anti-TNF-α mAb neutralizes 200 μg of human rTNF-α, and 1 μg/ml anti-IL-2 neutralizes 50 ng/ml rIL-18, 5 ng/ml rIL-15, 1000 U/ml rIFN-γ, and 100 U/ml rIL-2, respectively. Purified mouse myeloma IgG1 and IgG2a proteins and rabbit serum purchased from ICN Pharmaceuticals (Costa Mesa, CA) were used as an isotype-matched control Ig for the mAbs and as a control serum for the IL-2 Ab, respectively, and were shown not to alter the IFN-γ production or cytotoxicity of NK cells in our experiments. The following reagents were commercially obtained: polymixin B (Sigma Chemical, St. Louis, MO); Dynabeads M-450 CD3, CD14, CD19, and anti-mouse IgG (Dyan, Oslo, Norway); mouse anti-human mAbs directed CD3, CD14, CD16, CD19, CD25, D30, D38, D69, CD91, CD97, D134, CD43, CD47, CD48, DDA-DR, and HLA-AbC (Pharmingen; Becton Dickinson, San Diego, CA, and/or DAKO, Glostrup, Denmark); FITC- or PE-labeled anti-CD3, anti-CD14, anti-CD19, and anti-CD56 (Pharmingen); and goat anti-mouse Ig (DAKO, Becton Dickinson Immunocytometry Systems, San Jose, CA, or Caltag, San Francisco, CA).

Preparation of BCG-derived DNA and the synthetic oligo-DNA

A single-stranded oligo-DNA-rich fraction designated MY-1 was extracted from BCG as described previously (5). MY-1 does not contain any detectable cell wall components. We purchased the oligo-DNAs from Nishinbo (Tokyo, Japan), who prepared them using an Expedite Model 8909 Nucleic Acid Synthesis System (PerSeptive Biosystems, Framingham, MA). The endotoxin level in the synthetic oligo-DNAs was less than 50 pg/100 μg when measured by the Limulus test (Seikagaku, Tokyo, Japan), which specifically detects endotoxin. The sequences of the oligo-DNAs are presented in Figs. 4 and 9 and in Table II.

Cell preparations

Isolation of PBMC. PBMC were isolated from the venous blood of healthy volunteers by 60% osmolarity-adjusted Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. Platelets were removed from the PBMC suspension or the density-fractionated cells by centrifugation on Nyco Prep 1.063 (Daiichi Pure Chemicals, Tokyo, Japan), who prepared them using an Expedite Model 8909 Nucleic Acid Synthesis System (PerSeptive Biosystems, Framingham, MA). The endotoxin level in the synthetic oligo-DNAs was less than 50 pg/100 μg when measured by the Limulus test (Seikagaku, Tokyo, Japan), which specifically detects endotoxin. The sequences of the oligo-DNAs are presented in Figs. 4 and 9 and in Table II.

Preparation of nonadherent cells (NAC). PBMC suspended in complete medium were incubated in plastic dishes for 2 h at 37°C in 5% CO2 in a humidified atmosphere. After the plastic adherence was repeated at least twice, the cells that floated up by gentle agitation of the dishes were recovered, loaded on a nylon wool fiber (Polysciences, Warrington, PA) column, and washed twice at 2 h at 37°C in 5% CO2 in humidified air. The cells that passed through the nylon wool column were collected as NAC depleted of Mos/Md and B cells.

Purification of NK and T cells. NAC were layered over a discontinuous density gradient composed of 42.9% (F1), 46.2% (F2), 50.0% (F3), 54.5% (F4), and 60% (F5) or F1, F2, and 52.6% (F3/F4) of osmolarity-adjusted Percoll, and centrifuged for 30 min at 1500 or 1250 rpm, respectively, at room temperature. The cells layered on F3-F4 or F3/F4 were collected as the small granular lymphocyte (GLL)-rich (morphologically 60-90% of GLL), and those on F5 were collected as the T cell fraction (>99% CD3+ determined by flow cytometry (Fig. 10)). When T cell purity was not sufficient as determined by flow cytometry, B cells and Mos/Mds were removed with the use of M-450 CD19 and M-450 CD14, respectively, or by cell sorting (Epics Elite, Beckman Coulter, Fullerton, CA) with FITC-conjugated CD14, CD16, and CD19 mAbs (PharMingen). NK cells were purified by the LGL-rich fraction by negative or positive selection. In the negative selection, the LGL-rich population was depleted of Mos/Mds and T/B cells by the serial use of M-450 CD14, M-450 CD3, and M-450 CD19 magnetic beads or by an indirect method using anti-CD14, anti-CD3, and anti-CD19 mAbs as the first Abs, then with M-450 goat anti-mouse IgG or M-450 sheep anti-mouse IgG magnetic beads as the second Abs. The immunomagnetic depletion was repeated at least twice in each method. In the positive selection, indirect immunomagnetic separation was performed with a combination of anti-CD56 mAb and M-450 IgG after the repeated depletion of Mos/Mds, using M-450 CD14 to avoid the trapping of Mos/Mds in the NK cell population, which may be caused by phagocytosis of the immunobeads or nonspecific cell aggregation. Cells obtained by these methods contained >97% CD56-positive cells as determined by flow cytometry (Fig. 1A) and <1% Mos/Mds as evaluated with nonspecific esterase (Muto Pure Chemical, Tokyo, Japan) or flow cytometric analysis of CD14 expression. In some experiments, NK cells were isolated with FITC-conjugated CD56 mAb (PharMingen) by cell sorting (Epics Elite). NK cells purified by CD56-positive selection in the immunomagnetic separation method were used after 6 h of incubation at 37°C in 5% CO2 to detach the beads but were used without removing the beads when cells were stimulated with anti-CD16 mAb. Both procedures for the NK cell purification did not alter the responsiveness of the NK cells to oligo-DNAs.

