RESEARCH COMMUNICATION

The survival of ingested lactoferrin in the gastrointestinal tract of adult mice

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Lactoferrin is an 80 kDa major protein component of mammalian colostral whey. The antimicrobial active centre of lactoferrin, lactoferricin (Lfcin), may also be an important determinant of the interaction between lactoferrin and specific receptors on lymphocytes. We have documented the survival *in vivo* of ingested lactoferrin in the gastrointestinal tract of adult mice by surface-enhanced laser desorption/ionization affinity MS. Various kinds of degraded lactoferrin fragments were detected as molecular-ion peaks corresponding to Lfcin after being captured by an affinity capture device, hydrolysis *in situ* and laser desorption/ionization. No evident molecular-ion peaks of Lfcin were observed upon analysis of faeces from mice fed commercial milk, whereas lactoferrin fragments containing the Lfcin region were detected at concentrations in the order of at least pmol/g in the faeces of mice fed milk enriched with lactoferrin at 40 mg/ml. These results suggest that ingested lactoferrin would survive transit through the gastrointestinal tract as partially degraded forms containing the receptor-binding region(s) as well as the antimicrobial active centre.

INTRODUCTION

Lactoferrin, an iron-binding glycoprotein of the transferrin family, is prominently found in mammalian colostrum and mature milk. It is also present in several other biological fluids and in the granules of neutrophils [1–3]. Lactoferrin has been associated with a wide variety of biologically important processes, including host defence, regulation of cell function, cell growth and cell differentiation [4]. Specific lactoferrin receptors have been found on peripheral blood cells [5], liver cells [6] and cells lining the intestinal tract [7]. Although it had been thought that the ability of lactoferrin to bind and sequester iron prevents microbial growth, a bactericidal domain, which is distinct from the iron-binding region of the protein, has been identified and named lactoferricin® (Lfcin) [8]. Bovine, human and porcine Lfcin were identified from the respective lactoferrins hydrolysed by porcine pepsin *in vitro* [8,9]. It now appears that the Lfcin sequence may be an important determinant of the interaction between lactoferrin and its receptor on lymphocytes [5]. Furthermore, the interaction of lactoferrin with certain glycosaminoglycans and lipopolysaccharide may require a sequence located within or near the Lfcin domain [10,11].

Lactoferrin is remarkably resistant to proteolytic degradation. Spik et al. [12] have found that lactoferrin in a form retaining its iron-binding capability is excreted in the faeces of human infants. The urinary excretion of intact lactoferrin of maternal origin has been documented in pre-term infants 6 weeks old [13,14]. Given its wide variety of biological functions and unusual stability against proteolysis, pharmaceutical and food-related applications of lactoferrin purified on an industrial scale, including its use in infant formulae [15–17], as a chemopreventor of carcinogenesis [18,19] and as an immunopotentiator [20], have lately attracted considerable attention. However, the metabolic fate of lactoferrin is still poorly understood, especially when orally administered in adults.

Recently we have developed a surface-enhanced laser desorption/ionization (SELDI) affinity assay for lactoferrin and its fragments containing the Lfcin region [9,21,22]. This assay eliminates the influence of contaminants and quantifies the analyte without any possible cross-reactivities, which always arise using conventional methods, such as ELISA and Western-blotting methods. In this report, we employed the SELDI affinity assay to accurately detect various kinds of degraded fragments of lactoferrin. The results document the survival of lactoferrin fragments containing the receptor-binding domain(s) in the gastrointestinal tract of adult mice when orally administered.

MATERIALS AND METHODS

Materials

Bovine Lfcin was purified as described previously [8]. Since commercial bovine lactoferrin shows batch-to-batch variation in iron saturation, and since its enzymic digestibility is influenced by the degree of iron saturation, a single lot of lactoferrin produced by Morinaga Milk Industry (Kanagawa, Japan), with an iron saturation level of 25%, was used for the feeding experiments.

Feeding and sampling

Male Balb/c SPF mice that were 6 weeks of age were obtained from Nihon SLC (Shizuoka, Japan). They were initially allowed free access to a commercial pelleted diet (F-2; Funabashi Farms Co., Chiba, Japan) and tap water for 7 days. The mice were divided randomly into four groups of 5 or 6 mice each. The first two groups of mice were fed commercial bovine milk and the other two groups were fed bovine milk with bovine lactoferrin

Abbreviations used: Lfcin, lactoferricin; MALDI, matrix-assisted laser desorption/ionization; SELDI, surface-enhanced laser desorption/ionization.

