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lactoferrin sialic acid
milk

Heterogeneity of Human Lactoferrin Due to Differences in Sialic Acid Content

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Extract

Differences in sialic acid content and not genetic polymorphism determine the observed electrophoretic heterogeneity of human lactoferrin, the iron-binding protein of external secretions. Lactoferrin molecules have at least one to four sialic acid residues. The asialo derivative is electrophoretically homogeneous.

Speculation

As with other nonhuman iron-binding proteins from various species, human lactoferrin from several secretions was shown to have varying sialic acid content. A regulatory role of sialic acid in the regulation of secretion and function of lactoferrin is postulated.

Introduction

Lactoferrin, the iron-binding protein of exocrine tissue secretions, was isolated from human milk and duodenal fluid. This purified lactoferrin was observed to be electrophoretically heterogeneous. The basis for this charge heterogeneity was studied and found to be due to differing sialic acid content of protein molecules.

Materials and Methods

A procedure for the isolation of various forms of lactoferrin with protein of different net charges was devised utilizing cation exchange chromatography (CMC) [26] followed by Sephadex G-100 gel filtration. In a representative experiment, 500 ml human milk serum [27] obtained from pooled frozen milk from three donors contained 680 mg lactoferrin [10]. The milk serum was dialyzed against 0.05 M acetate buffer, pH 5.3, and adsorbed to a 2.5-cm by 50-cm CMC column equilibrated with the same buffer maintained at 4°. The effluent was monitored for protein by absorbance at 280 mμ and for lactoferrin using anticolostral protein and antilactoferrin antisera. Ten column volumes of the initial buffer were allowed to flow through the CMC. After two protein peaks were obtained which did not contain lactoferrin, an ionic gradient of 0.1–0.2 M NaCl in the acetate buffer was applied for elution. A peak containing IgA was eluted, followed by a long low protein peak (approximately 5 column volumes) containing lactoferrin. To obtain lactoferrin molecules of different net charges the first half of the peak containing lactoferrin was pooled and designated *fraction A*, and the second half, *fraction B*. After completion of the gradient, lactoferrin, designated *C*, was sharply eluted with 1.0 M NaCl. The three lactoferrin fractions were separately purified using a 2.5-cm by 100-cm Sephadex G-100 gel filtration column. The conditions of this chromatography were: (*1*) the solvent was 1.0 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0; (*2*) temperature was 4°; (*3*) flow rate was 10 ml/hr; (*4*) fraction size was 5.0 ml. The final product yielded 35 mg *fraction A*, 250 mg *fraction B*, and 316 mg *fraction C*, which accounted for 88% of the lactoferrin present in the original milk sample.

The molecular weight of lactoferrin was determined

by the method described by Whitaker [24]. A 2.5-cm by 100-cm column of Sephadex G-200 was calibrated with 1.0-ml samples of purified equine cytochrome *c*, human serum albumin, and rabbit IgG globulin containing 5.0 mg/ml. The solvent was 0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA (pH 7.0), the flow rate was 10.0 ml/hr, and the temperature was 4°. With this method, the molecular weight of lactoferrin was found to be 96,000.

Results

Purity and antigenic identity of the lactoferrin preparations were demonstrated by a single precipitin line following immunoelectrophoresis (Fig. 1*A*) and immunodiffusion (Fig. 1*B*) with goat antihuman colostral protein antiserum. The purity was further confirmed when a goat and a rabbit immunized with purified lactoferrin in Freund's adjuvant produced antisera giving single precipitin bands with human colostrum.

Immunoelectrophoresis (Fig. 1*A*) showed that lactoferrin *A* migrated fastest toward the anode. *Fraction C*, eluted last from the column, had the slowest migration, and *fraction B* had intermediate mobility. Polyacrylamide gel electrophoresis [4] resolved each fraction into two to three separate protein bands (Fig. 1*C*). The fastest moving bands, called 1, 2, and 3, were found in *fraction A*. These findings were confirmed by vertical starch gel electrophoresis [17, 18]. Attempts to fractionate the lactoferrin into preparations yielding single bands by polyacrylamide gel electrophoresis were unsuccessful. The sialic acid content [22] of the three lactoferrin fractions was measured using neuraminic acid, dried over P_2O_5 at 0.5 mm Hg pressure at 4° to a constant weight, as a standard. The most negatively charged lactoferrin, *A* (Table I), contained 4.09 μM sialic acid, *fraction B* contained 3.38 μM sialic acid, and *fraction C* contained 2.31 μM sialic acid/μM lactoferrin.

