

SAFETY AND IMMUNOGENICITY OF AN INTRANASAL ANTHRAX VACCINE IN NEW ZEALAND WHITE RABBITS

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ABSTRACT

LigoCyte's intranasal dry powder anthrax vaccine* (Vaccine), based on recombinant protective antigen (rPA), protects against both the anthrax toxin and the infectious disease. The Vaccine offers the potential for long-term stability and protects rabbits against inhaled anthrax. The purpose of this study was to evaluate the potential toxicity of the Vaccine following four repeated intranasal doses (N+1 design based on a clinical regimen of three vaccinations) to New Zealand White rabbits. Sixty rabbits were assigned to one of three groups (10 animals/sex/group), and administered placebo, adjuvant-excipient, or Vaccine. Four days following the last dose, half were euthanized and necropsied, and the remaining half were euthanized and necropsied after a 1-month recovery interval. Parameters evaluated include mortality, clinical observations, body weights, food consumption, body temperature, nasal/pharyngeal examinations, ophthalmologic examinations, clinical pathology, C-reactive protein and serum protein electrophoresis, immunology, gross pathology, organ weights, and histopathology. There was no toxicity of the Vaccine; rPA-specific IgG and TNA responses were observed in vaccinated animals but not in animals treated with the adjuvant/excipient or placebo. In animals sacrificed four days after the final dose, minimal heterophilic infiltrates and apical blebbing of the respiratory epithelium lining the nasal cavity and minimal luminal exudate was present in animals treated with the adjuvant-excipient (alone or in combination with the Vaccine). Changes in nasal histopathology were not present in recovery animals indicating full recovery of the nasal epithelium (i.e. reversibility of the effect) and the observation was therefore not considered adverse. There were several adjuvant-excipient-related increases but they were of no toxicological significance and were fully recoverable. In conclusion, there were no adverse effects on potential target organs and no evidence for delayed onset of toxicity. Based on the findings, the No Observed-Adverse-Effect-Level (NOAEL) is a total dose of 1200 µg rPA contained in LigoCyte Anthrax Vaccine when dosed four times to both nostrils.

*The Vaccine incorporates chitosan. This application of chitosan (ChiSys®) has been licensed from Archimedes Development, Ltd.

INTRODUCTION

Anthrax is an acute disease caused by the *Bacillus anthracis*, and spores (which are the mode of transmission) can be produced *in vitro* and used as a biological weapon. Depending on the route of infection, anthrax disease can occur in three forms: cutaneous, inhalational, and rarely, gastrointestinal. There is currently only one FDA-licensed human anthrax vaccine in the United States.

LigoCyte is one of several companies working on a new anthrax vaccine for people. The current vaccine is complicated to administer and has adverse side effects. LigoCyte's intranasal anthrax vaccine incorporates recombinant Protective Antigen (rPA) which is the same anthrax toxin protein that is currently being studied in human clinical trials. In anthrax disease, toxin produced as a byproduct of the infection is one of the primary causes of death. The dry powder formulation offers the potential for long-term stability, and intranasal administration helps to stimulate a better response in mucosal tissues such as the lung, where anthrax infections begin following inhalation of the bacterial spores. The vaccine has been shown to provide protection in rabbits from exposure to inhaled anthrax. In that study rabbits who raised immune responses to only the anthrax toxin survived, but became ill during the course of the study. Rabbits receiving the vaccine survived without becoming ill, demonstrating control of the infectious process.

The purpose of this study was to evaluate the potential toxicity of LigoCyte's Anthrax Vaccine following four repeated intranasal doses on Study Days (SD) 1, 29, 57, and 85 to New Zealand White rabbits during a 12-week study interval, and to assess the persistence, reversibility, or delayed onset of any effects after a four-week no-treatment recovery interval. Parameters evaluated include mortality, clinical observations, body weights, food consumption, body temperature, nasal/pharyngeal examinations, ophthalmologic examinations, clinical pathology, C-reactive protein and serum protein electrophoresis, immunology, gross pathology, organ weights, and histopathology.

