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14. ABSTRACT

The lysate of *Leishmania tropica* promastigotes is a complex mixture of substances, including proteins in the range of 8 kDa to 70 kDa. In *Leishmania* naïve adult humans, the lysate can cause sensitization manifest by the conversion of a negative to positive delayed-type hypersensitivity (DTH) skin test. This was observed on the third skin test with 30 ug and 50 ug doses of the crude lysate administered intradermally at monthly intervals. Fractionation of the lysate identified dominant proteins at 8 kDa, 20 kDa, and 56-58 kDa. Skin tests in *L. tropica* sensitized guinea pigs with each of these fractions revealed significant differences in the reactivity of the fractions. The 8 kDa fraction, which contained approximately 95% of the lysate mass, failed to elicit a positive DTH response. Conversely, the 20 kDa and 56-56 kDa fractions tested at 1.0 ug, 3.0 ug and 10.0 ug protein elicited strongly positive skin tests comparable in size and intensity to a 50 μg dose of the crude promastigote lysate. In *Leishmania* naïve guinea pigs, the crude lysate and the 8 kDa, 20 kDa and 56-58 kDa fractions all exhibited sensitizing properties at the doses studied. Similar tests were not conducted in *Leishmania* naïve humans.

15. SUBJECT TERMS

LtSTA = *Leishmania tropica* Skin test Antigen

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1. INTRODUCTION

Skin test antigens of *Leishmania* have been used to demonstrate the absence of prior infection with the parasite. Skin test antigens that are sensitizing are of limited value in clinical and epidemiological settings that require repeat testing. The skin test antigen of *Leishmania tropica* (LtSTA) developed by Allermed was intended to be used to screen military and civilian personnel for infection with *Leishmania* during and after multiple deployments to *Leishmania* endemic regions. With the discovery that 30 μg and 50 μg doses of LtSTA were capable of sensitizing *Leishmania* naïve adults after two previous skin tests, it was no longer possible to achieve the primary objective of licensing a multiple-use skin test antigen from the whole promastigote lysate. Rather the emphasis was placed on attempting to separate and remove sensitizing components of the lysate from the product. This report details the work that has been done in evaluating fractions of the lysate relative to their ability to elicit a positive delayed-type hypersensitivity response in *L. tropica* sensitized guinea pigs.

2. BACKGROUND

2.1 SOURCE MATERIAL

Allermed was awarded Contract by the United States Government to develop and license with the Food and Drug Administration a skin test antigen of *Leishmania tropica* (LtSTA). The United States Army believed that this product would be useful in screening military personnel for infection with *Leishmania* following service in regions of the world in which the parasite is endemic.

Prior to beginning this project, Allermed had successfully licensed a skin test antigen of the yeast *Candida albicans* (Candin®) for use in the detection of cell-mediated immunity. For this reason, Allermed was recognized by the U.S. Army as uniquely qualified to develop a *Leishmania* skin test antigen.

The history of *Leishmania tropica* strain WR# 1063 which was provided to Allermed by the U.S. Army is as follows:

Strain WR#1063 was isolated in November 1990 from a bone marrow biopsy sample obtained from a U.S. soldier who acquired viscerotropic leishmaniasis in extreme East Central Saudi Arabia during the Operation Desert Storm (ODS) (Kreutzer et al., 1993. *Am J Trop Med Hyg 49(3)*: 357-363). During clinical evaluation, this patient was shown to be negative for antibodies to the Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV), and Hepatitis C Virus (HCV). This patient was treated for leishmaniasis and remained healthy and without serologic evidence of HIV, HBV, or HCV.

Strain WR#1063 was characterized as *Leishmania tropica* by isoenzyme analysis in Dr. R.D. Kreutzer's laboratory in the Biology Department of Youngstown State University, Youngstown, OH in April, 1991.

Strain WR#1063 was subcultured as strain WR#1063C in the laboratory of Dr. Max Grogl, Dept. of Parasitology, Division of Experimental Therapeutics (Bldg No. 500), Walter Reed Army Institute of Research (WRAIR), Forest Glen Annex, Silver Spring, MD. This subculture (WR#1063C) also was sent to Dr. Kreutzer for isoenzyme analysis. Subculture WR#1063C was subsequently cloned by Dr. Max Grogl producing clone WR#1063:C1A; this clone (WR#1063:C1A) was cryopreserved (Aug 6th, 1992), and stored in the WRAIR cryobank in the Division of Experimental Therapeutics (Bldg No. 500) at WRAIR.

Allermed received two cryopreserved vials of WR#1063:C1A from WRAIR. Upon receipt of the two vials, one vial was placed in liquid nitrogen storage and the other vial was used to establish a supply of *L. tropica* WR#1063:C1A promastigotes for product development and for the generation of a Master Cell Bank of the parasite at Allermed.

2.2 CHARACTERIZATION OF CELL BANKS

Work that was completed on the master and working cell banks of *Leishmania tropica* (WR1063) addressed the recommendations of the guidance document for industry entitled "Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications"(2010). The tests that were performed on isolate WR1063 are shown below. The results of the tests confirmed the speciation of the parasite and absence of the adventitious agents in the cell banks.

Description
Speciation of *Leishmania* Parasites by Isoenzyme Analysis
Assays for *Leishmania* virus RNA Performed on *Leishmania tropica* Seed Material (WR1063), Cell Banks, Raw Material & FBS using TaqMan RT-PCR also Nuclear Hybridization
Assays for Mycoplasma Detection Performed on *Leishmania tropica* Seed Material (WR1063), Cell Banks, Raw Material & FBS: "Points to Consider" Using Indirect Staining and Direct Microbiological Assay
Test for the Presence of Non Agar Cultivable Mycoplasma using Vero Cell Culture Assay
Assays Performed on *Leishmania tropica* Cells Beyond the Production Age Limit: Test for the Presence of Agar Cultivable Mycoplasma
Assay for the Presence of Bovine Viruses in *Leishmania tropica* Parasites

2.3 FORMULATION STUDIES

Formulation studies included the use of several different buffers ranging in pH from ~6-8. Additionally, each combination was formulated with a low (0.0001%) and high (0.01%) concentration of Tween 80. Saline, phosphate, and bicarbonate buffers were formulated with 0.7 mg/mL protein (assayed by Ninhydrin). A high concentration of protein was used to challenge the matrix of the solution to maintain solubility. Samples were stored at 4ºC, 25ºC, and 35ºC. Protein and pH were tested at time zero and at 1 month for samples stored at 4ºC and 25ºC and after 3 months for samples

stored at 35ºC. The presence or absence of precipitation and color change were observed for all temperatures on a weekly basis.

The most stable product formulation was a phosphate buffer system as formulated below:

12.5mM Na_2HPO_4
12.5mM NaH_2PO_4
8.5% NaCl
0.4% Phenol
1.0% Glycerol
0.01% Polysorbate 80

2.4 MANUFACTURE OF PRODUCTION LOTS

LtSTA lots were manufactured using the formula shown above. The release data for stability lots were used to establish in-process and final acceptance criteria for clinical lots, and stability data obtained with the lots were used to determine product stability for the period of time between date of manufacture and completion of clinical trials. Parameters measured included visual inspection, pH, SDS-PAGE, potency, sterility, and protein, phosphate, chloride and phenol content. Visual inspection indicated that the product was slightly opalescent after filling, but became clear after a few days. SDS-PAGE indicated that the banding pattern produced by *L. tropica* was consistent. Data points were measured over a period of 6 years.

2.4.1 Production Process

The LtSTA manufacturing process was developed from a series of carefully controlled studies beginning with the selection of the cultivation medium, passage of the organism from the working cell bank to production cultures, growth of parasite in Celstirs, harvesting and washing promastigotes, microfluidization of promastigotes, heating the lysate at 60°C, and formulation of the drug product.

The assays used in measuring salts and preservative in LtSTA were used routinely at Allermed in the testing of currently licensed products. The data obtained from the use of these procedures in the testing of LtSTA were consistent from lot to lot, which demonstrated that the procedures were capable of yielding uniform results if the parameters of the test remain unchanged.

The general method of manufacture of the drug substance and drug product was consistent throughout the development process. Aeration was added to Celstir cultures to increase the yield of the parasite and the procedure was validated, but no other procedural changes were introduced in the manufacturing process of the promastigotes lysate.

2.4.2 Cultivation and Harvest of Promastigotes

A single vial of the *Leishmania tropica* working cell bank was withdrawn from liquid nitrogen storage. The frozen cell bank was reactivated by aseptically transferring the parasite pellet to 5 mL of Schneider's Medium with 10% Fetal Bovine Serum (FBS) contained in a T25 flask. Percent viability was determined microscopically by counting non-viable cells and recording the results in the batch record.

The T25 flask was observed for promastigotes in the mid to late log growth phase 24 hours after

reactivation. The cell concentration was determined and the volume of inoculum was calculated (≥4 x10^6) prior to being transferred to a T150 flask containing 60 mL of Schneider's medium. Earlier studies used 50 mL of Schneider's at this step, but the amount of medium was changed to 60 mL to increase the culture volume in the flask. Approximately 48 hours after a T150 flask was inoculated, the culture was observed for promastigotes in mid to late log growth phase. Cell concentration was determined and inoculum volume was calculated as described above. The promastigotes were then transferred to two T300 flasks, each containing 450 mL of Schneider's Medium. Earlier studies used 500 mL of Schneider's Medium, but the volume was reduced to 450 mL, due to the possibility of the culture being exposed to the cap of the flask and creating a potential contamination risk. Samples were withdrawn from each flask and placed in T25 flasks to observe for promastigotes in mid to late log growth phase. If acceptable growth was observed, the contents of the flasks were combined, cell concentration determined and the inoculum volume was calculated. The inoculum volume was then placed into a 3-liter spinner flasks (Celstir®) containing 1.5 liters of Half Schneider's medium with 5% FBS. The Celstirs were fitted with a double hose-barbed apparatus with sterile vent filters as illustrated below.

Celstirs were placed on magnetic stir plates set at 80 rpm and medical grade air was supplied through the vent filter directly into the medium at approximately 1 bubble per second. Approximately 72 hours after the Celstir cultures were initiated; samples were withdrawn from each Celstir culture and placed in T25 flasks to observe the stationary growth phase of development. Both cell concentration and total cell numbers were determined. The cells were harvested and then washed with sterile saline by centrifugation. Bioburden samples were taken prior to the final centrifugation run. The pellet was stored at -80 °C until released for further manufacturing.

