Showa Univ. J. Med. Sci. 9(1), 39~44, June 1997

**Original**

# Salivary Lactoferrin in Neonates with Chorioamnionitis

Katsufumi OTSUKI, Aki YODA, Kazuhiro HIROSE, Yukiko SHIMIZU, Hiroshi SAITO and Takumi YANAIHARA

**Abstract:** Lactoferrin, an iron-binding glycoprotein, has been found to have various biological functions in various tissues. Although, many studies have been reported on salivary lactoferrin in adults and children, little is known regarding lactoferrin in salivary fluid of neonates, especially under conditions of chorioamnionitis (CAM). Salivary lactoferrin concentrations in neonates gradually increased as the days advanced and when breast feeding started, the levels elevated significantly. Salivary lactoferrin levels in neonates measured on the day of delivery in the group delivered with chorioamnionitis were $3.4\pm0.5\ \mu g/ml$ and were significantly higher ($p<0.05$) than those without chorioamnionitis ($0.8\pm0.3\ \mu g/ml$). The changes in the lactoferrin concentrations of salivary fluids in neonates were demonstrated. Furthermore, the elevation of salivary lactoferrin concentrations in neonates in response to the amniotic infection suggested the novel function as a host defense against infections.

**Key words:** lactoferrin, neonate, salivary fluid, amniotic infection, chorioamnionitis

## Introduction

Lactoferrin, an 80 kDa iron-binding glycoprotein was first isolated from human milk[1-3] and other biological fluids, e.g., urine[3], saliva in adult[4] and amniotic fluid[5]. Lactoferrin consists of a single polypeptide chain and two iron-binding sites per molecule[6].

Many biological functions of lactoferrin have been reported, including bacteriostatic effect[7-11], iron absorption[12,13], lymphocyte growth-promoting effects, and product regulation of macrophages, granulocytes, and neutrophils[14,15]. Inhibition of lipid oxidation has also been reported[16,17]. The bacteriostatic effect of lactoferrin is one of the significant physiological functions in human milk. High concentration of lactoferrin in colostrum might be one factor that explains why breast-fed infants rarely suffer from intestinal infections. Therefore, it has been suggested that lactoferrin plays a possible role in the defense against local mucosal infections[18,19]. In addition, lactoferrin has been found in amniotic fluid[5] and suppresses the production of inflammatory cytokines, tumour necrosis factor alpha, and interleukin 6[20] both *in vivo* and *in vitro*.

Although several studies of salivary lactoferrin in adults[21,22] and in children have been reported[23], there is no information about salivary lactoferrin in neonates, especially those delivered under conditions of amniotic infection. Amniotic infection causes the degradation of chorioamnion with the invasion of granulocytes and intrauterine fetal infection. Therefore,

*Department of Obstetrics and Gynecology, Showa University School of Medicine,* 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan.

in the present study, lactoferrin concentrations were measured in the saliva of neonates who had been delivered with and without chorioamnionitis (CAM), and the possible role of salivary lactoferrin is discussed.

## Materials and Methods

*Subjects*

Subjects were 18 full-term neonates (9 boys and 9 girls) who had been delivered without obstetric complications. Saliva was collected daily and the subjects were observed for 4 to 5 days. Saliva was collected on the day of birth from neonates (4 boys and 3 girls) who had been delivered under conditions of CAM, which had been diagnosed pathologically[24]. There was no significant difference between two groups in gestational age at birth (normal; 39.5±1.1 weeks; CAM (+); 38.1±1.7 weeks) or body weight (normal; 3,118±319 g; CAM (+); 2,955±377 g). No neonates had complications after birth. The children were fed 5% sugar water until breast feeding was started, and physiological weight losses were less than 7%.

The purpose of the study was explained and informed consent was obtained from all parents.

*Sampling*

To minimize possible effects of the circadian sleep-awake cycle and feeding on lactoferrin levels, the first saliva sampling of neonates was performed between 19:00 and 20:00 on the day of birth. Saliva sampling was performed every day until breast feeding started.

A dental cotton roll (1 cm in diameter, 3 cm in length) was used to collect saliva from the infants according to the method previously reported[25]. Saliva sampling was continued for about 5 to 10 minutes, and if necessary, unsweetened lemon crystals were given to obtain sufficient amount of saliva[26]. Saliva was kept frozen at −80°C until assayed.

