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Comparative studies on *in vitro* expression and *in vivo* immunogenicity of supercoiled and open circular forms of plasmid DNA vaccines

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Abstract

Here we use tests for *in vitro* expression and *in vivo* immunogenicity to compare the biological activity of supercoiled and open circular forms of plasmid DNA vaccines. The different forms of vaccine DNA revealed no differences in the expression of mRNA or protein following DEAE-Dextran-assisted transfection of cultured cells. In contrast, supercoiled DNA was 3-times more effective than open circular DNA at priming a MVA-boosted CD8 T cell response following intramuscular saline injections in mice. Thus, under our experimental conditions, measurements for supercoiled vaccine DNA provided a more accurate assessment of the potential to prime a CD8 response than tests for expression in transiently transfected cells.

Keywords

DNA vaccine; Supercoiled DNA; Open circular DNA; Potency tests

Introduction

As DNA vaccines are being entered into clinical trials in humans, manufacturers are working to develop assays that accurately define the potency of different production lots of plasmid DNA. The plasmid DNAs that constitute DNA vaccines are produced in bacteria as supercoiled, or covalently closed circular (CCC) plasmid DNA. Degradation of the purified supercoiled product can be initiated by enzymatic nicking or chemical processes such as free radical oxidation[1]. Single strand nicking results in the relaxation of supercoiled DNA into open circular DNA (OC). Localized double-strand nicking, such as done by restriction

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endonucleases, cleaves the DNA and results in the formation of linear DNAs. In purified preparations of DNA, OC DNA is the most frequent initial degradation product for supercoiled DNA. The US Food and Drug Administration (FDA) recommends that vaccines contain at least 80% supercoiled plasmid[2]. This requirement is based on an understanding that supercoiled content is a key indicator of plasmid quality and that supercoiled plasmid has superior biological activity as compared to other plasmid forms [3].

Two DNAs were used in our studies: pGA2/JS7 and pGA1/JS8. pGA2/JS7 expresses Gag, Pol, Env, Tat, Rev, and Vpu of HIV-1 by subgenomic splicing of a modified HIV-1 proviral DNA [4]. pGA2/JS7 is currently being used as a DNA prime for a modified vaccinia Ankara (MVA) boost in phase 1 human trials conducted by the HIV Vaccine Trials Network. pGA1/JS8 expresses a codon-optimized Gag gene[4]. Both plasmids were used for *in vitro* studies. *In vivo* immunogenicity tests were conducted with pGA1/JS8 because pGA2/JS7 is not immunogenic in mice (unpublished observations). The *in vitro* studies were done with DEAE-Dextran assisted transfections to enhance the entry of DNA into cells. The *in vivo* immunogenicity studies were conducted using a DNA prime followed by a MVA boost to achieve levels of CD8 T cell responses that could be accurately measured[5] and mimic the conditions being used in human trials..

The goal of this study was to determine whether supercoiled DNA has superior biological activity to open circular DNA. Both supercoiled and open circular DNAs contain the complete genetic sequence of a plasmid. Thus, given successful entry into cells, both should be able to express vaccine inserts.

Our results reveal supercoiled and open circular DNAs having different biological activities for *in vivo* immunizations but indistinguishable expression patterns in transiently transfected cell cultures. In the immunization studies, the supercoiled DNA had 3-times higher ability to prime an MVA boost than the OC DNA. We conclude that determination of the amount of supercoiled plasmid DNA in a vaccine preparation is a better predictor for *in vivo* immunogenicity than the levels of plasmid expression in transiently transfected cells.

