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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