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Enhanced early innate and T cell-mediated responses in subjects immunized with Anthrax Vaccine Adsorbed Plus CPG 7909 (AV7909)

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ABSTRACT

NuThrax™ (Anthrax Vaccine Adsorbed with CPG 7909 Adjuvant) (AV7909) is in development. Samples obtained in a phase Ib clinical trial were tested to confirm biomarkers of innate immunity and evaluate effects of CPG 7909 (PF-03512676) on adaptive immunity. Subjects received two intramuscular doses of commercial BioThrax® (Anthrax Vaccine Adsorbed, AVA), or two intramuscular doses of one of four formulations of AV7909. IP-10, IL-6, and C-reactive protein (CRP) levels were elevated 24–48 h after administration of AV7909 formulations, returning to baseline by Day 7. AVA (no CPG 7909) resulted in elevated IL-6 and CRP, but not IP-10. Another marker of CpG, transiently decreased absolute lymphocyte counts (ALCs), correlated with transiently increased IP-10. Cellular recall responses to anthrax protective antigen (PA) or PA peptides were assessed by IFN-γ ELISpot assay performed on cryopreserved PBMCs obtained from subjects prior to immunization and 7 days following the second immunization (study day 21). One-half of subjects that received AV7909 with low-dose (0.25 mg/dose) CPG 7909 possessed positive Day 21 T cell responses to PA. In contrast, positive T cell responses occurred at an 11% average rate (1/9) for AVA-treated subjects. Differences in cellular responses due to dose level of CPG 7909 were not associated with differences in humoral anti-PA IgG responses, which were elevated for recipients of AV7909 compared to recipients of AVA. Serum markers at 24 or 48 h (i.e. % ALC decrease, or increase in IL-6, IP-10, or CRP) correlated with the humoral (antibody) responses 1 month later, but did not correlate with cellular ELISpot responses. In summary, biomarkers of early responses to CPG 7909 were confirmed, and adding a CpG adjuvant to a vaccine administered twice resulted in increased T cell effects relative to vaccine alone. Changes in early biomarkers correlated with subsequent adaptive humoral immunity but not cellular immunity.

1. Introduction

NuThrax™ (Anthrax Vaccine Adsorbed with CPG 7909 adjuvant) (AV7909) is a post-exposure prophylaxis (PEP) anthrax vaccine candidate being developed to accelerate the immune response and minimize the number of injections needed to confer protective immunity. AV7909 contains AVA bulk drug substance as a source of Protective Antigen (PA) immunogen, aluminum hydroxide, and the toll-like receptor 9 (TLR9) agonist CPG 7909 (PF-03512676). Administration of AV7909 stimulates the immune system to produce toxin-neutralizing antibodies directed to PA, a component of anthrax toxins [1]. Human CpG biomarkers can become the basis for *in vitro* assays that are useful during vaccine development.

Human serum biomarkers affected by CpG adjuvants include increased IP-10 and IL-6 [2], IL-12, MCP-1 and IFN-α [3] as well as enhanced antibody responses [1,4,5]. Also reported were transiently decreased absolute lymphocyte counts (ALCs) and C-reactive Protein (CRP) after subcutaneous (SC) administration [3,6,19], *in vitro* interferon-gamma (IFN-γ) production by peripheral blood mononuclear cells (PBMC) obtained after *in vivo* CpG treatment [4], increased T cell expansion [7], increased circulating T cells and NK cells after intra-venous (IV) administration [6] and increased $CD8^+$ T cells. *In vitro* responses to CpG2006 or CPG 7909 included enhanced IL-10, IL-6, IFN-γ [8], IL-8 [9] by human plasmacytoid dendritic cells, as well as increased PBMC production of IL-6, IL-10, IFN-α, IFN-γ, and IP-10 [9,10] and enhanced $CD8^+$ T cells developed from PBMC [9,11].

The contributions of cell-mediated immune responses to the production of anthrax toxin-neutralizing antibodies remain to be defined. Although human T cell epitopes within the PA molecule,

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Table 1
Vaccine formulations, study design and number of donor samples.

Study arm	AV7909 formulation	Active ingredients and vaccine amounts	Subjects per arm (N)[a]	ELISpot subjects per arm[b] (N)	ELISA subject samples per arm (N)
1	None	AVA 0.5 mL (BioThrax®)	17	15	14–15[c]
2	1, or F1	AVA 0.5 mL + CPG 7909 0.5 mg	18	15	17
3	2, or F2	AVA 0.5 mL + CPG 7909 0.25 mg	17	15	16
4	3, or F3	AVA 0.25 mL + CPG 7909 0.5 mg	19	18	17–18[d]
5	4, or F4	AVA 0.25 mL + CPG 7909 0.25 mg	18	16	18
6	None	None	15	14	14–15[e]

[a] The group sizes (n = 15–19) for samples used for determinations of early biomarkers were the same as number of enrolled subjects reported in [14], with the exception that a subject in Arm 1 was included for early biomarker determinations that did not continue in the study beyond Day 7.
[b] ELISpot testing was performed using PBMC from blood collected on Days 0 and 21 if meeting criteria that Subject's Day 0 and D21 samples passed Trypan blue viability.
[c] One Subject's ELISA samples were not available on Day 70.
[d] One Subject's ELISA samples were not available on Day 28.
[e] One Subject's ELISA samples were not available on Day 42.

restricted by 2 different HLA allotypes were identified using tetramer guided epitope mapping [12,13], neither these epitopes nor other peptides have been tested previously for capacity to induce T cell recall responses in PBMC from recipients of anthrax vaccines.

