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CpG-OLIGONUCLEOTIDES FOR CANCER IMMUNOTHERAPY : REVIEW OF THE LITERATURE AND POTENTIAL APPLICATIONS IN MALIGNANT GLIOMA

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1. ABSTRACT

Bacterial DNA and synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODNs) are strong activators of both innate and specific immunity, driving the immune response towards the Th1 phenotype. CpG-ODNs have been successfully used in several experimental models of allergies or infections and are now entering clinical trials for these diseases. In this review, we will focus on their potential applications in cancers. CpG-ODN can be used alone to activate locally the innate immunity and trigger a tumor-specific immune response, overcoming the need for identification of a relevant tumoral antigen. Other promising approaches combined CpG-ODN with tumor antigens, monoclonal antibodies or dendritic cells. Preclinical models have shown impressive results and several clinical trials are on-going worldwide. So far, the toxicity observed in humans appeared limited, and objective responses have been observed in a few patients. In malignant gliomas, intra-tumoral injections of CpG-ODN represent a practical approach. Indeed, human gliomas display a locally invasive pattern of growth and rarely metastasize, making local treatment clinically relevant.

2. INTRODUCTION

Recently, bacterial DNA has been reported to be a strong activator of both innate and specific immunity, driving the immune response towards the Th1 phenotype. These immunogenic properties have been linked to unmethylated CpG dinucleotides (CpG motifs) contained in bacterial DNA. Synthetic phosphorothiotate oligodeoxynucleotides (ODNs) containing such motifs display similar immunological effects. The immunogenic properties of ODNs containing CpG motifs (CpG-ODNs) have been successfully used in several experimental models of allergies, bacterial, or viral infections (1-3). In humans, preliminary results of a phase I study confirmed the potency of CpG-ODNs as an adjuvant in an hepatitis B vaccine (4), and several phase I and II trials are on-going in allergies and other vaccine against infectious diseases (5). In this review, we will focus on the potential applications in cancer immunotherapy. Initial studies showed that when a tumor antigen is known, CpG-ODNs can be used as an adjuvant in a vaccination protocol. To overcome the need for identification of a relevant tumoral antigen, we recently developed an approach in which CpG-ODN are used alone to activate locally the innate immunity and trigger a tumor-specific immune response.

3. CpG MOTIF

3.1.Definition of CpG motif

The imunogenic properties of DNA were first discovered in 1984 when DNA extracts from Mycobacter tuberculosis were reported to activate NK cells and

Table 1. Effects of some phosphorothioates ODNs on human B-cells proliferation *in vitro*.

	Human B-cell proliferation (thymidine incorporation: cpm+/-SD)		
	Assay 1	**Assay 2**	**Assay 3**
Control	48 +/- 15	69 +/- 16	224 +/- 133
TGACTGTGAAGGTTCGAGATGA	90 +/- 47	NP	447 +/- 87
TC GTCGTT TT GTCGTT TT GTCGTT (2006)	2 934 +/- 695	1 155 +/- 324	16 284 +/- 947
TA AACGTT TTT AACGTT TTT GACGTC TT	5 083 +/- 255	1 967 +/- 238	30 167 +/- 2130
TA AACGTT AT AACGTT AT GACGTC AT	4 350 +/- 157	1 701 +/- 148	17 008 +/- 1475
TA AACGTT CT AACGTT CT GACGTC CT	4 241 +/- 665	1 709 +/- 173	24 183 +/- 2263
TA AAGGTT CT AACGTT CT GACGTC CT	1 560 +/- 315	NP	NP
TA AAGGTT CT AACCTT CT GACGTC CT	1 182 +/- 225	NP	NP

Lymphocytes from surgically resected tonsils (3 different patients) were incubated with sheep blood cells activated with AET(2-aminoethyl isothiouronium bromide), then centrifuged on a ficoll gradient to retrieve B-lymphocytes. B-cells (100 000 / wells) were dispensed in triplicate into 96 well plates and cultivated for 72 hours with 2µg/ml ODN. An ODN without CpG motif was added as a negative control. The ODN 2006 has been described as a very potent ODN to stimulate human B-lymphocytes and dendritic cells (12) and was used as a positive control. The cells were then pulsed with 50µCi/ml of tritiated thymidine for 18 hours, and the radioactivity was measured. NP, not performed.

sometimes induce tumor regression (6). Subsequent works showed that the immunogenic properties were due to the presence within the bacterial DNA of CpG sequences (7-8), which are suppressed and methylated in vertebrate DNA (9). Interestingly, synthetic oligodeoxynucleotides containing CpG motifs display similar properties than bacterial DNA. Their biological activity is further increased if they are rendered nuclease resistant by a phosphorothioate backbone modification (10). The biological activity of CpG motifs also depends upon the 3' and 5' flanking bases, and several immunostimulatory sequences have been defined such as hexameric palindromes containing CpG motifs (11) or 5'- pur-pur-CG-pyr-pyr hexameric sequences (8). Interestingly some authors have reported that the optimal CpG motif for activating human cells might be different from the effective mouse sequence (5'-GTCGTT versus 5'-GACGTT) (12,13). However, by screening a large number of ODNs, we found that some ODNs containing the palindromic hexamers 5'-AACGTT or 5'-GACGTC displayed optimal immunostimulatory activity on human B-lymphocytes (table 1) and were also very potent to stimulate murine macrophages or lymphocytes (personal data).

