Successful Treatment of Intracranial Gliomas in Rat by Oligodeoxynucleotides Containing CpG Motifs

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ABSTRACT

Phosphorothioate oligodeoxynucleotides with CpG motifs (CpG-ODNs) activate various immune cell subsets and induce production of numerous cytokines. To evaluate whether CpG-ODNs can induce rejection of established tumors, Lewis rats were inoculated intracerebrally with syngeneic CNS-1 glioma cells and subsequently injected with CpG-ODNs into the tumor bed. Although all of the control rats (n = 14) died within 23 days, 88% of the animals (n = 8) treated with a single CpG-ODN injection 5 days after tumor inoculation showed long-term survival (>90 days; P < 0.002). CpG-ODNs increased tumoral infiltration with macrophage/microglial cells, CD8, and natural killer lymphocytes. CpG-ODN-cured animals were further protected against a second tumor challenge. CpG-ODNs had no effect on a s.c. CNS1 tumor in nude mice, which suggested that CpG-ODN is not directly cytotoxic and that immunostimulation is required for the antitumoral effect. These findings suggest that intratumoral injections of CpG-ODNs represent a new immunotherapeutic approach in human gliomas, which overcome the need for the selection and purification of a tumoral antigen.

INTRODUCTION

Bacterial DNA can activate in vivo various immune cell subsets, including B cells, macrophages, or NK cells, and induce production of a wide variety of cytokines, such as tumor necrosis factor-α, IFN-γ, or IL-12 (1–4). These immunogenic properties have been linked to a much higher frequency of unmethylated CpG dinucleotides (CpG motifs) in bacterial DNA than in vertebrate DNA (5). CpG motifs flanked by two 5′ purines and two 3′ pyrimidines appear as the most potent immunostimulatory sequences (5). Synthetic ODNs containing such hexamers have immunological effects similar to those seen with bacterial DNA and are promising vaccine adjuvants (5–7).

Malignant glioma patients exhibit depressed in vitro and in vivo reactivity of peripheral blood and tumor-infiltrating lymphocytes (8, 9), which has been attributed to the decreased IL-2 sensitivity of CD4+ lymphocytes (10) and local secretion, by glioma cells, of the immunosuppressive factors, transforming growth factor-β2, prostaglandin E2, and IL-10 (11–13). Treatment with an immunostimulatory agent that would reverse this immunosuppressive tumor environment might allow the rejection of glioma cells by the immune system. Thus, we evaluated whether an ODN with a CpG-ODN could lead to glioma rejections. Immunocompetent rats were inoculated with syngeneic CNS1 glioma cells and subsequently treated with CpG-ODNs directly into the tumor bed to bypass the blood-brain barrier (14).

MATERIALS AND METHODS

Oligonucleotides. Purified single-stranded ODNs were purchased from Genset (Paris, France). The sequences used in this study were CpG-ODN, 5′-TGACTGTAAGCTTCGAGATGA, which contains two CpG dinucleotides, one being part of an immunostimulatory sequence (5′-AACGTT), and IMM-ODN, 5′-TGACTGTAAGCTTCGAGATGA, in which both CpG motifs had been mutated, as described by Roman et al. (6). Lipopolysaccharide levels in the ODNs, assessed by the Limulus assay, were <1 ng/μg (BioWhittaker, Emerainville, France).

Glioma Cell Line and in Vitro Toxicity Assays. The murine glioma cell line CNS-1 (15), kindly provided by Dr. W. F. Hickey (Hanover, NH), was cultivated in RPMI 1640 supplemented with 10% FCS (Boehringer, Meylan, France). For toxicity assays, 10,000 CNS-1 cells were plated in 25-cm² culture flasks, and ODNs were added (at 5 × 10⁻⁶ or 5 × 10⁻⁷ M) on day 1 of culture. The cells were harvested on day 3 by trypsinization and counted on a Malassez hematocytometer. Cell viability was then checked by trypan blue exclusion. The experiments were performed in triplicate, and results were expressed as the means ± SD.

