Comparison of human lactoferrins from milk and neutrophilic leucocytes

Relative molecular mass, isoelectric point, iron-binding properties and uptake by the liver

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Human lactoferrins isolated from neutrophilic leucocytes and milk by CM-Sephadex chromatography were similar in M_r (76000) and pI (8.7). Upon acidification, both proteins released their two Fe^{3+} ions/molecule in a similar biphasic way. Both proteins intravenously injected into mice were cleared from plasma at the same rate. The maximal uptakes by the liver, which occurred 5 min after injection, were inhibited to the same extent by milk lactoferrin used as a competitor.

Lactoferrin, also called lactotransferrin (Montreuil & Mullet, 1960), is an iron-binding glycoprotein secreted by glandular epithelia (Masson *et al.*, 1966, 1969), and one of the major constituents of the specific granules of neutrophilic leucocytes (Baggiolini *et al.*, 1970), the only source of plasma lactoferrin (Hansen *et al.*, 1975). Milk lactoferrin is a single polypeptide chain (M_r 76000), homologous to transferrin (Mazurier *et al.*, 1983). Both proteins have two domains, which can be separated by mild proteolysis, each capable of reversibly binding one molecule of Fe^{3+} (Bluard-Deconinck *et al.*, 1978). However, the affinity of apolactoferrin for iron is higher than that of transferrin (Aisen & Leibman, 1972), especially at low pH (Montreuil *et al.*, 1960).

In the iron economy of the body, the role of transferrin as plasma carrier responsible for the exchange of iron between the various tissues is well known, whereas the role of lactoferrin remains a matter of speculation. Milk lactoferrin binds to macrophages *in vitro* and its iron is subsequently recovered in the ferritin iron stores of these cells (Van Snick & Masson, 1976; Van Snick *et al.*, 1977). This led to the proposal that hyposideraemia, commonly found during inflammation, is mediated by lactoferrin released from neutrophils (Van Snick *et al.*, 1974). We have previously shown that milk lactoferrin, when intravenously injected into mice, is rapidly cleared from the blood by the liver, where the protein accumulates in the Kupffer and endothelial cells (Retegui *et al.*, 1984; Courtoy *et al.*, 1984). The uptake of milk lactoferrin is critically dependent on the integrity of the protein moiety and does not require sugar recognition (Moguilevsky *et al.*, 1983, 1984).

All the studies reported above were made with lactoferrin isolated from milk, a convenient source.

Ambruso & Johnston (1981) reported that leucocyte lactoferrin had a higher enhancing effect on the free-radical production by neutrophils than had milk lactoferrin, an observation suggesting the existence of isolactoferrins. However, in the present study, we failed to detect any significant differences between leucocyte and milk lactoferrins with respect to M_r , pI, stability of the iron-protein complex at acid pH and uptake by the liver.

Experimental

Materials

CM-Sephadex, Sepharose 4B, Dextran T500 and carrier ampholytes (Pharmalyte, pH range 8–10.5) were from Pharmacia Fine Chemicals, Uppsala, Sweden. Acrylamide, bisacrylamide, ammonium persulphate, proteinase substrate gel tablets and Dowex AG1-X8 (100–200 mesh; Cl^- form) were from Bio-Rad Laboratories, Richmond, CA, U.S.A. Dowex 2 X8 (100–200 mesh; Cl^- form) was from Fluka A.G., Buchs, Switzerland. Trasylol (5400 kallikrein-inactivating units/mg) was from Bayer Werk, Elberfeld, W. Germany, and the M_r marker proteins (M_r 29000–170000) were from Serva, Heidelberg, W. Germany. Twice-crystallized elastase from pig pancreas (34 units/mg) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other reagents were of the highest grade commercially available. A Packard 5230 γ -radiation counter was used for counting radioactivity.

Purification of lactoferrin from milk

Lactoferrin was isolated from human milk by chromatography on CM-Sephadex (Querineau *et al.*, 1971). The iron content of native lactoferrin was 9% of its binding capacity, as calculated from the A_{460} .

Radiolabelling

Milk and leucocyte lactoferrins were radioiodinated (with Na^{125}I , carrier-free; from Amersham International, Amersham, Bucks., U.K.) by the chloramine-T procedure (McConahey & Dixon, 1966). Their specific radioactivities ranged from 5 to $10 \mu\text{Ci}/\mu\text{g}$.