Table I. Sialic acid content of human milk lactoferrin[1]

Sample	Pooled milk	Donor *JS*	Donor *EK*	Donor *BM*
Fraction A	4.09	4.67	3.92	4.01
Fraction B	3.38	3.75	3.75	3.80
Fraction C	2.31	2.47	2.67	2.20
A + *B* + *C* at pH 4.6	3.50			
A + *B* + *C* at pH 4.6	3.50			
A + *B* + *C* at pH 8.0	3.76			
A + *B* + *C* at pH 8.0	3.71			

[1] The sialic acid content of pooled and individual samples of fractionated milk lactoferrin was analyzed by the method of Warren [22]. The results are expressed as micromoles of sialic acid per micromole of lactoferrin, assuming the molecular weight of the protein to be 96,000 [24].

To ascertain that the differing sialic acid values were not due to genetic variations, lactoferrins *A*, *B*, and *C* from three individuals, *JS*, *EK*, and *BM*, were isolated. All of these lactoferrin samples demonstrated similar electrophoretic mobilities and sialic acid content as pooled milk (Table I).

To verify that the heterogeneity was not a result of the isolation procedure, lactoferrin was obtained containing all of the forms by DEAE-cellulose chromatography and gel filtration (Sephadex G-100) of whole human milk [15] that had not been subjected to the isoelectric precipitation of casein. Following dialysis of whole milk, a sample containing 100 ml was applied to DEAE column (5 by 50 cm) [28] at 0.001 M potassium

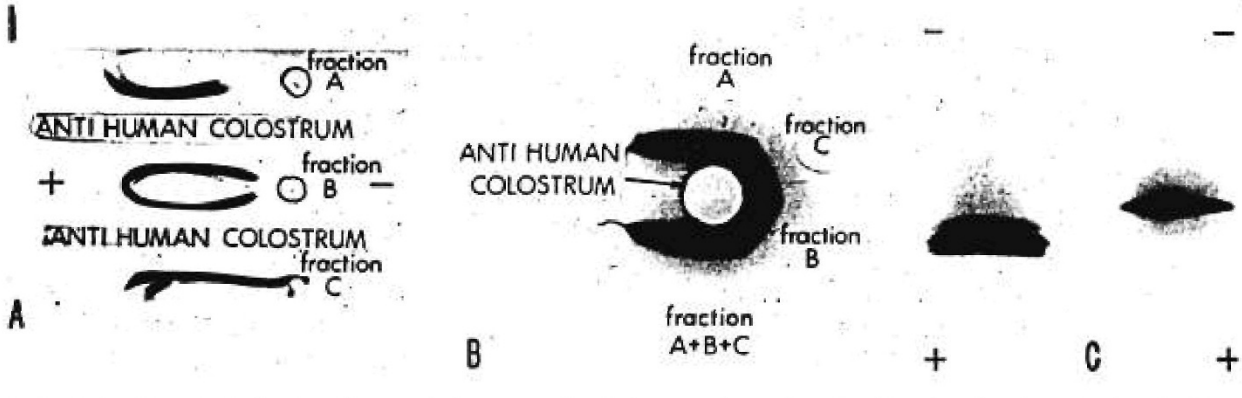

Fig. 1. Analysis of lactoferrin fractions from pooled human milk. *A:* Immunoelectrophoresis of the three fractions developed with goat antihuman colostral protein serum. *B:* Immunodiffusion of the three fractions (2.0 mg/ml protein solutions) showing antigenic identity. *C:* Polyacrylamide gel electrophoresis of lactoferrin *fraction B* (*left*) and *fraction B* after neuraminidase treatment (*right*). At least three proteins are observed in the native form. Following neuraminidase treatment only one protein band with a slower anodal mobility than any untreated protein was observed.

phosphate, 0.001 M EDTA, pH 7.5, at 4° and eluted with 0.05 M NaCl. The lactoferrin-containing fractions were further purified by gel filtration using Sephadex G-100 (*vide supra*). Aliquots of this lactoferrin were dialyzed at pH 4.6 and pH 8.0 for 48 hr. The sialic acid content of these two preparations were almost identical (Table I).