2.4.3 Preparation of the Drug Substance

A frozen pellet of *L. tropica* promastigotes was removed from -80°C storage and suspended in sterile Saline with 0.0001% Tween 80 (SALT). While one technician thawed the pellet, a second technician set up and prepared to microfluidize the material in a Class II safety cabinet. The flow rate was determined by running the previously sterilized microfluidizer at operating pressure for one (1) minute using the sterile SALT solution and collecting the volume in a graduated cylinder. The processing time was calculated using flow rate and a ten (10) pass production scheme. Once the pellet was completely thawed, the sterile SALT solution was removed from the microfluidizer and replaced with the promastigotes suspension.

Microfluidization occurred for approximately 5 minutes. The lysate was collected in sterile centrifuge tubes and centrifuged for 30 minutes at 18,500 xg. The supernatant was decanted into a sterile glass bottle and the pellet was discarded. The lysate was then heated in a water bath for 30 minutes at 60°C. The material was cooled to 10 °C, poured into sterile centrifuge tubes and centrifuged at 18,500 xg for 30 minutes. The supernatant was retained and the pellet discarded. The supernatant was aseptically filtered using a 0.2µm cellulose acetate filter and retained in a sterile 500 mL bottle. Samples were withdrawn for non-viability testing, protein analysis and SDS-PAGE. The lysate was stored at 2-8°C until released for further manufacturing.

2.4.4 Preparation of the Bulk Drug Product

The drug product was prepared by aseptically combining the drug substance (promastigotes lysate) with an equal volume of 2X phosphate diluent. The lysate and 2X phosphate diluent were mixed for 10 minutes using a magnetic stirring device. The protein concentration of the diluted lysate was determined and the lysate was further diluted to the desired protein concentration (0.5 mg/mL) with 1X phosphate diluent containing10% Tween 80. The solution was again mixed for 10 minutes and resampled for protein content to ensure that the correct dilution was made. Phenol, present as the preservative in both diluting buffers, was measured at this point in the manufacturing process.

The bulk drug product was tested for bioburden and aseptically filtered using a sterile 0.2µm cellulose acetate filter and peristaltic pump. Following filtration, samples were taken for the bulk sterility test, in-process protein determination, and in-process potency testing. The bulk drug product was stored at 1-5 °C until further processed in final containers.

2.4.5 Preparation of Final Drug Product

The preparation of the final drug product involved dispensing the bulk drug product into final containers. This process was performed in a dedicated, limited access, classified area. A vigilant and responsive program for environmental monitoring was followed to assess the effectiveness of the cleaning and sanitization procedures. The filling, capping and assembly of final containers occurred in Class 100 workstations furnished with VLF HEPA filters which were located in Class 10,000 rooms.

To ensure product sterility, operators were trained to follow aseptic processing procedures. Personnel involved in the filling operations were trained in gowning, aseptic technique, hygiene, and cleanroom behavior. Prior to entering the aseptic processing area, operators dressed in sterile clean room

garments were assisted in donning sterile sleeves and gloves and assisted in entering the sterile processing room. The aseptic processing of LtSTA involved the following procedures:

Staging for sterile filling began with the removal of the carboy containing the sterile bulk drug product from 1-5 °C storage and transferring it to the entrance of the filling room. The outer plastic bag was removed and the bulk product was then taken into the filling room where the second plastic bag was removed under a Class 100 VLF HEPA hood. Sterile tubing connected to the dispensing pump was then aseptically placed in the bulk product. At this point in the process, the bulk drug product was ready to be dispensed into final containers.

At the start of the filling operation, temperature and relative humidity readings from a Met-One particle counter and differential pressure from a magnehelic gauge were recorded in the Batch Production & Control Record (BPCR).

During the fill, positive air pressure in the Filling Room was continuously monitored by an electronic Honeywell Multitrend Recorder. The temperature (range 64-76 °F) and humidity (range ≤65%) also was read and recorded in the BPCR.

Environmental monitoring of the air for viable particles was achieved using a SMA MicroPortable viable particle counter. The SMA particle counter was located next to the containers being filled. Sequential sampling of 10^3 ft of air onto Trypticase Soy Agar (TSA) plates placed within the SMA unit was accomplished with a new TSA plate challenged every thirty (30) minutes. After incubating the plates at 20-25 °C for 7 days, the results were recorded into the BPCR and reviewed by a Quality Control Unit (QCU) member.

Environmental monitoring of the air for total particles was achieved using a MET One laser particle counter Model A2408 equipped with an eight (8) foot transit tube. During filling operations the MET One probe was placed next to the containers being filled. The sampled volume was 1.0 ft^3 collected over 1 minute. Particle counts ≥0.5µm were recorded at five minute intervals during the filling operation. The raw data were included in the BPCR.

The filling operation was a seamless, four stage process: (1) bulk product was pumped from a sterile holding vessel through sterile Wheaton 3 mm ID silicone tubing; the tubing was fitted with a six inch dispensing nozzle that was used to deliver a uniform volume to each container; a dedicated operator filled the containers; (2) a second dedicated operator applied stoppers to the filled containers using sterile forceps, (3) a third dedicated operator applied seals to the filled and stoppered containers, (4) a fourth dedicated operator crimped the vials. All operations were performed under a Class 100 VLF HEPA hood.

Throughout the filling operation a technician was responsible for staging materials, supplying operators with packaging components and removing finished product from the filling table. A microbiologist was responsible for air, surface and personnel monitoring, as well as recording the lot numbers of all packaging components used in the fill in the BPCR. A production supervisor directed the filling process to ensure adherence to proper procedures and aseptic technique.

At the conclusion of the fill, TSA/Lecithin/Polysorbate-80 touch plates were used to sample the sleeves and gloves of dedicated operators. The plates were incubated for three to five days at 20-25°C then transferred to 30-35°C for three additional days; the results were read and recorded. The QCU was responsible for the analysis of results and for determining if follow-up was required. Deviations from established procedures were documented and justified, or the product was discarded.

2.4.6 Quality Control Testing of Final Drug Product

The range of values for the components of LtSTA was based on the results of product testing of seventeen lots. The inherent error of each assay used in measuring the concentration of excipients and other attributes of LtSTA was reflected in the value obtained for the assay. The sensitivity and specificity of the assay and the variability associated with the performance of the assay were reflected in the final result of the test procedure. The high and low values (range) for the excipients are summarized below.

2.4.7 Range Of Test Results For Excipients And pH For Seventeen Lots Of LtSTA

Test	***Low Value***	***High Value***
Phenol	0.40 %	0.43 %
Glycerin	0.87 %	0.94 %
Sodium Chloride	0.87 %	0.92 %
pH	6.8	6.9
BSA	21.0 ng/mL	106.0 ng/mL
Phosphate	2.16 mg/mL	2.76 mg/mL

2.5 Clinical Development

During the course of developing LtSTA, three clinical trials were conducted. A Phase I dose-response safety trial was conducted in 2005-2006 in which four groups of eight adult volunteers per group were skin tested with four different concentration of LtSTA. In 2007 a Phase II dose-response safety/efficacy trial was conducted in Tunisia. This study involved 100 adult volunteers. Based on the results of the 2007 trial, a Phase IIB dose dependent sensitization study was conducted in 2008-2009. In this study, 41 adult volunteers completed the trial. Summaries of the Phase I, Phase II, and Phase IIB trials are reported below.

2.5.1 Phase I Dose-Response Safety Trial

This phase I clinical trial was designed as a placebo-controlled, dose escalation study using four concentrations of LtSTA. Four cohorts of eight healthy adult volunteers without previous known exposure to *Leishmania* were enrolled in the study. Each cohort had a gender ratio of 50:50. Candin® and *Trichophyton* allergenic extract (1:1000 w/v) were used to qualify subjects as DTH reactive and were administered at least 1 week prior to LtSTA administration. A saline control also was administered with the DTH control antigens. LtSTA was supplied to the investigational site at a target concentration of 120 µg/0.1 mL (acceptable limits were 110 µg/0.1 mL to 130 µg/0.1 mL Ninhydrin protein). This 120 µg product was diluted in the clinic to prepare the 20, 40 and 80 µg doses per 0.1 mL. For all skin tests, 0.1 mL was injected intradermally into the volar surface of the forearm.

Beginning with the smallest dose of 20 µg the dosage of LtSTA was increased in each cohort. Intradermal injections of placebo and saline control were administered concurrently with LtSTA

Initially, eight healthy volunteers (Cohort 1) received 0.1 mL ID of LtSTA containing 20 µg protein/0.1 mL. Based on the absence of clinically significant adverse reactions in the volunteers of Cohort 1 during a two-week observation period, 0.1 mL of the 40 µg protein/0.1 mL concentration of LtSTA was administered to Cohort 2, and so on through Cohort 4. Each of the volunteers were followed for two weeks to assess local and systemic reactions. Subjects were required to complete a daily adverse event diary during the two-week observation period. Blood and urine samples were obtained from each study participant before skin testing and 2 weeks after the administration of LtSTA to perform laboratory tests for measures of test article tolerability. Tests for infectious agents were performed prior to admission into the study. Volunteers were enrolled sequentially as they qualified and consented to participate.

The four cohorts, each consisting of eight adult volunteers without a history of exposure to *Leishmania* parasites, were skin tested with four ascending doses of LtSTA. Recordings were made at 24 and 48hrs for induration at the skin test site. Positive and negative controls were used to more clearly define the skin response to LtSTA.

Induration ≥5 mm was not observed to any of the four ascending doses of LtSTA at 24 and 48 hrs. A local inflammatory response to the highest dose of 120 µg was observed in two subjects at days 9 and 16 after skin testing. This response disappeared after several days in both subjects. Topical steroid cream was administered to one subject to promote resolution.

2.5.2 Phase II Dose-Response, Safety/Efficacy Trial

To achieve the objectives of the study it was necessary to evaluate LtSTA in a population of individuals residing in a geographic area in which leishmaniasis occurs.

Studies in guinea pigs conducted by Allermed demonstrated the presence of cross-reactivity between *L.major* and *L.tropica.* Guinea pigs sensitized to *L.major* were skin tested with LtSTA and found to have positive DTH reactions to the antigen. This finding was of particular interest in that the vast majority of *Leishmania* cases diagnosed in U.S. Military personnel serving in Afghanistan and Iraq were infected with *L.major.*

To evaluate LtSTA in *L.major* infected persons the study was conducted in Sidi Bouzid, Tunisia. The study site was established in 1992 in collaboration with WHO/TDR through a research strengthening grant to the Epidemiology Department of Institut de Tunis, Tunisia. The site is located in central Tunisia in the focus of cutaneous leishmaniasis, 250 kilometers away from Tunis.

The clinical trial was designed to first determine a dose of LtSTA that could be used to identify persons with present or past infection with *L.major*. This was done by evaluating the skin test response to four concentrations of the antigen in persons with active CL. From the results of this research, a best fit dose-response line was constructed and the dose estimated to elicit a 15 mm

induration response was calculated. This dose (30 μg) was evaluated for sensitivity and specificity in persons with and without a history of CL caused by *L.major*.

