0031-3998/90/2705-0525$02.00/0
PEDIATRIC RESEARCH

Vol. 27, No. 5, 1990
Printed in U.S.A.

Iron Is Not Required in the Lactoferrin Stimulation of Thymidine Incorporation into the DNA of Rat Crypt Enterocytes

BUFORD L. NICHOLS, KATHRYN S. McKEE, AND HELMUT A. HUEBERS

USDA/ARS Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine and Texas Children's Hospital, Houston, Texas 77030

ABSTRACT. Lactoferrin has been identified as a factor in human colostrum that accounts for increased incorporation of thymidine into the DNA in an *in vitro* rat crypt enterocyte bioassay. We have examined lacatoferrin-stimulated thymidine incorporation by comparing the effects of iron-free lactoferrin (apolactoferrin) with those of iron-saturated lactoferrin (diferric lactoferrin) under conditions that inhibit the transfer of iron between these iron-binding proteins in the bioassay system. In addition, we have compared the dose-response relationships of diferric lactoferrin and apolactoferrin. The results demonstrated that lactoferrin, independent of iron-binding states, promoted the incorporation of thymidine into the DNA of rat crypt enterocytes. These observations suggest a previously unreported nutritional role for lactoferrin that is independent of its iron-binding capacity. (*Pediatr Res* 27: 525–528, 1990)

Abbreviations

DFO, desferrioxamine
ATF, apotransferrin
HLF, human lactoferrin
ALF, apolactoferrin
DLF, diferric lactoferrin
FPLC, fast protein liquid chromatography

Lactoferrin is a glycoprotein of 78 000 D which binds 2 mol of iron (1). Milk lactoferrin is believed to function in the gastrointestinal tract of newborn mammals by facilitating the transport of iron from milk and by suppressing the growth of enteric bacteria that require exogenous iron (2). Recent observations that human lactoferrin stimulates thymidine incorporation into rat crypt enterocytes (3, 4) suggest that lactoferrin may have a nutritional function in addition to its gastrointestinal function. Lactoferrin has been reported by various authors to play a role in the modulation of the immune system and to stimulate cell division (5–15) (Table 1). Although the function of lactoferrin in the immune system is independent of the presence of iron in regard to lymphocyte adherence (7), mast cell degranulation (9), and the inhibition of granulocyte peroxidation of membranes, studies of immortalized cell lines indicate that iron is a requirement for the lactoferrin trophic effect (15). Investigations in other cell lines contradict this finding and have failed to clarify whether the trophic effects of lactoferrin are dependent on the presence of iron (16).

Our studies were conducted to determine whether the stimulation of thymidine incorporation into the DNA of crypt enterocytes is dependent on the presence or absence of iron in lactoferrin.

Received July 28, 1989; accepted January 11, 1990.

Correspondence and reprint requests: Buford L. Nichols, Jr., M.D., Children's Nutrition Research Center, 1100 Bates Street, Houston, TX 77030.

Publication of the USDA/ARS Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine and Texas Children's Hospital, Houston, TX. Funded in part with federal funds from the U.S. Department of Agriculture, Agricultural Research Service under Cooperative Agreement number 58-7MN1-6-100. The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

MATERIALS AND METHODS

Animals. Male Sprague Dawley rats (Harlan Industries, Indianapolis, IN) were housed under standard conditions. They received rat food and water *ad libitum*. The eight animals used in this experiment weighed 418 ± 24 g.

Isotopes and chemicals. Radioiron (^{59}Fe) was purchased from Dupont de Nemours & Co., Inc. (Hoffman Estates, IL) as ferrous sulfate, 13–22 μCi/μg of iron, dissolved in 0.5 M HCl (1 Ci = 3.7×10^{10}Bq). Tritiated thymidine (methyl-^{3}H, 60–90 Ci/mm) was obtained from ICN Radiochemicals (Irvine, CA). Ferrous ammonium sulfate, $(NH_4)_2\,Fe(SO_4)_2 \times 6\,H_2O$, ammonium sulfate, $(NH_4)_2SO_4$, DFO, sodium chloride, sodium bicarbonate, monobasic sodium phosphate, NaH_2PO_4, human ATF (purity ≥98%), human diferric transferrin (purity ≥98%) and Tris-buffer were obtained from Sigma Chemical Co. (St. Louis, MO). Chelex-100 was a product of BioRad Laboratories (Richmond, CA). FCS and Trowell's T-8 medium were purchased from Gibco Laboratories (Chagrin Falls, OH). DE and CM Sepharose fast-flow iron exchange resins were products of Pharmacia (Piscataway, NJ).

