



## CpG-oligodeoxynucleotide rejection of a neuroblastoma in A/J mice does not induce a paraneoplastic disease

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### Abstract

Oligodeoxynucleotides containing CpG motifs (CpG-ODN) are powerful immunostimulating agents that are currently entering clinical trials in various human diseases. Concerns exist about potential auto-immune diseases triggered by such treatment. We thus investigated whether tumor rejection induced by CpG-ODN treatment could lead to a harmful auto-immune reaction against the nervous system (neurological paraneoplastic disease) at the time of acute tumor rejection, or in long-term surviving animals. Mice bearing established neuroblastomas were treated with intra-tumoral injections of CpG-ODN, resulting in tumor inhibition and tumor rejection in one-third of the animals. Immunocytochemistry and Western blot studies revealed no specific anti-neuronal antibodies. None of the animals developed neurological disabilities and histological studies of the nervous system were normal. CpG-ODN can therefore trigger neuroblastoma rejection without inducing neurological paraneoplastic disease. © 2002 Published by Elsevier Science Ireland Ltd.

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Independently of any antisense effects, CpG motifs included in DNA or synthetic oligodeoxynucleotides (CpG-ODN) are specifically recognized by the Toll-like receptor 9 [7], leading to a strong macrophage and B-cell activation, which drives the immune response towards the Th1 phenotype [10]. Such ODN have potential therapeutic applications in various human diseases such as allergies and infections [8,9]. In cancer, CpG-ODN have been successfully used either in combination with a tumoral antigen [17] or alone as a locally injected immunostimulatory agent. We have previously reported that eradication of established neuroblastomas or gliomas can be achieved by intra-tumoral injections of CpG-ODN and that tumor rejection was associated with the priming of a long-lasting anti-tumor immunity [2,3].

Since CpG-ODN are nowadays entering clinical trials, auto-immune diseases potentially induced by CpG-ODN become a major concern, given their potent immunostimulatory activity. We therefore investigated whether the immune response associated with tumor rejection could lead to a harmful auto-immune attack against the nervous system. This question was particularly relevant because a subset of patients with cancer develop neurological paraneoplastic syndromes characterized by inflammation of the nervous system, indolent tumor growth and an immune reaction against antigens shared by the nervous system and the tumor cells. *High-titer* antibodies against these antigens are sometimes identified in the patients' sera and are strongly associated with paraneoplastic syndromes [13]. In particular, neuroblastoma patients sometimes develop a neurological paraneoplastic syndrome, and auto-antibodies against the HuD neuronal antigen are found in some of these patients' sera [4].

In this study, we investigated whether mice undergoing neuroblastoma rejection with CpG-ODN treatment show any evidence of paraneoplastic disease. Central (brain)

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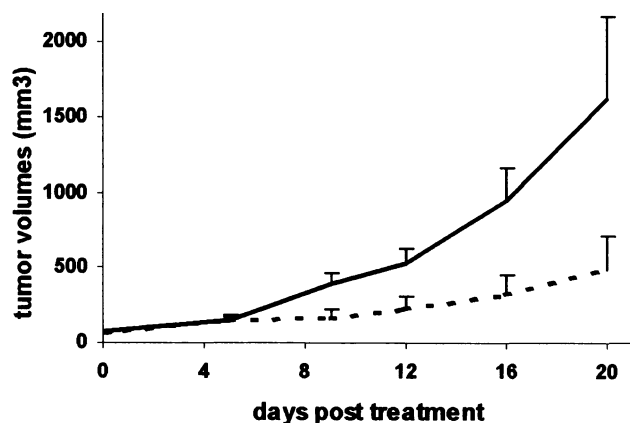


Fig. 1. Neuro2a tumor volumes (mean + SEM) in A/J mice bearing Neuro2a tumors of 5 mm diameter and injected subcutaneously around the tumor three times a week for 2 weeks with 25  $\mu$ l sodium chloride (black line,  $n = 6$ ), or 25  $\mu$ g CpG-ODN (dashed line,  $n = 7$ ). Days are the number of days after initiation of treatment.

and peripheral (intestinal Peyer plaques) nervous systems were thus examined histopathologically and mice sera were screened for anti-neural antibodies by immunocytochemistry and Western blotting.