MATERIALS AND METHODS

Test Animals and Husbandry

New Zealand White rabbits weighing between 2.1 and 2.9 kg and at approximately 16 months of age were obtained from Covance Research Products (Denver, PA), and acclimated to laboratory conditions for 8 days prior to the first dose. Animals were food fasted on the day of arrival, and given increasing amounts of Certified Global Harlan Teklad Laboratory Diet 2030 for the first two days after arrival and *ad libitum* thereafter. Environmental controls for the animal room were set to maintain a temperature of 16 to 22°C, a relative humidity of 30 to 70%, and a 12-hour light/12-hour dark cycle. In addition to standard husbandry procedures, animals were provided with toys as environmental enrichment.

Test and Control Articles

Table 1. Test Materials

Name	Supplier	Purity	Description
LV02 Anthrax Vaccine 150 µg rPA	LigoCyte Pharmaceuticals, Inc. Bozeman, MT	100% (SDS-PAGE and HPLC)	Dry powder
Placebo (lactose)	LigoCyte Pharmaceuticals, Inc. Bozeman, MT	Assumed 100%	Dry powder
Adjuvant/Excipient (Chitosan, MPL, Mannitol)	LigoCyte Pharmaceuticals, Inc. Bozeman, MT	Assumed 100%	Dry powder

rPA – Recombinant Protective Antigen

MPL – (3-Q-desacyl-4'-monophosphoryl lipid A)

Vaccine, placebo, and adjuvant/excipient were provided in single-use dry-powder intranasal devices sealed in foil pouches. All materials were used as received. Duplicate samples of Vaccine, placebo and adjuvant/excipient were collected at the time of the first and last dose administration and sent to LigoCyte for analysis.

Experimental Design

Animals were accepted into the randomization pool based upon prestudy body weights, physical examinations, and ocular examinations. Males and females were randomized separately, and 10 animals/sex/group were assigned as presented in Table 2.

Table 2. Study Design

Group	Treatment	Nominal Dose rPA (µg/nostril)	Total Dose rPA (µg)
1	Placebo	0	0
2	Adjuvant/Excipient	0	0
3	Vaccine	150	300

Animals were dosed via the intranasal route of administration on SD 1, 29, 57, and 85. Doses of placebo, adjuvant/excipient, and Vaccine were administered using a Valois monopowder nasal delivery device. Doses of placebo were administered to the right nostril and doses of adjuvant/excipient and Vaccine were administered to each nostril using one delivery device per nostril. Anesthesia was not required.

Animals were observed as shown in Table 3.

Table 3. Animal Observations/Measurements

Procedure	Frequency of Testing
Cageside Observations	≥ 2 Daily
Clinical Observations	Prior to each dose, weekly all other times, and at termination
Body Weight	Prior to each dose, weekly all other times, on the day prior to necropsy (unfasted) and at termination (fasted)
Food Consumption	Daily, except when interrupted for study-related events
Body Temperature	Prior to dosing on SD 1, 29, 57, and 85, 6 hours (± 15 minutes) following each dose, and once daily on SD 2-7, 30-35, 58-63, 86-91, and 113
Ophthalmologic Examination	Prior to first dose and prior to necropsy (all surviving animals at each necropsy interval)
Nasal/ Pharyngeal Examination	Prior to each dose, 3 hours ± 15 minutes following each dose, weekly at all other times, and prior to each necropsy (Recovery animals did not have exams performed at the terminal necropsy.)

Cageside observations included observation for mortality, moribundity, general health and signs of toxicity. Clinical observations included evaluation of skin and fur characteristics, eye and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and somatomotor and behavior patterns.

Ophthalmologic observations were conducted using indirect ophthalmoscopy and slit-lamp biomicroscopy (as needed) following administration of 1% Tropicamide[®] mydriatic solution.

Blood was collected for clinical pathology evaluation as shown in Table 4.