Preparative isolation of HLF. Lactoferrin was isolated from human milk after sufficient amounts of ferrous sulfate were added to saturate the specific sites of the protein. Thawed human milk (950 mL) was centrifuged in four 230-mL plastic centrifuge bottles at 10 000 × *g* for 90 min at 4°C in a J2-21M centrifuge equipped with a JA-14 fixed-angle rotor (Beckman Instrument, Inc., Palo Alto, CA). The skim milk or colostrum was separated from the top fat cake by decanting and filtering through a double layer of a grade 373 (qualitative, fluted) filter (Baxter Scientific Products, McGaw Park, IL) and a Whatman no. 1 filter (Whatman International Ltd., Maidstone, UK). Thirty mL of sodium bicarbonate (1 M, pH 9.1) were added to the filtrate (750 mL), then 20 μL of $^{59}Fe^{2+}$ solution (200 μCi-mL). Thereafter, 3 mL ferrous sulfate solution (2 mg Fe^{2+}/0.01 N HCl mL) were added slowly as the mixture was stirred at room temperature. The sample was incubated at 37°C for 15 min, then diluted with an equal volume of distilled water. Three 1-mL aliquots were withdrawn and counted to determine the initial total radioactivity, which was normalized to 100%.

The sample of skim milk was loaded onto a CM-Sepharose

fast flow column (2.6 cm × 90 cm; equilibrated with 0.05 M Tris-HCl buffer, containing 1 M NaCl, pH 8.3) at a flow rate of 4.5 mL/min using a peristaltic pump P-3 (Pharmacia, Uppsala, Sweden). After the sample was loaded, the column was eluted with a linear salt gradient (0 to 1 M NaCl in 0.05 M Tris-HCl buffer; total volume, 1250 mL) at a flow rate of 1.0 mL/min. The UV absorption was recorded at $\lambda = 254$ nm and $\lambda = 280$ nm throughout the run using a dual path monitor UV-2 (Pharmacia, Uppsala, Sweden). One-mL aliquots were withdrawn from each of the 100 fractions (fraction volume, 12.5 mL) collected; ^{59}Fe-radioactivity was counted in a gamma counter; and samples were separated by FPLC using a Mono S column (Pharmacia).

The ^{59}Fe-radioactivity-containing peak fractions eluted with the sale gradient were combined and concentrated to 50 mL. Thereafter, they were dialyzed against 4 vol of 0.05 M Tris-HCl buffer (pH 8.3) using a stirred ultrafiltration cell model 8400 equipped with a YM-10 membrane (Amicon, Danvers, MA). The dialysate was then applied at 4.5 mL/min to an anion exchange, fast flow DEAE-Sepharose column (bed volume 2.6 cm × 90 cm) that had been equilibrated previously with 0.05 M Tris-HCl buffer containing 0.4 M NaCl (pH 8.3). Thereafter, the column was eluted with a linear salt gradient (0.0 to 0.1 M NaCl) in 0.05 M Tris-HCl buffer (pH 8.3) at a flow rate of 1.0 mL/min. The UV absorption was recorded continuously throughout the run, and the ^{59}Fe-radioactivity was counted in 1-mL aliquots, as described above.

Preparation of ALF and DLF. Complete saturation of the isolated lactoferrin with iron was confirmed by an E465/E280 ratio of 0.045 (15). Treatment with 1 mg/mL DFO in 0.1 M phosphate buffer of pH 4.12 at 37°C for 4 h permitted complete iron release from lactoferrin to DFO. The iron-free form of lactoferrin, ALF, was isolated after removal of the ferrioxamine-iron by extensive diafiltration against 1 M phosphate buffer (pH 4.1) in an Amicon diafiltration cell, using iron-free reagents prepared by treatment with Chelex 100 (17). The biochemical integrity of the ALF (Fig. 2) was verified by a spectrophotometric titration technique (18). An increase in color development was observed at $\lambda = 465$ nm in a Cary 2690 spectrometer (Varian, Sunnyvale, CA). At the point of saturation. The E465/E280 ratio was determined. Ferrous ammonium sulfate (273.4 μg iron/mL, pH 2.0) was used in these saturation studies. This spectrophotometric titration method also was used to verify the biochemical integrity of the commercial ATF.

