



28-Day repeated dose oral toxicity of recombinant human holo-lactoferrin in rats

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ABSTRACT

Recombinant human holo-lactoferrin (holo-rhLF) was orally administered, via gavage, to Wistar rats at 1000, 500 and 100 mg/kgbw/day for 28 days. The test article, holo-rhLF, was expressed in rice grain, extracted, purified and saturated with iron. During the 28-day period, animals were examined for evidence of toxicity. On day 29, the animals were exsanguinated, examined for gross pathology, and tissues preserved for histopathology. There were no deaths caused by holo-rhLF and in-life physical signs were generally normal. Although statistical differences were noted in some hematology, clinical chemistry and heart/body weight ratios, they were of questionable biological significance. A significantly greater total iron binding capacity (TIBC) was detected in the blood of male animals dosed with holo-rhLF. Serum was analyzed for the presence of IgG and IgE antibodies; demonstrating low levels of IgG antibodies to the human protein, but no increase in IgE antibodies. There was no increase in serum lactoferrin levels. The results of the 28-day oral administration demonstrate a lack of toxicity of holo-rhLF in rats. There were no treatment related, toxicologically relevant changes in clinical signs, growth, food consumption, hematology, clinical chemistry, organ weights or pathology. The no observed adverse effect level (NOAEL) is greater than 1000 mg/kg/day.

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1. Introduction

Lactoferrin is an 80 kDa glycoprotein member of the transferrin family of iron binding proteins. Human lactoferrin is a single chain polypeptide of 692 amino acids (Lonnerdal and Iyer, 1995). Lactoferrin is ubiquitous in the human body. Lactoferrin occurs naturally in tears, saliva and other epithelial cell secretions as well as in the granules of circulating neutrophils (Lin et al., 2001; Ohashi et al., 2003; Pupek et al., 2003). The concentration of human lactoferrin (hLF)¹ is highest in breast milk, from 5 to 7 mg/mL in colostrum to 1–3 mg/mL in mature milk (Lonnerdal, 1985).

Since the discovery of lactoferrin by Sorensen more than 60 years ago, studies have been performed *in vitro*, in experimental animals, and in humans to analyze the functions of lactoferrin (Sorensen and Sorensen, 1939; Brock, 1980, 2002). Lactoferrin has been shown to exhibit anti-bacterial, anti-viral, anti-fungal and anti-parasitic activities (Naidu, 2000; Shimazaki, 2000). It has also demonstrated activity as an anti-inflammatory/anti-oxidant agent (Gutteridge, 1989; Morris et al., 1995; Baveye et al., 1999).

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¹ Abbreviations used: EDTA, ethylene diamine tetracetate; ELISA, enzyme linked immunosorbent assay; hLF, human lactoferrin; Holo-rhLF, recombinant human holo-lactoferrin.

The commercial use of human lactoferrin has not been developed due the prohibitive cost of lactoferrin isolated from breast milk. The production of recombinant proteins in rice provides a method for production of large volumes with low cost. Recombinant proteins are targeted to the protein body of the maturing grain for safe expression and accumulation through specific promoters and signal peptides. Recombinant human lactoferrin (rhLF) has been expressed in rice at significantly higher levels than in other transgenic plant systems, reaching 0.5% of grain weight (Nandi et al., 2002). The target protein is extracted from the rice and kilograms of rhLF have been purified to near homogeneity and compared to native human lactoferrin (Nandi et al., 2005). The purified rhLF can be saturated with iron to form holo-rhLF containing 1.4 mg iron/gram of protein.

Expression of human lactoferrin in rice is an attractive approach for producing proteins for oral delivery. Rice is among the first foods recommended for introduction to infants and is used in allergenicity exclusion diets; it has good nutritional value and low allergenicity. Lactoferrin is not a toxin and has no sequence homology with any toxins listed in the SWISS-PROT and TrEMBL databases. Since human lactoferrin is a component of breast milk and there is also exposure to lactoferrin from tears and saliva, it is not anticipated that the addition of recombinant human lactoferrin to the diet would result in any toxicities. In order to support the use of iron saturated lactoferrin in foods, the safety was evaluated in a 28-day oral administration study in rats to examine possible

toxicity, a similar study was done using recombinant human lactoferrin with the iron removed (Cerven et al., 2008). The study was conducted according to the standards set forth in the Organization for Economic Cooperation & Development (OECD) Guidelines for Testing of Chemicals, #407, adopted 7/27/95. The oral route of exposure was selected since human exposure is expected to be via this route. The selection of animal species, general study parameters and duration of the study was made according to the OECD Guidelines.