2. Material and Methods

2.1 Preparation and analysis of DNA

OC plasmid DNAs were generated by digestion of predominantly CCC DNAs with *N. Bst*NI (New England Biolabs, Beverly, MA) at 55°C using 0.5 – 1 unit of enzyme for each microgram of DNA. DNA was collected by ethanol precipitation and the pellets washed with cold 70% ethanol and allowed to air-dry. Pellets were then resuspended in DNA resuspension buffer and the DNA quantified on a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). For the pGA2/JS7 analysis reported in this paper, DNA samples were diluted in loading buffer and separated on a 0.8% agarose gel in 1X TAE buffer containing 40 mM sodium acetate. Electrophoresis was carried out at 1.75 V/cm for approximately 24 hours. Buffer was changed once at approximately 18 hours after the start of electrophoresis. The gel was stained for approximately 3 hours with Syto60 dye (Invitrogen, Carlsbad, CA) diluted 1:10,000 in deionized water. The Syto60-stained gel was washed in deionized water, and then scanned on an Odyssey infrared imager (Li-Cor, Lincoln, NE). pGA1/JS8 preparations were analyzed by agarose gel electrophoresis using a very similar method with slight variations in reagents, electrophoresis conditions and staining times. Two independent preparations of pGA1/JS8 were used for the *in vivo* immunogenicity studies: one for DNAs used in experiment 1 and a 2nd preparation for DNAs used in experiments 2 and 3 (see Results for independent experiments).

2.2 DEAE-Dextran assisted transfections

All transfections were conducted in duplicate in 6-well plates (Corning Labware, Corning, N.Y.) coated with poly-D-lysine (BD Biosciences, San Jose, CA) at a concentration of 2 µg/cm². 293T cells were seeded onto coated plates at a density of 1.5×10^6 cells/well and incubated for about 20 hrs. In preparation for transfections, DNA samples were serially diluted in sterile PBS in a volume of 800 µL. 40 µL of 10 mg/mL DEAE-Dextran (Promega, Madison, WI) was added to each tube, and the DEAE-Dextran-DNA combinations were mixed gently. After washing cells twice with 1XPBS, DEAE-Dextran-DNA mixtures were added to wells at 200 µL/well. Mock samples were included with DEAE-Dextran but no DNA. Cells were incubated with DEAE-Dextran mixtures for 30 minutes at 37°C, 5% CO₂, and rocked gently every 10 minutes. 30 minutes after addition of DEAE-Dextran mixtures, DMEM/2 (DMEM + 2% FBS, no antibiotic) was added to plates at 1.5 mL/well. Plates were incubated for 1 hour at 37°C, 5% CO₂, supernatants were aspirated, and 1.2 mL DMEM/2 was added to each well.

After two days further incubation, culture supernatants and cells were harvested. Culture supernatants were aspirated and frozen immediately at -70°C prior to analysis by ELISA. Cell monolayers were harvested by trypsinization and cell suspensions were transferred into microcentrifuge tubes. Cells were pelleted by centrifugation and resuspended in 1 mL of cold 1X PBS. 4/5 of each cell suspension was transferred into a 5-mL polystyrene tube for staining for FACS analyses. These cells were pelleted, resuspended in cytofix/cytoperm fixation buffer (BD Biosciences, San Jose, CA) and stored at 2-8°C. The remaining 1/5 of each cell suspension was pelleted, and PBS was aspirated. RNA was prepared from each sample using a ChargeSwitch Total RNA Cell Kit (Invitrogen, Carlsbad, CA). RNA samples were frozen at -70°C.

Cell staining and flow cytometry—Fixed cells were washed twice with FACS Perm/Wash (BD Biosciences, San Jose, CA). Cells were stained with the anti-Gag antibody KC57-FITC (Beckman-Coulter, Fullerton, CA) diluted 1:100 in FACS Perm. Staining was performed for approximately 30 minutes at 4° C in the dark. Cells were washed twice with FACS Perm, then once with PBS + 2% FBS. Cells were pelleted again and resuspended in PBS + 1% formaldehyde. Cells were stored at 2-8°C prior to flow cytometry. Cells were analyzed on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA). 100,000 cells from each sample were collected. Data files were analyzed in FlowJo (TreeStar, Ashland, OR). Gating analysis was used to determine the percentage of each sample positive for Gag expression.