Isolation of cells

Blood (400 ml) was collected from normal human volunteers in 80 ml of 0.15 M-NaCl containing 10 g of EDTA/l. We also used as starting material packed white cells obtained by leucopheresis from patients with chronic myeloid leukaemia and having at least 6×10^4 cells/ μl , with 90% of late-maturation forms (promyelocytes to mature neutrophils). The cells, concentrated in 150 ml of plasma, were mixed with 300 ml of phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) and with 90 ml of a 60 g/l solution of Dextran T500 in phosphate-buffered saline containing 10 g of EDTA/l.

Erythrocytes were sedimented at 1 g for 1 h at 4°C (Böyum, 1968) and the supernatant was centrifuged at 3000 g-min. The remaining erythrocytes were haemolysed by resuspension of the pellet in 10 ml of 0.14 M- NH_4Cl containing 0.01 mM-EDTA (acid form) and incubation for 30 min at 4°C.

Purification of lactoferrin from leucocytes

After a washing step with phosphate-buffered saline, the leucocytes from 400 ml of blood were resuspended in 1 M-NaCl/0.1 M-sodium phosphate buffer, pH 8, at a concentration of 10^8 cells/ml and disrupted by six subsequent freezings and thawings. Cell debris were removed by centrifugation at 9×10^6 g-min in a Beckman Ti50 rotor at 4°C. The supernatant was dialysed against 10 mM-sodium phosphate buffer, pH 7.0, centrifuged at 4×10^5 g-min at 4°C and mixed with 3 ml of CM-Sephadex in the same buffer. After gentle overnight agitation by rotation, the gel was poured into a 45 mm $\times$ 10 mm column, successively washed with 10 mM- and 20 mM-sodium phosphate buffer, pH 7.0, and with 20 mM-sodium phosphate buffer containing 0.25 M-NaCl. The proteins were eluted stepwise by 10 ml of 0.33 M- and 10 ml of 1 M-NaCl in 20 mM-sodium phosphate buffer, pH 7.0, and

lactoferrin was assayed in various fractions by radioimmunoassay or particle-counting immunoassay (Masson *et al.*, 1981), with purified milk lactoferrin as standard. The material eluted in the 0.33 M- and 1 M-NaCl fractions was iodinated and, after characterization, used as such or rechromatographed under the same conditions after dialysis against 10 mM-sodium phosphate buffer, pH 7.0. Lactoferrin eluted by 1 M-NaCl contained more iron than did lactoferrin eluted by 0.33 M-NaCl (see the Results section). In some experiments, to remove contaminating proteinases, we incubated the labelled material with Trasylol-Sepharose overnight (Baugh & Travis, 1976). Trasylol (25 mg) was coupled to 6-aminohexyl-Sepharose 4B (8 ml of packed gel) activated with glutaraldehyde (Cambiaso *et al.*, 1974). Proteolytic activity was measured by digestion of casein in agar gel (Bjerrum & Bøj-Hansen, 1975). In all preparations, more than 97% of the radioactivity was precipitated by 10% (w/v) trichloroacetic acid.

Electrophoresis

Electrophoresis was run through polyacrylamide-gel rods (100 mm $\times$ 5 mm). For electrophoresis in the presence of sodium dodecyl sulphate (0.15%) without prior reduction of the samples, we used a 5%-polyacrylamide gel and a constant current of 5 mA/gel (Weber & Osborn, 1969). A mixture of proteins of M_r 29 000–170 000, used as standards, was run in parallel and stained with Coomassie Blue.

Polyacrylamide-gel electrophoresis without sodium dodecyl sulphate was run under non-denaturing conditions in β -alanine/acetic acid buffer, pH 4.7, in a 7.5%-polyacrylamide gel, at 5 mA/gel (Reisfeld *et al.*, 1962). When the dye front reached the bottom of the gel, gels were frozen, cut every 2 mm and counted for radioactivity.

