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Divergent Therapeutic and Immunologic Effects of Oligodeoxynucleotides with Distinct CpG Motifs


Immune stimulatory oligodeoxynucleotides (ODN) with unmethylated CpG motifs are potent inducers of both innate and adaptive immunity. It initially appeared that a single type of optimal CpG motif would work in all applications. We now report that specific motifs of CpG ODN can vary dramatically in their ability to induce individual immune effects and that these differences impact on their antitumor activity in different tumor models. In particular, a distinct type of CpG motif, which has a chimeric backbone in combination with poly(G) tails, is a potent inducer of NK lytic activity but has little effect on cytokine secretion or B cell proliferation. One such NK-optimized CpG ODN (1585) can induce regression of established melanomas in mice. Surprisingly, no such therapeutic effects were seen with CpG ODN optimized for activation of B cells and Th1-like cytokine expression (ODN 1826). The therapeutic effects of CpG 1585 in melanoma required the presence of NK but not T or B cells and were not associated with the induction of a tumor-specific memory response. In contrast, CpG 1826, but not CpG 1585, was effective at inducing regression of the EL4 murine lymphoma; this rejection was associated with the induction of a memory response and although NK cells were necessary, they were not sufficient. These results demonstrate that selection of optimal CpG ODN for cancer immunotherapy depends upon a careful analysis of the cellular specificities of various CpG motifs and an understanding of the cellular mechanisms responsible for the antitumor activity in a particular tumor. The Journal of Immunology, 2001, 167: 4878–4886.

In 1893, Dr. William Coley, a New York surgeon, developed a crude bacterial extract that became known as “Coley’s toxins” (1, 2). Coley administered his toxins to 894 patients with biopsy-proven carcinomas and sarcomas; he achieved a remarkable 45% five-year survival in these otherwise inoperable or untreatable patients (1, 2). The various ingredients of Coley’s toxins were not well characterized but it can be surmised that the toxin must have contained several immunomodulatory microbial compounds, including endotoxin, bacterial cell wall components, and bacterial DNA.

It has recently become apparent that bacterial DNA has potent immunostimulatory properties. Vertebrate DNA has low levels of CpG dinucleotides and most of the cytokine residues are methylated. In contrast, the genomes of bacteria, viruses, and retroviruses are not methylated and may or may not show CpG suppression (3, 4). It is now well established that humans and other vertebrates may detect unmethylated CpG dinucleotides in particular base contexts (“CpG motifs”) as a sign of danger or infection. Such CpG DNA, or synthetic oligodeoxynucleotides (ODN) containing these CpG motifs, activate dendritic cells, monocytes, and macrophages to secrete Th1-like cytokines and to induce Th1 T cell responses, including the generation of cytotoxic T lymphocytes (5–11). In conjunction with adherent cell-derived cytokines, CpG DNA also directly activates B cells to proliferate, secrete IL-6, and Ig and to have increased cell surface expression of costimulatory molecules (3, 9, 15). In contrast to LPS and other immune stimulatory agents that have been evaluated, CpG DNA shows an extremely strong pattern of Th1-like cytokine induction and can even be used for immunotherapy of Th2-mediated allergic disease in experimental models (16–18). Teleologically, it seems likely that immune recognition of CpG DNA has evolved as a defense against intracellular pathogens, thus providing a rationale for the observed skewing of its effects toward Th1 activation (19).

Since Th1-like immune activation and NK cells are thought to be desirable for cancer immunotherapy (20, 21), we tested a variety of CpG ODN to identify which type of CpG motif is optimal for activating these responses. There was little correlation between the ability of a CpG motif to induce cytokine secretion and its ability to induce NK activity. Moreover, although several CpG ODN were active as sole immunotherapeutic agents in two tumor models, different motifs were optimal in each model. CpG ODN 1585 was optimal against B16 melanoma and its effects were dependent on NK cells. CpG ODN 1826 was optimal in a lymphoma model and its effects appeared to require NK (early) and T cells (late). These results illustrate the potent therapeutic potential of various CpG motifs and suggest that distinct CpG motifs can be custom-tailored for each desired immune effect.

