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Adding synthetic oligodeoxynucleotides containing unmethylated CpG motifs to Anthrax vaccine adsorbed (AVA), the licensed human vaccine, increases the speed and magnitude of the resultant Ab response. Ab titers persist in the protective range for >1 year, significantly longer than in animals vaccinated with AVA alone. Unexpectedly, a majority of mice immunized with CpG-adjuvanted AVA maintained resistance to anthrax infection even after their Ab titers had declined into the subprotective range. The survival of these animals was mediated by the de novo production of protective Abs by high affinity memory B cells re-stimulated immediately after challenge. Thus, a previously unrecognized benefit of CpG oligodeoxynucleotides adjuvants is their ability to expand the long-lived memory B cell population. Current findings demonstrate that CpG-adjuvanted AVA mediates protection both by stimulating a strong/persistent serum Ab response and by generating a high-affinity long-lived pool of memory B cells. The Journal of Immunology, 2008, 181: 5785–5790.

B. anthracis is an aerobic Gram-positive bacterium found naturally in wild and domesticated animals (1). B. anthracis spores are highly resistant to environmental degradation and, upon germination, produce a tripartite toxin that reduces the ability of the host’s immune system to eliminate the pathogen (1). Abs against protective Ag (PA)3 can neutralize the toxin, inhibit spore germination, and improve the phagocytosis/ killing of spores by macrophages (2–5). Thus, vaccines targeting PA provide an effective and relatively inexpensive means of reducing susceptibility to anthrax (6).

Anthrax vaccine adsorbed (AVA) is the sole vaccine licensed to prevent human anthrax in the US. It is prepared by adsorbing the culture filtrate of an attenuated toxinogenic nonencapsulated strain of B. anthracis (V770-NP1-R) onto aluminum hydroxide (7). AVA induces a strong anti-PA response through a series of six immunizations over 18 mo followed by yearly boosts (8). Repeated vaccination with AVA has been linked to a variety of adverse events (9–11).

Anthrax spores designed for aerosol delivery were released in the US by bioterrorists in 2001, causing morbidity, mortality, and widespread panic (12). This event underscored the need for a vaccine that induced protective immunity more rapidly than AVA and maintained protection without repeated boosts (12). One strategy to achieve these goals involved adding synthetic oligodeoxynucleotides (ODN) containing immunostimulatory “CpG motifs” to AVA (13–15). CpG ODN interact with TLR 9 expressed by B cells and plasmacytoid dendritic cells (16–19), improving Ag presentation and triggering the production of Th1 and proinflammatory chemokines and cytokines (including IFN-γ, IL-6, IL-12, IL-18, and TNF-α) (16, 17, 20, 21). Studies in mice, monkeys and humans verify that CpG ODN both accelerate and magnify the immune response elicited by AVA (13–15). However, neither the duration nor mechanism(s) underlying CpG-mediated improvements in protection are fully characterized.

In the current work, large numbers of mice vaccinated with AVA plus CpG ODN were followed long term and their response to anthrax challenge monitored. Results show that CpG-adjuvanted AVA induced the production of significantly higher anti-PA titers that persisted in the protective range for significantly longer (>1 year) than AVA alone. Unexpectedly, many mice immunized with CpG-adjuvanted AVA (but not AVA alone) remained resistant to infectious challenge even after their anti-PA titers fell into the subprotective range. A modification of the splenic fragment technique (SFT) was used to identify the mechanism underlying this persistence of protection. Results indicate that vaccination with CpG-adjuvanted AVA preferentially generated a large pool of high affinity memory B cells. This finding establishes a novel benefit of CpG ODN adjuvants and has profound implications for the criteria used to assess the efficacy of future anthrax vaccines.

