

**LOCAL AND SYSTEMIC TOXICITY OF THREE
INFLUENZA VACCINES FOLLOWING
INTRAMUSCULAR INJECTION
TO NEW ZEALAND WHITE RABBITS**

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ABSTRACT

The purpose of this study was to evaluate the toxicity of three influenza vaccines when administered to rabbits. The three vaccines differed with respect to the position of the influenza HA head with the flagellin fusion partner. Twelve/sex/group received the control and vaccines by IM injection on Study Day (SD) 1, 22, and 43. The vaccines had no effect on mortality, clinical observations, ophthalmology, hematology and urinalysis variables, organ weights, or histopathology. STF2R3.HA1 and STF2R3.2xHA1 had no effect on Draize scores, body weight changes, food consumption, and clinical chemistry. Body temperatures were higher 6 hr after the first dose but were not adverse. On SD 23, there was an increase in mean C-reactive protein values in animals receiving these vaccines. Following administration of the first dose of STF2.HA1 vaccine test article-related erythema and edema were noted but were not adverse. Decreases in absolute body weights and food consumption also occurred in both sexes following the first dose of this vaccine at both dose levels but the effects were not adverse. As with the other two vaccines, STF2R3.HA1 and STF2R3.2xHA1, body temperatures were elevated 6 hr after dosing on SD 1 but were not adverse. Changes in serum globulin and mean albumin/globulin ratios also occurred but were not adverse. There were also test article-related increases of mean fibrinogen and activated partial thromboplastin values, and a decrease in mean prothrombin time in animals that received STF2.HA1 but none of these changes was considered adverse. C-reactive protein also increased following the first administration of this vaccine. Samples collected prior to dosing and on SD 23, 46, and 68 were analyzed in HA-specific and STF2-specific IgG ELISA, as well as hemagglutination inhibition (HAI), and the results suggest that the rabbit model is an appropriate immunological model for all three vaccine test articles. In conclusion, administration of the three vaccines was well tolerated and no adverse effects occurred at the highest administered doses.

INTRODUCTION

Currently marketed influenza vaccines are based on a development, production and vaccination strategy that has not changed significantly in the past five decades. Due to the seasonal nature of the disease and the genetic instability of the virus, it is necessary to formulate a new influenza vaccine each year based on an epidemiological prediction of the strains most likely to be circulating in the human population in the next winter's flu season. Current vaccines are formulated with hemagglutinin (HA) as the viral antigen (the component of the virus, usually a protein, that serves as a target for an immune response). Due to slow development and production cycles, there is general concern that traditional vaccines may not consistently meet the demands of seasonal influenza or potential pandemic virus outbreaks. The recent appearance of a novel H1N1 (nH1N1) virus has highlighted the importance of rapid response to emerging pandemic threats. VaxInnate has developed an influenza vaccine production system in bacteria that permits rapid development and manufacturing on a large scale within a short period of time.

The purpose of this study was to evaluate the potential local and systemic toxicity of three candidate vaccines when administered three times to the New Zealand White rabbit over a 43-day study period. On completion of the treatment period, designated animals were held for a 25-day treatment-free period in order to evaluate the reversibility of any findings. The vaccines evaluated in the present study comprise the protective globular head subunit of the HA monomer fused covalently to *S. typhimurium* flagellin. The first (STF2.HA1) has the globular head fused to the C terminus of flagellin, the second (STF2R3.HA1) has the head fused in place of domain 3 (D3) of flagellin, and the third (STF2R3.2xHA1) has the head in both positions.

