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Human Lactoferrin Stimulates Thymidine Incorporation into DNA of Rat Crypt Cells

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ABSTRACT. In a search for dietary factors that might stimulate enterocyte proliferation, we developed an assay for thymidine incorporation into DNA using harvested crypt cells from mature rat small intestine. Human colostrum stimulated a significant increase in thymidine incorporation into rat crypt cell DNA during a 60-min period of incubation. When the protein with biological activity was purified to a single peak by sequential ion exchange and gel filtration chromatography, it was found to have the characteristics of lactoferrin. The protein was identical to lactoferrin standards by sodium dodecyl sulfate polyacrylamide gel electrophoresis, isoelectric focusing, and double-diffusion immunologic precipitation. All available human lactoferrins stimulated thymidine uptake and all reacted with a lactoferrin polyclonal antibody. Human lactoferrin appears to be a potent activator of thymidine incorporation into DNA in incubated rat crypt cells, a nutritional function not previously reported. (*Pediatr Res* 21: 563–567, 1987)

Abbreviations

M_r, molecular weight
EGF, epidermal growth factor
PBS, phosphate-buffered saline
T8, Trowell's T8 medium
HPLC, high-performance liquid chromatography
SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
cpm, counts per minute
pI, isoelectric point

Studies in the pig, dog, and rat indicate that the gastrointestinal tract matures more rapidly if the newborn animal is suckled (1–3). The nutritional significance of these observations lies in the principle that the structure of ingested protein may have biological significance beyond the dietary requirement for amino acids. Based on these observations, *in vitro* fibroblasts and other cell lines have been used to test for the presence of growth-promoting factors in milk. Mammary secretions from goats, sheep, cows, and humans have been found to stimulate the proliferation of various cell lines growing in culture (4–6). A portion of the activity in fibroblast culture can be attributed to EGF, a 6000-M_r mitogen present in many mammalian secretions (7). However, other factors with mitogenic activity have been identified, *e.g.* polypeptides with M_r of approximately 100,000 and 30,000 (5).

None of the cell lines used for the bioassay of mitogenic factors exhibit polarity and none has brush-border membranes. Assuming that specificity may exist in intestinal cells, we developed a bioassay based on harvested rat crypt enterocytes. With this assay, we have confirmed the presence of mitogenic activity in human colostrum. We report the details of the bioassay, the isolation of lactoferrin as a mitogenic dietary factor present in human milk, and compare the enterocyte assay with the fibroblast assay system used by Klagsbrun (5).

METHODS

Isolation of crypt cells. Pathogen-free male Sprague Dawley rats (Harlan Industries, Houston, TX) were housed under standard conditions for at least 2 wk. They were fed rat food *ad libitum*. At the time of the study, the rats weighed 375 to 475 g. After the animals were anesthetized with pentobarbitol (50 mg/kg body weight), the jejunum and ileum were removed before the animals were killed. The mucosal cells were sequentially harvested at 37° C using the method of Harrison and Webster (8) as modified by Bronstein *et al.* (9) in which high frequency vibration at low amplitude is used to shake off mucosal cells. The crypt-cell fraction collected between 12 and 18 min of vibration was washed twice in Dulbecco's PBS, suspended in Trowell's T8 medium (Gibco Laboratories, Long Island, NY), and used immediately.

Differential counts of the harvested cells were made in some experiments using Wright-Giemsa and Papanicolaou stains. Trypan blue exclusion tests also were done on freshly harvested and incubated cells.