Cell culture and ELISA

NK, NAC, or T cells were placed in 96-well plates (round-bottom plates for NAC and NK cells and flat-bottom plates for T cells (Corning Glass Works, Corning, NY) and cultured in complete medium at 37°C in a humidified atmosphere with 5% CO2 under the conditions described in Results. IFN-γ secreted in the culture supernatants was measured by an ELISA kit (Cytoscreen Immunoassay Kit) (Biosource International). The lower limit of detection for human IFN-γ was 4 pg/ml.

Cytotoxicity assay

NK cells were cultured with K562 cells at the indicated E:T ratios in triplicate, for 4 h at 37°C in 5% CO2. The activity of lactate dehydrogenase released from damaged cells into the culture medium was measured by a cytoxicity detection kit (lactate dehydrogenase) (Boehringer Mannheim, Mannheim, Germany), and the cytototoxicity is expressed here as a percentage of target cell lysis.

Flow cytometry analysis

Flow cytometry analysis was performed on a EPICS XL (Beckman Coulter). Cells were incubated with mAbs, followed by washing and labeling with FITC or PE-conjugated goat anti-mouse Ig. Data were obtained in a logarithmic scale.

Statistical analysis

Data were analyzed with the Wilcoxon signed rank test, ANOVA, or Student’s t test. Differences in the results were considered significant at p < 5%.

Results

BCG-DNA. MY-1, directly induces NK cells, but not T cells, to produce IFN-γ

To determine the cell type(s) that is responsive to BCG-DNA, MY-1, to produce IFN-γ, we separated LGL and T cells from NAC. When these two cell factions were cultured for 24 h at a concentration of 2 × 106 cells/ml, only the LGL fraction produced IFN-γ in the presence of MY-1 (Table I, experiments 1 and 2). NAC which contain 20-30% of NK and 70-80% of T cells, produced IFN-γ in response to MY-1 when the cell density was increased to 4 × 106 cells/ml (experiment 2), whereas the T cell fraction did not produce IFN-γ even when cultured at 1 × 107 cells/ml (experiment 3) or for longer periods (data not shown). Polymyxin B, a LPS inhibitor, did not affect the MY-1-induced IFN-γ production, and DNase treatment of MY-1 abolished the IFN-γ-inducible activity (data not shown).
We then purified CD56\(^+\) cells from the LGL fraction (Fig. 1A) and tested their responsiveness to MY-1. As shown in Table I (experiment 3), NK cells produced IFN-\(\gamma\) in response to MY-1. The doses of MY-1 necessary to induce the maximum amount of IFN-\(\gamma\) were between 12.5 and 50 \(\mu\)g/ml in the culture of NK cells (Fig. 1B). IFN-\(\gamma\) production in the culture with MY-1 was first observed at 18 h and increased thereafter (Fig. 2). The amounts of IFN-\(\gamma\) produced without MY-1 at 24-h culture were mostly below 4 pg/ml and did not exceed 13 pg/ml in any NK cell sources examined. These results show that NK cells are responsive to MY-1 in terms of IFN-\(\gamma\) production.

To prove that the MY-1-induced IFN-\(\gamma\) production is caused by a direct action on NK cells, we added neutralizing concentrations of mAbs against IL-12 or TNF-\(\alpha\) to the culture of NK cells in the presence or absence of oligo-DNA. In this experiment, instead of MY-1, g10GACGA (synthetic oligo-DNA) was used because of its potent ability to induce IFN-\(\gamma\) (see below). As shown in Fig. 3, neither anti-IL-12 nor anti-TNF-\(\alpha\) mAb influenced the IFN-\(\gamma\) production by NK cells cultured with or without g10GACGA. The combined addition of mAbs against IL-12 and TNF-\(\alpha\) also did not inhibit the production of IFN-\(\gamma\). No inhibitory effect of these Abs on the IFN-\(\gamma\) production was observed when 10 \(\mu\)g/ml g10GACGA was applied for NK cell stimulation. In addition, 1–10 \(\mu\)g/ml mAbs to IL-18, IL-15, or IFN-\(\alpha\) did not alter the level of IFN-\(\gamma\) production induced by the oligo-DNA (data not shown).

