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Importance of Avidity for an Endogenous Drug Carrier: An Antibody Carrier for CpG Oligonucleotides

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Abstract: In animal models, successful anticancer monotherapy with CpG oligodeoxynucleotide (ODN) has been limited to the intratumoral and peritumoral routes of administration. To overcome this limitation, we developed a delivery system utilizing an endogenous antibody as a carrier for CpG ODNs. When a 1:1 conjugate of 2,4-dinitrophenyl (DNP) to a CpG ODN was administered to tumor-bearing mice that were preimmunized against DNP, intravenous (iv) administration successfully inhibited tumor growth (Palma, E.; Cho, M. J. *J. Controlled Release* **2007**, *120*, 95−103). In the present studies, we reproduced the iv results and showed that a DNP derivative of a controlled ODN with scrambled nucleotide sequence failed in the same model. Perhaps more significantly, contralateral subcutaneous (sc) routes of administration also suppressed tumor growth. However, in a separate experiment, when the anti-DNP titer level was low, the antitumor effect was abolished, supporting the importance of the avidity involved in the complexation. With the low titer, a significant fraction of injected dose must have existed as unbound that is subject to rapid clearance. The present study justifies chemically cross-linked immune complexes such that the CpG ODN cannot dissociate in the body after administration.

Keywords: Endogenous carriers; immune complexes; avidity; CpG ODN; pharmacokinetic and pharmacodynamic; solid tumors

Introduction

The human immune system reacts against bacterial DNA that contains abundant unmethylated CpG dinucleotide sequences as a "danger signal" and responds with innate as well as antigen-specific adaptive immunity. These responses can be harnessed to fight against tumor growth using a small nucleic acid that contains the CpG dinucleotide motif.[2] In essence CpG-containing ODNs mimic a local inflammation. Indeed intratumorally or peritumorally administered CpG ODNs of about 20 nucleotides suppress tumor growth.[3] However, in phase II human clinical studies, the iv route was ineffective[4] while sc administration produced only marginal effects.[5] In many aspects these disappointing observations mirror the failure of systemic cytokines in

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treating tumors, reflecting the lack of their paracrine function in the tumor microenvironment.[6,7] Thus, the pharmacokinetic (PK) requirement in CpG ODN delivery includes a sustained and targeted delivery to solid tumors. At a (sub)cellular level, its target is not tumor cells but the endosomal Toll-like receptor 9 of dendritic cells in the vicinity.[2]

Naturally occurring endogenous carriers such as hemoglobin in erythrocytes have evolved to near perfection through evolution. Binding and dissociation of its ligand oxygen to and from the protein are cooperative, dictated by oxygen pressure. Similarly the release of fatty acids bound to albumin critically depends on the serum level of the ligand via step dissociation involving several binding sites, each with different binding affinity. When xenobiotics latch onto these biopolymers, blood-borne particulates, or cells, it is generally viewed as undesirable since it affects serum PK and biodistribution of the drug often in unexpected ways. On the other hand, the endogenous carriers can be exploited for drug delivery as shown with albumin[8] and immunoglobulin.[9,10] These two major proteins in human circulation show almost an identical serum $t_{1/2}$ of approximately 20 days. Such a long $t_{1/2}$ originates from protective recycling via so-called Brambell receptor of FcRn primarily on endothelium.[11]

One can envision that the PK of a drug molecule will mimic that of an endogenous carrier itself if the drug binds the latter with a high affinity. This was found to be the case for small hapten molecules that cannot induce immune clearance via cross-linking.[1,12,13] Thus in mice that were preimmunized with DNP as a model hapten, DNP derivatives of a CpG ODN showed a serum $t_{1/2}$ as long as 3 to 8 days. This in turn allowed the complex to accumulate at tumor tissue resulting in desirable pharmacological outcome.[1] Altered PK and biodistribution due to binding to endogenous carriers can indeed alter the pharmacodynamics (PD) of a therapeutic agent as has previously been shown.[1] In the following experiments, mice were immunized against DNP such that they produce endogenously circulating anti-DNP antibodies. Some mice were subsequently treated with a therapeutic CpG ODN which had been chemically conjugated to DNP. This article shows the different pharmacodynamic effects of DNP−CpG dependent on varying levels of the endogenous carrier.