*Chemicals*

Anti-human lactoferrin IgG from sheep and anti-human lactoferrin peroxidase conjugate from sheep were obtained from The Binding Site. (Birmingham, England). Lactoferrin from human milk was purchased from Sigma, Co. (St. Louis, Mo, USA).

All other reagents were of analytical grade.

*Lactoferrin measurement*

Human lactoferrin in saliva was measured with enzyme-linked immuno-sorbent assay (ELISA) of the sandwich type modified with the method reported by Camile and Irwin[27]. The method is illustrated in Fig. 1.

Polyvinyl microplates (Immuno Plate MaxiSorp F96, Nunc, Roskilde, Denmark) were coated with anti-human lactoferrin IgG from sheep. The coated plates were left for 2 hours at 37°C in a moisture chamber before use. To prevent non specific binding, gelatin was added to the plates. Aliquots of saliva samples were diluted 100 to 500 times with phosphate buffered saline. Incubation was performed for 1 hour at 37°C. Plate was washed and purified anti-human lactoferrin peroxidase conjugate from sheep was added and successively incubated for 1 hour. Plates were washed and left for 20 minutes at room temperature. The intensity of the green color was measured with an ELISA Reader (International Reagents Corporation, Tokyo, Japan). Assays were performed in duplicate. Inter and intra coefficients of variation were 4.7 and 7.0%, respectively. The acceptable assay range lies

Abbreviations: CAM: chorioamnionitis, ELISA: enzyme-linked immuno-sorbent assay.

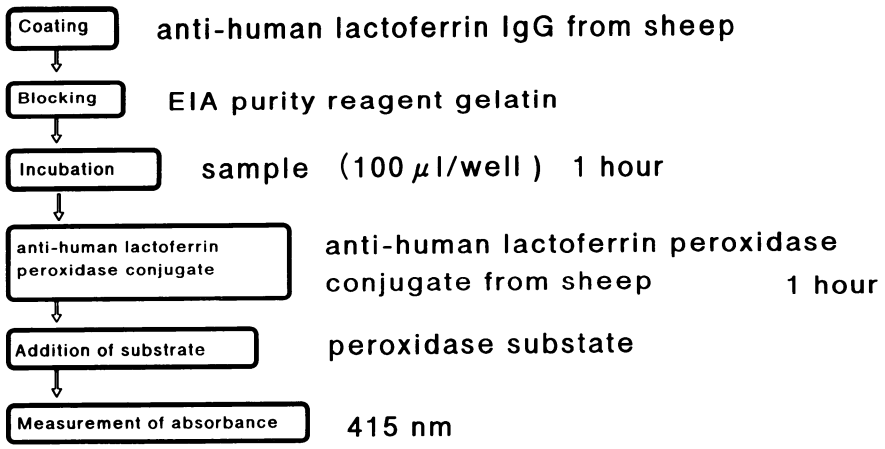


Fig. 1. Measurement of salivary lactoferrin by ELISA (enzyme-linked immuno-sorbent assay) of the sandwich type, a modification of the method reported by Camille and Irwin[27].

Table 1. Changes of salivary lactoferrin levels in neonates after birth.

| Days after birth | No. of samples | Lactoferrin (μg/ml) |
|---|---|---|
| 0 | 18 | 0.8±0.3 |
| 1 | 18 | 2.5±3.2[a] |
| 2, 3 | 11 | 4.1±4.1[a] |
| 4, 5 | 7 | 5.7±1.8[a] |
| breast feeding | 18 | 34.1±10.1[b] |

a; $p<0.05$ v.s. day 0, b; $p<0.01$ v.s. 1 day before.

between 5 and 250 ng/ml.

*Statistics*

The data are presented as mean±S.D. Statistical analysis was performed by means of unpaired Student's *t*-test. In all analyses, $p<0.05$ was considered to indicate statistical significance.

## Results

Changes in salivary lactoferrin concentrations in neonates after birth are shown in Table 1 and Figure 2. Lactoferrin levels in salivary fluid on the first day were 0.8±0.3 μg/ml, and the concentrations increased significantly on the 1st day of life and gradually increased as days passed. After breast feeding started, salivary lactoferrin levels in all neonates increased significantly, to 34.1±10.1 μg/ml ($p<0.01$). The salivary lactoferrin levels of neonates on the day of birth in the CAM (+) group were 3.4±0.5 μg/ml and were significantly higher ($p<0.05$) than those in normal neonates (0.8±0.3 μg/ml) (Fig. 3).