2. Materials and methods^{2,3}

2.1. Test substance

The recombinant human holo-lactoferrin used in the study was prepared from rice grain expressing the protein. The rice is de-husked and milled to 100-mesh powder using standard food industry procedures. Recombinant human lactoferrin is extracted from ground rice powder using 0.02 M phosphate buffer with 0.3 M sodium chloride, pH 6.5. After 1.5–3.5 h of extraction, the insoluble rice residuals are separated from the liquid phase by centrifugation. The liquid phase, containing the soluble target protein, is filtered and concentrated. The concentrate is further purified to an isolate using ion exchange chromatography. The concentrate is loaded on a column of SP Sepharose Big Bead Media (Amersham), the column is washed and the bound lactoferrin eluted with 0.8 M sodium chloride. The eluate is diafiltered (30 kDa) to remove excess salt and concentrated (Nandi et al., 2005). Prior to lyophilization the lactoferrin is saturated with iron to produce recombinant human holo-lactoferrin (Nandi et al., 2002). In the material used for the study the lactoferrin was 92% w/w, bound iron concentration was 1.2 mg/g, and total iron was 1.95 mg/g.

2.2. Animals and treatment

Seven-week-old Wistar albino rats were received from Ace Animals, Inc., Boyertown, PA. Following a 7-day equilibration period, 20 healthy male and 20 healthy female rats were randomly selected and assigned to treatment and control groups using a computer randomization process. The body weight range immediately prior to the start of dosing was 223–282 g for males and 170–248 g for females.

The animals were housed 1/cage in suspended stainless steel wire bottom cages and individually identified by a uniquely numbered eartag. The individual cages were identified with a cage card indicating the MB project number, holo-rhLF dose level, date of study initiation, animal number and sex. ANIPADSTM paper bedding, placed beneath the cages, was changed three times/week. Fresh Certified PMI Rodent Chow Diet #5002M (Meal) was provided *ad libitum* except for the fasting period on the day prior to termination. Fresh well water, which is analyzed four times per year for impurities, was available *ad libitum*. During the time frame of this study, there were no aqueous impurities found which would affect this toxicologic evaluation. The animal room had a 12-h light/dark cycle but was not maintained as pathogen free. The temperature of the animal room was 20–22 °C for the majority of the study. Transient episodes of 15–25 °C were noted during the period when replacement animals were the only animals on study. This did not affect the outcome of the study since the animals all appeared normal during that period. The humidity generally ranged from 20 to 63%; with only short transient periods below 20%.

Holo-rhLF or control vehicle (saline) was administered orally, once daily, by gavage using a syringe and 16 gauge ball-tipped feeding needle. There were four groups with 5 female and 5 male animals in each group. Animals received holo-rhLF in saline at levels of 0, 100, 500 or 1000 mg/kg bw/day in a volume of 4 mL/kg. Due to the macroingredient category inclusion of these proteins in foods and drinks, the high dosage level was determined as a level which would not result in nutritional imbalance (Borzelleca, 1996; Munro et al., 1996). The holo-rhLF was mixed with saline to the proper concentration and stored refrigerated when not in use. Individual dose volumes were adjusted weekly based on body weight.

2.3. Animal observations

All animals were observed once daily for toxicity and pharmacologic effects and twice daily for morbidity and mortality. Body weights were recorded immediately pretest, weekly, and on the day prior to study termination. Food consumption was calculated each week using weight of remaining food and adjusted for spillage. On day 28, animals were fasted overnight and on day 29 animals were anesthetized with ether prior to being euthanized by exsanguinations from the aorta. Whole blood in EDTA containers was shipped on ice; citrated plasma and serum were separated from cells by centrifugation and shipped on dry ice overnight to AniLytics, Inc., Gaithersburg, MD, for analysis.

2.3.1. Hematology and serum/plasma chemistry

Hematology measurements (ABX Pentra 60) included: red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count (WBC), differential leukocyte count, platelet count. Prothrombin time was analyzed using a STA compact instrument.

Serum clinical chemistry (Hitachi 717) included: sodium, potassium, chloride, magnesium, calcium, phosphorus, globulin, alanine aminotransferase (SGPT), aspartate aminotransferase (SGOT), alkaline phosphatase, gamma glutamyl transpeptidase (GGT), glucose, BUN, creatinine, total cholesterol, triglycerides, total bilirubin, albumin, total protein, sorbitol dehydrogenase, iron, unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), and serum transferrin.