Table 4. Clinical Pathology

Parameter	Chemistry	Hematology	Coagulation ^a	C-reactive Protein ^b and Serum Protein Electrophoresis	Urinalysis
Collection Day	SD -2, 2, 30, 58, 89, 113		SD -2, 2, 16, 28, 30, 36, 56, 58, 64, 84, 89, 91, 113	SD -2, 2, 30, 58, 89, 113	SD -2, 2, 30, 58, 89, 113
Collection Method	Medial auricular artery				Clean catch pan
Volume Collected	≥1.2 mL	≥1.2 mL	≥1.8 mL	≥1.5 mL	Total volume recorded
Tubes Used	Serum separator	K ₂ EDTA	Sodium citrate	Serum separator	Urintek

a - The coagulation parameter, APTT, was evaluated starting on SD 16.

b - C-reactive protein samples (1.0 mL) were also collected on SD 28, 56, and 84.

Blood samples for serology (1.0 mL) were collected prior to the first dose and then weekly until termination. Blood samples were processed to serum and shipped to the Sponsor for analysis.

Termination and Postmortem Procedures

Necropsies were performed on SD 89 (main phase) and SD 113 (recovery phase). The gross necropsy included examination of the external surface of the body, all orifices, the cranial, thoracic, and abdominal cavities, and their contents. Organs were weighed as soon as possible after dissection; paired organs were weighed together. Tissues were preserved in 10% neutral buffered formalin (NBF) with the exception of eyes (with optic nerve) and testes (with epididymides) which were preserved in modified Davidson's fixative for 24-72 h and subsequently transferred to 10% NBF. Two bone marrow smears were prepared from the sternum. Protocol-required preserved tissues were embedded in paraffin, sectioned, and stained with hematoxylin. All slides from main phase animals and slides of the nasal cavity from the recovery animals were evaluated by a board certified veterinary pathologist.

RESULTS

Stability and Dose Verification Analysis

Dose analysis results indicated that samples of placebo, adjuvant/excipient, and Vaccine collected at the time of first and last use met acceptance criteria. These materials were therefore considered to be stable for the duration of the study and acceptable for dosing.

Mortality

Four females were found dead and one male was sacrificed in a moribund condition during the study clinical observations and/or cageside observations noted prior to the unscheduled deaths are summarized in Table 5. The cause of death was nephropathy, and was unrelated to the administration of test article.

Table 5. Summary of Early Deaths

Group/ Sex	Animal	Treatment	Day of Death	Type of Death	Clinical and/or Cageside Observations
1/M	10441	Placebo	49	Moribund euthanasia	Cold to touch, labored respiration, and low posture
1/F	10453	Placebo	100	Found dead	Cold to touch, labored respiration, yellow mucoid discharge from anus, and thinness
1/F	10454	Placebo	63	Found dead	Abrasions on the mouth and yellow discharge from anus
2/F	10475	Adjuvant/ Excipient	75	Found dead	None
3/F	10495	Vaccine	98	Found dead	Thinness

Clinical pathology observations in all of these animals included elevated mean serum BUN and creatinine. Microscopically, moderate to marked nephropathy was seen in the kidneys of all five animals. Microscopic findings were consistent with uremia and included mineralization in numerous extra-renal tissues (all five animals), erosion of the gastric mucosa (one animal only), and a gallbladder ulcer (one animal only). Minimal to marked fibrous osteodystrophy was observed in the

femur and nasal cavity of all five animals and is consistent with renal osteodystrophy.

Animal Disposition and Clinical Observations

Treatment with the Vaccine had no effect on clinical or cageside observations. The few observations were considered unrelated to administration of the Vaccine because they are common observations in the laboratory rabbit or were noted in control animals.

Nasal/Pharyngeal Examinations

Administration of the Vaccine, adjuvant/excipient, and placebo resulted in findings of red discoloration, discharge (yellow or white) from the nose, and swelling during the nasal/pharyngeal examinations; the majority of these findings were noted during the 3-hour postdose examinations following the second, third, and fourth doses.

Body Weight, Body Weight Changes, and Food Consumption

Treatment with the Vaccine had no effect on body weights, body weight changes, or food consumption.

Body Temperature

Males and females dosed with the Vaccine had significantly higher body temperatures on all dosing days which at times persisted through the following day but remained within normal ranges. Animals dosed with adjuvant/excipient also had significant increases in body temperatures during the 6-hour postdose measurements following the first three doses but values remained with normal ranges.