Tumor Implantation and in Vivo Treatment. For intracerebral tumor implantation, anesthetized 6- to 7-week-old male Lewis rats (CERJ, Lyon, France) were placed in a stereotactic frame, and a burr hole was drilled 2 mm posterior and 4 mm lateral to the bregma. The brain was punctured by a Hamilton syringe, and the needle was inserted 5 mm deep. Viable glioma cells (1 × 10⁵), suspended in 10 μl of RPMI...
1640, were injected. The ODNs, dissolved to the appropriate concentration in 7 μl of saline, were injected similarly.

Six-week-old male nude mice (CERJ, Lyon, France) were injected s.c. with 10^5 CNS1 cells into the right flank. Five days later, mice were injected into the tumor bed either with 50 μl sodium chloride or 100 μg of ODNs dissolved in 50 μl of saline. Tumor volumes were assessed with a caliper every 4 days using the formula: \( \pi/6 \times \text{length} \times \text{width}^2 \) (16).

**Histology/Immunohistochemistry.** For histology and histochemistry analysis, the rats were killed just before the expected date of death. After induction of anesthesia, the animals underwent intracardiac perfusion with 2% paraformaldehyde in PBS. The brains were then fixed for 2 h in the same fixative and either embedded in paraffin for staining with H&E or snap-frozen and stored at −80°C. For immunohistochemistry, frozen sections (10 μm) were thawed, incubated at room temperature for 1 h with 10% normal goat serum (Jackson ImmunoResearch, West Grove, PA), and subsequently incubated for 2 h with the primary antibody. The following primary antibodies (all from Serotec, Oxford, UK) were used: OX8 (1:100) directed against CD8-positive T cells; 3.2.3 (1:100) against NK cells, and ED1 (1:500) against microglial cells and macrophages. The sections were incubated additionally for 1 h with FITC-conjugated goat antimouse IgG1 (1:50; Clini- sciences, Montrouge, France) and were examined under a Leica fluorescent microscope. Quantitative analysis of labeled cells was performed using two different sections for each sample. In each section, the number of positive cells in three random fields measuring 0.135 mm² were counted by an investigator who was blinded to the animal history.

**Statistics.** Statistical significance for survival was assessed using the Kaplan-Meier analysis. Comparisons of cellular infiltrates were done by the Mann-Whitney U test.

**RESULTS**

**Injections of CpG-ODNs into Intracerebral Glioma.** In the first set of experiments, each rat was inoculated intracerebrally with 10^5 CNS1 cells and then injected 1, 5, or 9 days later in the same location with 100 μg of CpG-ODN in 7 μl of vehicle or with 7 μl of vehicle alone on day 1 as control (Fig. 1). The control animals injected with vehicle (n = 14) showed a median survival time of 15 days, all dying within 23 days. Survival was increased in CpG-ODN-treated animals with long-term survival (>90 days) in 67% (n = 6; P < 0.01), 88% (n = 8; P < 0.002), and 29% (n = 7; P < 0.07), respectively, for rats treated on days 1, 5, and 9, whereas all of the control animals (n = 14) died within 23 days.

Survival curves of Lewis rats inoculated intracerebrally with CNS1 glioma cells and injected into the tumor bed with various doses of CpG-ODN or sodium chloride (----) on day 1. Long-term survival (>90 days) was increased to 60% (n = 5; P < 0.03), 40% (n = 5; P = 0.22), and 0% (n = 5; P, not significant), respectively, for rats treated with 50 (----), 10 (---), and 1 μg (----), whereas all of the control animals (n = 5) died within 3 weeks.

**Fig. 3** Survival curves of rats (n = 5) previously cured by CpG-ODN injections (----) and subjected to a second intracranial tumor challenge with 10^6 CNS1 cells, 12 weeks after completion of the initial treatment. All of them survived without any treatment, whereas all of the control rats (----, n = 4) died (P < 0.001).

None of the surviving animals exhibited neurological disabilities, and histopathological studies of six animals killed on day 90 showed no tumor cells but showed enlarged ventricles in the vicinity of the former tumor site. Tumor growth was found to be the cause of death for all of the nonsurviving animals.

To assess the minimal dose of required CpG-ODNs, rats were injected intracerebrally 1 day after CNS1 tumor graft with 50, 10, or 1 μg of CpG-ODN in 7 μl of vehicle (Fig. 2). Long-term survival (>90 days) was observed in 60% (n = 5; P < 0.03), 40% (n = 5; P = 0.22), and 0% (n = 5; P, not significant) for rats treated with 50, 10, and 1 μg, respectively, whereas all of the control animals injected with the vehicle died (n = 5).

