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The Effects of Physical and Chemical Modulating Agents on the Induction of Vaccine-Induced Immune Responses

Andrea Nicole Ryan
University of Connecticut - Storrs, andrea.ryan@huskymail.uconn.edu

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ABSTRACT

After the development of the viral-based prostate cancer vaccine, Ad5-PSA, much research has been orientated to help enhance the induced immune response by combining the vaccine with physical and chemical modulating agents, more specifically the polymers polyethylenimine (PEI), chitosan, and chitosan coated with CD3 complex antibodies; all previously shown to stimulate an immune response as isolated gene carriers. To compare the vaccine-induced immune responses between the naked vaccine and the polymer-vaccine combinations, a mouse model using the ovalbumin- specific Ad-OVA vaccine was tested using intracellular cytokine staining (ICS), tetramer staining, and cytotoxic T-cell lymphocyte assays to measure the activation of CD8+ T-cells, interferon gamma proteins (INFγ), and the induced cytotoxicity to ovalbumin. The Ad-OVA vaccine combined with both chitosan and chitosan with CD3 complex antibodies, both natural polymers, were found to induce similar immune responses to the naked vaccine while the vaccine combined with the synthetic polymer, PEI, diminished the immune response.

INTRODUCTION

History of Cancer. Although not yet identified as the epidemic we know today, ancient fossil and mummies pre-date the existence of cancer to ancient Egypt in approximately 1600 B.C. It was not until 400 B.C. that the “Father of Medicine” Hippocrates coined the term cancer, referring to the crab-like extensions from the disease. Labeled as “incurable” in ancient manuscripts, the

doctors of the Renaissance were the first to suggest that surgery may be effective if the tumor was “movable.” The development of pathology of the 19th century first allowed causes of death to be associated with the pathologic state of the corpse, opening the door for numerous theories on the cause of cancer. With the 19th century contributing anesthesia and the use of X-rays and the 20th century providing the discovery of the actual composition and structure of DNA, cancer can no longer be presumed as incurable, with numerous treatments available including radiation therapy, chemotherapy, and hormone therapy discussed below [39].

Current Treatments. Radiation therapy has been used since its development in the 19th century as a local therapy against cancerous areas of the body. Using high energy rays to damage DNA, reproducing cancer cells are killed due to the high frequency of their replication. Although induced DNA damage also targets normal cells, they are able to repair DNA damage due to the much slower growing cycle than that of cancer cells. Major side effects are related to the area of exposure including skin reactions, hair loss, fatigue, and nutritional problems, but gradually lessen after treatment is complete. Radiation treatment is normally used in combination with other types of cancer treatments [35].

Perhaps the most common method of cancer treatment, chemotherapy uses alkylating agents or other toxins to destroy rapidly dividing cancer cells in the body by destroying their DNA. Known as systemic therapy, these agents are able to travel and target cancer throughout the body after administration either

orally, intravenously, directly in a targeted body cavity, or intra-arterially. Since these toxins are unable to distinguish between cancerous and healthy cells, side effects commonly occur in other normal body cells that proliferate frequently such as in the hair, skin, and digestive tract [38].

After it was observed that removal of the ovaries severely decreased the onset of breast cancer in women, hormone therapy became widespread treatment for cancers such as breast and prostate cancer, which are highly dependent on the amount of estrogen and testosterone in the body, respectively [39]. This therapy blocks hormones from acting on cells, prevents the host from producing the hormone, or eliminates the receptors for the hormone on the host cell. Because the effects are widespread, this treatment is also considered systemic with drawbacks mainly caused by the lack of hormone in the body. Women under estrogen therapy experience menopause symptoms while men under testosterone therapy experience hot flashes, nausea, a loss of sex drive, and in severe cases, impotence [36].

Perhaps the most modern treatment after the development of molecular biology in the 20th century, gene therapy targets the mutations in the host genes that contribute to the development of cancer. Targets for this therapy vary as some replace missing or malfunctioning genes, such as the “tumor suppressor gene,” while others hope to use the host immune system to trigger an attack on a gene inserted into the body. To efficiently transport these genes, vectors must be used, such as deactivated viruses unable to replicate within the host. The most common side effect of such therapy is the body’s rejection to this foreign

vector-gene system causing possible fever, decrease in blood pressure, vomiting, and headache [37]. It is this type of therapy that the remainder of this paper focuses on, combining the prostate specific antigen (PSA) gene with a deactivated adenovirus and other polymer vectors in hopes of mounting an immune attack against prostate cancer.

Prostate Cancer. Cancer is defined as a malignant, invasive growth caused by abnormal cell division, most likely occurring after excision of a primary tumor and traveling (metastasizing) to other sites in the body [5]. In the United States, prostate cancer is the most common cancer and the second deadliest cancer among men. The American Cancer Society predicts that in 2008, 186,320 men will be diagnosed and, of those diagnosed cases, 26,660 will prove lethal [2]. While the average age of patients suffering from prostate cancer between 1973 and 1987 was 67 years old, the incident of the disease increases drastically at approximately 50 years of age, with 1 in every 4 men showing symptoms of prostate cancer [3]. While no known causes have been identified, approximately 5-10% of cases are predicted to have a strong inherited component, with over 90% of all cancers observed to contain some type of genetic mutation [4, 5]. Ethnicity plays a minor role with twice as many African American males suffering from the disease compared to European-decent, and Asians, American Indians, and the Latino communities contributing the lowest incidence [4].

