Antimetastatic Effect of CpG DNA Mediated by Type I IFN

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ABSTRACT

The mechanisms involved in the antimetastatic effect of CpG-containing DNA were investigated in a mouse model of experimental metastasis. Tumor cell colony formation in lungs or livers of mice after i.v. inoculation with syngeneic fibrosarcoma or thymoma cells was determined. The i.v. injection of plasmid DNA or synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs before tumor cell application strongly inhibited metastasis. Because synthetic CpG-ODN was not directly tumor cytotoxic, the target cells for this CpG-ODN effect were determined. The cytotoxic activity on standard natural killer (NK) targets as well as on fibrosarcoma cells of splenic NK cells and NKT cell-containing liver mononuclear cells derived from CpG-ODN-treated mice was strongly enhanced. Participation of NK/NKT cells in the CpG-ODN-induced antimetastatic effect was demonstrated by reduction of the antimetastatic effect in mice depleted of NK/NKT cells and β2-microglobulin-deficient mice. Neutralization of interleukin 12, interleukin 18, and IFN-γ did not interfere with the CpG-induced antimetastatic effect. However, in sera of CpG-ODN-treated mice, high levels of IFN-α were detected, and in IFN-α/β receptor-deficient mice, the CpG-ODN-induced antimetastatic effect was strongly reduced. These data indicate that CpG-ODNs activate NK/NKT cells for antimetastatic activity indirectly via IFN-α/β receptor activation. The exploitation of the stimulatory activity of CpG-ODN for the innate immune system might be a useful strategy for antimetastatic therapy.

INTRODUCTION

Bacterial products like lipopolysaccharide are known to modulate the immune response in a rather nonspecific manner (1). Accumulating data suggest that bacterial DNA likewise plays an important role in the activation of the immune system of mice (2). Unmethylated CpG-dinucleotide-containing sequences (CpG-ODNs) were found to be responsible for the stimulatory effect of bacterial DNA (3–9). The most active immune stimulatory sequences in mice contain a palindromic sequence with an unmethylated CpG-dinucleotide that is flanked at the 5′-end by two purines and at the 3′-end by two pyrimidines. The receptor by which CpG-ODN activates immune cells has been identified as TLR9 (10). Antigen-presenting cells such as macrophages, dendritic cells, and B cells become directly activated by CpG-ODN or CpG-dinucleotide-containing sequences (CpG-ODNs) and induce a Th1 immune response (11–19). In the mouse model, CpG-ODN caused a significantly increased Th1 cytokine production and reduced lung inflammation (32, 33). However, in other models, CpG-ODN caused systemic inflammatory responses and primed inflammatory cells for lipopolysaccharide-induced toxicity (11, 34, 35) and induced arthritis (36).

We investigated the effect of CpG-containing DNA in metastasis. The complete process of hematogenous metastasis comprises a series of events from dissociation of single tumor cells from the primary tumor, intravasation into the circulation, dissemination, to the extravasation into the target tissue. There, individual tumor cells form metastases, provided the tumor cells proliferate, and vascularization is initiated to supply the growing metastases with the required nutrients. The process is very selective so that only a few tumor cells are successful in forming metastases (37). The blood passage is especially critical for tumor cell survival because tumor cells are exposed to physical stress by shear forces and confronted with leukocytes in the blood. Therefore, a strong influence on the metastatic process can be expected by activation of antimetastatic functions of cellular mechanisms in the blood during tumor cell colonization.

Here we report on an antimetastatic effect after immune modulation with CpG-containing DNA observed in a mouse model of experimental metastasis. Extravasation and tumor cell colonization into the target tissues were strongly inhibited by pretreatment with CpG-containing plasmid DNA or CpG-ODN. Activation of NK and NKT cells as well as IFN-α/β receptor activation was found to be critical for the CpG-ODN-induced antimetastatic effect.