**Table I. Effect of BCG-derived DNA, MY-1, on IFN-\(\gamma\) production by NK and T cells**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Fractionated Cells</th>
<th>Cell Densities</th>
<th>IFN-(\gamma) Produced (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Without MY-1</td>
</tr>
<tr>
<td>1</td>
<td>LGL</td>
<td>2 × 10^7/ml</td>
<td>10.5 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>T cells</td>
<td>2 × 10^7/ml</td>
<td>&lt;4</td>
</tr>
<tr>
<td></td>
<td>NAC</td>
<td>2 × 10^7/ml</td>
<td>&lt;4</td>
</tr>
<tr>
<td>2</td>
<td>LGL</td>
<td>2 × 10^6/ml</td>
<td>&lt;4</td>
</tr>
<tr>
<td></td>
<td>T cells</td>
<td>2 × 10^6/ml</td>
<td>&lt;4</td>
</tr>
<tr>
<td></td>
<td>NAC</td>
<td>2 × 10^6/ml</td>
<td>&lt;4</td>
</tr>
<tr>
<td>3</td>
<td>CD56(^+) cells in LGL</td>
<td>2 × 10^6/ml</td>
<td>&lt;4</td>
</tr>
<tr>
<td></td>
<td>CD56(^+) cells in LGL</td>
<td>2 × 10^6/ml</td>
<td>58.7 ± 5.6*</td>
</tr>
<tr>
<td></td>
<td>T cells</td>
<td>2 × 10^6/ml</td>
<td>&lt;4</td>
</tr>
<tr>
<td></td>
<td>1 × 10^7/ml</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

* Data are representative of eight experiments performed with similar results. In each experiment, cells were fractionated from PBMC obtained from different donors and cultured at indicated cell densities for 24 h with or without 50 \(\mu\)g/ml MY-1. Data are the means ± SD (n = 3). *\(p<0.01\) compared with the respective controls without MY-1.

**FIGURE 1.** A. Purity of NK cells. NK cells isolated and used in the present study contained >97% CD56\(^+\) cells and <1% of CD14\(^+\) and/or CD19\(^+\) cells. The cells shown in A contained 98.9% of CD56\(^+\) cells and 0.4% of CD14/CD19\(^+\) cells. B. Dose-response effects of MY-1 and synthetic oligo-DNA, g10GACGA, on the IFN-\(\gamma\) production by NK cells. NK cells were cultured in triplicate at 3 × 10^6/ml for 24 h with various concentrations of MY-1 or g10GACGA. IFN-\(\gamma\) concentrations in the culture supernatants are presented as the mean ± SD. **, \(p<0.01\) compared with the control culture with medium alone. Similar results were obtained in three other separate experiments.

**FIGURE 2.** Time course studies of MY-1-induced IFN-\(\gamma\) production by NK cells. NK cells were cultured in triplicate at 3 × 10^6/ml for 12, 18, 24, and 48 h in the presence or absence of 50 \(\mu\)g/ml MY-1. Data are means ± SD. This analysis was repeated three times with very similar results. **, \(p<0.01\) compared to the respective controls without MY-1.
FIGURE 3. Effect of mAbs against IL-12 or TNF-α on g10GACGA-induced IFN-γ production. NK cells (2 × 10^6/ml) were cultured in triplicate for 24 h with medium, 50 μg/ml g10GACGA, 500 pg/ml rIL-12, or 200 pg/ml rTNF-α in the presence of the mAbs against IL-12 or TNF-α, mouse IgG1, or medium alone. Anti-IL-12 and anti-TNF-α mAbs and mouse IgG1 were added at concentrations of 1, 0.1, and 1 μg/ml, respectively. Data are means ± SD. NS, not significant compared with the corresponding controls with medium, g10GACGA, rIL-12, or rTNF-α alone. §, p < 0.01 compared with the value with medium alone. **, p < 0.01 compared with the value with rIL-12 alone. The results are representative of three independent experiments performed with cells obtained from different individuals. α, anti.

Synthetic oligo-DNAs composed of palindrome sequences containing CG motif(s) induce IFN-γ production by purified NK cells

To determine the effective sequence(s) in MY-1 for human NK cells, 30-mer synthetic oligo-DNAs possessing various palindrome sequences were first tested. These oligo-DNAs are analogues of BCG-4a (12), the sequence of which was chosen from the cDNA encoding 64-kDa heat shock protein AgA of BCG. GACGTC in BCG-4a was replaced with different types of hexamer palindromes. These included nine active palindromes which enhanced NK cytotoxicity more strongly than GACGTC in mice and one inactive form (17). The ability of each 30-mer oligo-DNAs to induce IFN-γ production was compared at a concentration of 5 μM (almost equivalent to 50 pg/ml for each oligoDNA). The sequences containing ATCGAT, TCGCGA, CGTACG, CGGCCG, and GACGTC (named ATCGAT-30, TCGCGA-30, CGTACG-30, CGGCCG-30, and GACGTC-30, respectively) were shown to induce IFN-γ production by human NK cells as potently as 10 U/ml IL-2 or 10 pg/ml IL-12 (see Fig. 8). The amount of IFN-γ detected in the culture with 30-mer oligo-G (g30) appeared slightly higher than that in the control culture, but the increase was not significant.