Disease Progression. Prostate cancer progresses very slowly through various phases: prostatic intraepithelial, neoplasia (tumor growth), localized,

metastases, and finally to hormone refractory [5]. If diagnosed early with the cancer localized in the prostate, treatment options include: radical prostatectomy (removal of the prostate), external radiotherapy, brachytherapy (otherwise known as interstitial radiotherapy), and prolonged observation and surveillance of the disease progression. While successful prostatectomy should result in undetectable prostate-specific antigen (PSA) levels after 3 weeks, 5 years post operation have shown rapid increase in PSA levels in 15-40% of all cases. Of those patients diagnosed with advanced or metastasized prostate cancer, anti-testosterone therapy and other hormone therapies remain the only option [8]. Removal of the prostate is not suggested at this level of diagnosis due to the increased chance of metastasizing the cancer. No other treatment options exist once a tumor becomes hormone refractory and no longer responds to hormone therapy [6] and once metastasized, the cancer is ultimately incurable due to uncontrollable tumor growth [7].

Prostate-Specific Antigen (PSA). Due to the lack of cancer treatment available once tumors become androgen-independent or metastasized, much research has been dedicated to the development of a novel immunotherapy, able to attack prostate cancer both localized to the prostate and throughout the body. As mentioned previously, prostate-specific antigen (PSA) is a single-chain glycoprotein secreted by both healthy and cancerous epithelial cells in the prostate gland [7]. Due to its delectability at low qualities in adult males, serum PSA is the most commonly used marker for prostate cancer malignancies, with research being conducted currently to induct a doubling-time test as a more

appropriate gage compared to the current static marker of PSA levels [9]. PSA expression increases from benign prostate epithelium tissue to cancerous tissue, with the intensity of PSA expression being directly proportional to the malignancy of the cancer [3, 8]. Yet, since the expression of PSA is limited to the prostate and since the organ, responsible for liquefying seminal clot after ejaculation, is nonessential for survival, PSA exists as the best candidate for targeted immunotherapy of prostate cancer [4, 8]. Unfortunately, the tissue-specific (not tumor-specific) self-antigen remains poorly immunogenic due to the host immune tolerance, with studies showing PSA-specific CD8+ T-cells exist in the body regardless of the presence of cancer [4].

Immunotherapy. The main challenge of prostate cancer immunotherapy is to override the host tolerance to PSA. Gene therapy aims to introduce an effector gene (PSA) that can stimulate greater amounts of CD8+ T cells producing cytotoxic T lymphocytes (CTL) and interferon γ (IFNγ) activity to help control prostate cancer progression [5]. Prostate tumor cells have low antigenicity (ability to stimulate and interact with antibodies), which suggests that MHC class I tumor-associated antigen evasion is critical for the tumor growth. Since naked DNA is immediately degraded by the host immune response, the aid of gene delivery vectors and adjuvants are required to ultimately transfer recombinant PSA-DNA into human cells to achieve an anti-tumor, vaccinated effect.

Viral Gene Vectors. Classified as viral or non-viral, the ideal vector must be highly specific to the target cell with efficient DNA delivery, remain nontoxic,

non-immunogenic, non-mutagenic, and for economic purposes be cheap and easily manipulated *in vitro* [10]. Due to their high efficiency of DNA transfer, viral vectors such as retroviruses, adenoviruses, vaccinia, and herpes simplex viruses are used in the majority of therapy research. Adenoviruses offer many advantages such as their easy manipulation *in vitro*, the infection ability in both dividing and non-dividing cells, and the lack of DNA incorporation into the host genome, by-passing the previously mentioned concerns of mutagenicity. Adenoviruses also offer no viral proteins and therefore limit the viral immune response [8, 10]. With high affinity attachment to cell surfaces, adenoviruses can internalize and translocate into the nucleus with protein expression detectable within 6 hours post injection [11].

Non-Viral Gene Vectors. Non-viral gene vectors can be synthetic or natural vehicles for the protection and introduction of DNA into a target cell [11]. Benefits include but are not excluded to: capacity to target specific receptors without undesired attachment, an unlimited DNA-cargo capacity, and the potential to minimize the host immune response. Popular non-viral vectors include polycation complexes, whose positively charged backbone allows easy interaction and condensing of the gene, which can be transported to into the nucleus more efficiently. The neutralization of the overall charge can help enable binding to specific target cells without interacting with host systemic clearance mechanisms, such as macrophages. Disadvantages of non-viral vectors include high toxicity, poor storage stability *in vivo*, and much lower transfer efficiency than viral-based vectors. Such vectors include polyethylenimine (PEI), chitosan,

and the coating of polymers with CD3 antibodies, each of which will be discussed in greater detail later in this report.