Still, the biological activity of a given hexamer is strongly modulated by the remaining sequences within the oligonucleotide. Inhibitory motifs have been described such as over representation of 5'-CCG, 5'-GCGGG or 5'-ACGGG motifs (14,15) or poly-G sequences in a phosphorotioate (but not phosphodiester or chimeric) backbone oligonucleotide (16). On the other hand, repeating several times an immunostimulating CpG motif within an ODN enhances its immunostimulatory activity (12). In our experience, oligonucleotides comprising several repeats of 5'-AACGTT or 5'-GACGTC motifs are among the most efficient (table 1).

Recently, it was suggested that a distinct family of immunostimulating CpG-ODNs might exist. Some specific sequences (so called A-type) might preferably induces IFN alpha secretion by APC and activation of NK-cells and gamma delta T cells, in contrast to the "classical" CpG-ODNs (so-called B-type) which rather induce secretion of IL12 and B-cell proliferation (17-21). Difference in sequences between both families are still unclear and the identification of the specific receptor(s) will probably help to define the requirements for a specific immunostimulation.

Backbone modifications also seem to play an important role, as non specific *in vivo* effects have been described. Phosphorothioates ODN can stimulate B-cells proliferation and induce long-lasting effects such as lymphadenopathy (22,23). CpG motifs in nuclease-resistant phosphorothioate backbones have enhanced B-cell stimulatory properties but reduced NK-cell stimulation.

Methylation of the cytosine within CpG-motifs abrogates the immunostimulating properties (8). Interestingly, methylation of CpG-motifs within promoter region of genomic DNA is critical in mammalian gene expression, but there is no evidence that mammalian DNA can be immunostimulatory due to a failure or loss of methylation. That methylation of CpG-motifs is involved in such different biological processes (immune properties of DNA extracts and modulation of promoter regions) is astonishing and the relationship, if any, between both is unclear.

3.2.Biological activity of CpG motifs

Bacterial DNA or CpG-ODNs display pleiotrophic effects on the immune system (24). They directly activate B-cells and dendritic cells in mammalian species, and also macrophages in mouse and rats.

On B-cells, CpG-ODNs are directly mitogenic, induce secretion of a number of cytokines such as IL6 or IL10 (25,26), prevent apoptosis triggered by surface antigen-receptor cross linking or other apoptotic agents (27,28) and promote Ig secretion (8).

Dendritic cells (DCs) are directly activated by CpG-ODNs to express co-stimulatory molecules (CD40, CD80, CD86) and secrete a wide variety of cytokines such as TNFα, interferons, interleukin (IL)-6 or IL-12 (29-31), allowing activation of T-lymphocytes. Most interestingly,

CpG-ODNs are able to "license" DCs to directly prime CD8 T cells by a T-helper cell-independent mechanism (32). CpG-ODN's effects on antigen processing are complex: after an initial phase of enhancement, both antigen processing and MHC-II expression are down modulated (33-35). All dendritic cells are not directly sensitive to CpG-ODNs. CD4+ peripheral blood DC in humans include 2 major subsets of cells: the plasmacytoid dendritic cell (PDC) and the myeloid DC (MDC). While PDC are primary targets for CpG-ODNs, MDC are indirectly activated by PDC-derived cytokines (19).

Direct activation of T-cells by CpG-ODNs is unlikely. However, cytokine secretion by dendritic cells activate T-cells (36,37). Secretion of IL12 and IFN gamma drive the T-cell differentiation towards the Th1 phenotype and can even redirect established Th2-biased to Th1 immune responses (38). In addition, upon TCR ligation, CpG-ODNs can induce IL2 receptor, IL2 secretion and increase cytolytic activity of the T-cells (39).

NK cells are strongly activated by CpG-ODNs but NK cells are not primary targets of CpG-ODNs as initially thought (16). Actually, NK cell activation depends upon cytokine secretion by DCs, among which IL12, TNFalpha and IFN (40). Some authors have reported that phosphorothioate stabilized ODNs have lower potential for inducing NK activation than do chimeric ODNs in which only the extremities of the ODN are stabilized (16).

3.3.Intracellular mechanism

Immune stimulation of dendritic and B-cells requires entry of the ODNs or DNA into the cell (41,42). There is no evidence for a specific CpG receptor on the cell surface. Bacterial DNA or CpG-ODNs are probably taken up within cells by endocytosis and then acidified and degraded within endosomes, a process which can be inhibited by chloroquine or bafilomycin A (28,42). Internalized CpG-ODNs rapidly activate multiple signaling transduction pathways, including the activation of reactive oxygen species and the mitogen-activated protein kinase (MAP) pathways, leading to the activation of various transcriptional regulatory proteins such as nuclear factor κB and AP-1 (29,43,). These signaling pathways culminate in the transcription of multiple cytokines and proto-oncogenes, which are thought to mediate the other immune effects of CpG-ODNs.

