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Production and characterization of recombinant human lactoferrin

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Chapter 9

Sub-chronic (13-week) oral toxicity study in rats with recombinant human lactoferrin produced in the milk of transgenic cows

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Abstract

The oral toxicity of recombinant human lactoferrin (rhLF) produced in the milk of transgenic cows was investigated in Wistar rats by daily administration via oral gavage for 13 consecutive weeks, 7 days per week. The study used four groups of 20 rats/sex/dose. The control group received physiological saline and the three test groups received daily doses of 200, 600 and 2000 mg of rhLF per kg body weight. Clinical observations, growth, food consumption, food conversion efficiency, water consumption, neurobehavioural testing, ophthalmoscopy, haematology, clinical chemistry, renal concentration test, urinalysis, organ weights and gross examination at necropsy and microscopic examination of various organs and tissues were used as criteria for detecting the effects of treatment. Overall, no treatment-related, toxicologically significant changes were observed. The few findings that may be related to the treatment (lower cholesterol in high-dose females, lower urinary pH in high-dose males and females and very slightly higher kidney weight in high-dose females) were considered of no toxicological significance.

Based on the absence of treatment-related, toxicologically relevant changes, the no-observed-adverse-effect level (NOAEL) was considered to be at least 2000 mg/kg body weight/day.

Keywords: Recombinant human lactoferrin; Oral administration; Rats; Repeated dose toxicity study

1. Introduction

Human lactoferrin (hLF) is a single-chain metal-binding 77-kDa glycoprotein that belongs to the transferrin family (Anderson et al., 1989). Lactoferrin (LF) consists of two highly homologous lobes, designated the N- and C-lobe, each of which can bind a single ferric ion concomitantly with one bicarbonate anion (Anderson et al., 1989). The molecule is found in milk, tears, saliva, bronchial and intestinal secretions as well as in the secondary granules of neutrophils (Nuijens et al., 1996). Extensive in vitro and in vivo studies showed LF to have antibacterial, antifungal, antiviral and anti-inflammatory activities. On the basis of these activities, LF is postulated to be involved in the innate host defence against infection and severe inflammation, most notable at mucosal surfaces such as those of the gastrointestinal tract (Nuijens et al., 1996). Antimicrobial activities of LF include bacteriostasis by iron deprivation (Reiter et al., 1975), bactericidal activity by destabilization of the cell-wall (Ellison et al., 1988; Ellison and Giehl, 1991) and antiviral activity by inhibition of viral infection (van der Strate et al., 2001). Anti-inflammatory actions of LF include inhibition of hydroxyl-radical formation (Sanchez et al., 1992), of complement activation (Kijlstra and Jeurissen, 1982) and of cytokine production (Zucali et al., 1989) as well as neutralization of lipopolysaccharide (LPS; Lee et al., 1998). Besides antimicrobial activity, LF has been

Abbreviations: LF, lactoferrin; hLF, human LF; rhLF, recombinant hLF; bLF, bovine LF; NOAEL, no-observed-adverse-effect level.

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shown to promote the growth of Bifidobacterium species, the predominant bacteria of the intestinal flora of healthy breast-fed infants (Petschow and Talbott, 1991). In addition, LF has been shown to promote the growth of intestinal cells both in vitro (Nichols et al., 1987) as well as in vivo (Zhang et al., 2001), which may be mediated through binding to specific receptors (Ashida et al., 2004).

Most of the biological actions of LF are mediated by the sequestration of iron or by a positively charged domain located in the N-terminus which binds to negatively charged ligands such as LPS (Appelmelk et al., 1994), DNA (He and Furmanski, 1995) and heparin (Mann et al., 1994), as well as to specific receptors (Ashida et al., 2004; Ziere et al., 1993; Legrand et al., 1997). The release of a N-terminal fragment from LF by pepsin action yields a potent bactericidal peptide (lactoferricin) against Gram-positive and -negative bacteria, yeast and molds (Tomita et al., 1994).

A wide variety of applications of LF in human health care are possible due to the diverse biological actions of the molecule. Both bovine LF (bLF) and hLF could be used as a component of nutritional products aimed at the prevention and treatment of gastro-intestinal tract infection and inflammation. In nutraceutical applications, hLF may be preferred over bLF as it is less susceptible to proteolysis by digestive proteases like trypsin (Brines and Brock, 1983; van Veen et al., 2004) which is relevant as LF may have to survive the harsh environment of the gastro-intestinal tract.

Recently, we reported the production of recombinant hLF (rhLF) in the milk of transgenic cows (van Berkel et al., 2002). Comparative studies between rhLF and hLF from human milk revealed almost identical protein structures, identical iron-binding and release properties and, despite differences in N-linked glycosylation, similar effectiveness in various infection models (van Berkel et al., 2002; Thomassen et al., 2005). Here we report on toxicological studies of rhLF in rats, which were orally dosed rhLF for 13 consecutive weeks. Based on the absence of treatment-related, toxicologically relevant changes, the no-observed-adverse-effect level (NOAEL) is considered to be at least 2000 mg/kg body weight/day.