## Discussion

Salivary lactoferrin levels in neonates were demonstrated. Salivary lactoferrin levels in

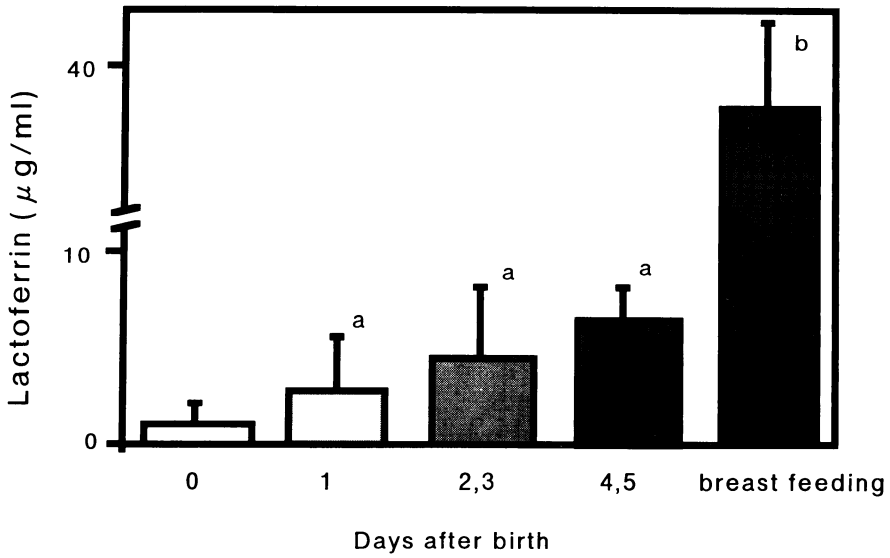


Fig. 2. Changes of salivary lactoferrin levels in neonates after birth. a; $p<0.05$ v.s. day 0, b; $p<0.01$ v.s. 1 day before.

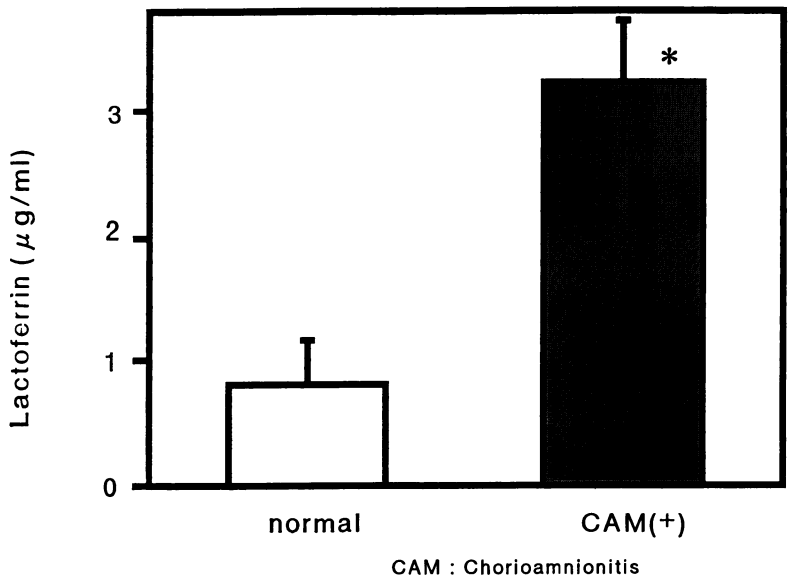


Fig. 3. Lactoferrin levels in salivary fluids with and without chorioamnionitis. CAM(⁻); n=18. CAM(⁺); n=7. Values are expressed as mean±S.D., *; $p<0.01$.