Effect of nonpalindrome oligo-DNAs on IFN-γ production by NK cells

The sequences of immunostimulatory DNAs that have been extensively studied by other investigators do not have the hexamer palindrome. In mice, gagaacgcgcgtcgc (1643) is mitogenic to B cells (33), and tccatgacgttcctgat (1668) induces not only B cell activation (33) but also the production of inflammatory cytokines by lymphocytes (18, 20). Furthermore, tctccagcgtgcgccat (1758, antisense hBcl-2) activates NK cells (37). In humans, ttgcttccttctctct (2105) directly activates B cells (34). We tested whether these sequences are effective for human NK cells to induce IFN-γ production. Unlike the active sequences mentioned above, they did not induce IFN-γ production of human NK cells; these sequences were tested at concentrations ranging from 0.2 to 20 μM, by extending the culture periods to 5 days. The representative data (IFN-γ amount, pg/ml) from three separate experiments with NK cells, which were purified by cell sorting and cultured at 3 × 10^6/ml for 3 days in the presence of 1643, 1668, 1758, 2105, AACGTT-30, g10GACGA, or medium alone, were 20.0 ± 3.6 (mean ± SD, n = 3), 25.4 ± 4.8, 26.2 ± 5.1, 18.4 ± 1.8, 75.4 ± 15.4, 163.7 ± 25.2, and 18.8 ± 3.6, respectively. Further, replacement of

NGCCTTGC (g10CGGTC) showed no IFN-γ-inducing effect on NK cells. The oligo-G introduced at the position of the extrapalindrome sequences containing ATCGAT, TCGCGA, CGTACG, CGGCCG, and GACGTC (named ATCGAT-30, TCGCGA-30, CGTACG-30, CGGCCG-30, and GACGTC-30, respectively) were shown to induce IFN-γ production, but ACAACGTTGT (g10ACAAC) did not. The doses of g10GACGA capable of inducing the plateau level of IFN-γ production by NK cells were from 6.3 to 50 μg/ml (10 μg/ml is almost equal to 1 μM) (Fig. 1). The level of IFN-γ production at 24-h culture with 10 μg/ml of g10GACGA was comparable with those induced by 10 U/ml IL-2 or 10 pg/ml IL-12 (see Fig. 8). The amount of IFN-γ detected in the culture with 30-mer oligo-G (g30) appeared slightly higher than that in the control culture, but the increase was not significant.

FIGURE 4. Responsiveness of NK cells to synthetic oligo-DNAs. NK cells (2 × 10^6/ml) were cultured in triplicate for 40 h with medium, MY-1 (50 μg/ml), or various kinds of 30-mer synthetic oligo-DNAs (5 μM, almost equivalent to 50 μg/ml for each oligoDNA). Data are means ± SD. + and **, p < 0.05 and p < 0.01, respectively, compared with the control culture with medium alone. Similar results were obtained in two other separate experiments.
the AACGTT motif of AACGTT-30 with the immunostimulatory core sequence, PuPuCGPyPy, i.e., AACGCT, AACGTC, or AACGCC, and with other sequences containing one thymine at the 3'-side of AACG, such as AACGAT, AACGGT, AACGTA, and AACGTG, did not induce IFN-\(\gamma\) production (data not shown).

**Oligo-DNA-induced IFN-\(\gamma\) production participates in the enhancement of NK activity**

We tested whether oligo-DNAs can affect the cytotoxicity of purified NK cells. As shown in Fig. 5, when NK cells were cultured with MY-1 or g10GACGA, the ability to lyse K562 cells was enhanced and the enhancement was prominent in the culture with g10GACGA. The 30-mer homooligo-G, g30, used as a control DNA, did not alter the cytotoxic activity. These results indicate that the enhanced cytotoxicity is directly elicited by purified NK cells cultured with synthetic oligo-DNAs. To examine the role of IFN-\(\gamma\) induced by oligo-DNAs in the augmentation of NK activity, we added a neutralizing mAb against IFN-\(\gamma\) to the NK cell culture in the presence or absence of g10GACGA. The ability of g10GACGA to enhance NK activity was diminished by the addition of anti-IFN-\(\gamma\) mAb (Fig. 6). Similarly, MY-1 enhanced NK cells and the enhancement was inhibited in the presence of the anti-IFN-\(\gamma\) mAb (data not shown).