2. Material and methods

2.1. Production of rhLF

The production of rhLF from the milk of transgenic cows has been described previously (van Berkel et al., 2002). Briefly, a genomic hLF sequence under control of regulatory elements from the bovine αS_1 casein gene, was introduced into the bovine germline. The resulting transgenic cattle lines showed rhLF expression levels between 0.4 and 2.5 g/L (van Berkel et al., 2002). Various batches of the test-substance were produced by freeze-drying of the LF fraction (containing rhLF and bLF), extracted from mature transgenic milk using S Sepharose (van Berkel et al., 2002). The purity of rhLF was assessed by SDS-PAGE, analytical Mono S chromatography and specific ELISAs for hLF and bLF (van Berkel et al., 2002). The purity of rhLF in the LF batches was about 95%; the amount of bLF was about 4%. Absorbance measurements revealed the LF to be saturated with iron for about 7%.

2.2. Animals

The study was performed in compliance with Good Laboratory Practice and according to current FDA and OECD Guidelines for toxicity testing (FDA, 1982; OECD, 1998). The study was conducted with 85 male and 85 female SPF Wistar outbred (Crl:(WI)WU BR) rats (Charles River Deutschland, Germany). Pre-test neurobehavioural testing was conducted in the 13-week study on animals of 5–6 weeks of age. At the start of the treatment period the rats were approximately 7 weeks old. Body weights at the start of the treatment ranged from 140.9 g to 187.0 g (mean 158.4 g) in males and from 131.0 g to 168.1 g (mean 147.2 g) in females. The animals were housed under conventional conditions in one room, in macrolon cages, with sterilized wood shavings as bedding material, 5 rats per cage, separated by sex. The room was ventilated with about 10 air changes per hour and was maintained at a temperature of 22 ± 4 °C. The room was set at a relative humidity of 30–70%. Lighting was artificial with a sequence of 12 h light and 12 h dark. Water and powdered diet (Rat & Mouse No. 3 Breeding Diet, RM3; SDS Special Diets Services, England) were provided ad libitum.

2.3. Administration of rhLF, experimental groups and dose levels

Recombinant hLF was administered by oral gavage as a dilution in physiological saline (0.9% NaCl) once daily for at least 90 consecutive days. The rats of the various groups were dosed with different concentrations of the test substance in the vehicle, to ensure a constant dose-volume of 10 ml per kg body weight per day at all dose levels. Controls were treated with the vehicle only. Once per week the dose volumes were adjusted to the latest recorded body weight for each individual rat, to maintain a constant dose level in terms of the animal's body weight. Fresh dilutions of the test substance in the vehicle were made daily, just prior to treatment. The rhLF was dissolved in warm physiological saline (approx. 37 °C). Four groups of 20 males and 20 females each were used, viz. one vehicle control group and three test groups receiving 200, 600 or 2000 mg rhLF per kg body weight per day for 13 consecutive weeks. The concentrations in the dosing solutions were corrected for the slight differences in purity of the various batches of rhLF.

2.4. Observations, measurements and examinations

2.4.1. General clinical signs were observed daily

Body weights and food consumption were recorded weekly and water consumption was recorded over 4-day periods in weeks 1, 6 and 12 of the study.

Neurobehavioural testing was conducted in 10 rats/sex/group. Arena testing was conducted prior to the first exposure and then once weekly up to and including week 12. Signs noted included changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, autonomic activity, gait, posture, response to handling and presence of clonic or tonic movements, stereotypies and bizarre behaviour. At the end of the study, Functional Observational Battery (FOB) tests and spontaneous motor activity measurements were performed in week 13 (Moser et al., 1997). Food and water were not available during this testing.

Ophthalmoscopic observations were made prior to the start of treatment in all animals and towards the end of the treatment period in all surviving animals of the control group and the high-dose group. Eye examinations were carried out using an ophthalmoscope after induction of mydriasis by a solution of atropine sulphate.

At necropsy at the end of treatment, blood samples were taken from the abdominal aorta of 10 rats per sex per group, whilst under CO_2/O_2-anaesthesia. K_2-EDTA (haematology) or heparin (clinical chemistry) were used as anticoagulants. Fasting glucose was determined shortly before the end of the treatment period in blood collected from the tip of the tail. As required by FDA and OECD Guidelines, haematology and clinical

chemistry parameters were determined according to well established methods (FDA, 1982; OECD, 1998).

Shortly before the end of the treatment, 10 rats per sex per group (the same as those used for haematology and clinical chemistry) were deprived of water for 24 h and of food during the last 16 h of this period. During the last 16 h of deprivation, the rats were kept in stainless steel metabolism cages (one rat per cage) and urine was collected. The concentrating ability of the kidneys was investigated by measuring the volume and density of the individual samples. Urinalysis was conducted as required by FDA and OECD Guidelines (FDA, 1982; OECD, 1998).

