Addition of an Anti-DNA Antibody Enhances Plasmid DNA Vaccination Against Herpes Simplex Virus

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1/03/04
Addition of an Anti-DNA Antibody Enhances Plasmid DNA Vaccination Against Herpes Simplex Virus

Amy Cupples

Senior Thesis submitted to the Department of Microbiology and University Honors Department at The University of Tennessee

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Abstract

Plasmid DNA vaccines are being developed for many human and animal diseases with some notable success, however, the formulations of these vaccines need to be adjusted to provide a more immunogenic effect with a practical dose volume. The approach to this problem adopted in this study is targeting the DNA vaccine to antigen presenting cells (APCs) through a pathway that stimulates the induction of a CD8+ T cell based immune response. An anti-DNA antibody is employed to selectively deliver the plasmid to APCs via their antibody-binding Fc receptors. It is hoped that the antibody-DNA complex vaccine will outperform the DNA alone in increasing uptake and expression of Herpes Simplex Virus antigen, thereby leading to a more robust immune response.
Introduction

Conventional vaccines have been successful in stimulating humoral immune responses against many diseases, but it has been found that stimulating a CD8⁺ T cell (a.k.a. Cytotoxic T lymphocyte or CTL) mediated immune response with a vaccine that is both safe and effective often proves to be a greater challenge. In the case of intracellular parasites and some viruses such as Herpes Simplex Virus-1 (HSV-1), an antibody based response is not sufficient for protection; a CD8⁺ T cell based response is necessary. The worldwide prevalence of lethal diseases for which no effective vaccine is yet available have stimulated creative new approaches to vaccinology, such as plasmid DNA vaccination. The benefits of the DNA vaccine approach include an increased safety profile (due to the fact that no live virus is used) and the ability to stimulate a CD8⁺ T cell response to the specific proteins of interest. The major drawback at this point in time is its relative inefficiency. Impractical volumes are required in order to stimulate a fully protective immune response. The current focus is on increasing immunogenicity so that these vaccines can one day be used in humans.

DNA vaccination utilizes a plasmid encoding a gene for an antigenic protein under the control of a strong viral promoter, such as the CMV promoter. DNA vaccines work on the premise that the plasmid is taken up into cells, transcribed, and translated into protein that can then be processed and presented on MHC Class I molecules for activation of CD8⁺ T cells. With few exceptions, all cells in the body process and present peptides made inside the cell on MHC Class I, and in fact, this is how virally infected cells are identified. Professional antigen presenting cells (APCs) are specially equipped to activate the immune system both through the MHC Class I dependant pathway described
above as well as through an exogenous protein (MHC Class II) processing pathway. APCs are designed to prime the immune system, and express costimulatory surface molecules and secrete inflammatory cytokines to that end. Giving the cells a plasmid DNA blueprint for viral protein makes the cell appear to be infected by that virus, stimulating an immune response.

The first hint that such an approach might be feasible came in 1990 when it was shown that injection of naked DNA into skeletal muscle lead to in vivo expression of the encoded protein for up to two months [1]. Later, other groups successfully used this strategy for immunizations in mice [2,3]. Ulmer’s group provided protection from influenza by administering a plasmid encoding Influenza A nucleoprotein, then challenging with a different strain of Influenza A [3]. This is remarkable even today.

HSV-1 infection is notoriously difficult to prevent with vaccination. Many approaches center around administering the HSV-1 glycoprotein B (gB) or genes encoding it in plasmid DNA vectors. gB contains major CD8+ T cell and CD4+ T cell epitopes. In the C57Bl/6 mouse, we know that the major CD8+ T cell epitope is gB498-505 and has the sequence SSIEFARL. Whether administered exogenously or produced in the vaccine transfected cell, gB is then processed down to various peptide epitopes, including SSIEFARL. It has been shown in a similar system that administering the SSIEFARL epitope can in fact stimulate a CD8+ T cell response and that a CTL response directed against just one epitope can protect against HSV-2 challenge [4]. Studies from our own group have found that gB encoding plasmid vaccines coadministered with DNA encoding IL-12 or IL-18 improves protection against a lethal Herpes Simplex Virus-1 challenge [5]. IL-12 and IL-18 are cytokines which polarize the immune response toward a Th1
dominated, cell mediated (CTL) response rather than a Th2 humoral (antibody based) immune response. Additionally, other members have speculated that the addition of genes encoding IL-15 and/or IL-23, critical cytokines involved in the maintenance of memory T cells, may boost the level of memory CD8+ T cells [6]. Furthermore, other members of our lab have demonstrated that including DNA containing CpG sequences into a peptide vaccine induces CD8+ T cell based immunity and resistance to viral challenge [7]. CpG sequences are unmethylated bacterial DNA stretches which activate pattern recognition molecules, namely TLR-9, on APCs stimulating them to produce a more robust killer cell based response. In this case, we see that certain DNA can itself serve as an adjuvant.