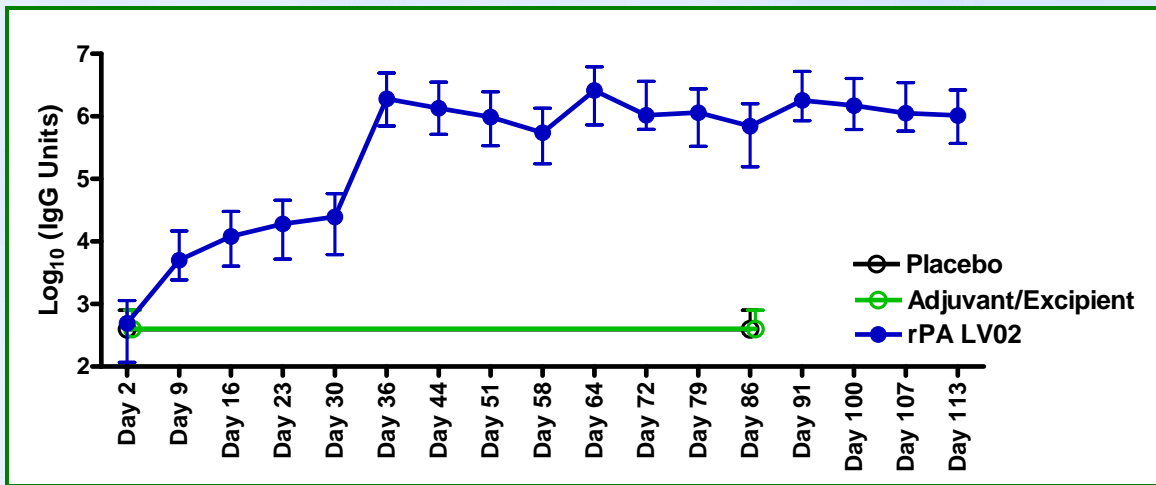
Ophthalmology

Treatment with the Vaccine did not result in ocular changes and there was no evidence of delayed toxicity.

Immunology

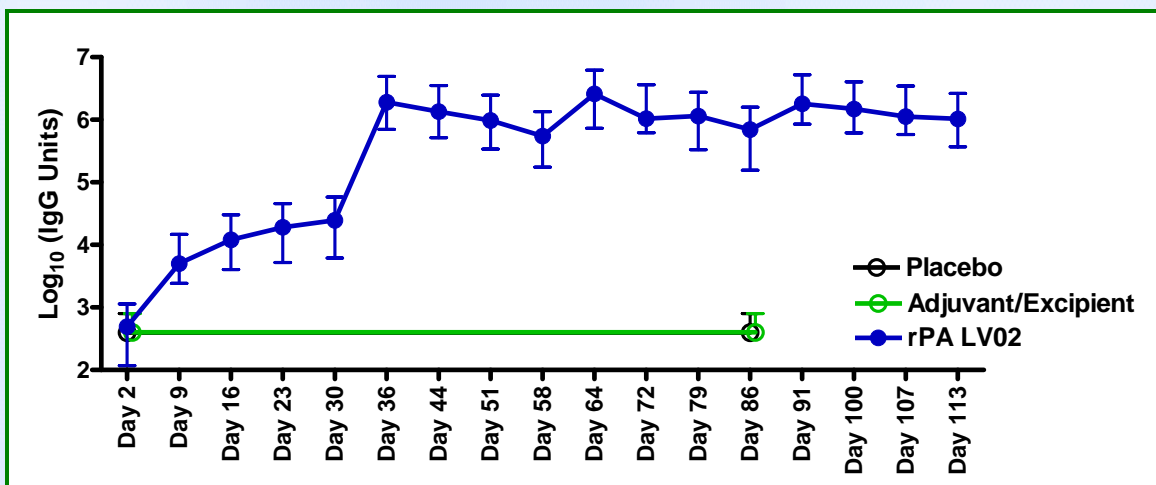
The first measurable rPA-specific serum IgG responses in vaccinated animals were seen on SD 9. Serum levels of rPA-specific IgG rose following each boost. However, by the third boost the effect was less dramatic, indicating that the IgG response was approaching a maximum limit (Figure 1).