2.4 Antigen capture ELISA for Gag

Sheep anti-p17 Gag antibody (NIH AIDS Research and Reference Reagent Program, Germantown, MD) was diluted 1:1,000,000 in HEPES coating buffer (10 mM HEPES, 150 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, pH 7.5). High-binding EIA plates (Thermo Labsystems, Milford, MA) were coated overnight with the diluted anti-Gag antibody at 100 µL/well. Plates were washed three times with 200 µL/well PBST (phosphate buffered saline containing 0.1% Tween-20), then duplicate samples of culture supernatants were added to the plates at 100 µL/well. A negative control (DMEM) and a positive control (SF2 pr55 Gag, also from the NIH reagent bank, diluted in DMEM) were included on each plate. Culture supernatants were incubated on the plates overnight at 2-8°C. Plates were washed three times with 200 µL/well PBST. Plates were blocked with 100 µL/well PBST + 1% BSA for 1 hour at room temperature. Plates were washed once with 200 µL/well PBST. The mouse-anti-Gag antibody H12-5C was diluted to 1 µg/mL in PBST + 0.25% BSA, then added to plates at 100 µL/well. Primary antibody incubation was performed for 90 minutes at room temperature. Plates were washed three times with 200 µL/well PBST. Anti-mouse-AP conjugate was diluted 1:500 in PBST + 0.1% BSA, then added to the plates at 100 µL/well. The conjugate incubation was performed for 1 hour at room temperature. Plates were washed three times with 200 µL/

well PBST. pNPP substrate solution (p-nitrophenyl phosphate, Pierce, Rockford, IL) was added to the plates at 100 μ L/well. Plates were read on a Versamax plate reader (Molecular Devices, Sunnyvale, CA) set to 405 nm.

2.5 Quantitative RT-PCR

RNA preps were thawed, then added to reverse transcriptase (RT) master mix containing 1X TaqMan buffer A, 5 mM MgCl₂, 1 mM dNTP's, 2.5 μ M random hexamer primers, 1 unit/reaction MultiScribe reverse transcriptase (absent in –RT control reactions), and 0.4 unit/reaction RNase inhibitor. RT and PCR reagents were obtained from Applied Biosystems (Foster City, CA). Reaction volume was 20 μ L, and 10 μ L of RNA prep was used in each reaction. Reverse transcription was performed on an ABI 7500 real-time PCR instrument. Custom primers and FAM-labeled probes were obtained from Eurogentec (San Diego, CA). Reaction conditions were as follows: 25°C for 10 minutes, 42°C for 30 minutes, and a final denaturation of 99°C for 5 minutes. PCR master mix was prepared and added to RT reactions. PCR reaction conditions were 1X TaqMan buffer A, 4 mM MgCl₂, 0.25 μ M each of forward and reverse primers, 0.1 μ M probe, and 1.25 units/reaction AmpliTaq Gold polymerase. Final reaction volume was 50 μ L. Cycling was performed as follows: initial denaturation of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. PCR was performed on an ABI 7500 real-time PCR instrument.

2.6 In vivo immunogenicity test

Six to 8 wk-old BALB/c mice (H2-K^d) were purchased from Charles River Laboratories (Wilmington, MA). Mice were injected with 100 μ L of saline (mock) or 100 μ L of saline containing 10 or 100 μ g of CCC or OC forms of pGA2/JS8 DNA. 50 μ L was injected intramuscularly by needle and syringe into each quadriceps. Two weeks after priming mice were bled to enumerate the frequency of Gag-specific CD8 T cells elicited by the DNA prime. Four weeks after priming, mice were boosted by injecting a single quadriceps with 0.1 mL of PBS containing 1×10^6 pfu of MVA/HIV62[6]. Mock controls again received buffer. Splenocytes were harvested for the determination of the frequencies of Gag-specific CD8 T cells at 1 week following the boost. Following both the prime and boost, responding CD8 T cells were quantified by staining blood (post prime) or splenocytes (post boost) with a H2-K^d tetramer recognizing the immunodominant Gag CD8 epitope AMQMLKETI. In some experiments, responding splenocytes were also quantified using intracellular cytokine staining for IFN- γ and IL-2 expression following stimulation with the AMQMLKETI peptide. Both procedures were conducted on samples from individual mice using previously described conditions for tetramer staining and intracellular cytokine analyses [7]. For both tetramer and intracellular cytokine staining, stained cells were quantified as a % of total CD8 T cells following acquisition of data using a FACSCalibur flow cytometer and analysis of data using FlowJo software.