Abbreviation used in this paper: ODN, oligodeoxynucleotide.

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Materials and Methods

Mice

Virus-free 4- to 6-wk-old C57BL/6 (B6), SCID, and beige mice, as well as IL-2, IL-4, IFN-γ, IL-12, TAP, perforin, and β2-microglobulin knockouts (all on a C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME); type I IFNR (IFN-IR) knockout mice (on a 129J background) were generously supplied by Dr. T. Waldschmidt (Department of Pathology, University of Iowa, Iowa City, IA). All mice were housed in the specific pathogen-free facility at the University of Iowa Animal Care Unit.

Oligodeoxynucleotides

Phosphorothioate-modified ODN and chimeric ODN were provided by Coley Pharmaceutical Group (Wellesley, MA). Chimeric ODN have phosphodiester linkages; we have previously shown this arrangement to be optimal (3, 22). The sequences used are provided in Table I. ODN were found to have undetectable endotoxin levels using the limulus amebocyte lysis assay (BioWhittaker, Walkersville, MD; lower detection limit, 0.1 endotoxin units/ml). For in vitro assays, ODN were diluted in TE buffer (10 mM Tris (pH 7.0) and 1 mM EDTA) and stored at −20°C. For in vivo use, ODN were further diluted in PBS (0.1 M PBS, pH 7.3) and stored at 4°C. All dilutions were conducted using pyrogen-free reagents.

Cytokines and B cell proliferation

Spleens were removed from 6- to 12-wk-old female C57BL/6 mice and cultured at 5 × 10^6/ml with the indicated ODN for 4 h (TNF-α) or 24 h (IL-6, IFN-γ, IL-12), the supernatants were harvested, and cytokines were detected by ELISA as previously described (14, 23). To evaluate CpG-induced B cell proliferation, spleen cells were depleted of T cells with anti-Thy-1.2 and complement treatment; viable cells were obtained by centrifugation over Lympholyte M (CEDARLANE Laboratories, Hornby, Ontario, Canada) and cultured with the indicated ODN. At 44 h, the cultures were pulsed for 4 h with 1 μCi of [3H]thymidine as described previously (3).

Tumor models

The B16.F1 melanoma and EL4 T cell lymphoma cell lines, which are syngeneic to B6 mice, were maintained in vitro (24, 25). Tumor cells were harvested and injected, i.p. (B16.F1) or s.c. (EL4), into B6 mice at the indicated time points i.p. at doses of 100,000 cells/mouse, unless otherwise indicated. Mice were checked daily for tumor growth and for survival. There were 5–10 mice in each group, unless otherwise indicated. NK cell depletion was done as previously described (26).

Statistical analysis

Survival curves were estimated using the Kaplan-Meier method (27). Hypotheses were tested using the log rank test (28). All statistical tests were performed using the SAS system (SAS System for Windows, version 8.0; SAS Institute, Cary, NC).

Table I. Identification of CpG ODN with distinct immune profiles

<table>
<thead>
<tr>
<th>ODN</th>
<th>Sequence</th>
<th>Backbone</th>
<th>IL-6*</th>
<th>IL-12</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>NK* (LU)</th>
<th>B Cell†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1826</td>
<td>TCCATGACGGTCTCTCTACGTT</td>
<td>S</td>
<td>10,574</td>
<td>8,674</td>
<td>319</td>
<td>14,183</td>
<td>0.91</td>
<td>154</td>
</tr>
<tr>
<td>1982</td>
<td>TCCAGGACGGTCTCTCTACGTT</td>
<td>S</td>
<td>0</td>
<td>257</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>ND</td>
</tr>
<tr>
<td>1880</td>
<td>TCCATGACGGTCTCTCTACGTT</td>
<td>SOS</td>
<td>2,129</td>
<td>18,170</td>
<td>317</td>
<td>4,469</td>
<td>2.72</td>
<td>52</td>
</tr>
<tr>
<td>1585</td>
<td>GGGGTCAACGCTGAGGAGG</td>
<td>SOS</td>
<td>0</td>
<td>2,365</td>
<td>1,476</td>
<td>641</td>
<td>5.38</td>
<td>2</td>
</tr>
<tr>
<td>2118</td>
<td>GGGGTCAACGCTGAGGAGG</td>
<td>SOS</td>
<td>0</td>
<td>0</td>
<td>1,421</td>
<td>578</td>
<td>0.00</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Because of their greater nuclease resistance, results for ODN with S backbones are shown using a concentration of 0.3 μg/ml, while ODN with SOS backbones were used at a concentration of 3 μg/ml. The data shown are representative of four separate experiments and dose titrations from 0.03 to 30 μg/ml.