To assess whether CpG-ODNs could also act on a distant intracerebral tumor, rats were grafted with 10^5 CNS1 cells into the right cerebral hemisphere in two different locations (4-mm
distance) and also were injected 5 days later in only one of the
two locations with 50 μg CpG-ODN in 7 μl of vehicle or in 7 μl of vehicle alone. The control animals (n = 6) all died within
25 days, whereas long-term survival (>90 days) was seen in
44% of those treated with CpG-ODNs (n = 7; P < 0.05).

Second Challenge with CNS1 Cells in Rats Cured by
CpG-ODNs. Twelve weeks after completion of the initial
treatment, some of the surviving animals (n = 5) that had not
been killed for histological evaluation were subjected to a sec-
ond intracranial tumor challenge with 10⁶ CNS1 cells. All of
them survived without any treatment, whereas all of the control
rats (n = 4) died (P < 0.001; Fig. 3). Histopathological studies
did not reveal any evidence of residual tumor cells in the
surviving rats.

Intratumoral Treatment with an ODN That Lacked the
CpG Motifs. To assess whether the tumor rejection observed
with CpG-ODNs was dependent on the CpG motifs, rats were
grafted intracerebrally with 10⁵ CNS1 cells and then injected
24 h later in the same location with 7 μl of vehicle (n = 5) or
with 100 μg of IMM-ODN that lacked the CpG motifs (n = 5).
IMM-ODN injections did not affect median survival time when
compared with controls (14 versus 15 days), and all of the rats
in both groups died.

In Vitro Toxicity of CpG-ODNs. To investigate whether
the effect of CpG-ODNs could be mediated by a direct toxicity
on glioma cells, 10,000 CNS1 cells were plated in 25-cm²
culture flasks. CpG-ODN or IMM-ODN was added at two
different concentrations (5 × 10⁻⁶ or 5 × 10⁻⁷ M)on day 1 of
culture, and the cells were counted on day 3. No inhibition of
proliferation was seen in either of the concentrations tested
when compared with the controls (mean ± SD: 97,000 ± 1,500
for control; 73,000 ± 1,000 and 88,000 ± 1,500 for 5 × 10⁻⁶
M and 5 × 10⁻⁷ M CpG-ODN, respectively; 77,000 ± 2,000 and
99,000 ± 1,000 for 5 × 10⁻⁶ M and 5 × 10⁻⁷ M CpG-ODN,
respectively).

Treatment with CpG-ODNs in Nude Mice. To further
assess that the effects of CpG-ODN did not result from a direct
cytotoxicity on CNS1 cells but rather involved the immune
system, nude mice that had been implanted s.c. with 10⁵ CNS1
cells were divided into two groups (n = 4 in each) and were
injected on day 5 at the same location with a single injection of
either 50 μl of sodium chloride or 100 μg of CpG-ODN. No
significant differences in tumor volumes measured on day 12
were seen between the two groups (mean tumor volume ± SE:
628 ± 158 mm³ for saline; 613 ± 74 mm³ for CpG-ODN; P = 0.56).

Fig. 4  Histological analysis of tumors in rats implanted on day 0 with CNS1 cells, injected with saline (a and c) or CpG-ODN (b and d) on day 5,
and killed on day 6. CNS1 tumor cells invade the normal parenchyma in a control rat (a), whereas in a CpG-ODN-treated animal (b), viable tumor
cells are surrounded by an area of pyknotic cells and a cicatricial tissue, where macrophages and lymphocytes are seen but where tumor cells are
sparse. Normal parenchyma is seen on the right. Inset, enlargement of the pyknotic area showing apoptotic cells (H&E staining, ×200; inset, ×400).
Immunohistochemical analysis (×400) with OX8 antibodies showing higher tumoral infiltration with CD8 T lymphocytes in CpG-ODN-injected
animals (d) than in control rats (c).
Recruitment of Macrophage/Microglial, NK, and CD8 Tumor-infiltrating Cells by CpG-ODN Injections. To study the effects of CpG-ODN on tumors, rats implanted on day 0 with CNS1 were injected with CpG-ODN or with saline on day 5. The rats were killed on day 6, and the brains were removed for histological evaluation. On H&E-stained brain tissue sections, CpG-ODN-treated animals showed intratumoral infiltration and large areas of apoptotic cells at the periphery of the tumors, whereas the controls showed less infiltration and no apoptosis (Fig. 4). The number of OX8- (CD8 T cells), 3.2.3- (NK cells), and ED1- (macrophages and microglial cells) positive tumor-infiltrating cells in CpG-ODN-treated animals (■) and in control rats (□; mean per 0.135-mm² field ± SE): The rats were implanted on day 0 with CNS1 cells, injected with CpG-ODN or saline on day 5, and killed on day 6 (+, P < 0.05).