Crypt cell bioassay. Isolated crypt cells were used in an assay to measure ^{3}H-thymidine incorporation into DNA. T8 medium used for incubation contained 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum (Gibco Laboratories), and 10 μCi/ml ^{3}H-thymidine. Incubation tubes were placed in a Dubnoff shaking water bath at 37° C under 95% O_2 and 5% CO_2. The tubes contained 0.85 ml medium and 0.05 ml of tested proteins in saline and were preincubated for 1 h before adding 4 to 5×10^5 cells in 0.10 ml T8. After incubation for 1 h, the tubes were placed on ice and 49 ml of PBS without Ca and Mg (1.0 mM EDTA and 18 mM NaCl) was added. The tubes were centrifuged for 10 min at $300 \times g$ and the supernatant decanted. The cells were lysed by adding 0.03 ml 10% (w/v) sodium dodecylsulfate in 0.60 ml of buffer (10 mM Tris HCl, 100 mM NaCl, 1.0 mM EDTA, pH 7.4) and the tubes were shaken gently for 15 min at room temperature. The samples were deproteinized by adding 0.03 ml of 10 mg/ml crude protease (Sigma Chemical Company, St. Louis, MO) and incubated for 30 min at 37° C.

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DNA was determined in deproteinized samples using the method of Labarca and Paigen (10). The buffer used was PBS without Ca and Mg at pH 7.4 with 2 M NaCl and 1.5 g/liter EDTA. Calf thymus DNA type I was used as the standard. Hoechst 33258 (Sigma Chemical Company) was used at a concentration of 20 ng/ml. Each acrylic cuvette contained 20 μl of sample or standard and 3.5 ml of buffer with dye. The samples were excited at a wavelength of 354 nm and emission was measured at 450 nm. The assay was linear over the range used.

Radioactivity incorporated into DNA was measured using a modification of the method of Lanford and Butel (11). Glass microfiber filter paper strips (2.54 × 20.32 cm) were pretreated with a solution of 1% bovine serum albumin with 0.05 M thymidine and 0.5 M NaCl and dried 24 h at 37° C. Each strip was spotted with 300 μl of deproteinized cell digests and dried overnight at 37° C. The paper strips were agitated using an orbital shaker for 10 min in successive washes of cold 10% trichloroacetic acid, 5% trichloroacetic acid, and 95% ethanol. After drying under warm air, the strips were rolled up, put into plastic minivials, and covered with 5.25 ml Scintiverse-II (Fisher Scientific, Houston, TX). cpm were determined using a Mark III Scintillation Counter (TM Analytic, Elk Grove Village, IL).

Fibroblast bioassay. BALB/c 3T3 mouse embryo cells were grown in Eagle's medium with 10% fetal calf serum, 2 mM glutamine, 4500 mg D-glucose/liter, 50 U/ml penicillin, and 50 μg/ml streptomycin. At passage 86, the cells were treated with trypsin and resuspended at 5 × 10^4/ml in medium. Cells (200 μl) were placed in each well of a 96-well plate (Falcon no. 3072, Becton Dickinson and Co., Oxnard, CA). The cells were incubated at 37° C in 7% CO_2 and were allowed to quiesce. After stimulation with lactoferrin or EGF (25 μl/well) 25 μl ^{3}H-thymidine at 160 μCi were added. Four replicates at each concentration were made. Human AB serum (200 μg/ml stock) (Gibco) was used as a positive control and 0.9% NaCl as a negative control. All dilutions were made in 0.9% NaCl. After incubation for 48 h, a Skatron cell harvester (no. 700 Skatron, Inc., Sterling, VA) was used to transfer the cells to filter paper. The papers were dried, punched out, and counted in 3 ml Scintiverse II.

Milk collection and processing. Human colostrum was obtained up to 5 days postpartum. Colostrum and mature milk were collected from individual donors using Egnell breast pumps (Egnell, Inc., Cary, IL) and frozen immediately at −20° C. Subsequently, donor samples were thawed, pooled, and refrozen (−20 or −70° C). Colostrum used in the bioassay was thawed and centrifuged at 1000 × *g* for 10 min. The acellular infranatant was added at 5% v/v to the incubation medium.