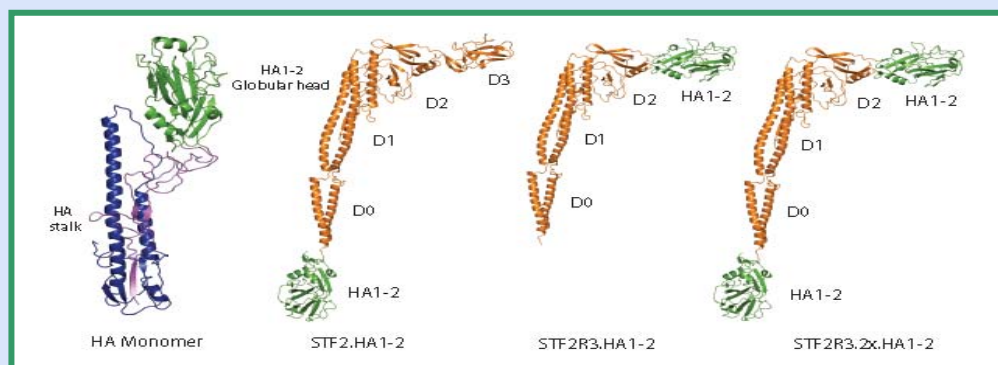


Figure 1. Diagrams of the HA monomer (left) and the three vaccine constructs. The globular head subunit of the HA monomer is shown in green, flagellin is shown in gold with the different flagellin domains labeled.

MATERIALS AND METHODS

Test Animals and Husbandry

New Zealand White rabbits weighing between 2.2 and 3.2 kg and 13-14 weeks of age were obtained from Harlan Sprague Dawley (Oxford, MI), and acclimated to laboratory conditions for 7 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	Lot/Batch No.	Supplier	Purity
STF2.HA1	128A809037-1	VaxInnate Corporation Cranbury, NJ	98%
STF2R3.HA1	128B809035-1		96%
STF2R3.2xHA1	128C809036-1		97%
Suspension buffer F147	147809032 147810001		Not supplied

The control article was used as received and dispensed on each day of dosing. Each vaccine was aseptically prepared on each day of dosing by mixing the required volume of the appropriate vaccine with the required volume of control article to achieve the correct concentration. Following preparation, the formulations were mixed to ensure visual uniformity.

Experimental Design

Animals were initially accepted into the randomization pool based upon pre-study body weights, cage side observations, physical examinations, and ocular examinations. Males and females were randomized separately, and assigned to study as presented in **Table 2**.

Table 2. Study Design

Group	Treatment	Dose Level ($\mu\text{g}/\text{Dose}$)	Dose Volume (mL/animal)
1	Control Article	0	0.5
2	STF2.HA1	4	0.5
3	STF2.HA1	8	0.5
4	STF2R3.HA1	8	0.5
5	STF2R3.HA1	16	0.5
6	STF2R3.2xHA1	8	0.5
7	STF2R3.2xHA1	16	0.5

Animals were administered test or control articles via intramuscular injection to the right hind limb on SD 1 and 43 (separate site used) and to the left hind limb on SD 22. The injection sites were shaved and marked as needed to visualize the site. Animals were divided into cohorts based on the test article they received; each cohort was housed in a separate animal room and dosing was staggered according to cohort assignment. Animals in Cohort 1 received STF2.HA1, animals in Cohort 2 received STF2R3.HA1, and animals in Cohort 3 received STF2R3.2xHA1. Control animals were distributed evenly amongst the cohorts.

The dose formulations and control article were maintained at room temperature throughout the dosing procedure, and used within 8 hours of formulation.

Animals were observed as shown in **Table 3**.

Table 3. Animal Observations/Measurements

Procedure	Frequency
Cageside Observations	≥ twice daily and on SD 1 and 15 within 4 ± 0.5 h of the time of dosing
Clinical Examinations	Prior to each dose, 24 ± 2 hours after each dose, weekly thereafter, on the day prior to necropsy and at termination
Body Weights	Prior to each dose, for 2 consecutive days following each dose, weekly thereafter, the day prior to necropsy (unfasted, all animals) and on the day of necropsy (only animals scheduled for necropsy, fasted)
Food Consumption	Daily except when interrupted for study-related events
Ophthalmologic Examinations	Prior to necropsy (all surviving animals at each interval)
Body Temperature	On the day prior to each dose, 6 hours ± 15 minutes after each dose and 24 ± 0.5 hours after each dose. If any temperature vales were greater than 40°C, daily body temperatures were collected until the temperature returns below 40°C
Dermal Draize Observations	Prior to each dose, for 7 consecutive days after each dose (only the site being dosed), weekly thereafter (all sites), and at termination (all sites)

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

Ocular exams were conducted using indirect ophthalmoscopy and slit-lamp (as needed) following administration of a mydriatic solution. Body temperatures were collected rectally using either the Thermalert TH-5 or the Filac F-1500 temperature unit.