MATERIALS AND METHODS

Animals. Specific pathogen-free female 5–12-week-old C3H/HeN, C3H/HeJ, DBA/2, C57BL/6, and SCID mice were obtained from Charles River (Charles River, Sulzfeld, Germany). IFN-α/β receptor-deficient mice and control littermates were bred in the animal facility of the Deutsches Krebsforschungszentrum (Heidelberg, Germany). IFN-α/β receptor-deficient mice derived from the original line 129Sv/Ev IFNAR (38) were crossed to C57BL/6, and the progeny of the second backcross to the parental C57BL/6 strain were bred in the animal facility of the Deutsches Krebsforschungszentrum. Mice deficient in β2-m (39) were kindly provided by I. Autenrieth (Max von Pettenkofer-Institut, München, München, Germany).

Tumor Cells. CFS1 is a lacZ gene-transduced methylcholanthrene-induced fibrosarcoma cell line of C3H/HeN mouse origin (40). ESB-L-NA-10s is a lacZ gene-transduced ESB cell line derived from the highly metastatic subline of the methylcholanthrene-induced DBA/2 lymphoma L5178YE (41). YAC1 Moloney virus-induced lymphoma cells were used as target cells for the in vitro NK cytotoxicity assay. All cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 5 mm HEPES, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (all from Life Technologies, Inc., Eggenstein, Germany) in a humidified atmosphere containing 5% CO2 at 37°C. YAC1 cell culture medium also contained 50 μg/ml β-mercaptoethanol (Merck, Darmstadt, Germany).

Reagents. Monoclonal rat antimouse IL-2 receptor β (TM-β1) antibodies were used as described by Tanaka et al. (42). NK-depleted mice were generated by treatment with 1 mg of TM-β1 one week before the experiment. Mice received 1 mg of purified polyvalent rabbit antihuman IL-18 IgG (43) or an equal amount of rabbit IgG (Sigma Chemical Co., Deisenhofen, Germany) i.p. 1 day before ODN treatment.

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3 The abbreviations used are: ODN, oligodeoxynucleotide; NK, natural killer; IL, interleukin; TLR, Toll-like receptor; Th, T helper; DOTAP, 1-(2,3-dioleoyloxyl)pro- pyl)-N,N,N-trimethylammoniummethyl sulfate; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; HPF, high-power field; β2-m, β2-microglobulin, BCG, Bacille Calmette Guerin.

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Anti-IL-12 antiserum (Ref. 40; 100 μl) or anti-IFN-γ monoclonal antibody (Ref. 44; 100 μg; kindly provided by W. Falk, Department of Internal Medicine I, University of Regensburg, Regensburg) were given i.p. 2 h before the ODN treatment.

The following synthetic single-stranded phosphorothioate-modified cyto- sine-methylated or unmethylated ODNs were used: (a) CpG-ODN, 5′-TCTATGGAAAAACGCTTCTCGGCGG-3′; and (b) control-ODN, 5′-TCTATTGAAAAGGTCTTTGGGCGGG-3′ (Ref. 15; MWG Biotech, Munich, Germany).

The following plasmids were used in the metastasis experiments: empty pUC18 (Boehringer Mannheim) and pUC18/Kan (15) and pEF-BOS (45) with three different inserts (lacZ encoding β-galactosidase and p35 or p40 encoding the p35 or p40 chain of IL-12). The plasmids were isolated and purified with a plasmid purification kit (Qiagen, Hilden, Germany). Additional plasmid DNA purification was performed by electrophoresis in TRIS acetate EDTA-agarose gel. For isolation of the plasmid DNA from the gel, the Quiaex Kit was used (Qiagen). Proteinase K (Boehringer Mannheim) was used for protein degradation as described by the manufacturer.

**Preparation of Mice with DNA.** For antimetastatic treatment, 30 μl of the cationic liposome preparation (DOTAP; Boehringer Mannheim) per mouse were mixed with 5 μg of plasmid DNA or ODNs (if not otherwise indicated) in 150 μl of PBS in a polystyrene tube for 10 min with a glass syringe before injection into the tail vein 6 h (if not otherwise indicated) before the injection of the tumor cells.