At the end of the treatment period, all animals were subjected to a complete gross necropsy. The animals were killed by exsanguination from the abdominal aorta under CO_2/O_2-anaesthesia and then examined grossly for pathological changes. A large number of organs and tissues were excised, weighed, collected and preserved, as required by FDA and OECD guidelines (FDA, 1982; OECD, 1998).

The tissues to be examined microscopically were embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Histopathological examination was performed on all animals of the control group and of the high-dose group (including the animal that was killed *in extremis*). In addition, the lungs, liver, kidneys and gross lesions were examined microscopically in all rats of the intermediate dose-groups.

The statistical procedures included analysis of covariance, (non-parametric) analysis of variance and the Fisher's exact probability test, where appropriate.

3. Results

No treatment-related clinical signs were observed. One high-dose female was killed *in extemis*, due to posterior paralysis. This condition was not considered related to treatment.

Body weights were similar among the groups throughout the study. Food consumption and food conversion efficiency were similar among the groups throughout the study. The mean weekly food consumption ranged from 18.3 to 19.5 g/rat/day in males and from 12.6 to 12.9 g/rat/day in females. The mean weekly food conversion efficiency was 0.14 g weight gain/g food consumed in males and 0.07 g weight gain/g food consumed in females.

Water consumption and urinalysis results are shown in Table 1. Water consumption was similar among the groups throughout the study. Urinary density was statistically significantly higher in males of the high-dose group. Urinary pH was statistically significantly decreased in males and females of the high-dose group. Urinary crystals were statistically significantly increased in males of the low- and

Table 1
Water intake and urinary findings in rats after 13 weeks of repeated oral administration of recombinant human lactoferrin (rhLF)

		Water intake (ml/day)	Volume (ml)	Density (kg/l)	Apprnc-U	pH-U	Prot-U (0-3)	Gluc-U (0-4)	Keton-U (0-3)	Oc.Bld-U (0-3)	Urobil-U (0-4)	Biliru-U (0-3)
Males												
Control	Mean	27.3	4.8	1.036	Yellow	7.2	1	0	0	0	0	0
	sem		0.5	0.002								
	n		10	10	10	10	10	10	10	10	10	10
0.2 g/kg	Mean	29.2	4.5	1.042	Yellow	7.0	1	0	0	0	0	0
	sem		0.4	0.003								
	n		10	10	10	10	10	10	10	10	10	10
0.6 g/kg	Mean	28.9	5.0	1.038	Yellow	7.3	1	0	0	0	0	0
	sem		0.4	0.002								
	n		10	10	10	10	10	10	10	10	10	10
2.0 g/kg	Mean	29.0	4.4	1.048**	Yellow	6.6**	1	0	0	0	0	0
	sem		0.5	0.003								
	n		10	10	10	10	10	10	10	10	10	10
Females												
Control	Mean	22.5	2.4	1.050	Yellow	6.4	0	0	0	0	0	0
	sem		0.2	0.003								
	n		10	10	10	10	10	10	10	10	10	10
0.2 g/kg	Mean	23.0	3.0	1.049	Yellow	6.5	1	0	0	1	0	0
	sem		0.5	0.004								
	n		10	10	10	10	10	10	10	10	10	10
0.6 g/kg	Mean	21.9	2.6	1.049	Yellow	6.2	0	0	0	0	0	0
	sem		0.3	0.004								
	n		10	10	10	10	10	10	10	10	10	10
2.0 g/kg	Mean	23.4	2.3	1.057	Yellow	5.8*	0	0	0	0	0	0
	sem		0.2	0.002								
	n		10	10	10	10	10	10	10	10	10	10

Statistics: One-way analysis of variance followed by Dunnett's multiple comparison tests; $^{*}P < 0.05$, $^{**}P < 0.01$; or in case of non-continuous parameters: Kruskal–Wallis non-parametric analysis of variance followed by Mann–Whitney U-tests; $^{*}P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.002$.
Apprnc-U: Appearance urine; pH-U: pH urine; prot-U: Protein in urine; Gluc-U: Glucose in urine; Keton-U: Ketones in urine; Oc.Bld-U: Occult blood in urine; Urobil-U: Urobilinogen in urine; Biliru-U: Bilirubin in urine.