† Cytokine values are measured by ELISA in picograms per milliliter and represent the levels in supernatants of C57BL/6 spleen cells (5 × 10^6/ml) cultured for 4 h (TNF-α assays) or 24 h (all other cytokines) with or without the indicated ODN.

‡ NK lytic activity is expressed in LU per one million cells. One LU was defined as the number of effector cells needed to cause 30% specific lysis of YAC-1 tumor target cells. We have tested several hundred ODN for their ability to stimulate NK cell lytic activity and have found 1585 to be the strongest inducer of such activity, but nearly identical results are seen with ODN in which the central palindromic (TCAACGTTGA) is replaced by other stimulatory palindromes, such as TCGACGTTGA, TCATCGATGA, GACGCAGTC, or TGGTGAGC (not shown). Generally, 1585 induced a NK activity equivalent to that induced by IL-2.

§ B cell proliferation was measured by [3H]thymidine incorporation of spleen cells as previously described (3). Data presented are stimulation indices.
various amounts of the B16 tumor i.p. on day 0. The CpG or control ODN was administered on days 0, 3, and 7 and weekly thereafter. As shown in Fig. 1, we found that the CpG ODN 1585 significantly prolonged (with a \( p < 0.001 \) for all of Fig. 1 panels comparing 1585 vs PBS or non-CpG ODN) survival of mice that received the very high challenge dose of \( 1.5 \times 10^6 \) tumor cells (Fig. 1A). When smaller numbers of tumor cells were used, the mice survival improved progressively with a resultant 80% long-term survival of the mice that received \( 1 \times 10^4 \) tumor cells and the CpG ODN 1585 (Fig. 1D). It is worth noting that the duration of survival of mice receiving PBS only was almost identical regardless of the tumor dose tested in Fig. 1, demonstrating the high tumorigenic potential of the B16 tumor. Using a \( 5 \times 10^3 \) tumor challenge dose and pooling identical experiments, we found that, using a pool of 25 mice with PBS challenge, the mean survival time was 25.16 ± 0.84 (mean ± SEM) days, the mean survival of the control CpG group was 35.53 ± 2.12, and all 25 mice in the 1585 group were long-term survivors (i.e., 0% mortality). We next attempted to determine the optimal dose and frequency of CpG ODN. B6 mice were given \( 5 \times 10^3 \) tumor cells on day 0 and then were given only one injection, on day 0 as well, of various doses of CpG ODN 1585 or control ODN (2118). As shown in Fig. 2, 100 \( \mu g \) of 1585 was optimal and resulted, in this experiment, in a 100% survival. Interestingly, higher doses (300 \( \mu g \)) were less effective and resulted in a survival rate similar to that of lower doses (30 \( \mu g \)).

**CpG-mediated protection against melanoma challenge is independent of T or B cells**

CpG ODN have been shown to stimulate a variety of immune cell types. For example, some CpG ODN are very potent mitogens for B cells and can induce the activation of APC (3, 7, 8, 42, 43). It has

![FIGURE 1. CpG therapeutic potential is dependent on tumor dose. B6 mice were given the indicated number of B16 melanoma cells on day 0. On days 0, 3, 7, 10, and 14 and weekly thereafter (for a total of 2 mo), the mice received 100 \( \mu g \) of the indicated ODN or control. There were five mice in each group. This is representative of at least 10 similar experiments. Analysis of each experiment yielded a \( p < 0.001 \) when comparing CpG to non-CpG ODN. Analysis of all pooled experiments (which would bring the total number of mice to 50) again showed a \( p < 0.001 \).](image1)

