

# Safety, Pharmacokinetic, Immunogenicity, and Pharmacodynamic Responses in Healthy Volunteers Following a Single Intravenous Injection of Purified Staphylococcal Protein A

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## Abstract

A single-dose study was conducted to characterize the safety, pharmacokinetic, immunogenicity, and pharmacodynamic activity of highly purified Staphylococcal protein A (SPA), a native bacterial protein with immune-modulatory activity. Twenty healthy adults received a single intravenous dose of either 0.3  $\mu\text{g}/\text{kg}$  ( $n = 8$ ) or 0.45  $\mu\text{g}/\text{kg}$  ( $n = 8$ ) of SPA or placebo ( $n = 4$ ). Changes in C-reactive protein and neopterin were used as markers of immune activation. All treatment-related AEs were of mild severity. Twelve of 16 active-dosed subjects developed detectable anti-protein A antibodies after dosing. These subjects had notably more rapid plasma clearance of SPA even prior to development of detectable titers. A transient post-dose decrease in circulating lymphocytes was observed as a notable pharmacodynamic effect, but was not correlated with plasma clearance or AUC. In peripheral blood mononuclear cells, SPA dosing increased transcription of multiple genes regulated by type-I interferons, and up-regulation of several of these genes correlated with the degree of lymphopenia seen 24 hours after dosing. This study demonstrates the safety and tolerability of small intravenous doses of SPA and delineates acute and transient pharmacodynamic effects not previously reported.

## Keywords

Staphylococcal protein A, pharmacokinetics, pharmacodynamics, gene expression

Staphylococcal protein A (SPA) is an immunomodulatory virulence protein produced by many strains of *Staphylococcus aureus*. In addition to binding the IgG Fc region, SPA also binds with equal or higher affinity to the heavy chain CDR2 domain of the 20–30% of human Igs that utilize the VH3 gene to code this region. Thus it also binds to the IgM antigen receptor of all Vh3 B-cells.<sup>1,2</sup> We discovered that in vitro exposure of human macrophages to SPA at concentrations as low as 10–50 ng/mL inhibit, both phagocytosis of opsonized platelets as well as the secretion of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and the up-regulation of CD16 and CD40 after stimulation by bacterial endotoxin.<sup>3</sup> This activity appears to involve inhibitory signaling via Fc receptors. Studies of very low parenteral doses of SPA in the mouse collagen arthritis model showed this treatment to reduce disease activity in a manner comparable to etanercept, a soluble TNF-receptor.<sup>3</sup> Unlike the activity of etanercept, the suppression of disease activity is not abrogated when mice form antibodies to SPA. This suggests that a limited number of intravenous (IV) injections of an appropriate dose of SPA might be capable of modulating deregulated immunity and decreasing disease activity in a variety of autoimmune diseases.

A 12-week toxicology study found the “no observed adverse effect level” for weekly IV doses of highly

purified SPA in Cynomolgus monkeys to be  $>25 \mu\text{g}/\text{kg}$  (Bernton et al, unpublished observation). However, other published non-clinical studies report that at doses resulting in exposures approximately 10–1,000 times higher than those demonstrated in the GLP toxicology studies, the injection of commercially produced protein A causes immune-complex formation, complement activation, peripheral B-cell deletion in mouse, rat, and rabbit animal models and mast cell degranulation in human basophils or skin.<sup>4–7</sup>

Prior to the current study, a legacy study was performed evaluating the safety and pharmacokinetics of single doses of 2, 5, 10, and 20  $\mu\text{g}$  of SPA in healthy volunteers.<sup>8</sup> In this study, 6 of 28 active-dosed subjects reported a total of 12 treatment-emergent adverse events (TEAEs) with an

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onset on the dosing day. These included mild or moderate pyrexia, back pain, somnolence, heartburn, headache, nausea, chest discomfort, myalgia, chills, and fatigue. The incidence of these AEs and of increases in C-reactive protein and serum neopterin increased with increased SPA plasma  $C_{max}$ . This reactogenicity was later ascribed to trace amounts of co-purified staphylococcal host-cell protein (S-HCP) in the product. Consequently, downstream purification of the SPA drug product was modified to greatly reduce the amount of co-purifying S-HCP. The current healthy volunteer phase I study was performed to re-evaluate the safety, reactogenicity, pharmacokinetic (PK), and pharmacodynamic effects of the “second-generation” SPA drug product, to investigate whether inflammatory responses to drug were associated with the intrinsic superantigen properties of SPA, or were due to trace amounts of *S. aureus* contaminants.

## Methods

### Drug Product

A highly purified form of the native SPA (PRTX-100) was prepared from cultures of *S. aureus* strain A676 using Good Manufacturing Practices (GMP). This form of SPA is a 47 kDa bacterial protein comprising five homologous 58–61 amino acid Ig binding domains.<sup>2,3</sup> SPA was harvested from bulk cultures of *S. aureus*. Following initial microfiltration, three chromatographic purifications steps were followed by final formulation and microfiltration to produce bulk drug product. Product identity and purity (>98%) were determined by SEC HPLC, reversed-phase HPLC, and SDS-PAGE. The product met FDA requirements for endotoxin content by limulus assay, residual DNA, and for bioburden. Enterotoxin B content measured by enzyme immunoassay (EIA) was undetectable.

Following the legacy phase I study, a sensitive EIA to detect non-SPA S-HCP was developed and qualified. Using this assay, the initial clinical lot of drug product was found to have approximately 800 ppm (0.08%) of S-HCP. After changes in downstream chromatographic purification, the residual S-HCP was reduced 80-fold to <10 ppm. This “second-generation” drug substance was used to prepare drug product for this single-dose phase I study.<sup>3</sup>

### Phase I Study Design

The protocol for this double-blind, randomized, placebo-controlled, sequential single-dose escalation study was approved by Central Institutional Review Board, IntegReview, and the study (NCT00517855) was performed in 2007 at the Buffalo Clinical Research Center in Buffalo, NY, USA. The primary objective was to evaluate the safety and PK of a single IV injection of SPA in healthy volunteers of both genders. Standard clinical laboratory

parameters and evaluation of AEs were the primary safety endpoints. PK sampling was conducted until 96 hours post-dose.