For isoelectrofocusing (Låås & Fast-Johansson, 1979) we used a 5%-polyacrylamide gel containing 6.25% (v/v) Ampholines with a linear range of pH 8.5–10. The gels were focused at 2 mA/gel for 5 h at 4°C. To measure the pH and count radioactivity, we immersed each slice of the gel overnight in 1 ml of water.

Immunoprecipitation

Radiolabelled lactoferrin preparations were incubated for 2 h at 37°C with 1 mg of goat anti-human milk lactoferrin IgG (produced as described by Baggiolini *et al.*, 1970) in 1 ml of phosphate-buffered saline, followed by overnight incubation at 4°C with 1 ml of 4 M- $(\text{NH}_4)_2\text{SO}_4$. After centrifugation at 12 000 g-min, the radioactivities of the tube and of the supernatant were counted.

Clearance and uptake experiments

These are described elsewhere (Retegui *et al.*, 1984). Briefly, the tracer in 100–200 μ l of 0.15 M-NaCl was injected into the tail vein of male BALB/c mice (about 13 weeks old). A few seconds before the indicated times, the mice were anaesthetized with diethyl ether, and 0.5 ml of blood was collected from the orbital venous plexus. The mice were killed and the livers excised and counted for radioactivity. The radioactivity of the total plasma was calculated on the assumption that the plasma volume represented 6.7% of the body weight.

Dissociation of the iron–lactoferrin complexes

We studied the dissociation of the iron–lactoferrin complexes by the method of Mazurier & Spik (1980), with slight modifications. Briefly, the milk and leucocyte fractions (100 μ g of lactoferrin/ml) eluted by 0.33 M-NaCl from CM-Sephadex were saturated after dialysis at 4°C against 0.1 M-sodium citrate/0.1 M-sodium bicarbonate buffer, pH 8.6, with radioactive [^{59}Fe]ferric citrate (27 mCi/mg; IRE, Fleurus, Belgium) diluted in the same buffer. ^{59}Fe -saturated lactoferrins were then transferred in 100 μ l portions into three sets of ten tubes containing 900 μ l of 0.1 M-sodium citrate buffer adjusted to various pH values ranging from 3.5 to 8.5. After incubation for 5 h at room temperature, the pH was checked and free iron was retained by small Dowex 2 X8 columns (in 1 ml automatic-pipette tips) in the corresponding citrate buffer. After lactoferrin deposition, the columns were washed with three 1 ml portions of the appropriate citrate buffer. The results were calculated from the radioactivity of the starting lactoferrin solution, the eluate and the resin, and expressed as percentage of the maximal iron binding at pH 8.6.

Results

Purification of human leucocyte lactoferrin

In a typical experiment we found that the soluble leucocyte extract that was obtained from 400 ml of blood (1.2×10^9 neutrophils) contained 107 mg of total protein assayed by the Lowry method and 3.4 mg of lactoferrin assayed by particle-counting immunoassay (Masson *et al.*, 1981). Precipitation occurred during dialysis at 4°C against 10 mM-phosphate buffer, pH 7.0, before CM-Sephadex chromatography. A large fraction corresponding to 60% of the total lactoferrin content was lost in this precipitate. After the chromatographic step, 0.32 mg of lactoferrin was recovered in the 0.33 M-NaCl fraction and 0.13 mg in the 1 M-NaCl fraction.

Purified preparations were analysed by immuno-precipitation. Goat anti-(milk lactoferrin) immuno-

globulins precipitated 92% of radiolabelled leucocyte lactoferrin and 88% of milk lactoferrin, whereas immunoglobulins from a non-immunized goat caused 11% and 21% precipitation of leucocyte and milk lactoferrin respectively.

Electrophoresis and isoelectric focusing

^{125}I -labelled leucocyte and milk lactoferrins migrated to the same position in polyacrylamide-gel electrophoresis under non-denaturing conditions, in β -alanine/acetic acid buffer, pH 4.5 (Fig. 1a).