**Oligo-DNA enhances the expression of CD69 molecule on NK cells**

To identify the activation-associated molecule(s) which would be induced by oligo-DNA, we examined the expression of CD25, CD69, and CD94 molecules and HLA-ABC on cultured NK cells. As shown in Fig. 7, NK cells strongly expressed the CD69 molecule when cultured with g10GACGA with 2.44 ± 0.56 (mean ± SD, \(n = 3\)) times more in the percent positive cells than in those cultured with medium alone. Expression of other molecules such as CD25 and CD94 was unchanged by the culture with g10GACGA. In these experiments, the fluorescence of NK cells stained with HLA-ABC was always intensified by the culture with oligo-DNA (e.g., medium: 502 ± 17, g10GACGA: 590 ± 19, and IL-2 as a positive control: 705 ± 17 as expressed by the mean intensity ± SD), indicating the autocrine stimulation of NK cells by IFN-\(\gamma\) induced by g10GACGA.

**Figures**

**FIGURE 5.** Effect of synthetic oligo-DNAs on NK activity. NK cells were cultured in triplicate for 24 h with 50 \(\mu\)g/ml MY-1, synthetic oligo-DNAs, g10GACGA and g30, or medium alone. NK activities are expressed as percent lysis (mean ± SD) at the indicated E:T ratios. ***, \(p < 0.01\) compared with the value with medium alone at the respective E:T ratios. The oligo-DNA-enhanced NK activity was similarly reproduced in experiments that were repeated three times using cells from different individuals.

**FIGURE 6.** Effect of anti (α)-IFN-\(\gamma\) mAb on g10GACGA-enhanced NK activity. NK cells were cultured in triplicate for 44 h with medium, 50 \(\mu\)g/ml g10GACGA, or 400 pg/ml rIFN-\(\gamma\) in the presence of 1 \(\mu\)g/ml mouse IgG2a or anti-IFN-\(\gamma\) mAb. NK activities are presented as percent lysis at E:T = 12, 6, and 3 (mean ± SD). ***, \(p < 0.01\) compared with the value with medium alone. §, \(p < 0.01\) compared with the respective control values without anti-IFN-\(\gamma\). No significant difference was observed between the values with anti-IFN-\(\gamma\) plus g10GACGA or rIFN-\(\gamma\) and the value with anti-IFN-\(\gamma\) plus medium. The results shown are representative of three experiments with similar results.

**FIGURE 7.** Oligo-DNA enhances the expression of CD69 in NK cells. NK cells were cultured for 36 h with and without 1 \(\mu\)M g10GACGA, washed, and stained for CD69 surface expression.

**MY-1/oligo-DNA-induced IFN-\(\gamma\) production by NK cells is enhanced in the presence of IL-2, IL-12, or anti-CD16 mAb**

We then tested the ability of NK cells to produce IFN-\(\gamma\) in response to oligo-DNAs in the presence of IL-2, IL-12, or anti-CD16 mAb to examine the influence of the activation status of NK cells.
on their responsiveness to oligo-DNAs. g10GACGA was used in this experiment because of its potent activity. As shown in Fig. 8, g10GACGA could induce IFN-γ production by NK cells in the absence of the stimuli. The addition of IL-2, IL-12, or anti-CD16 mAb to this culture significantly enhanced the IFN-γ production. The increase was synergistic in the culture with IL-2, whereas in the culture with IL-12 or anti-CD16 mAb, the increases were additive. Therefore, the activated NK cells appear to be more susceptible to oligo-DNAs in terms of IFN-γ production, especially with IL-2 stimulation.

Effect of different palindrome sequences on IL-2-activated NK cells

With IL-2, MY-1 also enhanced IFN-γ production by NK cells (Fig. 9). We then examined the effect of different palindrome sequences, which often occur in MY-1 (17), on the IFN-γ production by NK cells in the presence of IL-2, in a manner similar to that used for unactivated NK cells. The synthetic oligo-DNAs that induced IFN-γ production by unactivated NK cells, i.e., ATCGAT-30, GACGTC-30, TCGCGA-30, CGTACG-30, and CGGCCG-30, and those that showed weak or modest abilities to induce IFN-γ production by the unactivated NK cells, i.e., ACGGCT-30, CGATCG-30, CGGCCG-30, and AAGCTT-30 all enhanced the IFN-γ production by NK cells in the presence of IL-2. When the IFN-γ-inducing activity of these palindromes was expressed as a percentage of the control in six separate experiments, the order of potency was as follows: AAGCTT (432 ± 95 pg/ml, mean ± SE), ACCCGT (408 ± 48, CGTACG (376 ± 42), ACGGCT (376 ± 72), GCCGCC (320 ± 32), CGATCG (259 ± 37), TCGCGA (256 ± 35), ATCGAT (249 ± 37), CGGCCG (246 ± 22), and GACGTC (238 ± 40). That of MY-1 was 397 ± 34. These values were not statistically different. Unlike those in the culture of unactivated NK cells, however, AAGCTT was the most potent and ACGGCT the weakest palindrome in the culture of IL-2-activated NK cells. In these results, there was a striking difference in the sequence pattern of the induction of IFN-γ production from those observed in the unactivated NK cells. That is, an oligo-DNA with the ACCCGGT palindrome (which was inactive in unactivated NK cells) was able to induce IFN-γ in the presence of IL-2. One of these data is shown in Fig. 9 (experiment 1) as the amount of IFN-γ produced in the cell culture supernatant. A palindrome that contains GC instead of CG, AAGCTT, showed no effect on the IFN-γ production (experiment 2).