Isolation from colostrum. The thawed skim colostrum (100 ml) was collected after a 30-min centrifugation at 10,000 × *g*. Sufficient HCl was added to lower the pH to 4.3. The acidified colostrum was incubated 60 to 90 min at 37° C and was centrifuged at 30,000 × *g* for 70 min at 4° C. The supernatant was collected and dialyzed at 4° C against 0.5 M sodium acetate buffer with 0.2 M NaCl (pH 4.2) using tubing with a molecular weight cutoff of 12,000 to 14,000. After dialyzing 5 h, the sample was transferred to fresh buffer and dialysis was continued overnight. The gel (SP-Sephadex C-50, Pharmacia, Inc., Piscataway, NJ) was equilibrated in the dialysis buffer. Dialyzed colostrum was mixed with an equal volume of gel and allowed to stand for 15 min with occasional stirring. Unbound protein was removed by washing, using a Buchner funnel and a small volume of buffer. The filtrate was mixed with fresh gel and allowed to bind. Both gels were eluted with the same buffer containing 0.5 M NaCl. The eluates were combined and diluted with buffer to 0.2 M NaCl, and rechromatographed using fresh gel. The final eluate was placed in dialysis tubing with a molecular weight cutoff of 3500. Proteins were concentrated by coating the tubing with dry polyethylene glycol 8000. When the sample was 10% of the original volume, 10 ml was applied to a gel filtration column. Separation of proteins was done at 4° C on a 1.5 cm × 70 cm column of Sepharose Cl-6B-200 (Pharmacia, Inc.) using 0.5 M sodium acetate buffer with 0.5 M NaCl (pH 4.2) as the eluant. The column was calibrated using a mixture of thyroglobulin, γ-globulin, ovalbumin, myoglobin, and vitamin B_{12} (Gel Filtration Standards, Bio-Rad Laboratories, Richmond, CA). Fractions (3.5 ml) were collected every 22 min. Fractions containing protein (optical density at 280 nm) were dialyzed against distilled water or 1.0 M NaCl and concentrated using Centricon-30 microconcentrators (Amicon Corporation, Danvers, MA). These fractions were stored frozen at −20° C. Protein concentration was measured using Coomassie blue G-250. Bovine serum albumin was used as a protein standard.

Isolation from mature milk. Mature human milk was pooled, divided into aliquots, and stored at −20° C. Lactoferrin was isolated by the procedure of McKenzie (12). The milk was thawed and centrifuged at 16,000 × *g* for 30 min at 3° C. The skim was removed and placed into an ice bath. Ammonium sulfate (26.4 g/dl) (Sigma Chemical Company) was added gradually to the skim milk which was stirred mechanically during a 30-min period. The solution was stirred an additional 90 min with the temperature maintained at 0 to 3° C. The precipitate was removed by centrifugation at 14,600 × *g* for 35 min at 3° C. The

Table 1. *Crypt cell bioassays of trophic factors*

Proteins added to medium (5% v/v)	No. of assays	% stimulation of ^{3}H-thymidine incorporation
Controls (T8)	46	100 ± 7*,†
Colostrum, pooled	4	120 ± 9‡
Colostrum, individual donors	41	136 ± 15‡
Colostrum, casein-free	13	137 ± 14‡
Fractions of colostrum (4 mg/ml)	14	139 ± 17‡
Lactoferrin standards (4 mg/ml)	13	130 ± 13‡
Lactoferrin from milk (4 mg/ml)	4	136 ± 15‡
EGF (0.04 μg/ml)	6	95 ± 12
EGF (0.20 μg/ml)	6	97 ± 7
EGF (1.00 μg/ml)	6	98 ± 13
EGF (5.00 μg/ml)	6	87 ± 13

* Mean ± SD.

† Normalized to control values.

‡ Significantly different from control ($p < 0.02$) by Dunnett's procedure.