A total of 100 volunteers, ages 18-65 years, inclusive of both genders were involved in the study. Volunteers were recruited from the local community by non-coercive means. Twenty volunteers with active cutaneous leishmaniasis (CL) were enrolled in the titration phase of the study. Forty volunteers with a history of CL within the past 24 months and forty volunteers with no history of active CL were enrolled in the sensitivity/specificity phase of the study.

The decision to conduct the trial in an *L.major* endemic area was based on laboratory data from guinea pigs which showed DTH cross-reactivity between the two species. The hypothesis that *L.tropica* antigen could detect prior exposure to *L.major* in humans was proven valid in subjects with active and healed CL caused by *L.major*. Dose-response testing of persons with active CL with LtSTA concentrations of 10 μg, 20 μg, 40 μg, and 80 μg demonstrated 100% reactivity. As anticipated, the size of the DTH skin test response increased with the corresponding increase in dose; however, the test was positive at all four dose levels. From these data, a dose of 30 μg was selected to evaluate the sensitivity of LtSTA in subjects with healed *L.major* CL. This work revealed that 85% of persons with a history of CL within the past 24 months were skin test positive to LtSTA. Conversely, testing LtSTA in persons residing in an endemic area for *L.major*, but without histories of CL, demonstrated a high level of product specificity. In this population LtSTA did not elicit positive skin tests in 97% of the subjects tested. In a follow-up investigation, six subjects with histories of healed CL who failed to react to the 30 μg dose of LtSTA were skin tested with a 50 μg dose. All six subjects had positive skin tests to the 50 μg dose. This finding confirmed that sensitivity to cross-reacting components also was present in these individuals, but required a higher concentration of LtSTA to evoke a measureable induration response.

The reactivity pattern observed with ascending doses of LtSTA between 10 and 80 μg in subjects with active CL demonstrated the levels at which cross-reactivity occurs between *L.tropica* and *L.major*, i.e. in persons with current disease where sensitivity might be most pronounced, a relatively small dose (10 μg) of the product was capable of evoking a positive DTH response. However, with the passing of time, sensitivity apparently decreased in some individuals as reflected in the skin test patterns of the six subjects who were negative to the 30 μg dose, but positive to the 50 μg dose.

2.5.3 Phase II Sensitization Trial

This clinical trial was conducted for the purpose of evaluating the safety of administering three doses of LtSTA and to investigate the sensitizing capacity of the skin test antigen in *Leishmania* naïve adult volunteers. Three doses of LtSTA at concentrations of 15 μg, 30 μg and 50 μg were administered at 30-day intervals to participants. Each participant received either a 15 μg, 30 μg or 50 μg dose throughout the study.

The results of skin tests with the 15 μg dose of LtSTA were as follows: twelve subjects received one intracutaneous dose. Eleven subjects received two intracutaneous doses and eight subjects received three intracutaneous doses. No positive DTH skin tests were observed to LtSTA or the placebo and saline controls. A 3 mm induration response to LtSTA was observed after the first dose in one subject, but induration was not observed to LtSTA in this subject after the second and third tests.

The results of skin tests with the 30 μg dose of LtSTA were as follows: twenty-seven subjects received one intracutaneous dose. Twenty-four subjects received two intracutaneous doses and twenty-three subjects received three intracutaneous doses. A 9 mm induration response to LtSTA was observed in one subject with the third skin test. All others failed to convert from a negative to positive skin test after three intradermal injections of a 30 μg dose of LtSTA.

The results of skin tests with the 50 μg dose of LtSTA were as follows: eleven subjects received one intracutaneous dose. Ten subjects received the second and third dose of the antigen. The 50 μg dose elicited a 11.5 mm induration response in one subject on the third skin test. Further evaluation of this dose was believed to be unnecessary, since a positive DTH response in one member of this cohort demonstrated that the 50 μg dose was capable of sensitizing individuals that were immunologically naïve to *Leishmania* antigen.

2.5.4 Notable Findings Regarding Clinical trials

1. LtSTA containing 20 μg to 120 μg/0.1 mL of Ninhydrin protein was administered to *Leishmania* naïve human volunteers without causing serious adverse events.
2. A 30 μg dose of LtSTA detected cellular hypersensitivity in 85% of persons with a history of cutaneous leishmaniasis caused by *L. major* within the past 24 months.
3. A 30 μg dose of LtSTA did not detect cellular hypersensitivity in 97% of persons without a history of cutaneous leishmaniasis caused by *L. major.*
4. A 50 μg dose of LtSTA detected cellular hypersensitivity in persons with a history of cutaneous leishmaniasis caused by *L. major* who were skin test negative to a 30 μg dose of LtSTA.
5. Three repeat doses of 30 μg and 50 μg of LtSTA were safely administered to *Leishmania* naïve human volunteers. However, one study subject converted from skin test negative to skin test positive with the 30 μg dose on the third skin test and one subject converted from skin test negative to skin test positive on the third skin test with the 50 μg dose.
6. Adverse events in study participants following a skin test with LtSTA were largely expected events that are known to occur following the administration of a skin test antigen. Local AE included itching, swelling and pain at the site of the skin test. These events were mostly mild and occurred within the first 48 hours after skin testing. Systemic events included body ache, weakness, dizziness and nausea. The cause of these events is not known, since a positive DTH control, placebo and saline were administered concurrently with LtSTA. Unlike local AE where the reaction site can be identified with a specific article, it was not possible to identify the article responsible for a systemic AE in these studies.

2.6 PROTEIN CHARACTERIZATION OF LTSTA

Leishmania skin test antigens have been prepared from suspensions of whole or fragmented promastigotes, or lysates of promastigotes. The rationale for using preparations that are representative of the whole organism has been based on the assumption that sensitization could involve more than a single component or antigen. For this reason, LtSTA was developed using the entire lysate of the promastigotes of *L.tropica.* However, due to the observation that repeat doses of LtSTA at concentrations of 30 µg and 50 µg resulted in sensitization of naïve individuals, a decision was made to fractionate the lysate with the intention of removing potentially sensitizing components. To accomplish this, SDS-PAGE preparations were made of the drug substance (whole lysate) and drug product (LtSTA) and the predominant protein bands were identified and subsequently sequenced.

In the SDS-PAGE studies reported in Allermed's IND application, polyacrylamide gels were stained with silver nitrate. With this procedure, protein bands were observed at 70 kDa, 51 kDa, 25 kDa, 20 kDa and 8 kDa. Minor variations of these bands were observed in lots manufactured throughout the development of LtSTA. Coomassie Blue stained preparations revealed dominant bands at 67 kDa, 58 kDa, 30 kDa, 20 kDa and 8 kDa. In comparing gels stained with silver nitrate and Coomassie Blue, it appeared that the 70 kDa and 67 kDa bands represented the same components; this also was true for the 51 kDa and 56 kDa bands and the 25 kDa and 30 kDa bands. For this reason, the 67 kDa, 56 kDa, 30 kDa, 20 kDa and 8 kDa bands were isolated from Coomassie Blue stained gels and the proteins analyzed using NanoLC-ESI-MS/MS peptide sequencing technology. This work was performed by ProTech, 2550 Boulevard of the Generals, Norristown, PA., 19403. The results of these studies revealed that the 8 kDa band was ubiquitin (mixture of nucleic acids and peptides); the 20 kDa band consisted of threonine peptidase and iron superoxide dismutase; the 30 kDa band consisted of NGG1 interacting factor 3-like protein; the 56 kDa band contained trypanothione reductase, carboxypeptidase, metallo-peptidases dihydrolipoamide dehydrogenase, and the 67 kDa band consisted of cysteine peptidase, aminopeptidase, and two metallo-peptidases.

3. BODY OF REPORT

3.1 STATEMENT OF WORK

The identification of several major proteins in the lysate of *L. tropica* using NanoLC-ESI-MS/MS peptide sequencing technology provided a basis for evaluating the importance of these compounds in the delayed hypersensitivity response observed in guinea pigs sensitized to the promastigotes of *L. tropica*.

Work performed during this reporting period involved further study of the major and minor proteins in *L. tropica* promastigote lysate and the role of these proteins in eliciting a positive delayed hypersensitivity (DTH) response in sensitized guinea pigs after intracutaneous administration. Two objectives were considered: (1) isolation of a DTH active fraction that could be used as a skin test antigen, and (2) separation of the sensitizing components of the lysate from the DTH active fraction.

3.2 KEY ACCOMPLISHMENTS

3.2.1 Fractionation of LtSTA by Fast Protein Liquid Chromatography

Through experimentation with several different SDS-PAGE gel staining reagents and kits [Coomassie Blue R-250, SilverSNAP stain kit (Pierce), and SilverXpress stain kit (Invitrogen)] a procedure was identified that resulted in high resolution, and crisp banding patterns. Using this system, SDS-PAGE profiles of the drug product from multiple batches were compared; the results showed that several bands were consistently present between lots of the drug substance as shown below:

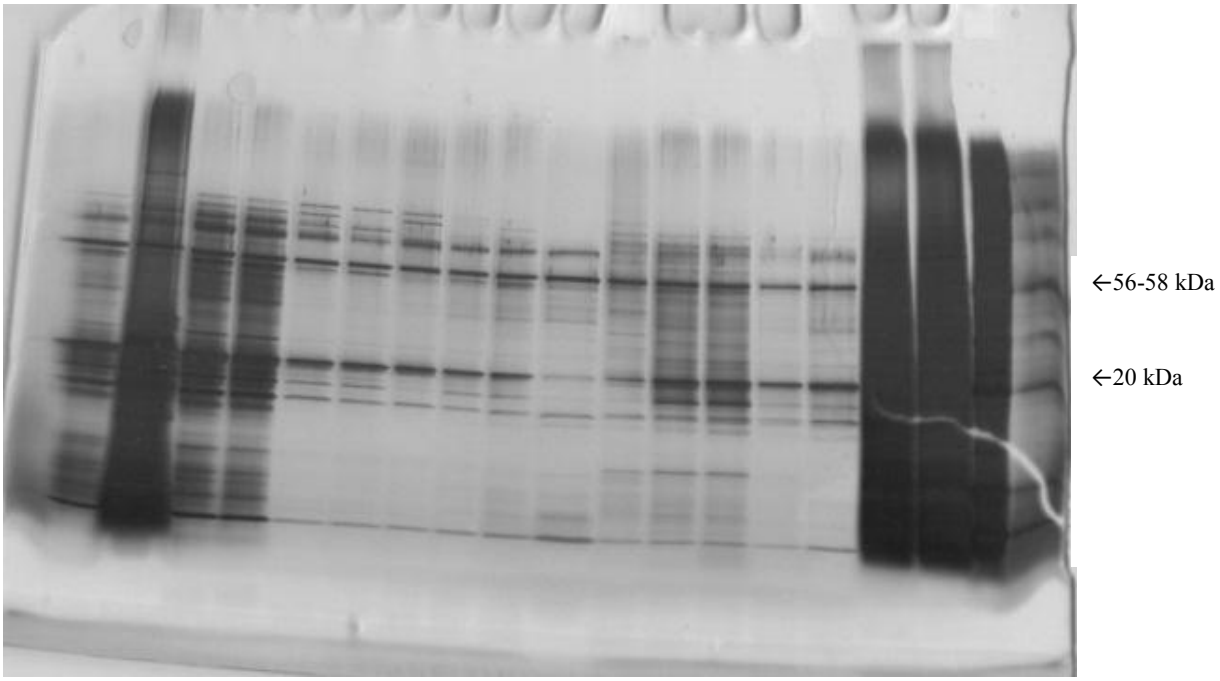

Figure 1. Silver-stained proteins separated by SDS-PAGE. Multiple batches showed very similar protein banding patterns between 8 kDa and 70 kDa.