To ascertain whether iron content affected the heterogeneity, lactoferrin was dialyzed at pH 2.0 for 12 hr at 4° [3, 6]. The resulting iron-free preparation showed the same heterogeneity.

The asialo derivatives of the three lactoferrin fractions were prepared by incubating 0.5 mg of each fraction with 0.1 ml neuraminidase containing 50 units [29] in a dialysis sac with 0.01 M $CaCl_2$-0.05 M $NaCH_3O_2$, pH 5.2 [1], as the solvent and outer fluid for 17 hr at 37°. These derivatives yielded single bands of protein with identical mobility by polyacrylamide gel electrophoresis (Fig. 1*C*), slower than the slowest naturally occurring lactoferrin. Therefore, it may be concluded that all lactoferrin molecules contain at least one sialic acid residue and that there is no genetic polymorphism detectable by these techniques.

Lactoferrin fractions *A*, *B*, and *C* were isolated by this technique from the duodenal fluid of one human. Polyacrylamide gel electrophoresis, immunoelectrophoresis, and immunodiffusion studies revealed the same heterogeneity, relative quantities, and antigenic identity of the three fractions as milk lactoferrin.

Pooled and three individual saliva samples gave heterogeneous precipitation bands by immunoelectrophoresis with antilactoferrin and anticolostral antisera, identical with those observed in milk lactoferrin.

Discussion

Difference in sialic acid content characterizes the iron-binding proteins of many vertebrates [1, 25]. In the rabbit [1], milk and serum transferrins have identical protein moieties and are electrophoretically heterogeneous. Neuraminidase treatment yields an electrophoretically homogeneous protein. In the domestic fowl [25], the iron-binding proteins conalbumin and serum transferrin have three electrophoretic forms. Stepwise reduction of the mobilities of these three bands is obtained by neuraminidase. In humans, serum transferrin [14] is homogeneous but can be resolved into four electrophoretically slower components by neuraminidase treatment. In human cord serum, these slower bands are present, probably due to an immature enzyme mechanism in fetal liver for the stepwise addition of sialic acid to fetal transferrin. Human cerebrospinal fluid has two electrophoretic varieties corresponding to the slowest and fastest transferrin. Neuraminidase treatment of polymorphic variants of human transferrin provides evidence that sialic acid is not involved in this genetic variation. Direct measurement of sialic acid of these electrophoretic variants of iron-binding proteins, however, has not been previously reported. We have found lactoferrin to be similar to the iron-binding proteins previously studied in that differences in the heterogeneity can be explained by differences in sialic content only.

Human lactoferrin was first identified in 1960 [3, 5, 6, 13] and its physical and chemical properties were characterized. Lactoferrin is found in external secretions such as bile [16], saliva, semen, bronchial secretions, and milk, but not in human serum including the sera of lactating females [3]. There is no relation or identity among peptide sequences between human lactoferrin and transferrin [3, 6, 19]. Fluorescent antibody studies suggest that lactoferrin is synthesized in bronchial and salivary glands [11, 21]. In the rabbit [8], there is no transport of labeled serum transferrin to milk [7]. Other evidence suggests that lactoferrin may also be an intracellular constituent [12] of circulating leukocytes.

Sialic acid has widespread distribution in the animal organism [23] but its biologic function for proteins is unknown. It does not appear to be related to iron-binding by lactoferrin [3, 14, 16], or the resistance of lactoferrins to intestinal protease [19].

Since the carbohydrate moiety has been implicated in the transport of one type of glycoprotein (immunoglobulins) across the cell membrane [20], and since all lactoferrin contains at least one sialic acid residue, a possible role for sialic acid may be in the transport of this protein across the cell membrane. The occurrence of lactoferrin in heterogeneous form suggests the presence of an enzyme mechanism in human secretory glands for the stepwise addition of sialic acid to a protein molecule. Such an enzyme mechanism has been demonstrated in mammalian and human mammary tissue [2, 9]. Since sialic acid is a terminal residue of the carbohydrate chain of many glycoproteins, it may be necessary for extracellular transport of these proteins.

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