Materials and Methods

Reagents

Phosphorothiate CpG ODN 1555 (GCTAGACGTAGCGT) and 1466 (TCAACGTGTTAGC) as well as control ODN 1612 (GCTAGAGCT TAGCGT) and 1471 (TCAAGCTTGA) were synthesized at the Food and Drug Administration Center for Biologics core facility. All ODN were free of endotoxin and protein contamination. AVA was obtained from BioPort, rPA was provided by U.S. Army Medical Research Institute of Infectious Diseases and prepared as described (22). The toxigenic (pXO1+), non-encapsulated (pXO2−) Sterne vaccine strain spores of B. anthracis were obtained from the culture collection of U.S. Army Medical Research Institute of Infectious Diseases. Spores were prepared and stored as previously described (23).

Animals

Specific pathogen-free female A/J mice were obtained from the National Cancer Institute. They were housed in microisolator cages in a barrier environment and initially vaccinated at 8–12 wk of age. All animal experiments were conducted using Animal Care and Use Committee approved protocols, and challenge studies were performed in a BSL-2 facility.
Immunization and challenge studies

Mice were immunized i.p. with 2–10 μl of AVA ± 20 μg of CpG ODN in a final volume of 50 μl. These vaccine doses were selected on the basis of preliminary studies demonstrating that 2 μl of AVA induced a detectable but suboptimal IgG anti-PA response, whereas 10 μl of AVA induced a response that protected most mice from low dose anthrax challenge (13). Mice were bled monthly by tail nicking and serum stored at −20°C until use. To evaluate the effect of serum Ab titers on protection, animals were challenged as early as 10 wk (at the height of their Ab response) or as late as 20 mo post vaccination. Immunization times were staggered so that animals with different initial Ab titers could be challenged and studied simultaneously. Challenge consisted of Sterne strain anthrax spores suspended in 0.1 ml of sterile PBS administered i.p. at dose of 5–100 LD₅₀ (1 LD₅₀ = 1.1 × 10⁸ Sterne strain spores). Survival was monitored for 21 days.

Splenic fragment technique

A/J mice were immunized with 2–10 μl of AVA ± 20 μg of CpG ODN. Spleens were aseptically removed from animals whose Ab titers fell into the subprotective range. Each organ was diced into 50-lm² fragments which were cultured in individual wells of a 96-well microtiter plate (Costar). Fragments were maintained in RPMI 1640 media supplemented with 10% FCS, 50 μg/ml streptomycin, 0.3 μg/ml glutamine, 1 μM nonessential amino acids, 1 μM sodium pyruvate, 10 mM HEPES, and 10⁻³ M 2-ME in a 5% CO₂ incubator. For the first 2 days of culture, medium was supplemented with 10⁻³–10⁻¹ M rPA. Culture supernatants were replaced every 3 days and spent supernatants assayed for IgG anti-PA Ab content by ELISA.

IgG anti-PA and avidity assays

IgG anti-PA Ab titers were monitored as described (15). In brief, 96-well microtiter plates (Immulon 2HB; Thermo Labsystems) were coated with 1 μg/ml rPA in PBS at 4°C overnight. The plates then were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20. Plates were washed, and overlaid with serially diluted serum or undiluted splenic fragment culture supernatants for 2 h at room temperature. The plates were washed and, for avidity assays, overlaid for 15 min with 200 μl of 6 M urea. Bound Abs were detected after washing by adding HRP-labeled goat anti-mouse IgG (Southern Biotechnology Associates) followed by ABTS substrate (Kirkegaard & Perry Laboratories). Ab titers were determined by comparison to a standard curve generated using pooled sera from hyperimmunized (Southern Biotechnology Associates) followed by ABTS substrate (Kirkegaard & Perry Laboratories). Ab titers were determined by comparison to a standard curve generated using high-titered anti-PA serum. All serum samples were analyzed in duplicate.

Statistics

Differences in the kinetic development of anti-PA immune responses were determined by two-way ANOVA. Differences in the IgG anti-PA response induced by various vaccine-adjuvant combinations were assessed by one-way ANOVA. Differences in survival were evaluated using χ² analysis of Kaplan-Meier curves. Correlation coefficients were determined by linear regression analysis. The frequency of positive wells in the splenic fragment assay was calculated based on the assumption of a binomial distribution and evaluated using the z test.