To evaluate the skin test activity of the proteins identified in polyacrylamide gels at 67 kDa, 56 kDa, 30 kDa, 20 kDa, and 8 kDa, the drug substance was fractionated with fast protein liquid chromatography (FPLC) using a Superdex 200 10/300 GL column (GE Healthcare Life Sciences).

Preliminary studies using FPLC and a Nanodrop spectrophotometer (Thermo Scientific) showed that over 95% of the mass of the product was contained in a column fraction corresponding to components 8 kDa. This fraction was identified as "Ubiquitin" by mass spectrometry and by U.V. absorption at 260/280. The fraction identified as flow through (FT) had an absorption ratio of 1.66 which was indicative of DNA rather than protein. The 260/280 ratio of pure DNA is 1.8 whereas protein is 0.5-0.8. The 260/280 ratio of the FT (8 kDa) fraction and the drug substance were essentially the same. This finding demonstrated the mass dominance of the 8 kDa fraction in the drug substance even though previous densitometry studies had shown that the 8 kDa fraction accounted for approximately 40% of the total protein banding on polyacrylamide gels stained with Coomassie blue dye. In contrast, the 260/280 ratios of the fractions identified as shoulder (26 kDa), 11 mL (67 kDa), 13 mL (56-58 kDa), 15 mL (20 kDa) and the (above 8 kDa concentrate) were consistent with the 260/280 ratio of proteins as summarized in Table 1 below.

Table 1. Ultraviolet Light Analysis of LtSTA Drug Substance Showing 260/280 Ratios for Fractions.
Samples 1-9 (First Run) Samples 10-18 (Second Run)

#	Sample ID	User name	Date and Time	Protein Conc.	Unit	A280	260/280	Sample Type
1	FT	AlphaImager	3/8/2012 11:52:25 AM	9.841	mg/ml	9.841	1.66	1 Abs = 1 mg / mL
2	Initial	AlphaImager	3/8/2012 11:53:36 AM	9.925	mg/ml	9.925	1.65	1 Abs = 1 mg / mL
3	Blank	AlphaImager	3/8/2012 11:54:20 AM	-0.008	mg/ml	-0.008	-2.11	1 Abs = 1 mg / mL
4	Aggregate	AlphaImager	3/8/2012 11:55:14 AM	0.530	mg/ml	0.530	1.09	1 Abs = 1 mg / mL
5	Shoulder	AlphaImager	3/8/2012 11:56:16 AM	0.279	mg/ml	0.279	0.89	1 Abs = 1 mg / mL
6	11ml	AlphaImager	3/8/2012 11:57:10 AM	0.079	mg/ml	0.079	0.97	1 Abs = 1 mg / mL
7	13ml	AlphaImager	3/8/2012 11:57:51 AM	0.860	mg/ml	0.860	0.72	1 Abs = 1 mg / mL
8	15ml	AlphaImager	3/8/2012 11:58:33 AM	0.375	mg/ml	0.375	0.73	1 Abs = 1 mg / mL
9	8+conc	AlphaImager	3/8/2012 11:59:04 AM	3.287	mg/ml	3.287	0.74	1 Abs = 1 mg / mL
10	FT2	AlphaImager	3/8/2012 12:01:35 PM	9.852	mg/ml	9.852	1.65	1 Abs = 1 mg / mL
11	Initial2	AlphaImager	3/8/2012 12:02:16 PM	9.966	mg/ml	9.966	1.65	1 Abs = 1 mg / mL
12	Blank2	AlphaImager	3/8/2012 12:03:33 PM	-0.003	mg/ml	-0.003	-7.03	1 Abs = 1 mg / mL
13	Aggregate2	AlphaImager	3/8/2012 12:04:20 PM	0.564	mg/ml	0.564	1.07	1 Abs = 1 mg / mL
14	Shoulder2	AlphaImager	3/8/2012 12:04:58 PM	0.254	mg/ml	0.254	0.91	1 Abs = 1 mg / mL
15	11mL2	AlphaImager	3/8/2012 12:05:41 PM	0.088	mg/ml	0.088	0.94	1 Abs = 1 mg / mL
16	13mL2	AlphaImager	3/8/2012 12:06:28 PM	0.855	mg/ml	0.855	0.70	1 Abs = 1 mg / mL
17	15mL2	AlphaImager	3/8/2012 12:06:57 PM	0.374	mg/ml	0.374	0.71	1 Abs = 1 mg / mL
18	8+conc2	AlphaImager	3/8/2012 12:07:40 PM	3.268	mg/ml	3.268	0.74	1 Abs = 1 mg / mL

In an attempt to identify which proteins in the drug substance were involved in the DTH response, the drug substance was fractionated as follows:

Drug substance containing 3.5 mg/mL Ninhydrin protein was filtered and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-30 membrane (Millipore). Centrifugation at 4000 x g for 10 minutes was performed four times to ensure complete removal of all high molecular weight proteins. The flow through (FT) was collected and run over a Superdex 200 10/300 GL column to confirm that all high molecular weight peaks were removed. No peaks higher than the 8 kDa peak (19.96 mL fraction) were detected in the FT using FPLC (Figure 2). This material was considered the 8 kDa fraction with a peak 19.96 mL.

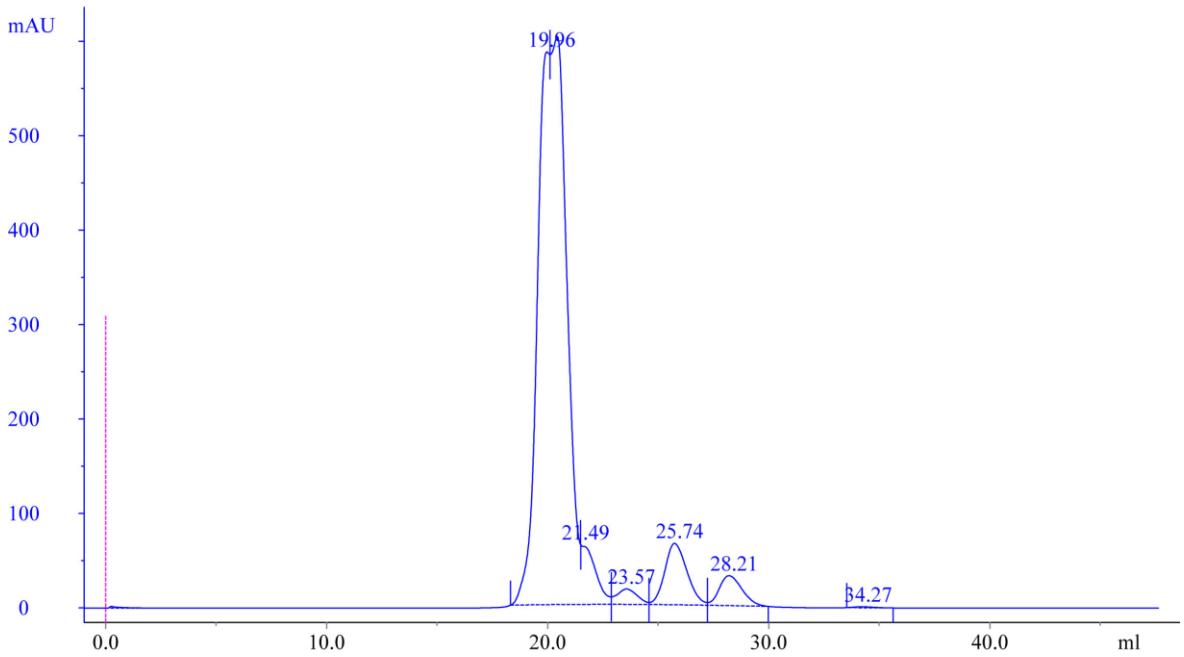

Figure 2. FPLC analysis of the 8 kDa and below fraction. UV absorbance of 1 mL fractions from the Superdex 200 column. The 20 mL peak corresponded to molecules that were 8 kDa. All other peaks in this sample were smaller than 8 kDa.

The protein retentate collected from filtration and concentration steps above was pooled and resuspended in sterile Dulbecco's PBS (DPBS) to a final volume of 250µl. The protein retentate was then run over a Superdex 200 10/300 GL column and all fractions above 8 kDa (below 20.16 mL fraction) were collected (Figure 3).