![FIGURE 2. CpG dose response. B6 mice were given \( 5 \times 10^3 \) B16 cells on day 0. On the same day, the mice received a single injection of the indicated ODN at the indicated dose. The mice were then observed for survival. There were five mice in each group. This is representative of two similar experiments.](image2)
also been suggested that CpG ODN can activate T lymphocytes (10, 11). The ability of CpG ODN to activate APC could potentially promote the induction of CTL against the tumor. To determine whether T or B cells were required for this protection, C57BL/6 SCID mice were treated with CpG ODN 1585 and challenged with the B16 melanoma using the same regimen as in Fig. 1. Similar survival rates were seen in CpG-treated SCID mice compared with wild-type mice at each tumor dose (Fig. 3). These data demonstrate that neither T nor B cells are necessary for the observed survival induced by CpG ODN in this model. Interestingly, not all CpG ODN were equally effective. When the CpG ODN 1826 (optimal B and dendritic cell-inducing motif; Table I and data not shown) and the CpG ODN 2006 (also optimal for B and dendritic cell activation) were tested, using SCID (Fig. 4) or B6 (data not shown) mice, we found CpG 1585 to be optimal whereas CpG 1826 was intermediate and CpG 2006 was similar to the non-CpG control ODN. Also shown in Fig. 4 is that, when 5 × 10^6 tumor cells were used, CpG ODN 1585 induced a 100% survival of the SCID mice, similar to the immunocompetent B6 mice. In data not shown, TAP-1 and β2-microglobulin knockout mice were protected by CpG 1585 to a similar degree as normal B6 mice, again suggesting that CD8 and NKT lymphocytes are not needed for the protective effect.

**Lack of immunologic memory in survivors**

The data shown above demonstrated that B and T cells are not necessary for the observed therapeutic effect. It remained possible, however, that T cells might play a role in tumor protection in immunocompetent mice. We reasoned that if this were the case, we should be able to demonstrate immunological memory; i.e., mice that survived the tumor challenge should be able to reject a second tumor challenge in an accelerated fashion. Therefore, mice that had been treated with CpG ODN 1585 and failed to establish tumor (tumor free for >100 days after the initial tumor challenge) were rechallenged later with 1 × 10^6 tumor cells each. The tumor challenge dose was given 2–3 mo after the last dose of CpG ODN to allow for the protective CpG ODN effect to “wear off.” Age-matched control B6 mice were also given the same dose. As shown in Fig. 5, mice previously “cured” by CpG ODN had a slightly prolonged survival (with a mean survival time of 38.9 days compared with 26.0 days for the control mice) but there were no long-term survivors and no “cures.” Thus, CpG ODN-mediated resistance to the B16 melanoma does not lead to the generation of significant Ag-specific memory responses. Indeed, in data not shown, adoptive transfer of splenocytes from cured mice failed to protect naive mice against B16 tumor challenge.

**NK cells are essential for the CpG 1585 protective effect in B16 melanoma**

The data presented so far strongly suggested that T cells and B cells are not necessary for the observed therapeutic effect, the implication being that NK cells are the cells of import. However, SCID mice, in addition to having normal NK cells, have normal APC including dendritic cells. We, therefore, examined whether NK cells were necessary for the CpG ODN effect. SCID mice were given 100 μg of anti-NK1.1 Ab, or control Ig, on days −4 and −1.

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**FIGURE 3.** T and B cells are not necessary for CpG therapeutic effect. SCID mice (on a B6 background) received the indicated amounts of B16 cells or ODN in a schedule identical to that in Fig. 1. There were five mice in each group. These data are representative of at least five similar experiments.

**FIGURE 4.** Relative efficacy of various CpG ODN in SCID mice. SCID mice received 5 × 10^5 B16 cells on day 0. The indicated ODN were given, at 100 μg/mouse in a schedule identical to that of Fig. 1. This is representative of three similar experiments with five mice per group in each experiment.