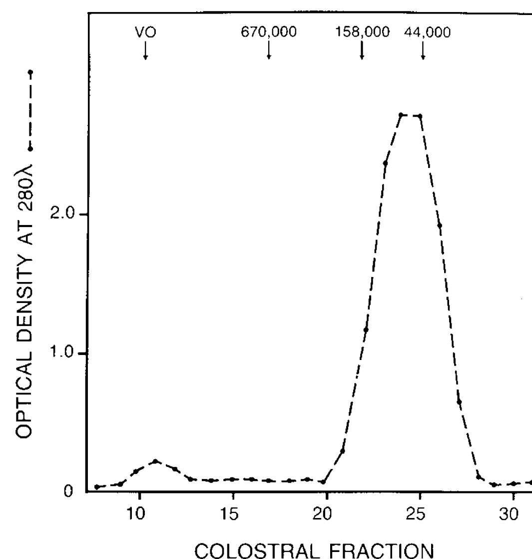

Fig. 1. Gel filtration of colostral protein isolated by ion chromatography: the elution volume and M_r of protein standards are shown at the *bottom* and *top* of the *graph*.

supernatant fluid was dialyzed in 0.5 M sodium acetate and 0.5 M sodium chloride (pH 4.2) at 4° C. The solution was again centrifuged at 14,600 × *g* for 35 min at 3° C. The supernatant was treated by gel filtration as previously described.

HPLC. Molecular weight was determined by size exclusion chromatography using a 9.4 × 250 mm GF-250 column (DuPont Company, Wilmington, DE) which was equilibrated to 50 mM Tris, 2 M NaCl, pH 8.0. The column was calibrated using gel filtration standards (Bio-Rad Laboratories) and was eluted isocratically at a flow rate of 1.0 ml/min using a Hewlett-Packard 1090A HPLC apparatus with UV detection at 280 nm. All samples were 0.45 μ filtered (Millipore, Bedford, MA) before chromatography.

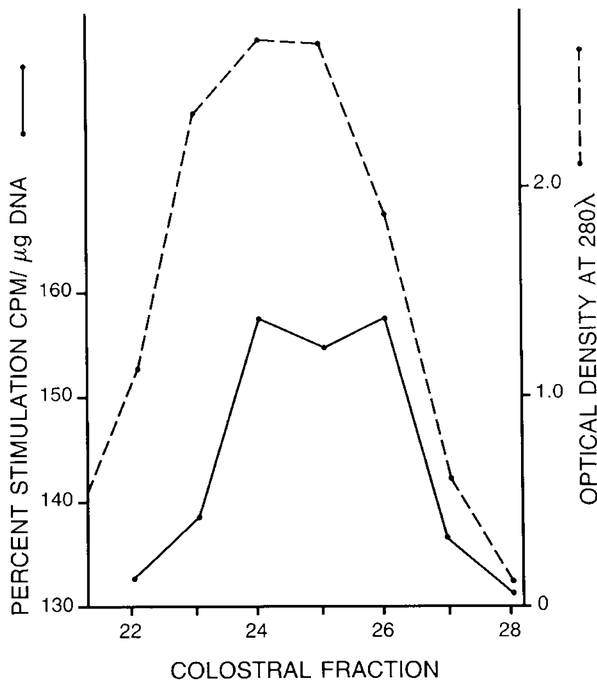

Fig. 2. Crypt cell bioassay—gel filtration fractions from Figure 1 tested in duplicate (200 μg/ml). All were more stimulatory than controls (100 ± 7.2).

Electrophoresis and immunodiffusion. SDS PAGE was performed using the method of Laemmli (13) on 7.5% total acrylamide gels with 2.67% cross-linker. The electrophoresis was run overnight at room temperature with a constant 40 V. SDS PAGE high molecular weight standards (Bio-Rad Laboratories) were used. The method of Warnick *et al.* (14) was used for isoelectric focusing. The gel had 7.5% total acrylamide and 2.67% cross-linker in 8 M urea and was run at 10° C. Immunologic tests of identity were performed using Ouchterlony's comparative double-diffusion combined system (15). A polyclonal antibody to human lactoferrin (Cooper Biomedical, Inc., Malvern, PA) was used. The gels and plates were stained with Coomassie blue.