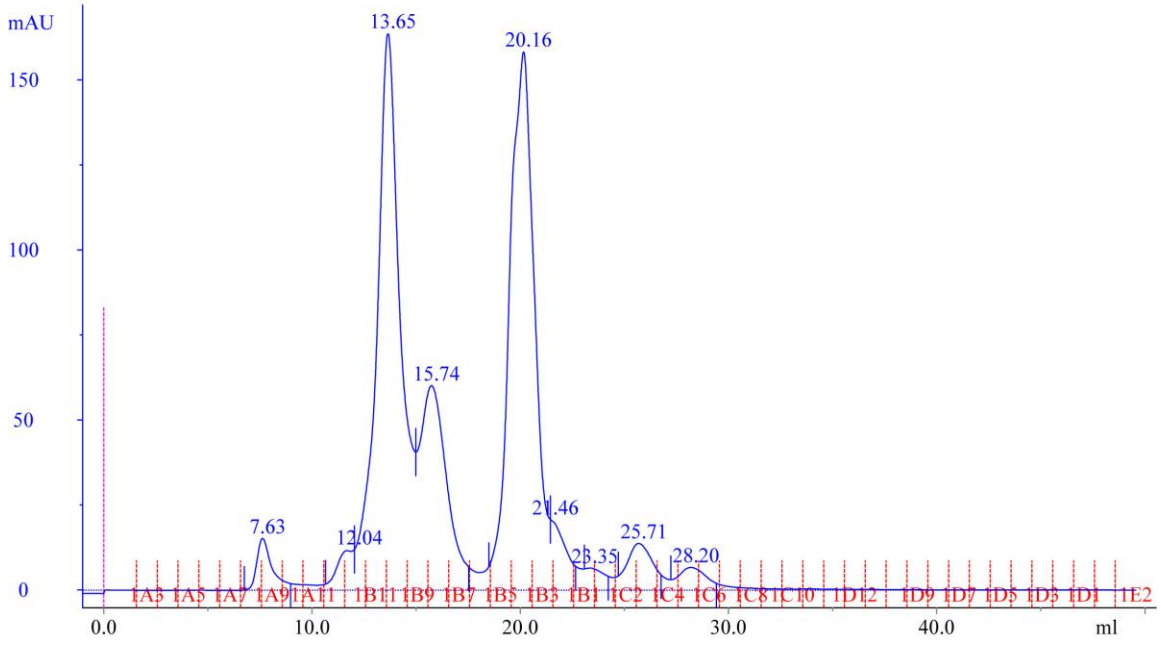

Figure 3. FPLC analysis of the protein retentate after the first filtration and concentration step. UV absorbance of 1 mL fractions from Superdex 200 column. The 20 mL peak corresponded to molecules that are 8 kDa. All peaks that migrated faster through the column (7.63 mL, 12.04 mL, 13.65 mL, and 15.74 mL) were collected as the above 8 kDa fraction.

The remainder of the above 8 kDa fraction was pooled and reconcentrated to serve as the combined above 8 kDa fraction. A portion of the combined above 8 kDa fraction again passed through the Superdex 200 10/300 GL column to confirm that the proper peaks were collected. In addition to the dominant peaks discussed above, a number of smaller peaks below 10 kDa were also seen in the above 8 kDa fraction, which indicated that protein degradation was occurring in the sample. See Figure 4 below.

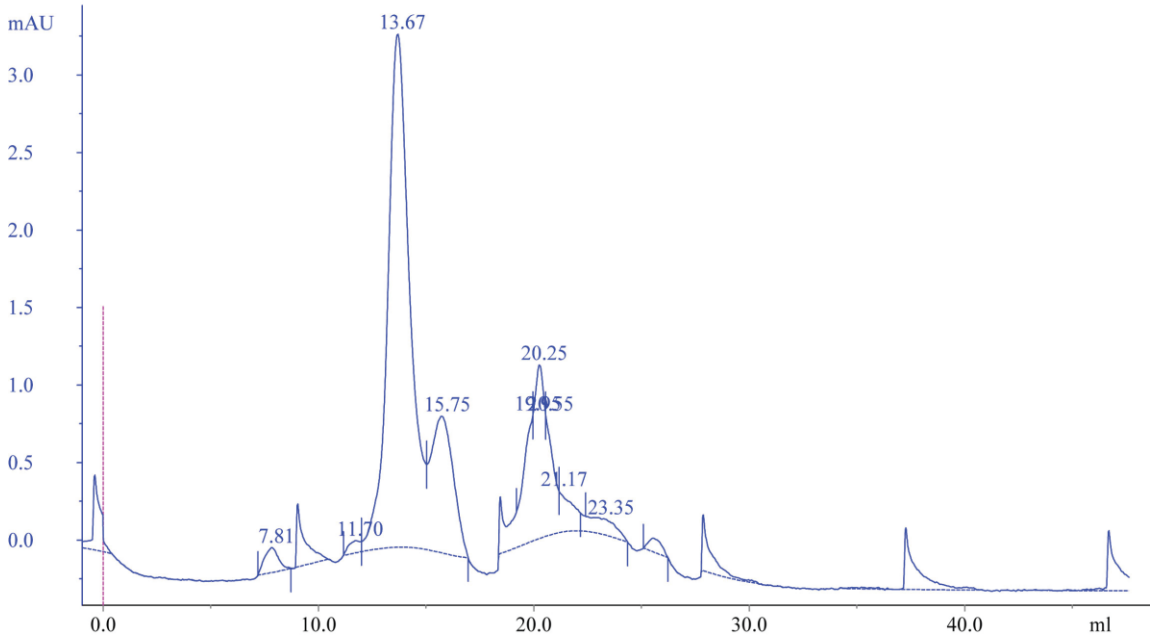

Figure 4. FPLC analysis of the above 8 kDa fraction. UV absorbance of 1 mL fractions from the Superdex 200 column. The 20 mL peak corresponded to molecules that were 8 kDa. The peaks representing molecules larger than 8 kDa were present in this fraction along with smaller degradation peaks.

Both the above 8 kDa fraction and the 8 kDa fraction were analyzed by UV spectrophotometry to (approximately) quantify the amount of protein in each fraction. The above 8 kDa fraction possessed a 260/280 ratio of 0.6 indicating that this fraction was pure protein. In contrast, the 8 kDa fraction had a 260/280 ratio > 1.5 indicating significant DNA contamination.

As previously mentioned, over 95% of the mass of the product was contained in the fraction corresponding to 8 kDa as determined by FPLC and the Nanodrop spectrophotometer (Thermo Scientific). The two fractions were also analyzed for protein using the bicinchonic acid (BCA) method. The relationship between the amount of protein in the two fractions and the lot of drug substance from which the fractions were obtained is reported in the table below.

Table 2. BCA Analysis of LtSTA the Drug Substance, The Above 8 kDa Fraction, and The 8 kDa Fraction. Number represents µg/mL of BCA reacting material.

	Whole Drug Substance	Fraction Above 8 kDa	Fraction 8 kDa
Replicate	Concentration (µg/mL)		
1	183.4	13.5	175.2
2	223.3	13.9	214.1
Average	203.4	13.7	194.7
Standard Deviation	28.214	0.283	27.506
% CV	13.87	2.06	14.13

3.2.2 Delayed Hypersensitivity Response to Fractions of *L.tropica* promastigote Lysate

The skin test activity of the 8 kDa fraction and the fraction containing all moieties with a molecular mass greater than 8 kDa were evaluated in Hartley hairless guinea pigs (HA-HO IAF) that had been sensitized to *L.tropica* promastigotes. The non-fractionated lysate drug substance and the buffers used in the fractionate process were tested concurrently on the same animals. The results of this experiment are shown below:

Article Tested	Skin Test Result
Drug Substance	Positive
8 kDa Fraction	Negative
Above 8 kDa Fraction	Positive
Buffer Control*	Negative

*Dulbecco PBS

This experiment was repeated with serial 10-fold dilutions of the 8 kDa fraction, above 8 kDa fraction and the drug substance. Four sets of sensitized guinea pigs were skin tested with the three preparations. Each set consisted of six animals. The results are shown in the following table:

Table 3. Mean Induration (mm) in Guinea Pigs Sensitized with *Leishmania tropica* and Tested with Fractions of Drug Substance, as well as Non-Fractionated Drug Substance.

	Above 8 kDa		8 kDa		Drug Substance	
	Dose (µg)	Induration (mm)	Dose (µg)	Induration (mm)	Dose (µg)	Induration (mm)
1:100	1.1	9	4.1	0	3.8	2
1:1,000	0.11	6	0.41	1	0.38	2
1:10,000	0.011	3	0.041	1	0.038	2
1:100,000	0.0011	0	0.0041	0	0.0038	0

The results of this work revealed the following: The above 8 kDa fraction was significantly more potent than the drug substance (whole promastigote lysate). A positive skin test was observed with 1:100 and 1:1,000 dilutions of the fraction. The drug substance did not elicit a positive test at those dilutions, and the 8 kDa fraction was not skin test positive at any dilution tested.

FPLC analysis of the drug substance is illustrated in the Figure below. Ultraviolet absorbance of the effluent from a Superdex 200 column resulted in dominant peaks at 13.79 mL and 15.78 mL points in the collection process. The portion of the fraction directly under the peak was collected to best represent the components corresponding to 67 kDa, 56-58 kDa, and 20 kDa. The profile of the drug substance above 8 kDa is shown in figure (5) below with the corresponding SDS-PAGE bands and sample numbers.

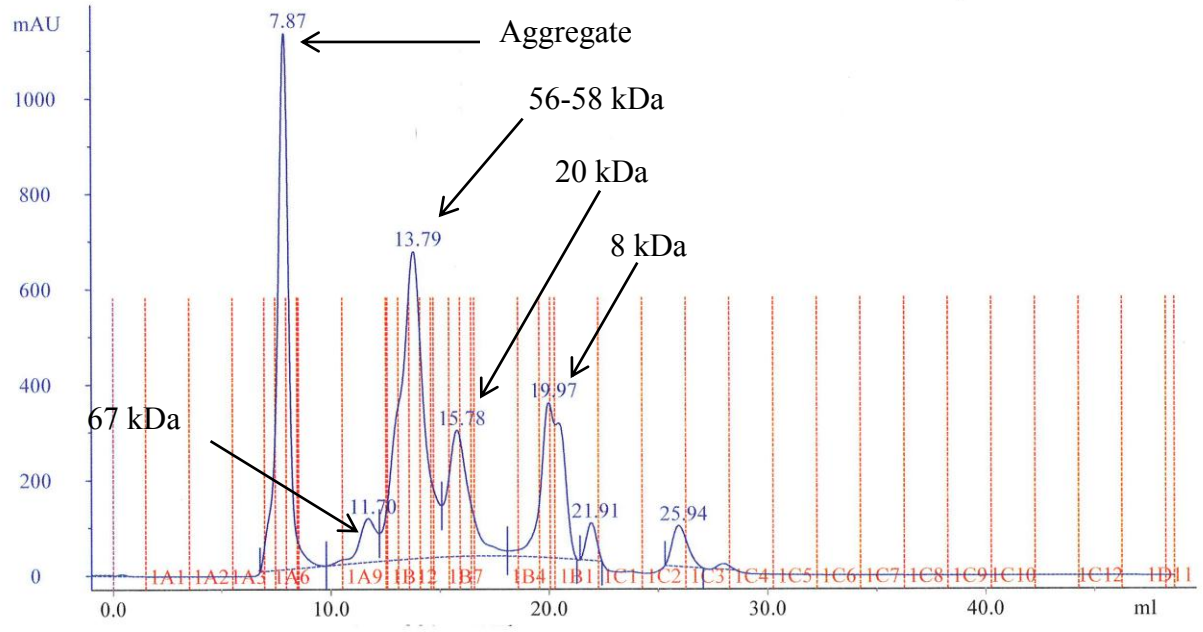

Figure 5. FPLC Profile of *L.tropica* Drug Substance Showing Major and Minor Peaks

The molecular mass of the protein in each peak is shown with arrows. The peak at 7.87 mL is the Aggregate which represents a mixture of components.