Table 2
Haematological findings in rats after 13 weeks of repeated oral administration of recombinant human lactoferrin (rhLF)

		RBC 10E12/l	HB (mmol/l)	PCV (l/l)	MCV (fl)	MCH (fmol)	MCHC (mmol/l)	Reticulocyte/ 1000	Thromboc 10E9/l	PTT (s)	Eosinoph (%)	Neutroph (%)	Lymphoc (%)	Monocyt (%)	Basophil (%)
Males															
Control	Mean	8.19	9.6	0.421	51.5	1.17	22.8	37.3	958	42.7	0.9	6.7	91.5	0.8	0.1
	sem	0.15	0.1	0.006	0.8	0.02	0.1	1.5	34	0.5	0.3	1.2	1.2	0.2	0.1
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.2 g/kg	Mean	8.10	9.6	0.422	52.1	1.18	22.8	36.5	948	42.7	0.6	6.1	92.6	0.7	0.0
	sem	0.10	0.1	0.007	0.5	0.01	0.1	0.8	18	0.9	0.2	1.2	1.2	0.3	0.0
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.6 g/kg	Mean	8.03	9.3	0.411	51.3	1.16	22.6	39.2	975	41.4	0.6	8.3	90.6	0.5	0.0
	sem	0.07	0.1	0.003	0.5	0.01	0.1	2.4	21	0.7	0.2	1.0	0.9	0.2	0.0
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
2.0 g/kg	Mean	8.00	9.5	0.417	52.1	1.19	22.8	36.8	940	41.5	0.6	7.6	91.7	0.4	0.0
	sem	0.14	0.2	0.007	0.5	0.01	0.1	1.3	20	0.4	0.3	1.8	1.8	0.2	0.0
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Females															
Control	Mean	7.45	9.4	0.408	54.8	1.27	23.1	46.4	815	34.6	0.3	7.2	92.0	0.4	0.1
	sem	0.09	0.1	0.005	0.6	0.01	0.2	1.8	24	0.5	0.2	1.5	1.7	0.2	0.1
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.2 g/kg	Mean	7.69	9.5	0.408	53.1	1.23**	23.2	39.6	748	34.9	0.8	9.3	89.4	0.4	0.1
	sem	0.10	0.1	0.004	0.3	0.01	0.1	1.0	15	0.5	0.3	2.3	2.3	0.2	0.1
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.6 g/kg	Mean	7.56	9.3	0.407	53.9	1.24*	22.9	43.7	803	34.3	0.6	11.1	87.4	0.9	0.0
	sem	0.08	0.1	0.004	0.5	0.01	0.1	1.7	16	0.5	0.3	2.8	2.9	0.3	0.0
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
2.0 g/kg	Mean	7.51	9.4	0.409	54.5	1.26	23.1	43.6	797	36.0	0.4	8.6	90.7	0.3	0.0
	sem	0.10	0.1	0.006	0.5	0.01	0.1	2.3	22	0.4	0.2	1.3	1.3	0.2	0.0
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Statistics: One-way analysis of variance followed by Dunnett's multiple comparison tests; $^{*}P < 0.05$, $^{**}P < 0.01$; or in case of non-continuous parameters: Kruskal–Wallis non-parametric analysis of variance followed by Mann–Whitney U-tests; $^{*}P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.002$.
RBC: Red blood cells; HB: Haemoglobin; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; Reticulo: Reticulocytes; Thromboc: Thrombocytes; PTT: Prothrombin time; Eosinoph: Eosinophils; Neutroph: Neutrophils; Lymphoc: Lymphocytes; Monocyt: Monocytes; Basophil: Basophils.

Table 3
Clinical chemistry findings in rats after 13 weeks of repeated oral administration of recombinant human lactoferrin (rhLF)

