Vol. 101, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1981 Pages 88-95

CALCIUM-DEPENDENT POLYMERIZATION OF LACTOFERRIN.

Robert M. Bennett, Grover C. Bagby and Julianne Davis
Department of Medicine, University of Oregon Health Sciences Center
Portland, Oregon 97201

Received May 29, 1981

SUMMARY

Lactoferrin is known to have a molecular weight of approximately 77,000. In this study, we show that it undergoes polymerization in calcium-containing fluids and that the predominant species is a tetramer. Preliminary evidence is presented to indicate that the tetramer is also found in human serum, tears and breast milk. When the lactoferrin monomer and the tetramer were tested for biological activity in a system that is known to be highly sensitive to an inhibitory effect of lactoferrin - the production of granulocyte monocyte colony-stimulating activity - it was found that only the monomeric form of lactoferrin was inhibitory.

INTRODUCTION

Estimates of the molecular weight of human lactoferrin have shown certain inconsistencies - reported values ranging from 75,000 to 95,000 (1,2,3). There are several reports which suggest that lactoferrin may undergo a variable degree of polymerization in biological fluids. Tabak et al. (4) have noted changes in the apparent molecular weight of lactoferrin recovered from the saliva of a patient with parotitis; a polymeric form was observed during the acute stage and this changed to an apparent dimer, and then a monomer, as the inflammation subsided. Harmon et al. (5) described somewhat similar findings in the milk of an experimentally-induced infection of a bovine mammary gland; at the height of the infection, the molecular weight of the lactoferrin was consistent with a trimer and this decreased as the infection subsided. Others have indicated that lactoferrin complexes are present in bovine lacteal secretions (2,6,7). Whether such complexes result from an interaction of lactoferrin with other macromolecules or are due to self-

0006-291X/81/130088-08$01.00/0

association, has not been entirely resolved; however, there is accumulating evidence that lactoferrin readily associates with acid macromolecules (8,9,10).

In this communication we described a calcium-dependent self-association of lactoferrin. Evidence is presented that such self-association results in the formation of lactoferrin tetramers and that these are the predominant form of lactoferrin in certain biological fluids. Preliminary findings implicate a possible biological role for the monomeric form of lactoferrin, but not the tetramer in the feedback control of granulopoiesis.

MATERIALS AND METHODS

Lactoferrin was isolated from pooled human breast milk as previously described (11). Its purity was verified by polyacrylamide gel electrophoresis and immunoelectrophoresis using troughs containing antiserum to whole human serum and human breast milk (11,12). It was sterile on routine cultures and endotoxin-free in the limulus assay. From the E_{465} 1% it was 85% iron-saturated. As previously outlined, lactoferrin was labelled with ^{125}I by the chloramine T method to a specific activity of 23.8 $\mu Ci/\mu g$; nanogram quantities of lactoferrin were measured by a solid phase radioimmunoassay (11). Gel chromatography employed a Sephacryl S-300. Flow was in an upward direction at a rate of 15 ml/hr. The buffer systems were: a) phosphate-buffered saline (0.15 M, pH 7.4) plus tetra sodium EDTA (10 mM), and b) borate buffer (0.2 M, pH 7.4) plus $CaCl_2$ (10 mM). Fractions (5 ml) were collected and the A_{280} measured. The column was calibrated with markers of defined molecular weight.

Rate zonal density gradient sedimentation was performed in a Beckman preparative ultracentrifuge using a SW56Ti rotor; all runs were at 4°C. Linear sucrose gradients were generated from 10% to 40% with a final 200 µl "cushion zone" of 60% sucrose. The sucrose solution was supplemented with 10 mM Ca++ (as $CaCl_2$). Each rate zonal run was calibrated by including one tube containing markers of known molecular weight (bovine serum albumin, aldolase, catalase, ferritin and thyroglobulin).

Electroimmunodiffusion was performed with human breast milk, saliva and tears. Electrophoresis (constant current of 20 mA for 90 min) was into a 1% agarose gel containing 0.5 ml of high avidity rabbit anti-human lactoferrin antiserum per 15 ml of agarose. In one experiment, the agarose was made up in a barbital buffer (0.04 M, pH 8.6) containing 10 mM Ca++ and, in another experiment, it was made up in a calcium deficient buffer containing 10 mM tetra sodium EDTA.