To evaluate the skin test reactivity of the main protein groups (20 kDa, 56-58 kDa) on the guinea pigs sensitized to *L.tropica* promastigotes, each fraction was normalized to 0.1 mg/mL and three-fold dilutions were prepared, resulting in 0.1 mL dose of 10 µg, 3.0 µg, 1.0 µg, 0.3 µg. The 67 kDa fraction was not included in this study, since this fraction was not consistently present in all lots of drug substance. As a control, reference LtLRS02 was administered concurrently in the same animals. The 50 µg dose of the reference was diluted to bring the amount of protein administered more closely to that of the lysate fraction. The 8 kDa fraction was omitted from the study, since previous studies had shown that the 8 kDa did not elicit a DTH skin test in sensitized animals. The results of this work are summarized in Table 4.

Table 4. Skin tests with fractions in Guinea pigs Sensitized to *L.tropica* Promastigotes. Numbers represent mean mm induration at 24 Hrs.

Fraction	Dose (µg)	Mean Induration (mm)	
56-58 kDa		Fraction	Reference (LtLRS02)
1:1	10	12.7	NT*
1:3	3	9.2	8.7
1:9	1	5.0	6.0
1:27	0.3	4.7	3.3

20 kDa		Fraction	Reference (LtLRS02)
1:1	10	10.7	NT*
1:3	3	8.3	7.3
1:9	1	6.3	5.7
1:27	0.3	4.7	3.3

* NT=Not Tested

In a separate study, three-fold dilutions of the 8 kDa, 20 kDa and 56-58 kDa fractions were compared with the promastigote lysate with the 8 kDa fraction removed. All fractions were normalized to 0.1mg/mL before being diluted with normal saline. The results of the work are illustrated in Figure 6.

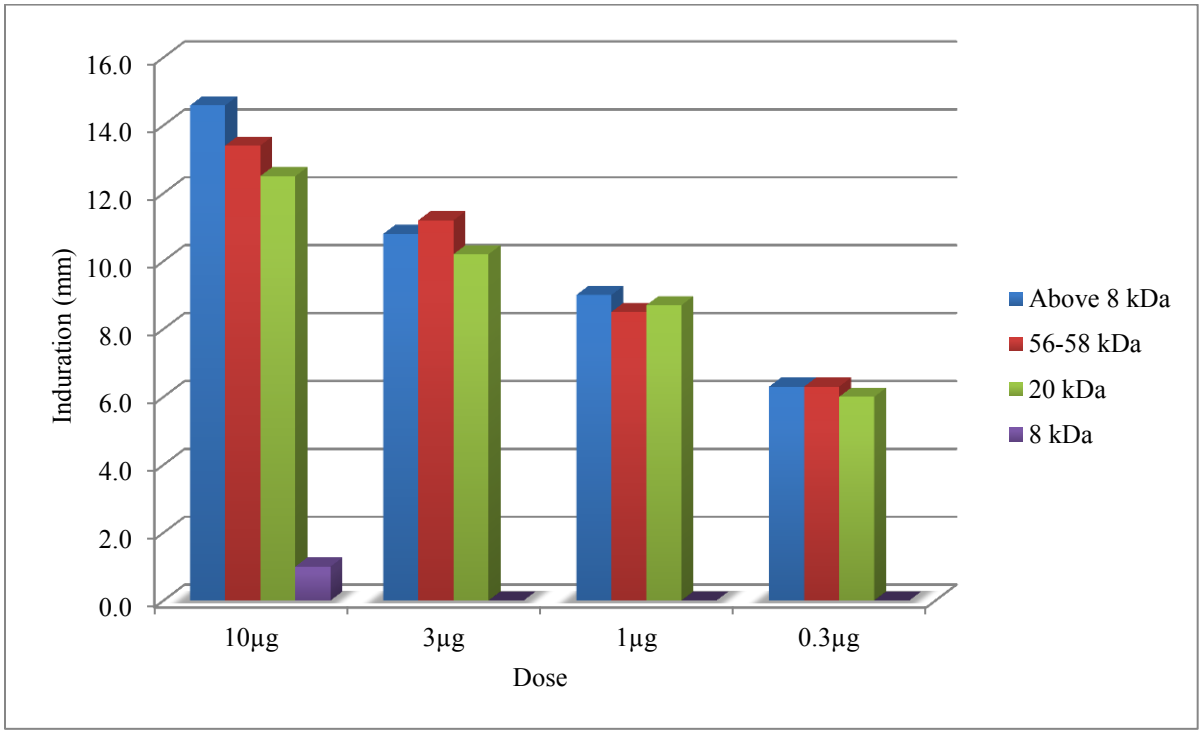

Figure 6. Skin Test Response to Three-fold Dilutions of *L.tropica* Drug Substance Fractions in Guinea Pigs Sensitized to the Promastigotes of *L.tropica*. Starting Protein Concentration of 0.1mg/mL (10 µg Dose)

As shown in the Figure 6, the 8 kDa failed to elicit a positive DTH skin test at any dilution. The skin test response to the 20 kDa, 56-58 kDa, and the fraction containing all components with a molecular mass above 8 kDa (Above 8 kDa). This finding suggested that both the 20 kDa and 56-58 kDa are important immunologically in the DTH response.

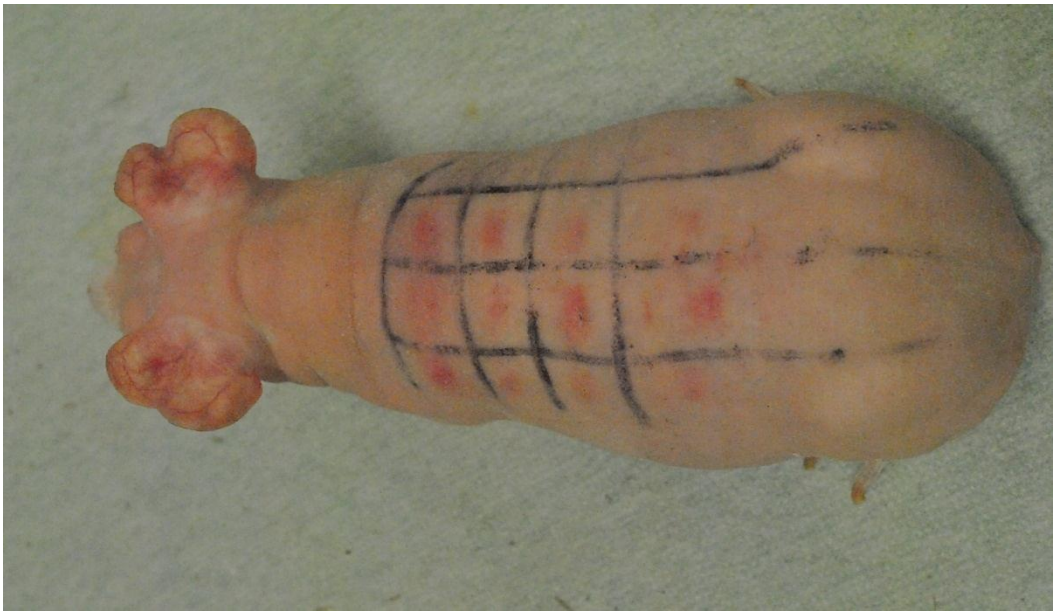

Figure 7. Skin Test Reactions to Three-fold Dilutions of Fractions of *L.tropica* Drug Substance in a Guinea Pig Sensitized to the Promastigotes of *L.tropica.* (1) Top row, 8 kDa Fraction (2) Second from top, 20 kDa Fraction (3) Third from top, 56-58 kDa Fraction, and (4) Bottom row, Lysate Fraction containing all components with a molecular mass greater than 8 kDa (Above 8 kDa).

As shown in Figure 7, the 8 kDa fraction (1) failed to elicit a positive DTH skin test which confirmed earlier findings that this fraction did not contain skin test active components. Conversely, the 20 kDa (2) and 56-58 kDa (3) fractions were both highly reactive and did not differ from each other in terms of the size of the skin test response elicited by each fraction. Both fractions contained other proteins as shown in Figures 7 and 8, but the 20 kDa and 56-58 kDa components were the dominant proteins in the fractions and were the only proteins visible at a concentration of 1 µg. The above 8 kDa (4) fraction containing all components with a molecular mass greater than 8 kDa gave essentially the same skin test response as the 20 kDa and 56-58 kDa fractions.

To continue the composition of the 20 kDa, 56-58 kDa and above 8 kDa fractions, SDS-PAGE was performed on each fraction with silver stain. The results of this work are shown in Figures 8, 9, and 10. At concentrations of 10 µg, other protein bands were present in the 20 kDa and 56-58 kDa fractions. However, banding associated with the non-identified components were less prominent than the bands representing 20 kDa protein (Figure 8) and 56-58 kDa protein (Figure 9). This difference was magnified in more dilute samples. At the 1.0 µg level, the only bands that were clearly visible were the 20 kDa and 56-58 kDa bands respectively.

Protein bands in the drug product (LtSTA01) at a concentration of 50 µg were less conspicuous than the bands present in the 20 kDa, 56-58 kDa and above 8 kDa fractions. The dominant band in the drug product was the 8 kDa band which was not present in the 20 kDa, 56-58 kDa and the above 8 kDa samples.

Figure 8 shows the dominance of the 20 kDa protein in the 10 µg sample, 3 µg sample and 1 µg sample. Other proteins were present in the 10 µg and 3 µg sample, but only the 20 kDa component is visible at the 1 µg dilution.

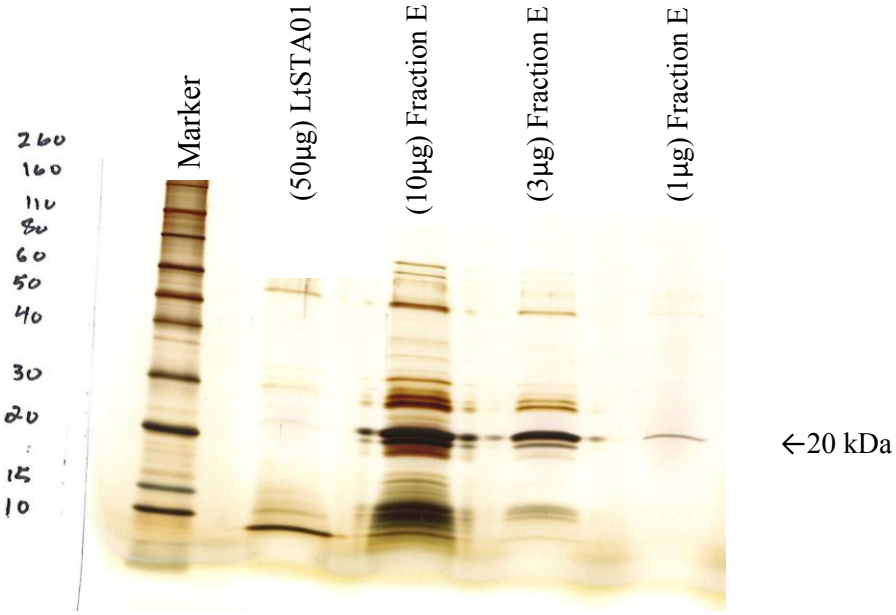

Figure 8. SDS-PAGE of 20 kDa Fraction E. 10 µg, 3 µg, and 1 µg dilutions silver stained. Drug Product was LtSTA01.