Serum C-reactive protein (CRP) and neopterin were evaluated at multiple time points to assess potential immune activation or downstream effects of cytokine release after dosing. Hemolytic complement (CH50), C3a-des-arg, and C1q were also serially evaluated to detect any complement activation or immune complex formation. The immunophenotype of circulating PBMCs was evaluated to describe post-dose alterations. Serial evaluations of the “in vitro bleeding time” were conducted with the Platelet Function Analyzer (PFA)-100 to look for any evidence of a pharmacodynamic effect on platelet function. Samples were obtained pre-dose and at 1, 2, and 7 days post-dose. An exploratory gene-expression analysis was also performed on a subset of 10 volunteers to evaluate differential gene expression in PBMC at 4 and 24 hours following active or placebo dosing.

Since a maximum tolerated dose (MTD) of SPA was not identified in the previous phase I study, the dose cohorts in this study received either 0.30 or 0.45  $\mu\text{g}/\text{kg}$  (approximately 20 or 30  $\mu\text{g}$  of drug product). In each cohort, eight subjects received SPA and two subjects received placebo.

### Safety Parameters

Vital signs, 12-lead ECG, physical examination, hematology, chemistry, urinalysis, and treatment-emergent AEs (TEAEs) were monitored. Vital signs were recorded pre-dose and at 15, 30, and 60 minutes, and 8, 12, and 24 hours post-dose. Triplicate ECG's were performed pre-dose and at 4 and 24 hours post-dose. Serum chemistry, CBC, and urinalysis were performed pre-dose and at 8, 24, 48, 72, and 96 hours post-dose. AE's were assessed on an ongoing basis. Volunteers were confined to the CPRU for the first 96 hours post-dose. Adverse events were assessed by the PI as unrelated, probably related, or possibly related. AE severity was graded using the NCI CTCAE version 3.

### Pharmacokinetics

Drug was injected IV over a 10–20-second period into the side-arm port of a rapidly flowing saline IV line. Blood samples for PK analysis were collected in heparinized tubes pre-dose and at 2, 15, 30 minutes and 1, 2, 4, 6, 8, 12, 16, 24, 36, 48, 72, 96, 120, 144, 216, and 312 hours post-dose from all subjects. Separated plasma was frozen at  $-70^{\circ}\text{C}$  until shipped on dry ice to the bioanalytical laboratory for analysis. SPA concentrations were determined after a minimum 1/100 sample dilution using an enzyme-linked immunosorbent assay (ELISA) developed and validated by Prevalere Life Sciences (Whitesboro, NY, USA). The lower limit of quantification in plasma for the assay is 0.55  $\text{ng}/\text{mL}$ .<sup>9</sup>

### Immunogenicity

Serum samples were collected for immunogenicity at pre-dose, and at Days 7, 14, 30, and 60. The anti-SPA titer was determined using a validated ELISA method at Prevalere Life Sciences. In brief, this method utilizes SPA bound to wells on a polystyrene plate. After blocking of non-specific binding of IgG by SPA with 20% pig serum, a 1/100 dilution of samples, negative controls, or calibration samples were incubated in the wells. Rabbit anti-SPA was used for positive controls and calibration samples. Bound anti-SPA antibodies were detected using biotinylated pig anti-rabbit or anti-human Ig, after incubation with Streptavidin-HRP conjugate. An optical density upper limit was established using 34 serum samples from untreated controls, and used to establish a cut-point for each run, based on assay calibration samples. Samples that were positive for anti-SPA antibodies by cut-point analysis were confirmed by isotype analysis.<sup>10</sup>

A sensitive EIA was developed and qualified by Dr. Robert Hamilton at the Johns Hopkins Allergy and Asthma Center, Baltimore, USA, and was used to detect IgE antibodies to SPA. The lower limit of detection (LLD) for this assay was 1 ng/mL. All samples positive by cut-point analysis at Prevalere Life Sciences were also evaluated in this assay. In this assay, protein A was coated on ELISA plates and porcine IgG was used to block its binding of antibodies or reagents via their Fc regions. A unique feature of the assay was its heterologous calibration curve. Since there were no positive human IgE anti-protein A sera available, a surrogate reference curve was developed by using the anti-human IgE Fc drug, Omalizumab (Genentech, South San Francisco, CA). This human IgG1 antibody is bound by the protein A coated on the plate (without addition of porcine IgG) and is then free to bind human IgE from a calibration serum which is analyzed at multiple dilutions. This allowed for the construction of a multi-point IgE calibration curve. The assay cutoff point which defines the optical density above which a specimen is identified as containing IgE anti-protein A was determined statistically based on the 95% upper confidence limit by analyzing the sera from 20 volunteers (not exposed to SPA) in 10 assays.

### Pharmacodynamics

CH<sub>50</sub>, C3a-des-arg, C1q assays were performed pre-dose and 24 hours post-dose and on Day 7. CRP (using the Roche high-sensitivity autoanalyzer assay) and serum neopterin (by commercial EIA) were assessed pre-dose on Day 1 and post-dose on Days 2, 3, 4, 5, 7, 10, 14, 30, and 60. Mast cell tryptase was measured by EIA. Complete blood counts (CBCs) performed at 8, 24, 48, 72, and 96 hours after dosing allowed serial evaluation of the absolute lymphocyte count. Immunophenotypes were determined by flow cytometry using standard reagents to detect CD20, CD14, CD3, CD56, CD16, CD4, and CD8.