In addition, an aliquot of serum was shipped to the sponsor to test for lactoferrin levels and antibodies to lactoferrin. Lactoferrin levels were assayed by an ELISA sandwich assay, as previously described, using a standard curve of purified lactoferrin from 10 to 70 ng/mL (Nandi et al., 2002). The serum was diluted 1:1 with assay buffer.

The presence of IgG and IgE antibodies to human lactoferrin was determined using ELISA plates coated with recombinant human lactoferrin. Sera were diluted 1:4 in assay buffer and added to plates coated with recombinant human lactoferrin using a sandwich protocol based on the ELISA for lactoferrin levels. Antibody presence was detected using a second step addition of rabbit anti-rat IgG-HRP (horse radish peroxidase) (DAKO, Carpinteria, CA) or sheep anti-rat IgE-HRP (Bethyl Labs, Montgomery, TX).

2.3.2. Necropsy and histopathology

All rats underwent a complete necropsy following blood sample collection that included examination of external surfaces of the body, all orifices and the cranial, thoracic and abdominal cavities and their contents. Organs were trimmed and weights obtained for the following organs: adrenals (2), kidneys (2), liver, testes (with epididymides) (2), thymus, spleen, brain and heart. Organ to body weight ratios were calculated for these tissues. The following tissues were preserved in 10% neutral buffered formalin:

² This study meets the Good Laboratory Practice requirements of EPA 40 CFR parts 792 and 160, FDA 21 CFR Part 58.

³ Studies were conducted at MB Research, Spinnerstown, PA.

esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, liver, adrenals, thyroid/parathyroid, pancreas, lung/trachea, kidneys, bladder, testes/epididymides, seminal vesicles, uterus, ovaries, prostate, vagina, aorta (thoracic), heart, bone marrow (sternal), lymph nodes (sub-mandibular, mesenteric), spleen, thymus, brain (multiple sections), pituitary, peripheral nerve(s), spinal cord (three levels), any gross lesions or masses (if present), and the ear tag area. All the preserved tissues from the control (0 mg/kg), the high-dose group (1000 mg/kg) were paraffin embedded, sectioned, stained with hematoxylin and eosin and examined microscopically. Histopathologic preparations and microscopic analyses were performed by W. Ray Brown, DVM, Ph.D., Research Pathology Services, Inc., New Britain, PA.

2.4. Statistical analysis

All non-discrete data from clinical chemistry, hematology, organ weights, body weights, organ/body weight ratios, and food consumption were tabulated with means and standard deviations. An Analysis of Variance (ANOVA) was performed on absolute organ weights, body weights and organ/body weight ratios. Parametric data was analyzed using ANOVA techniques with the Tukey-Kramer post hoc test. Paired means were analyzed using a Student's *t*-test. Instat® Version 2.0 software was used for statistical analysis. The results were evaluated based on the relationship between the dose levels and incidence or severity of responses. An estimate of the No Observed Adverse Effect Level (NOAEL) was made.

3. Results

3.1. Survival and clinical signs

There was no mortality attributed to any effect of holo-rhLF. Five female animals, across all groups, died within the first two weeks of the study due to inadvertent gavage accidents. All animals were replaced and dosed according to a schedule similar to the other animals, i.e., a full 28 days. The reported data includes the replacement animals. In-life observations showed minimal instances of abnormal physical signs, the most common being chromorhinorrhea (one male control, one female—1000 mg/kg) and localized alopecia on the limbs (one male—500 mg/kg and one female—100 mg/kg).

3.2. Food consumption and body weights

There were no significant differences in body weight among dose levels in either male or female animals at any time point in the study, illustrated in Fig. 1.

There were no differences in mean food consumption among the dose levels. Mean daily food consumption ranged from 24.7 ± 1.09 to 27.5 ± 3.18 g/rat/day in males and 17.8 ± 2.59 to 21.9 ± 1.17 g/rat/day in females.