**Experimental Metastasis.** CFS1/lacZ tumor cells [after detachment with a 0.05% trypsin-0.02% EDTA solution (Life Technologies, Inc.) and ESb-L.NA-10s tumor cells were washed twice. CFS1/lacZ (1 × 10^6) or ESb-L.NA-10s cells (2 × 10^5) in 0.2 ml of medium were injected into the lateral tail vein of mice. Animals were killed by cervical dislocation on day 3 after CFS1/lacZ or ESb-L.NA-10s tumor cell inoculation. To quantify micrometastases, crossections of the lungs from mice injected with CFS1/lacZ cells or crossections of the livers from mice injected with ESb-L.NA-10s cells, respectively, were fixed in 2% formaldehyde/0.2% glutaraldehyde (Merck) in PBS for 10 min at 4°C. After washing with PBS, the sections were incubated in X-gal staining solution consisting of 1 mg/ml X-gal [AppliChem (Darmstadt, Germany)] and 0.05% trypsin-0.02% EDTA solution (Life Technologies, Inc.) in PBS for 3–12 h at 37°C. The organs were rinsed with PBS and stained with eosin. Metastases were determined by counting the metastatic foci microscopically using a measuring grid. Per organ tumor colonies in at least six different sections were counted. The results are given as the mean ± SD of CFS1 colonies/mm² or ESb-L.NA-10s colonies/HPF (×40).

**NK/NKT Cytotoxicity Assay.** Cytotoxic activity was tested in a standard 4-h ^51^Cr release assay. Briefly, splenic effector cells were cultured at E:T ratios of 200:1, 100:1, 50:1, and 25:1 with ^51^Cr-labeled YAC1 or CFS1 target cells [5 × 10^5 cells/ml labeled with Na^51^Cr (100 μCi; New England Nuclear DuPont, Bad Homburg, Germany) for 90 min] in quadruplicates for 4 h at 37°C. Label release into the supernatant was used to calculate the cytotoxicity: percentage of cytotoxicity = (a − b)/(c − b) × 100, where a represents the cpm released in the test well, b represents the spontaneous release from target cells without the addition of effector cells, and c represents the total release.

NK cell-containing liver mononuclear cells were isolated as described by Watanabe et al. (46) after liver perfusion of anesthetized mice. For determination of the cytotoxic activity, the isolated cells were used at E:T ratios of 10:1 and 5:1.

**IFN Assay.** Mouse sera were assayed for IFN activity using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test as described previously (47). Briefly, monolayers of L929 cells (American Type Culture Collection; CCL1) were incubated overnight with 2-fold dilutions of the prediluted sera and then infected with murine encephalomyocarditis virus (0.1 plaque-forming unit/cell). Twenty-four h later, cells were incubated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co.) for 4 h. IFN-dependent protection from viral cytopathic effect was measured after lysis of cells with 2-propanol/50 mM HCl by colorimetric analysis of alcohol-soluble dye at 570 nm using an ELISA reader (Multiskan MS; Labsystems). To determine the type of IFN responsible for antiviral protection, the serum samples were adjusted to an activity of 50 IU and incubated for 90 min at room temperature with excess amounts of neutralizing monoclonal antibodies to mouse IFN-α, IFN-β (48), or IFN-γ (44). Residual antiviral activity in the diluted sera was then assayed as described above.

**Statistics.** Results are expressed as the mean values ± SD from at least three mice per group. Each finding was reproduced in at least three independent experiments. P values were determined using Student’s t test.

**RESULTS**

**Antimetastatic Effect of Plasmid DNA.** To test the effect of CpG-containing DNA on the metastatic process, mice received a mixture of liposome transfection reagent and increasing amounts of plasmid DNA i.v. Such treatment 3 days before CFS1 tumor cell injection inhibited lung tumor colonization in syngeneic C3H/HeN mice dose dependently (Fig. 1A). Although the reduction of plasmid DNA-induced ESb-L.NA-10s liver tumor colonization in tumor cell-syngeneic DBA/2 mice was less predominant (Fig. 1B), these data suggest that the inhibition of metastasis is independent of the tumor cell line, mouse strain, and target organ. Five plasmid DNAs containing different cDNAs (coding for the p35 or p40 of IL-12 or for the lacZ gene) or empty vectors (pUC18 or pUC18/Kan) gave very similar results (data not shown). Liposomes by themselves did not affect the metastatic process but enhanced the antimetastatic effect of the plasmid DNA to a level similar to that of pretreatment with IL-12 (Table 1).