Serial evaluations of the “in vitro bleeding time” were conducted with the PFA-100 to look for any evidence of a pharmacodynamic effect on platelet function. The PFA-100 is a system in which citrated whole blood is aspirated at high shear rates through disposable cartridges containing an aperture within a membrane coated with collagen and either epinephrine (CEPI) or ADP (CADP), then determining the time until aperture closure by platelet plug. Reference ranges for normal platelet function have been established for both conditions.<sup>11</sup> PFA-100 testing was performed pre-dose and at 1, 2, and 7 days post-dose.

### Gene Expression

PBMCs were isolated on Ficoll gradients from fresh whole blood samples obtained at pre-dose, and 4 and 24 hours post-dose. Isolated PBMCs from 30 samples were lysed in RNA STAT-60 (AMS Bio, Lake Forest, CA) and RNA was extracted according to manufacturer's instructions. RNA samples were assessed for quantity and quality by standard spectrophotometry and Agilent Bioanalyzer analysis. High-quality RNA samples were used with the Affymetrix 3'-Amplification Two-Cycle cDNA Synthesis kit to generate double-stranded cDNA. This material was further amplified and labeled with the GeneChip® Expression 3'-Amplification IVT Labeling Kit to generate biotinylated cRNA samples that were hybridized to Affymetrix Human Genome U133 Plus2.0 Arrays. Arrays were processed and scanned according to the Affymetrix Expression Analysis Technical Manual. The 30 samples analyzed were obtained from each subject pre-dose, and 4 and 24 hours post-dose. Three subjects received placebo and seven received active SPA. The seven SPA-treated subjects were categorized as “high responders” (n = 4, all with a  $\geq 40\%$  decrease in absolute lymphocyte count [ALC] at 24 hours post-dose) or “low responders” (n = 3, all with a  $\leq 20\%$  decrease in ALC).

Genes that had statistically significant up- or down-regulation of PBMC transcripts at 4 or 24 hours post-dose relative to pre-dose in the seven active-treated but not the three placebo-treated subjects were used to generate a list of “top 20 genes” for further analysis. Top 20 genes in the overall sequence set were retained on the list if their gene expression ratio changes between post- and pre-dose were at least fourfold and their *P*-values from paired *t*-tests were  $< 0.05$ . The genes of interest from the seven active-treated subjects categorized as four “high responders” and three “low responders” were also evaluated for their correlation with this biomarker.

### Statistical Analysis

Non-compartmental methods were used to estimate the PK endpoints. Semi-log plots were constructed of the individual plasma SPA concentration versus time profiles for each subject. Area under the curve (AUC) values were calculated by log-linear trapezoidal integration using the

KINETICA™ version 2.0.1 validated computer program. The terminal half-life was calculated as  $\ln(2)/\lambda_z$  where  $\lambda_z$  was the calculated slope of the terminal portion of the log plasma concentration versus time curve. Extrapolation of the AUC from the last measured plasma concentration to infinity was calculated as  $C_{p_n}/\lambda_z$  where  $C_{p_n}$  is the last measured analyte plasma concentration. Mean residence time (MRT) was calculated as  $[(AUMC_{0-\infty}/AUC_{0-\infty}) - T_{inf}/2]$ , where  $T_{inf}$  is the dose infusion duration (0 hour). Plasma clearance (CL) was calculated as  $Dose/AUC_{0-\infty}$ , and the terminal phase distribution volume ( $V_z$ ) was calculated as  $Dose/(AUC_{0-\infty} \times \lambda_z)$ . The steady-state distribution volume ( $V_{ss}$ ) was calculated as  $[Dose \times AUMC_{0-\infty}/(AUC_{0-\infty} \times AUC_{0-\infty})] - [(T_{inf} \times dose)/2 \times AUC_{0-\infty}]$ . The “apparent” volume of the central compartment ( $V_c$ ) was estimated as  $dose/C_{p_{0extrap}}$  where  $C_{p_{0extrap}}$  is the zero time extrapolated plasma SPA concentration value estimated from the initial log-transformed plasma concentration versus time curve using the KINETICA™ Version 2.0.1 Software Program. At least three time points in the terminal phase and an  $r^2$  value  $\geq 0.8$  were required to calculate  $\lambda_z$ .

The non-parametric Kendall tau rank correlation statistic was used to test for significant correlations between exposure ( $C_{max}$ , AUC) and post-dose changes in ALC.

## Results

### Safety and Tolerability

The occurrence of TEAEs and treatment-related AEs (TRAEs) in the current study are summarized in Supplemental Table S1. All the TEAEs were of mild severity. Four of the four placebo-treated subjects had TEAEs of which three had TRAEs. Eight of the eight subjects in the 0.30  $\mu\text{g}/\text{kg}$  treatment group had TEAEs of which six subjects had TRAEs. Three of the eight subjects in the 0.45  $\mu\text{g}/\text{kg}$  treatment group had TEAEs of which one subject had a TRAE. The most commonly affected system organ class (SOC) for all treatment groups was nervous system disorders followed by musculoskeletal and connective tissue disorders. Headache was the most frequently reported AE, affecting six of eight subjects in the 0.30  $\mu\text{g}/\text{kg}$  treatment group, three of four subjects in the placebo treatment group, and one of eight subjects in the 0.45  $\mu\text{g}/\text{kg}$  treatment group. Myalgia was the second most frequently reported AE affecting four of eight subjects treated with 0.30  $\mu\text{g}/\text{kg}$  SPA, two of four placebo subjects, and none of the subjects receiving 0.45  $\mu\text{g}/\text{kg}$  SPA. There were no anaphylactoid reactions or rashes experienced during this study. No fevers occurred. The AEs of moderate severity comprised headache (two subjects), back pain (one), diarrhea (one), and nausea (one), and all were considered unrelated to study treatment because of time of onset ( $>72$  hours after dosing) or alternative plausible causes.

In this study, peri-dosing AEs related to immune reactivity of SPA or mild cytokine release (fever, myalgias, GI upset, headache) occurred less frequently than in the legacy trial, despite higher doses of SPA, and did not show a dose response.