3.3. Hematology

Results of hematological parameters are shown in Table 1. Significant differences were noted in two hematological parameters (red blood cell count and mean corpuscular hemoglobin) between male control animals and males in the 500 and 100 mg/kg groups. Mean red blood cell count was statistically higher ($p \leq 0.05$) than control in the 500 and 100 mg/kg groups, but values fell within the normal reference range values for rats, included in Table 1. Mean corpuscular hemoglobin was statistically lower in the 500 mg/kg group than in controls, but also within normal reference range. There were no significant differences involving the high-

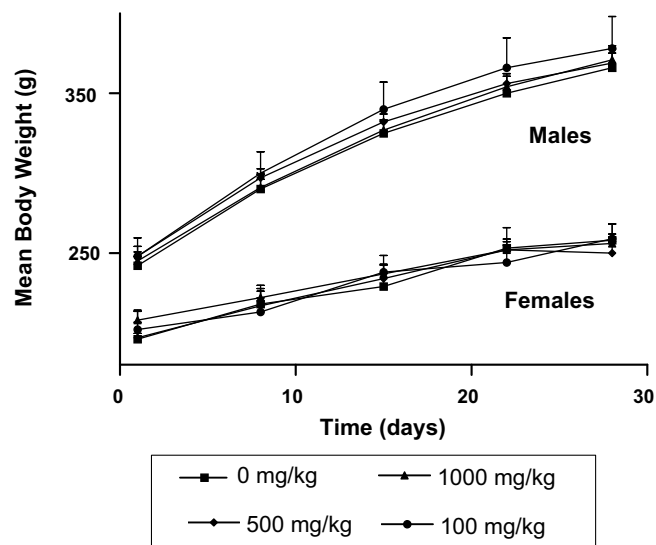


Fig. 1. Mean body weight for male and female rats orally administered holo-rhLF for 28 days by gavage at the indicated doses ($n = 5$ animals per group). The data are means $\pm$ standard deviation for each group.

dose males compared to control or any of the female groups. The differences seen are of questionable biological significance since the mean values fall within the normal reference range values for rats and the lack of involvement of high-dose animals.

3.4. Clinical chemistry

Results of clinical chemistry analysis are in Table 2. There were few significant differences noted in clinical chemistry parameters for the male test animals. Sodium was higher than control at the 500 and 100 mg/kg, but well within the normal range for sodium. Total bilirubin was significantly lower in all groups compared to control. The significance of the reduced bilirubin concentrations is of questionable importance given the lack of variance in the individual values, which apparently caused the statistical significance, and the fact that there were no consistent dose-related relationships. The mean and standard deviation bilirubin value for the control group was 0.2 ± 0.1 ; the mean and standard deviation was 0.1 ± 0.0 for each of the three test groups (100, 500, 1000 mg/kg). Total iron binding capacity (TIBC) showed an increase in all groups compared to control and unsaturated iron binding capacity was higher in the 500 and 100 mg/kg groups; however, there was no significant increase in serum transferrin levels. All groups, including controls, showed serum iron levels below the normal range.

In the females, the only significant difference in any clinical chemistry parameter was the sodium concentration in the medium and low-dose groups and again not dose-related and within the normal range for sodium.

3.5. Necropsy

At necropsy there were no changes attributable to the administration of holo-rhLF in males or females. The following observations were made, but considered to be incidental. In the control group eight of ten animals were normal; chromodacryorrhea was noted in one male and a brown area in the posterior area of the lungs in one female. In the 500 mg/kg dose one male had a distended cecum. In the 1000 and 100 mg/kg dose groups all animals appeared normal.

