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# Transcriptional analysis of protective antigen-stimulated PBMC from non-human primates vaccinated with the anthrax vaccine absorbed

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**Abstract**

The transcriptional responses in recombinant protective antigen (PA)-stimulated peripheral blood mononuclear cells (PBMCs) from Anthrax Vaccine Absorbed (AVA)-vaccinated rhesus macaques were evaluated using Affymetrix HGU133 Plus 2.0 GeneChips. PBMCs from animals vaccinated at 0, 4, and 26 weeks were harvested at week 30, stimulated with PA, and RNA isolated. The expression of 295 unigenes was significantly increased in PA-stimulated compared to non-stimulated PBMCs; no significant decrease in gene expression was observed. These upregulated transcripts encoded for proteins functioning in both innate and adaptive immunity. Results were corroborated for several genes by real-time RT-PCR. This study provides information on the potential underlying transcriptional mechanisms in the immune response to PA in AVA-vaccinated rhesus macaques.


*Keywords:* Anthrax; Vaccine; Protective antigen; Non-human primate

## 1. Introduction

The etiologic agent *Bacillus anthracis* is a Gram-positive, non-hemolytic, spore-forming, facultative anaerobic bacterium that can cause inhalation, cutaneous, and gastrointestinal anthrax in animals and humans. The spores formed by *B. anthracis* are highly resistant to many environmental conditions; therefore, these spores may be used as a biological weapon due to their potential lethality and ability to be easily produced and disseminated. Concerns regarding the use of *B. anthracis* as a biological weapon of war or bioterrorism have prompted the need for further development of vaccines and therapeutics to counteract or protect against the effects of anthrax.

Current human anthrax vaccines include the U.S. licensed Anthrax Vaccine Absorbed (AVA-Biothrax, BioPort, Lansing, MI) and the U.K. licensed anthrax vaccine. AVA consists primarily of *B. anthracis* V770-NP1-R protective antigen (PA) adsorbed to aluminum hydroxide. The UK vaccine is a potassium aluminum phosphate-precipitated *B. anthracis* Sterne strain ($34F_2$) culture filtrate (anthrax vaccine precipitated, AVP). Next generation anthrax vaccines have focused primarily on the immunogenic *B. anthracis* components PA and lethal factor (LF), which are proteins that contribute to bacterial evasion of the host innate immune response [1–4]. In addition to AVA and AVP, additional immunization approaches have included recombinant PA, plasmid DNA encoding full-length and truncated forms of PA and/or LF, and cationic lipid-formulated plasmid DNA encoding PA and LF [5–10]. Various animal models have been used for efficacy testing of potential anthrax vaccines including mice, guinea pigs, hamsters, rabbits, and non-human primates. The protective response of anthrax vaccines, however, varies widely among these species [5–24]. Characterizing anthrax vaccine efficacy in humans presents an ethical issue due to the very

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low prevalence of naturally occurring human anthrax and thus the inability to conduct well-controlled clinical trials. This issue is the greatest challenge facing the evaluation of current and newly-developed vaccines against anthrax.

To date, the production and kinetics of anti-PA antibody titer and toxin neutralizing antibody titer have been the primary focus for evaluating immune protection against anthrax infection. However, the immune response against pathogenic microorganisms is dynamic and involves the interaction of coordinated responses occurring at the molecular, cellular, and tissue levels. Therefore, it is possible that changes in the underlying molecular mechanisms associated with a robust immune response could be monitored to characterize immune protection against anthrax. Studies have provided detailed information of the underlying transcriptional responses to bacterial infection showing expression patterns that are characteristic of the development of innate and adaptive immunity [25–30]. In the present study, we used the Human HGU133 Plus 2.0 array (approximately 38,500 genes) for transcriptional analysis of genes encoding proteins associated with immune activation in PA-stimulated PBMCs from AVA-vaccinated non-human primates.

