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REPORT TITLE: Environmental safety study of a cold-adapted DISEASE vaccine trivalent (CAD-T) vaccine formulation in turkeys.

SUMMARY: The study was performed to determine whether turkeys could be productively infected with the vaccine viruses comprising a live, attenuated, cold-adapted DISEASE virus trivalent (CAD-T) vaccine. To evaluate this possibility, turkeys were inoculated orally with a CAD-T vaccine formulation that contained the following cold-adapted DISEASE virus strains: 1) EXAMPLE NAME/20/99, 2) EXAMPLE NAME2/05/97, and 3) EXAMPLE NAME3/504/2000, with each virus present at 7 ± 0.5 Log_{10} focus-forming units (FFU)/dose in a stabilizing buffer. Three groups of 10 animals each were included in the study; the control group received buffer alone, and the other 2 groups received virus. Throat swabs were collected daily following inoculation. One group of animals inoculated with virus was sacrificed on Day 4; a second group of virus-inoculated animals and the control group were sacrificed on Day 7. Intestine, lung, and excreta samples were collected at the time of sacrifice. Intestinal samples were homogenized and analyzed for viral load by plaque formation on Madin-Darby canine kidney (MDCK) cells. Virus was not detected in the intestinal samples from inoculated animals. These results demonstrate that productive replication was not established in turkeys for the attenuated, cold-adapted, temperature-sensitive, DISEASE virus strains in the vaccine.

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1.0 INTRODUCTION

DISEASE viruses have been isolated from a wide variety of animals. As all virus subtypes have been found in aquatic birds, they are thought to be a reservoir of virus for other species (Hatta and Kawaoka 2002). Serological analysis has identified 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes in aquatic birds; however, the number of viral subtypes in mammals is more limited (Hatta and Kawaoka 2002). DISEASE A viruses that replicate in mammals show a restricted combination of HA and NA types. Three HA types (H1, H2, and H3) and 2 NA types (N1 and N2) are primarily associated with disease in humans. Genetic studies of pandemic strains suggest that avian viruses, particularly those maintained by waterfowl, play a key role in the emergence of new viral strains (Subbarao and Katz 2000).

Transmission of DISEASE viruses between avian and mammalian species is restricted by a number of viral and host factors. Avian viruses tend to replicate better at higher temperature and lower pH than their mammalian counterparts, consistent with the higher body temperature of birds (~40^{o}C) compared with mammals (Baigent and McCauley 2003). In avian species, the primary site of replication of DISEASE virus is the intestinal tract, whereas in humans the viral replication is restricted to the respiratory tract.

Experimental infections of ducks with human virus have demonstrated that human viruses fail to replicate in ducks (Hatta, Halfmann et al. 2002). However, transmission of DISEASE A virus from poultry to humans was observed in 1997 (Hatta and Kawaoka 2002). Eighteen cases of DISEASE disease in humans were attributed to an H5N1 strain of DISEASE that was transmitted directly from poultry to humans in Hong Kong. Similarly, in 1999, a DISEASE virus of H9N2 subtype was isolated from children in Hong Kong. In 2000–2002, H5N1 viruses emerged in the poultry markets of Hong Kong, but the chickens were sacrificed before any human cases appeared. The ability of the virus to be transmitted directly from avian species to humans raised concerns about the avian population serving as a reservoir for DISEASE viruses and the potential for their involvement in causing a future pandemic (Hatta and Kawaoka 2002).

While pandemics have resulted from introduction of HA and/or NA genes by reassortment from an animal-derived virus into the genetic background of a currently circulating human virus (Baigent and McCauley 2003), the pathogenicity of the resultant reassortant viruses is a polygenic trait. The HA and NA surface glycoproteins play a central role in the successful adaptation of a virus to a new host (Baigent and McCauley 2003), with a major determinant of host range being the affinity of the viral HA for the host cell sialic acid (SA)-containing receptor. In general, DISEASE viruses bind to SA receptors in the mammalian respiratory tract and avian intestinal tract. In the avian host, SA is joined to sugar chains through an α2-3 linkage, and viruses isolated from birds have a high affinity for sugar with this linkage. Both avian and equine DISEASE viruses preferentially bind to SA-α2-3-galactose, and the α2-3 receptor is present in epithelial cells of duck intestine and horse trachea (Suzuki et al 2000). This could explain the apparent direct transmission of an avian DISEASE A virus to horses in China (Guo, Wang et al. 1992).

Cells in the human respiratory tract have α2-6 SA linkages. Viruses circulating in humans have mutations in HA which result in loss of affinity for α2-3 receptors and a concomitant increase in α2-6–binding efficiency (Horimoto and Kawaoka 2001; Baigent and McCauley 2003). Porcine tracheal epithelial cells contain both SA-α2-3 and α2-6-galactose linkage receptors (Suzuki et al 2000), resulting in swine susceptibility to infection from both avian and human DISEASE viruses. Hence, the pig is a prime source of reassortant viruses (Zhou, Seene et al. 1999) with human pandemic potential (Hatta and Kawaoka 2002).