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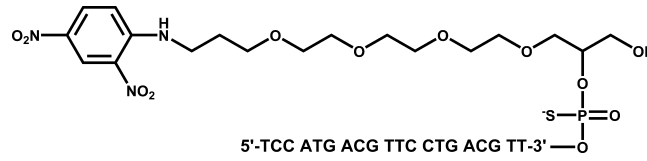
Experimental Section

Materials. All reagents were purchased from commercial sources and were used without further purification unless noted otherwise. Molecular biology-grade water, phosphate buffered saline (PBS), Hank's balanced salt solution (HBSS), thimerosal, Tween 20, and complete (CFA) and incomplete (IFA) Freund's adjuvants were all purchased from Sigma-Aldrich (St. Louis, MO). Hammersten-grade casein was purchased from Research Organics (Cleveland, OH). Dinitrophenylated keyhole limpet hemocyanin (DNP-KLH) and DNP-albumin were purchased from Calbiochem (San Diego, CA). Nonheparinized microcapillary tubes and Nunc MaxiSorp 96-well plates were purchased from Thermo Fisher Scientific (Waltham, MA). *O*-Phenylenediamine dihydrochloride (OPD) and an accompanying buffer were from Pierce (Rockford, IL). Goat anti-mouse IgG-peroxidase conjugate was purchased from Southern Biotech (Birmingham, AL). All culture media components were from Invitrogen (Carlsbad, CA).

BALB/c mice, 5 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed following the NIH guidelines, and all procedures were approved by the UNC Institutional Animal Care and Use Committee. BALB/c-derived colon carcinoma CT26 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and cultured in Dulbecco's modified Eagle medium with low (1 g/L) glucose supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Shown below is the nucleotide sequence of the CpG ODN, known as CpG 1826 in the literature, and the structure of its DNP derivative. A control ODN used, designated as DNP-ss, has the same nucleotide composition as the CpG 1826 but does not have any CpG motif: 5′-TCCAGGACTTCTCTCAGGTT-3′. This sequence is commonly referred to as 1982 in the literature.[14] These ODN and their DNP derivatives were synthesized with a phosphorothioate backbone by Dr. Rowshon Alam of the Nucleic Acid Core Facility of the Program Project Grant in Pharmacodynamics of Genes and Oligonucleotides at the University of North Carolina at Chapel Hill.



Vaccine Preparation and Active Immunization. Exact procedures in detail can be found elsewhere.[1] To prepare the primary vaccine, 20 mg of DNP-KLH was dissolved in 10 mL of endotoxin-free water to give a 2 mg/mL solution. Prior to vaccination, an equal volume of CFA was added. The mixture was emulsified by vortexing at maximum rpm for 15 min using a multitube vortexer (VWR, West Chester,

PA). Secondary vaccines were prepared similarly, but using IFA instead of CFA.

BALB/c mice were immunized intraperitoneally with 0.2 mL of freshly prepared primary vaccine. A booster injection was given 2 weeks later using 0.2 mL of the secondary vaccine. Anti-DNP antibody titer was monitored by enzyme-linked immunosorbent assay (ELISA) as described below. In the first series of experiments, another booster injection was given 5 weeks after the first booster to maintain a high antibody titer.

Antibody Titer. In both series of experiments, 4 mice were selected at random for antibody titer bleeds and were used throughout the entire duration of the experiment. Samples were collected before immunization to establish background antibody levels and were then collected every 2 weeks. Blood samples, each 50 μL, were collected by tail clip into nonheparanzied microcapillary tubes. Blood samples were allowed to clot at room temperature (RT) for up to 1 h and were then centrifuged at 12000g for 5 min. Serum fractions were collected and diluted 10-fold in PBS. Anti-DNP IgG titers were determined by ELISA as described elsewhere.[1] Briefly, 96-well plates were first coated with DNP-BSA solution/carbonate buffer at pH 9.6 and incubated overnight at 4 °C. Wells were washed in triplicate with PBS containing 0.1% Tween 20 and then blocked with casein/PBS for 2 h at RT. Wells were then washed again in triplicate and incubated with 50 μL of serially diluted serum samples in PBS and incubated for 2 h at RT. After a triplicate wash, wells were incubated with of goat anti-mouse IgG-peroxidase conjugate in PBS for 2 h at RT. Wells were washed in triplicate and incubated with 125 μL per well of a 1 mg/mL OPD solution for 20 min at RT. The colorimetric reaction was terminated by the addition of a sulfuric acid solution. Absorbance at 492 nm was measured on a Bio-Rad 3550 Microplate Reader. Best-fit sigmoidal curves were obtained from plotting absorbances vs log dilution factors using GraphPad Prism 4 (La Jolla, CA). Titer levels were obtained as EC_{50} values from the midpoint of each sigmoidal curve.

