Improvements in the Prevention and Treatment of Anthrax Infection

Dennis M. Klinman and Debra Tross

Cancer and Inflammation Program, National Cancer Institute, Frederick MD 21702, USA

Abstract

Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs mimic the immunostimulatory activity of bacterial DNA. CpG ODN are finding use as vaccine adjuvants based on their ability to increase the speed, magnitude and duration of vaccine-specific immune responses. We conducted studies examining the capacity of CpG ODN to accelerate and prolong the protection induced by AVA (Anthrax Vaccine Adsorbed). Co-administering CpG-adjuvanted AVA with Dalbavancin, a long-activity antibiotic, protected naive mice from both the immediate and long-term threat posed by exposure to anthrax spores. A majority of animals immunized only once with CpG-adjuvanted AVA maintained resistance to anthrax infection for more than one year, even after their Ab titers declined to sub-protective levels. This survival was mediated by the de novo production of protective Abs by high affinity long-lived memory B cells that were generated by vaccination with CpG-adjuvanted AVA.

Abbreviations: Ab; antibody, Ag; antigen, APC; antigen presenting cell, AVA; Anthrax Vaccine Adsorbed, AVP; Anthrax Vaccine precipitated, LD50; 50% lethal dose, ODN; oligodeoxynucleotide, rPA; recombinant protective antigen

© 2009 Elsevier B.V. Open access under CC BY-NC-ND license.

Keywords: anthrax, vaccine, adjuvant

1. Immunomodulatory properties of CpG ODN

Synthetic oligodeoxynucleotides (ODN) containing immunostimulatory “CpG motifs” interact with Toll-like receptor 9 to initiate an immunostimulatory cascade that culminates in the maturation, differentiation and/or proliferation of multiple cell types, including lymphocytes, dendritic cells, NK cells, monocytes and macrophages [1-7]. Together, these secrete cytokines and chemokines that create a pro-inflammatory (IL-1, IL-6, IL-18 and TNF) and Th1-biased (IFNg and IL-12) immune milieu [3;4;7-11]. In humans, TLR9 is primarily present within human B cells and plasmacytoid DC, while in mice multiple cells of the myeloid lineage (including monocytes, macrophages and DC) express TLR 9 and directly respond to CpG stimulation [12-14].

2. Anthrax as a biothreat agent

Bacillus anthracis is an aerobic gram-positive bacterium found naturally in wild and domesticated animals [15]. Several features of anthrax make it a “pathogen of choice” for bioterrorists seeking to cause widespread mortality, morbidity and panic [16]. The spores of B. anthracis are highly resistant to environmental degradation, allowing them to...
be transported under ambient conditions and remain infectious for prolonged periods after release [15;15]. When inhaled, these spores induce a serious often lethal infection[15;17]. Aerosolized spores can persist in the host for months before germinating, therefore posing both a long-term and immediate threat to exposed individuals [17-21].

3. Use of CpG ODN to improve vaccine-induced protection against anthrax.

Antibiotics are used to treat the infection caused when an immunologically naive (ie, non-vaccinated) individual is exposed to anthrax. B. anthracis is susceptible to a wide variety of antibiotics that interfere with the organism’s ability to replicate and evade host defenses [22;23]. Antibiotic therapy must be initiated shortly after exposure, as efficacy diminishes as toxemia progresses (in part because the bacteria’s toxin reduces immune function) [24].

By comparison, vaccination represents the most effective and least costly method of reducing susceptibility to infection [17]. In the United States,AVA (Anthrax Vaccine Adsorbed) is the sole anthrax vaccine licensed for human use. AVA is manufactured by adsorbing the culture filtrate of the attenuated toxigenic non-encapsulated V770-NP1-R strain of B. anthracis onto aluminum hydroxide [25]. AVA induces protective immunity primarily by stimulating the production of Abs against ‘protective antigen’ (PA), a critical component of the tripartite anthrax toxin. Anti-PA Abs inhibit spore germination, improve the phagocytosis/killing of spores by macrophages, and directly neutralize the toxin [26-29]. While never tested for efficacy against inhalational anthrax in humans, AVA reduced the incidence of cutaneous anthrax in wool workers and prevented disease following systemic and aerosol challenge in animals, including non-human primates [30;31] (reviewed in [32]).

AVA requires a series of 6 immunizations over 18 months to induce and maintain the production of antibodies that neutralize the PA [33]. Thus, AVA induces immunity too slowly to protect naive individuals exposed to anthrax or “first responders” dispatched to sites of anthrax infection [31;34]. Anthrax spores designed for aerosol delivery were released in the US by bioterrorists in 2001, causing morbidity, mortality, and widespread panic [16]. That event underscored the need for a vaccine that elicited protective immunity more rapidly than AVA and maintained protection without repeated boosts [16]. One strategy to achieve those goals involved adding CpG ODN to AVA. The ability of CpG ODN to promote Th1 responses and induce the maturation and activation of professional antigen presenting cells supported their use as vaccine adjuvants [35-38]. Subsequent studies established that CpG ODN both accelerated and magnified the immune response elicited by AVA [39-41].