## 2. Materials and methods

### 2.1. Animals and vaccination

Rhesus macaque (*Macaca mulatta*) non-human primates (NHP) approximately 3.0 kg or larger were purchased from Covance Laboratories (Princeton, NJ). The NHPs were in good health, free of malformations, and exhibited no signs of clinical disease. NHPs were pair-housed in stainless steel cages on racks equipped with automatic watering systems. The animals were maintained at controlled temperature, RH (30–70%), and under a light/dark cycle of 12 h each per day using fluorescent lighting. Animals were provided Certified Monkey Chow® biscuits (PMI Feeds, Inc., St. Louis, MO) twice daily with water provided ad libitum. NHPs were vaccinated intramuscularly (IM) with 0.5 mL (equivalent to human dose) undiluted Anthrax Vaccine Adsorbed (AVA; BioPort, Inc., Lansing, MI) at week 0 and boosted at 4 and 26 weeks. NHPs injected IM with 0.5 mL of 0.9% saline at the same week intervals were used as negative controls for vaccination. In conducting research using animals, the investigator(s) adhered to the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council (National Academy Press, 1996).

### 2.2. PBMC isolation and stimulation

At week 30, 4 weeks after the third vaccination, AVA-vaccinated (two female and four male) and saline control animals (three female) were anesthetized following an IM injection of 3–6 mg/kg Telazol®. Whole blood was collected from each NHP into 8 mL CPT™ Vacutainer™ (CPT) tubes. The CPT tubes were centrifuged at $1750 \times g$ for 45 min at room temperature. Following centrifugation, plasma layers were discarded and the PBMCs were harvested and transferred to sterile 15 mL polypropylene tissue culture tubes containing 5 mL calcium/magnesium-free Dulbecco's phosphate-buffered saline (PBS; 2.67 mM KCl, 1.47 mM $KH_2PO_4$, 137.93 mM NaCl, 8.06 $Na_2HPO_4$; pH 7.4). Tubes were mixed by inversion three to five times and centrifuged at approximately $200 \times g$ for 7 min at room temperature to pellet the cells. The cell pellets were washed with calcium/magnesium-free PBS, and red blood cells were lysed using erythrocyte lysis solution (168 mM $NH_4Cl$, 1 mM $KHCO_3$, 0.1 mM $Na_4EDTA$; pH 7.3). PBMCs were counted using a hemacytometer and resuspended to a concentration of approximately $1.25 \times 10^6$ cells/mL in RPMI 1640 with GlutaMax™ and HEPES (Gibco BRL, Gaithersburg, MD) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, and 10% heat-inactivated autologous plasma.

The PBMCs ($1.0 \times 10^6$ cells/well) were stimulated, in duplicate, in 24-well tissue culture plates in RPMI media with and without 1.25 μg/mL recombinant PA. The cells were incubated at 37 °C in a humidified incubator under 95% air and 5% $CO_2$ for 24 h. Following incubation, the duplicate cultures were pooled and the PBMCs were pelleted by centrifugation. Each cell pellet was resuspended in TRIzol Reagent (Invitrogen, Carlsbad, CA), frozen, and stored at −70 °C until isolation of total RNA.

### 2.3. RNA isolation and cDNA synthesis

Total cellular RNA was isolated from PBMC cell pellets using TRIzol Reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol with the addition of PhaseLock Gel (5 Prime 3 Prime, Inc., Boulder, CO) during centrifugation to allow separation of the phenol-chloroform phase. Following chloroform extraction, total RNA was precipitated in isopropanol, washed with ethanol, suspended in a nuclease-free solution, and stored at −70 °C until used for cDNA synthesis. Total RNA was quantified spectrophotometrically based on an absorbance at 260 nm of one equal to an RNA concentration of 40 μg/mL.

### 2.4. Transcript analysis

Total RNA (5 μg) was used to synthesize double-stranded cDNA using the Superscript II kit (Life Technologies, Gaithersburg, MD). The cDNA served as a template to synthesize biotin-labeled antisense cRNA using a BioArray™ RNA Transcript Labeling Kit (T7; Enzo Diagnostics, Farmingdale, NY). Labeled cRNA was fragmented and hybridized to the Human HGU133 Plus 2.0 array (containing approximately 38,500 genes) as described in the Affymetrix GeneChip® protocol (Affymetrix, Santa Clara, CA). Each