**FIGURE 5.** Lack of immunological memory in surviving mice. Long-term survivors; i.e., B6 mice that failed to establish tumor after a primary challenge with various numbers of B16 cells (these mice received CpG on day 0 of the initial tumor challenge and days 3, 7, 10, and 14 and weekly for a total of 2 mo) were rechallenged with 1 × 10^6 B16 cells i.p. 2–3 mo after the last dose of CpG. There were 13 mice in this group; there were 7 mice in the age-matched controls.
On day 0, the mice were give $2 \times 10^4$ B16 cells i.p.; some mice also received CpG ODN 1585 as above. Anti-NK1.1 Ab was given i.p. twice weekly until the end of the experiment (we have previously shown that this regimen results in near total depletion of NK cells (26)). As seen in Fig. 6, the control mice whose NK cells were depleted died more rapidly than the control mice that received the control Ig. Similarly, the mice that received CpG ODN 1585 and anti-NK1.1 had 100% mortality in a time frame identical to the control mice. The mice that received CpG ODN 1585 and control Ig had a 100% survival. These data demonstrate that it is indeed the NK cells that are responsible for the observed therapeutic effect in this model. To confirm these results further, normal B6 mice were depleted of NK cells and the effect of CpG ODN was examined. In data not shown, we found that NK cell depletion in the wild-type mice also resulted in abolishing the CpG protective effect.

Possible mechanisms of CpG induction of NK cell activity
It is clear from the above data that the effects of CpG in the B16 melanoma model are dependent on NK cells. It remained to be seen, however, as to whether CpG acted by inducing cytokine secretion by NK cells and/or dendritic cells or whether the protective effect was actually mediated by the lytic capacity of NK cells. We first examined the ability of CpG to induce NK cell killing activity in vivo in various knockout mice. CpG was injected in the footpad and the draining lymph nodes were obtained 2 days later and used as effectors against YAC-1 tumor target cells (we have previously shown (13) that this approach was optimal for the in vivo induction of NK cell killing activity by CpG). As shown in Fig. 7, CpG was able to induce NK cell activity in normal B6 mice as well as in IL-2, IL-4, IFN-γ, IL-12, and β2-microglobulin knockout mice. Interestingly, mice whose receptor for type I IFN has been inactivated (IFN-I-R) did not augment their NK cell activity. These data suggested that the induction of type I IFNs may be the pivotal step in the protection conferred by CpG. It has been known for some time that type I IFN can augment the lytic potential of NK cells without necessarily inducing their proliferation (44). We sought to determine whether NK lytic activity is necessary for rejection of the B16 melanoma. We tested beige mice whose fresh NK killing activity is barely detectable but this activity can be readily augmented with the proper stimulation (45, 46). As shown in Fig. 8A, CpG induced significant protection in beige mice with 80% long-term survival ($p < 0.0001$). Perforin knock-out mice (whose NK cells cannot kill except through the relatively less efficient Fas pathway; Ref. 47), on the other hand, had only 20% long-term survivors. These findings strongly suggest that it is the ability of CpG ODN to augment the killing activity of NK cells, perhaps by the induction of type I IFN, that is responsible for the rejection of B16 melanoma in this model.

CpG ODN-induced regression of established melanoma
The toughest challenge for any type of tumor therapy is to cure established tumors, rather than simply preventing the generation of a new tumor. To test whether CpG ODN treatment could accomplish this, mice were challenged with $1 \times 10^5$ B16 melanoma cells on day 0; $100 \mu$g of CpG ODN 1585 or control ODN 2118 was given starting 3 days afterward. The ODN were also given on days 7, 10, and 14 and weekly thereafter for 2 mo. As shown in Fig. 9, treatment with the CpG ODN 1585 led to a 60% survival rate, while the mice given a control ODN or PBS all died between days 20 and 41 (1585 vs PBS had a $p < 0.01$, 2118 vs PBS had a $p$ of 0.8 and 2118 vs 1585 had a $p$ of 0.006). The surviving mice lived with no evidence of recurrent tumor for $>100$ days, when the experiment was terminated.