Table 1

Hematological findings after 28 days of oral repeat doses of holo-rhLF

Parameter	Unit (ref range)	Males ^a				Females ^a			
		0	100	500	1000	0	100	500	1000
Dose	mg/kg/d								
WBC	10 ³ /μL (3–17)	8.2 ± 2.5	8.1 ± 2.1	9.2 ± 3.9	9.3 ± 1.2	4.8 ± 1.1	5.9 ± 2.3	6.2 ± 2.3	5.2 ± 1.3
RBC	10 ⁶ /μL (5–10)	8.0 ± 0.13	8.53 ± 0.32*	8.57 ± 0.08*	8.34 ± 0.33	7.81 ± 0.43	7.83 ± 0.56	8.05 ± 0.36	8.25 ± 0.3
Hb	gm/dL (11–19)	15.8 ± 0.3	16.4 ± 0.5	16.3 ± 0.3	16.0 ± 0.8	15.2 ± 0.5	15.2 ± 0.8	15.8 ± 0.7	15.7 ± 0.5
Hematocrit	% (35–57)	45.6 ± 1.3	47.5 ± 1.6	46.9 ± 1.0	46.5 ± 2.1	42.3 ± 1.9	42.5 ± 2.3	44.1 ± 1.9	44.3 ± 1.5
Mean corpuscular vol	fL (46–65)	57.2 ± 1.6	55.8 ± 0.8	54.8 ± 1.8	55.8 ± 0.8	54.2 ± 1.3	54.6 ± 1.7	54.8 ± 1.1	53.6 ± 0.5
Mean corpuscular Hb	pg (18–23)	19.8 ± 0.4	19.3 ± 0.2	19.0 ± 0.6*	19.2 ± 0.4	19.5 ± 0.4	19.4 ± 0.7	19.6 ± 0.6	19.0 ± 0.4
Mean corpuscular Hb conc.	% (31–40)	34.7 ± 0.3	34.6 ± 0.3	34.6 ± 0.3	34.5 ± 0.3	35.9 ± 0.3	35.7 ± 0.5	35.7 ± 0.6	35.6 ± 0.4
Platelet	10 ³ /μL (200–1500)	883 ± 23.7	920 ± 85.1	996 ± 97.4	890 ± 44.4	947 ± 86.2	1115 ± 264	1086 ± 53.8	992 ± 35.8
Prothrombin time	sec (<28.8)	18.3 ± 3.6	18.0 ± 1.3	18.9 ± 3.6	17.7 ± 1.8	19.0 ± 1.9	18.0 ± 1.0	18.5 ± 1.4	16.8 ± 1.2
Seg. neutrophils	10 ³ /μL (0.39–4.42)	0.61 ± 0.19	0.60 ± 0.27	0.63 ± 0.15	0.83 ± 0.31	0.40 ± 0.14	0.62 ± 0.5	0.59 ± 0.29	0.40 ± 0.2
Lymphocytes	10 ³ /μL (1.95–14.11)	7.15 ± 2.11	7.07 ± 1.69	8.09 ± 3.43	7.96 ± 1.05	4.18 ± 0.99	4.92 ± 1.88	5.32 ± 1.96	4.58 ± 1.05
Eosinophils	10 ³ /μL (0–0.68)	0.08 ± 0.08	0.04 ± 0.02	0.06 ± 0.03	0.07 ± 0.04	0.06 ± 0.02	0.07 ± 0.03	0.10 ± 0.07	0.06 ± 0.03
Monocytes	10 ³ /μL (0–0.68)	0.25 ± 0.08	0.27 ± 0.14	0.33 ± 0.18	0.29 ± 0.1	0.15 ± 0.04	0.18 ± 0.11	0.15 ± 0.08	0.10 ± 0.06
Basophils	10 ³ /μL (0–0.17)	0.09 ± 0.05	0.10 ± 0.04	0.13 ± 0.1	0.11 ± 0.01	0.04 ± 0.01	0.07 ± 0.05	0.08 ± 0.04	0.05 ± 0.02

WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin.

^a Five animals per group.* Significantly different from control ($p \leq 0.05$).**Table 2**