The apparent M_r of both ^{125}I -lactoferrins was determined by polyacrylamide-gel electrophoresis in the presence of 0.15% sodium dodecyl sulphate (Fig. 1b). A major peak of radioactivity was detected in the region corresponding to M_r 76 000, a value found for milk lactoferrin by Querinjean *et al.* (1971). A small additional component of higher M_r was detected in milk lactoferrin and one of low M_r (25 000–30 000) in leucocyte lactoferrin. This low- M_r contaminant was probably a serine proteinase, as it was retained on a Trasyol–Sephadex column. Cathepsin G and elastase have M_r values of 24 000 and 30 000 respectively (Travis *et al.*, 1980). With pig pancreatic elastase as a standard we found a proteolytic activity corresponding to 5.7 units/ml (protein concentration 62.5 μ g/ml). After passage through the Trasyol–Sephadex column, no activity was detected in the effluent, which contained 38.5 μ g of protein/ml.

Isoelectric focusing in polyacrylamide gel of both lactoferrins showed a major peak at pH 8.7. The curve of milk lactoferrin was asymmetric, with a shoulder and a trail on the acidic side of the major peak. Two minor components, of pI 8.2 and 8.4, were visible in the leucocyte lactoferrin preparation (Fig. 1c).

Stability of iron–lactoferrin complexes

We compared the stabilities of the leucocyte and milk ^{59}Fe -lactoferrin complexes that were formed at pH 8.6 with the stability of the iron–transferrin complex by measuring the release of radioactive iron upon progressive acidification in citrate buffer (Fig. 2). The two binding sites of transferrin simultaneously and completely released their iron at pH 7.0, whereas both lactoferrins released their first Fe^{3+} ion between pH 6.5 and 5.5 and the second between pH 4.5 and 3.5.

Clearance and liver uptake after intravenous injection

Like ^{125}I -labelled milk lactoferrin (Retegui *et al.*, 1984), ^{125}I -labelled leucocyte lactoferrin injected intravenously into mice was cleared from the plasma with a half-life of a few minutes and accumulated in the liver, where radioactivity reached a peak at 5 min after injection (Fig. 3). The

Fig. 1. Polyacrylamide-gel electrophoresis of lactoferrins

(a) Electrophoresis at pH 4.5; (b) electrophoresis in the presence of sodium dodecyl sulphate; (c) isoelectric focusing. A load of 8 ng (60 nCi) of the 0.33 M-NaCl fraction of CM-Sephadex of ^{125}I -labelled milk lactoferrin (upper panels) or leucocyte lactoferrin (lower panels) was used. The relative fraction number is the ratio of the electrophoretic mobility of the radioactive material to that of the dye front.

Fig. 2. Dissociation of iron-lactoferrin complexes. Samples of milk (○) and leucocyte (●) lactoferrins and of transferrin (△), after saturation with ^{59}Fe at pH 8.6, were incubated in citrate buffer at various pH values. Unbound ^{59}Fe was retained on Dowex, as described in the text.

decay of radioactivity in the liver was found to differ from one lactoferrin preparation to the other. Leucocyte lactoferrin that had been eluted from CM-Sephadex at 0.33 M-NaCl had a more rapid decay than that eluted at 1 M-NaCl. Such a difference could be explained not only by possible contamination, aggregation or alteration of the proteins, but also by difference in its iron content, as iron-saturated lactoferrin, which is eluted at higher salt concentration (Masson, 1970), is more resistant to intracellular catabolism than is apolactoferrin (Van Snick *et al.*, 1977). The radioactivity in the liver 5 min after injection was proportional to the purity or structural integrity of lactoferrin assessed by immunoprecipitation. With 100% immunoprecipitable lactoferrin, 80% was recovered in the liver 5 min after injection. In competition experiments (Fig. 4) the uptake of ^{125}I -labelled milk lactoferrin (8 ng) was inhibited to 50% by 2.5 mg of unlabelled milk lactoferrin. For leucocyte lactoferrin, such an inhibition required 5 mg of unlabelled milk lactoferrin. Such a result

Fig. 3. Clearance and uptake of lactoferrin by mouse liver. Trace amounts (8 ng, 60 nCi) of ^{125}I -lactoferrin, purified from milk (eluted at 1 M-NaCl) (a) or leucocytes [respectively eluted at 1 M-NaCl (b) or 0.33 M-NaCl (c)] were injected intravenously into mice and the radioactivity was measured in the plasma and the liver at various times after injection. Results are means $\pm$ S.D. (bars) for at least three determinations.

would suggest that leucocyte lactoferrin had more affinity for the liver than did milk lactoferrin. However, when the leucocyte lactoferrin that had been passed through the Trasyol-Sephadex column was used as a tracer, the competition curve was identical with that of milk lactoferrin (Fig. 4).