Lactoferrin and EGF standards. Standard proteins were purchased from the Sigma Chemical Company as freeze-dried preparations. Human lactoferrin was dissolved in 2 M NaCl. Aliquots were stored at −20° C and 2 M NaCl was diluted to 0.15 M immediately before use. Mouse salivary gland EGF used in the crypt cell assay was dissolved in distilled water, stored frozen, and diluted 1/10 with T8 before use. EGF used in the fibroblast assay was stored frozen in 2 M NaCl.

Calculations. Results from the crypt cell bioassay were expressed as cpm/μg DNA for each incubation tube. At least five controls were run in each assay and the mean of the results was chosen to represent 100% basal stimulation. All values from each experiment were normalized to the mean basal stimulation. Results from the fibroblast bioassay were expressed as cpm/well. Statistical significance of the stimulation was assessed using Dunnett's procedure which compares all means with a control (16).

RESULTS

Crypt cell response to human colostral proteins. Microscopic examination of stained smears from the 12- to 18-min fraction of the harvested mucosal cells indicated the presence of 0 to 2% lymphocytes. The bioassay, however, was not dependent on the presence of lymphocytes. This fraction was free of columnar enterocytes. The cells were 96% viable by Trypan blue exclusion immediately after harvesting and 80% viable after 60 min of incubation.

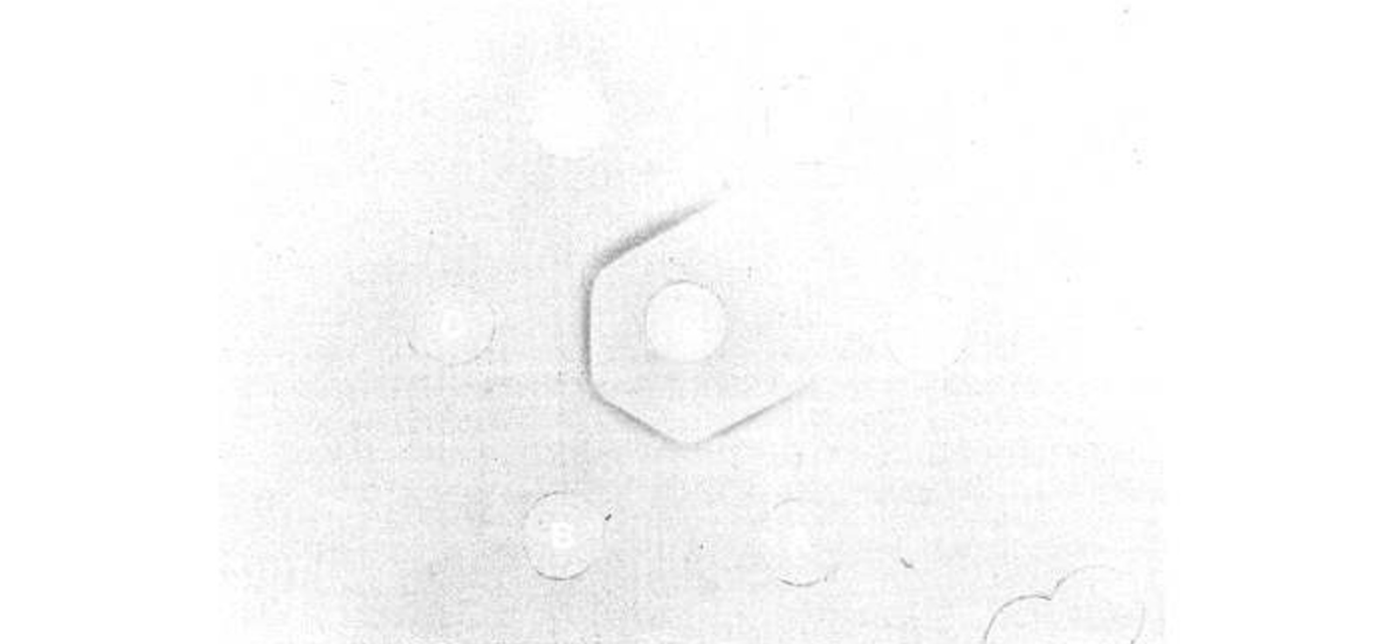

Fig. 3. Ouchterlony plate—two human lactoferrin standards were in *wells A* and *B*. Fractions 23 and 24 from gel filtration were in *wells C* and *D*. Bovine lactoferrin standards were in *wells E* and *F*. Antibody to human lactoferrin was in the *center well*.