Analysis of laboratory results showed no significant trends or abnormalities, with the exception of white blood cell count which revealed a transient decrease in ALC in some subjects. Lymphocyte counts that were decreased at 24 or 48 hours post-dose had returned to baseline by Day 7. These are discussed below as pharmacodynamic responses to SPA.

No clinically significant individual laboratory results were noted, with the exception of a 34-year-old African-American subject in the 0.3  $\mu\text{g}/\text{kg}$  dose group who experienced transient grade 3 neutropenia. This subject's absolute neutrophil count was  $1.0 \times 10^9/\text{L}$  pre-dose,  $3.3 \times 10^9/\text{L}$  at 24 hours post-dose, and  $0.9 \times 10^9/\text{L}$  at 72 hours post-dose. At 96 hours post-dose, the subject's neutrophil count was  $1.6 \times 10^9/\text{L}$  and ranged between  $1.0 \times 10^9/\text{L}$  and  $1.3 \times 10^9/\text{L}$  for the remaining duration of the study. This subject also reported mild post-dose myalgia, headache, and lethargy and was the only subject on study who had elevations of CRP and neopterin beyond the upper limit of normal. This subject's absolute lymphocyte count was  $3.0 \times 10^9/\text{L}$  at pre-dose and decreased to  $1.0 \times 10^9/\text{L}$  at 24 hours post-dose,  $1.5 \times 10^9/\text{L}$  at 48 hours post-dose, and then remained between  $1.9 \times 10^9/\text{L}$  and  $2.8 \times 10^9/\text{L}$  over the remainder of the study. No other laboratory abnormalities were noted for this subject.

### Immunogenicity

Sera from all 20 subjects were analyzed for anti-SPA antibodies. Three of eight subjects who received 0.3  $\mu\text{g}/\text{kg}$  and seven of eight subjects who received 0.45  $\mu\text{g}/\text{kg}$  SPA (overall, 63% of subjects receiving SPA) developed anti-SPA antibodies above the cut-point threshold for screen and this was confirmed for at least one time point by isotype analysis. Two subjects had anti-SPA antibodies by screen, which were not detectable in isotyping EIAs. Two out of 20 subjects in this study had pre-existing antibodies to protein A. No placebo treated patients developed de novo isotype positive ADAs. All immunogenicity samples were also evaluated with a sensitive EIA developed to measure IgE antibodies to protein A (LLD = 1 ng/mL). No IgE antibodies binding to protein A were detected.

Five subjects had IgM anti-SPA as the only detectable isotype, three subjects had IgG as the only detectable isotype, two subjects had IgA and IgG isotypes, and two subjects had IgM and IgG isotypes. In six samples, two or more isotypes were detected. Two subjects had IgG anti-SPA as early as Day 7. Multiple isotype expression, in some cases within 7 days of a single treatment with SPA, implies a recall response rather than a naïve response to

SPA in many of these subjects. This suggests prior immunization due to staphylococcal exposure may not be uncommon in the general population. No IgE Abs were detected pre- or post-dose in any subjects, suggesting that while protein A can elicit an antibody response, it does not appear to be allergenic.

#### Pharmacokinetics

No subjects had detectable plasma levels (LLD of 0.55 ng/mL) of SPA beyond 96 hours post-dose. In preliminary analysis of pharmacokinetic data, it was obvious that SPA plasma clearance parameters were much higher in individuals who later developed detectable anti-SPA titers. As shown in Table 1 and Supplemental Table S2, three of eight subjects dosed at 0.3  $\mu\text{g}/\text{kg}$  and seven of eight subjects dosed at 0.45  $\mu\text{g}/\text{kg}$  developed anti-protein A antibodies by Day 30 post-dose. Examination of the 0.3  $\mu\text{g}/\text{kg}$  shows clear differences in AUC, half-life, CL, and MRT, which correlated with eventual antibody status. Because all but one of subjects that received 0.45  $\mu\text{g}/\text{kg}$  SPA developed anti-protein A antibodies, a similar analysis could not be made (Subject 2007 in the 0.45  $\mu\text{g}/\text{kg}$  dose group initially tested screen positive for the presence of antibodies to SPA but subsequent immunophenotyping assays failed to confirm the initial findings). This analysis suggested that development of post-dose antibody titers was associated with meaningful differences in the clearance of the previous single dose of SPA. Figure 1 shows the mean plasma concentration–time curves for each dose group in this study, stratified by ultimate anti-SPA antibody status.

Therefore, PK parameter analysis was conducted by subgroups, according to whether the subject eventually

developed antibodies to SPA. PK parameters are summarized in Table 1 and Supplemental Table S3. Within each dose group, half-life and terminal CL showed marked variability between subjects, even when subjects were sub-grouped by anti-product antibody status.

The steady-state distribution volume ( $V_{ss}$ ), which ranged from 5 to 6 L across dose groups, is close to theoretical blood volume.

#### Pharmacodynamics

**High-Sensitivity C-Reactive Protein (hs-CRP) and Serum Neopterin.** Post-dose changes in hs-CRP and neopterin are summarized in Table 2. In order to compare the reactogenicity of the current drug product with reduced host-cell protein to that found in the legacy phase I study, the data from this study is included in Table 3. In contrast to the legacy study, where higher exposures to SPA were associated with post-dose elevations of both serum CRP and neopterin, in the current study only one subject (0.3  $\mu\text{g}/\text{kg}$  dose) had an elevated CRP with a maximum increase from baseline of 16.4 mg/L at 48 hours post-dose. Only this subject had a marked increase in neopterin with a maximum increase from baseline of 18.8 nmol/L at 48 hours post-dose. This subject also had the highest number of peri-dosing AEs that included headache, nausea, myalgia, and fatigue. All increases in neopterin and CRP had returned to the normal range by 96 hours post-dose.