Discussion

Neutrophilic leucocytes contain about $3 \mu\text{g}$ of lactoferrin per 10^6 cells (Masson *et al.*, 1969).

Fig. 4. Competition for liver uptake of lactoferrins. Iodinated milk lactoferrin (○), leucocyte lactoferrin (△) and leucocyte lactoferrin passed through the Trasyol-Sephadex column (●) [8 ng (60 nCi) of each] were injected into mice without or with increasing amounts of unlabelled milk lactoferrin as a competitor. Liver uptake was measured 5 min after injection and expressed as a percentage of the value obtained in the absence of competitor. The uptakes at 5 min reached 73% of the injected dose for ^{125}I -labelled milk lactoferrin, 74% for ^{125}I -labelled leucocyte lactoferrin and 60% for ^{125}I -labelled leucocyte lactoferrin after adsorption on Trasyol-Sephadex. Results are means $\pm$ S.D. (bars) for at least three determinations.

However, we recovered only 10% after CM-Sephadex chromatography, the major part being lost in the precipitate formed during dialysis of the leucocytic extract against 10 mM-phosphate buffer, pH 7.0. We presume that lactoferrin, because of its high pI, binds to nucleic acids (Bennett *et al.*, 1983) or other acidic macromolecules, these complexes being insoluble at low ionic strength.

The pI value (8.7) that we found for leucocyte and milk lactoferrins corresponded to that previously reported for milk lactoferrin by Roberts *et al.* (1972), who found additional peaks of pI 8.4 and 9.1. Because a single peak of pI 9.3 was recovered after digestion with neuraminidase, these authors attributed the extra peaks to variants differing in their sialic acid content. We did not detect the pI 9.3 fraction, but found more-acidic components.

The iron-lactoferrin complexes dissociated similarly at acid pH whatever their origin. The dissociation curves could be superimposed on

those obtained by Mazurier & Spik (1980) when they used a dissociation medium of high ionic strength (10.2) and containing EDTA.

As described previously (Moguilevsky *et al.*, 1984), the uptake of lactoferrin by the liver is strictly dependent on the integrity of the protein. Here we observed that removing a contaminant from the leucocyte lactoferrin preparation used as tracer enhanced the inhibitory effect of the competing protein, milk lactoferrin. This observation led to at least two possible interpretations. On the one hand, leucocyte lactoferrin had more affinity for the liver than did milk lactoferrin, and the passage of the preparation through the Trasylol-Sephadex column altered the protein. On the other hand, the contaminant retained on the column had itself a strong affinity for the liver. In the latter case the contaminant could have been taken up either by the lactoferrin-binding sites on the sinusoidal cells or by any liver receptors. Uptake by common binding sites seems more likely, because the uptake of leucocyte lactoferrin could be inhibited by milk lactoferrin, although by higher doses of competitor, to the same extent as the uptake of radiolabelled milk lactoferrin. An informative experiment would have been the use of leucocyte lactoferrin as a competitor against labelled milk lactoferrin. However, insufficient leucocyte lactoferrin was available for such an experiment.

In the Results section we have concluded, on the basis of its binding to Trasylol-Sephadex and its molecular size, that the contaminant could be elastase or cathepsin G. These two proteinases have been shown by Campbell (1982) to compete with milk lactoferrin for the same binding sites on macrophages. The dissociation constants were 1.7×10^{-6} M for lactoferrin, 4.0×10^{-7} M for elastase and 2×10^{-7} M for cathepsin G. Therefore it is tempting to believe that these two proteinases compete with lactoferrin also for the liver binding site.

In conclusion, we failed to detect differences between milk and leucocyte lactoferrins, and we wonder whether the differences observed by Ambruso & Johnston (1981) in the catalysis of the production of hydroxyl radicals by leucocyte and milk lactoferrins could not be explained by the presence in the leucocyte lactoferrin preparation of elastase or cathepsin G, which have been found to enhance the release of oxygen metabolites by macrophages (Speer *et al.*, 1984).

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