As exploratory endpoints in the clinical trial designed to investigate the safety and immunogenicity of intramuscular (IM) administration of AVA formulated with CPG 7909 adjuvant [14], IP-10, IL-6, C-reactive protein (CRP), and ALC were evaluated in blood samples obtained from human AV7909 recipients and compared to AVA recipients. To investigate T cell responses to PA protein, PBMC samples from immunized subjects were re-stimulated *in vitro* with a mixture of predicted HLA class II restricted PA peptide epitopes or with recombinant PA (rPA) and were visualized as IFN-γ-producing cells using an enzyme-linked immunospot (ELISpot) technique. The potential correlations of these markers with subsequent serum IgG anti-PA responses (present manuscript), and toxin neutralizing antibody responses [14] were evaluated.

2. Materials and methods

2.1. Study design

A randomized double-blinded clinical study ("EBS.AVA.201/DMID 10-0013"; Trial # NCT01263691) [14] was conducted in compliance with the Declaration of Helsinki and ICH guidelines, under an investigational new drug (IND) application. After the nature and possible consequences of the study were fully explained to subjects, informed consent was obtained. Four formulations of AV7909 contained either 0.5 mL or 0.25 mL of AVA with either 0.25 or 0.5 mg of CPG 7909. A full dose of AVA (0.5 mL) was administered as a comparator vaccine. Saline served as placebo vaccine. Table 1 lists vaccine formulations, doses, and sample sizes for each of 6 treatment groups, and an explanation if the sample size differed from the number of subjects who completed the study [14]. An equivalent number of male and female subjects were included across the arms of the study; demographic information is available in the Hopkins et al. paper [14].

Samples (101) were collected during subject visits at 0, 1, 2, and 7 days after the first dose of vaccine to evaluate CRP, IP-10, and IL-6 levels, as well as changes in ALC. Both CRP, measured with high-sensitivity nephelometry assay (Roche Diagnostics, Indianapolis, IN) and ALC (derived from the CBC) were performed commercially (ACM Global Laboratory, Rochester, NY). IP-10 and IL-6 ELISAs are described below. Cellular responses were evaluated 7 days after the second administration of vaccine. Antibody responses were evaluated to determine anti-PA IgG levels in serum samples collected on Day 0, 14, 28, 42, and 70 (this paper) and toxin-neutralizing antibody (TNA) levels [14].

2.2. IFN-γ enzyme-linked Immunospot assay (ELISpot)

Prior to the first vaccine dose, and 7 days after the second vaccine dose (study day 21), PBMC were isolated from venous blood samples, and stored in liquid nitrogen vapors at SeraCare Life Sciences (Gaithersburg, MD). For ELISpot controls: stimulants were phytohaemmaglutinin (PHA; mitogen, control for viability, Sigma, St Louis, MO) and CEF I peptide pool (Cellular Technology Ltd; Shaker Heights, OH) representing HLA Class I-restricted peptides from cytomegalovirus, Epstein Bar virus and influenza virus (CEF). Recall antigens were rPA (Emergent BioSolutions, Gaithersburg, MD) or a pool of 10 PA-derived peptides (PAps) (ProImmune, Oxford, UK). Sequences for PAps were selected on the basis of (1) high binding scores calculated by SYFPEITHI [15] and PROPRED [16] in silico programs, (2) predicted binding by multiple HLA Class II types, (3) low hydrophobicity and (4) absence of cytotoxicity to naïve PBMC. Stimulation by PAp mixture was performed with a final concentration of 10 μg/mL of each peptide. PAp

Table 2
Amino acid sequences of predicted HLA class II-restricted PA-derived peptide epitopes and the restricting HLA haplotypes.

Peptide number	Amino acid sequence	Predicted HLA class II preferences
1[a]	LGYYFSDLNFQAPMVVTSS	DRB1*01, DRB1*04, DRB1*11, DRB1*15
2	TVDVKNKRTFLSPWI	DRB1*04, DRB1*08, DRB1*11, DRB1*13
3[a]	SSTVAIDHSLSLAGE	DRB1*03, DRB1*15
4[a]	NANIRYVNTGTAPIYNV	DRB1*01, DRB1*03, DRB1*04, DRB1*07, DRB1*08, DRB1*11, DRB1*13
5	LDKIKLNAKMNILIR	DRB1*03, DRB1*11, DRB1*13
6[a]	KMNILIRDKRFHYDR	DRB1*08, DRB1*11, DRB1*13
7	RYDMLNISSLRQDGK	DRB1*04, DRB1*11, DRB1*13
8	DKDIRKILSGYIVEI	DRB1*04, DRB1*07, DRB1*15
9[a]	KLPLYISNPNYKVNV	DRB1*01, DRB1*03, DRB1*07, DRB1*11, DRB1*13
10	ISSLRQDGKTFIDFK	DRB1*03, DRB1*11

[a] This sequence, predicted independently with the in silico programs, is identical to or has significant sequence homology with published class-II restricted PA peptides [13], detected using tetramer staining.

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