The study outlined in this paper seeks to prove the hypothesis that complexing plasmid DNA to an anti-DNA antibody will provide more efficient targeting of the vaccine to APCs, leading to increased uptake and expression of viral antigen and therefore a more robust immune response when compared to the plasmid DNA administered alone. APCs express Fc receptors on their cell surface which serve the purpose of bringing antibody-bound molecules into the cell for processing and presentation (Figure 1). We wish to exploit this mechanism to target the vaccine to APCs and achieve the goals described above.

Materials and Methods

Mice

Four- to five-week-old C57BL/6 (H-2b) mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). In conducting the research described in this work we
adhered to the *Guide for the Care and Use of Laboratory Animals* as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences of the National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

*Peptides*

The HSV gB (amino acids 498 to 505) peptide SSIEFARL and the chicken ovalbumin (aa 257 to 264) peptide SIINFEKL were synthesized and supplied by Research Genetics (Huntsville, AL).

*Cell lines*

Vero (African green monkey kidney cell line) was used for growing of viral stocks and MC38 was used as a target cell (C57BL/6 adenocarcinoma, H-2b). All cell lines were cultured in Dulbecco’s Modification of Eagle’s Medium (Mediatech, VA) supplemented with 10% heat-inactivated fetal bovine serum, 5 mg/L of gentamicin sulfate, and 2 mM L-glutamine. T cell stimulation assays were carried out in 25 mM Hepes buffered RPMI-1640 media (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum, 5 mg/L of gentamicin sulfate, 0.05 mM 2-ME, and 2 mM L-glutamine.

*MRSS-1 production and purification*

MRSS-1 is a B cell hybridoma which makes a mouse IgG3 isotype anti-DNA antibody (ATCC, Manassas, VA) [8]. The hybridoma was grown in roller bottles in
DMEM 5% FCS (Mediatech, Herndon, VA and Hyclone, Logan, UT, respectively). The cells were centrifuged out, then the supernatant was filtered using Whatman paper (Middlesex, UK) and was frozen at -20°C until a later date. The supernatant was then thawed and mixed 1:1 with a saturated ammonium sulfate solution and allowed to sit at 4°C overnight for precipitation of immunoglobulins and other high-molecular weight proteins. The precipitated proteins were passed over a protein L column (Pierce, Rockford IL) to isolate mouse Ig by affinity chromatography. The concentration of the antibody was determined by UV spectroscopy.

*Plasmid DNA preparation*

The plasmid pcDNA-gB was created as described by Manickan et al.[9]. It was propagated in *E. coli* DH5-a cells and grown in LB broth (both Invitrogen, San Diego, CA) and purified with an Endofree Plasmid Mega kit (Qiagen, Valencia, CA).

*DNA/Antibody binding assay*

pcDNA-gB was bound to a standard ELISA plate (Corning, Acton, MA) at [DNA]=5 μg/ml, 100ul/well using ReactiBind (Pierce, Rockford, IL) to bind the DNA to the plate. MRSS-1 was then added at various concentrations. Negative control wells contained non-specific mouse IgG (Southern Biotech Associates, Birmingham, AL). All wells were done in duplicate and the ELISA was performed as previously described [9].
T cell activation assay

An in vitro T cell activation assay was performed by first allowing splenic adherent cells (splenocytes adhered to plastic tissue culture plates for 2 h @ 37°C and then washed, scraped, and counted), consisting mainly of macrophage and DC, to take up plasmid DNA (pcDNA-gB), express protein, and present processed peptides to T cells. Antigen sources were as follows: 0.1 μg/ml of MRSS-1 anti-DNA antibody was incubated with 0.1 μg/ml of pcDNA-gB for 1 h @ 37°C (total vol 100 μl). This complex was then added to 500,000 (total vol 50 μl) splenic adherent cells in a 96 well U-bottom plate and allowed to incubate for 24 h 37°C, 5% CO₂. Plasmid DNA without antibody as well as a group with an irrelevant control antibody were also included. For a positive control adherent splenocytes were either transfected with the same amount of pcDNA-gB as above using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen), or gB498-505 peptide (1 μg/ml) was used. Following the 24 h incubation 50,000 (total vol 50 μl) T cell hybridoma specific for gB498-505, clone 2E2 [10] (provided by F. Carbone) were added and the incubation was continued for another 24 h. Supernatant was then collected and tested for IL-2 production by ELISA.