Effect of nonpalindrome sequences on IL-2-activated NK cells

It has been reported by Chace et al. (22) that oligo-DNAs act on mouse NK cells in the presence but not in the absence of IL-12. Activated NK cells may be more susceptible for oligo-DNA stimulation, regardless of the particular contexts such as a palindrome with internal CG or the PuPuCGPyPy sequence. Then two bases of AACGTT were replaced at the 3′-side with theoretically possible dinucleotides containing one thymine, to test for the IFN-γ-inducing ability using IL-2-activated NK cells. As shown in Fig. 9 (experiment 3), activated NK cells responded to the sequences containing CG irrespective of particular contexts such as the palindrome or PuPuCGPyPy sequence. Among them, the sequences with TT, CT, AT, TC, and TA at the 3′-side of AACG were more potent.

Activated T cells can be induced by MY-1 and oligo-DNAs to produce IFN-γ

Resting T cells did not respond to MY-1 (Table I). However, the synergism between oligo-DNAs and IL-2 observed in NK cells prompted us to examine the responsiveness of activated T cells to MY-1 and synthetic oligo-DNAs. We stimulated purified T cells (Fig. 10A) with Dynabeads M-450 CD3, which is able to activate T cells (manufacturer’s information), and evaluated their IFN-γ production.
production in response to MY-1 and g10GACGA. As shown in Fig. 10B, T cells produced IFN-γ in the presence of M-450 CD3, and this production was significantly enhanced by the addition of MY-1 or g10GACGA. A 10-μg/ml concentration of g10GACGA was almost equipotent to 100 U/ml IL-2 for the induction of IFN-γ in M-450 CD3-stimulated T cells. The effect of g10GACGA on the IFN-γ production was not influenced by the addition of anti-IL-2 Ab to these cultures (Fig. 10C), indicating that oligo-DNA-induced IFN-γ production is independent of IL-2 production by activated T cells.

The expression of CD25, CD69, CD94, HLA-ABC, CD30, CD38, CD71, CD94, CD97, CD134, CDw137, and HLA-DR was also tested as to whether specific activation marker(s) are induced by oligo-DNA. Anti-CD3 stimulation of T cells expressed higher levels of these molecules, and further enhancement was not observed when examined at 24 and 48 h of cultures with MY-1 or g10GACGA (data not shown).

Effective sequences to induce IFN-γ production by activated T cells

To seek out the effective sequences for activated T cells, the sequences involved in the induction of IFN-γ production were examined in a manner similar to that used for NK cells. All oligo-DNAs that contained hexamer palindromes with CG motif(s), except for ACCGGT, induced IFN-γ production by M-450 CD3-stimulated T cells. When the activity was presented as a percentage of the control in six independent experiments, the order of potency among the active palindromes was as follows: CGGCCG (273 ± 57 pg/ml mean ± SE); TCGCGA (223 ± 45); AGCGCT (220 ± 38); AACGTT (218 ± 13); ATCGAT (173 ± 19); CGATCG (166 ± 19); GCGCGC (163 ± 6); CGTACG (160 ± 21); and GACGTT (132 ± 6). That of MY-1 was 233 ± 12. These values were not statistically different, but one of the weakly active sequences for IL-2-activated NK cells, CGGCCG, was distinctly active for activated T cells. A representative result of these experiments is shown as the amount of IFN-γ in the culture supernatant in Fig. 11. A non-CG palindrome, CG of which was replaced with GC, i.e., AAGCTT-30, showed no effect on IFN-γ production (Fig. 11 and Table II).

We then examined the activities of nonpalindrome oligo-DNAs. Although replacement of AACGTT of AACGTT-30 by nonhexamer palindromes such as AACCCT, AACGTC, or AACGTA exhibited IFN-γ inducing activity, when replaced by AACCCT or AACGTC, the activity was very weak or completely undetectable. AACCCT remained inactive (Table II). The substitution of GC for CG in the active motifs abolished their activities (Table II). However, the oligo-DNA, 1643, which contains AACCCT, did not induce IFN-γ production. Then, AACCCT, AACGTC, and AACGTA were flanked by oligo-G to determine whether a backbone sequence changes the activity of these sequences. In the oligo-G-flanked sequences, only AACGTT (g12AAC) showed potent activity (Table II), and this seemed most potent for activated T cells among the sequences tested. To examine whether the IFN-γ-inducing activity of g12AAC is modified by the methylation of CD3-stimulated T cell culture. C. Effect of anti(α)-IL-2 Ab on the MY-1-induced IFN-γ production. M-450 CD3-activated T cells (a mixture of 2 × 10^6/ml T cells and 2 × 10^6/ml M-450 CD3) were cultured for 24 h with medium, 50 μg/ml MY-1, or 100 U/ml IL-2 in the presence or absence of anti-IL-2 Ab. Data are means ± SD. **, p < 0.01 compared with the control value with IL-2 alone. NS, not significantly different compared with the corresponding controls with medium or MY-1 alone.
FIGURE 11. Effect of different sequences of palindromes on IFN-γ production by M-450 CD3-stimulated T cells. T cells (2 × 10^6/ml) were cultured in triplicate for 24 h with medium, 50 μg/ml MY-1, or 5 μM oligo-DNAs that contain different sequences of palindrome or nonpalindrome, in the presence of 2 × 10^6 particles/ml M-450 CD3. The sequences of oligo-DNAs are listed in Figs. 3 and 8. The results shown are representative of six independent experiments with cells obtained from different donors. The IFN-γ concentrations in culture supernatants are expressed as the mean ± SD.