The respective biological activities of lactoferrin monomer and lactoferrin polymer were assayed in a system which is highly sensitive to an inhibitory effect of lactoferrin on the production of in vitro colony stimulating activity for granulocytes (GM-CSA). In brief, human low-density bone marrow cells were cultured in 0.9% methylcellulose layered onto a feeder layer of peripheral blood mononuclear cells in 0.5% agar in alpha medium (Gibco) supplemented with 15% lactoferrin-depleted fetal calf serum. The feeder layer contained either lactoferrin monomer, lactoferrin polymer or no

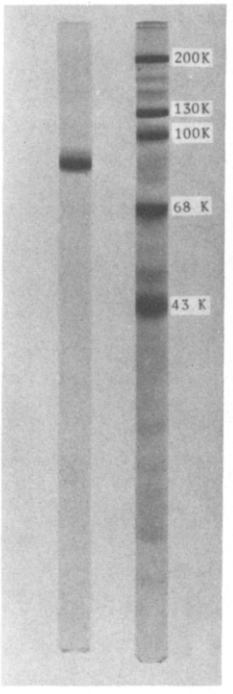


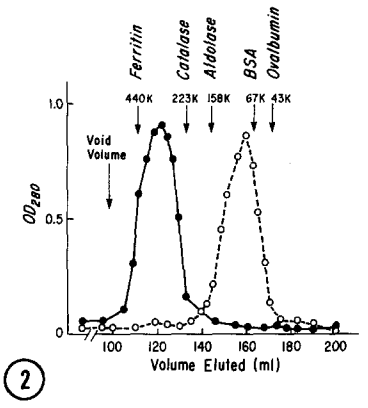
FIG. 1 - Lactoferrin (20 μg in 20 μl) was subjected to electrophoresis in a 7.5% polyacrylamide gel. Molecular weight markers, myosin (200 K), beta-galactosidase (130 K), phosphorylase (100 K), bovine serum albumin (68 K) and ovalbumin (43 K) were electrophoresed at the same time. The system employed a phosphate buffer (0.01 M, pH 7.0 containing 1% SDS) and electrophoresis was performed at a constant current (8 m amps) for three hours. A semilog plot of the distances migrated of the markers versus their molecular weight was used to estimate the molecular weight of the lactoferrin; a value of approximately 76,000 was obtained.

lactoferrin. Colonies and clusters were counted on the seventh day of culture, as has been previously described (13).

RESULTS AND DISCUSSION

1. Lactoferrin Characterization

Polyacrylamide gel SDS electrophoresis of the lactoferrin used in these experiments revealed a single protein band migrating on a position consistent with a molecular weight of approximately 76,000 (Figure 1). After iodination with ^{125}I, trace amounts (2 ng) were found to be free of aggregates as assessed by the elution profile over a Sephacryl S-300 column equilibrated with phosphate-buffered saline. The (^{125}I) lactoferrin was reactive with



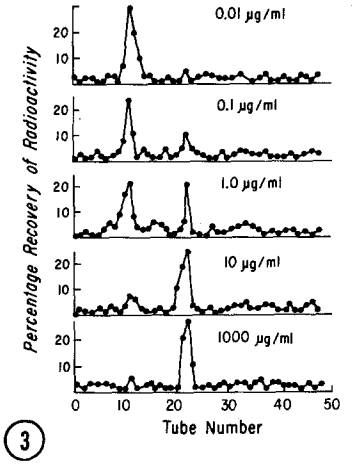


FIG. 2 - Lactoferrin (1 ml, 10 mg/ml) was chromatographed over a column of Sephacryl S-300 (95 x 2.5 cm) with an upward flow of 15 ml/hr. In the first experiment (discontinuous line), the eluant was phosphate-buffered saline (0.15 M, pH 7.4) supplemented with tetra sodium EDTA (10 mM). In the second experiment (continuous line), the eluant was a borate buffer (0.2 M, pH 7.4) supplemented with calcium chloride (20 mM). The column was calibrated with markers of a defined molecular weight (bovine serum albumin, aldolase, catalase, ferritin and thyroglobulin). In the absence of calcium, a single peak eluted at a position consistent with a MW of 76,000. With a calcium supplemented-buffer, a single peak eluted at a position consistent with a MW of 300,000. The ratio of polymer to monomer is 3.95:1, suggesting that lactoferrin exists as a tetramer in calcium-containing fluids.