ELISPOT

ELISPOT assay for IFN-γ secreting cells was performed as previously described [7], except for slight modifications. Briefly, 96-well filter plates (Millipore HA) were coated with capture antibody for IFN-γ (RA-6A2) (BD Pharmingen) ON @ 4°C (BD Pharmingen) (2 μg/ml) in PBS. Plates were then washed with sterile PBS and blocked
with culture media containing 10% FCS. Splenocytes from a single cell suspension were incubated at various effector:stimulator ratios. 10^5 irradiated, SIINFEKL or SSIEFARL-pulsed irradiated (3000 rads), syngeneic splenocytes were used as stimulators. Serial 2-fold dilutions of effectors were then incubated with stimulators starting with 5 x 10^5 and ending with 6.25 x 10^4. 20 U/well of IL-2 was also added to the culture. Cells were then incubated for 48 h at 37°C, 5% CO₂. Wells were then washed with PBS, followed by PBS-Tween. Biotin anti-mouse IFN-γ (XMG1.2) (BD Pharmingen) (1 μg/ml in PBS 3% BSA) was then added to each well and incubated ON @ 4°C. Wells were then washed with PBS-Tween and streptavidin-conjugated peroxidase (Jackson Laboratories, Bar Harbor, ME) in PBS 3% BSA was added to each well (1 μg/ml) for 30 min @ 37°C. Wells were then washed with PBS-Tween and developed using 3-amino-9-ethylcarbazole (AEC) (Sigma) in 0.1 M acetate buffer pH 5.0, containing 0.05% H₂O₂. Reactions were allowed to proceed for 10 min and were ended by extensive washing with dH₂O. Plates were allowed to dry and then counted on a stereo microscope.

**CTL assay**

The CTL assay was performed as described earlier [7]. Briefly, effector cells generated after a 5 d in vitro expansion (with SSIEFARL-loaded, irradiated splenocytes) were analyzed for their ability to kill major histocompatibility complex (MHC)-matched antigen-presenting targets (H-2b). The cells were mixed with the target at various ratios and incubated for 4 h. The targets included ^51^Cr-pulsed MHC-matched SSIEFARL-pulsed as well as control SIINFEKL-pulsed MC38 target cells. Percent specific lysis was then calculated according to the following formula:
ELISA was performed as previously described [7]. Briefly, 96-well EIA/RIA plates were coated with capture antibody for IL-2 (BD Pharmingen) ON @ 4°C (2 μg/ml) in 1 M Na₂HPO₄. The wells were then washed with PBS-Tween and supernatant taken after 24 h was added and the plates were incubated ON @ 4°C. For detection wells were washed with PBS-Tween and biotin anti-mouse IL-2 (BD Pharmingen) (1 μg/ml in PBS 3% BSA) was then added to each well and incubated for 1h @ RT. Wells were then washed with PBS-Tween and streptavidin-conjugated peroxidase (Jackson Laboratories, ME) in PBS 3% BSA was added to each well (1 μg/ml) for 30 min @ RT. Plates were then washed and developed using ABTS (Sigma). ELISA readings were taken on an automated ELISA reader (SpectraMAX 340, Molecular Devices, Sunnyvale, CA).

Immunizations

90μg/mouse pcDNA-gB was complexed to 10μg/mouse MRSS-1 for 1 h @ 37°C. This preparation was then split in half and administered to both of the tibialis anterior muscles of the mouse on day 0. Negative controls included pcDNA-gB alone and pcDNA-gB + control mouse IgG (Southern Biotech Associates, Birmingham, AL). Both underwent the same incubation as the experimental preparation. Tibialis anterior administration of 10⁶ pfu recombinant vaccinia virus expressing gB (VV-gB) served as a positive control.
Statistical analysis

The data were analyzed by using a 2-tailed student’s \( t \) test, and \( p \) values less than .05 were deemed significant.

Results

MRSS-1 binds to the pcDNA-gB vaccine

Before using the complexed vaccine for study, it was necessary to demonstrate that the antibody purified from MRSS-1 does, in fact, bind pcDNA-gB. Following the ELISA based binding assay protocol described above, a dose dependant binding curve was found to exist for the MRSS-1 but not for the negative control antibody (Figure 2). Binding increased consistently up to the 1.0 \( \mu \text{g/ml} \) antibody concentration, after which the binding curve plateaus.

pcDNA-gB + MRSS-1 activates a T cell hybridoma specific for the gB 498-505 SSIEFARL epitope