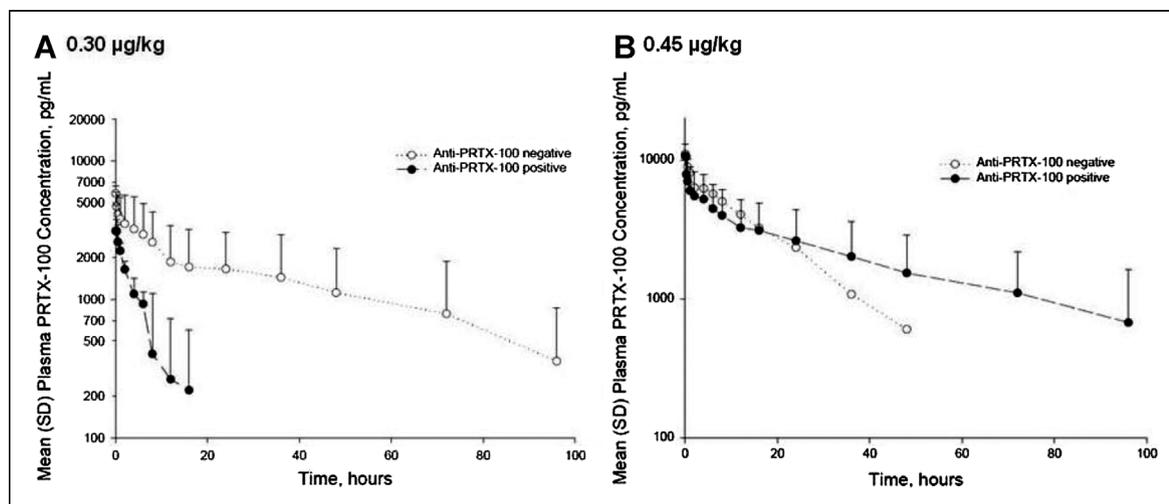
**Post-Dose Decreases in ALC.** A post-dose decrease in ALC was observed in active-dosed subjects (Table 3). This response was noted as early as 8 hours after dosing. Decreases in the ALC were maximal at 24 hours. The mean percentage changes at 8 hours post-dose were 7% and –30% in placebo- and active-treated subjects,

**Table 1.** Selected Pharmacokinetic Parameters for Plasma SPA Following IV Administration by Dose and Ultimate Anti-Product Antibody Status

Parameter	$C_{max}$ (pg/mL)	$AUC_{0-n}$ [(pg/mL) $\times$ hour]	$AUC_{0-\infty}$ [(pg/mL) $\times$ hour]	$\lambda_z$ ( $\text{hr}^{-1}$ )	Half-life (hour)	MRT (hour)
0.30 $\mu\text{g}/\text{kg}$ anti-SPA, eventual antibody negative subjects						
Mean	6,028	121,223	184,222	0.0330	28.3	40.8
SD	606	119,291	140,271	0.0242	14.3	21.4
GM	6,003	62,326	126,645	0.0279	24.9	35.4
N	5	5	4 <sup>a</sup>	4 <sup>a</sup>	4 <sup>a</sup>	4 <sup>a</sup>
0.30 $\mu\text{g}/\text{kg}$ anti-SPA, eventual antibody positive subjects						
Mean	3,739	12,392	18,586	0.168	5.9	8.4
SD	800	6,455	10,092	0.114	4.4	5.8
GM	3,685	11,416	16,994	0.141	4.9	7.1
N	3	3	3	3	3	3
0.45 $\mu\text{g}/\text{kg}$ anti-SPA, eventual antibody negative subject (N = 1)						
Mean	11,044	136,245	147,761	0.0526	13.2	18.7
0.45 $\mu\text{g}/\text{kg}$ anti-SPA, eventual antibody positive subjects <sup>b</sup>						
Mean	11413.5	223,264	310,944	0.0245	41.4	55.9
SD	1647.3	116,115	239,069	0.0143	27.1	37.1
GM	11314.1	195,507	251,026	0.0210	35.3	47.5
N	6	6	6	6	6	6

<sup>a</sup>Terminal half-life could not be accurately determined in one subject.

<sup>b</sup>One subject deleted from these tabulations as outlier (>3 SD from mean for CL, AUC, and MRT).



**Figure 1.** Mean (SD) plasma SPA concentrations in subjects receiving (A) 0.30 µg/kg or (B) 0.45 µg/kg intravenous SPA with and without later detection of anti-SPA antibody formation.

respectively. At 24 hours post-dose these changes were  $-11\%$  and  $-34\%$ , respectively. The mean ALC change from baseline at 24 hours post-dose differed significantly between placebo and pooled SPA subjects ( $-0.03$  vs.  $-0.76$ ,  $P < .05$ ,  $t$ -test). However, by 96 and 120 hours after dosing, there was no meaningful difference in the ALC between the placebo and active-dosed subjects.

The subject with the most profound post-dose lymphopenic response ( $-67\%$  at 24 hours) also had elevated CRP and neopterin as well as peri-dosing AEs (headache, nausea, myalgia, drowsiness). Thus, all three of these acute responses to SPA appear to move in parallel in an individual with a more pronounced pharmacodynamic response to SPA.

The short-lived lymphopenic response to higher exposure with SPA most likely reflects vascular margination or redistribution of lymphocytes, such as is seen after dosing with glucocorticoids or type-1 interferons.<sup>13,14</sup> Immunophenotyping pre-dose and at 24 hours post-dose (data not shown) demonstrated that this decrease represented both CD20 B-lymphocytes and CD4+ and CD8+ T-lymphocytes, but not NK cells. This decrease in

circulating cells was thought to represent lymphocyte margination or redistribution from circulation to tissue compartments secondary to SPA exposure.

**Platelet Function.** No effect was observed with SPA dosing on the PFA closure time (Supplemental Table S4), a measure of platelet aggregation and white thrombus formation by whole blood after ex vivo platelet activation.

**Mast Cell Tryptase, C3a-des Arg, CH50, and C1q.** Mast cell tryptase was measured pre-dose and at 2 hours post-dose. No abnormal elevations were associated with active dosing (data not shown).