FIG. 3 - Varying concentrations of cold lactoferrin were mixed with tracer (^{125}I), lactoferrin (2 ng) and 20 µl of the mixture subjected to rate zonal density sedimentation (108,000 x g at 4°C for 24 hr). Each run consisted of five tubes containing varying concentrations of cold lactoferrin and one tube containing molecular weight marker. Sucrose gradients (10-40% with a 60% buffer zone) were supplemented with calcium chloride (10 mM). Fractions were collected from the top downward. Two peaks were obtained, one corresponding to a polymer of MW 310,000, and the other corresponding to the monomeric form of lactoferrin (MW 77,000). The ratio of the MW of the polymer to that of the monomer is 3.97:1. This experiment also shows that as the concentration of lactoferrin approaches 0.01 µg/ml (10^{-9} M), there is a progressive dissociation of the polymer.

anti-lactoferrin antibodies, the reaction being inhibited in a dose-dependent manner by the addition of cold lactoferrin, as assessed by a solid phase radioimmunoassay (11).

2. Gel Chromatography

One ml of lactoferrin (10 mg/ml) was chromatographed over Sephacryl S-300 equilibrated with phosphate-buffered saline supplemented with 10 mM tetra sodium EDTA. Monitoring the eluate at 280 nm revealed only one peak at an

Table 1: Human serum and human breast milk were chromatographed over a Sephacryl S-300 column. The eluates were monitored for lactoferrin by radioimmunoassay. The major peak for both fluids corresponded to a MW of approximately 300,000 with a smaller peak at 76,000. In the case of breast milk, a third peak was observed at the void volume. This was not analyzed further but probably represented the complexing of lactoferrin to acidic macromolecules.

Sample	Lactoferrin Recovered (ng/ml)		
	Void Volume	300 K Peak	76 K Peak
Serum	0	>300	130
Breast milk	41	55	28

elution volume of 161 ml (Figure 2). When the same experiment was performed with the same column equilibrated with a 0.2 M borate buffer supplemented with calcium chloride, a single peak eluted at 122 ml (Figure 2). The two elution peaks of the lactoferrin corresponded to molecular weights of 76,000 and 300,000. In complementary experiments, magnesium did not promote a self-association of lactoferrin. These results indicate that lactoferrin self-associates in the presence of calcium, with a molecular weight most consistent with tetramerization. Using the same size column and the calcium-supplemented buffer, human serum (2 ml) and diluted human breast milk (2 ml) were chromatographed. Eluates were tested for the presence of lactoferrin by radioimmunoassay. The results are shown in Table 1; it is seen that most of the lactoferrin eluted in a position corresponding to a molecular weight of approximately 300,000. In the case of breast milk, a third peak was present at the void volume; this peak may represent the complexing of lactoferrin to acidic macromolecules (7), but this was not explored any further in this study.

3. Rate Zonal Density Sedimentation

Cold lactoferrin at varying concentrations was mixed with tracer (^{125}I) lactoferrin (2 ng) and 20 µl of the mixture was layered onto sucrose gradients. Each run consisted of five tubes containing lactoferrin and one tube containing molecular weight markers. The sedimentation profile showed predominantly lactoferrin polymer at high lactoferrin concentrations (Figure

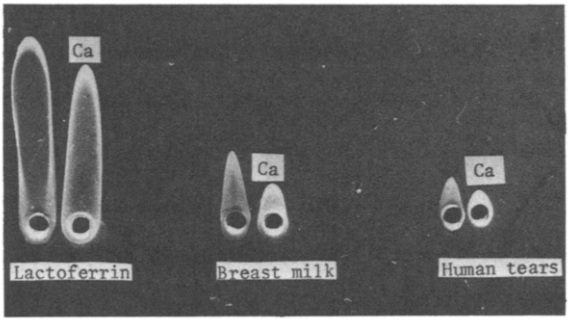


FIG. 4 - Lactoferrin, human tears and human breast milk were electrophoresed into a 1% agarose gel containing 0.5 ml of high avidity rabbit-anti-human lactoferrin antiserum. Electrophoresis was in a barbital buffer (0.04 M, pH 8.6) and two runs of exactly 90 minutes each were performed (constant current 20 mA). In the first run, the buffer was supplemented with tetra sodium EDTA (10 mM); in the second run, the buffer was supplemented with calcium chloride (10 mM). It is seen that the pure lactoferrin and both secretions show a restricted mobility when run in the calcium-supplemented buffer (for the sake of clarity, the "rockets" from the calcium-supplemented buffer were cut out and placed by the side of the respective "rocket" in the calcium-depleted buffer).