3. Results

3.1 Production and characterization of different forms of DNA

Open circular and linear forms of pGA2/JS7 and pGA1/JS8 were generated from preparations of DNA that were predominantly supercoiled by digestion with *N. Bst*NI I, a site specific nuclease that cleaves only one strand of a double stranded DNA and has a cleavage site outside of the expression cassettes of the test plasmids. Agarose gel electrophoresis revealed the expected forms of plasmid DNA. For pGA2/JS7, the linear DNA migrated at 9.5 kb, the open circular DNA at 14.2 kb and the supercoiled DNA at 12.7 kb (Figure 1). For all preparations, the linear and OC forms contained almost exclusively the desired forms of DNA, whereas the supercoiled DNA exhibited small amounts of OC and supercoiled dimer DNAs (Figure 1).

3.2 Tests for biological activity in cell culture

In vitro tests revealed no differences in expression of the supercoiled and OC forms of pGA2/JS7 and pGA1/JS8 DNAs (Figure 2 and data not shown). Expression tests were conducted on 293T cells transiently transfected with a 10,000-fold range of DNA doses, from 10^{-1} ng to 1×10^3 ng of DNA. The results of these analyses revealed that our antigen capture ELISA was the least sensitive assay for expression, scoring for Gag expression between 100 and 1000 ng of DNA per transfection (Figure 2A). The FACS had intermediate sensitivity, scoring for Gag expressing cells for transfections with 50 to 250 ng of DNA (Figure 2B). The RT-PCR had the greatest sensitivity, scoring Gag mRNA expression for transfections with from 5 to 50 ng of DNA (Figure 2C). In all cases, dose response curves showed a good linear range. For the ELISA assay, the highest tested dose of DNA was still on the linear range. Similar results were found in repeated experiments and using lipofectamine-assisted transfections (data not shown).

3.2 Tests for immunogenicity in vaccinated mice

In contrast to the indistinguishable expression of the OC and CCC forms of plasmid DNAs in transiently transfected cells, the supercoiled form had consistently higher ability than the OC form to prime an MVA-boosted CD8 T cell response in mice (Figure 3). Three separate experiments were conducted to test the ability of OC and CCC forms of pGA1/JS8 DNA to prime an MVA boost. Each experiment used 100 μ g of DNA delivered at time 0 to prime the immune response and 1×10^6 pfu of recombinant MVA/HIV62 delivered at week 4 to boost the response (Figure 3A). The MVA expressed the same Gag gene as the DNA. One experiment included 10 as well as 100 μ g of DNA. All experiments were in BALB/c mice and all inoculations were intramuscular injections into the quadriceps (see Materials and Methods for detail). Assays for responding CD8 T cells were conducted on PBMC at 2 weeks following the DNA prime and on splenocytes at one week following the MVA boost (Figure 3A).

Elicited CD8 T cells were detected by testing for CD8 T cells recognizing the immunodominant AMQMLKETI epitope restricted by the H2-K^d haplotype of BALB/c mice. These assays included tetramer staining, which was conducted on unstimulated cells, and intracellular cytokine staining, which was conducted following stimulation with the AMQMLKETI peptide. For both assays, frequencies of responding cells were quantified as % of total CD8 T cells. The results of both assays showed good agreement. These analyses revealed the DNA prime eliciting low frequencies of tetramer staining cells that underwent marked increases with the MVA boost (Figure 3B). Most mice within individual groups had frequencies of tetramer staining cells that clustered within a 10-fold range of values (Figure 3C). Consistent with prior studies on the effect of DNA dose on the height of MVA-boosted CD8 T cell responses[7], CD8 responses primed by 100 μ g of OC or CCC DNAs were much higher than responses primed by 10 μ g doses showing that studies had been conducted in the active range for a dose response (data not shown).