Study of CpG ODN in the EL4 T cell lymphoma model
To assess whether the above results can be generalized to other tumors, we examined the effect of CpG ODN in a lymphoma model using the EL4 tumor cells, which are also syngeneic to B6 mice. Similar to B16, EL4 cells are resistant to killing by fresh NK cells but are susceptible to killing by activated NK cells. B6 mice were inoculated s.c. with $10^7$ EL4 cells on day 0 and treatment with $100 \mu$g of several different CpG ODN given i.p. weekly beginning on day 2 (i.e., days 2, 9, 16, etc.). All mice developed tumor by day 12. However, tumor growth was slower in those mice treated with CpG ODN 1826 (data not shown) and overall survival was significantly prolonged (Fig. 10A). In repeat studies, between 30 and 50% of mice treated with CpG ODN in this manner rejected the tumor and remained tumor free thereafter (Fig. 10B). A number of interesting differences were found when comparing results from the B16 model to those from the EL4 model. The CpG ODN 1585, which was more effective than the CpG ODN 1826 in the B16 model, was not effective in the EL4 model. Unlike the B16 tumor, it appeared that the EL4-challenge surviving mice were able to develop memory since they failed to develop tumor upon rechallenge (arrow in Fig. 10B), suggesting a memory response.

NK cells are necessary but not sufficient in EL4
To assess the relative contribution of NK and T cells for the rejection of EL4 induced by the CpG ODN 1826, SCID mice were
examined. In contrast to the B16 model, no antitumor activity against EL4 was seen (Fig. 11), suggesting that T cells are necessary and that NK cells, if needed, are not sufficient for the therapeutic effect. Surprisingly, however, NK cell depletion, using anti-NK1.1 as above, in normal B6 mice abrogated the protective effect of CpG (Fig. 11). Thus, in contrast to the B16 model, both NK and T cells are required for the antitumor effect in the EL4 model.

Discussion

Innate immunity is deceptively simple. Phylogenetically older than its adaptive counterpart, innate immunity uses pattern recognition receptors to recognize biologically relevant pathogens (48, 49). It is now well recognized that innate immunity triggers a cascade of events that may have a profound effect on adaptive immunity (48, 49). In recent years, it became apparent that CpG DNA can be a potent inducer of innate immunity and may promote the generation of adaptive immunity, functioning as a potent vaccine adjuvant in mice and primates (5, 6, 9, 10, 30, 31, 50–60). CpG ODN can activate dendritic cells, macrophages, monocytes, and NK cells. CpG ODN have also been shown to induce B cells to proliferate and to secrete Ig. Moreover, CpG ODN have been shown to favor CpG ODN and its ability to activate B cells and dendritic cells, and its ability to induce cytokine production (3, 8, 22, 62) (Table I). On the other hand, the phosphorothioate backbone greatly improves the stability of an optimal CpG motif and its ability to activate B cells and dendritic cells, and its ability to induce cytokine production (3, 8, 22, 62) (Table I). Since the beneficial stabilizing effects of the phosphorothioate backbone can be obtained by modification of just the 5′ and 3′ ends of the ODN (22), we have tested this type of chimeric ODN backbone. Poly(G) sequences consist of four or more consecutive guanines in a row, or two or more regions of three consecutive guanines, and are known to bind to the macrophage scavenger receptor and to improve ODN uptake and IFN-γ production (63), but to interfere with cell proliferation (64, 65).

The experiments reported in this manuscript indicate that the combination of a CpG motif with a chimeric backbone along with poly(G) sequences on the 5′ and 3′ flanks yields an ODN which has relatively little B cell-, TNF-α-, or IL-12-stimulating effects, but which is extremely active at inducing NK cell lytic activity (Table I). NK cells have long been thought to be important in tumor surveillance. By virtue of their ability to secrete IFN-γ, among other cytokines, NK cells are also thought to influence adaptive immunity (66, 67). Melanoma is a tumor model that has lent itself to the examination of various models of immunotherapy. For human melanoma, several tumor-specific Ags have been described and are known to induce T cell responses (68, 69). On the other hand, the B16 murine melanoma is thought to be amenable to treatment both by activated T or NK cells (37, 39).