Results

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 >1 yr. A/J mice were selected for evaluation because these animals i) mount strong Ab responses to AVA plus CpG ODN, ii) are highly susceptible to challenge by attenuated Sterne strain anthrax spores due to a defect in their complement cascade, and iii) generate an anti-PA response to AVA plus CpG ODN similar to that of other species, including Man (13, 15, 24–26). Consistent with previous reports, adding CpG ODN increased the mean Ab response when compared with AVA alone by >10-fold (from a GMT of 185,000 to 3,250,000, p < 0.01; Fig. 1) (14, 25). Over time, anti-PA titers declined in both groups of vaccinated mice. The half-life of the serum IgG anti-PA response was virtually identical between groups (AVA alone: 38.2 days vs AVA plus CpG ODN: 37.6 days, see dashed lines in Fig. 1). The rate of decline in Ab titers slowed after 1 yr (perhaps reflecting intermittent activation of memory B cells). Of note, IgG anti-PA titers remained significantly higher in the group vaccinated with CpG-adjuvanted AVA vs AVA alone for the duration of the study (p < .01; Fig. 1).

Correlation between IgG anti-PA titers and protection from Sterne strain anthrax spore challenge

A/J mice immunized with 2–10 μl of AVA were challenged i.p. up to 6 mo later with 5–100 LD₅₀ of B. anthracis Sterne spores. Consistent with previous studies, survival correlated with serum IgG anti-PA titers at the time of challenge. More animals succumbed to infection as the challenge dose was increased, and a higher serum anti-PA titer was required to survive (Fig. 2, 80% anti-PA titer prior to challenge dose).
translating anti-PA titers were challenged simultaneously. As expected, animals with circulating anti-PA titers in the "protective range" (i.e., in excess of 1:16,000) and represent the combined results of two similar but independent experiments. *p < .05 vs AVA alone.

Additional animals were vaccinated with 10 μl of AVA ± CpG ODN and their Ab titers monitored for up to 20 mo. As seen in Fig. 3, every mouse immunized with CpG-adjuvanted vaccine maintained Ab titers >1:16,000 for at least 1 year, whereas titers fell below this level in half of the mice immunized with AVA alone within 6 mo (p < .001).

**Memory B cells contribute to vaccine-induced protective immunity**

To determine whether IgG anti-PA titers accurately predicted susceptibility to infection over time, mice were immunized with 2–10 μl of AVA ± CpG ODN. To compensate for differences in the magnitude of the initial IgG anti-PA response induced by specific vaccine/adjuvant combinations (see Fig. 1), multiple independent groups of mice were vaccinated over a 10-mo period. Ab titers were monitored monthly in every animal. Mice with serum Ab titers in a broad but overlapping range were selected from among those vaccinated with AVA alone vs AVA plus CpG ODN and represent the combined results of two similar but independent experiments.

Survival following 5 LD_{50} vs 26% survival following 100 LD_{50}, p < 0.001) (15). Results from this experiment showed that serum IgG anti-PA titers in excess of 1:16,000 generally protected against the highest spore challenge examined (100 LD_{50}). This Ab titer was taken as a "protective baseline" and used to evaluate the duration of protective immunity in individual mice over time.