Using knock-out mice, Hemmi recently identified the Toll-like receptor (TLR9) as a major component in CpG-ODNs recognition (45). The Toll-like receptor (TLR) family is a phylogenetically conserved mediator of innate immunity that is essential for microbial recognition (46). Specific TLR9 expression have been demonstrated in CpG-ODN sensitive cells, such as B-lymphocytes and plasmacytoid DCs (47). TLR-9 has a transmembrane domain but the intracellular localization of TLR9 remains controversial, and the proofs of the direct binding of CpG motifs to TLR9 are still lacking.

A role for the DNA-dependent protein kinase (DNA-PK) in mediating immune activation by CpG-ODNs has also been suggested (48). DNA-PK was previously known to be activated by double-stranded DNA (dsDNA) structures, but the authors found that the optimal activation of DNA-PK activity required the presence of unmethylated CpG motifs, and was reduced by DNA methylation. Surprinsingly, CpG-ODNs are still immunostimulant in severe combined immunodeficient (SCID) mice, which have weak DNA-PK catalytic function (49) and further investigations are required to clarify this issue.

4. APPLICATIONS OF CPG-ODNS IN CANCER

CpG-ODNs pleiotrophic immune activity provides a unique opportunity to design new immunotherapeutic regimens in cancer. Activation of NK cells and macrophages can be used in the purpose of either stimulating innate immunity within the tumor mass or to stimulate antibody-dependant cell cytotoxicity (ADCC). Activation of dendritic cells and CpG-ODNs' ability to drive the immune response towards the Th1 phenotype, can be advantageously used to promote tumor specific cytotoxic T lymphocytes (CTL).

4.1.CpG-ODNs alone

Selection and purification of a relevant tumor antigen is usually a limiting step in cancer immunotherapy. To overcome this problem, CpG-ODNs alone could be directly injected into the tumor, expecting that the immune system will select by itself the most relevant antigens. In addition, local immunostimulation by CpG-ODNs could also activate innate immunity (NK cells and macrophages) which can directly kill tumor cells.

The validity of such approach was first suggested with DNA extract of Mycobacterium bovis (MY-1), which injections allowed rejection of subcutaneous tumor graft in mice models (6). As the activity of MY-1 was dependent on the presence of bacterial DNA, it is likely that the anti-tumor effect of MY-1 was actually related to immunostimulatory CpG motifs included in bacterial DNA. This concept was finally proven in a neuroblastoma model, in which we showed that peritumoral injections of a synthetic ODN containing a CpG motif induced complete tumor rejection in a majority of mice, and triggered a long-term specific immunity against a subsequent tumor challenge (50). Moreover, the antitumor efficacy of such approach was greatly improved by the usage of phosphorothioate modification of the phosphodiester backbone, making the ODN nuclease resistant (Figure1).

Subsequent studies by our team and others have confirmed the antitumor effects of CpG-ODNs alone in various models, such as glioma (51,52) mesothelioma, fibrosarcoma, lung carcinoma, melanoma or acute myeloid leukemia AML (53-57) and it is likely that direct injections of CpG-ODNs are efficient in almost all solid tumors (Figure 2). CpG-ODNs or bacterial DNA can also be successfully used in liposomal preparations which protect them against nuclease degradation and increase their immunostimulating properties (58-61).

The situation in B-cell malignancies is more complex as CpG-ODNs have a direct effect on these cells.

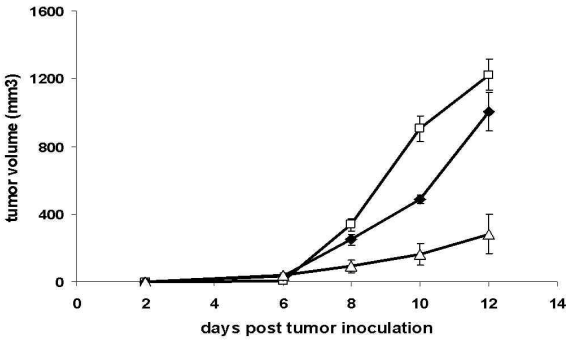

Figure 1. Neuro2a tumor volumes (mean + SEM) in A/J mice injected 2 days after tumor implantation either into the tumor bed with sodium chloride (—□—, n=6), or 50 µg of phophodiester CpG-ODN (—♦—, n=6), or 50 µg phosphorothioate CpG-ODN (—Δ—, n=6). Days are the number of days after tumor implantation. Tumor growth was assessed with the formula: Vol.= length x width x width x π)/6. While all animals injected with saline developed fast growing tumors, treatment with CpG-ODN resulted in a inhibition of tumor growth which was more pronounced when CpG-ODN were stabilized by phosphorothioate backbone modification ($p<0.001$) (personal data). (CpG-ODN: 5'- GACTGTGAACGTTCGAGATGA).