Ad5-PSA Vaccine. In the late 1990's, scientists at the University of Iowa developed a PSA-recombinant, replication-deficient viral vector, the Adenovirus5-PSA (Ad-PSA), that generated strong PSA-specific anti-tumor immune responses in a prostate cancer mouse model [7, 16]. Directly proportional to the antigen-specific tumor destruction [5], CD8+ T cells were identified as the primary effector cells, capable of challenging subcutaneous injections of prostate cancer cells but unable to act on pre-existing mouse tumors [7, 14]. Phase I clinical trials, mainly responsible for determining the safety, dosage, and effectiveness of the vaccine, were a great success in patients with hormone refractory prostate cancer [5]. Research was further expanded in 2006 as the effective Ad-PSA was first combined with known adjuvant CpG-ODN to increase the tumor protection of prostate cancer. With further support of the enhancement of CD8+ T cell cytotoxic T lymphocyte (CTL) activity of the PSA-bearing tumor cells, both *in vitro* and *in vivo*, this study stimulated further research into a hybrid vector of the adenovirus and a non-viral adjuvant [12]. In order to model the prostate cancer on transgenic mice, the recombinant type-5 adenovirus carrying the ovalbumin (Ad-OVA) gene was created [13]. Unfortunately, the major factor restricting the success of the adenovirus as a gene vector is the host neutralizing anti-adenovirus antibodies, since most humans and animals have been exposed to the virus, mainly due to its collaboration with the common cold [15].

To help increase the half-life of the viral vaccine by avoiding neutralizing host antibodies, it was proposed to coat the vaccine in polymers as protection [16]. Research on hybrid vectors consisting of PEI/DNA combinations further encompassed by the adenovirus vector have unfortunately been halted by the same host immune obstructions observed in viral vectors [11]. Therefore, the focus of this study was to observe the stimulation of OVA-specific CD8+ T cells using the Ad-OVA vaccine, coated with the three polymers listed previously: PEI, chitosan, and chitosan coated with CD3 antibodies (chito@CD3).

Barriers in Gene Delivery. Known barriers to non-viral gene transfer include: i) surviving the extracellular matrix before targeting the cell, ii) delivery to the cytoplasm from the endosome, iii) transversing the cytoplasm to the nucleus, and iv) disassociation from the DNA for transcription [17, 22, 23]. Starting in the extracellular matrix, the positive charge of most non-viral vectors can accumulate and interact with non-specific targets such as red blood cells and other organs prior to target contact [23]. DNA must stay well protected and compacted from enzymes and DNA nucleases. Upon contacting the targeted cell ligand, the polyplex must enter by endocytosis, a fete determined by size, hydrophobicity, polymer type, and surface charge [18], which progresses into a late state before fusion with acidic lysosomes and degradation. Therefore, the vector must be able to escape the endosome to avoid further degradation after lysosome fusion. The vector must then transverse the cytoplasm to the nucleus and travel across the nuclear pore complex while disassociating from the contained gene [11, 17].

The following three polymers have been identified as potential non-viral vectors due to described characteristics overcoming some of the barriers listed above.

PEI. Polyethylenimine (PEI) is a polycationic, synthetic polymer that has had success both *in vivo* and *in vitro* with gene delivery as a plasmid carrier [19]. Due to the positive charge of the polymer, PEI is able to compact negatively-charged DNA very tightly, allowing easy protection and transversion through the extracellular matrix and the cytoplasm. Since the DNA phosphate backbone provides an overall negative charge, the PEI/DNA complex as a whole allows a nearly neutralized charge, avoiding attachment to red blood cells and untargeted organs extracellularly. Due to the primary, secondary, and tertiary amine groups repeated throughout the PEI backbone, PEI acts as a pH buffer and allows lysis of the endosome by becoming a “proton sponge” [21, 22, 24]. At neutral pH, PEI captures protons moving into the endosome causing chloride ion influx, ultimately causing osmotic swelling, bursting, and escape from the endosome [22, 23]. Therefore, the PEI/DNA complex is able to escape lysosome degradation and move through the nuclear pore complex with its tightly compacted carrier plasmid [19]. Unfortunately, high transfection at large molecular weight PEI corresponds to high toxicity due to adaptive immune responses to free polymers at high concentrations and is limited by low transfection efficiency compared to viral transfection, due to mitotic activity required for gene uptake [23, 25]. Linear PEI resulted in higher viability and transfection over branched PEI [21] and was therefore used in this experiment.