Despite this general trend, a subset of mice was identified that survived infection despite having anti-PA titers 10-fold below the protective baseline (bracketed data, Fig. 4). Almost all of these survivors had been immunized with CpG-adjuvanted AVA (8/16 vs 1/18 immunized with AVA alone, p < 0.01) The mechanism underlying the survival of animals with low serum IgG anti-PA titers was investigated. Mice immunized with 2 or 10 μl of AVA ± CpG ODN were challenged up to 18 mos later, after their serum anti-PA titers had fallen into the subprotective range. Serum was collected from all animals before and 3 days after challenge (as susceptible mice began to die by day 4). Consistent with the pattern noted above, 8/19 mice immunized with CpG-adjuvanted AVA survived infection vs 0/17 mice vaccinated with AVA alone (p < 0.01). Survival was not a function of circulating anti-PA Ab titers before challenge, since i) the geometric mean titer of protected vs unvaccinated mice was similar and ii) anti-PA titers in all mice were substantially below the protective baseline of 1:16,000. Of interest, IgG anti-PA titers rose in the cohort of mice that survived infection (average increase 3.2-fold by day 3) but not among those that succumbed to infection (p < .02, Fig. 5). These results suggest that the rapid production of IgG anti-PA Abs was protecting hosts with initially low circulating anti-PA titers.
they were of relatively low affinity (Fig. 6). Cells from mice vaccinated with AVA alone responded to stimulation thus were of high affinity, whereas none of these CpG-adjuvanted vaccine donors were typically of high affinity (i.e., responded to low Ag concentrations), whereas none of the splenic fragments from donors vaccinated with AVA alone were of high affinity at day 3 ($p < .01$). These results suggest that vaccination with CpG-adjuvanted AVA generates a significantly larger and higher affinity population of memory B cells than AVA alone.

### Discussion

Producing effective vaccines against conventional and bioterror pathogens is an important goal of biomedical research. Toward that end, immunostimulatory adjuvants such as CpG ODN are being evaluated for their ability to improve the immunogenicity of vaccines targeting anthrax, smallpox, HIV, and other infectious agents (reviewed in Ref. 32). Previous research established that CpG ODN accelerated and magnified AVA-induced immunity in mice, macaques, and humans (13–15, 26). Current results confirm and extend these findings by demonstrating that CpG ODN extend the duration of protective immunity through 1 year. They further document that vaccination with CpG-adjuvanted AVA generates a large and long-lived population of high affinity memory B cells that respond so rapidly to challenge that they protect otherwise susceptible animals. AVA requires six immunizations delivered over 18 mo to induce and maintain protective Ab titers in humans, a regimen associated with deleterious side effects including joint pain, gastrointestinal disorders, and pneumonia (9–11). Preclinical and phase I clinical studies show that adding CpG ODN to AVA increases serum IgG anti-PA titers by 6- to 20-fold (26). As seen in Fig. 1, the serum half-life of anti-PA Abs was similar in mice vaccinated with AVA vs CpG-adjuvanted AVA. However, protection persisted significantly longer in recipients of CpG-adjuvanted vaccine due to their initially higher anti-PA titers (Fig. 3).

Multiple mechanisms have been proposed to explain the ability of CpG ODN to improve AVA immunogenicity. Unmethylated CpG DNA directly trigger immune cells that express TLR 9, initiating an innate immune response characterized by the production of proinflammatory and Th1 cytokines/chemokines capable of promoting the development of adaptive humoral responses (17, 20, 21, 33). CpG ODN also induce the functional maturation of professional APCs (34, 35). The increased availability of such “help” may explain why CpG-adjuvanted AVA induces protection more rapidly than AVA alone and generates a larger and more avid population of memory B cells. The presence of such help may also facilitate the development of a protective secondary response. It is well established that serum anti-PA Abs protect against infection, and that resistance is maintained by repeated re-immunization (36–38). However, the literature provides examples of...
animals remaining resistant to infection after their serum Ab response has waned (39). We therefore examined the susceptibility of mice to challenge after their anti-PA titers fell into the subprotective range. Surprisingly, half of the mice immunized with CpG-adjuvanted AVA with anti-PA titers 10-fold below the protective baseline survived a 100 LD<sub>50</sub> Sterne strain spore challenge (Figs. 4 and 5). This contrasted with only 1/35 mice with the same Ab titer that had been immunized with AVA alone (p < .01). The survival of mice with subprotective titers did not correlate with the maximal Ab titer achieved following vaccination, time post vaccination, or dose of AVA (p > 0.45 for each parameter). Rather, protection correlated with how rapidly the host mounted a humoral response following pathogen challenge (Fig. 5). Specifically, IgG anti-PA titers rose rapidly in mice that survived but were unchanged in animals that succumbed.