		Gluc (mmol/l)	ALP (U/l)	ALAT (U/l)	ASAT (U/l)	GGT (U/l)	TP (g/l)	Album (g/l)	A/G ratio	Urea (mmol/l)	Creatin (μmol/l)	Bili-Tot (μmol/l)	Cholest (mmol/l)	Triglyc (mmol/l)	Phos-lip (mmol/l)	Ca (mmol/l)	K (mmol/l)	Na (mmol/l)	Cl (mmol/1)	Inorg-P (mmol/l)
Males																				
Control	Mean	3.88	134	54	48	0.2	71	44	1.66	7.6	28	0.4	1.88	1.55	2.02	3.13	5.1	151	100	2.26
	sem	0.07	7	2	2	0.1	1	0	0.03	0.3	0	0.1	0.09	0.18	0.07	0.03	0.1	0	0	0.07
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.2 g/kg	Mean	3.85	117	48	48	0.1	72	45	1.62	7.5	27	0.2	1.71	1.44	1.89	3.12	5.1	152	100	2.20
	sem	0.05	6	3	1	0.1	0	0	0.02	0.2	0	0.1	0.08	0.10	0.05	0.02	0.1	0	0	0.06
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.6 g/kg	Mean	3.88	116	49	50	0.0	70	43*	1.56	7.6	27	0.4	1.83	1.69	2.00	3.07	5.2	151	100	2.14
	sem	0.12	6	2	2	0.0	1	0	0.06	0.3	1	0.1	0.09	0.29	0.10	0.02	0.1	0	0	0.07
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
2.0 g/kg	Mean	4.01	115	53	55	0.1	72	44	1.58	7.4	27	0.5	1.79	1.44	1.93	3.13	5.2	151	100	2.29
	sem	0.21	5	2	2	0.1	1	0	0.03	0.2	1	0.1	0.06	0.17	0.08	0.04	0.1	1	0	0.08
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Females																				
Control	Mean	3.74	127	72	78	0.6	73	49	2.11	7.0	39	1.7	2.26	1.32	2.55	3.03	5.2	152	99	2.35
	sem	0.09	8	4	3	0.1	1	1	0.05	0.3	1	0.3	0.09	0.20	0.09	0.04	0.1	1	1	0.12
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.2 g/kg	Mean	3.64	117	66	73	0.5	73	50	2.19	7.0	37	1.6	2.18	1.48	2.58	3.07	5.0	150	100	2.51
	sem	0.09	7	3	4	0.1	1	0	0.05	0.2	1	0.2	0.09	0.36	0.10	0.03	0.1	0	1	0.06
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.6 g/kg	Mean	4.01	138	64	75	0.5	74	50	2.09	6.8	37	1.5	2.04	1.31	2.34	3.05	4.9	151	100	2.42
	sem	0.15	8	3	3	0.1	1	0	0.06	0.3	1	0.2	0.05	0.21	0.06	0.03	0.1	0	1	0.09
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
2.0 g/kg	Mean	3.49	128	69	78	0.6	71	48	2.06	7.2	37	1.5	1.97*	1.09	2.30	3.06	5.2	150	100	2.62
	sem	0.09	10	3	3	0.1	1	1	0.04	0.2	1	0.1	0.07	0.13	0.09	0.03	0.1	1	0	0.10
	n	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Statistics: One-way analysis of variance followed by Dunnett's multiple comparison tests; $^{*}P < 0.05$, $^{**}P < 0.01$; or in case of non-continuous parameters: Kruskal–Wallis non-parametric analysis of variance followed by Mann–Whitney U-tests; $^{*}P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.002$.

Gluc: Glucose; ALP: Alkaline phosphatase; ALAT: Alanine aminotransferase (GPT); ASAT: Aspartate aminotransferase (GOT); GGT: Gamma glutamyl transferase; TP: Total protein; Album: Albumin; A/G Rati: Albumin/globulin ratio; Urea: Urea in plasma; Creatin: Creatinine; Bili-Tot: Bilirubin (total); Cholest: Cholesterol (total); Triglyc: Triglycerides; Phos-lip: Phospholipids; Ca: Calcium; K: Potassium; Na: Sodium; Cl: Chloride; Inorg-P: Inorganic phosphate.

mid-dose groups (data not shown). Further semi-quantitative and microscopic urinary observations were similar among the groups (data not shown).

The results of functional observational battery (FOB) testing did not show significant changes that were considered to be related to treatment. A minor statistically significant difference between groups was found for hindlimb gripstrength in females (ANOVA, $F = 2.94$, $p < 0.05$). However, post-hoc group comparisons showed that none of the treated groups was significantly different from the control group.

With respect to motor activity, analysis of variance of the total distance moved of males indicated a significant difference between groups (ANOVA, $F = 3.16$, $p < 0.05$). Analysis of variance indicated also a significant difference between groups for the number of movements of males (ANOVA, $F = 3.06$, $p < 0.05$). However, post-hoc group comparisons showed that none of the treated groups was significantly different from the control group for both measures. For females a significant difference between groups was found for the mean velocity (ANOVA, $F = 2.91$, $p < 0.05$). Also in this case, post-hoc group comparisons showed that none of the treated groups was significantly different from the control group. Finally, no effects on habituation were observed. Overall, no treatment-related changes were observed during the neurobehavioural testing of males and females at arena testing during the study and FOB and motor activity assessment at the end of the study. Therefore, no evidence was obtained for a neurotoxic potential of the test substance.

Ophthalmoscopic observations revealed no treatment-related ocular changes.

Results of the haematological examinations are given in Table 2. Mean Corpuscular Haematology was statistically significantly lower in females of the low- and mid-dose groups. No other statistically significant changes were observed.

Results of the clinical chemistry examinations are given in Table 3. Albumin was statistically significantly lower in males of the mid-dose group. Cholesterol was statistically significantly lower in females of the high-dose group. No other statistically significant changes in clinical chemistry parameters were observed.