Since we have shown that CpG ODN activate NK cells both in vivo and in vitro (13), we examined their effect on the survival of mice with melanoma. The data presented in this report clearly demonstrate that, by virtue of their ability to activate NK cells, some CpG ODN are quite effective, as the sole therapeutic agent, in preventing the development of B16 melanoma and, more interestingly, at rejecting an established B16 tumor. As little as one dose of CpG ODN 1585 is as protective against tumor challenge as repeated doses (Fig. 2). Moreover, there appeared to be nonlinear dose response since a 300-μg dose of CpG was less effective than

![FIGURE 8.](asdasdasd) NK lytic activity is needed for ODN effect. Beige mice or perforin knockout mice (both on a B6 background) received $1 \times 10^4$ B16 melanoma cells and ODN 1585 as described in the legend to Fig. 1. There were 5 mice each in the beige group and 10 mice in the perforin group.

![FIGURE 9.](asdasdasd) Effect of CpG on established tumor. B6 mice received $1 \times 10^4$ B16 cells on day 0. The indicated ODN were given on days +3, 7, 10, and 14 and weekly thereafter for a total of 2 mo. This experiment is representative of two similar experiments.
doses of 100 or 30 μg. This is similar to our previous findings with CpG in a Listeria model and probably relates to the observation that high doses of CpG prime mice for the sepsis syndrome (70–72).

Interestingly, it appears that the protection afforded by CpG ODN against the B16 melanoma is mediated by innate rather than adaptive immunity since SCID mice rejected the tumor just as efficiently as normal mice. Moreover, no “memory” appears to have developed in the cured immunocompetent mice. In data not shown, we were not able to transfer protection by adoptive immune transfer using spleen cells from the cured immunocompetent mice. All of these results point to innate immunity as being the system of import in this model.

The two major cellular components of innate immunity are NK cells and dendritic cells. In theory, dendritic cells could be the cells of import in this model. However, our data with the in vivo depletion of NK cells, clearly demonstrate that NK cells are the essential cells. These results do not exclude the possibility that DC may help to activate NK cells as shown in other antitumor models where NK cells can slow tumor growth, but do not cause tumor regression (73). Interestingly, it has been suggested that it is the NKT, rather than the classical NK cells, that are the cells of import in rejecting the B16 melanoma in an IL-12-induced tumor rejection model (37, 41). Our data suggest that NKT (equivalent data obtained in TAP-1 and β2-microglobulin knockout mice; data not shown) cells are not necessary for the therapeutic effect of CpG ODN in this model.

It appears that IL-12 is also not necessary for the therapeutic effect in this model. Using SCID mice, we found IL-12 (which is dependent on NKT cells for its therapeutic effect (37, 41)) to be less efficient than CpG ODN at rejecting the B16 tumor and we had no long-term survivors (data not shown). Moreover, the ability of a CpG ODN to induce IL-12 did not correlate with its ability to stimulate NK cell activity or to improve long-term survival. These data suggest that the antitumor effect of CpG ODN in this model is not mediated through the IL-12 pathway and that the IL-12 pathway is not essential for the CpG ODN beneficial effect. Indeed, IL-12 knockout mice gave a response similar to the normal B6 mice (data not shown), suggesting that IL-12 is not necessary for the protective effect. Surprisingly, the induction of IFN-γ secretion also did not correlate with CpG-induced protection, suggesting that the therapeutic effects in this model may be mediated through distinct NK activation pathways. Our studies with the various knockout mice indicate that type I IFN may be the cytokine of import since IFN-I-R knockout mice were the only cytokine/cytokine receptor knockout mice that failed to augment their NK cell activity in response to CpG both in vivo (Fig. 7) or in vitro (data not shown). Moreover, it appears that it is the lytic potential of NK cells (rather than their ability to secrete various cytokines) which is protective in this model, since perforin knockout mice yielded significantly less long-term survivors (20 vs 80%). NK cells can kill through the perforin pathway or through the Fas/Fas ligand pathway with the perforin pathway being dominant in cytokine-activated NK cells (47). It is possible that the 20% survival seen in the perforin knockout mice is due to killing through the Fas pathway, although we were unable to demonstrate significant staining of B16 with anti-CD95 Ab nor were we able to demonstrate augmentation of CD95 on B16 melanoma cultured with CpG in vitro (data not shown). Moreover, we examined the effect of CpG on the expression of other surface markers on B16 cells after culture with CpG ODN for 1–3 days; we found no change in the expression of