Chitosan. A natural cationic polysaccaride, chitosan is derived from chitin, found in the exoskeletons of crustaceans such as crawfish and insects [17, 28], and became popular in pharmaceuticals due to its biocompatibility, non-toxicity, and biodegradability confirmed *in vivo* in mice models [26, 27]. The high viscosity and positive charges form strong interactions with negatively-charged residues, and have been found to increase the half-time of antigen clearance, allowing more exposure *in vivo* [27]. Compared to PEI, chitosan contains limited buffering capacity but also minimal cytoxicity. Instead of becoming a "proton sponge", at physiological pH, chitosan becomes insoluble, ensuring that the chitosan/DNA complexes created at low pH remain physically stable without chemical crosslinking, and giving physical protection from nuclease attack [29]. Chitosan has been applied to vaccines in a powder form with success facilitating immunization through nasal administration [18] and, in combination with a hydrogel, chitosan has enhanced tumor-specific CD8+ T cell immunity post vaccination [26]. Due to its high retention of antigen, chitosan has been labeled an ideal vaccination scenario targeting the sight of inflammation while generating an adaptive immune response [28].

CD3 Complex Antibodies. The CD3 complex consists of glycoproteins linked to the antigen receptors exclusively on T lymphocytes [31, 33]. CD3 antibodies have been used to activate peripheral T cell independent of antigen presentation [30]. Depending on the cell-line, the incorporation of a CD3 ligand has resulted in a 1,000 fold increase in transfection efficiency [23]. It has been demonstrated that CD4 T cells activated *in vitro* by CD3 antibodies were able to

generate antigen-specific memory CD4 T cells in antigen-free adoptive hosts, even after prolonged absence of antigen exposure [30]. INFγ was also produced in high quantities in adoptive hosts. CD3 antibodies have also been found to stimulate effector CD4+ T cells to suppress both proliferation and cytokine production in target prostate cells [32]. In other studies, antiCD3 molecules have had success with initial activation through the cell cycle, but only in the presence of accessory cells [31]. Yet, other results indicate that CD3 antibodies do not initiate CTL lysis but rather inhibit target-cell recognition in the absence of Fc receptors; this indicates that the CD3 complex does not play a role in the initial killing of the target cell but acts in the CTL recognition of the antigen [34]. This study uses the antibodies to target the vaccine to the CD4 T cell to stimulate an adaptive immune response, along with the protection characteristics already listed for polymer chitosan.

Immune Cells Targeted. Intracellular staining, tetramer staining, and cytotoxic T lymphocyte (CTL) assays were performed to quantify the immune response for each vaccine group. In the intracellular staining, rat IgG2a-FITC labeled anti-mouse CD8a antibody was used to stain cells for the CD8 surface marker and rat IgG1-PE labeled anti-mouse IFNγ was used to stain for intracellular IFNγ expression [6], mainly to identify an inflammatory response to the vaccine. MHC:peptide tetramer complexes are stained to identify that the response has been antigen-specific since tetramer complexes display a distinct epitope to the immune system. Both sets of staining were analyzed using flow cytometry (FACS), which causes the dyed cellular receptors to excite and

fluoresce as they pass through the laser light and are scattered [1]. CTL assays were used to identify the ability of the stimulated T lymphocytes to kill targeted cells producing PSA. By injecting target cell lines, one producing PSA and a negative control, with Cromium-51 isotope and incubated with stimulated T cells, the amount of lysed target cells can be measured by the amount of radiation released using a gamma counter [1].

MATERIALS AND METHODS

Animals. C57/BL/6 (H-2b) mice, approximately 6 to 8 weeks of age, were purchased from National Cancer Institute (Bethesda, MD). All protocols were in accordance to regulations and recommendations by UIACCU. The mice were maintained in filtered cages at the VA Animal Research Facility before and during use.

Vaccines and vaccination. C57/CL/6 mice were injected with doses of adenovirus-OVA, adenovirus-LacZ (containing a gene encoding for β-galactosidase), polyethylenimine, chitosan, chitosan coated with CD3 antibodies, the adenovirus-OVA combined with each of the above polymers, and the adenovirus-LacZ combined with each of the above polymers; eleven groups consisting of two mice per group were vaccinated. The mice were injected with a dose of 10^8 pfu of the vaccine; Ad-OVA and Ad-LacZ were created in the method explained in Elzey et al. [7] and were obtained from the University of Iowa Gene Vector Core. The adenovirus and polymer vaccine combination were prepared

by the pharmacology lab of Dr. Aliasger Salem and Dr. Zhang Xueqing at the University of Iowa. Each combination was mixed prior to vaccination.

Spleen Harvest, Processing, Staining. The spleens of mice were obtained from immunized mice 14 days post vaccination. Two mice were sacrificed in order to obtain two spleens per group.

In order to the harvest the spleen, the mouse was sacrificed using carbon dioxide gassing. Placed on its right side, the fur was wetted with ethyl alcohol and a 2 cm incision with scissors was made through the fur, with the fur pulled back using forceps. Another small incision was made in the inner layer of the skin and the spleen was identified by the dark, elongated, flat characteristic of the organ located on the left side of the mouse. The spleens from each group were placed in 5% FBS/PBS.