CG, we synthesized methylated g12AAC in which CG inside the palindrome was methylated. As shown in Table II, the methylated g12AAC did not induce the IFN-γ production. Other well-investigated immunostimulatory oligo-DNAs, 1668, 1758, and 2105, which activate mouse spleen cells or human B cells, had little activity for anti-CD3-stimulated T cells (Table II).

Discussion

In this study, we demonstrated that both BCG-derived DNA (MY-1) and synthetic oligo-DNAs directly induce human NK cells and activated T cells to produce IFN-γ and that the autocrine IFN-γ enhances NK activity. Our present study reveals that the oligo-DNA responsiveness of human IFN-γ-producing cells differ with that of mice in terms of the sequence requirement and that the effective sequences are different according to the cell types and/or the activation status. Furthermore, the IFN-γ-inducing activity of the oligo-DNA was somehow interdependent on the presence of CG, the context of the core motif of CG, and its outer flanking sequences.

To exclude an indirect action caused by contaminated Mos/Mds, we purified the NK and T cell fractions to more than 97% CD56^+ and 99% CD3^+ cells, respectively, and less than 1% Mos/Mds. We also added mAbs against IL-12, TNF-α, IL-15, IL-18, or IFN-α to these cell cultures with oligo-DNAs to determine whether these cytokines produced by contaminated Mos/Mds (<1%) were involved in the IFN-γ production. However, the levels of IFN-γ production remained unchanged. Indeed, none of these cytokines was detected in the culture supernatants of the NK or T cell population (data not shown). For instance, the concentration of IL-12 measured by ELISA was less than the detectable dose (1 pg/ml) which was not sufficient to induce IFN-γ production by these cells in our preliminary experiments. Furthermore, g10GACGA induced TNF-α production in the same culture conditions; however, the level was too low (2.86 ± 1.70 pg/ml in 40-h culture, mean ± SD) to induce IFN-γ production (Ref. 45 and our observation). Our unpublished data suggest that TNF-α detected in the NK cell culture with g10GACGA may be produced by NK cells.

Table II. Effect of oligo-DNAs containing CG, GC, or methylated CG on the IFN-γ production by activated human T cells

<table>
<thead>
<tr>
<th>Oligo-DNA</th>
<th>IFN-γ Amount in the Culture Supernatant (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td></td>
</tr>
<tr>
<td>AAGCTT-30</td>
<td>119.7 ± 15.4*</td>
</tr>
<tr>
<td>AAACGCT-30</td>
<td>126.7 ± 15.8*</td>
</tr>
<tr>
<td>AACGTA-30</td>
<td>57.3 ± 8.3</td>
</tr>
<tr>
<td>AAGCTA-30</td>
<td>58.3 ± 7.8</td>
</tr>
<tr>
<td>AACGTG-30</td>
<td>66.5 ± 11.3</td>
</tr>
<tr>
<td>AACGTC-30</td>
<td>58.0 ± 5.0</td>
</tr>
<tr>
<td>AACGTG-30</td>
<td>66.5 ± 11.3</td>
</tr>
<tr>
<td>AACGTC-30</td>
<td>14.0 ± 5.0</td>
</tr>
<tr>
<td>AACGTA-30</td>
<td>58.3 ± 7.8</td>
</tr>
<tr>
<td>AACCT-30</td>
<td>131.9 ± 19.4*</td>
</tr>
<tr>
<td>AAGCT-30</td>
<td>65.9 ± 5.0</td>
</tr>
<tr>
<td>AACGCT-30</td>
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<tr>
<td>AAGGCCT-30</td>
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<tr>
<td>AAGCCT-30</td>
<td>48.0 ± 6.0</td>
</tr>
<tr>
<td>AAGCT-30</td>
<td>114.0 ± 9.6*</td>
</tr>
<tr>
<td>BCG-DNA fraction</td>
<td>MY-1</td>
</tr>
</tbody>
</table>

Note: T cells were cultured in triplicate at 2 × 10^6/ml for 21 h with medium, 50 μg/ml MY-1, 5 μM oligo-DNAs containing certain sequences (underlined) with CG, GC, or methylated CG (bold c), in the presence of 2 × 10^6 particles/ml M-450 CD3. The results are representative of four experiments with cells obtained from different donors with similar results. The IFN-γ concentrations in culture supernatants are expressed as means ± SD.