The CAD-T vaccine is being developed for the prevention of illness caused by Type A and Type B DISEASE viruses. The vaccine consists of three cold-adapted, temperature-sensitive, attenuated reassortant DISEASE strains: two Type A, subtypes H1N1 and H3N2; and one Type B. The vaccine is formulated as a mixture of the 3 strains containing 7 ± 0.5 Log_{10} FFU of each virus in one human dose. The vaccine was tested in avian and mammalian species. The oral route of administration was used in avian subjects to accommodate the size and structure of the nose and the intranasal route of administration was used in mammalian subjects, as this is the method to be used in human clinical medicine. Ferrets, which are naturally susceptible to human

DISEASE A and B viruses, have served as models in studies on DISEASE pathogenesis, transmission and immunity (Herlocher, Elias, et al. 2001; Zitlow, Rowe, et al. 2002), and were used as a positive control in studies on the CAD-T vaccine.

The attenuated viruses comprising the CAD-T vaccine exhibit a temperature-sensitive phenotype, which would restrict growth in the higher body temperature of the avian. This quality, in addition to the difficulty in establishing growth of human viruses in birds, suggests that replication of the viruses in the CAD-T vaccine would not be supported in turkeys.

2.0 OBJECTIVE

The objective of this study was to determine if turkeys could become infected with the viruses in the CAD-T vaccine.

3.0 RESPONSIBILITIES

The "in-life" portion of the study was conducted at CONTRACTOR2 Bio-Labs under study protocol number XXX-1234. Tissue samples were shipped to EXAMPLE PHARMA Research, Vaccines Research Discovery for analysis of viral load. Throat swabs were shipped to CONTRACTOR Laboratories (North Yorkshire, UK) for analysis of viral load.

4.0 MATERIALS AND METHODS

4.1 Materials

The test and control substances were provided to CONTRACTOR2 Bio-labs by EXAMPLE PHARMA Pharmaceuticals, Sometown, PA as prepared dose solution in pre-filled syringes. The test substance was the CAD-T vaccine, lot number 7-6167-003B. The control substance was Gelatin Placebo for CAD-T vaccine, lot number 7-8022-001A. The test substance consisted of the following virus strains: EXAMPLE NAME/20/99, EXAMPLE NAME2/05/97 and EXAMPLE NAME3/504/2000 at a concentration of 7 ± 0.5 Log_{10} FFU of each virus strain per 0.2-mL human dose. The control buffer consisted of sucrose, phosphate, glutamate, acid-hydrolyzed gelatin, and arginine.

4.2 Methods

4.2.1 EXAMPLE PHARMA Procedures

Thirty, 2.5-week-old, Nicholas cross turkeys were used in the study. Serum from each animal was tested with the enzyme-linked immunosorbent assay (ELISA) for pre-existing immunity to DISEASE virus; all animal results were negative. On study Day 1, 0.2 mL of either vaccine (groups 1 and 2) or control substance (group 3) was administered via oral gavage.

Animals were sacrificed on study Day 4 (group 1) or study Day 7 (groups 2 and 3). Approximately 0.5 g each of lung, large intestine, and excreta were removed and placed into 2.0-mL of phosphate-buffered saline (PBS) plus 0.14% bovine serum albumin (BSA) and then frozen at –20^{o}C. Samples were shipped to EXAMPLE PHARMA Research on dry ice.

4.2.2 Sample Preparation

Tissue samples were stored at –80^{o}C by EXAMPLE PHARMA Research until processed for virologic analyses. Each tissue sample was diluted to a final volume of 10 mL with virus stabilization buffer, which consisted of PBS with glutamine, arginine, and hydrolyzed gelatin. The tissue was homogenized on ice using an Omni Homogenizer at a setting of 6 for 60 seconds, followed by 30 seconds at a setting of 8. Homogenates were clarified by centrifugation at 500x g for 15 minutes in a tabletop centrifuge. Aliquots of the tissue extracts were dispensed (5 x 1 mL), and four were flash-frozen immediately in a dry ice/ethanol bath and stored at –80^{o}C. The remaining aliquot was analyzed for viral load by plaque assay.

4.2.3 Plaque Assay

Plaque assay testing was performed on the tissue type most likely to have virus replication, intestinal tract in avian and respiratory tract in mammalian species. The assay was not performed on further tissues unless the initial tissue sample tested positive.