The injection sites were evaluated according to the Draize scoring scale.

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

Table 4. Clinical Pathology and Serology

Procedure	Time Points	Sample Volume (mL)	Collection Device
Chemistry	Prior to initiation of dosing, on SD 3, and prior to necropsy (all surviving animals)	≥1.2	Serum separator tubes
Hematology		≥1.2	Tubes containing dipotassium EDTA
Coagulation	Prior to initiation of dosing, on SD 3, 8, 24, and prior to necropsy (all surviving animals)	≥1.8	Sodium citrate tubes
C-Reactive Protein, Serum Protein Electrophoresis	Prior to initiation of dosing, on SD 2, 3, 23, 24, and prior to necropsy (all surviving animals)	≥1.5	Serum separator tubes
Urinalysis	Prior to necropsy (all surviving animals)	Up to 12 mL	Clean catch pan
Serology	Prior to each dose, 24 ± 0.5 hours following each dose, on SD 3 and 24 and at termination (all surviving animals)	≥1.0	Serum separator tubes

Note: In addition antibody analysis was conducted on SD 23, 46, and 68. Residual serum collected for CRP analysis was used.

Termination and Postmortem Procedures

On SD 46 for main phase animals and SD 68 for recovery animals, all surviving animals were euthanized by injection of sodium pentobarbital followed by exsanguination. A gross necropsy, which included examination of the external surface of the body, all orifices, injection sites, the cranial, thoracic, and abdominal cavities, and their contents, was performed. 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 harderian glands, lacrimal glands, eyes (with optic nerves) and testes (with epididymides) which were preserved in modified Davidson's fixative and subsequently transferred to 10% NBF. Protocol-required preserved tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissues from main phase animals in the control group and high dose groups (Group 1, 3, 5, and 7) and gross lesions from all other animals were examined by a board-certified veterinary pathologist.

RESULTS

Animal Disposition and Clinical Observations

Treatment with STF2.HA1, STF2R3.HA1, or STF2R3.2xHA1 had no effect on mortality, clinical examinations or cage side observations. All observations were incidental and are common findings in laboratory-housed New Zealand White rabbits.

Dermal Draize Observations

Treatment with STF2R3.HA1, or STF2R3.2xHA1 had no effect on dermal Draize observations. However, minimal to mild erythema and minimal edema were observed in males and females following the first dose of STF2.HA1 vaccine. Although not considered adverse, the observations were test article-related.

Body Weight and Body Weight Change

Treatment with STF2R3.HA1 or STF2R3.2xHA1 had no adverse effect on body weights or body weight changes. All animals treated with STF2R3.HA1 or STF2R3.2xHA1 gained weight throughout the course of the study and there were no significant differences in the total absolute body weight change for males or females during the SD 1-45 study interval.

During the SD 1-2 study interval statistically significant decreases in absolute body weights occurred in males administered STF2.HA1 vaccine at both 4 µg and 8 µg; female body weights were also lower but the decreases were not statistically different from the controls. These decreased body weight changes were correlated with decreased food consumption for the same interval in both males and females. Although test article-related the effects were not considered adverse because they did not persist and because the absolute total body weight gain during the treatment or recovery phase was not significantly different from that of the control.

Food Consumption

Overall, treatment with STF2.HA1, STF2R3.HA1, or STF2R3.2xHA1 had no adverse effect on food consumption. No significant differences in total food consumption occurred throughout the dosing interval (SD 1-45). Significantly lower food consumption from SD 1 to 2 in males and females treated with 8 µg STF2.HA1 (Group 3) was test article-related and correlated to body weight decreases for the same interval.