* p < 0.01 compared with the control value with medium alone.
cells rather than by Mos/Mδs. These facts thus indicate that the effect of Mos/Mδs contaminating the NK or T cell fraction at <1% was negligible in the IFN-γ production.

It has been reported in mice that bacterial DNA or oligo-DNA does not induce IFN-γ production or NK enhancement when purified NK cells or Mo/Mδ-depleted nonadherent cells are used without additional stimuli as the responders (19, 22, 27). In humans, these DNAs directly activated NK cells (present study). This implies that mouse and human NK cells behave differently in response to the DNA stimulation. It is unclear as to what caused the difference in the responsiveness of NK cells to oligo-DNAs between our present study and the others. In B cells, a differential requirement regarding the oligo-DNA sequence between humans and mice has been observed (33, 34). The sensitivity of NK cells to oligo-DNA sequences may also, therefore, be different between mice and humans. The PuPuCGPyPy sequences that were immunostimulatory for mouse spleen cells (33), such as AACGCT or AACGTC, did not directly activate mouse NK cells (27).

In the present study, these sequences did not act directly on human NK cells either. Although the hexamer palindromes with CG dinucleotide(s) also did not activate mouse NK cells (9, 12, 14, 19, 27), they were active for human NK cells in our study. Human NK cells thus appear sensitive to oligo-DNA, especially when the particular sequences such as the CG palindromes are present. For Mos/Mδs, hexamer palindromes behave actively regardless of the species (12, 14, 15, 19, 23, 28, 46). In this study, these sequences were shown to be effective also for activated human T cells to enhance IFN-γ production. The palindromic sequences containing the CG motif may therefore be some of the most potent sequences for immunocompetent cells involved in the induction of IFN-γ production in humans.

Among the CG-oligo-DNAs tested in this study, the favorable sequences for the IFN-γ induction differed with the cell lineage and/or its activation status. For example, in palindromes, ATC GAT was more effective in unactivated NK cells than in activated NK cells, and vice versa in AACGTT. Further, the weakly active CGGGCG in activated NK cells was distinctly active for activated T cells. More importantly, ACCGCT, one of the palindromic sequences that was inactive for unactivated NK cells turned out to be active for IL-2-activated NK cells. Because in humans ACCGTT, hexamer palindromes such as CGATCG (g10GACGA) or could conversely cause the suppression of IFN-γ production by perturbing the interacting signals toward the IFN-γ production in other types of palindrome such as AACGTT in NK cells. This may also be one of the reasons why oligo-G-flanked AACGTT (19) or TCAACGTTGA (27) did not activate the murine NK cells. Changes of the IFN-γ-inducing activity by oligo-G flanking was further observed when activated T cells were targeted: the activity of the palindrome AACGTT was greatly enhanced by the oligo-G flanking; and that of the nonpalindrome AACGCT, AACGTC, or AACGTA was decreased to negligible levels. The flanking sequence thus appears to influence the oligo-DNA activity in different ways according to the sequence of the core motif and also to the lineage and/or activation status of the target cells, in terms of IFN-γ production.

We previously reported that the biological activity of palindromes is triggered after their entry into the cells (46, 49). As shown in other typical cells (50, 51), oligo-DNA containing palindrome with CG may be located in endosomes and in the nucleus once it is taken up by NK or activated T cells. However, the mechanisms by which oligo-DNA induces IFN-γ production in these cells remain to be identified. Yi et al. (25) showed that oligo-DNA directly increases the transcriptional activity of IL-6 promoter, suggesting an interaction of oligo-DNA with responsive elements. Stacey et al. (29) and Sparwasser et al. (30) demonstrated that oligo-DNA modulates the activity of transcription factors. As an alternative mechanism, a certain structure of oligo-DNA may interact, as a charged structure, with second messenger-delivered signals which are involved in IFN-γ production, because the calf thymus-DNA structure activates p68 kinase, which has specific ATP-binding sites (52). This mechanism should be examined in the oligo-DNA-induced IFN-γ production in human NK or activated T cells, because the expression of CD69 Ag, which was reported to be involved in signal transduction (53), was enhanced by oligo-DNA in NK cells.

A clinical trial with MY-1 has been performed in Japan to assess its efficacy as an immunotherapeutic agent for malignant diseases, with positive results (54). Our present results imply that not only MY-1 but also the immunogenic synthetic oligo-DNAs induce multiple immune responses in vivo including NK cell activation.
and the possible induction of cytotoxic T cells, both of which are major components of the immune defense system against neoplasms. If NK or T cells are activated under some circumstances, the in vivo action of these oligo-DNAs may be augmented under those circumstances. Further studies are required to identify physiological potentiators of oligo-DNAs for the better therapeutic efficacy of these agents.

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References