Prevention of iron mobility in bioassay. Crypt cells were isolated as reported previously (3) using high-frequency, low-amplitude vibration of gut segments everted over rods and suspended in buffer at 37°C. The crypt cell fraction was washed and the cells were incubated, as described (3), in the presence of ^{3}H-thymidine. After incubation, the cells were deproteinized and DNA assayed fluorometrically. Radioactivity in the DNA was determined by spotting on glass microfiber strips followed by trichloroacetic acid and alcohol washes as reported (3). Results were expressed as cpm/μg DNA as a percent of control (at least 10% of the incubation tubes were controls).

DFO (10 μg/mL) was added to the incubation medium (T8 buffer containing 10% FCS) to prevent iron loading of the ALF from any contaminating iron in the medium during *in vitro* incubation with rat crypt cells. The *in vitro* exchange of iron was studied by analyzing the radioactivity of ^{59}Fe-binding components before and after a 1-h incubation with rat crypt cells at pH 7.4. The protein components were isolated after the assay on a mono S column by FPLC at pH 8.3. In the first experiment, 200 μg/mL ^{59}Fe diferric transferrin and 200 μg/mL ALF were added in the presence of 10 μg/mL DFO. In a second experiment, 200 μg/mL ATF and 200 μg/mL ^{59}Fe-DLF were incubated in the presence of 10 μg/mL DFO. The radioactivity of the isolated iron-binding factors was analyzed in a gamma counter (Minaxi 5000 series, Packard Instrument Co., Inc., Downers Grove, IL) before and after a 1-h assay procedure to determine if radioactivity was transported from diferric to apo forms of transferrin or lactoferrin. ATF was also added to other assays as an internal standard and quality control reagent.

We conducted a series of experiments to test the relative assay potency of lactoferrin in its apo and diferric forms in the presence of 10 μg/mL DFO and 200 μg/mL of ATF. The effect of DFO and ATF in basal and lactoferrin-supplemented thymidine uptake into DNA by incubated rat crypt cells was evaluated. The response to fresh ALF and DLF was then compared in the assay at 200 μg/mL and at lower concentrations.

Statistical procedures. *T* tests with Bonferroni's adjustment for multiple comparisons and analysis of variance were used. Dunnett's procedure for comparing multiple treatments with a control was also used. Regression analyses were performed.

RESULTS

Purity and biological integrity of lactoferrin. The purity of the HLF preparation in its iron-loaded form was documented by an E465/280 ratio of 0.045 (16) and an E465/412 ratio of 1.3. After dialysis at pH 4.2 for 4 h at 37°C, all the characteristic pink color of the DLF disappeared. No iron could be detected in this preparation by the bathophenantholine method (19). A spectrophotometric titration study of the ALF preparation conducted at pH 8.3 and monitored at 465 nm showed a linear increase until saturation was indicated by a plateau (Fig. 1). At the point of saturation, the E465/280 ratio was 0.045 (the same value determined for the diferric preparation before the cycle of iron removal and resaturation), which documented the integrity of the iron-binding sites of the ALF and ATF preparations. The

Table 1. *The immune functions and trophic effects of lactoferrin*

	APO active	Reference
Immune functions of lactoferrin		
Inhibits complement deposits	?	5
Promotes polymorphonuclear cells adhesiveness	?	6
Lymphocyte adherence	+	7
Lymphocyte activation	?	8
Mast cell degranulation	+	9
Peroxidation of membranes		10
Peroxidation of membranes	+	11
Suppressed myelopoiesis		12
Suppression of IgM production	?	13
Trophic effects of lactoferrin		
Adenocarcinoma (HT 29)	?	14
B cell lines (human)		15
B cell lines (mouse)	?	15
T cell lines (human)	?	15
Enterocytes	?	3