After the spleen was harvested, it was grinded used frosted slides into single-cell suspensions. The suspensions were washed, centrifuged at 150 x g and suspended in 1 ml of ACK buffer for one minute, the latter for the lysis of contaminating red blood cells. The cells were again washed and centrifuged with the cell suspensions ran through a cell strainer and again centrifuged. The cells were resuspended in 10 ml of 5% FBS/PBS, counted, and 2 x 10^7 cells were placed into 96 well round-bottom plates and 24 well flat-bottom plates used for the intracellular staining and the cytotoxic T-lymphocyte assays, respectively.

The cells were centrifuged and were incubated for 15 minutes at 4 C with Fc-block to reduce the non-specific adherence of antibody molecules. Following a wash the cells were respectively stained respectively with rat IgG2a-FITC

labeled anti-mouse CD8a for CD8 T-cells, PE-Cy5 for CD3 T-cells, and rat IgG1-PE labeled anti-mouse IFNγ for INFγ staining. Each group contained unstimulated, SINFEKL (American Peptide Company; Tetramer antibody), and P/I (15 μL PMA/ 15 μL Iomycin/ 70 μL PBS; positive control group), all stained with the antibodies listed above.

The CTL assay plates were set up with 500 μL target cell line, 100 μL spleenocytes, 500 μL IL-2 (Grant; to allow lymphocytes to remain viable), and 900 μL CTL media. After 5 days incubation, Crominum-51 was added, the wells serially diluted, and the supernatants were evaluated using a COBRA™ II, Auto-gamma counter (Packard Instrument Company, IL).

For the tetramer assay, each group was stained with the PE-Cy5 and FitC antibodies listed above, incubated for 30 minutes with tetramer (Beckman Coulter), and then fixed with PFA. After the above preparation, the ICS and tetramer plates were analyzed using FACS discussed above.

Antibodies. All antibodies were purchased from eBiosciences and BioLegend.

Flow Cytometric Analysis. Flow cytometric analysis was performed collecting 1 x 10^5 events and data were analyzed with Flow Jo 6.4 (Tree Star, Stanford) software.

RESULTS

Expression of Antigen-Specific INFγ+CD8+ T-Cells. In an effort to first understand how each vaccination group differed in their immunological response elicited by the respected adenovirus-OVA (Ad-OVA), adenovirus-LacZ (Ad-LacZ), or in combination with polyethylenimine (PEI), chitosan, or chitosan coated with CD3- antibodies (chito@CD3), spleen lymphocytes (2 spleens per group) 14 days post vaccination were stained for OVA-specific IFNγ+ CD8+ T-cell. Interferon γ is a protein synthesized post-viral infection and so-named due to its interference in the viral reproduction. Interferon γ is unique from interferons α and β, since it is not directly induced but produced later as a major contributor in the adaptive immune response to foreign pathogens [1].

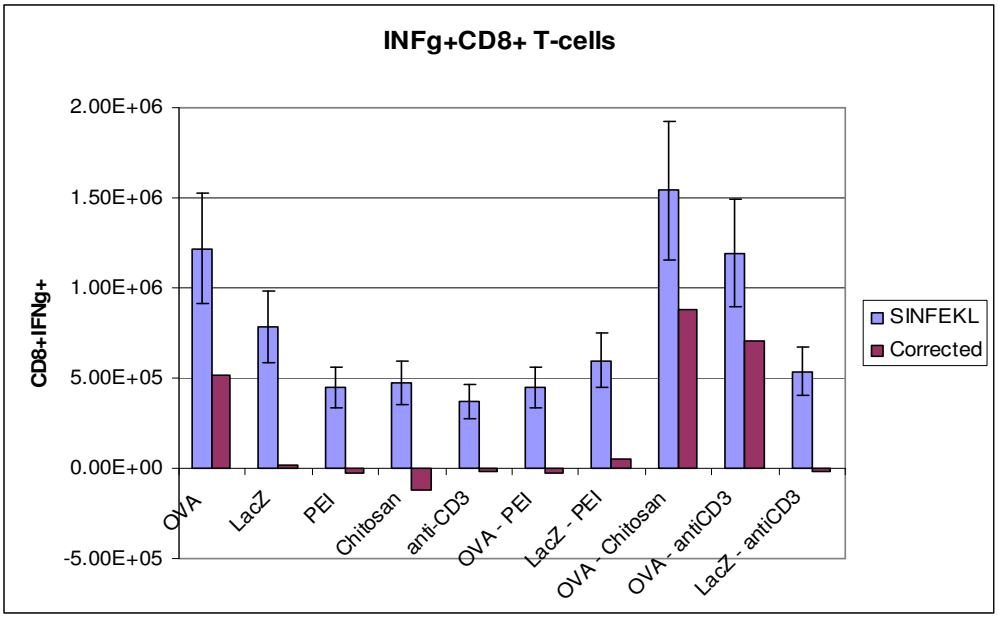

Figure 1. CD8+INFg+ T-cells activated by the vaccinated groups.

Using flow cytometric analysis to identify the dual positive T-cells for each group, Figure 1 was generated to identify the overall trend of every vaccination group.