Tumor Inoculation, Tumor Measurements and Dosing Schedule. Four to 5 weeks after primary vaccination, DNP-immunized mice were inoculated with 2.5×10^5 CT26 cells suspended in 0.1 mL of HBSS into the right flank. Tumors were monitored daily and measured every 2 or 3 days with an electronic digital caliper. Tumor volumes were calculated based on the formula for an ellipsoid: length $\times$ width^2 $\times$ π/6. Mice were euthanized when tumors exceeded 2.0 cm in any dimension. Treatments began 9 days after inoculation when tumor sizes were between 75 and 150 mm^3.

In the first series of experiments, 24 mice were divided evenly into 3 groups: DNP-ss via iv (negative control), DNP−CpG via iv (positive control), and DNP−CpG via sc (test). Mice of the iv group were given a bolus dose that is equivalent to 100 μg of CpG ODN in 100 μL of PBS via the tail vein. Mice of the sc group were given the same dose into the left flank (contralateral to the tumor). Three doses were given in 7-day intervals followed by 3 additional doses at 3-day intervals for a total of 6 doses.

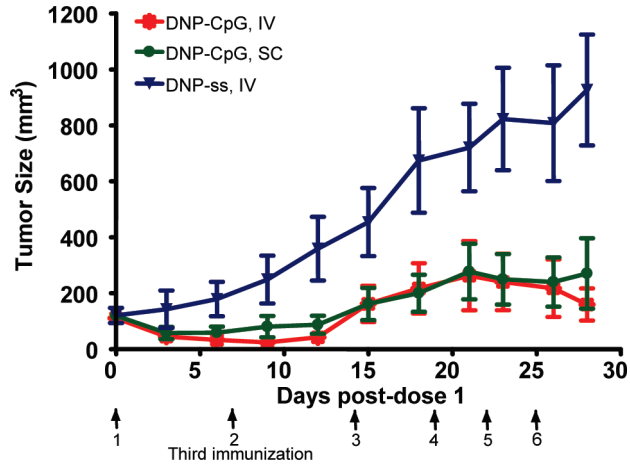


Figure 1. Comparison of tumor growth inhibition between sc and iv dosing of DNP−CpG in DNP-immunized, CT26 tumor-bearing mice among: iv dosing of DNP-ss (negative control, $n = 10$), iv dosing of DNP−CpG ($n = 10$), and sc injection of DNP−CpG ($n = 10$). Tumor volumes were plotted against time in days after administration of the first dose. Data are expressed as means ± SD.

In the second series of experiments, only the sc route of administration was tested with 29 mice in 3 groups: DNP-ss (negative control, $n = 9$), free CpG (test, $n = 10$), and DNP−CpG (test, $n = 10$). Five doses equivalent to 100 μg of CpG ODN in 100 μL of PBS were administered every 5 days. Tumor monitoring, measurements, and animal euthanasia were as described above.

Statistical Analysis. Using Van der Waerden normal scores, a nonparametric pairwise comparison test was performed to determine if there were statistical differences in tumor volumes between each group at each day. Statistical analyses were performed with the SAS statistical software by Dominic T. Moore of the UNC Lineberger Comprehensive Cancer Center Biostatistics and Data Management.

Results and Discussion

As shown in Figure 1 and as found in an earlier study,[1] iv administration of DNP−CpG 1826 conjugate exhibited antitumor activity in DNP-immunized mice. With the control DNP−CpG 1982, which do not have any CpG motif but with the same nucleotide composition, tumor growth proceeded. Most notable is that sc administration of DNP−CpG was not only highly effective but also comparable to the iv route. When tumor burden was low, almost complete tumor regression was observed with only a few doses. When tumor growth rate noticeably increased, dosing frequency was increased to every 3 days and once again partial tumor regression was achieved. Note that, in this experiment, a third immunization was made to keep the anti-DNP titer high throughout the treatment schedule. The omission of additional control groups of nonimmunized and immunized mice treated with PBS was warranted based on previous studies.[1] Furthermore, the DNP hapten has been thoroughly studied and exhibits no anticancer effects thereby deeming further DNP-related test groups to be unnecessary.