A possible antimetastatic effect of potentially contaminating bacterial endotoxin in the liposome-DNA mixture was excluded by using endotoxin-resistant C3H/HeJ mice. No difference in tumor colonization was observed compared with endotoxin-sensitive C3H/HeN mice (data not shown). Neither purification of the plasmid DNA using preparative agarose gel electrophoresis nor treatment with protease K reduced its antimetastatic effect in the CFS1 tumor cell model (data not shown).

**Unmethylated CpG Motifs Are Essential for Inhibition of Metastasis.** Unmethylated CpG motifs have already been shown to be the functional principle of immune-stimulatory DNA (3–6, 8). To obtain evidence that CpG motifs are responsible for the antimetastatic effect, we tested the effect of CpG-containing single-stranded synthetic phosphorothioate-modified ODNs (CpG-ODN) that have been shown to exert adjuvant activity (15). As with plasmid DNA, lung colonization by CFS1 tumor cells (Fig. 2A) and liver colonization by ESb-L.NA-10s tumor cells (Fig. 2B) were inhibited in mice pretreated
The relevance of the enhanced NK/NKT cell cytotoxicity on tumor colony formation was evaluated by using β2m-deficient mice that have reduced numbers of NKT cells and impaired NK cytotoxicity (39, 51, 52) and by using NK/NKT cell-depleted mice. NK/NKT cell depletion was achieved by treatment with a monoclonal antibody to the IL-2 receptor β chain TM-β1 (42). The effectiveness of the antibody treatment on NK/NKT cytotoxic activity was ascertained in the conventional NK assay comparing the lytic activity of spleen cell preparations and liver-derived NKT cells from TM-β1-treated mice and control mice. No splenic NK or liver NKT cell cytotoxic activity on YAC1 targets was measurable 7 days after the antibody treatment (data not shown). The number of CFS1 micrometastases in such NK/NKT cell-depleted mice was increased about 20-fold compared with untreated mice. Therefore, $5 \times 10^4$ instead of $1 \times 10^6$ CFS1 tumor cells were injected to obtain comparable tumor colony

### Table 1: Antimetastatic effect of IL-12 or plasmid DNA and transfection reagent on CFS1 tumor cell colonization

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Transfection reagent</th>
<th>Tumor colonies/mm²</th>
<th>% inhibition</th>
</tr>
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<tbody>
<tr>
<td>PBS</td>
<td>-</td>
<td>12.3 ± 5.7</td>
<td>98</td>
</tr>
<tr>
<td>rmIL-12</td>
<td>-</td>
<td>0.3 ± 0.4</td>
<td>98</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>+</td>
<td>0.3 ± 0.2</td>
<td>98</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>-</td>
<td>4.2 ± 4.2</td>
<td>66</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>13.0 ± 3.2</td>
<td>&lt;0</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of CPG-ODN on metastasis. A, C3H/HeN mice were treated i.v. with graded amounts (as indicated) of CPG-ODN in DOTAP transfection reagent 6 h before tumor challenge. Tumor colonies (mean ± SD) were counted in lung sections (n = 3; P = 0.001 for 0 versus 5.0 μg of CPG-ODN). B, CPG-ODN pretreatment (5 μg/mouse) before i.v. injection of ESb-L.NA-10s T-cell lymphoma cells reduced liver metastasis. Tumor colonies (mean ± SD) were counted in liver sections (n = 3; P = 0.0004 for 0 versus 5.0 μg of CPG-ODN).