C3a-des Arg is the stable metabolite of C3a that is formed by proteolytic activation of complement component C3. No significant increases in C3A des-Arg or decreases in CH50 were observed 24 hours post-dose and 7 days after SPA treatment, suggesting that no activation or consumption of complement took place (data not shown). However, due to the lack of an early post-dose sampling time point, transient complement activation might have occurred and been undetected.

The C1q assay detects circulating immune complexes in serum samples. This measurement was made pre-dose

**Table 2.** Post-Dose Changes in hs-CRP and Neopterin After a Single Dose of SPA

Parameter	Legacy phase I study <sup>a</sup>		Current study		
	20 µg <sup>b</sup>	5 and 10 µg	0 µg/kg	0.3 µg/kg	0.45 µg/kg
Subjects with post-dose CRP > ULN	4/7 (57%)	5/14 (36%)	0/4 (0%)	1/8 (12.5%)	0/8 (0%)
Mean increase <sup>c</sup>	7.4	3.4	-0.25	1.9	0.58
Subjects with post-dose neopterin > ULN	5/7 (71%)	5/14 (36%)	0/4 (0%)	1/8 (12.5%)	0/8 (0%)
Mean increase <sup>c</sup>	10.4 <sup>c</sup>	2.4 <sup>c</sup>	-0.50 <sup>c</sup>	1.58 <sup>c</sup>	1.59 <sup>c</sup>

<sup>a</sup>From PRTX-100 Investigators Brochure.

<sup>b</sup>Twenty micrograms of total dose equals approximately 0.3 µg/kg.

<sup>c</sup>Mean increase from baseline at 48 hours post-dose (ULN for hsCRP = 10 mg/L, ULN for neopterin = 10 nmol/L).

**Table 3.** Absolute and Percentage Change From Baseline in the Absolute Lymphocyte Count ( $\times 10^9/L$ ) At 8, 24, 48, and 96 hours After Treatment

Study day, timepoint	Placebo (N = 4)	SPA 0.30 $\mu\text{g}/\text{kg}$ (N = 8)	SPA 0.45 $\mu\text{g}/\text{kg}$ (N = 8)	SPA total (N = 20)
<b>Baseline</b>				
Mean (SD)	2.28 (0.43)	2.13 (0.77)	2.31 (1.00)	2.22 (0.87)
Median (range)	2.25 (1.8, 2.8)	2.05 (1.2, 3.5)	2.10 (1.4, 4.4)	2.04 (1.2, 4.4)
<b>Day 1, 8 hours post-dose</b>				
Change from baseline				
Mean (SD)	0.15 (0.39)	-0.61 (0.30)	-0.70 (0.69)	-0.66 (0.51)
Median (range)	0.25 (-0.4, 0.5)	-0.55 (-1.1, -0.3)	-0.40 (-2.3, -0.1)	-0.45 (-2.3, -0.1)
Mean decrease (%)	7	-29	-30	-30
<b>Day 2, 24 hours post-dose</b>				
Change from baseline				
Mean (SD)	-0.25 (0.17)	-0.75 (0.59)	-0.78 (0.49)	-0.76 (0.52)
Median (range)	-0.25 (-0.4, -0.1)	-0.50 (-2.0, -0.3)	-0.75 (-1.7, -0.1)	-0.55 (-2.0, -0.1)
Mean decrease, %	-11	-35	-34	-34
<b>Day 3, 48 hours post-dose</b>				
Change from baseline				
Mean (SD)	-0.30 (0.26)	-0.50 (0.50)	-0.70 (0.57)	-0.60 (0.53)
Median (range)	-0.30 (-0.60, 0)	-0.35 (-1.5, -0.1)	-0.60 (-1.8, -0.1)	-0.40 (-1.8, -0.1)
Mean decrease (%)	-13	-24	-30	27
<b>Day 5</b>				
Change from baseline				
Mean (SD)	-0.43 (0.31)	-0.56 (0.47)	-0.49 (0.67)	-0.53 (0.56)
Median (range)	-0.50 (-0.7, 0)	-0.40 (-1.3, -0.2)	-0.30 (-2.0, 0.1)	-0.35 (-2.0, 0.1)
Mean decrease (%)	-19	-26	21	-24

and at 24 hours and 7 days post-dose; all values were within the normal range (data not shown).

#### PK/Pharmacodynamic Correlations

SPA  $C_{\text{max}}$  values ranged from 3,167 to 12,419  $\text{pg}/\text{mL}$ . In Supplemental Table S5,  $C_{\text{max}}$  and AUC values for all subjects are displayed with their corresponding anti-protein A antibody status and with the post-dose changes in the ALC.

Using the Kendall tau correlation statistic, the changes in lymphocyte counts post-dose showed no significant correlation with either the subjects'  $C_{\text{max}}$  or AUC, suggesting that over this range of exposures (AUC range: 11,839–776,426  $\text{pg}/\text{mL} \times \text{hour}$ ), this pharmacodynamic effect was unaffected by more rapid clearance of SPA in individuals who developed anti-SPA antibodies (Supplemental Figure S1).

#### Gene Expression Profiling and Serum Pharmacodynamic Markers

SPA gene expression patterns were different at 4 and 24 hours after dosing. At 4 hours after dosing with SPA but not placebo (data not shown), expression of the CCL20 gene, also known as MIP-3 $\alpha$ , was up-regulated more than 10-fold. This chemokine can be involved in the extravasation and tissue localization of T and B lymphocytes and immature dendritic cells.<sup>15</sup> Increased gene expression was also seen for the chemokine CCL4 or MIP-1 $\alpha$ , a product of NK cells

and monocytes, and gene expression for CCRL2, an orphan chemokine receptor, was increased. Chemokine expression could be related to the altered lymphocyte trafficking post-dose and could explain the decreased number of circulating lymphocytes.