Statistical analysis of data from the three independent immunogenicity studies revealed that the CCC DNA was 3.2 times more effective than the OC DNA at priming a MVA-boosted CD8 T cell response ($p=0.017$) (Table 1). This difference in efficiency resulted in geometric mean frequencies of 5.7% as opposed to 1.76% of total CD8 T cells being vaccine-elicited cells for the AMQMLKETI CD8 epitope in Gag. The frequencies of responding CD8 T cells for each experiment were first tested for inter-experiment consistency. Analysis of variance used log-transformed data and the Wilcoxon test for pairwise comparisons between-experiments. No consistent experimental differences were found for the MVA-boosted responses among the groups receiving OC DNA priming or among the groups receiving CCC DNA priming. Data for all 3 experiments were then pooled and tested for differences in the ability of the two DNAs to prime the CD8 response using the t-test.

4. Discussion

Due to their novelty, plasmid DNA vaccines lack well-established methods for potency testing. In a 2005 draft guidance for industry, “Considerations for Plasmid DNA Vaccines for Infectious Disease Indications”, the FDA allows developers considerable flexibility in the selection of potency assays during early product development. Such assays include tests on transiently transfected cells for vaccine expressed mRNA or proteins. The FDA also recommends that as product development advances that evidence be provided that any *in vitro* potency test directly correlates with an *in vivo* immunogenicity test.

To test the comparability of candidate *in vitro* and *in vivo* potency tests, we directly compared the biological activity of supercoiled DNA with that of its most frequent early degradation product, open circular DNA. Following transient transfections in cell culture, both of these forms of DNA were indistinguishable in their biological activity. However, when tested in mice for the ability to prime a MVA-boosted CD8 T cell response, the supercoiled form was 3 times more effective than the open circular form.

Differences in the *in vitro* and *in vivo* potency tests likely reflected differences in the efficiency of the introduction of DNA into cells in the two test systems. For the *in vitro* tests, DNA was transfected into cells using DEAE-dextran to increase the efficiency of transfection. For *in vivo* tests, DNA was inoculated in saline. Open circular (relaxed) DNA is a less condensed structure than supercoiled DNA. DEAE dextran, a positively charged molecule, assists transfection by complexing with negatively charged DNA to give it a more neutral or positive charge to facilitate entry into cells through negatively charged phospholipid membranes. DEAE-Dextran was chosen for use as a transfection reagent in lieu of more efficient cationic lipid reagents because the DNA-DEAE-Dextran complexes were considered more likely to reflect differences in DNA topology than liposome complexes formed by cationic lipid reagents. Nonetheless, the complexes of DEAE dextran with open circular and supercoiled DNA appear to have made these two DNA forms comparably efficient at entering cells. In contrast, in the *in vivo* test, the OC DNA was not as effective as the supercoiled DNA. This could have been due to the more open structure of OC DNA resulting in a poorer ability to enter cells and/or a higher susceptibility to degradation than supercoiled DNA.

Our immunogenicity results using saline injections of an enzymatically generated form of OC DNA are in agreement with results using saline injections of an OC DNA generated during temperature-induced degradation of plasmid DNA[8]. In this study in cats, saline injections of a rabies vaccine DNA containing large proportions of OC DNA were less effective at eliciting Ab, CD4 T cell and protective responses than supercoiled DNA. Thus we think it likely that our results for the elicitation of CD8 T cells by DNA primes followed by an MVA boost will be generalizable to other vaccine DNAs delivered as saline injections. However, our findings may not apply to particle or liposome-mediated delivery where both delivery and stabilization of DNA are likely different than in saline injections[9].