Fig. 3. Unmethylated CpG motifs mediate the antimetastatic effect. A, the requirement for CpG motifs was tested by pretreating C3H/HeN mice with ODN containing GpG motifs instead of CpG motifs in DOTAP transfection reagent 6 h before CFS1 lung colonization was initiated (n = 5; P = 0.002 for 0 versus 5.0 μg of CPG-ODN). B, the effect of the methylation status of CPG-ODN was determined by comparing unmethylated versus cytosine-methylated CPG-ODN in CFS1 metastasis experiments (n = 5; P = 0.02 for GpG-ODN versus methylated CPG-ODN).

Fig. 4. Effect of CPG-ODN on NK and NKT cell activity. C3H/HeN mice were treated with 5 μg of CPG-ODN or GpG-ODN in DOTAP transfection reagent 24 h before spleen cell (left panels) and liver peripheral blood mononuclear cell cytotoxicity (right panels) was determined in a standard Cr release assay on YAC1 (top panels) and CFS1 (bottom panels) target cells.
numbers in the lungs. CpG-ODN pretreatment was not as effective in mice depleted of NK/NKT cells as compared with equally pretreated control mice (Fig. 5A). Confirming this result, β2m-deficient mice that lack NKT cells and have reduced splenic NK activity also responded with a less pronounced antimetastatic effect to CpG-ODN pretreatment (Fig. 5B).

**Role of Cytokines in the Antimetastatic Effect of CpG-ODN.** Neither neutralization of IL-12 or IL-18 with polyclonal antibodies nor neutralization of IFN-γ with monoclonal antibodies abolished the antimetastatic CpG-ODN activity, indicating that none of these cytokines is involved (Fig. 6, A–C). However, in mice deficient for the IFN-α/β receptor, CpG-ODN treatment was not able to induce a significant reduction of tumor colonization (Fig. 6D). We therefore inoculated wild-type mice with CpG-ODN and determined IFN activity in the sera after 6 and 12 h. As shown in Table 2, high levels of IFN were detected in CpG-ODN-treated mice in comparison with control animals. Neutralization experiments using monoclonal antibodies to IFN-α, IFN-β, and IFN-γ clearly revealed that IFN-α is responsible for the antiviral activity in these sera (data not shown).

**DISCUSSION**

Bacterial DNA from *Mycobacterium bovis* (BCG) and randomly chosen synthetic ODNs with hexameric palindromic sequences from cDNA encoding BCG proteins have been shown to be active compounds in BCG tumor therapy (53–55). For CpG-ODN 1668, which contains a GACGTT motif and is therefore different from the CpG-ODN used in this study, the activation of immune cells via TLR9, a member of the phylogenetically conserved receptors mediating innate immunity, has been demonstrated (10). Among the many immune-stimulatory effects, CpG-ODN are also known to enhance immune-stimulatory effects, CpG-ODN are also known to enhance antiviral activity in these sera (data not shown).

**Fig. 6. Involvement of cytokines in the antimetastatic CpG-ODN effect.** Monoclonal or polyclonal antibodies were used to neutralize IL-18, IFN-γ, or IL-12. A, polyclonal anti-IL-18 IgG (1 mg/mouse; n = 5; P = 0.002 and 0.003 in control and anti-IL-18-treated mice, respectively). B, monoclonal IFN-γ antibodies (100 μg/mouse; n = 5; P = 0.0001 and 0.0003 in control and anti-IFN-γ-treated mice, respectively). C, polyclonal anti-IL-12 serum (100 μl/mouse; n = 3; P = 0.002 and 0.001 in control and anti-IL-12-treated mice, respectively). The appropriate control substances were applied to C3H/HeN mice before the antimetastatic CpG-ODN effect was evaluated. D, to determine the involvement of type 1 IFN, CFS1 lung colonization experiments were performed with homologous IFN-α/β receptor-deficient mice heterozygous littermates [IFNα/β−/−] or with nonhomozygous littermates [IFNα/β−/−]. Mice were inoculated i.v. with unmethylated CpG-ODN (5 μg/mouse) or with the methylated compound (control). Serum was prepared from blood samples taken after 6 and 12 h and tested for IFN activity. Each value represents the IFN serum level of an individual mouse.