Among survivors, serum anti-PA titers did not reach protective levels (>1:16,000) until >10 days post challenge (by which time virtually all susceptible mice had died). This suggests that the cumulative Ab response over the course of infection, rather than solely at the time of challenge, determines host survival. This interpretation is consistent with results from an earlier study showing that mice challenged shortly after vaccination (when serum anti-PA titers were low) survived infection if their anti-PA response was rising toward protective levels (14).

We hypothesize that mice with low serum Ab levels can survive infection if their high-affinity memory B cells respond rapidly to the Ag released following challenge. This possibility could not be evaluated in vivo due to the rapid demise of AVA-vaccinated animals. Instead, the frequency and speed with which PA-specific memory B cells responded to Ag stimulation ex vivo was examined using the SFT. The SFT maintains the splenic microenvironment, thereby facilitating the detection of Ag-specific memory B cells (27, 28). Splenic fragments from all vaccinated mice secreting anti-PA Abs within 6 days of culture with rPA, unlike those from naive mice (Fig. 6). However, three important differences were noted between the response of splenic fragments from mice immunized with AVA alone vs AVA plus CpG ODN. First, significantly more memory B cells were present in the spleens of mice vaccinated with the CpG-adjuvanted vaccine (p < .05; Fig. 6). 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 (Fig. 6). Finally, these B cells responded to lower concentrations of rPA, and produced Ab of higher affinity, that those from mice vaccinated with AVA alone (Fig. 6). 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 (Figs. 4–6). Although several mechanism(s) might contribute to the rapid activation of memory B cells from mice immunized with the CpG-adjuvanted vaccine, data suggest that a critical factor is their high affinity for PA. As seen in Fig. 6, significantly more memory B cells from CpG-adjuvanted animals i) responded to low concentrations of rPA and ii) produced high affinity Abs, than those from mice immunized with AVA alone.

A detailed analysis of the SFT results showed marked intra-animal variability in the response of CpG-adjuvanted mice. Specifically, splenic fragments from approximately one-third of donor mice behaved like those from AVA-vaccinated animals: they contained relatively few anti-PA secreting B cells and these were primarily of low affinity. By comparison, splenic fragments from the majority of mice vaccinated with CpG-adjuvanted AVA contained large numbers of memory B cells, many of which secreted Abs of such high affinity that they remained bound to their target Ag despite treatment with 6 M urea (which strips low affinity Abs from rPA) (25). We speculate that the latter group of mice are those destined to survive challenge. Thus, while other immune elements (such as Ag-responsive T cells) might also contribute to protection, current findings suggest that an important but previously unrecognized goal of anthrax vaccine development should be the generation long-lasting high-affinity memory B cells.

Conventional phase III clinical trials are designed to test whether a vaccine reduces the risk of human infection. Serious technical and ethical limitations prevent the conduct of such studies for vaccines targeting bioterror pathogens. Recognizing this problem, the Food and Drug Administration developed an “animal rule” that allows surrogate markers of protection derived from animal challenge studies to be substituted for evidence of clinical efficacy in human licensure decisions. Vaccine-induced anti-PA Abs correlate with survival from anthrax challenge in multiple animal models and thus represent one such marker (13, 36–38). However, current results indicate that high affinity memory B cells also reduce host susceptibility to infection. Thus, relying on serum anti-PA Ab levels alone for licensure decisions could underestimate the protection conferred by novel vaccines. This leads us to suggest that second and third generation anthrax vaccines should also be evaluated for their ability to generate a durable pool of high-affinity memory B cells.

Disclosures
The authors have no financial conflict of interest.

References
The authors have no financial conflict of interest.


