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Stochastic humoral immunity to *Bacillus anthracis* protective antigen: Identification of anti-peptide IgG correlating with seroconversion to Lethal Toxin neutralization

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ABSTRACT

A substantial fraction of individuals vaccinated against anthrax have low to immeasurable levels of serum Lethal Toxin (LeTx)-neutralizing activity. The only known correlate of protection against *Bacillus anthracis* in the currently licensed vaccine is magnitude of the IgG response to Protective Antigen (PA); however, some individuals producing high serum levels of anti-PA IgG fail to neutralize LeTx *in vitro*. This suggests that non-protective humoral responses to PA may be immunodominant in some individuals. Therefore, to better understand why anthrax vaccination elicits heterogeneous levels of protection, this study was designed to elucidate the relationship between anti-PA fine specificity and LeTx neutralization in response to PA vaccination. Inbred mice immunized with recombinant PA produced high levels of anti-PA IgG and neutralized LeTx *in vitro* and *in vivo*. Decapeptide binding studies using pooled sera reproducibly identified the same 9 epitopes. Unexpectedly, sera from individual mice revealed substantial heterogeneity in the anti-PA IgG and LeTx neutralization responses, despite relative genetic homogeneity, shared environment and exposure to the same immunogen. This heterogeneity permitted the identification of specificities that correlate with LeTx-neutralizing activity. IgG binding to six decapeptides comprising two PA epitopes, located in domains I and IV, significantly correlate with seroconversion to LeTx neutralization. These results indicate that stochastic variation in humoral immunity is likely to be a major contributor to the general problem of heterogeneity in vaccine responsiveness and suggest that vaccine effectiveness could be improved by approaches that focus the humoral response toward protective epitopes in a greater fraction of vaccinees.

1. Introduction

Bioterrorism is a real and ongoing threat that was heightened by the malicious release of *Bacillus anthracis* spores through the U.S. postal system in 2001. Consequently, adequate protection from anthrax infection through vaccination remains an important concern for military forces. These issues have prompted careful review of the safety and efficacy of currently licensed anthrax vaccines over the last decade.

Anthrax infection has three main forms: cutaneous, gastrointestinal, and inhalational, each with its own route of entry and associated mortality [1]. The primary route associated with bioterrorism is inhalational anthrax, which has a projected mortality rate of at least 40% even with modern standard medical care [2]. The major virulence factors of this highly pathogenic, spore-forming, Gram-positive rod are a poly-D-glutamic acid capsule and a tripartite toxin [1]. The tripartite toxin consists of three proteins: Protective Antigen (PA), Lethal Factor (LF), and Edema Factor (EF) [1]. After binding the widely expressed anthrax receptors on host cells, PA forms pores that enable entry of the EF and LF toxin components into the cells [3,4]. LF is a zinc-dependent metalloprotease that cleaves mitogen-activated protein kinase kinases [5], and EF is a calmodulin-dependent adenylate cyclase [6]. While none of these proteins are toxic individually, the combination of PA and LF makes

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Lethal Toxin (LeTx), and the combination of PA and EF makes Edema Toxin (EdTx) [1]. These toxins subvert the host immune system, which helps to establish *B. anthracis* infection and permit excessive bacterial growth.

Experimental anthrax vaccines that generate neutralizing antibodies to anthrax LeTx are sufficient for protection from challenge with virulent *B. anthracis* in animal models [7]. The most widely used human vaccines, Anthrax Vaccine Precipitated (AVP; [8,9]) and Anthrax Vaccine Adsorbed (AVA; [9,10]) are alum-based preparations of culture filtrates from toxigenic, non-encapsulated strains of *B. anthracis*. IgG antibodies directed to PA are the only reported correlates of the toxin-neutralizing antibody response to these vaccines [11,12]. The only human anthrax vaccine approved in the United States is AVA, or Biothrax®. The AVA vaccination schedule consists of intramuscular injections at 0, 1, 6, 12, and 18 months and requires yearly boosters [12–15]. We previously observed significant variation in the human response to the AVA vaccine, with only half of vaccinees producing measurable levels of neutralizing antibodies [12,16]. Sera from 7.5% of vaccinees produced high titer anti-PA IgG antibodies but failed to measurably neutralize LeTx *in vitro*, suggesting that some individuals may produce immunodominant responses to non-protective PA epitopes [12,16]. Additional disadvantages include the requirement for large containment and production facilities, high incidence of adverse reactogenicity at the injection site, onerous immunization schedule and possible batch-to-batch variation.