![Fig. 5. Involvement of NK/NKT cells in the antimetastatic CpG-ODN effect.](image)

A: Control Mice

B: TM-β1-treated Mice

**Fig. 5. Involvement of NK/NKT cells in the antimetastatic CpG-ODN effect.** A, C3H/HeN mice were depleted of NK/NKT cells by treatment with TM-β1 antibodies (42) 7 days before the antimetastatic effect of CpG-ODN on CFS1 lung colonization was investigated (n = 3; P = 0.001 and 0.02 for control versus 1.0 μg of CpG-ODN and P = 0.002 and 0.005 for control versus 5.0 μg of CpG-ODN in control and TM-β1-treated mice, respectively). B, the antimetastatic CpG-ODN effect was examined in β2m-deficient mice [β2m−/−] and compared with wild-type C57B/6 control mice (n = 4; P = 0.02 and 0.02 for control versus 1.0 μg of CpG-ODN and P = 0.008 and 0.006 for control versus 5.0 μg of CpG-ODN in control and β2m−/− mice, respectively).

**Table 2. Serum IFN levels in mice after CpG-ODN injection**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>After 6 h</th>
<th>After 12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG-ODN</td>
<td>2560</td>
<td>1920</td>
</tr>
<tr>
<td>CpG-ODN</td>
<td>640</td>
<td>1920</td>
</tr>
<tr>
<td>CpG-ODN</td>
<td>720</td>
<td>480</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;5</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

NK activity and antibody-dependent cellular cytotoxicity (8, 20–24, 56). Here, we demonstrate that experimental metastasis in mice was strongly inhibited after pretreatment with different plasmid DNAs or CpG-ODN. The use of two different tumor cell lines forming tumor colonies in either the lungs or liver indicated that the effect is not restricted to a certain mouse strain, tumor cell line, or target organ. Pretreatment with GpG-ODN and methylated CpG-ODN was not effective, showing that unmethylated CpG motifs are essential for the effectiveness of bacterial DNA (3–8, 13). Due to the short observation time in our tumor colonization experiments, the influence of CpG-ODN on further tumor colony growth remains to be established.

After the central CpG-dinucleotide motif turned out to be essential for both the antimetastatic effect and the described adjuvant effect (9, 15), we compared the required CpG-flanking sequences. For that
Purpose, plasmid DNA was generated (pUC/Kan) that is reportedly free of sequences defined as classical immune-stimulatory sequences and has been shown to exert no adjuvant activity (15). This plasmid DNA still exerts a strong antimetastatic effect in our model, although it is devoid of palindromic sequences containing a CpG-dinucleotide motif flanked at the 5′-end by either AA, GA, or AG and at the 3′-end by either TT, TC, or CT, respectively. This indicates that additional sequences in bacterial DNA that differ from the originally defined immune-stimulatory sequences exert stimulatory activity in vivo. Indeed, sequence analysis confirmed that the pUC18/Kan plasmid contains one palindromic CpG-containing sequence flanked by a 5′ GG and a 3′ CC dinucleotide. More interestingly, one nonpalindromic CpG-containing sequence (GAGCCT) that has been reported to be active on macrophages in vitro as well as in vivo for IL-12 but not for tumor necrosis factor induction (13) is present in pUC18/-KAN and thus might explain the observed antimetastatic effect. All other plasmids used in this study have an ampicillin resistance containing three classical CpG motifs like the one that was used as CpG-ODN. These findings demonstrate that interpretation of in vivo gene transfer experiments should be made with caution because obviously different CpG-ODNs can interfere with therapeutic intentions (57).