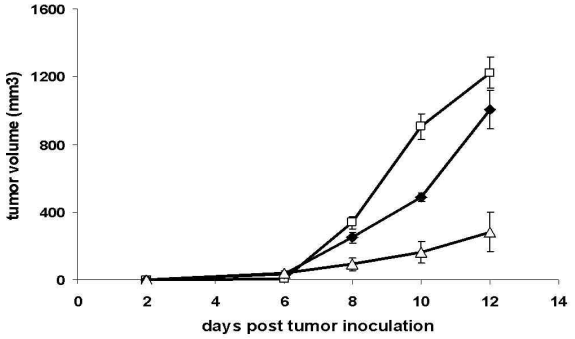

Figure 2. antitumor effect of an immunostimulating oligonucleotide on the B16 melanoma model. C57Bl6 mice were injected sub-cutaneously into the right flank with 100 000 melanoma cells. On day 2, 5 and 9 after tumor inoculation, mice were injected at the tumor site either with saline (—□—, n=6) or 50µg of CpG-ODN (—▲—, n=6). While all animals injected with saline developed fast growing tumors, treatment with oligonucleotide CpG-ODN resulted in a dramatic inhibition of tumor growth when compared to controls ($p<0.001$) (personal data). (CpG-ODN: 5'- GACTGTGAACGTTCGAGATGA).

CpG-ODNs can increase the immunogenicity of these tumor cells by promoting the expression of costimulatory molecules (CD20, CD40, CD80, CD86, CD54), IL2 receptor (CD25) and MHC class I and II (62-64). On the other side, CpG-ODNs have been reported to stimulate the proliferation of lymphoma cells and to protect them against apoptosis triggered by various agent (65). Concerns therefore exist that CpG-ODNs might protect malignant cells against the immune attack, as long as the treatment is administered. Altogether, a positive effect of CpG-ODNs alone was reported on a few lymphoma models (54,56), although the antitumor effect was very modest in another case (66).

The optimal dose and schedule probably depends upon the tumor types. In almost all murine models, distant injections of CpG-ODNs are less efficient than local treatment, suggesting that CpG-ODNs exert their effects locally and must reach a sufficiently high concentration at the tumor site (50,55). However, systemic injections of CpG-ODNs in cancer might have some value, especially in hematological malignancies. Iterative injections appeared superior to a single dose in some cases (50,55), while a single injection of 50µg CpG-ODN was sufficient to induce tumor rejection in a glioma model (51). Efficient dosage ranges from 20 to 100µg per injections, with a dose-effect relationship (51). Interestingly a 300µg dose of CpG-ODN was less effective than doses of 100 or 30µg in a melanoma model (56) suggesting a non-linear response, but this finding should be confirmed in other models.

The reasons for these discrepancies are not clear, but probably reflect either the different levels of immunogenicity of the tumor models or the mechanisms by which the tumor is rejected. Indeed, one could speculate that iterative injections are needed when tumor cells are killed by innate immunity, while in tumors which are rejected by specific immunity, one or several injections might be sufficient to trigger the immune response as is the case for vaccination.

All studies have pointed out the key role of both the innate and the adaptive immune system in tumor rejections. No direct cytotoxicity of CpG-ODNs has been detected *in vitro* (50,51) and CpG-ODNs had no effect on expression of class I or class II MHC, CD40, CD40l, CD28, CD80 or CD86 on B16 melanoma cell *in vitro* (56).

NK cells depletion diminished or abrogated CpG-ODNs antitumoral effect (50,53-57). As NK cells are not primary targets for CpG-ODNs, tumor infiltrating macrophages and dendritic cells probably play a critical role in NK cell activation. NK cell activation seem to depend on the secretion of type-I interferon but not IFNγ, IL12, IL2, IL4 (54,56). Type I IFN is known to activate the cytolitic potential of NK cells (67). Experiments in knock-out mice suggested that NK cells kill their target more by a direct cytolitic activity with perforin, rather than by their ability to release various cytokines (56). Other component of the innate immune response probably play a role in tumor rejection since CpG-ODNs still display antitumor effect in SCID-beige mice which lack B, T and NK cells. Increased tumoral infiltration with neutrophils have also been observed but not fully investigated (53, personal data). The role of macrophages has been highlighted in a murine model, in which macrophages inactivation by silica injections reduced the anti-tumor effects (52).

Specific immunity also plays a critical role as demonstrated by the reduced efficacy of CpG-ODNs in nude or SCID mice (52,56). Experiments demonstrated that CpG-ODNs induced maturation and migration of DC into the draining lymph nodes *in vivo* (55), in accordance with a

previous paper showing the migration of Langerhans cells from skin (68). CD8+ cells are needed to achieve optimal effects in most cancer models (53,55-56). Depleting *in vivo* CD4+ cells in a murine model did not significantly impaired the antitumor effects (53), probably because CpG-ODNs allow DCs to directly prime CD8 T cells by a T-helper cell-independent mechanism (69). However, the priming of long term immunity, reported in almost every model tested so far, demonstrates that memory CD4+ cells are probably involved. This long-term immunity is tumor specific, as rats cured from a 9L glioma were not protected against another syngenic glioma cell line (52). The role played by antibodies in tumor inhibition deserves further studies. Antibody-dependant cell cytotoxicity (ADCC) has been suggested in a lymphoma model (70), but not in solid tumors.