The ideal anthrax vaccine would generate a rapid, long-lasting protective response in all immunized individuals with infrequent boosters and little reactogenicity. Approaches to improve the anthrax vaccine have included the use of better-defined recombinant protective antigen [17,18], inclusion of experimental adjuvants [19,20] and reduction in the number of priming vaccinations [21,22]. A better understanding of the biological factors that influence vaccine responsiveness would additionally bolster vaccine development. For example, identification of protective and non-protective epitopes may permit refinement of the PA vaccine to target the most protective epitopes and eliminate highly immunogenic but non-protective epitopes. Genetic polymorphisms that affect vaccine responsiveness may contribute to variation in the generation of protective humoral immunity following vaccination. Genetic associations have been reported to influence the humoral responses to smallpox [23] and measles [24,25] vaccination in humans and production of protective levels of neutralizing anti-retroviral antibodies in mice [26]. Studies measuring the heritability of vaccine responses in monozygotic twins also reveal a significant contribution of non-genetic factors to variation in humoral vaccine responsiveness [27]. However, the extent to which such non-heritable variation is governed by the environment or stochastic features of adaptive immunity is unknown.

2. Materials and methods

2.1. Mice

Six-8 week-old A/J strain mice were purchased from Jackson Laboratories (Bar Harbor, ME), and housed in specific pathogen-free conditions at the Oklahoma Medical Research Foundation Laboratory Animal Resources Facility. The Oklahoma Medical Research Foundation Institutional Animal Care and Use Committee approved all mouse experiments.

2.2. Production of recombinant (r)PA protein

rPA was produced as an amino-terminal His_6-tagged protein [28] using methods previously described [28,29].

2.3. Immunization and blood sampling

Mice were immunized subcutaneously with rPA (50 μg/0.1 mL/injection) emulsified 1:1 in complete Freund's adjuvant (CFA; Difco, Lawrence, KS) on day 0, then boosted with rPA (50 μg rPA/0.1 mL/injection) emulsified 1:1 in incomplete Freund's adjuvant on either days 10, 24 and 38, or 14, 28 and 42. Control mice were immunized with PBS/adjuvant only on identical schedules. Blood samples were collected 4 days after each boost.

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed as described previously except that plates were coated with 1 μg/well rPA instead of rLF [29]. Positive signals were defined as OD values exceeding 3 standard deviations (SD) above the mean OD for 1:100-diluted control samples from mice immunized with adjuvant alone. Anti-PA titer was defined as the inverse of the last serum dilution giving a positive signal.

2.5. In vitro LeTx neutralization assay

Inhibition of LeTx activity was determined as previously described except assays used either a 1:1 ratio of rPA:rLF or a 3:1 ratio of rPA:rLF (1:1 ratio = 50 ng/well rPA + 50 ng/well rLF; 3:1 ratio = 75 ng/well rPA + 25 ng/well rLF; final well volume, 100 μL) [29,30] as indicated in the Figure legends. Neutralization titer was defined as the inverse of the last serum dilution giving a positive signal. For assays using 1:1 rPA:rLF, a positive signal was defined as ≥50% cell viability. For assays using 3:1 rPA:rLF, a positive signal was defined as OD values exceeding 4 SD above the mean OD of diluted samples from PBS/adjuvant-immunized mice. This corresponded to cell viability values ≥37%.

2.6. In vivo LeTx challenge

rPA and control mice were challenged on day 120 with 3X LD_{50} dose of LeTx for A/J mice (300 μg PA and 125 μg LF [29]). Mice were monitored for 5 days and mortality recorded. Survival curves were compared using the Mantel–Cox test.

2.7. Solid-phase humoral epitope mapping

Decamer peptides overlapping by 8 amino acids and spanning the entire length of the PA protein (GenBank accession number AAA22637) were covalently synthesized onto polyethylene solid phase supports using a 96-well format as previously described [31]. Peptides were incubated with serum dilutions of 1:200. Detection of bound antibodies utilized peroxidase-labeled goat anti-mouse IgG and SureBlue Reserve TMB substrate (both from KPL, Gaithersburg, MD) in a modified ELISA protocol [31]. An epitope was defined as a region of high antibody binding in which two or more consecutive solid-phase peptides exhibited ELISA OD_{450} values greater than or equal to the average OD_{450} plus 5 standard deviations (SD) of a known low response region (PA amino acids (aa) 97–126 or aa 497–526).

2.8. Data analysis

Differences in antibody binding to particular decapeptides were determined by 2-tailed *t*-test, with *P* values of ≤0.05 considered statistically significant. Increases of peptide binding activity ≥0.5 OD were considered biologically significant.