DISCUSSION

These data show that rejection of a syngeneic intracerebral glioma can be achieved by intratumoral injections of CpG-ODNs. Although none of the control animals survived the tumor challenge, >85% of the rats treated 5 days after the tumor inoculation showed long-term survival and tumor eradication. Moreover, increased survival was seen in rats bearing two separate tumors but treated only at one tumor site, which suggests that the treatment may also be active on tumors located at some distance from the CpG-ODN injections, although a diffusion of CpG-ODN through the cerebral spinal fluid cannot be ruled out. We have recently reported that CpG-ODN injections could also induce rejection of syngeneic neuroblastoma in mice through an immune mechanism (16). The efficacy of CpG-ODNs in both gliomas and neuroblastomas suggests that the antitumoral properties of CpG-ODNs may be applied to several types of cancers, the spectrum of which remains to be defined. Intratumoral injections of the MY-1 extract of Mycobacterium bovis have been shown previously to inhibit tumor growth in murine models of carcinoma (17). Because the activity of MY-1 was dependent on the presence of bacterial DNA, at least part of the effects of MY-1 might be attributable to the immunostimulatory CpG motifs included in bacterial DNA. However, MY-1 failed to improve survival in a mouse glioma model (18). The superior efficacy of CpG-ODNs described in the present study may be related to the amplification of immunostimulatory sequences and the enhanced stability of ODNs (19) when compared with crude bacterial DNA.

The mechanisms responsible for the antitumoral effects could result from a direct cytotoxicity of CpG-ODNs. However, no cytotoxicity on CNS1 cells was detected in vitro, and their antitumoral effects were abrogated in nude mice, which strongly suggests that eradication of glioma cells were immune-mediated. The increased tumoral infiltration with macrophage/microglial cells, CD8, and NK lymphocytes seen after CpG-ODN injections when compared with the control group support this hypothesis. Interestingly, rats that were cured by CpG-ODN injections were protected additionally against a new tumor challenge, which showed that a long-term immunity was primed. CpG-ODN has been reported to induce endogenous release of IL-12 by macrophages (4). Because IL-12 displays antitumor effects in murine glioma models (20, 21), the CpG-ODNs’ antitumoral effects could be mediated, at least in part, by IL-12 secretion. CpG-ODNs carry out the advantage over IL-12 alone to trigger a sustained expression of IL-12 for at least 8 days (22), whereas the half-life of exogenous IL-12 is <10 h (23).

Despite the susceptibility of the Lewis strain to develop experimental acute encephalomyelitis, rats injected with CpG-ODN showed no short- or long-term neurological impairment. Histological studies of the brains of cured animals showed no abnormalities other than enlarged ventricles in the vicinity of the original tumor sites. Direct injections of CpG-ODN in normal rat brains yielded only minimal necrosis along the needle tracks (data not shown). Although additional toxicological studies are mandatory, CpG-ODN injections seemed thus far to have no deleterious effect on the normal brain parenchyma.

CpG-ODNs are promising adjuvants for immunization against selected antigens such as hepatitis B (6, 24) or for immunization against a tumor antigen in a murine lymphoma model (25). Our data show that direct injections of CpG-ODN alone into tumors represent a simple means of achieving therapeutic effects without the need for selection and purification of tumor antigens. Human gliomas display a locally invasive pattern of growth and rarely metastasize, which makes local treatment clinically relevant. Intratumoral injections of CpG-ODN, therefore, may represent a new immunotherapeutic approach in gliomas and warrant additional studies in other malignancies.
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