Both innate and specific immunity are therefore required to achieve optimal anti-tumor effects. It is interesting to point out that activation of innate immunity is local, and lasts as long as the treatment is administered. Thus innate immunity probably plays a major role only in the early phase of local tumor rejection, before a specific immunity can be primed. The ability of the CpG-ODNs to mount an immune response will be critical to achieve complete tumor rejection for metastatic cancers. Preliminary results of an on-going clinical trial in metastastic melanoma have shown objective response at the site of injection, but not on distant tumor sites (71). Design of optimal CpG-ODN sequences and regimen therefore represent the main challenge for clinical applications in disseminated cancers. Insights into the immune mechanisms needed for tumor lysis will probably lead to customized regimen in each type of cancer, as illustrated in some models in which B-type CpG-ODNs were more potent than a NK-optimized ODNs, but not in others (56,57).

4.2.CpG-ODNs combined with antigens or antibodies

When a tumor antigen is known, a specific immunity can be triggered using CpG-ODNs as an adjuvant in a classical vaccination protocol. Successful immunization has thus been reported in a lymphoma model, leading to protective immunity against a lethal tumor challenge (66). CpG-ODNs combined with tumoral antigen induced a higher titer of antigen-specific antibodies than did complete Freud's adjuvant, and were associated with less toxicity (66). In an other model, CpG-ODN increased several fold the CTL response to immunization with various synthetic peptides, and protein vaccination in combination with CpG was effective in reducing the growth of antigen-transfected tumors (72). CpG-ODNs were successfully used to generate specific CTL in mice immunized against tumor-derived synthetic peptide analog of MART-1/Melan-A(26-35) (73). Adding GM-CSF can further increase the adjuvant properties of CpG-ODNs, especially when a fusion protein is created with the tumor antigen and GM-CSF. A single immunization with CpG-ODN and antigen/GM-CSF fusion protein 3 days before tumor inoculation prevented tumor growth in a lymphoma model (74).

Another promising approach combines CpG-ODNs and monoclonal antibodies targeted against tumor antigens. CpG-ODNs stimulate NK cells and macrophages, hence enhancing antibody-dependant cell cytotoxicity. Indeed, combination of CpG-ODNs with monoclonal antibodies was effective in preventing tumor growth in the 38C13 B cell lymphoma model (70,75). Clinical trials combining CpG-ODNs with Herceptin® in breast cancer or Rituxan® in non-Hodgkin's lymphoma are on-going.

Recently, an original approach uses CpG-ODNs as an inducing agent in cancer cells for a target molecule against which a cytotoxic antibody exists. CpG-ODNs can increase expression of the IL2 R (CD25) on the surface of B-CLL, and to a lesser level on normal B-lymphocytes, and render B-CLL more sensitive to a recombinant anti-CD25 immunotoxin (62,76).

Unfortunately, relevant tumor antigen are rarely identified in cancer, therefore limiting the clinical usefulness of these approaches. Moreover, the tumor might select antigen-negative cells and escape the immune response. Immunization against not one but several tumor antigens might be needed to achieve significant clinical effects.

4.3.CpG-ODNs combined with dendritic cells

CpG-ODNs cause simultaneous maturation of immature and activation of mature dendritic cells to produce cytokines *in vitro* and to induce activation of cytolytic CD8 cells *in vivo* (34,35,77). Stimulation of freshly isolated T helper cells with syngeneic A20 lymphoma cells and APCs in the presence of CpG-ODNs generated large numbers of tumor-specific Th1 cells, which were able to eradicate disseminated lymphomas and provided lifelong protection (78). In mice pretreated with Flt3 ligand which expands *in vivo* the numbers of immature DCs, the injections of a tumor Ag and CpG-ODNs generated Ag-loaded DCs *in situ* that can induce potent antitumor immunity (79).

4.4.Tolerance

Very limited data are available on the safety of CpG-ODNs treatment in cancers. CpG-ODNs are powerful immuno-stimulating agents and can therefore potentially trigger or exacerbate auto-immune diseases. In cancer immunotherapy, the fear for auto-immune reactions is particularly relevant because a subset of patients with cancer do spontaneously develop paraneoplastic syndromes. For example, neurological paraneoplastic syndromes are characterized by inflammation of the nervous system, indolent tumor growth and an immune reaction against antigens shared by the nervous system and the tumors cells. We have recently shown that CpG-ODNs can successfully trigger an immune response leading to rejection of established neuroblastoma in mice without inducing a neurological paraneoplastic disease (80). In an intracranial glioma model, no toxicity of CpG-ODNs injections was seen, despite the susceptibility of the Lewis strain to develop experimental allergic encephalomyelitis (EAE) (51).

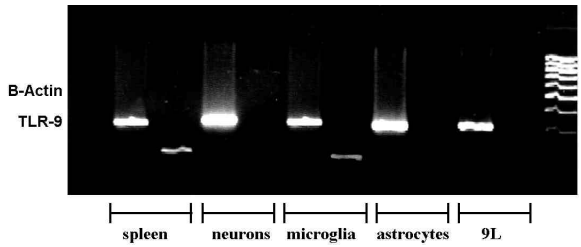

Figure 3. TLR9 mRNA is expressed in microglial but not in neurons or glial cells. Purified populations of microglial cells, astrocytes or neurons, (obtained from embryonic (E14-E17) OFA Wistar rat as described in (109, 110)), and rat glioma cells (9L) were extracted for mRNA. Actin and TLR9 gene expression was assessed by RT-PCR using the appropriate primers. Spleen mRNA was used as a positive control for actin and TLR9 expression. All subgroups of cells did express mRNA for actin, but only microglial cells (and spleen) did show expression of TLR9. (personal data).