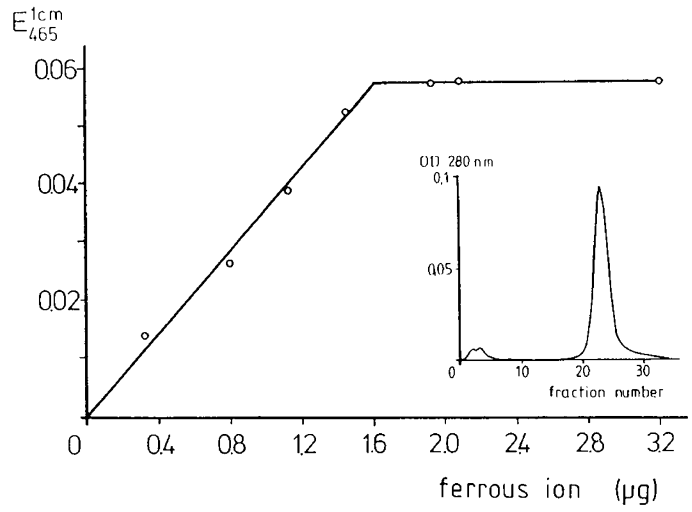

Fig. 1. Spectrophotometric behavior of human ALF. ALF (1.15 mg/mL) was dissolved in 0.05 M Tris/HCl buffer (pH 8.3) that contained 0.01 M sodium-bicarbonate. Iron was added as ferrous ammonium sulfate (10 μg/mL, pH 2.0) and the color development of λ = 465 nm was measured in a Cary 2390 double beam spectrophotometer. *Inset:* purity control of the iron-saturated HLF preparation on a mono S column by FPLC at pH 8.3 (see text). Elution was conducted with a 0.5 to 1.0 M linear salt gradient in 0.05 M Tris/HCl buffer. A total of 34 1-mL fractions was collected. The UV absorption was recorded at λ = 280 nm.

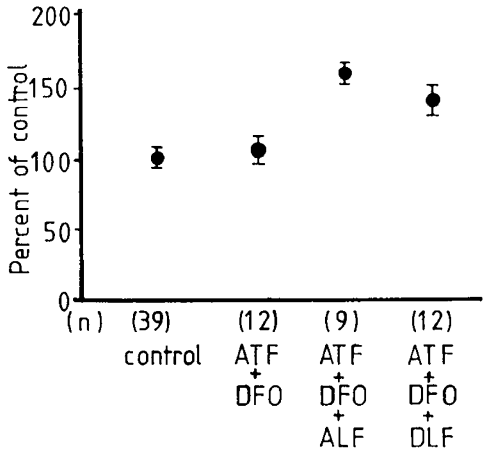

Fig. 2. Thymidine incorporation into rat crypt cells as affected by lactoferrin in its apo- or diferric form. In the control, the incorporation was normalized to 100%. ATF, 200 μg/mL; DFO, 10 μg/mL; ALF, 200 μg/mL; DLF, 200 μg/mL. These are results from eight experiments. *T* tests with Bonferroni's adjustment for multiple comparisons were used. Control and ATF + DFO were not significantly different. All other comparisons were significantly different from control and ATF + DFO ($p < 0.0001$).

purity of fresh human DLF was documented further by the results of FPLC on a mono S column (see *inset* in Fig. 1). The addition of human ATF to the test system in a concentration of 200 μg/mL had no effect on basal thymidine incorporation (Fig. 2).

Prevention of iron exchange in bioassay. The addition of the specific iron chelator DFO to the culture system in concentrations as high as 200 μg/mL did not interfere with the basal incorporation of thymidine into the crypt cells. However, a concentration of DFO in the medium as low as 10 μg/mL (Fig. 2) effectively blocked iron loading of the ALF from added ^{59}Fe transferrin during the 1-h incubation (Fig. 3, *left panel*). DFO at this concentration, however, did not remove iron from a ^{59}Fe-