3). The polymer sedimented in a position corresponding to a molecular weight of 310,000 and the monomer at 78,000. The ratio of polymer to monomer is 3.97:1, suggesting that the predominant species of polymer is a tetramer. This experiment also indicates that there is a concentration-dependent dissociation of the tetramer form in favor of the monomer; at a concentration of about 0.01 μg/ml (10^{-9} M) lactoferrin is predominantly in a monomeric form.

4. Electroimmunodiffusion

When lactoferrin was electrophoresed into an anti-LF agarose gel containing calcium (10 mM), its migration was inhibited in comparison to a similar experiment performed when omitting calcium. A similar inhibition of migration was also observed for human tears, saliva and breast milk (Figure 4). One interpretation of these results is that the calcium-dependent polymerization of lactoferrin in these secretions retards its migration through the agarose.

5. Inhibition of Granulocyte Colony-stimulating Activity

One of the few known biological effects of lactoferrin is its ability to inhibit the production of GM-CSF in vitro. This effect has been observed at concentrations as low as 10^{-17} M (14). Lactoferrin (1 mg in Mcoys medium) was separated into polymer and monomer fractions by passage over a Sephacryl S 300 column. The monomer (2.0×10^{-10}M) and polymer (1.7×10^{-6} M) were added to a feeder layer of human peripheral blood mononuclear cells which underlayed human low-density bone marrow cells cultured in 0.9% methylcellulose. In five separate experiments, the lactoferrin monomer inhibited the development of granulocyte colonies by from 32 to 60%, as compared to control cultures without lactoferrin. The lactoferrin polymer had no inhibitory effect on granulocyte colony formation until it was diluted out to a concentration of 10^{-9} M or less; at this concentration it had dissociated into monomer. This result may be of relevance to the apparent lack of feedback control that is seen in patients with chronic myeloid leukemia. Such patients have high levels of circulating lactoferrin (11,14), which our experiments predict will be mainly in the form of a tetramer and hence ineffective in restraining granulopoiesis.

ACKNOWLEDGEMENTS

The authors wish to acknowledgement the helpful advice and criticism of Dr. D.A. Rigas of the Department of Biochemistry, University of Oregon Health Sciences Center, Portland, Oregon.

REFERENCES

1. Queringjean P, Masson PL, Heremans JF (1971). Eur. J. Biochem. 20:420-425.
2. Oram JD, Reiter B. (1968). Biochim Biophys Acta 170:351-365.
3. Schade AL, Pallavicini C, Wiessman U (1968). Protides Biol. Fluids Proc. Colloq. Bruges 16:619-
4. Tabak L, Mandel ID, Herrera M, Baurmash H (1978). J. Oral Path. 1:97-99.
5. Harmon RJ, Schanbacher FL, Ferguson LC, Smith LC (1976). Infect. Immun. 13: 533-542.
6. Groves ML (1965). Biochim. Biophys. Acta 100:154-162.
7. Smith KL, Conrad HR, Porter RM (1971). J. Dairy Sci. 54:1427-1435.
8. Hekman A (1971). Biochim. Biophys. Acta 251:380-387.
9. Malmquist J, Johansson BG (1971). Biochim. Biophys. Acta 236:38-46.

10. Castellino FJ, Fish W, Mann J (1970). J. Biol. Chem. 245:4209-4275.
11. Bennett RM, Mohla C (1976). J. Lab. Clin. Med. 88:156-166.
12. Bennett RM, Kokocinski (1978). Brit. J. Haematol. 39:509-521.
13. Bagby GC, Gabourel JD (1979). J. Clin. Invest. 64:72-82.
14. Broxmeyer HE, Smithyman A, Eger R, Myers P, De Sousa M. (1978). J. Exp. Med. 148:1052-1067.