In human, preliminary data on ongoing clinical trials in melanoma and basal cell carcinoma (iterative injections every week) have shown that clinical response are observed above 1mg per injections, with limited side effects, consisting of local inflammation and flu-like syndromes (71).

5. LOCAL CpG-ODNs TREATMENT IN MALIGNANT GLIOMAS

5.1.Glioma and immunity

Recent advances have shown that the brain can both prime (afferent response), and be reached (efferent response) by the immune system. Microglia, endothelial cells, capillary pericytes, and occasionally astrocytes, all can act as Antigen Presenting Cells (APC) (81). Microglia can express MHC class I, MHC class II and co-stimulatory molecules B7-1 (82,83), secrete activating cytokines such as IFNgamma, IL1, IL6 and GM-CSF (84), display phagocytic activity in cultures (81), and cluster CD4+ T-cells *in vitro* (85). Capillary pericytes share common functions with microglia, but are in addition capable of migration, making them good candidates for APC functions.

In addition, the ability of lymphocytes to invade normal brain parenchyma have been clearly demonstrated in various studies, using graft-vs-host disease, brain melanoma metastasis or beta-galactosidase expression under the GFAP promoter (86-88). To cross the BBB, lymphocytes require activation prior to entry into the CNS, but antigen specificity is not necessary.

Evidence have been found for cell-mediated anti-tumor activity in glioma patients (89). The identification of tumor-infiltrating lymphocytes (TIL) in malignant glioma (90) also argues for the existence of glioma-specific and tumor-associated antigens. Among potential tumor-associated antigens (TAA), the extra-cellular matrix-associated Ags GP240 and Tenascin, the membrane-associated ganglioside molecules, the mutated p53, and the over-expressed deletion variant of the Epidermal Growth Factor Receptor (EGFRvIII) have been identified (91), although none of them have been shown to be the target of TIL (90).

However, patients with malignant glioma exhibit depressed *in vitro* and *in vivo* immune responsiveness, explaining why the tumors are not spontaneously rejected. Patients generally exhibit abnormal delayed hypersensitivity to such Ags as Mycobacterium tuberculosis or Candida Albicans and poor T-cell responsiveness to mitogens such as PHA, Concanavalin A or phorbol ester (92). These altered immune responses have been attributed to decreased IL-2 sensitivity of CD4+ lymphocytes (93,94) and local secretion, by glioma cells, of the immunosuppressive factors TGFβ2, prostaglandin E2 and possibly Fas-L (95,96). Cytokines (IL-6, IL-10) that may shift immunity to the less effective humoral Th2 responses are also secreted by gliomas (97).

5.2.CpG-ODNs as an immonutherapeutic approach in malignant gliomas

Secretion by glioma cells of immunosuppressive cytokines provides the rationale for the usage of CpG-ODNs as a local immunostimulating agent. Indeed, such approach offers the unique opportunity to stimulate both the innate and the specific immunity, increasing the efficiency of the immune system both to trigger an anti-tumor immunity (afferent arm) and to boost its anti-tumor efficacy (efferent arm):

- afferent arm: CpG-ODNs can stimulate microglia and antigen presenting cells within the brain (cf infra). By locally stimulating APC, CpG-ODNs treatment allow the immune system to select by itself one or several antigens, therefore overcoming the need for the physician to select and purify a relevant antigen. Moreover, CpG-ODNs can switch a poorly efficient Th2 anti-tumor immune response towards a Th1 response, thought to be more efficient in cancer immunotherapy. In addition, CpG-ODNs induce secretion of IL12 or IFN gamma, which can both counteract the immunosuppressive effect of TGF beta or IL10.

- efferent arm: CpG-ODNs activate macrophages and NK cells which can kill directly tumor cells. Moreover, induced secretion of interferon gamma by CpG-ODNs, could increase the expression of MHC class I by glioma cells, hence making them more susceptible to cell-mediated cytotoxicity.

5.3.TLR9 expression within the nervous system

Usage of CpG-ODNs as a local immunostimulating agent in brain tumors requires the presence within the brain or within the tumor of CpG-ODNs sensitive cells. CpG-ODNs effects are thought to be mediated by the Toll-like receptor 9 (45). TLR9 expression was not directly detected in the brain using nothern-blot study (45), probably because only a small subset of cells do express the TLR9. Indeed, using purified subsets of nervous system cells, we were able to demonstrate TLR9 expression in microglial cells but not astrocytes, neurons or glioma cells (Figure 3). In addition, human gliomas are