Using the SINFEKL as the OVA peptide-specific antibody, the number of stimulated T-cells was corrected by subtracting the unstimulated stained T-cells from each group to give an accurate depiction of the adaptive immune response. Using LacZ as a negative control to the OVA-induced immune response, the Ad-OVA vaccine elicited a greater adaptive response compared to the LacZ. It should also be noted that the PEI, chitosan, and anti-CD3 chitosan polymers individually produced no INFγ+CD8+ T-cell immune response compared to that generated by the negative control, suggesting no adjuvant effects. Error bars of ±25% were included in Figure 1 to compensate for the different in stimulated T cell values of the two spleen harvests and staining assays done in duplicate for this experiment.

To further analyze the selected hybrid vectors of Ad-OVA and polymer, Figure 2 includes only the OVA, LacZ, and the OVA combination with each polymer.

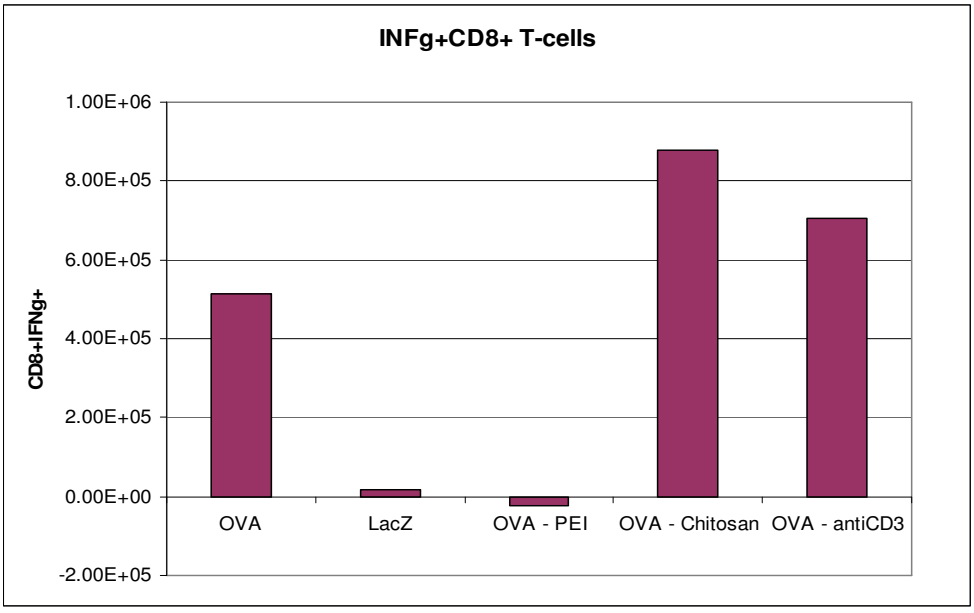

Figure 2. INFγ+CD8+ T-cells activated by each OVA-specific group.

From the graph, it can seen that Ad-OVA, OVA-Chitosan, and OVA-Chito@CD3 all generated INFg+CD8+ immune responses, with OVA-chitosan contributing to more than double the amount of activated T-cells than the Ad-OVA vaccine alone. Ad-OVA combined with PEI actually gave a diminished immune response, stimulating less T-cells than the Ad-LacZ vaccine and less than the unstimulated T-cells stained, as identified by the negative number above. Since IFNγ induction is post-viral infection, it is probable that the synthetic PEI polymer was destroyed and filtered through the body before the adenovirus-OVA could be exposed to elicit the stained immune response.

It should be noted that due to ulcerations at the injection sites, the mice injected with the LacZ-chitosan combination were sacrificed early and could not be used for comparison in this report. Therefore, a possible false positive of OVA-chitosan by contamination must be acknowledged as a possible cause of the stimulated immune response.

Expression of Antigen-Specific Tetramer+CD8+ T-Cells. To compare the immediate immune response elicited by each vaccination group, spleens lymphocytes 14 days post-vaccination were stained for the OVA-specific MHC Class I Tetramer.