As seen in Supplemental Table 4, at 24 hours after dosing, transcription of 21 genes are up-regulated in PBMCs by at least twofold after treatment with SPA but not placebo. Gene expression for CX ligand 10, also called interferon-induced protein 10 (IP-10), is up-regulated 10-fold. LR8 is up-regulated eightfold, and serpin-peptidase inhibitor fourfold. Of the 21 up-regulated mRNAs, 11 are genes (marked with an asterisk in S-IV) known to be regulated by the interferon-stimulated response element (ISRE).<sup>16</sup>

Several SPA-modulated genes were highly predictive for the magnitude of lymphopenic response at 24 hours after dosing. To identify gene expression patterns in SPA response, unsupervised hierarchical cluster analysis was performed on the pre-dose to post-dose intensity ratios of all genes across the three subjects with a  $\leq 20\%$  decrease in ALC and the four subjects with a  $\geq 40\%$  decrease. The results of this clustering analysis are presented in heat-map format with the ratio data colored on  $\log_{10}$  scale (Figure 2). The up-regulated genes most associated with post-dose lymphopenia were tryptophanyl tRNA synthetase, IFP 35, IFIP 6, 2–5 oligoadenylate synthetase, clusterin, and serpin peptidase inhibitor. These are all genes regulated by type-1 interferons. Notably, no increase in expression was

**Table 4.** Genes With Greater Than Twofold Changes From Baseline at 24 hours After SPA Dosing and Unique to Active Dosing

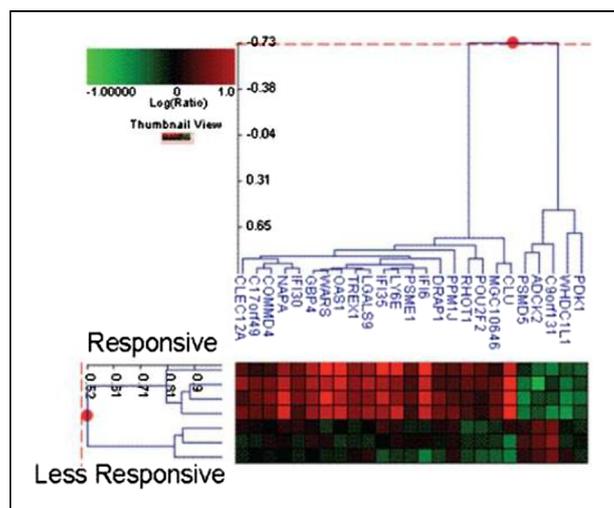
Fold change	P-value	Sequence description
11.9 <sup>a</sup>	0.0497	Chemokine (C-X-C motif) ligand 10
8.3	0.0399	Folate receptor 3 (gamma)
8.1 <sup>a</sup>	0.0334	LR8 protein
4.7 <sup>a</sup>	0.0121	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)
3.4 <sup>a</sup>	0.0768	Interferon-induced protein with tetratricopeptide repeats 1
3.1 <sup>a</sup>	0.0080	Tryptophanyl-tRNA synthetase
3.1 <sup>a</sup>	0.0458	2'-5'-oligoadenylate synthetase 3, 100 kDa
3.0	0.0002	1-acylglycerol-3-phosphate O-acyltransferase 3
2.8	0.0062	Lymphocyte antigen 6 complex, locus E
2.8	0.0254	Guanylate binding protein 4
2.6 <sup>a</sup>	0.0134	2'-5'-oligoadenylate synthetase 1, 40/46 kDa
2.5	0.0004	N-ethylmaleimide-sensitive factor attachment protein, alpha
2.5	0.0942	Lipocalin 2 (oncogene 24p3)
2.5	0.1179	Myomesin (M-protein) 2, 165 kDa
2.4	0.0133	Aldehyde dehydrogenase 3 family, member B1
2.3 <sup>a</sup>	0.1069	2'-5'-oligoadenylate synthetase-like
2.3	0.0215	Fibroblast growth factor (acidic) intracellular binding protein
2.3 <sup>a</sup>	0.0237	Interferon, alpha-inducible protein 6 (IFIP6)
2.3 <sup>a</sup>	0.0136	Interferon-induced protein 35
2.2	0.0130	Chromosome 1 open reading frame 57
2.2 <sup>a</sup>	0.0095	2'-5'-oligoadenylate synthetase 2, 69/71 kDa

<sup>a</sup>Gene known to be responsive to type-I interferons (N = seven active dosed subjects, three placebo subjects).

observed for any genes coding type 1 interferons at either 4 or 24 hours, although plasmacytoid dendritic cells, the main source of type 1 interferon production, are rare in peripheral blood and, thus, are poorly represented in the mRNA extracts from PBMC.

When serum samples were assayed for the chemokines IP-10 and CCL20, many but not all active-dosed volunteers had 2- to 10-fold elevations at 24 hours post-dose compared with pre-dose, a pattern not seen in

placebo-dosed volunteers (data not shown). The correlation between post-dose increase in IP-10 and the percentage decrease in lymphocyte count after dosing was significant by the Kendall Tau statistic ( $P < .02$ ). The eight SPA-dosed subjects with the greatest change in ALC post-dose (mean -42%) had a mean increase of 420% and 422% for IP-10 and CCL20, respectively, at 24 hours post-dose. The eight SPA-dosed subjects with the lowest changes in ALC post-dose (mean -23%) had mean increases of 105% and 144%, respectively. This suggests that the changes observed in interferon-regulated gene expression after SPA dosing can be confirmed by measurement of two of the gene-products in blood samples and could mediate altered lymphocyte trafficking. In four placebo-treated subjects and the six active-dosed subjects with the greatest post-dose increases in IP-10 and CCL20, blood samples at 0, 6, and 24 hours post-dose were assayed for beta- and alpha-interferon, but no elevations in blood levels of these cytokines were associated with SPA dosing.



**Figure 2.** Heat map presentation of gene expression for genes correlated with low or high lymphopenic response 24 hours after SPA dosing.