Clinical chemistry findings after 28 days or oral repeat dose of holo-rhLF

Parameter	Unit (ref range)	Males ^a				Females ^a			
		0	100	500	1000	0	100	500	1000
Sodium	meq/L (141–151)	145 ± 0.5	149 ± 1.0*	147 ± 1.3*	146 ± 1.1	143 ± 2.3	147 ± 1.5*	147 ± 2.6*	146 ± 2.3
Potassium	meq/L (5–7.6)	4.6 ± 0.2	4.8 ± 0.2	4.8 ± 0.3	4.7 ± 0.4	4.6 ± 0.3	4.7 ± 0.2	4.8 ± 0.3	4.9 ± 0.4
Chloride	meq/L (84–109)	103.4 ± 1.7	104.2 ± 1.3	103.2 ± 1.6	103.8 ± 1.3	103.8 ± 1.3	105.2 ± 1.6	105.4 ± 1.7	104.6 ± 1.9
Calcium	mg/L (9.4–13.8)	10.3 ± 0.4	10.5 ± 0.3	10.4 ± 0.3	10.2 ± 0.2	10.2 ± 0.2	10.4 ± 0.4	10.5 ± 0.2	10.4 ± 0.3
Phosphorus	mg/L (7.9–10.7)	8.9 ± 0.4	9.1 ± 0.9	9.2 ± 0.3	9.0 ± 0.6	8.4 ± 0.8	9.3 ± 0.2	9.3 ± 0.6	8.6 ± 0.9
Magnesium	meq/L (1.3–3.7)	2.2 ± 0.1	2.3 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	2.4 ± 0.2	2.4 ± 0.1	2.4 ± 0.1
SGPT	U/L (20–56)	26 ± 5.0	30 ± 6.3	32 ± 5.2	30 ± 5.5	22 ± 3.1	23 ± 2.9	22 ± 4.7	24 ± 7.1
SGOT	U/L (68–192)	106 ± 14.4	127 ± 28.1	143 ± 44.9	123 ± 19.8	112 ± 16.3	128 ± 38.5	126 ± 17.6	132 ± 34.4
Alkaline phosphatase	U/L (17–183)	157 ± 21.7	143 ± 43.6	158 ± 32.2	128 ± 21.6	87 ± 11.8	86 ± 18.8	89 ± 7.3	95 ± 26.8
GGT	U/L (0–4)	2 ± 0	2 ± 1	2 ± 1	2 ± 1	3 ± 1	3 ± 1	3 ± 1	3 ± 0
Glucose	mg/dL (74–319)	139 ± 11.6	140 ± 8.9	142 ± 3.5	146 ± 26.4	127 ± 12.1	119 ± 11.9	127 ± 10.5	124 ± 27.2
BUN	mg/dL (12–23)	13 ± 1.3	13 ± 1.5	12 ± 0.8	14 ± 3.0	14 ± 1.5	15 ± 2.4	15 ± 4.0	15 ± 0.8
Creatinine	mg/dL (0.5–1)	0.52 ± 0.0	0.52 ± 0.0	0.56 ± 0.1	0.58 ± 0.0	0.58 ± 0.1	0.58 ± 0.0	0.6 ± 0.1	0.54 ± 0.1
Total cholesterol	mg/dL (48–96)	56 ± 7.9	65 ± 5.6	62 ± 8.9	60 ± 4.6	58 ± 8.0	75 ± 12.3	70 ± 16.3	59 ± 17.4
Triglycerides	mg/dL (3–104)	55 ± 26.8	62 ± 17.5	46 ± 14.5	50 ± 10.8	29 ± 7.3	33 ± 3.9	30 ± 8.5	29 ± 9.1
Total bilirubin	mg/dL (0–0.5)	0.2 ± 0.1	0.1 ± 0.0*	0.1 ± 0.0*	0.1 ± 0.0*	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Albumin	g/dL (2.9–6)	4.2 ± 0.1	4.4 ± 0.2	4.4 ± 0.1	4.2 ± 0.1	4.4 ± 0.1	4.4 ± 0.3	4.6 ± 0.2	4.5 ± 0.2
Total protein	g/dL (4.8–9.2)	6.3 ± 0.3	6.6 ± 0.3	6.5 ± 0.1	6.4 ± 0.4	6.2 ± 0.4	6.2 ± 0.1	6.4 ± 0.2	6.4 ± 0.4
Globulin	g/dL (1.1–3.8)	2.1 ± 0.3	2.2 ± 0.1	2.1 ± 0.1	2.2 ± 0.4	1.8 ± 0.3	1.8 ± 0.3	1.8 ± 0.1	1.8 ± 0.3
Sorbitol dehydrogenase	u/L (13–37)	12 ± 2.6	14 ± 0.9	14 ± 2.7	13 ± 2.2	21 ± 4.5	32 ± 12.7	24 ± 7.1	22 ± 9.2
Iron	μg/dL (137–380)	111 ± 40.0	92 ± 31.1	131 ± 50.2	120 ± 39.1	268 ± 90.9	252 ± 90.8	228 ± 32.6	234 ± 81.3
UIBC ^b	μg/dL (na)	211 ± 96.1	334 ± 43.0*	336 ± 46.0*	314 ± 31.7	141 ± 100.1	168 ± 90.8	194 ± 74.0	172 ± 79.9
TIBC ^b	μg/dL (na)	323 ± 63.5	426 ± 49.2*	468 ± 24.3*	435 ± 30.1*	409 ± 54.0	419 ± 32.7	422 ± 43.0	406 ± 49.5
Serum transferrin ^b	mg/dL (na)	346 ± 32.9	320 ± 56.7	339 ± 43.1	321 ± 36.0	329 ± 45.9	301 ± 26.7	318 ± 42.3	309 ± 42.7

SGPT, alanine aminotransferase; SGOT, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; BUN, blood urea nitrogen; UIBC, unsaturated iron binding capacity; TIBC, total iron binding capacity.

^a Five animals per group.^b Reference range not available.* Significantly different from control ($p \leq 0.05$).