The T cell activation assay demonstrated that the pcDNA-gB + MRSS-1 complex vaccine was capable of stimulating a T cell hybridoma which recognizes the SSIEFARL epitope. Moreover, the response to the pcDNA-gB + MRSS-1 was three-fold higher than that of the pcDNA-gB alone (Figure 3). Adherent splenocytes loaded with pcDNA-gB alone secreted an amount of IL-2 indistinguishable from background (16pg/ml) whereas those loaded with pcDNA-gB + MRSS-1 secreted an amount which is three times background (48pg/ml IL-2). The lipofectamine and gB\textsubscript{498-505} peptide positive controls
were included to verify that the assay worked, though it should be noted that neither is a vaccine candidate in this study. gB 498-505 has been evaluated in other studies as a vaccine component along with adjuvants such as heat shock proteins and alum with some success [11][Pack, unpublished data].

\emph{pcDNA-gB + MRSS-1 stimulates IFN-\(\gamma\) secreting cells}

The ELISPOT assay measures the relative number of activated inflammatory cells, identified by their secretion of the inflammatory cytokine IFN-\(\gamma\), between treatment groups. We observed a more than five-fold increase in the number of IFN-\(\gamma\) secreting cells in the pcDNA-gB + MRSS-1 group (82 ± 50 SFU) over the group which received pcDNA-gB alone (14 ± 6 SFU). This shows that the pcDNA-gB + MRSS-1 vaccine can not only stimulate a response, it also elicits a more potent response than pcDNA-gB alone and that this response is of the desired Th1 type. This response was less robust than the gold standard live vaccinia virus encoding gB, which gave 631 ± 273 SFU, but the live virus vaccine has problems of its own, namely a decrease in safety.

\emph{Specific killing of SSIEFARL loaded target cells is increased with the pcDNA-gB + MRSS-1 vaccine}

When we examine the ability of splenocytes from the various groups to kill peptide loaded target cells, we are able to quantitatively see the efficacy of the vaccine in stimulating a CTL response. In this case, we saw approximately a two-fold increase in CTL specific lysis of target cells in the pcDNA-gB group versus the group which received pcDNA-gB alone (Figure 4). At an effector : target ratio of 25:1, splenocytes
from pcDNA-gB + MRSS-1 vaccinated mice exhibited 31% specific killing of target cells (the peak of the response curve) whereas splenocytes from pcDNA-gB vaccinated mice exhibited only 15% specific killing. This two fold difference in responses is consistent all the way down to an effector to target ratio of 1.5:1. It should also be noted that the pcDNA-gB + negative control antibody response was, at 13% specific killing at an effector to target ratio of 25:1, comparable to that of the pcDNA-gB alone all the way down the dilution series. pcDNA-gB + MRSS-1 even stood up reasonably well when compared to the gold standard live vaccinia virus encoding gB, which exhibited 52% specific lysis at its peak. A negative control containing all the same groups and target cells loaded with an irrelevant negative control peptide, SIINFEKL, demonstrated the expected negligible amount of CTL activity (data not shown).

Discussion

Various strategies are employed to increase immunogenicity or skew the response toward Th1/inflammatory and CTL mediated immunity vs. Th2/humoral immunity. In viruses subject to rapid mutation, multiple antigens corresponding to different strains may be encoded, as in a recent influenza vaccine trial [12]. Genetic adjuvants (i.e. genes for cytokines, transcription factors, growth factors, or costimulatory molecules) may be added to enhance the response or skew it toward a Th1 or Th2 bias [13]. Additionally, CpG motifs in the plasmid DNA activate TLR-9 on plasmacytoid DCs, stimulating them to produce type I interferons and activate NK cells [14]. Chemical adjuvants such as alum are also often employed to increase immunogenicity by creating an “antigen depot” to slow the release of antigen [15]. The administration of the vaccine in a “prime-boost”
strategy by following the DNA vaccine with a viral vector vaccine is another popular and effective technique [13].

Additionally, dendritic cells (DCs), a subset of the APC population, have been shown to mature upon binding of an immune complex to its Fc receptor, after which the DC presents the antigen on both MHC Class I and Class II [16]. The DC is capable of stimulating both CD8$^+$ and cognate helper CD4$^+$ T cell responses. APCs express higher levels of costimulatory molecules than the average cell, making them better able to activate CD8$^+$ and CD4$^+$ T cells. In light of this, as well as the cross-priming phenomenon exhibited by DCs, many of the plasmid DNA vaccine optimization strategies in current studies involve targeting the DNA to APCs and/or promoting an immune response which is more cellular rather than humoral.

One such study uses a targeting protein from Reovirus to ensure that mucosally administered DNA is targeted to M cells which deliver it to the subepithelial space; an area of high immune cell concentration [17] [18]. Another used bacterial “ghosts” derived from Manheim hemolytica to target encased plasmid DNA to APCs (via the previously described exogenous pathway) and serve as natural adjuvants [19].