Absolute and relative organ weights are shown in Tables 4 and 5. Absolute adrenal weights were higher in males of the low- and high-dose groups, absolute kidney weights

Table 4
Absolute organ weights (g) in rats after 13 weeks of repeated oral administration of recombinant human lactoferrin (rhLF)

		TermBW (g)	Thyroid (g)	Adrenals (g)	Kidneys (g)	Thymus (g)	Brain (g)	Spleen (g)	Heart (g)	Liver (g)	Testes (g)	Epididy (g)
Males												
Control	Mean	385.7	0.023	0.044	2.24	0.381	1.87	0.675	1.17	12.92	3.21	1.31
	sem	5.9	0.001	0.001	0.04	0.023	0.02	0.015	0.02	0.27	0.07	0.02
	n	20	20	20	19	20	20	20	20	20	20	20
0.2 g/kg	Mean	405.7	0.022	0.048*	2.35	0.439	1.90	0.709	1.25	13.98*	3.32	1.36
	sem	5.8	0.001	0.001	0.04	0.022	0.02	0.015	0.03	0.29	0.08	0.03
	n	20	19	20	20	20	20	20	20	20	20	20
0.6 g/kg	Mean	411.4*	0.024	0.047	2.41**	0.405	1.93	0.718	1.27	14.11*	3.25	1.33
	sem	7.4	0.001	0.001	0.03	0.021	0.02	0.018	0.03	0.31	0.10	0.02
	n	20	20	20	20	20	20	20	20	20	20	20
2.0 g/kg	Mean	397.5	0.023	0.048*	2.37*	0.385	1.91	0.693	1.23	13.76	3.28	1.33
	sem	6.4	0.001	0.001	0.04	0.020	0.02	0.014	0.02	0.36	0.07	0.02
	n	20	20	20	19	20	20	20	20	20	20	20
Females											Ovaries (g)	Uterus (g)
Control	Mean	229.1	0.020	0.060	1.42	0.279	1.76	0.463	0.80	7.50	0.080	0.691
	sem	3.3	0.001	0.001	0.02	0.009	0.01	0.013	0.01	0.17	0.002	0.053
	n	20	20	20	20	20	20	20	20	20	20	20
0.2 g/kg	Mean	232.4	0.020	0.060	1.51	0.288	1.76	0.447	0.79	7.78	0.079	0.687
	sem	2.7	0.001	0.001	0.03	0.012	0.01	0.012	0.01	0.24	0.002	0.045
	n	20	20	20	19	20	20	20	20	20	20	20
0.6 g/kg	Mean	231.6	0.021	0.063	1.50	0.297	1.74	0.458	0.82	7.88	0.084	0.703
	sem	2.8	0.001	0.002	0.02	0.010	0.01	0.013	0.02	0.22	0.003	0.061
	n	20	20	19	20	20	20	20	20	20	20	20
2.0 g/kg	Mean	233.0	0.020	0.063	1.53**	0.292	1.78	0.490	0.83	7.96	0.082	0.606
	sem	2.8	0.001	0.002	0.03	0.009	0.02	0.010	0.02	0.21	0.002	0.043
	n	19	19	19	19	19	19	19	19	19	19	19

Statistics: One-way analysis of variance followed by Dunnett's multiple comparison tests; * $P < 0.05$, ** $P < 0.01$.
TermBW: Terminal Body Weight.

Table 5
Relative organ weights (g/kg body weight) in rats after 13 weeks of repeated oral administration of recombinant human lactoferrin (rhLF)

		TermBW (g)	Thyroid (g/kg BW)	Adrenals (g/kg BW)	Kidneys (g/kg BW)	Thymus (g/kg BW)	Brain (g/kg BW)	Spleen (g/kg BW)	Heart (g/kg BW)	Liver (g/kg BW)	Testes (g/kg BW)	Epididy (g/kg BW)
Males												
Control	Mean	385.7	0.060	0.114	5.81	0.99	4.86	1.75	3.04	33.5	8.35	3.41
	sem	5.9	0.002	0.002	0.10	0.06	0.07	0.03	0.04	0.4	0.21	0.06
	n	20	20	20	19	20	20	20	20	20	20	20
0.2 g/kg	Mean	405.7	0.056	0.119	5.80	1.08	4.70	1.75	3.08	34.5	8.18	3.36
	sem	5.8	0.002	0.004	0.09	0.06	0.05	0.03	0.07	0.5	0.20	0.08
	n	20	19	20	20	20	20	20	20	20	20	20
0.6 g/kg	Mean	411.4*	0.057	0.116	5.88	0.98	4.72	1.75	3.09	34.3	7.96	3.26
	sem	7.4	0.002	0.003	0.08	0.04	0.07	0.04	0.06	0.5	0.28	0.07
	n	20	20	20	20	20	20	20	20	20	20	20
2.0 g/kg	Mean	397.5	0.059	0.122	5.99	0.97	4.84	1.75	3.09	34.5	8.30	3.36
	sem	6.4	0.002	0.003	0.06	0.05	0.08	0.03	0.04	0.6	0.24	0.07
	n	20	20	20	19	20	20	20	20	20	20	20
Females											Ovaries (g/kg BW)	Uterus (g/kg BW)
Control	Mean	229.1	0.086	0.265	6.20	1.22	7.72	2.03	3.50	32.8	0.351	3.04
	sem	3.3	0.004	0.008	0.08	0.04	0.09	0.06	0.08	0.8	0.011	0.25
	n	20	20	20	20	20	20	20	20	20	20	20
0.2 g/kg	Mean	232.4	0.088	0.258	6.49	1.24	7.60	1.92	3.38	33.5	0.342	2.96
	sem	2.7	0.004	0.006	0.10	0.05	0.09	0.04	0.04	1.0	0.010	0.19
	n	20	20	20	19	20	20	20	20	20	20	20
0.6 g/kg	Mean	231.6	0.090	0.270	6.46	1.28	7.54	1.98	3.56	34.0	0.360	3.04
	sem	2.8	0.005	0.007	0.07	0.04	0.06	0.05	0.05	0.8	0.012	0.27
	n	20	20	19	20	20	20	20	20	20	20	20
2.0 g/kg	Mean	233.0	0.086	0.269	6.58**	1.25	7.65	2.11	3.55	34.1	0.351	2.62
	sem	2.8	0.004	0.006	0.10	0.03	0.09	0.04	0.08	0.7	0.011	0.19
	n	19	19	19	19	19	19	19	19	19	19	19