As seen in Table I, adding CpG ODN to AVA increased the serum neutralizing titer of A/J mice by >10-fold [39]. The survival of vaccinated mice following anthrax spore challenge was also significantly improved by immunization with CpG adjuvanted AVA. In contrast, delaying the administration of CpG ODN until after AVA immunization yielded almost no booster effect, consistent with adjuvant activity requiring co-delivery with antigen (Table I). These murine findings were confirmed in studies of rhesus macaques, where co-administering CpG ODN with AVA induced a six-fold higher Ab response than AVA alone [42]. Serum from primates vaccinated with CpG-adjuvanted AVA transferred protection against anthrax spore challenge to murine recipients (Table I) [42]. A clinical trial examining the response of 69 normal healthy volunteers to 0.5 ml of AVA plus 1 mg of CpG ODN showed that the inclusion of CpG ODN significantly accelerated the induction of protective immunity and increased serum IgG anti-PA titers by 9-fold when compared to AVA alone (p < .05)[43].

Table I CpG ODN boost the response to co-administered AVA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% survival</th>
<th>TNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AVA alone</td>
<td>25</td>
<td>25 ± 7*</td>
</tr>
<tr>
<td>AVA plus CpG ODN (simultaneous)</td>
<td>80*</td>
<td>350 ± 48</td>
</tr>
<tr>
<td>AVA plus CpG ODN (delayed)</td>
<td>30</td>
<td>37 ± 6</td>
</tr>
</tbody>
</table>

A) Response of vaccinated A/J mice
B) Response of vaccinated rhesus macaques

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% survival</th>
<th>TNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AVA alone</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>AVA plus CpG ODN</td>
<td>50^*</td>
<td>420</td>
</tr>
</tbody>
</table>

A) A/J mice (12/group) were immunized once with 8 ul of AVA + 50 ug of CpG ODN either at the same time (simultaneous) or one day after (delayed) AVA delivery. Toxin neutralizing activity (TNA) was measured 3 weeks later. Mice were challenged with 60 LD50 of Sterne strain anthrax at 4 weeks [39].

B) Rhesus macaques (5/group) were immunized with 0.5 ml of AVA ± 500 ug of CpG ODN. Serum from each group of animals was pooled, and administered to naive A/J mice. Mice were challenged with 30 LD50 of Sterne strain anthrax one day later [42].

\[ p < .05 \]

4. Magnitude and duration of the IgG anti-PA response induced by CpG-adjuvanted AVA.

To evaluate the duration of the immune response induced by CpG-adjuvanted AVA, A/J mice were vaccinated and their serum IgG anti-PA titers monitored for >18 months. IgG anti-PA Abs capable of neutralizing anthrax toxin persisted at significantly higher levels in the group vaccinated with CpG-adjuvanted AVA vs AVA alone for the duration of the study (p. <.01, Fig 1) [44]. Survival following anthrax exposure correlated with serum IgG anti-PA titer, allowing for the identification of a “protective titer” that conferred resistance to high dose (100 LD50) spore challenge. As seen in Fig 1, every mouse immunized with CpG-adjuvanted vaccine maintained protective titers for at least one year, whereas titers fell below this level in half of the mice immunized with AVA alone within 6 months (p. <.001).

A/J mice were immunized i.p. with 10 ul of AVA alone (F) or adjuvanted with 20 ug of CpG ODN (M). Serum IgG anti-PA titers were monitored individually for each of >30 mice/group over time. Data reflect the percent of animals in each group with anti-PA titers in the “protective range” (> 1:16,000) and represent the combined results of two similar but independent experiments [44].

\[ *; p < .05 \] vs AVA alone.

5. Memory B cells contribute to vaccine-induced protective immunity.

Challenge studies showed that a subset of mice survived infection despite having IgG anti-PA titers <1/10th the protective baseline. Almost all of these survivors had been immunized with CpG-adjuvanted AVA (16/17) rather than
AVA alone (1/18, p <.001). Of interest, IgG anti-PA titers rose rapidly among mice that survived infection (average increase 3.6 fold by day 3, Table II) but not in those that succumbed to infection (p <.02)[44]. These findings suggest that animals with low anti-PA titers are able to survive challenge by rapidly activating their IgG anti-PA secreting memory B cell pool.