Cationic liposomes potentiated the CpG-ODN effect significantly without having any effect on metastasis themselves, supporting the finding that cellular uptake of ODN is necessary and important (12, 58). Exactly how liposomes facilitate the cellular uptake of the plasmid DNA or CpG-ODN and thereby improve the antimetastatic effect is not known (59). Because we could not detect any direct cytotoxic effect of CpG-ODN on tumor cells in our experimental settings, host-mediated antimetastatic mechanisms are suggested. In addition, the finding that pretreatment even 3 days before tumor cell inoculation was effective makes a direct antitumor toxicity very unlikely, considering the short half-life of DNA in vivo (60). Interestingly, the optimal point of time for treatment was dependent on the genetic background of the mice (i.e., −6 h for C3H/HeN mice and −24 h for C57BL/6 mice).

Enhanced antitumor activity due to activation of cytotoxic host effector cells could constitute a possible effector mechanism. Cytotoxic T cells do not seem to be the responsible effector cells because pretreatment of T-cell-deficient SCID mice with CpG-ODN also strongly reduced CFS1 lung tumor colonies (data not shown). Due to the short time course of our experiments, the development of tumor-specific CTLs or the involvement of B-cell-derived specific antitumor antibodies seems to be unlikely. However, both NK and NKT cell cytotoxicity on classical NK targets YAC1 and on CFS1 tumor cells were found to be strongly enhanced by CpG-ODN treatment. This is in agreement with reports in which NK cell activation has been described as one key feature of bacterial DNA and CpG-ODN action (3, 8, 20–24). In NK cell-depleted mice, the CpG-ODN-induced antimetastatic effect was reduced compared with that in normal mice, suggesting the involvement of NK cells in the antimetastatic mechanism by CpG-ODN. This is supported by the observation that CpG-ODN exhibited a stronger antimetastatic effect on NK-sensitive CFS1 cells than on NK-resistant ESB-L.NA-10s cells. Further support comes from the observation that β2m-deficient mice lacking αβ NKT cells (51, 52) are less susceptible to the CpG-ODN-induced antimetastatic effect. However, due to the complexity of the metastatic process, additional influences of CpG-ODN on interactions of tumor cells with endothelial cells, platelets, monocytes, and macrophages or the involvement of yet another T-cell subset cannot be excluded.

The NK/NKT cell-mediated antimetastatic effect was IL-12-, IL-18- and IFN-γ-independent. However, IFN-α/β receptor activation seemed to be essential for the antimetastatic effect. Weiner et al. (61) noted that depending on the nucleotides preceding and following the CpG motifs, some CpG-ODNs induce activation of antigen-presenting cells and NK cells along with production of IFN-α. In our model, high IFN-α production was found in CpG-ODN-treated mice. IFN-α has long been recognized as a potent activator of NK/NKT activity (62–64). Such enhanced cytotoxicity has been attributed to stimulation of perforin (65) and Fas ligand (66) expression on the cytotoxic effector cells. Thus, CpG-ODN might indirectly activate NK/NKT cells in a similar fashion, as has been shown for the stimulation of T cells via the induction of IFN-α production by antigen-presenting cells (67). The mechanism of this CpG-ODN antimetastatic effect may differ from the antimetastatic action of plasmid DNA.

Taken together, these data show that bacterial DNA and defined unmethylated single-stranded DNA sequences containing the CpG-dinucleotide motif flanked by two 5′ purines and two 3′ pyrimidines can exert strong antimetastatic activity. This CpG-ODN effect seems to be indirect: activation of antigen-presenting cells leading to the release of IFN-α is required to stimulate antitumoral NK/NKT activity via IFN-α/β receptor activation. Whether or not this activation is related to interaction with TLR9 as shown for the 1668 CpG-ODN (10) remains to be investigated. We can only exclude activation of the TLR4 by our CpG-ODN because C3H/HeJ mice expressing a functionally defective TLR4 protein still exert an antimetastatic effect similar to control mice. The growing comprehension of the molecular events by which CpG-ODN activates antitumoral effector mechanisms of the innate immune system might be useful for the development of antimetastatic treatment protocols. Further knowledge concerning the link to the adaptive immune system will reveal the therapeutic potential of CpG-ODNs as adjuvants for vaccination strategies for cancer.

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