## Discussion

SPA was well-tolerated overall in healthy adults at doses of 0.3 and 0.45  $\mu\text{g}/\text{kg}$ . This study was conducted after development of a sensitive staphylococcal host-cell protein EIA, and changes in drug substance manufacture which reduced HCP from 800 ppm (0.1%) to <40 ppm. It is likely that the trace HCP contaminants included

cell-wall constituents such as gram-positive lipoproteins, glycoproteins, and muramyl peptides, which are known pyrogens. This “second generation” product had a low incidence of peri-dosing AEs.

As seen in Table 3, the addition of hs-CRP and neopterin to standard safety laboratory evaluations allowed the reduced immune reactivity of the more purified product to be clearly demonstrated. The correlation in both studies of CRP and neopterin elevations with peri-dosing symptoms of cytokine release suggest these biomarkers provide a sensitive and quantitative method to assess the reactivity of novel biologics, and can supplement the data obtained from the reported adverse events. Transient CRP increases of similar magnitude have been reported in studies of influenza and pneumococcal vaccination.<sup>12</sup>

The transient lymphopenic response to SPA injection was not anticipated prior to the first study but proved a useful marker to demonstrate a systemic pharmacodynamic effect associated with very low doses of a novel biologic product. Whether this pharmacodynamic marker is related to the mechanism of action in autoimmune diseases is still unknown but it will be evaluated further in patient studies. The fact that post-dosing lymphopenic changes correlated with PBMC expression of certain chemokine and type-1 interferon-regulated genes provides potential insight into pharmacologic mechanisms. These chemokines can alter trafficking of peripheral lymphocytes, and post-dose lymphopenia is a well-known pharmacodynamic effect of type-1 interferons, which induce similar gene expression changes.<sup>13–15</sup>

SPA, a bacterial protein, was clearly immunogenic. In repeat-dose primate safety studies, development of detectable anti-protein A antibodies after six weekly doses was found to increase SPA clearance.<sup>16</sup> In fact, antibody formation after multiple doses decreased SPA plasma AUC of the last dose to approximately 30% of that seen after the first dose. Later detection of anti-SPA antibodies also predicted the clearance of a single dose of SPA in this human trial. A possible explanation for this association may be that many subjects who developed anti-protein A detectable by EIA have mounted an amnestic response after receiving a single dose. These subjects may have had levels of antibody prior to dosing that were too low to detect by EIA but were, nonetheless, able to affect the clearance of such a small amount of injected drug protein (0.3 or 0.45  $\mu\text{g}/\text{kg}$ ). The fact that Staphylococcal exposure is common and that two of 20 subjects in this study had detectable pre-dose antibodies to SPA is consistent with this explanation.

Significantly, no correlation was found between the SPA AUC and the pharmacodynamic marker of post-dose lymphopenia. It is hypothesized that SPA is pharmacologically active as a complex bound to Vh3 IgG antibodies, and antibody-mediated clearance from plasma

probably does not abrogate activity at cellular targets. Indeed, in mouse studies of collagen-induced arthritis, repeated doses of SPA continued to suppress disease activity *after* the development of mouse anti-protein A antibodies.<sup>3</sup> Obviously, only repeat-dose human studies in RA patients, examining repeated-dose immunogenicity, product clearance, and clinical efficacy endpoints, can fully resolve these questions.

These PBMC gene-expression data suggest that a single dose of SPA up-regulates interferon-regulated genes, without the induction of detectable type-1 interferons. A recent report by Dhodapkar et al<sup>17</sup> has demonstrated that Fc receptor-mediated stimulation of human dendritic cells and monocytes in the presence of blocking antibody to the inhibitory FcR gamma IIB receptor increases expression of interferon-regulated genes without inducing interferon production. Since the binding of SPA to monocytes and NK cells is dependent on the presence of human IgG and probably occurs via one or more Fc receptors,<sup>3</sup> it is possible that interactions of SPA with Fc receptors on peripheral monocytes and NK cells could mediate a similar effect on interferon-regulated genes. Any role that the acute induction of interferon-regulated genes may play in the longer-term immunomodulatory effects of SPA treatment remains to be determined. However, type-1 interferons have reported therapeutic activity in autoimmune diseases such as multiple sclerosis, immune uveitis, and Sjogrens syndrome,<sup>18–20</sup> and this activity may involve effects mediated by interferon-regulated gene products on regulatory T-cells.<sup>21,22</sup>

## Conclusions

This Phase I study characterizes the PK, safety, and PD activity of low IV doses of SPA. The data suggest that despite the likely prevalence of pre-existing antibodies in the general population, and the product's high-affinity binding to human IgG, this bacterial protein can be safely administered at these doses with no evidence of complement activation or immune-complex formation. In addition, the data suggest that acute PD effects were not obviated by the more rapid clearance, which was likely the result of low levels of anti-product antibodies. Use of biomarkers such as C3a des Arg, C1q, hs-CRP, neopterin, the PFA-100, and PBMC gene expression helped to better characterize the reactivity and pharmacologic activity of this drug. A phase 1b study of the product has now been completed in patients with rheumatoid arthritis, who received four weekly intravenous doses of up to 1.5  $\mu\text{g}/\text{kg}$ .<sup>23</sup>

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## Disclosures

Anissa Leh and Edward Bernton were employees of Protalex, Inc. All other authors were employees of Protalex contractors at the time of the study. Dr. Hamilton provided laboratory services under contract to Protalex.

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## Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

**Figure S1.** Graphic displays of AUC versus percentage change in ALC at 24 hours after SPA dosing shows no correlation.

**Table S1.** Summary of Treatment-Emergent (TEAEs) and Treatment-Related Adverse Events (TRAEs)

**Table S2.** Plasma SPA Clearance and Distribution Parameters by Dose and Ultimate Anti-Product Antibody Status

**Table S3.** Changes in PFA-100 Closure Times After SPA Dosing in Study B

**Table S4.** Serum C<sub>max</sub> and AUC Values With Corresponding Percent Change in ALC at 24 hours After Dosing