neonates gradually increased with the days progressed and when breast feeding was started extremely high levels of lactoferrin was observed. Salivary lactoferrin levels in neonates were similar to the levels reported previously in children and adults[22,23,27,28]. The salivary concentrations of lactoferrin in adults were reported to be 2–50 μg/ml[28]. On the other hand, Tenovuo et al.[22] and Lehtonen et al.[23] reported that the concentrations of salivary

lactoferrin measured by ELISA in children aged 0.8 to 3.8 years were 3.0±1.6 μg/ml and that in adults were 8.5±4.0 μg/ml. Takemori[29] reported that salivary lactoferrin levels in adults were 4.03±1.48 μg/ml. The lactoferrin concentrations in human milk showed several mg/ml. From our findings, the lactoferrin levels of salivary fluids in neonates were significantly ($p<0.001$) increased after the breast feeding started and the levels reached over the ten times higher than that collected before the breast feeding. To minimize possible effects of feeding on lactoferrin levels, all saliva samplings were performed at least 30 minutes prior to breast feeding in the present study. However, the contamination of the milk in these breast fed infants in saliva can not be completely ruled out.

Recently, the relationship between amniotic infection and preterm labor is well documented. Among several biological functions of lactoferrin proposed, the bacteriostatic effect of lactoferrin may be the most significant one. Chimura et al.[30] reported the decreased of lactoferrin levels in cervical mucous in case of chorioamnionitis. However, there is no report in regard to the lactoferrin levels in salivary fluid collected from the infant delivered with CAM. Therefore, in the present study, the lactoferrin concentrations in salivary fluids with CAM were measured. Significant high levels of salivary lactoferrin was observed.

Anti-microbial factors including lactoferrin in whole saliva of children were analyzed by Tenovuo et al.[22], and they reported that the lactoferrin concentrations in children prone to recurrent respiratory infections were compatible to those of healthy children[23]. Since lactoferrin inhibits the growth of bacteria and fungi *in vitro,* lactoferrin may be a possible contributor to defense against local mucosal infections[18,19]. Therefore we presumed that salivary lactoferrin in neonates might act as a local protection factor against infections. Most recently, we have measured the levels of lactoferrin in amniotic fluid in normal pregnancy, and the levels were compared with those complicated with infection. High lactoferrin concentrations in amniotic fluid were associated with the infection in this preliminary study (unpublished data). It has been proposed that lactoferrin might be involved in the protection of feto-placental allograft from immune rejection. At present, however, the physiological functions of salivary lactoferrin are not fully understood. Our results showing the increase of lactoferrin levels in salivary fluids with CAM, suggest that salivary lactoferrin in neonates may have some defense mechanisms against the intrauterine bacterial infections as a host defense against local infections.

**References**


1) Groves ML: The isolation of a red protein from milk. *J Am Chem Soc,* **82**: 3345–3350 (1960)
2) Masson PL and Heremans JF: Lactoferrin in milk from different species. *Comp Biochem Physiol,* **39B**: 119–129 (1971)
3) Nagasawa T, Kiyosawa I, Kuwahara K, Fukuwatari Y and Suzuki T: Changes of disc gel electrophoretic patterns of human milk protein with duration and temperature of storage of the milk, *J Dairy Sci,* **55**: 289–293 (1972)
4) Reitano S, Konttinen YT and Segerberg-Konttinen M: Distribution of lactoferroin in human salivary glands. *Histochemistry,* **66**: 285–291 (1980)
5) Niemela A, Kulomaa M, Vilja P, Tuohimaa P and Saarikoski S: Lactoferrin in human amniotic fluid. *Hum Reprod,* **4**: 99–101 (1989)
6) Crichton RR: Chemistry and biology of the transferrins. In: Inorganic Biochemistry of Iron Metabolism, Crichton RR (Ed), Ellis Horwood, New York, pp 101–119 (1991)
7) Arnold RR, Brewer M and Gauthier JJ: Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. *Infect Immun,* **28**: 893–898 (1980)
8) Staut J, Norell S and Harrington JP: Kinetic effect of human lactoferrin on the growth of *Escherichia*