MDCK cells were grown in minimal essential medium with Earle's Salts (MEM; Gibco-BRL, Inc., Gaithersburg, MD) supplemented with non-essential amino acids, sodium pyruvate, penicillin/streptomycin and heat-inactivated fetal bovine serum (10%). MDCK cells were seeded into 12-well dishes 24 to 48 hours prior to use and were confluent at the time of infection.

Ten-fold dilution series of organ tissue extracts were made in PBS supplemented with 0.2% BSA. Medium was aspirated from MDCK monolayers, and cells were washed twice with PBS. An aliquot of each virus dilution (100 uL) was adsorbed to cell monolayers for 1 hour at 33 ^{o}C, at

which time the inoculum was removed and 2 mL of agar overlay (MEM supplemented with non-essential amino acids, sodium pyruvate, penicillin/streptomycin, 0.2% BSA, and 0.6% [w:v] agar [Oxoid, LTD, Ogdensburg, New York]) was added to each well. After the agar solidified, culture plates were incubated for 3 days at 33°C in 5% CO_2. Virus plaques were visualized by staining with 0.1% crystal violet/32% methanol (w:v) after removal of the semisolid overlay. Plaques were counted in all wells having less than 50 plaques. The limit of detection in this assay is 50 plaque-forming units (PFU)/g of tissue. Reverse transcriptase polymerase chain reaction (RT/PCR) testing was not performed unless plaque assay testing was positive.

5.0 RESULTS AND DISCUSSION

Intestinal tissues harvested on study Days 4 and 7 from turkeys infected orally with a trivalent mixture of cold-adapted, attenuated DISEASE viruses were analyzed for the presence of DISEASE virus. Virus was not detected in intestinal tissue from any of these animals (Table 1). These results demonstrate that they are unable to support the replication of the cold-adapted, attenuated vaccine viruses in the CAD-T vaccine. Lung and fecal samples were not analyzed since virus was not detected in intestinal tissue.

Throat swabs were collected daily throughout the study. Swabs were placed in PBS plus BSA and stored at –20°C until analyzed. Samples were sent to a contract laboratory for analysis (CONTRACTOR Laboratories, North Yorkshire, UK). In order to test for virus, samples were placed on cells in tissue culture and observed for viral-induced cytopathic effect. Materials found in the samples caused disruption of the cell monolayer, including those from the placebo-controlled animals, rendering the results inconclusive. Therefore, they were not included in this study.

It is known that host restriction plays an important role in transmission of DISEASE strains between species. Typically, human viruses are unable to establish an infection in birds; therefore, it is unlikely that replication of the attenuated vaccine viruses would occur in turkeys. Also, since the viruses are temperature-sensitive, they would be expected to be restricted for growth in the elevated temperatures of the avian gut. The results demonstrate that transmission of CAD-T

vaccine human viruses to turkeys did not occur following oral inoculation, and that turkeys are not a potential reservoir for vaccine viruses in the environment.

6.0 CONCLUSIONS

Turkeys inoculated orally with a human dose of CAD-T vaccine did not establish a productive infection. Since this species does not support vaccine virus replication, it cannot be a reservoir for the live, cold-adapted, attenuated, DISEASE viruses in the CAD-T vaccine.

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8.0 Table 1.

Replication of CAD-T in turkeys infected by oral gavage

		Plaque Assay (Log PFU/g)			Real time RT/PCR (Log PFU equivalents/g)		
Group #/ Study Day Date	**ID #**	**Intestine Titer**	**Intestine GMT***	**Lung Titer**	**Intestine Titer**	**Intestine GMT***	**Lung Titer**
Group 1 Day 4 DATE	904	?	?value	ND	ND	ND	ND
	901	?		ND	ND		ND
	893	?		ND	ND		ND
	925	?		ND	ND		ND
	907	?		ND	ND		ND
	889	?		ND	ND		ND
	917	?		ND	ND		ND
	913	?		ND	ND		ND
	922	?		ND	ND		ND
	918	?		ND	ND		ND
Group 2 Day 7 DATE	894	?	?	ND	ND	ND	ND
	896	?		ND	ND		ND
	902	?		ND	ND		ND
	900	?		ND	ND		ND
	895	?		ND	ND		ND
	919	?		ND	ND		ND
	923	?		ND	ND		ND
	911	?		ND	ND		ND
	921	?		ND	ND		ND
	924	?		ND	ND		ND
Group 3 Day 7 DATE	891	ND	ND	ND	ND	ND	ND
	905	ND		ND	ND		ND
	898	ND		ND	ND		ND
	892	ND		ND	ND		ND
	888	ND		ND	ND		ND
	928	ND		ND	ND		ND
	910	ND		ND	ND		ND
	908	ND		ND	ND		ND
	912	ND		ND	ND		ND
	920	ND		ND	ND		ND

* geometric mean titer
ND = Not Done