Statistics: One-way analysis of variance followed by Dunnett's multiple comparison tests; * $P < 0.05$, ** $P < 0.01$.
TermBW: Terminal body weight.

were higher in males of the mid-dose group and in males and females of the high dose group, relative kidney weights were higher in females of the high-dose group and absolute liver weights were higher in males of the low- and mid-dose groups.

Results of histopathological examinations are shown in Table 6. No treatment-related gross lesions were observed at necropsy. All lesions observed were about equally distributed among the groups, or they occurred in a single animal only. The accessory lobe of the lung of the high-dose female animal that was killed *in extremis* (due to posterior paralysis), showed a large haemorrhage. Microscopic examination did not reveal any treatment-related changes. All changes observed were about equally distributed between

Table 6
Histopathological findings in rats ($N = 20$) after 13 weeks of repeated oral administration of recombinant human lactoferrin (rhLF)

Organ or tissue examined/changes found	Incidence of lesions							
	Males				Females			
	Control	Low-dose	Mid-dose	High-dose	Control	Low-dose	Mid-dose	High-dose
Brain/Hydrocephalus	–	–	–	–	–	1	–	–
Epididymides/focal mononuclear cell infiltrate	1	–	–	4	0	–	–	0
GALT (Peyer's patches)/focal calcification	1	–	–	0	0	–	–	0
Heart/cartilaginous metaplasia	1	–	–	0	–	–	–	–
Heart/focal subepicardial mononuclear cell infiltrate	2	–	–	3	–	–	–	–
Heart/focal myocardial mononuclear cell infiltrate	1	–	–	2	1	–	–	1
Kidneys/few proteinaceous casts	1	0	0	0	0	0	0	0
Kidneys/basophilic tubules	13	12	15	15	1	3	2	1
Kidneys/focal mononuclear cell infiltrate	1	2	0	1	0	1	0	0
Kidneys/cortical mineralization	0	0	0	0	2	0	0	0
Kidneys/ corticomedullary mineralization	0	0	0	0	2	3	4	3
Kidneys/pelvic (epithelial) mineralization	2	2	2	1	3	0	1	0
Kidneys/medullary mineralization	1	1	0	1	3	1	1	0
Kidneys/focal transitional cell hyperplasia	3	1	2	1	0	0	0	0
Kidneys/hydronephrosis	3	0	0	1	0	2	0	3
Kidneys/cysts	1	0	0	0	0	0	0	0
Kidneys/pyelitis	0	0	0	0	0	1	0	0
Liver/mononuclear cell aggregates/necrotic hepatocytes	10	7	7	11	9	9	12	10
Liver/periportal mononuclear cell infiltrate	0	0	0	1	0	0	0	0
Liver/vacuolated focus	0	0	0	1	0	0	0	0
Liver/focal hepatocellular necrosis	0	0	0	0	1	0	1	1
Lungs/focal alveolitis	0	0	0	1	0	0	0	1
Lungs/accumulation of alveolar macrophages	0	1	0	0	0	0	1	3
Lungs/focal pneumonia	0	0	0	0	0	0	0	1
Lungs/focal haemorrhage(s)	0	0	0	0	0	0	0	1
Lungs/perivascular polymorphonuclear leukocytic infiltration	0	0	0	0	0	0	1	0
Lungs/granulomatoma	0	0	0	0	0	0	1	0
Mesenteric lymph nodes/germinal centre development	8	–	–	5	9	–	–	10
Ovaries/focal mineralization					4	–	–	3
Ovaries/cyst(s)					3	–	–	4
Pancreas/focal mononuclear cell infiltrate	1	–	–	0	1	–	–	2
Pituitary/pars disalis cyst(s)	4	–	–	4	0	–	–	0
Prostate/focal mononuclear cell infiltrate	0	–	–	2				
Skin/focal acanthosis	0	–	–	0	1	–	1[a]	0
Skin/focal hypotrichosis	0	–	–	0	0	–	1[a]	0
Small intestines/focal enteritis	1	–	–	0	0	–	–	0
Spleen/increased extramedullary haematopoiesis	0	–	–	1	3	–	–	2
Subling. + submax. Salivary glands/focal parotid-type acini	3	–	–	2	2	–	–	3
Testes/seminiferous tubular atrophy	2	–	–	2				
Thymus/microhaemorrhage(s)	6	–	1[a]	1	0	–	–	3
Thymus/focal ductular structures	2	–	–	1	14	–	–	11
Thymus/cortical lymphoid depletion	0	0	0	0	0	–	–	1
Thyroid/focal mononuclear cell infiltrate	0	–	–	0	1	–	–	0
Trachea/bronchi/focal mononuclear cell infiltrate	0	–	–	2	1	–	–	0
Uterus/luminal dilatation					7	–	–	6