One important study described a method to increase targeting of a DNA vaccine to DC by inoculating mice with a plasmid encoding an antigen-Fc fusion protein [20]. Importantly, this fusion protein, once secreted, was taken up by DC via receptor mediated endocytosis and caused cross priming of the antigen. Typically, material obtained exogenously is presented on MHC Class II molecules for induction of a Th2 response. Cross priming is a phenomenon observed in DCs whereby exogenous antigens presented in certain contexts can be presented on MHC Class I molecules for the stimulation of a
CD8⁺ T cell response (Figure 6). Many postulate that DC cross-priming may be an important part of the mechanism by which DNA vaccines work to stimulate CTL responses [21]. The cross-priming pathway gives the plasmid DNA access to the cytoplasm for transcription, translation and processing of antigen though the MHC Class I endogenous pathway. Seeing the success of this targeting strategy along with evidence of the DC's importance in DNA vaccine success validated our own, which is, in essence, a more direct use of Fc receptor mediated uptake of a vaccine into DCs.

The study described in this paper represents preliminary data that suggests that the addition of the anti-DNA antibody MRSS-1 to the pcDNA-gB vaccine improves its immunogenicity. It can be said that all the data seems to point to an increased ability to prime T cells in vitro, a more robust CTL response, and a larger number of IFN-γ producing cells when MRSS-1 is added to the vaccine. Further studies need to be done in order to fully characterize and quantify the apparent improvements. It is possible that the effect seen is not due to more effective targeting, but to the maturation effects that immune complexes have on DCs [16]. RT-PCR could be performed on DCs in vitro to show that expression of the gB protein is increased when the pcDNA-gB is administered as an immune complex with MRSS-1. All the measurements of immune response shown here should be repeated at a later time point to examine memory responses. Additionally, the vaccine should be tested in a homologous boost and heterologous prime-boost regimen. Finally, the best test of any vaccine is a live virus challenge experiment. This will help to insure that the response generated is strong enough and of the correct type to be protective against infection. This will also allow for more evaluation of the long-term safety of the formulation.
If the vaccine were to be used in clinical veterinary or human practice, the formulation would need to be revised to use a sequence specific antibody to avoid the autoimmunity concerns typically associated with anti-DNA antibodies, which can cause diseases such as systemic lupus erythematosus [22]. A suitable antibody is already available; the Prat-Gay group has developed a hybridoma which produces antibodies which bind only a specific DNA sequence from HPV [23]. This sequence could be added to the plasmid DNA vaccine to give an epitope/ binding site for the antibody. As this sequence is not present in mammalian DNA, these antibodies should display no cross-reactivity with self DNA. The safety of DNA vaccines in general is still under review, with some believing that the injection of plasmid DNA into muscle leads to autoimmunity mediated by anti-DNA antibodies [24], where others feel that the administration of plasmid DNA does not cause autoimmunity, but does worsen the effect in those individuals predisposed to autoimmune disorders [25]. This study, and DNA vaccines in general, possess genuine potential, but pose difficult questions as well, which must be answered before these technologies can be useful to human or animal health.
Appendix: Figures
Figure 1. Schematic representation of plasmid DNA/anti-DNA antibody vaccination approach
Figure 2. MRSS-1 antibody binds plasmid DNA (pcDNA-gB) in a dose dependent manner. The negative control antibody does not.
Adherent Splenocytes loaded with:       IL-2 (pg/ml)

pcDNA-gB (0.5 mg/ml)                      16

pcDNA-gB (0.5 mg/ml) + MRSS-1 mAb (0.5 mg/ml)  48

pcDNA-gB (0.5 mg/ml) + mouse IgG (0.5 mg/ml)  15

MRSS-1 mAb alone                         17

Lipofectamine + pcDNA-gB                  1005

gB498-505 (1 mg/ml)                       1553

Figure 3. MRSS-1 antibody bound to pcDNA-gB activates a T cell hybridoma specific for gB498-505 (SSIEFARL).
Figure 4. MRSS-1 bound pcDNA-gB increases the number of IFN-γ secreting cells during the acute phase response as measured by ELISPOT. The difference between the number of IFN-γ secreting cells stimulated by pcDNA-gB alone versus pcDNA-gB + MRSS-1 is statistically significant.
Figure 5. CTL Activity is enhanced in the spleen of mice immunized with pcDNA-gB and MRSS-1 anti-DNA antibody
Figure 6. Schematic representation of the cross-priming pathway in dendritic cells
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