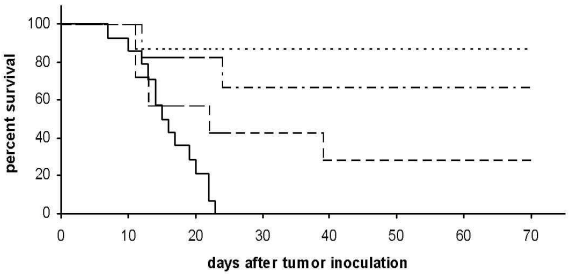

Figure 4. Survival curves of Lewis rats inoculated intracerebrally with CNS1 glioma cells and injected into the tumor bed with 50 µg of CpG-ODN on day 1 (–·–·–), day 5(·····), day 9 (– – –) or with sodium chloride (—). Survival was dramatically increased in CpG-ODN treated animals with a long term survival (>90 days) of 67% (n=6; p< 0.01), 88% (n= 8; p< 0.002) and 29% (n= 7; p< 0.07) for rats treated on day 1, 5 and 9 respectively, while all controls animals (n=14) died within 23 days (From (51)). (CpG-ODN: 5'- GACTGTGAACGTTCGAGATGA).

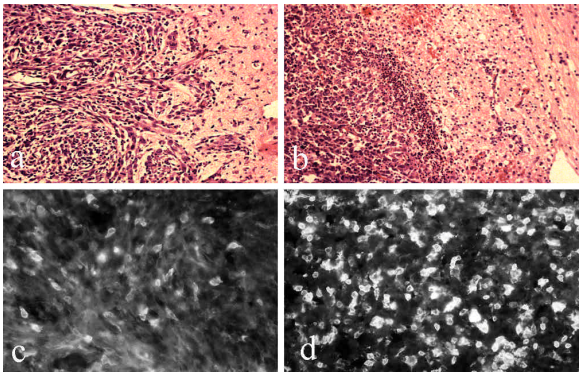

Figure 5. Histological analysis of tumors in rats implanted on day 0 with CNS1 cells, injected with saline (a, c) or CpG-ODN (b, d) on day 5, and sacrificed on day 6. CNS1 tumor cells invade the normal parenchyme in a control rat (a), whereas in a CpG-ODN treated animal (b), viable tumor cells are surrounded by an area of pycnotic cells and a cicatricial tissue where macrophages and lymphocytes are seen, but where tumor cells are sparse. Normal parenchyma is seen on the right (H&E staining, X200). Immunohistochemical analysis (X400) with OX8 antibodies showing higher tumoral infiltration with CD8 T-lymphocytes in CpG-ODN injected animals (d) than in control rats (c) (from (51)). (CpG-ODN: 5'-GACTGTGAACGTTCGAGATGA).

infiltrated with macrophages (98) which might also mediate the CpG-ODNs effects. Whether human tumor-infiltrating macrophages do express TLR9 is currently under investigation.

The expression of functional TLR9 by microglial cells is further supported by the pronounced microglial cells activation induced by intra-cerebral injections of CpG-ODNs (99). In this study, the authors also report on the astrocyte activation, which might actually be mediated by microglia, but this point deserves further studies. A recent study also reported activation of microglial cells *in vitro*, with induction of TNF-alpha, IL-12p40, IL-12p70 and NO, enhancement phagocytic activity and up-regulation of MHC class II, B7-1, B7-2, and CD40 molecules (100). In our experience, the number of ED1 positive cells (activated macrophages and microglia) seen 48 hours after intra-cerebral injections were 6 folds higher in CpG-ODNs healthy rats than in controls injected with saline (positive cells per field : 132+/-9 and 16+/-5, p< 0.05). Moreover, the activation area involved all the ipsilateral hemisphere with CpG-ODNs injections, but was limited around the needle track in controls. Similarly, injections of CpG-ODNs within an established intracerebral glioma up-regulated ED1 intratumoral positive cells (51).

5.4.Preclinical data in animal models

Promising data have been obtained in all syngenic murine glioma models tested so far. When 9L glioma cells were inoculated sub-cutaneously, local treatment with CpG-ODNs induced tumor growth inhibition, and complete tumor rejection in a significant subset of animal (52). Experiments with the poorly immunogenic RG2 glioma showed similar results (manuscript in preparation). Most interestingly, promising results have been obtained in an intracranial model of syngenic glioma (CNS1) with a single intratumoral injections of CpG-ODNs (51). While none of the control animals survived the tumor challenge, more than 85% of the rats treated five days after the tumor inoculation showed long term survival and tumor eradication (Figure 4). Rats which were cured by CpG-ODN injections were further protected against a new tumor challenge, showing that a long term immunity was primed. Moreover, increased survival was seen in rats bearing 2 separate tumors, but treated only at one tumor site. Surprisingly, bacterial DNA extracts (MY-1) which have previously shown antitumor effect in various malignancies, failed to improve survival in a mouse glioma model (101). The superior efficacy of CpG-ODNs in our models might be related to the amplification of immunostimulatory sequences and the enhanced stability of phosphorothioate ODNs when compared to crude bacterial DNA.

In the intracranial glioma model, CpG-ODN injections within the tumor mass induced tumor eradication with increased tumoral infiltration with activated macrophage/microglial cells, CD8 and NK lymphocytes when compared to the controls injected with saline (Figure 5). CpG-ODNs have been reported to induce endogenous release of IL12 by macrophages (49). The role of macrophages has been highlighted in the 9L model, in which macrophages inactivation by silica injections reduced the anti-tumor effects (52). Since IL12 displays anti-tumor effects in murine glioma models (102,103), the CpG-ODNs' antitumoral effects could be mediated, at least in part, by IL12 secretion. CpG-ODNs carry out the advantage over IL12 alone to trigger a sustained expression of IL 12 for at least 8 days (104), while the half-life of exogenous IL12 is less than 10 hours (105).