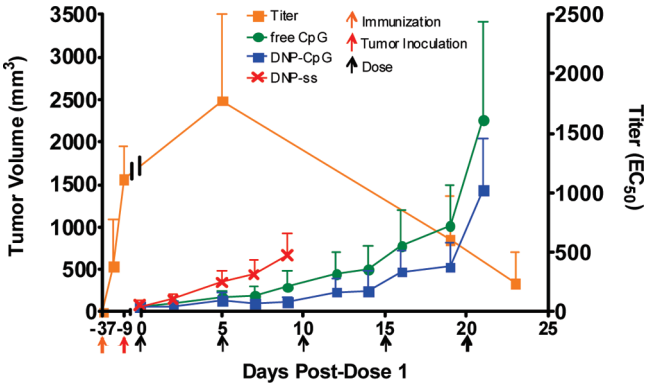


Figure 2. Comparison of tumor growth inhibition between free CpG and DNP−CpG during sc dosing in immunized mice. Tumor growth inhibition by subcutaneous administration of DNP-ss (negative control, n = 9), free CpG (n = 10), and DNP−CpG (n = 10) in DNP-immunized, CT26 tumor-bearing mice. Tumor volumes were plotted against time in days after administration of the first dose. Data are expressed as means ± SD. Anti-DNP IgG titer measurements are overlaid and expressed as means ± SD.

The ability of sc administration to achieve such a remarkable antitumor effect is, to the best of our knowledge, reported here for the first time in the literature and is attributed to multiple reasons. Since tissue antibody levels are normally in equilibrium with intravascular antibody levels at approximately half of the plasma concentration,[11] it is reasonable to believe that immune complexes can be readily formed at or near the injection site. The increase in apparent MW from approximately 7 kDa of DNP−CpG to 160 kDa for a 2:1 ODN:anti-DNP IgG immune complex should amplify the depot effect usually associated with an sc injection. This would allow for a prolonged and sustained release of immune-complexed DNP−CpG over time to the circulation. Drainage from the injection site to lymph nodes would occur via the lymphatic system. It is thus conceivable that DNP−CpG in immune complexes can be presented in a more direct manner to dendritic cells and macrophages in the lymph node, which would initiate the adaptive immune response leading to antitumor activity. One could further postulate a possible involvement of Fc gamma receptor on the target cell in the uptake of immune complexes.[15,16]

In a second series of experiments, results of which are shown in Figure 2, only the sc route of administration was employed and only one booster immunization was given. The failure of the sc administration of DNP-ss to inhibit tumor development was as expected, as in the case of iv administration of the same. This control group of mice was sacrificed earlier than the treatment groups following the guideline of Institutional Animal Care and Use Committee. In our protocol, the treatment group was allowed to develop tumors slightly larger than the control group. Tumors reached a maximum allowable size and quickly became ulcerated. Since only one booster was given, anti-DNP titers were high at the start of dosing, but started to fall between doses 2 and 5.

Consistent with the sc data on Figure 1, DNP−CpG 1826 was able to induce partial regression as long as the anti-DNP titer was high. However, the treatment failed to inhibit tumor growth as the titer decreased. When the titer dropped substantially on day 20 and beyond, the DNP−CpG treatments were not effective at all in suppressing tumor growth. At this point, tumor growth rate was rapid and mirrored the growth rate with the free CpG treatment group. Interestingly free CpG ODN also showed some antitumor effect upon sc injection. Although these mice were also immunized with DNP, immune complexes are unable to form because the ODN used was not derivatized with DNP. Free CpG ODN can also drain to the lymph node, albeit more rapidly than immune complexes, following the steps postulated earlier.

Although the DNP−CpG treatment group showed a slower tumor growth than the group treated with free CpG, the profiles of tumor growth observed with DNP−CpG and free CpG are remarkably similar to each other. This observation is attributed to the likelihood that DNP−CpG exists largely as unbound just like free CpG because of low anti-DNP titer after day 10. Note that there is only 8% difference in MW between DNP−CpG and CpG, and thus almost an identical rate of lymphatic transport is expected. Since this antitumor effect was not seen with iv data,[1] further study is required.

Statistical analysis indicates that tumor volumes were not statistically different when treatments began. Differences were deemed significant if p-values were less than 0.05. DNP−CpG already showed statistical significance on day 2 when compared to DNP-ss, but no statistical difference was seen with free CpG until day 7. Except for those days and at day 15, which was borderline significant, tumor volumes were statistically significant between DNP−CpG and free CpG.

In summary, the importance of antibody titer is evident in the two series of experiments. An inverse correlation was observed between antibody titer and tumor size. Partial tumor regression and higher inhibition of tumor growth observed with DNP−CpG in DNP-immunized mice reflect the enhanced antitumor effects of immune complexation over free CpG treatments. An implication for future studies is the importance of maintaining a high fraction of the ODN in the form of an immune complex.

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