Table II Memory response of vaccinated mice
A. Rapidity of memory B cell response

<table>
<thead>
<tr>
<th>Challenge outcome</th>
<th>IgG anti-PA titer</th>
<th>3 days post challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivors</td>
<td>411 ± 118</td>
<td>1,492 ± 442</td>
</tr>
<tr>
<td>Non-survivors</td>
<td>360 ± 61</td>
<td>356 ± 82</td>
</tr>
</tbody>
</table>

B. Analysis of memory B cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Frequency of PA-specific memory B cells/spleen</th>
<th>% high affinity B cells</th>
<th>Rapidity of Activation (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unimmunized</td>
<td>2 ± 2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>AVA alone</td>
<td>10 ± 3</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>AVA + CpG ODN</td>
<td>45 ± 4</td>
<td>62*</td>
<td>3</td>
</tr>
</tbody>
</table>

A) A/J mice were immunized i.p. with AVA + CpG ODN. Those mice with IgG anti-PA titers measured >6 months post vaccination in the range of 1:100 - 1:2,000 were challenged with 100 LD₅₀ of Sterne strain anthrax spores. Serum Ab titers are shown for each animal one week before (pre) and 3 days after challenge (post) (N = 8-26/group). Note that anti-PA titers rose .3-fold in animals that survived (p <.02), but did not change in animals that succumbed to infection. All of the surviving mice had been immunized with CpG adjuvanted AVA rather than AVA alone (p <.01) [44].

B) A/J mice were immunized with AVA + CpG ODN. Fragment cultures were established from the spleens of mice after their Ab titers had fallen into the sub-protective range. These fragments were stimulated ex vivo with high (>3x10⁻⁹ M) or low (<10⁻¹¹ M) concentrations of rPA. Results represent the frequency of IgG anti-PA secreting fragments (avg + SD) per spleen studied 3-6 days later. Data reflect the combined results from 6 independent experiments involving a total of 22 mice immunized with CpG-adjuvanted AVA, 17 immunized with AVA alone, and 8 naive mice [44].

*; p <.05, CpG adjuvanted AVA vs AVA alone.

This led us to examine the size, affinity and response characteristics of the memory B cells generated following vaccination with CpG-adjuvanted AVA. Results showed that 4-fold more PA-specific memory B cells were present in mice vaccinated with CpG-adjuvanted AVA than AVA alone (p <.05, Table II)[44]. Moreover, the Ab produced by these memory B cells was of considerably higher average affinity than by B cells from mice vaccinated only with AVA (p <.05, Table II) [44]. Kinetic studies showed that B cells generated by immunization with the CpG-adjuvanted vaccine responded to Ag significantly more rapidly than those from mice immunized with AVA alone (p <.01), consistent with their higher affinity receptors being triggered earlier in the infection process by lower amounts of Ag [44]. These results indicate that vaccination with CpG-adjuvanted AVA generates a significantly larger and higher affinity population of memory B cells than AVA alone.

6. Combination therapy for the prevention and treatment of anthrax infection.

The treatment of individuals recently exposed to anthrax and of “rapid responders” dispatched to sites of known pathogen release has two objectives: i) short-term protection against initial infection and ii) long-term protection against inhaled spores that can remain latent for months and then germinate in vivo. No single intervention can effectively eliminate both the immediate and long term threat posed by anthrax. However, both goals might be met by combining antibiotic treatment with vaccination[32,45,46]. Conceptually, antibiotics would protect exposed individuals from immediate disease caused by vegetative bacilli while the immune response elicited by vaccination would eliminate the threat posed by the delayed germination of latent spores (or repeat bioterror attack) [22,32,45,46].

Several studies demonstrated that administering AVA and treating twice daily with the antibiotic Ciprofloxacin could improve the survival of rodents and non-human primates challenged with anthrax [34,47]. Our group sought to identify a more effective vaccine/antibiotic combination that minimized the need for prolonged therapy. Our choice of
agents was predicated upon the observations that: i) a single dose of CpG-adjuvanted AVA elicited an immune response that protected mice against both aerosol and systemic anthrax challenge within 5-10 days of vaccination, significantly faster than AVA alone (p. <.01, [40]) and ii) a single dose of the long-acting lipoglycopeptide antibiotic Dalbavancin could protect against anthrax for >2 weeks in vivo, far longer than a single dose of Ciprofloxacin [48-50]. We therefore examined the activity of CpG-adjuvanted AVA combined with Dalbavancin.