Rat crypt cells incubated with human colostrum had higher rates of ^{3}H-thymidine incorporation into DNA than those of controls incubated with 5% (v/v) additional T8 (Table 1). Pooled colostrum, colostrum from individual donors, and casein-free colostral whey were stimulatory; EGF was not stimulatory (Table 1).

A protein fraction isolated by ion chromatography from colostral whey was subjected to molecular weight chromatography. Thirty-two fractions were collected from the Sepharose column. Fraction 24 (Fig. 1) contained 13.4 mg of protein and the total protein in fractions 21 to 27 was 51.6 mg. All protein fractions from the peak stimulated ^{3}H-thymidine incorporation (Fig. 2) to levels 30 to 60% higher than controls.

The color of the concentrated protein fractions from the gel filtration step was light pink. In the immunodiffusion tests using Ouchterlony plates, a line of identity was seen between proteins from the peak and antibodies to human lactoferrin (Fig. 3). Two human lactoferrin standards also formed lines of identity, but bovine lactoferrin standards did not react with the antibody.

Fig. 4. Isoelectric focusing—human lactoferrin standard in *lane 1* and fractions 24 and 25 from human colostrum in *lanes 2* and *3*.

Table 2. *3T3 bioassay of trophic factors*

Added to medium	^{3}H-thymidine incorporation cpm/well × 10^3	p*
Lactoferrin standards (μg/ml)		
400	11.6 ± 3.9†	<0.01
200	15.4 ± 3.5	NS
100	14.6 ± 2.3	<0.05
50	12.4 ± 3.1	<0.01
25	19.0 ± 5.4	NS
13	16.0 ± 3.1	NS
Lactoferrin from colostrum (μg/ml)		
400	14.4 ± 2.5	NS
200	18.2 ± 2.3	NS
100	21.1 ± 6.1	NS
50	17.7 ± 5.0	NS
25	16.7 ± 5.3	NS
13	21.3 ± 3.0	NS
Lactoferrin from milk (μg/ml)		
400	18.2 ± 3.6	NS
200	22.0 ± 5.4	NS
100	25.8 ± 5.7	NS
50	21.6 ± 5.6	NS
25	21.3 ± 5.1	NS
13	24.2 ± 4.7	NS
Epidermal growth factor (ng/ml)		
60	71.7 ± 30.7	<0.01
30	58.6 ± 11.4	<0.01
15	50.2 ± 9.2	<0.01
8	66.8 ± 28.6	<0.01
4	57.8 ± 9.6	<0.01
2	47.8 ± 12.2	<0.01
Negative control-saline (5% v/v)	20.2 ± 78	

* Based on Dunnet's procedure
† Mean ± SD

When tested by HPLC, fraction 25 appeared as a single peak with a molecular weight of approximately 79,000. Gel electrophoresis of the fractions and the human lactoferrin standards showed that the major band in all samples migrated at the same rate. Isoelectric focusing of two peak fractions and the human lactoferrin standard gave similar results (Fig. 4).

Crypt-cell and fibroblast response to lactoferrin. Two human lactoferrin standards were tested using the crypt-cell assay. Both were stimulatory (Table 1) as were skim human colostrum, combined protein fractions 21 to 28 isolated from colostrum, and lactoferrin from mature milk. Although none of the lactoferrins stimulated ^{3}H-thymidine uptake over negative control values (20.2 ± 7.8 × 10^3 cpm) in tests using 3T3 cells, EGF at 60 ng/ml resulted in a 75% increase (Table 2).