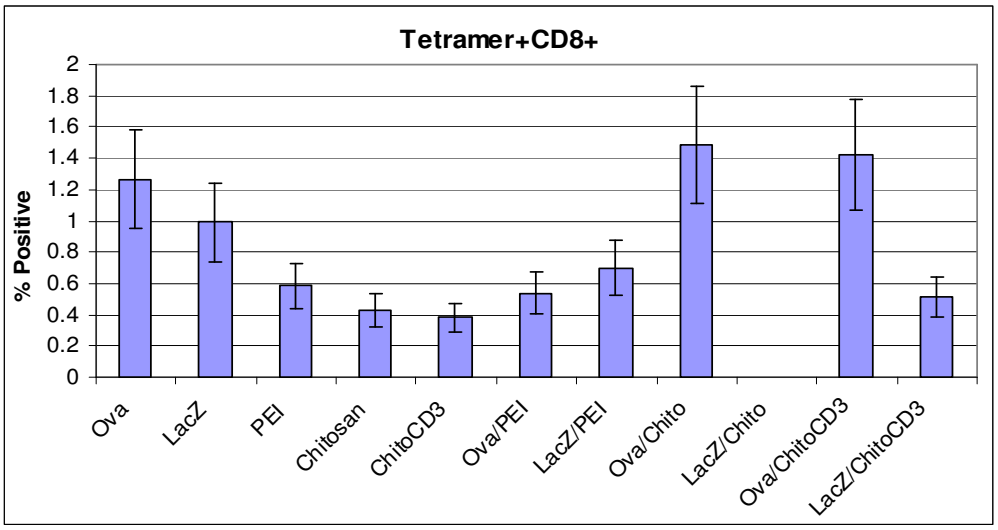

Figure 3. Tetramer+CD8+ T-cell activation for all vaccinated groups.

Figure 3 shows similar immune response to the intracellular cytokine staining for INFγ above. Since the assays were done in duplicate, error bars are provided at $\pm$25% to attribute to the discrepancies between trials. Identical to the INFγ staining, OVA, OVA-Chitosan, and OVA-Chito@CD3 all stimulated OVA-specific tetramer positive responses, with each individual polymer group resulting in comparably low percentages, implying they are not immunogenic. Although Figure 3 provides visual representation of the three groups providing the greatest stimulation, with the OVA-chitosan group providing only 0.5% higher positivity than the negative control, Ad-LacZ group, the immediate immune response after 14 days vaccination gives comparably weak immunization for the OVA-specific antigen.

Activation of OVA-specific peptide:MHC class I complex cytotoxic T-cells. To test the OVA-specific cytotoxicity, spleen lymphocytes 14 days post-vaccination were serial diluted with two cells line: EL4, negative control cell line, and EL7, producing ovalbumin proteins.

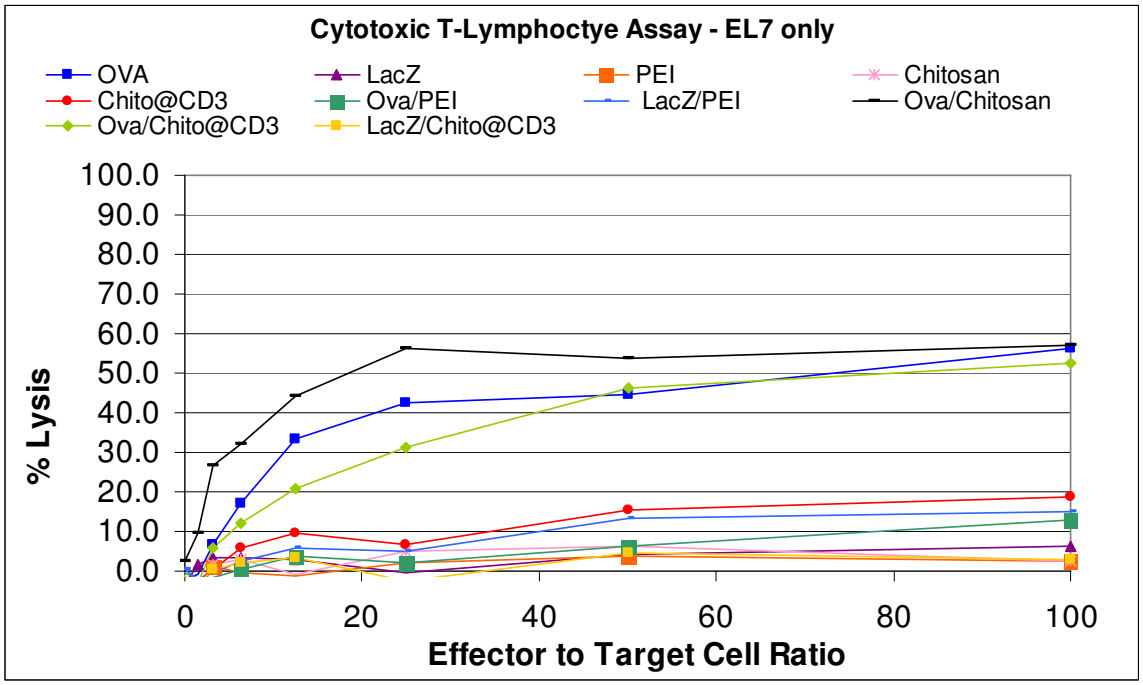

Figure 4. Activation of OVA-specific peptide:MHC class I complex cytotoxic T-cells.

Figure 4 shows the progression of cell lysis as the effector cells (spleen lymphocytes) become denser compared to the target cell line (EL4 or EL7). Concentrating on the top three data series belonging, from top to bottom at 20 E/T ratio, to OVA-Chitosan, OVA, and Ova-Chito@CD3, each series tended to peak between 50-60% lysis and began to plateau at 20 E/T ratio. Figure 4 recognizes that not only does the Ad-Ova vaccine and polymer combinations above elicit an adaptive immune response *in vivo*, they are able to directly lyse antigen-specific cells when combined *in vitro*.