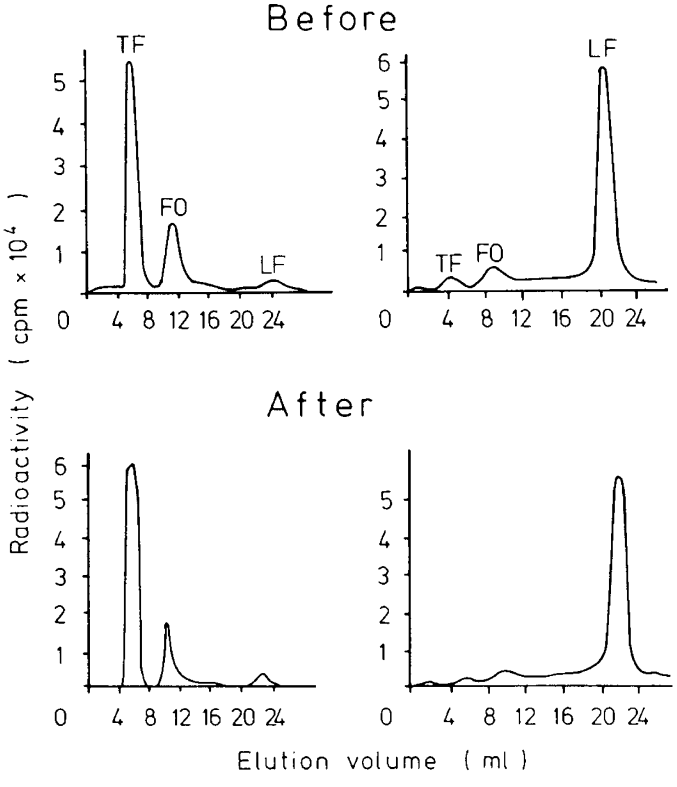

Fig. 3. Profile of ^{59}Fe-binding components before and after a 1-h incubation with rat intestinal crypt cells at 37°C. ^{59}Fe-human transferrin (*left*) or ^{59}Fe-HLF (*right*) was added to T8-FCS medium and separation was performed on a mono S column by FPLC at pH 8.3 (see text). The radioactivity of the separated iron binding fractions is indicated. TF, transferrin; FO, ferrioxamine; LF, lactoferrin.

tagged diferric preparation of lactoferrin, nor did it mediate transfer of radioiron to the ATF (Fig. 3, *right panel*).

Response to DLF. The results of experiments quantitating the effect of HLF on thymidine incorporation into rat crypt cells, with the addition of DFO and human ATF, are summarized in Figure 2. The addition of lactoferrin stimulated thymidine incorporation by approximately 50% and the stimulation was not blocked by the presence of the specific iron chelators, DFO, or human ATF. Human ALF and DLF supplemented at a concentration of 200 μg/mL were active in the bioassay. Nevertheless, we observed slightly lower, but statistically significant, mean values ($p < 0.0001$) for the diferric preparation (Fig. 2). In an effort to examine possible differences between the ALF and DLF preparations in greater detail, we studied the relationship between their concentrations and thymidine incorporation. As illustrated in Figure 4, no difference was detected in the thymidine uptake response between the two iron-loaded forms of HLF under conditions that precluded exchange of iron between the iron binders in the medium. We found significant thymidine incorporation into rat crypt cells with a lactoferrin concentration as low as 50 μg/mL.

DISCUSSION

After the discovery of lactoferrin in human milk in 1960, a multitude of functions was proposed for lactoferrin (5–15). Lactoferrin in the gastrointestinal tract of the newborn is believed to facilitate the transport of iron in milk and to suppress the growth of bacterial species that require exogenous iron. The batericidal activity of lactoferrin is almost completely eliminated by iron saturation of the protein (16). The presence of iron affects the activity of the many immunologic functions of lactoferrin (5–15).

Lactoferrin is a trophic factor in human colostrum that stim-

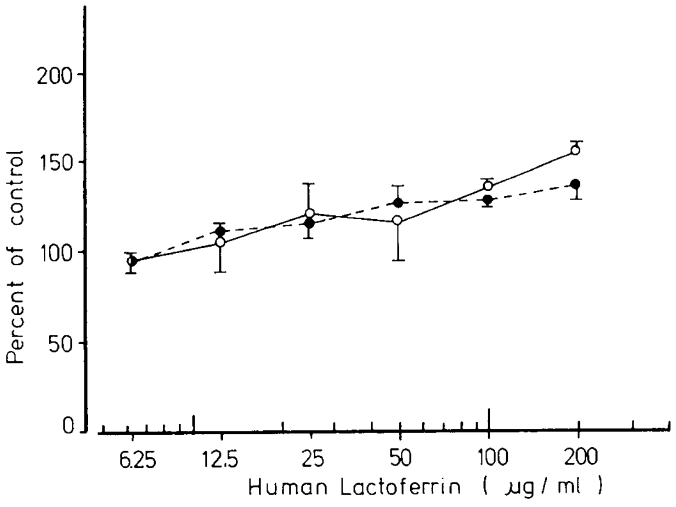

Fig. 4. Dose relationship of the thymidine incorporation into rat crypt cell DNA as stimulated by DLF (●) and ALF (○) in a representative experiment (control, $n = 6$; all others, $n = 3$). Using t test with Bonferroni's correction for multiple comparisons, no significant differences were found at any given dose. The separate regression analyses for ALF and DLF on cpm/µg DNA yielded slopes (β_1) of 0.270 and 0.163, respectively. Neither the slopes ($p = 0.071$) nor the intercepts ($p > 0.50$) were significantly different.

ulates thymidine incorporation into DNA in a rat crypt enterocyte assay (3 4), but the dependence of this stimulation on the presence of iron in lactoferrin has never been tested. The results of our study indicate that both DLF and ALF stimulate thymidine incorporation into crypt cell DNA without exchange of iron in the assay system. The assays were done in the presence of human ATF and DFO in the assay medium, which also blocked measurable iron flow.