The Neuro-2a cell line, a subclone of the C1300 murine neuroblastoma that was developed in A/J mice (CCL-131, American Type Culture Collection) was maintained in RPMI and 10% fetal bovine serum (Boehringer, Meylan, France). On the indicated day, A/J mice (Harlan laboratory, Lyon, France) were injected subcutaneously with  $10^6$

Neuro2a cells into the right flank. When the tumor diameter had reached 5 mm (approximately 10 days after tumor inoculation), mice were injected around the tumor either with saline or with a single-stranded phosphorothioate oligodeoxynucleotide (Eurogentec, Seraing, Belgium), containing two CpG dinucleotides, (5'-TGACTGT-GAACGTTTCGAGATGA-) as described elsewhere [2]. The tumor volumes were then assessed with a caliper every four days using the formula:  $\pi/6 \times \text{length} \times \text{width}^2$  [2]. The mice were examined weekly for abnormal behavior, obvious neurological deficits or drowsiness.

To investigate whether brain inflammation occurred during the acute phase of tumor rejection, mice bearing 5 mm tumors were injected three times a week for 2 weeks, either with 25  $\mu$ l saline ( $n = 6$ ) or with 25  $\mu$ g CpG-ODN ( $n = 7$ ). All the control animals treated with saline developed progressive tumors. Treatment with CpG-ODN induced tumor disappearance in three mice out of seven, and the tumor growth rate was reduced in the other animals when compared with mice treated with saline (mean tumor volume  $\pm$  SEM by day 20 after initiation of treatment:  $488 \pm 229$  versus  $1628 \pm 542$  mm<sup>3</sup>, for the CpG-ODN group and the control group, respectively,  $P = 0.002$ ; Fig. 1). None of the mice developed clinical impairment. The mice were sacrificed on day 20 and blood samples were collected. This delay of 20 days was chosen because control animals had to be sacrificed because of tumor growth, and because experiments in experimental allergic encephalitis (EAE) models have shown that maximal T-cell infiltrations and neurological symptoms occur within 3 weeks after

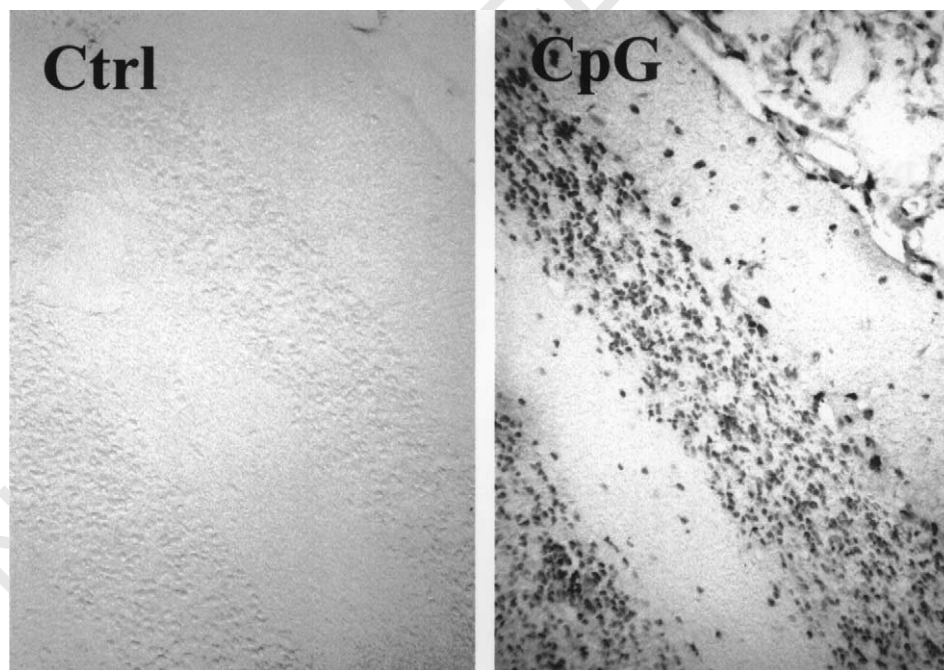


Fig. 2. Immunohistochemical staining by the serum of a CpG-treated mice (CpG), incubated on a cerebellar section of a Wistar rat at the dilution of 1:200, showing a reactivity in the granular and the molecular layer of the cerebellum, and in the choroid plexi (upper right). On opposite, the serum of a mouse treated with saline (Ctl) did not show any reactivity.

immunization [11]. After intra-cardiac perfusion with saline, brains and samples of intestine were surgically removed, immediately fixed for 4 h in 4% formalin, and embedded in paraffin. For each mouse, 3  $\mu$ m thick sections through the intestine, the hippocampus, the brainstem and the cerebellum were stained with hematoxylin–eosin, and studied by a pathologist (P.F.) who was blinded to the animal history. For all the animals, the brains showed no evidence of neuronal loss and/or gliosis and/or lymphocytic infiltration. Intestine had normal Peyer plaques and lymphoid follicles.