3.5.1. Organ weights

There were no significant differences in absolute organ weights between control and dosed groups. With respect to relative organ weights (g/kg body weight) only the heart/body weight ratio of 1000 mg/kg males was significantly less than the control males. This difference was 0.36 for 1000 dose males and 0.43 for control males. There were no other differences in the males and none in the females.

3.5.2. Histopathology

Microscopic examination was made of sections of the specified tissues from 10 male and 15 female rats. This included all animals from control group and the 1000 mg/kg group and 5 additional females which died from inadvertent gavage accident.

Results from the 5 additional females are not reported here as gavage accident was confirmed and the animals were replaced. The non-normal histopathological findings are summarized in Table 3. There were no test article related microscopic changes noted in any of the rats dosed with 1000 mg/kg/day of holo-rhLF for 28 days. There were a few microscopic changes observed in the various organs and tissues and these were considered to have occurred spontaneously. The changes in the esophagus, lung or heart were considered to have been related to aspiration of the control or test article or gavage accidents. None of the changes observed in these tissues were considered directly related to the vehicle or test article.

All other changes observed in the various organs and tissues generally occurred at single, low or similar frequencies among con-

Table 3

Summary of histomorphologic observations from control and 1000 mg/kg rats

Organ/tissue	Observation	Severity ^a	Animals with observation			
			C ^b , M	HD ^c , M	C ^b , F	HD ^c , F
Adrenal Glands	Accessory cortical nodule	P			1	
Epididymides	Infiltration, mononuclear cell, focal	1	1	2		
Esophagus	Necrosis, tunica muscularis, focal	1			1	1
Heart	Pericarditis, chronic	2			1	
Kidneys	Hyaline droplets, cortical tubules	2		1		
	Nephropathy, tubular, multifocal	1	2	1	1	
Liver	Hepatodiaphragmatic nodule	P			1	1
	Hyperplasia, bile duct, focal	1			1	
	Inflammation, chronic, multifocal	1			2	2
	Necrosis, focal	1			1	1
	Vacuolation, hepatocellular, multifocal	1			2	2
	Vacuolation, hepatocellular, periportal	1			1	2
		2			1	
Lung	Abscess, hilus	(P)			1	
	Granuloma, foreign-body, hilus	P	1		1	
		(P)				
	Inflammation, interstitial, multifocal	1	2	2		1
		2	1	1		
		3		2		
	Macrophages, alveoli, focal	1	1			1
		2				
	Microgranuloma	P	1	1		
	Pleuritis, fibrinopurulent	(3)			1	
Lymph Node, Mesenteric	Congestion	2			1	
Lymph Node, Submandibular	Hyperplasia, lymphocytic/plasmacytic	1	1	1	1	
		2		2		
Ovaries	Cystic bursa	P			1	
Pituitary	Cyst, pars intermedia	P			1	
Prostate	Atrophy, multifocal	1	1			
	Inflammation, chronic, focal	1	2	3		
Spleen	Hematopoiesis, extramedullary, increased	1		1		
Stomach	Dilation, mucosal glands	1	2	3		
Trachae	Dilation, glands	P	1			
	Inflammation, chronic, focal	1	1	2		1
Uterus	Distended, lumen	1			1	1
		3				1
Vagina	Mucification, mucosa	2			2	
		3			1	

1, minimal degree or amount of indicated change of lesion; 2, mild degree or amount of indicated change or lesion; 3, moderate degree or amount of indicated change or lesion; 4, marked degree or amount of indicated change or lesion; (P), microscopic finding(s) in tissue with gross/trimming observation.

^a P, indicated change or lesion present.

^b C = 0 mg/kg bw/day.

^c HD = 1000 mg/kg bw/day.

trol and high-dose males and females. They were considered to have occurred spontaneously.

3.6. Serum lactoferrin and lactoferrin antibody levels

At day 29 of the study, serum samples were prepared for analysis of serum lactoferrin levels and presence of antibody to lactoferrin and shipped to Ventria Bioscience, Sacramento, CA for analysis. Lactoferrin levels were assayed by an ELISA sandwich assay using a standard curve of purified lactoferrin from 10 to 70 ng/mL. Serum from all animals, diluted 1:4 with buffer, was tested and no lactoferrin was detectable in the serum. Rats have little or no lactoferrin in their milk (<50 µg/mL) and there is apparent non-specific binding of hLF to the small intestine brush border membranes (Suzuki et al., 2005). Human lactoferrin would not be expected to persist in the serum of rats fed holo-rhLF.