The results, therefore, indicate that lactoferrin stimulation of thymidine incorporation into DNA of rat crypt enterocytes is not dependent on the presence of bound iron in HLF. We suggest that the effect of lactoferrin in the crypt cell bioassay is part of a broader functional role for ALF, which is by far the most prevalent form of lactoferrin in human milk and colostrum (20).

Acknowledgments. The authors thank E. O. Smith, Ph.D., for statistical advice, J.D. Eastman for editorial assistance, and M. E. Lewis for preparation of the manuscript.

REFERENCES

1. Baker EN, Rumball SV, Anderson BF 1987 Transferrins: insights into structure and function from studies on lactoferrin. Trends Biochem Sci 12:350–353
2. Payne SM 1988 Iron and virulence in the family enterobacteriaceae. CRC Crit Rev Microbiol 16:81–111
3. Nichols BL, McKee KS, Henry JF, Putman M 1987 Human lactoferrin stimulates thymidine incorporation into DNA of rat crypt cells. Pediatr Res 21:563–567
4. Nichols BL, McKee KS, Henry JF, Nichols VN 1989 Human lactoferrin supplementation of infant formulas increases thymidine incorporation into DNA of rat crypt cells. J Pediatr Gastroenterol Nutr 8:102–109
5. Kulics J, Kijlstra A 1987 The effect of lactoferrin on complement mediated modulation of immune complex size. Immunol Lett 14:349–353
6. Oseas R, Yang H-H, Baehner RL, Boxer LA 1981 Lactoferrin: a promoter of polymorphonuclear leukocyte adhesiveness. Blood 57:939–945
7. Nishiya K, Hobwitz DA 1982 Contrasting effects of lactoferrin on human lymphocyte and monocyte natural killer activity and antibody-dependent cell-mediated cytotoxicity. J Immunol 129:2519–2523
8. Slater K, Fletcher J 1987 Lactoferrin derived from neutrophils inhibits the mixed lymphocytes reaction. Blood 69:1328–1333
9. Theobald K, Gross-Weege W, Keymling J, Konig W 1987 Inhibition of histamine release *in vitro* by a blocking factor from human serum: comparison with the iron binding proteins transferrin and lactoferrin. Agents Actions 20:10–16
10. Ambruso DR, Johnston RB 1981 Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions, and an enzymatic generating system. J Clin Invest 67:352–360
11. Gutteridge JMC, Paterson SK, Segal AW, Halliwell B 1981 Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. Biochem J 199:259–261
12. Gentile P, Broxmeyer HE 1983 Suppression of mouse myelopoiesis by administration of human lactoferrin *in vivo* and the comparative action of human transferrin. Blood 982–993
13. Duncan RL, McArthur WP 1981 Lactoferrin-mediated modulation of mononuclear cell activities I. Suppression of the murine *in vitro* primary antibody response. Cell Immunol 63:308–320
14. Amouric M, Marvaldi J, Pichon J, Bellot F, Figarella C 1984 Effect of lactoferrin on the growth of a human colon adenocarcinoma cell line—comparison with transferrin. In Vitro 20:543–548
15. Hashizume S, Kuroda K, Murakami H 1987 Cell culture assay of biological activity of lactoferrin and transferrin. Methods Enzymol 147:302–314
16. Ellison III RT, Giehl TJ, LaForce FM 1988 Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. Infect Immun 56:2774–2781
17. Greenberg RR, Kingston HM 1983 Trace element analysis of natural water samples by neutron activation analysis with chelating resin. Anal Chem 55:1160–1165
18. Bates GW, Schlabach MR 1973 The reaction of ferric salts with transferrin. J Biol Chem 248:3228–3232
19. International Committee for Standardization in Haematology 1978 Recommendation for measurement of serum iron in human blood. Br J Haemotol 38:291–294
20. Brines RD, Brock JH 1983 The effect of trypsin and chymotrypsin on the *in vitro* antimicrobial and iron-binding properties of lactoferrin in human milk and bovine colostrum. Biochim Biophys Acta 759:229–235