**FIGURE 10.** Effect of CpG in an EL4 tumor model. B6 mice received 1 × 10^7 EL4 tumor cells s.c. on day 0. Starting on day 2 and weekly thereafter, the mice received 100 μg of the indicated CpG i.p. A, Overall survival. B, Disease-free survival. The presence of tumor was monitored and the mice that developed tumor were scored as nondisease free. As is evident in B, all mice developed tumor early on but the tumor regressed only in mice that received CpG ODN, thus resulting in some mice being tumor-free survivors. The surviving mice were rechallenged with EL4 tumor s.c. (as indicated by the arrow) with no further administration of any CpG ODN; these mice failed to develop any tumor and continued to survive until the experiment was terminated. This experiment is representative of five similar experiments. A, 1826 vs PBS had a p < 0.0001; 1585 vs PBS had a p < 0.3; 1982 vs PBS had a p < 0.7; 1826 vs 1585 had a p < 0.0001.

**FIGURE 11.** NK cells are necessary but not sufficient in the EL4 tumor model. Normal B6 mice or SCID mice were used. For NK depletion, B6 mice received anti-NK1.1 Ab in vivo as described in the legend of Fig. 6; these mice are referred to as B6/-NK. Tumor and CpG administration were as described in the legend to Fig. 10. This experiment is a representative of three similar experiments. For wild-type mice, 1826 vs PBS had a p < 0.0001; for the NK depleted mice, 1826 vs PBS had a p < 1.0; and for 1826 in wild-type vs NK-depleted mice, the p <0.0001.
class I or class II MHC, CD40, CD40 ligand, CD28, CD80, or bacterial DNA are the 'effect seemed to be dependent on NK cells. Slowing the growth of a murine neuroblastoma model and that this (74) reported that a phosphorothioate CpG ODN was effective in presently under investigation. Effective therapeutic strategies based on these promising new cer therapy, and further dissection of the immunologic response to understanding of the antitumor immune responses needed for can-

ferent tumors and that one may need to design different CpG ODN

that different immunotherapeutic strategies will be required for dif-

 ineffective as antitumor agents. Importantly, our presented in this report clearly demonstrate that CpG ODN can be

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might play a role in this response as well. Since the CpG motifs

are clear that the lytic activity of NK cells is pivotal. It is likely that

through which NK cells contribute to the antitumor response in the EL4 model is different from that in the melanoma model, where it is clear that the lytic activity of NK cells is pivotal. It is likely that the role of NK cells in the EL4 model does not rely on their lytic potential but relies on their ability to secrete certain cytokines (such as IFN-γ, for example) which may facilitate the activation of T cells that lead to rejection of EL4. These possibilities are currently under investigation.

Interestingly, the results in these two models also differ from our previous findings in a murine B cell lymphoma model (35). In the B cell lymphoma model, CpG ODN alone were not effective at inhibiting tumor growth, although some CpG ODN were synergistic with antitumor mAb and gave an impressive survival rate of 80%. The 38C13 lymphoma used in that model is not susceptible to NK or activated NK cell lysis. Interestingly, Carpenter et al. (74) reported that a phosphorothioate CpG ODN was effective in slowing the growth of a murine neuroblastoma model and that this effect seemed to be dependent on NK cells.

For more than a century, it has been clear that bacterial extracts are the "missing link" to Coley's toxins, the data presented in this report clearly demonstrate that CpG ODN can be effective as antitumor agents. Importantly, our findings suggest that different immunotherapeutic strategies will be required for different tumors and that one may need to design different CpG ODN for inducing different types of desired responses. A more complete understanding of the antitumor immune responses needed for cancer therapy, and further dissection of the immunologic response to CpG ODN, should allow for the development of more rational and effective therapeutic strategies based on these promising new classes of potent immunologic agents.

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