IgG antibodies to lactoferrin were detected in all animals dosed with holo-rhLF, but not in a dose-dependent relation. The highest level of antibody was observed in the group receiving 100 mg/kg/day. In contrast, IgE antibody levels were equivalent to the control

animals. Fig. 2 gives the ELISA absorbance data for the IgG and IgE ELISAs.

4. Discussion

Lactoferrin is an iron binding protein and is recognized by a specific receptor in the human small intestine (Suzuki et al., 2005). In a study of young adult women, it was demonstrated that iron was equally well absorbed from lactoferrin and ferrous sulfate (Lönnerdal and Bryant, 2006). These results suggest recombinant human lactoferrin saturated with iron could provide a dietary source of iron. Since a gram of lactoferrin contains 1.4 mg of ferric iron, the low-dose level (100 mg/kg/day) selected for the toxicology study would provide about 10 mg of iron per day for a adult human.

The results demonstrate a lack of toxicity following dietary administration of recombinant human holo-lactoferrin at doses up to 1000 mg/kg bw/day for 28 consecutive days. The few minor changes found are discussed below. The statistically different hematological values in male rats of red blood cell count and mean corpuscular are considered of questionable biological significance.

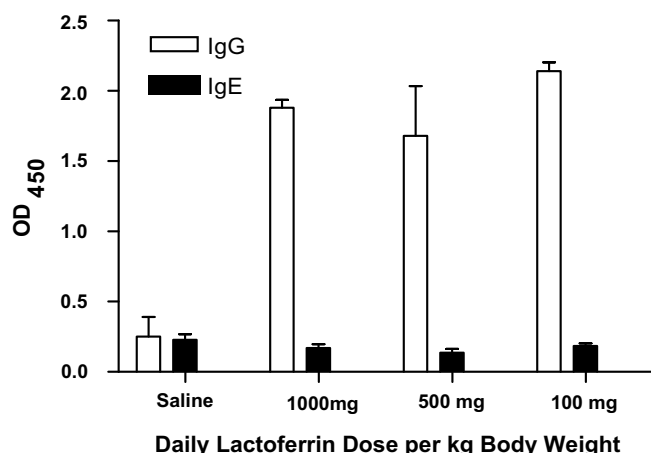


Fig. 2. IgG and IgE antibodies to lactoferrin in rat sera.

The differences, although statistically significant, are relatively minor when compared to control values and within the normal reference range values for rats; there is also no involvement of the high-dose males or the females.

Although there were significant differences in mean sodium (males and females) and total bilirubin (males), there was no consistent dose-related relationship and very small actual variation in values. Consequently, the differences are considered to be of questionable biological significance.

The differences found in the male rats for TIBC and UIBC are also of questionable biological significance. TIBC is an indirect measure of transferrin levels and no increase in transferrin levels was observed. In all groups of male rats, there were individuals with iron levels below the stated normal range. This included three animals in the control group, 2 in the 1000 mg/kg dose, 2 in the 500 mg/kg dose and 3 in the 100 mg/kg dose. There were no females with decreased serum iron. In humans, an increase in TIBC is associated with an increase in serum transferrin, a decrease in iron, and the presence of anemia. In this instance, there was no significant increase in transferrin or decrease in the red cell count, eliminating anemia as a result. The involvement of the control animals suggested this is not a pharmacological or toxicological effect of holo-rhLF.

There were no differences in absolute organ weights among any groups. The lower heart/body weight ratio for the high-dose males was considered incidental and not associated with any histopathological changes. The histopathological changes noted were distributed among treated and untreated groups and the incidences were within the range of normal background lesions.

Rats do not have lactoferrin in their milk; the role of lactoferrin being replaced by transferrin (Masson and Heremans, 1971). Based on the lack of normal exposure to lactoferrin, it is not unexpected that a low level IgG response to human lactoferrin would develop during repeated oral dosing. The lack of IgE antibody development, along with the absence of any histopathologic findings, suggest there is no allergenic sensitivity (Fig. 2).

In summary, the results of the 28-day oral administration demonstrate a lack of toxicity of holo-rhLF in rats. There were no

treatment related, toxicologically relevant changes in clinical signs, growth, food consumption, hematology, clinical chemistry, organ weights and pathology. The No Observed Adverse Effect Level (NOAEL) in male and female Wistar albino rats is considered to be greater than 1000 mg/kg/day.

Conflict of interest statement for authors

Delia Bethell is an employee of Ventria Bioscience. Ventria produces recombinant human lactoferrin and contracted MB Research for the study.

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