Yet, compared to assay trials non-specific to the scope of the report, a 60% lysis is a weak CTL response in comparison to the +80% lysis resulting from previous experiments with the Ad-OVA vaccine. This suggests that 14 day immunization may not allow enough proliferation of antigens-specific cytotoxic T lymphocytes. It should be noted that the duplicate trial for this assay was contaminated during preparation and could not be used for comparison in this report.

DISCUSSION

Administration with the Ad-OVA vaccine has shown to stimulate an immune response against ovalbumin in a mice model, yet the viral vaccine alone was not able to actively destroy antigen-specific prostate tissues without necessary accessory cells [13]. PEI has been proven to efficiently condense DNA and transfect into target cells, yet at lower transfection efficiency than the viral vectors [14]. PEI also has high toxicity directly related to high transfection efficiency [23, 25]. Chitosan, a natural polysaccharide, has been proven to also effectively deliver DNA to the target nucleus with lower toxicity and immunogenicity, but also lower effciency than the viral vectors [26, 27]. CD3 antibodies have been used previously in conjuction with PEI to target the CD3 complex unique to CD4 T cell, creating greater specificity and initiating proliferation into the cell cycle [23]. In this experiment, we investigated the possible adjuvant effects and short-term protection offered by each polymer complex to the vaccine-induced immune response using the Ad-OVA vaccine.

The results of this experiment show that each polymer complex individually offers no adjuvant effects to the stimulated immune response offered by the vaccine. Referring to Figures 1 and 3 above, the mice injection with only PEI, chitosan, and chito@CD3 did not stimulate the production of CD8+INFγ+ T cells or the antigen-specific tetramer complex compared to the negative control of the Ad-LacZ vaccination groups. These results suggest that without the ovalbumin gene present *in vivo*, even in the presence of foreign microparticles, the immune system is not able to stimulate an antigen-specific immune response.

Yet, in combination with the Ad-OVA vaccine, chitosan and chito@CD3 were able to both generate CD8+IFNγ+ T cell, CD8+Tet+, and cytotoxic T lymphocyte acitivity specific to the ovalbumin protein, while the combination of the vaccine with PEI actually diminished the immune response compared to the vaccine alone. Referring to Figure 2 above, chitosan provided the most effective protection and transport for the Ad-OVA vaccine, followed by the chito@CD3 complex. Although the chito@CD3 was designed as a highly specified polymer with T cell ligand specificity, it is possible that the interaction between antibody and CD3 complex inhibited further recognition by the vaccine instead of stimulating proliferation, which is consistent with other research [34].

Figure 3 mirrored the results found using intracellular staining, with chitosan and chito@CD3 both producing the stimulated of class I MHC:OVA-specific tetramer complexes on CD8+ T cells. Cytotoxicity was also activated by

the chitosan and chito@CD3 polymer combination with Figure 4 showing 50% target cell lysis at a 50:50 effector to target cell ratio.

The diminishment of immune response using PEI was mainly attributed to high toxicity and rapid clearance from the body. Because PEI was interesting with a neutral-charged adenovirus rather than directly with the negatively-charged DNA, it is possible that the overall positive charge caused interaction with other organs and molecules extracellularly, causing the polymer, along with the Ad-OVA inside, to be cleared from the host before the DNA was able to be transfected and an immune response mounted.

CONCLUSION

Although this experiment resulted in vaccine-induced immune responses to ovalbumin by combining the Ad-OVA vaccine with both chitosan and chito@CD3 polymers, it is unclear at what point in the transport from extracellular exposure to nuclear transcription the polymer aided in the transfection of the viral DNA. Since the polymers themselves were proven to have no adjuvant effects independent of the vaccine, it could be suggested that their main aid comes in protecting the virus from the harsh environment and from mounted host-immune tolerance. Since each vaccination group was sacrificed after 14 days, a time course study should be the next step in developing the effects of hybrid vector gene delivery systems. By sacrificing two mice from each vaccination group 7, 14, 21, and 28 days post vaccination, it will allow the assays to acknowledge

whether the polymer provides prolonged exposure and protection for the Ad-OVA vaccine or if another form of assistance was involved.

FUTURE DIRECTION

Although the polymer-vaccine combinations did not produce an increased immune response, it is possible that such polymers may contribute extended protection to the vaccine from immune attack, allowing longer exposure of the vaccine and possibly increasing its success over time. After the results of this experiment have been replicated and verified that the polymers indeed contain no adjuvant effects themselves, a time-course study should be pursued, sacrificing mice at staggering intervals such as 7, 14, and 21 days to observe how the induced immune response to PSA changes over time. If the amount of activated T cells increases with time, it may be concluded that polymer-vaccine combinations are successful in sustaining the vaccine in the harsh environment inside the host and should be further pursued in clinical trials. If such combinations of polymers and vaccines resulted in greater response to the Ad-PSA vaccine, our generation may see the decline of chemotherapy toxins and radiation exposure and an increase in specific gene therapies effective as systemic treatments for metastatic cancers, without the side effects of host rejection.

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