Body Temperatures

Treatment with STF2.HA1, STF2R3.HA1, or STF2R3.2xHA1 had no adverse effect on body temperatures. However, six hours after dosing on SD 1, Group 2, 3, 5, 6, and 7 males had statistically higher body temperatures when compared to the control and Group 2 and 3 females had significantly higher body temperatures. The elevation is not adverse because the temperatures did not exceed 40°C and did not persist.

Ophthalmology

Treatment with STF2.HA1, STF2R3.HA1, or STF2R3.2xHA1 had no effect on ophthalmology; the findings noted were considered incidental and unrelated to test article administration.

Clinical Pathology

The only test article-related effects on clinical chemistry and coagulation variables occurred on SD 3 that included an increase of mean globulin (GLOB) values with a decrease in mean albumin/globulin (A/G) ratios in animals given 8 µg STF2.HA1 vaccine (Group 3). Mean differences were statistically significant for GLOB (females) and A/G (males and females). All mean GLOB and A/G values were within the AVANZA Laboratories historical reference ranges. This test article effect was small in magnitude, not adverse, and compatible with systemic immune stimulation from vaccine test article.

There were also increases of mean fibrinogen (FIB) and activated partial thromboplastin (APTe) values in animals given 4 µg and 8 µg STF2.HA1 vaccine (Groups 2 and 3, respectively), and a decrease in mean prothrombin time (PT) values in Group 3 animals. The mean differences for FIB and PT were statistically significant in males and females. The mean differences for APTe values were statistically significant for Group 2 females and Group 3 males and females. The mean APTe values were within the AVANZA Laboratories historical reference ranges, but the mean FIB and PT values were above and below, respectively, the historical reference ranges. The test article effect on FIB was small in magnitude, not adverse, and compatible with systemic immune stimulation from vaccine test articles. In the absence of clinical signs or anatomic pathology evidence of hemorrhage or thromboembolism, the test article effects on PT and APTe were non-adverse.

There were no other test article-related effects on clinical pathology variables throughout the remainder of the study.

C-Reactive Protein

On SDs 2 and 3, there was a test article-related increase of mean C-reactive protein (CRP) values in animals given 4 µg and 8 µg STF2.HA1 vaccine (Groups 2 and 3, respectively). The test article effect was small in magnitude, not adverse, and compatible with systemic immune stimulation from vaccine test article.

On SD 23, there was a test article-related increase of mean C-reactive protein (CRP) values in animals given 8 µg and 16 µg STF2R3.HA1 (Groups 4 and 5, respectively) and STF2R3.2xHA1 vaccine (Groups 6 and 7, respectively). This test article effect was small in magnitude, not adverse, and compatible with systemic immune stimulation from vaccine test article.

There were no additional test article-related effects on C-reactive protein.

Serum Protein Electrophoresis

On SD 2, there was a test article-related decrease in mean albumin/globulin (A/G) ratio in females given 4 µg STF2.HA1 (Group 2) and 16 µg STF2R3.HA1 (Group 5) vaccines. This finding was the result of significant decreases in the mean percent (%) albumin (PE ALB) values and increases in mean % alpha globulin 1b, % alpha globulin 2, % beta globulin 1, % beta globulin 2 and % gamma globulin values. These changes were not observed in males. The mean A/G values in females given 4 µg STF2.HA1 and 16 µg STF2R3.HA1 vaccines were within the AVANZA Laboratories historical reference range for New Zealand White rabbits. These test article effects were small in magnitude, not adverse, and compatible with systemic immune stimulation and changes expected in acute phase reactant proteins with a vaccine test article.