SDS-PAGE of the 56-58 kDa sample is shown in Figure 9. Dominance of the 56-58 kDa is clearly apparent at the 10 µg, 3 µg, and 1 µg dilution levels. At the 1.0 µg dilution no other proteins are visible.

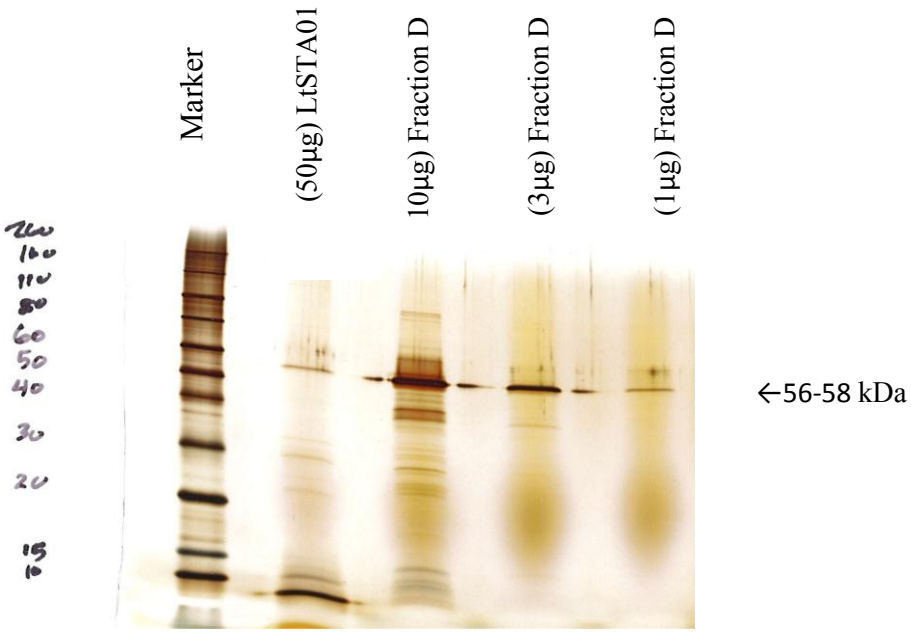

Figure 9. SDS-PAGE of 56-58 kDa Fraction. 10 µg, 3 µg, and 1 µg dilutions silver stained. Drug Product was LtSTA01.

SDS-PAGE of the fraction containing all components with a molecular mass greater than 8 kDa is shown in Figure 10. All proteins above 8 kDa were present in the 10 µg sample. Dominant bands are also present in the 3 µg sample and only the 56-58 kDa component is visible at the 1 µg dilution.

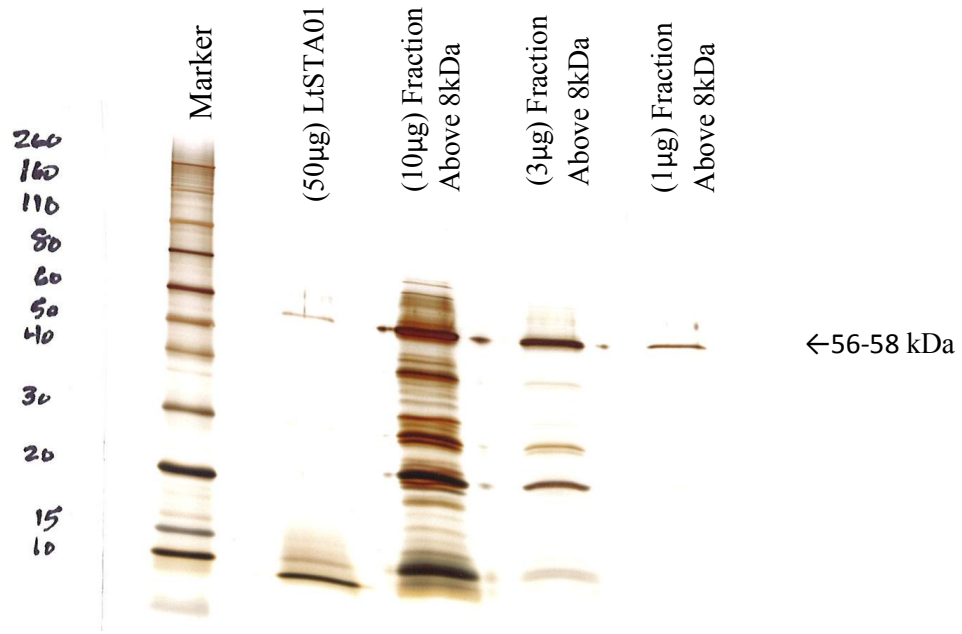

Figure 10. SDS-PAGE of Above 8 kDa Fraction. 10 µg, 3 µg, and 1 µg dilutions silver stained. Drug Product was LtSTA01.

3.2.3 Sensitizing Capacity of Lysate fractions

Clinical studies conducted with LtSTA indicated that the sensitization of naïve adult volunteers occurred at a higher incidence with larger doses of the skin test antigen. In Allermed's Phase I safety trial, 2/8 subjects who were skin tested with 120 µg dose had a positive DTH response at the original skin test site after 10-14 days. In Allermed's Phase II sensitization trial, 1/10 persons tested with 50 µg of LtSTA converted to a positive skin test after two previous negative tests and 1/23 persons converted from negative to positive on the third skin test with the 30 µg dose. These findings suggest that sensitization measured by conversion from a negative to positive skin test could be dose dependent.

In all clinical studies conducted by Allermed, the skin test material was the whole promastigote lysate adjusted to various concentrations based on the Ninhydrin protein nitrogen assay. When this assay was used to assess the protein content of the 8 kDa fraction and the fraction >8 kDa, the vast majority of Ninhydrin protein was found in the skin-test negative 8 kDa fraction. This finding and the observation that sensitization of naïve humans and guinea pigs could be related to the amount of protein administered suggested that the 8 kDa fraction might be acting as an adjuvant in the sensitization process.

To test this hypothesis, separate groups consisting of four naïve guinea pigs per group were skin tested by the intradermal administration of 0.1 mL of the preparations described below:

1. Drug Substance adjusted to 50 µg/0.1 mL.
2. 8 kDa Fraction adjusted to 50 µg/0.1 mL.
3. Above 8 kDa Fraction containing 1.1 µg/0.1 mL (this dose elicited a 9 mm skin test response equal to the response to 50 µg dose of drug substance).
4. 8 kDa Fraction adjusted to 50 µg/0.1 mL combined in equal parts with the above 8 kDa Fraction adjusted to 1.1 µg/0.1 mL.

Each preparation was administered intradermally once weekly until conversion from a negative to positive skin test occurred. The animals were observed after 24 hours for evidence of a DTH response at the test site. Positive DTH skin test responses were measured with a mm rule and recorded.

Protein determination and SDS-PAGE were performed on each preparation before testing began to insure the concentration and identity of the substance. The results of this experiment are shown in Table 5 below:

Table 5: Sensitizing Capacity of Lysate Fractions in Leishmania naïve Guinea Pigs

	Group 1 Above 8 kDa				Group 2 8 kDa				Group 3 8 kDa & > 8 kDa				Group 4 Drug Substance			
	Skin Test Results (mm)															
Guinea Pig #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
First Injection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Second Injection	5	0	8	6	0	0	0	0	5.5	7	6	3	0	0	0	0
Third Injection	4	3	6	7	8	6	5	3	9	5.5	6	10	13.5	12	3	3

In this experiment, erythema induration ≥5 mm was considered to be evidence of sensitization. Based on this criterion, no evidence of an inflammatory response occurred after the first injection of each substance. Three of the four animals injected with the above 8 kDa fraction (Group 1) showed a positive DTH response after the second injection. Three of the four animals that were injected with the 8 kDa fraction (Group 2) were positive on the third injection. Three of four animals receiving the 8 kDa fraction and the >8 kDa fraction (Group 3) were positive on the third skin test. Two of four animals receiving the drug substance were positive on the third skin test. This finding indicated that both the 8 kDa and above 8 kDa fractions were sensitizing in *Leishmania* naïve guinea pigs at the concentrations studied.

4. REPORTABLE OUTCOMES

4.1 SDS-PAGE of the whole lysate *L.tropica* promastigotes revealed dominant protein bands at 56-58 kDa, 20 kDa, and 8 kDa.

Separation of the 8 kDa fraction from the other fractions using a Superdex 200 column further revealed that the 8 kDa fraction contained the vast majority (95%) of Ninhydrin and BCA reacting material. However, based on the 260/280 UV data related to the 8 kDa fraction, it is probable that much of this material was protein fragments and nucleic acids.

4.2 FPLC studies of the lysate fraction above 8 kDa identified dominant peaks at 20 kDa and 56-58 kDa.

SDS-PAGE analysis of the dominant peaks showed the presence of other protein bands. However, the bands representing the 20 kDa and the 56-58 kDa were conspicuously dominant, indicating that the fractions consisted primarily of the proteins in the 20 kDa and 56-58 kDa range.

4.3 Skin tests in hairless guinea pigs sensitized to the promastigotes of *L.tropica* demonstrated differences in the DTH skin test activity of lysate fractions.

The 8 kDa fraction failed to elicit a positive DTH response in sensitized animals; whereas, skin tests with the lysate fraction containing proteins with a molecular mass greater than 8 kDa resulted in strongly positive DTH skin test reaction in the same animals.

4.4 Repeat skin tests with the 8 kDa fraction and the fraction containing proteins with a mass larger than 8 kDa resulted in negative to positive conversion when the two fractions were administered to *Leishmania* naïve guinea pigs.

Naïve guinea pigs skin tested with the whole promastigote lysate (drug substance) and with the 8 kDa fraction and above 8 kDa fraction, as well as with a combination of the 8 kDa and above 8 kDa fractions were skin test negative on the first test. However, after two of three intradermal injections conversion from a negative test to a positive test occurred with each preparation in some animals.