To further assess long-term safety, 12 mice bearing 5 mm Neuro2a tumors were treated with CpG-ODN. Four of them showed complete tumor rejection and remained tumor-free for more than 3 months. None of them showed neurological impairment on clinical examination. No evidence for active or previous paraneoplastic disease was found on histological examination of their brains.

For immunocytochemistry, naive anesthetized Wistar rats (Iffredo, Arbresle, France) underwent intra-cardiac perfusion with 2% paraformaldehyde in phosphate-buffered saline. The brains were surgically removed and further fixed for 2 h in the same fixative before being snap-frozen and stored at  $-80^{\circ}\text{C}$ . Frozen sections (10  $\mu$ m) of the cerebellum and brainstem were thawed, and subsequently incubated for 2 h with the mouse sera, at the dilution 1:200 in 10% goat serum. This dilution of 1:200 was chosen because only high-titer auto-antibodies are relevant in humans for the diagnosis of paraneoplastic diseases [13]. Sections were further incubated with a secondary anti-mouse IgG antibody (ICN-Biomedicals, Aurora, OH) and the reaction was revealed through an avidin–biotin system (Vectorlab, Burlingame, CA). The sera of two mice (both treated with CpG-ODN, one 20 days and one more than 3 months earlier) showed a reactivity against neurons, choroid plexus, and some glial cells (Fig. 2). However, this reactivity was weak, with antibody titers of 1:200 for the mouse treated 20 days before, and 1:500 for the other one.

Neuro2a cell extracts or purified recombinant HuD protein (kindly given by J. Dalmau, Rockville, IL), were subjected to electrophoresis on a 10% polyacrylamide sodium dodecyl sulfate gel, and transferred to nitrocellulose filters. Filters were blocked in 5% non-fat milk for 12 h at  $4^{\circ}\text{C}$ , cut into strips and incubated for 2 h at room temperature with the mouse sera diluted 1:200 in 10% goat serum. The strips were then incubated with a secondary anti-mouse IgG antibody and the reaction was revealed through the avidin–biotin system. On Western blot studies with Neuro2a extracts, no significant reactivity could be identified. No anti-Hu antibodies were seen on Western blot with HuD fusion protein.

Some studies have shown that ODN with CpG motif can promote auto-immune diseases such as collagen-induced arthritis [5,12], EAE [14] or Theiler's murine encephalomyelitis virus induced demyelinating disease [16]. Moreover, a recent study in mice has shown that systematically

administered CpG-ODN can up-regulate the expression of mRNA encoding pro-inflammatory cytokines in the brain [15]. We did not find in this study any clinical or histological evidence for paraneoplastic disease in any mice which rejected established tumors, neither in the acute phase of tumor rejection, nor in animals which rejected the tumors several months before.

Tumor rejection in this model critically depends on NK cell activation [2]. This involvement of innate immunity could partly explain why no remote effects of CpG-ODN therapy was seen in the nervous system, which theoretically require auto-immune CD4 or CD8-positive T-cells. However, T-cells are known to be also involved in tumor rejection. Indeed, nude mice, which only lack T-cells, eventually develop tumors despite an initial tumor growth inhibition, and CpG-ODN cured animals are protected against subsequent tumor inoculations suggesting the role of memory T-cells [2]. However, long-term surviving animals did not show any evidence of paraneoplastic disease. The absence of paraneoplastic disease in this model could result either from a specific tolerance of the neurons which do not express major histocompatibility complex, and are therefore not subject to CD4 or CD8 mediated cell toxicity, or from the selection by the immune system of a non-neural antigen. We can not exclude the possibility that this strain of mice is resistant to paraneoplastic disease, a disease for which no animal models exists.

Interestingly, two mice treated with CpG developed low-titer auto-antibodies, suggesting that an auto-immunity was triggered. However, these antibodies were at low titers and were not neuron-specific, reacting with choroid plexus and some glial cells. As neurological paraneoplastic diseases in humans are associated with high-titers of anti-neuronal antibodies, it is likely that these antibodies are not pathogenic [6]. It is also interesting to note that although the Hu antigen, which is expressed by neuro2a cells, was reported to be a potential target antigen for tumor immunotherapy in this model [1], no anti-Hu antibodies could be detected in our mice, suggesting that another tumor antigen was selected by the CpG immunotherapy.

Taken together, these data show that CpG-ODN can successfully trigger an immune response leading to rejection of established neuroblastoma in mice without inducing any neurological paraneoplastic disease, a fact that holds promise for clinical trials.

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