A potency test should support accurate measurement of the potential of a preparation of vaccine DNA to induce immune responses. Our *in vivo* immunogenicity tests required 25 mice per group to detect a 3-fold difference in the ability of supercoiled and OC DNA to prime an MVA boost. The geometric means obtained in these tests had wide confidence intervals reflecting the scatter in data points for the DNA-primed vaccine responses (see Table 1 and Figure 3).

Could the amount of vaccine DNA coupled with its per cent in a supercoiled form in a vaccine preparation be an appropriate potency test? Our data show that intramuscular saline injections of supercoiled DNA are more effective than intramuscular saline injections of OC DNA at priming a MVA-boosted CD8 response. Accurate measurement of supercoiled DNA can be done on agarose gels (Figure 1)[10], by high pressure liquid chromatography (HPLC)[10,11]

or by capillary gel electrophoresis[12]. The use of supercoiled DNA as a measure for vaccine potency would focus on quantifying the most active form of DNA for eliciting an immune response and provide a more accurate measurement for biological activity than tests for *in vitro* expression or *in vivo* immunogenicity. The *in vitro* expression tests, although having the potential to be highly accurate, fail to distinguish the activity of OC and CCC DNA under conditions of DEAE-dextran (or liposome)-assisted transfections. The *in vivo* immunogenicity tests are cumbersome and have wide confidence intervals even for test group sizes as large as 25, thus leading to potential errors in interpretation.

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Figure 1. Agarose gel electrophoresis displaying OC and CCC isoforms of pGA2/JS7 DNA
Different forms of DNA flanked by molecular weight markers. The CCC preparation pictured was used to generate the OC and linear forms displayed here. Molecular weight markers are the Bio-Rad 1-kb Molecular Ruler.

Figure 2. Results of tests for Gag expression following *in vitro* transfections of pGA2/JS7
 (A) Amount of protein expression determined in an antigen capture ELISA, (B) Transfection efficiency scored by FACS staining for Gag, (C) mRNA expression scored by quantitative RT-PCR. 293T cells were transiently transfected with the indicated concentrations of the OC and CCC forms of pGA2/JS7 Gag DNA. Analyses were done at ~2 days post transfection. For more detail see Materials and Methods.

Figure 3. *In vivo* Immunogenicity tests

(A) Schematic of trial design. Inoculations are given above and sampling times below the timeline. (B) Representative FACS scans showing examples of the different frequencies for tetramer staining cells for three mice in the different trial groups. The group being tested and the time in the trial are given above the schematics. The numbers within the FACScans are tetramer positive cells as a % of total CD8 T cells. (C) Results for one of 3 trials comparing the ability of OC and CCC DNA to elicit CD8 T cells to the immunodominant H2-K^d epitope in Gag. Data for each mouse is indicated by a different symbol. For more information see Materials and Methods.

Table 1

Summary and statistical analysis of CD8 responses to the immunodominant AMQMLKETI epitope for 3 independent immunogenicity tests in BALB/c mice¹.

Vaccination condition ²	Total Number of mice ³	Responding CD8, post-boosting		Probability
		Geometric mean ⁴	95% CI	
CCC/MVA	25	5.70%	3.25% ~ 10.00%	
OC/MVA	25	1.76%	0.79% ~ 3.89%	0.017
Mock/MVA	25	0.15%	0.10% ~ 0.23%	<0.0001
Mock/Mock	13	0.028%	0.016% ~ 0.047%	<0.0001

¹ See Results for explanation of analysis and Materials and Methods for details of the experiment.

² The vaccine used for the prime/the vaccine used for the boost

³ Number of mice pooled from 3 experiments

⁴ Geometric means for the frequencies of responding CD8 cells as a % of total CD8 T cells

⁵ Probability calculated using the t-test that the frequency of responding CD8 cells elicited by the indicated immunization is different from that elicited by CCC/MVA inoculations