5. CONCLUSIONS

Whole cell suspensions of *Leishmania* promastigotes, as well as soluble preparations of promastigotes have been used as skin test antigens for both diagnostic and epidemiologic purposes. In some instances, the antigen has been prepared from locally isolated strains of the parasite without consideration for appropriate controls or manufacturing methods. This has resulted in published information that is difficult to evaluate in terms of the reliability of the skin test in detecting sensitivity to the homologous agent, or to cross-reacting species. As a rule, maximum sensitivity and specificity of a skin test antigen occur when the antigen is prepared from the same agent that it is intended to detect. However, studies have shown that antigen made from one *Leishmania* species can be used to detect exposure to other species of the parasite.

In Allermed's Phase II study, *L.tropica* antigen (LtSTA) was employed to detect sensitivity to *L.major*. The decision to conduct the trial in an *L .major* endemic area was based on laboratory data from guinea pigs which showed DTH cross-reactivity between the two species. The hypothesis that *L.tropica* antigen could detect prior exposure to *L.major* in humans was proven valid in subjects with active and healed cutaneous leishmaniasis (CL) caused by *L.major*. Dose-response testing of persons with active CL with LtSTA concentrations of 10 µg, 20 µg, 40 µg, and 80 µg demonstrated 100%reactivity. As anticipated, the size of the DTH skin test response increased with the corresponding increase in dose; however, the test was positive at all four dose levels. From these data, a dose of 30 µg was selected to evaluate the sensitivity of LtSTA in subjects with healed *L.major* CL. This work revealed that 85% of persons with a history of CL within the past 24 months were skin test positive to LtSTA. Conversely, testing LtSTA in persons residing in an endemic area for *L.major*, but without histories of CL, demonstrated a high level of product specificity. In this population LtSTA did not elicit positive skin tests in 97% of the subjects tested. In a follow-up investigation, six subjects with histories of healed CL who failed to react to the 30 µg dose of LtSTA were skin tested with a 50 µg dose. All six subjects had positive skin tests to the 50 µg dose. This finding confirmed that sensitivity to cross-reacting components also was present in these individuals, but required a higher concentration of LtSTA to evoke a measurable induration response.

Based on the observation that LtSTA concentrations of 30 µg and 50 µg elicited positive DTH responses on the third intracutaneous injection of the antigen, the FDA specified that skin test at these does should not be administered more than one time in the same individual. Data obtained from the Phase I safety trial in which doses of 20 µg, 40 µg, 80 µg, and 120 µg were tested in *Leishmania* naïve persons demonstrated that absence of false positive DTH responses to the product following a single skin test. The data obtained in Allermed's Phase IIB investigation also showed that false positive responses did not occur after a single test at dose concentrations of 15 µg, 30 µg, or 50 µg. This finding showed that LtSTA can be used one time in the same individual without concern of a false-positive reaction.

Studies concerning the sensitizing properties of *Leishmania* skin test antigen have shown conflicting results. Weigle et al. [(1)] found that antigen containing 10 x 10^6/mL of merthiolate-fixed promastigotes divided between *L.panamensis* and *L.amazonensis* did not induce DTH reactions in 19 healthy *Leishmania* naïve adults when the antigen was administered two or three times.

Mayrink et al. [2] observed that 100 persons who were non-reactive to a skin test consisting of sonicated *Leishmania* promastigotes remained non-reactive to the same antigen when tested 3 months later.

Satti et al. [3] studied the effects of repeat skin tests in healthy volunteers residing in a leishmaniasis non-endemic area. The *Leishmnaia* antigen used in the study was made from *L. major* containing 5 x 10^5 promastigotes/0.1 mL. Subjects were given three doses of the antigen at 0, 30 and 90 day time points. Conversion from negative to positive DTH reaction was not observed in the six subjects included in the protocol. These investigators contrasted their findings with those of Nascimento et al.[4] who demonstrated DTH conversion using repeat doses of a *Leishmania* skin test antigen made from equal parts *L. braziliensis, L. amazonensis* and *L.guyanensis* at a protein concentration of 4.0 μg/0.1 mL.

Jose et al.[5] reported that twelve *Leishmania* naïve volunteers in Group I of their study who were skin tested with antigen containing 25 μg/0.1 mL of *L.amazonensis* protein converted from a DTH negative skin test to a DTH positive skin test. Their results were as follows: after 30 days a second skin test was positive in 33% of subjects; after 90 days, the skin test was positive in 67% of subjects.

In the Allermed's Phase IIB study, conversion from skin test negative to skin test positive after two intracutaneous tests administered at 30-day intervals was observed in one of 10 subjects tested with a 50 μg dose of LtSTA and in one of twenty-three subjects tested with a 30 μg dose of antigen. In eight subjects that received a 15 μg dose of the product, conversion from DTH negative to DTH positive was not observed. These findings and the findings of the other investigators underscore the importance of antigen concentration in defining the sensitizing capacity of the *Leishmania* skin test. Equally important is the number of subjects enrolled in sensitization studies. Sensitization from a 30 μg dose in this study was not demonstrated in the original cohort of 12 subjects, but was observed after additional subjects were added to the 30 μg group. Further study of the 15 μg dose would be required to fully evaluate the ability of repeat doses of 15 μg to convert *Leishmania* naïve individuals from DTH negative to DTH positive.

Skin test antigens of *Leishmania* have been used to demonstrate the absence of prior infection with the parasite. The test is commonly used to screen candidates in *Leishmania* vaccine trials. The skin test also has been used as a diagnostic aid in identifying persons with active disease, or as an epidemiological tool to identify persons with past exposure to the organism. Using a skin test antigen that is capable of sensitizing a recipient following single use is undesirable for vaccine studies, and a sensitizing antigen complicates the interpretation of a positive skin test when the product is used diagnostically or epidemiologically multiple times.

The skin test antigen developed by Allermed was intended to screen military and civilian personnel for infection with *Leishmania* during and after multiple deployments to *Leishmania* endemic regions. With the discovery that 30 μg and 50 μg doses of LtSTA were sensitizing to some individuals in a *Leishmania* naïve population, it was no longer possible to achieve the primary objective of licensing a multiple-use skin test antigen, with acceptable sensitivity and specificity, that was made from the whole lysate of *L.tropica* promastigotes. Rather, emphasis was placed on attempting to separate

sensitizing components of the lysate, if different, from the components responsible for the delayed-type hypersensitivity skin test response.

SDS-PAGE of the whole lysate *L.tropica* promastigotes revealed dominant protein bands at 56-58 kDa, 20 kDa, and 8 kDa. Separation of the 8 kDa fraction from the other fractions using a Superdex 200 column further revealed that the 8 kDa fraction contained the vast majority (>90%) of components that were reactive with the reagents used in the Ninhydrin and BCA methods of protein determination. However, based on the 260/280 UV data related to the 8 kDa fraction, it was apparent that much of this material consisted of fragments of proteins with Ninhydrin and BCA activity as well as nucleic acids. Skin tests with the 8 kDa fraction in guinea pigs sensitized to the promastigotes of *L.tropica* failed to elicit a positive DTH skin test response. Conversely, skin tests with the combined fractions above 8 kDa resulted in strongly positive DTH responses.

Partially purifying the lysate fraction above 8 kDa by FPLC revealed major peaks at 56-58 kDa and 20 kDa. SDS-PAGE analysis of these peaks showed that each peak also contained smaller amounts of other proteins. For this reason, it was not possible to know definitely if the 56-58 kDa and 20 kDa were acting in concert with other proteins in causing the DTH response to the fraction. What did appear to be clear was the importance of the 56-58 kDa and 20 kDa protein groups, since these bands were dominant in the drug substance and drug product and was present in all manufactured lots of the promastigote lysate.

In a separate study that included the 56-58 kDa, 20 kDa, 8 kDa fractions and a sample that included all proteins with a mass greater than 8 kDa, three-fold dilutions (10 μg, 3 μg, 1 μg, and 0.3 μg protein) of each fraction with a mass greater than 8 kDa gave very similar induration responses in sensitized guinea pigs. The induration response to the 56-58 kDa fraction was closely similar to the 20 kDa fraction and the combined fractions above 8 kDa. As expected, the sample containing the 8 kDa fraction did not elicit a positive skin test in the same sensitized animals. It is important to note that the induration responses to the 1μg and 3 μg doses of the 56-58 kDa, 20-26 kDa and the fraction containing all proteins larger than 8 kDa were equal in size and intensity to the induration response observed with the 50 μg dose of LtSTA manufactured from the whole promastigote lysate.

In a previous study Khabiri, Bagheri, and Assmar [6] found that a 56 kDa band in the lysate of *L.major* promastigotes was associated with a positive DTH skin test response in sensitized guinea pigs, but to a lesser degree than the whole-cell lysate. Furuya et al [7] fractionated the crude lysate of *L.panamensis* using sephacryl s-200 gel filtration. These investigations found that skin test active fractions in humans with cutaneous leishmaniasis contained proteins in the range of 66, 55, 45, 28, and 26 kDa. These fractions elicited DTH skin test reactions comparable to the reactions to the crude *L.panamensis* lysate at a dose of 10 μg protein.

Future development of a *L.tropica* skin test antigen should consider the intended use of the product. If single use is desired, the product can be made from the whole lysate of promastigotes. Allermed's study of persons with histories of cutaneous leishmaniasis caused by *L.major* within the past 24 months showed that a 50 μg dose of the lysate was skin test positive in these individuals.

If the skin test product is intended to be used multiple times, fractionation of the lysate and purification of the DTH active components should be considered. This could be accomplished by isolating a single DTH active protein and preparing a recombinant product that is non-sensitizing, or by removing sensitizing components of the lysate from those that are DTH active. Based on the results of the fractionation studies conducted by Allermed, it was possible to remove most of the Ninhydrin and BCA reacting material which was not DTH active by processing the crude lysate with an Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-30 membrane (Millipore) at 4,000 x g. This procedure, repeated four times separated DTH active components from those that did not elicit a DTH response. To confirm the composition of each fraction, the flow-through and the retentate were passed over a Superdex 200 10/300 GL column.

If the above procedure is done correctly, the flow-through should contain only components that are 8 kDa and smaller and the retentate should contain only components with a mass larger than 8 kDa. This procedure would allow the manufacture of a skin test antigen with less than 5% of the total protein in the crude promastigote lysate, which could provide a safer product with less ability to sensitize a *Leishmania* naïve person. Using the whole lysate, a 50 μg dose is believed to be both specific and sensitive in detecting prior infection with *L.major*. The same degree of specificity and sensitivity might be expected from a 1-3 μg dose of the lysate fraction containing only proteins above 8 kDa. A product of this type should be further explored in humans with prior infection with *L.major*, as well as in *Leishmania* naïve subjects to see if sensitization occurs after multiple skin test use.

6. REFERENCES

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