Altogether, CpG-ODNs as a local treatment appear promising in preclinical models, and fully justify a

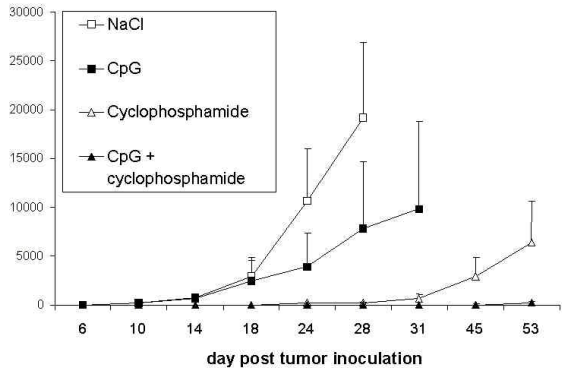

Figure 6 combination of cyclophosphamide and CpG-ODN. Fisher rats were inoculated sub-cutaneously with 100 000 9L glioma cells, injected I.P. with either saline or 50mg/kg cyclophosphamide on day 3, then injected at the tumor site with either saline or 50µg CpG-ODN on day 5. Both CpG-ODN and cyclophosphamide alone induced an inhibition of tumor growth, but maximal effects were obtained when both treatments were combined ($p < 0.05$) (personal data). (CpG-ODN: 5'- GACTGTGAACGTTCGAGATGA).

clinical trial in glioma patients. Moreover, human gliomas display a locally invasive pattern of growth and rarely metastasize, making local treatment clinically relevant.

5.5.Pharmacokinetic and toxicology of CpG-ODNs in the brain

The pharmacokinetic of synthetic oligodeoxynucleotides in the nervous system is poorly known. Diffusion after intra-cerebral injections occurs both within the brain parenchyme (a few millimeters around the site of injections) and into the CSF (106,107). The half life within the CSF is less than 1 hour. The half-life of phosphorothioate ODNs in the parenchyma is probably more related to the diffusion into the blood stream or the CSF than to a degradation in-situ which is limited. When radioactive ODNs are injected into the brain, the local radioactivity decrease by 85-95% in 10 hours in one study (108) and by 80% in 48 hours with a high-flow microinfusion technique (107).

Potential toxicity induced by CpG-ODN injections can be either acute, or delayed through an auto-immune mechanism. In rats, intracerebral injections of 10-200µg CpG-ODN induced activation of microglia, recruitment of macrophages and lymphocytes but no oedema or neurological symptoms. No similar data are available in humans. In an on-going clinical trial in melanoma patients (s.c. injections), adverses effects were limited to redness, swelling and flu-like symptoms with doses above 1 mg (71). Delayed toxicity through an auto-immune mechanism, such as angeitis or demyelinating diseases, can also theoretically be feared as CpG-ODNs are strong immunostimulating agents. However, we showed that despite the known susceptibility of the Lewis strain to develop EAE, rats injected with CpG-ODNs showed no short or long term neurological impairment. Histological studies of the brains of cured animals showed no other abnormalities than enlarged ventricles in the vicinity of the original tumor sites. No evidence for angeitis, diffuse inflammation or demyelinization was observed (51).

6. CONCLUSION AND PERSPECTIVES

Recent recognition of the potent immunostimulatory effects of specific sequences in DNA suggest that such agents may have potential applications in cancer immunotherapy, either alone to activate locally the innate immune responses and trigger an anti-tumor immune response, or combined with tumor antigens, monoclonal antibodies or dendritic cells.

Several clinical trials are on-going worldwide in melanoma, lymphoma, breast cancer uterine cervix carcinomas or renal carcinomas. A clinical trial in glioblastoma will be open for accrual in the next few months in Paris. So far, the toxicity observed in humans appeared limited, and objective response have been observed in a few patients (71).

In the next years, design of optimal CpG-ODN sequences, treatment schedules and new drug formulations allowing distant tumor killing represents the main challenge for clinical applications in cancers. For example, slow-release formulation of CpG-ODNs will be especially useful in cancers where a sustained activation of innate immunity is needed (mainly local cancers, sensitive to NK-mediated cytotoxicity). In contrast, regimens designed to trigger a specific immunity acting at distance will be needed for disseminated cancers.

At last, it is likely that combination of CpG-ODNs with other treatments will have clinical perspectives. Conventional treatment such as radiotherapy or chemotherapy can induce cells lysis and result in the release of tumor antigens. In addition, reducing the tumor burden would help the immune system to achieve complete tumor rejection. Preliminary experiments of CpG-ODNs and cyclophosphamide showed a synergistic activity (Figure 6). However, optimal dosages should be determined since high dose chemotherapy can induce immunodepression and perhaps counteract the immunotherapy. Combination of CpG-ODNs with other types of immunotherapy, such as monoclonal antibodies or dendritic cells, appears particularly promising.

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