*coli* 0111. *Int J Biochem,* **16**: 1043–1047 (1984)
9) Ellison RT III, Giehl TJ and Laforce MF: Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. *Infect Immun,* **56**: 2774–2781 (1988)
10) Suzuki T, Yamauchi K, Kawase K, Kiyosawa I and Okonogi S: Collaborative bacteriostatic activity of bovine lactoferrin with lysozome against *Escherichia coli* O111. *Agric Biol Chem,* **53**: 1705–1706 (1989)
11) Visca P, Dalmastri C, Verzili D, Antonini G, Chianocone E and Valenti P: Interaction of lactoferrin with *Escherichia coli* cells and correlation with anti bacterial activity. *Med Microbiol Immunol,* **179**: 323–333 (1990)
12) Cox TT, Mazurier J, Spik G, Montreuil J and Peters TJ: Iron binding proteins and influx of iron across the duodenal brush border. Evidence for specific lactotransferrin receptors in the human intestine. *Biochim Biophys Acta* ,**588**: 120–128 (1979)
13) Lonnerdal B: Iron in human milk and cow's milk—effects of binding ligands on bioavailability. In: Iron Metabolism in Infants, Lonnerdal B (Ed). CRC Pres, Boca Rato, FL, pp 87–107 (1990)
14) Broxmeyer H, Gentile P, Cooper S, Lu L, Juliano L, Piacibello W and Meyers LL: Functional activities of acidic isoferritins nad lactoferrin in vitro and vivo. *Blood Cels,* **10**: 397–426 (1984)
15) Birgens HS: The interaction of lactoferrin with human monocytes. *Dan Med Bull,* **38**: 244–252 (1991)
16) Gutteridge JMC, Paterson SK, Segal AW and Halliwell B: Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. *Biochem J,* **199**: 259–261 (1981)
17) Allen JC and Wrieden WL: Influence of milk proteins on lipid oxidation in aqueous emulsion. *J Dairy Res,* **49**: 240–262 (1982)
18) Rudney JD: Relationships between human parotid saliva lysozyme lactoferrin, salivary peroxidase and secretory immunoglobulin A in a large sample population. *Arch Oral Biol,* **34**: 499–506 (1989)
19) Cleveland MG, Bakos MA, Hilton SM and Goldblum RH: Amniotic fluid: the first feeding of mucosal immune factors. *Adv Exp Med Biol,* **310**: 41–49 (1991)
20) Machnicki M, Zimecki M and Zagulski T: Lactoferrin regulates the release of tumour necrosis factor alpha and interleukin 6 in vivo. *Int J Exp Pathol,* **74**: 433–439 (1993)
21) Rudney JD, Krig MA and Nuevar EK: Longitudial study of relations between human salivary antimicrobial proteins and measures of dental plaque accumulation and composition. *Archs Oral Biol,* **38**: 377–386 (1993)
22) Tenovuo J, Olli-Pekka JL, Anttis A, Pekka V and Pentti T: Antimicrobial factors in whole saliva of human infants. *Infect Immun* **51**: 49–53 (1986)
23) Lehtonen OPJ, Tenovuo J, Aaltonen AS and Vilja P: Immunoglobulins and innate factors of immunity in saliva of children prone to respiratory infections. *Acta Pathol Microbiol Immunol Scand Sect C Immunol,* **95**: 35–40 (1987)
24) Blanc WA: Pathology of the placenta, membranes, and umbilical cord in bacterial, fungal, and viral infections in man. In: Perinatal Diseases: International Academy of Pathology Monograph, Vol. 22, Blanc WA (Ed), Williams & Wilkins, Baltimore, pp 67–132 (1981)
25) Gunnar MR, Connors J and Isensee J: Lack of stability in neonatal adrenocortical reactivity because of rapid habituration of the adrenocortical response. *Dev Psychobiol,* **22**: 221–233 (1989)
26) Francis SJ, Walker RF, Rlad-Fahmy D, Hughes D, Murphy JF and Gray OP: Assessment of adrenocortical activity in term neonates using salivary cortisol determinations. *J Pediatr,* **1**: 129–133 (1987)
27) Dipaola C and Mandel ID: Lactoferrin concentration in human parotid saliva as measured by enzyme-linked immunosorbent assay (ELISA). *J Dent Res,* **59**: 1463–1465 (1980)
28) Benini L, Harvey RF, Piubello W, Lucchin L, Brocco G and Benini P: Lactoferrin concentration in the parotid saliva of patients with chronic pancreatitis. *Digestion,* **28**: 77–81 (1983)
29) Takemori Y: Studies on clinical application of the measurement of salivary lactoferrin levels and its significance. 1. Evaluation of parotid salivary lactoferrin in various diseases. *J Juzen Med Soc,* **97**: 789–801 (1988)
30) Chimura T, Hirayama T and Takase M: Lactoferrin in cervical mucos of patients with chorioamnionitis. *Jpn J Antibiot,* **46**: 318–322 (1993)

[Received February 3, 1997: Accepted February 17, 1997]