DISCUSSION

This investigation revealed that human lactoferrin from various sources stimulated thymidine incorporation into DNA by rat crypt enterocytes. Human milk is known to stimulate thymidine uptake in a variety of fibroblast cell lines. The factors responsible for the initiation of mitosis have been identified in part. EGF was the first described and is the best known active factor in human milk. Receptors at the plasma membrane of 3T3 fibroblasts bind EGF and internalize it for subsequent nuclear binding. This mechanism requires 12 to 14 h for completion when confluent fibroblast cultures are stimulated (17). In the assay with rat crypt cells, the DNA was harvested after only 1 h of incubation. The short incubation may be one reason for the failure of EGF to stimulate enterocytes. The cells in the crypt cell assay may have been conditioned *in vivo* by EGF before they were harvested for the *in vitro* bioassay (18).

Shing and Klagsbrun (19) have identified three factors in

human milk which stimulate cell proliferation. The 3T3 cell line responds *in vitro* to whole human milk and to purified fractions. Klagsbrun's fractions I and II and EGF accounted for 5, 20, and 75%, respectively, of the 3T3 stimulation by human milk. Both larger fractions are broken down to smaller M_r fractions under denaturing conditions. Fraction II is resolved by isoelectric focusing into two fractions with different pI. Based on the reported M_r and pI, none of Klagsbrun's fractions appear to be intact lactoferrin. The cathodic protein in his factor II may be a fragment from intact lactoferrin, but the relative resistance of lactoferrin to proteolysis makes this possibility unlikely. Assays with 3T3 cells confirmed (data not shown) that fractions of human colostrum stimulate thymidine incorporation in this cell line. Human lactoferrin, however, does not promote growth in the 3T3 bioassay which is sensitive to EGF (Table 2). The absence of sensitivity to human lactoferrin in the mouse 3T3 cell line explains why previous investigators have not observed its stimulation of thymidine incorporation in this fibroblast assay system. Species differences may account for this differential response. Another factor may be tissue specificity. Menard *et al.* (20) have demonstrated that mouse jejunum is insensitive to EGF.

Although the mechanisms by which lactoferrin may stimulate the production of DNA in crypt cells have not been described, a similar protein, transferrin, is known to have a stimulating effect in a variety of cell lines. The two proteins, however, are immunologically distinct. Transferrin is an essential component of highly defined tissue culture media with a requirement of less than 10 μg/ml for most cell lines. Whether this property of transferrin is attributable to the iron or to the apotransferrin protein has not been determined (21). In the cell lines studied thus far, an exclusive receptor is present for either lactoferrin or transferrin (22). Although transferrin and lactoferrin are not interchangeable, lactoferrin may have a role parallel to that of transferrin *in vitro* and *in vivo*.

Lactoferrin is a glycoprotein of known structure (23, 24) with a M_r of approximately 76,000 and a pI of 8.7 (25). It is present in human milk at a concentration ranging from 1 to 3 g/liter (26). Lactoferrin is known to have two functions in the gastrointestinal tract (21). It has an affinity for iron 300 times that of serum transferrin (27). Iron binding occurs on two sites of the molecule and persists after digestion of the fragments to approximately 40,000 M_r (28). The presence of lactoferrin is believed to account for the superior absorption of iron from human milk (29). Lactoferrin also has a bacteriostatic effect on organisms present in the human bowel. The antimicrobial activity is dependent upon the desaturation of iron binding sites (30, 31). Lactoferrin in human milk is less than 5% saturated with iron (26).

The biological significance of human lactoferrin-induced thymidine incorporation in rat crypt cell DNA has not been elucidated. What is clear, however, is that lactoferrin is inactive in mouse 3T3 cell lines which are sensitive to EGF (Table 2) and respond to Klagsbrun's fractions I and II and that EGF is inactive in the crypt cell bioassay sensitive to lactoferrin (Table 1). Whether lactoferrin plays a role similar to that documented *in vivo* for EGF in the regulation of mucosal growth in the intact animal must be determined. If lactoferrin is active *in vivo*, this trophic effect on intestinal mucosa will be the third nutritional function described for this human milk protein.

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