Figure 1. rPA Specific IgG Titers in Rabbits Immunized with LigoCyte’s Antrax Vaccine



Serum Toxin Neutralizing Activity (TNA) was measurable in vaccinated animals beginning on SD 36. No measurable quantities of rPA-specific serum IgG were detected in animals treated with the adjuvant/excipient or placebo (Figure 2).

Figure 2. TNA Levels in Rabbits Immunized with LigoCyte’s Antrax Vaccine



Clinical Pathology

There were no Vaccine-related treatment effects on clinical pathology parameters.

There were adjuvant/excipient-related effects on blood proteins, lipids, leukocytes, platelets, and plasma coagulation times. All adjuvant/excipient-related treatment effects were of no toxicologic significance. There were no effects at the SD 113 recovery period so the adjuvant-excipient treatment effects were fully recoverable.

Macroscopic Pathology and Organ Weights

There were no Vaccine- or adjuvant/excipient-related gross findings or changes in organ weights in main phase or recovery animals.

Histopathology

There were no Vaccine-related microscopic findings. Adjuvant/excipient-related microscopic findings were limited to the nasal cavity of animals treated with adjuvant/excipient. Minimal apical blebbing of respiratory epithelium (two Group 3 males and one Group 3 female) and minimal heterophilic (analogous to human neutrophilic) infiltrates (one Group 3 male and one Group 3 female) were observed in the respiratory epithelium lining the rostral-most section of the nasal cavity. Minimal exudate was observed in the lumen of the nasal cavity or nasolacrimal duct of two Group 2 males, one Group 3 male, and one Group 3 female, and was characterized by a mixture of amorphous material (interpreted as mucus) and cellular debris. Clinical observations of white nasal discharge in two Group 3 animals on study day 85 had no microscopic correlate. Because the heterophilic infiltrates, apical blebbing and exudates were of low severity and incidence, affected only a small portion of the total area of the nasal turbinate or cavity, and were recoverable, they were not considered adverse.

Minimal to moderate spontaneous nephropathy affected most of the animals in this study. The findings were incidental, because there was no Vaccine- or adjuvant/excipient-related increase in incidence or severity. The spontaneous nephropathy correlated with necropsy findings of pale and/or enlarged kidneys, and elevated mean serum BUN and creatinine. The ability to discern overt potential test article/excipient renal changes was not compromised by these renal changes. As with the spontaneous nephropathy, the other findings in both treatment and recovery animals were unrelated to treatment due to low incidence,

lack of correlation between genders, and/or because they were common findings in New Zealand White rabbits.

There were no Vaccine- or adjuvant/excipient-related microscopic findings in the nasal cavities of recovery rabbits, indicating irreversibility of adjuvant/excipient-related changes.

CONCLUSIONS

Five animals were either found dead or sacrificed in a moribund condition. The cause of death was spontaneous nephropathy and was unrelated to treatment with the Vaccine.

There were no effects of the Vaccine on mortality, clinical observations, body weights, food consumption, body temperature, nasal/pharyngeal examinations, ophthalmologic examinations, clinical pathology, C-reactive protein and serum protein electrophoresis, gross pathology, organ weights, and histopathology.

Measurable rPA-specific IgG and TNA responses were observed in vaccinated animals, whereas no rPA-specific IgG were detected in animals treated with the adjuvant/excipient or placebo.

Minimal heterophilic infiltrates and apical blebbing of the respiratory epithelium lining the nasal cavity and minimal luminal exudate was present in animals treated with the adjuvant/excipient (alone or in combination with the Vaccine). These observations are not considered adverse and were reversible.

There were adjuvant/excipient-related effects on blood proteins, lipids, leukocytes, platelets, and plasma coagulation times. All adjuvant/excipient-related treatment effects were of no toxicologic significance. There were no effects at the SD 113 recovery period so the adjuvant-excipient treatment effects were fully recoverable.

In conclusion, there were no target organs, no adverse effects, and no evidence for delayed onset of toxicity associated with administration of the Vaccine. Based on the findings, the No-Observed-Adverse-Effect-Level (NOAEL) of the Vaccine is 1200 µg rPA when dosed four times to both nostrils.