Antibody Analysis

Samples collected prior to dosing and on SD 23, 46, and 68 were analyzed in HA-specific and STF2-specific IgG ELISA. All samples, except Animal 10912 (6M), collected prior to dosing and Group 1 (F147 control) samples had HA-specific IgG levels below 2 µg/mL. In all treated groups, anti-HA specific IgG were observed on SD 23 (as shown in Figure 2), 46, and 68 at both levels for each vaccine test article. Additionally, anti-STF2 specific IgG were also observed on SD 23, 46, and 68. A small number of prebleed and Group 1 samples had

measurable anti-STF2 specific IgG levels ($>1.5 \mu\text{g/mL}$). This was most likely due to exposure to flagellin on commensal bacteria. These results suggest that the rabbit model is an appropriate immunological model for all three vaccine test article tested.

Serology

Serum samples were evaluated for the presence of antibody against H1N1 A/California/07/09 Influenza virus by hemagglutination inhibition assay (HAI). All pre-bleed samples tested (group 1-7) negative ($\text{GMT}=5$) for antibodies against A/California/07/09 (H1N1) virus. As shown in Figure 3, vaccines consisting of recombinant influenza hemagglutinin (rH) STF2.HA1 at $4\mu\text{g}$ (Group 2) and $8\mu\text{g}$ (Group 3), STF2R3.HA1 at $8\mu\text{g}$ (Group 4) and $16\mu\text{g}$ (Group 5), or STF2R3.2xHA1 at $8\mu\text{g}$ (Group 6) and $16\mu\text{g}$ (Group 7) all showed efficacy against influenza A/California/7/09 as demonstrated by a 40-fold or higher rise in antibody titer post vaccination and in comparison to control Group 1 on SD 46. This response was sustained through Day 68. The presence of HAI titers in the Day 46 and Day 68 sera samples from vaccinated groups (2-7) demonstrate that the vaccine has the ability to generate a humoral immune response to A/California/07/09 (H1N1) virus.

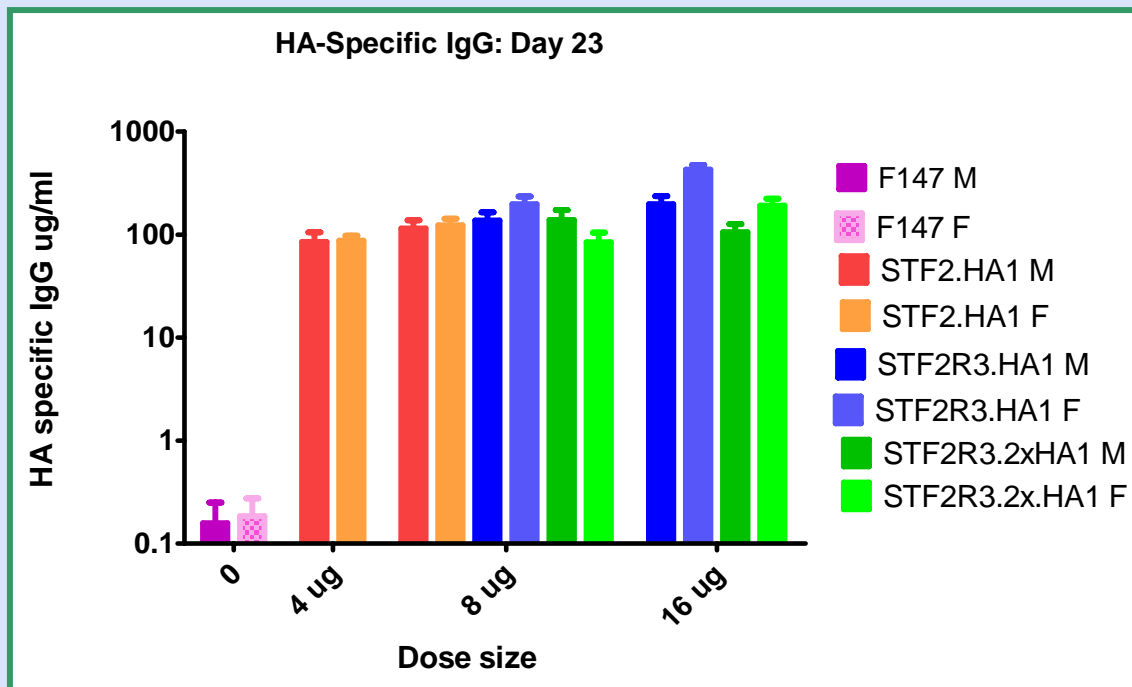


Figure 2: HA-specific IgG SD 23. Serum was tested in an ELISA using HA-coated plates. Specific IgG values were calculated by comparing OD values of serum dilutions against a standard curve of rabbit polyclonal IgG plotted using a 4-parameter logistic equation.