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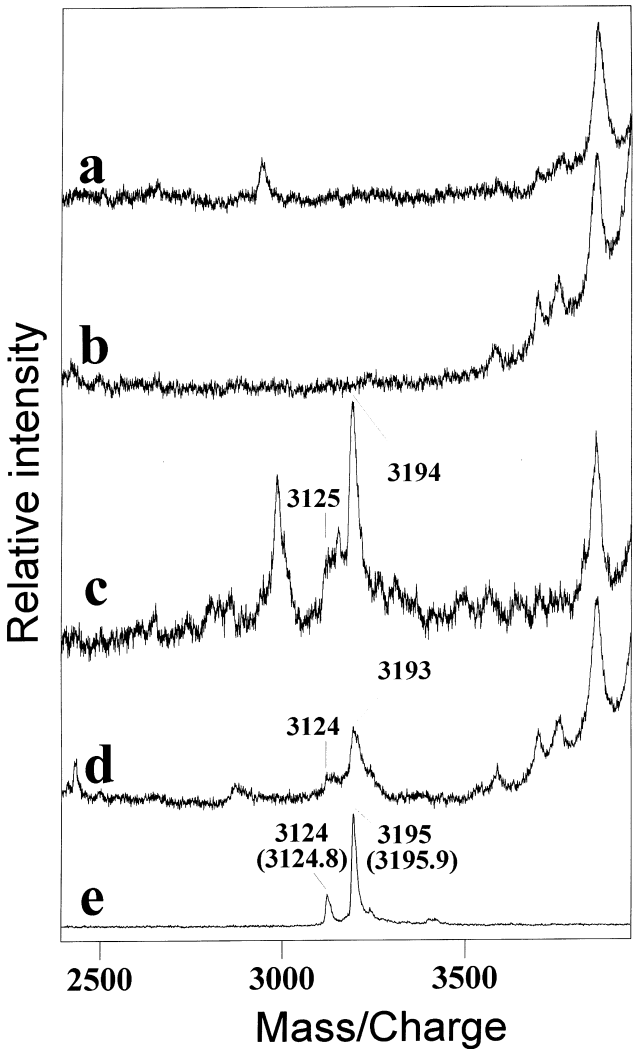

Figure 1 Direct evidence of the survival of lactoferrin fragments in the gastrointestinal tract of mice fed milk enriched with lactoferrin

Samples comprising extracts of faeces from mice fed bovine milk (**a**,**b**), or from mice fed bovine milk enriched with lactoferrin (**c**) were analysed by the SELDI affinity assay for lactoferrin fragments. In the case of the extracts of faeces from control mice (i.e. fed milk only), standard lactoferrin was added prior to analysis to determine the detection limit of the assay (**d**). Standard Lfcin was analysed by MALDI (**e**). The numbers and the numbers in parentheses represent observed mass and calculated average mass ($M+[H]^+$), respectively.

added at a final concentration of 4 %. The bovine milk or milk enriched with bovine lactoferrin was supplied *ad libitum* using a sterile bag and a sterile nozzle (AN pack and SE nozzle; Musashi Co., Saitama, Japan). The sterile bags containing the liquid diets were changed every 2 days. Fresh faeces were collected after feeding for 30 days and frozen at −20 °C until analysis.

SELDI affinity assay for lactoferrin fragments

A portion of faeces weighing 56–70 mg was suspended in PBS (145 mM NaCl/10 mM phosphate buffer, pH 7.2), containing 1 M NaCl, 6 M urea and 40 nM PMSF, by continuous vortex mixing for 30 min at 4 °C, and then centrifuged. To estimate the detection limit of this assay, 10 ng of standard lactoferrin was added to the suspension of faeces from animals fed milk only. Approx. 0.5 ml of the supernatant was collected, and this procedure was repeated twice. The supernatant was diluted with PBS containing 6 M urea and adjusted to 10 ml. To the samples, 20 μl of a 50 % suspension of Butyl-Toyopearl 650M (Tosohaas, PA, U.S.A.) was added followed by mixing at 4 °C overnight. The peptides captured by the gel were hydrolysed *in situ*, recaptured, and analysed by laser desorption time-of-flight MS as described previously (Kuwata, H., Yip, T. T., Tomita, M. and Hutchens, T. W., unpublished work). Because the spectra obtained from the faeces of two groups fed milk enriched with lactoferrin were very similar, only a representative spectrum is shown in Figure 1.