No abnormalities detected in: adrenals, aorta, caecum, cervical lymph nodes, colon, eyes, mammary glands, peripheral nerve, oesophagus, parathyroids, rectum, spinal cord, sternum with bone marrow, stomach, urinary bladder.

[a] Apart from kidneys, liver and lungs only gross lesions were microscopically examined in the intermediate dose groups.

the controls and the groups given the test substance, or occurred in a single or a few animals only. Moreover, they are common findings for the strain and age of rats used. Microscopy of the animal that was killed *in extremis* during the experiment revealed a pneumonia and haemorrhage of the lungs, focal hepatocellular necrosis and a cortical lymphoid depletion in the thymus (stress involution).

4. Discussion and conclusion

A wide variety of applications of human lactoferrin (hLF) in human health care are possible due to its antimicrobial and anti-inflammatory activities (Nuijens et al., 1996). In nutraceutical applications, hLF could be used as a component of products aimed at the prevention and treatment of gastro-intestinal tract infection and inflammation. In the present study the toxicity of recombinant hLF (rhLF) in Wistar rats was examined upon daily administration via oral gavage for 13 consecutive weeks up to a dose level of 2000 mg/kg body weight/day. Overall no treatment-related toxicity was observed. The few minor changes found are discussed below. The posterior paralysis observed in the female animal killed *in extemis*, was not considered related to treatment. Although the cause of the moribund condition could not be definitively established, it was most probably related to trauma possibly caused by the dosing procedure. The lower MCH in females of the low- and mid-dose groups was considered a chance finding, since a similar change was absent in the high-dose group. The lower albumin level in males of the mid-dose group was considered a chance finding in the absence of a similar change in the high-dose group. It cannot be excluded that the lower cholesterol level in females of the high-dose group was related to the treatment, but it was only minor and was well within the range of normal control values. For this reason the change was not considered of toxicological significance. The higher urinary density was related to the slightly (non-significant) lower urinary volume in males of the high-dose group. This change was considered of no toxicological relevance, since a decrease in urinary density is not an indication for impaired renal concentrating ability. Moreover, the change was minor and within the range of the normal control values. The lower urinary pH in males and females of the high-dose group was probably treatment-related. A similar change was previously observed in rats given bovine lactoferrin at the same dose level (2000 mg/kg/day) for 13 weeks (Yamauchi et al., 2000). The change was consistent with a non-specific decrease in pH, possibly as a result of high protein exposure and was considered of no toxicological relevance. The increase in urinary crystals in males of the low- and mid-dose groups was considered an incidental finding in the absence of a similar change in the high-dose group. The higher absolute adrenal weights in males of the low- and high-dose groups were considered incidental findings, since no significant differences were observed in relative adrenal weights and no histopathological changes were seen in this organ. The higher absolute kidney weights in males of the mid- and high-dose groups were not accompanied by significant changes in relative kidney weight or changes in renal histopathology. It cannot be excluded that the higher absolute and relative kidney weights in females of the high dose group were related to the treatment. However, the change was very slight (approx. 6%) and was neither accompanied by histopathological renal changes nor by changes in urinary or clinical chemistry parameters indicative of renal toxicity. For this reason, the changes in kidney weights were considered of no toxicological significance. The higher absolute liver weights in males of the low- and mid-dose groups were considered incidental findings, since a similar change was absent in the high-dose group. Moreover, relative liver weight showed no significant differences from controls.

It was concluded that based on the absence of treatment-related, toxicologically relevant changes in clinical signs, growth, food consumption, food conversion efficiency, water consumption, neurobehavioural parameters, ophthalmoscopy, haematology, clinical chemistry, renal concentrating ability, urinalysis, organ weights and pathology, the no-observed-adverse-effect level (NOAEL) was considered to be at least 2000 mg/kg body weight/day.

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