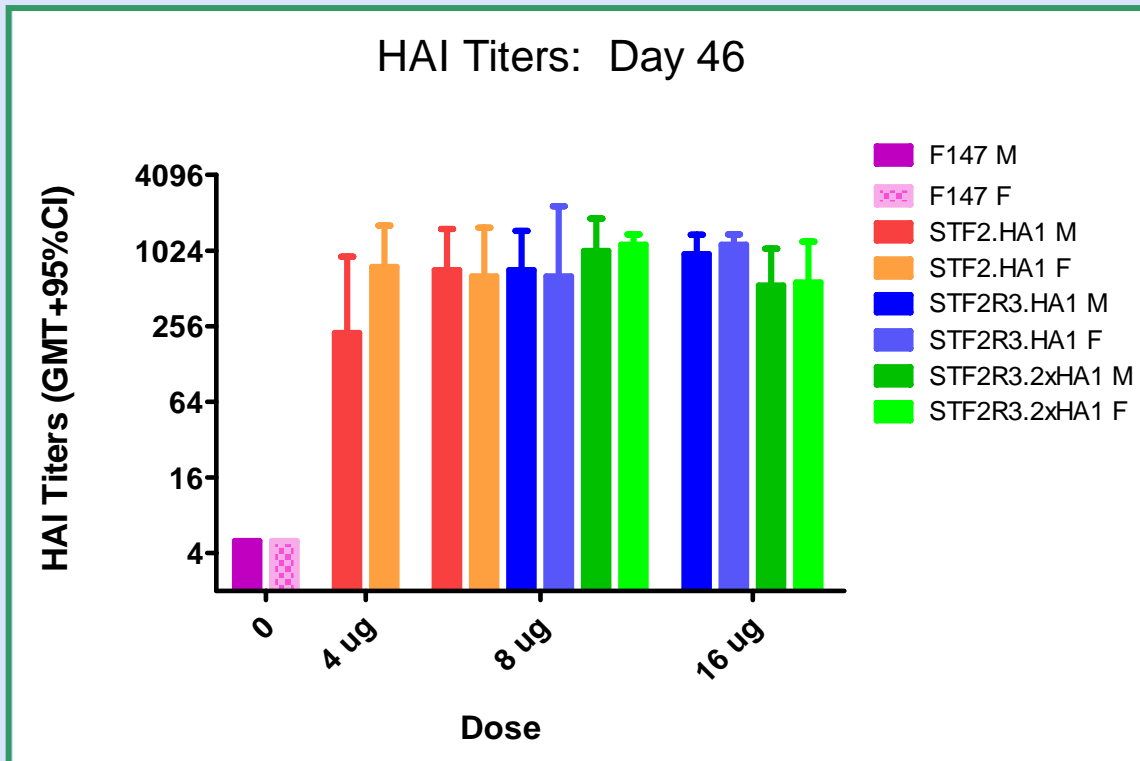


Figure 3: HAI titers on SD 46. Serum was tested in an HAI assay using A/California07/09 virus (Southern Research Institute, Birmingham, AL).

Macroscopic Findings and Organ Weights

There were no test article-related macroscopic findings or organ weights at any time point.

Histopathology

There were no test article-related microscopic findings noted in animals at either necropsy interval.

CONCLUSIONS

In conclusion, intramuscular administration of the three vaccines was well tolerated and did not result in any adverse effects at the highest administered dose. The presence of positive HAI titers in all vaccine groups after immunization confirms that the rabbit is an appropriate immunological model for influenza vaccines.