Peptide identification on the basis of mass was carried out using Protein Analysis Worksheets software (Dr. R. C. Beavis, New York University Medical Center, NY, U.S.A.). Each peptide was identified by molecular-mass matching with random fragments of bovine lactoferrin (EMBL database entry TFBOL, last update, 11 August 1995), allowing 0.1 % error in observed mass.

Instrumentation

Both matrix-assisted laser desorption/ionization (MALDI) and SELDI were performed using a modified Hewlett-Packard model 1700XP laser desorption time-of-flight mass spectrometer operated with a three-stage ion optic assembly at high voltage (30 kV). Laser pulses (3 ns) were generated at 10 MHz from a nitrogen laser (337 nm). Data were captured at 400 megasample/s with a digital oscilloscope (bandwidth 350 MHz).

RESULTS AND DISCUSSION

We first employed ELISA, Western blot and direct purification to detect the lactoferrin that survived degradation in the faeces from mice fed lactoferrin. Multiple forms of degraded lactoferrin and the existence of endogenous (i.e. murine) lactoferrin prevented the precise characterization of ingested lactoferrin (results not shown).

Using the SELDI affinity assay, various kinds of lactoferrin fragments containing the Lfcin region that bound to the affinity capture device were hydrolysed *in situ* and detected as Lfcin. When heterogenous lactoferrin is ingested, the Lfcin generated can be discriminated from other contaminants, including endogenous Lfcin, by means of the precise mass obtained by MS.

Figure 1 shows the SELDI mass spectra obtained upon analysis of faeces from mice fed milk or milk enriched with bovine lactoferrin at 40 mg/ml. Molecular-ion peaks corresponding to bovine Lfcin (i.e. Phe^{17}–Phe^{41} and Phe^{17}–Ala^{42} fragments of bovine lactoferrin) were clearly evident in the spectrum obtained for faeces from mice fed milk enriched with lactoferrin (Figure 1c). It has been reported that raw milk contains lactoferrin at a concentration of 0.1–0.2 mg/ml [23]. Employing the SELDI affinity assay, we were able to detect lactoferrin in commercial milk products, such as sterilized milk (results not shown). No evident molecular-ion peaks attributable to bovine Lfcin were observed in the spectra obtained for faeces from mice fed commercial milk only (Figures 1a and 1b).

The SELDI affinity assay for lactoferrin fragments was not accurately quantitative in this study because of the high complexity of the faeces samples, even when the peak intensity was normalized by comparison with an internal standard. However, lactoferrin added to the extract of the faeces from mice fed milk only was recovered and detected as Lfcin (Figure 1d). The corresponding amount of lactoferrin detected by this process was 10 ng/assay (10 ng/60 mg of faeces, or 167 ng/g of faeces, i.e. 2 pmol/g of faeces). Thus, lactoferrin fragments were present at concentrations in the order of at least pmol/g in the faeces from mice fed milk enriched with lactoferrin.

Interactions of lactoferrin with lipopolysaccharide, glycosaminoglycans and receptors on the surface of lymphocytes may require sequences located within or near the Lfcin domain, and Lfcin has been identified as the antimicrobial active centre of lactoferrin. This assay was effective in detecting lactoferrin fragments containing the Lfcin region. Our finding that lactoferrin fragments containing the Lfcin region survive degradation in the faeces of adult mice, and are present at concentrations in the order of pmol/g, seems to be significant, and this may explain why lactoferrin shows host-defensive effects when administered orally. To our knowledge, this is the first time that the survival of ingested lactoferrin or its active fragments has been documented without using any external label or tags. It is now important to examine the survival and absorption of lactoferrin in formula-fed and breast-fed infants, and even in adults such as geriatric patients consuming foods enriched with lactoferrin. Hopefully, continuing studies will yield further insight into the metabolic fate of lactoferrin in the gastrointestinal tract and in the blood stream after it has been absorbed.

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