In a well characterized murine challenge model [39;42], the anti-bacterial activity of Dalbavancin was not compromised by the co-administration of CpG-adjuvanted AVA, while vaccine-induced protection was not compromised by the co-delivery of Dalbavancin [34]. A single dose of Dalbavancin mixed with CpG-adjuvanted AVA provided significant protection when administered any time before through 3 days after anthrax spore challenge (Fig 2) [34]. This antibiotic/vaccine combination thus eliminated both the immediate and long term threat posed by anthrax exposure while minimizing the need for patients to reliably self-administer Ciprofloxacin twice daily for weeks - months.

**7. DISCUSSION**

DNA has multiple and complex effects on the immune system. CpG ODN trigger cells expressing TLR9 to initiate an immunostimulatory cascade culminating in the broad activation of the immune system and the production of Th1 and pro-inflammatory cytokines and chemokines [3;4;7-11].

The ability of CpG ODN to elicit a strong innate immune response is being harnessed to improve vaccine immunogenicity. Producing effective vaccines against conventional and biothreat pathogens is an important goal of such research. Towards that end, the utility of CpG ODN as an adjuvant for AVA was evaluated. Results show that CpG ODN both accelerate and magnify AVA-induced immunity in mice, macaques and humans [39-41;43]. Moreover, the use of CpG ODN improve the persistence of protective immunity through two mechanisms: i) maintaining Ab titers in the protective range for longer periods and ii) generating a large and long-lived population of high affinity memory B cells that respond rapidly to challenge to protect animals whose Ab titers have declined [44].

There were important differences in the memory B cell response of mice vaccinated with CpG-adjuvanted AVA vs AVA alone. First, significantly more memory B cells were present in the spleens of mice vaccinated with CpG-adjuvanted vaccine (p. <.05, Table II). Second, these cells responded more rapidly to Ag stimulation, producing anti-PA Abs by day 3 post stimulation vs day 6 in mice vaccinated only with AVA (Table II). Finally, these B cells responded to lower concentrations of Ag, and produced Ab of higher affinity, that those from mice vaccinated with AVA alone (Table

![Figure 2: Protection provided by vaccine/antibiotic combinations.](image-url)
These results are consistent with the in vivo observation that mice immunized with CpG-adjuvanted AVA responded rapidly to anthrax challenge by producing protective anti-PA Abs [44]. The mechanism by which CpG ODN promote the induction of a long-lived high-affinity memory B cell response is under active investigation.

Despite the long-lasting benefits of vaccination, no single strategy can neutralize the threat posed by the release of anthrax by bioterrorists. Although CpG-adjuvanted AVA significantly accelerates the induction of protective immunity when compared to AVA alone [39;39;40;40], it cannot protect immunologically naive individuals already exposed to anthrax. Rather, we find that a single dose of the long-acting antibiotic Dalbavancin co-administered with CpG-adjuvanted AVA provides seamless protection against both the immediate and long-term threat posed by anthrax infection (Fig 2).

The concept of treating individuals exposed to anthrax with antibiotics and then immunizing them is not novel. Indeed, a recent report by Vietri et al described the benefits of this strategy in macaques [47;47]. They vaccinated half of the animals with AVA, and treated all of the animals twice daily with oral Ciprofloxacin starting 2 hr after exposure. While 100% of the macaques that were vaccinated and treated with antibiotics survived infection by 1,000 LD50 of anthrax, 5/9 of the unvaccinated macaques succumbed to infection once antibiotic therapy was discontinued. We sought to improve upon these results by optimizing the antibiotic/vaccine combination.

Towards that end, we substituted CpG-adjuvanted AVA for AVA, since animal and human studies show that the adjuvanted vaccine induces protective immunity significantly faster than AVA alone [39;40]. In addition, we substituted a single dose of the long acting injectable lipoglycopeptide antibiotic Dalbavancin for twice daily oral Ciprofloxacin, thereby eliminating the need for continued patient compliance and eliminating the side effects associated with prolonged oral antibiotic usage [51-53][48-50]. Our studies were conducted in a well established murine model that enabled different treatment regimens to be evaluated at multiple time points relative to challenge [39;54]. This model provides immunogenicity results predictive of the response of other species (including humans) to vaccination and yields protection data relevant to both systemic and aerosol anthrax spore challenge [39;39;42;42;54;54].

The combination of CpG-adjuvanted AVA plus Dalbavancin significantly improved survival when delivered anytime before up to 3 days post challenge (Fig 2). Further evaluation of the safety and utility of this single-dose combination therapy for individuals exposed to (or at high risk of exposure) to anthrax appears warranted.

ACKNOWLEDGMENTS

This project was supported by funding from the intramural research program of the National Cancer Institute. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

Author disclosure statement:

DMK is an inventor / co-inventor on patents associated with CpG ODN. Rights to these patents are assigned to the US government.

REFERENCES


[27] Ivins BE. Molecular pathogenesis of Bacillus anthracis infection. Microbes Infect 1999 Feb;225:13–35.


