are associated with initiation of rapid cell proliferation or cellular response to hormonal stimulation (*1*-*3*). The association of increased polyamine biosynthesis during the period of enzymic maturation of the newborn rat intestinal mucosa is especially intriguing in this regard. Is this process of mucosal maturation in the newborn rat accompanied by the proliferation of a new population of cells, or by hormonally mediated maturation and gene reprogramming of an existing population of cells? The type of manipulation of polyamine biosynthesis used in our present studies could help clarify this aspect of cellular differentiation.

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16. We thank A. Sjoerdsma and P. McCann, Merrell Research Center, for the DFMO; M. A. Beaven, National Institutes of Health, for the [β-^{3}H]-histamine; J. H. Yardley for help with the histology; T. R. Hendrix and A. H. Owens, Jr., for advice; and G. Goodwin, W. Lubrich, J. Messersmith, and K. Wieman for technical assistance. This work was supported in part by grants 5-R01-18404, 1-P50-HL-19157-01, 5-T32-AM-07192-03, RR-5378, CA-15515, and CA-13525 from NIH. L.J.M. (CA-00112) and S.B.B. (CA-00027) are supported by NIH research career development awards.

16 April 1980; revised 27 June 1980

Epidermal Growth Factor Is a Major Growth-Promoting Agent in Human Milk

Abstract. *Human milk stimulates DNA synthesis in cell cultures in which growth has been arrested. The mitogenic activity of milk is neutralized by the addition of antibody to human epidermal growth factor. The results identify epidermal growth factor as a major growth-promoting agent in breast milk.*

Epidermal growth factor (EGF) is a small polypeptide mitogen (molecular weight ≃ 6000) detected in many mammalian species and isolated from mice (*1*) and humans (*2*). Although recent studies have focused on the mechanism by which EGF promotes the proliferation of cultured cells, the growth factor produces significant biological effects in the intact mammal, particularly the fetus and newborn. These effects include enhanced proliferation and differentiation (keratinization) of the epidermis (*1*, *3*), increased growth and maturation of the fetal pulmonary epithelium (*4*), stimulation of ornithine decarboxylase activity and DNA synthesis in the digestive tract (*5*), and acceleration of the healing of wounds of the corneal epithelium (*6*). Apparently unrelated to these mitogenic effects is the fact that EGF inhibits histamine- or pentagastrin-induced secretion of gastric acid (*7*).

Epidermal growth factor is present in many human body fluids including plasma, saliva, urine, amniotic fluid, and milk (*8*). Breast milk is important to the developing infant not only as a source of gross nutrients (protein, carbohydrate, and fat) and micronutrients (minerals and vitamins), but also as a source of molecules (immunoglobulins and hormones) that may have a more specialized role in ontogeny. Since human milk is mitogenic for cultured cells (*9*), we performed experiments to determine whether the growth-promoting activity of milk can be attributed to the presence of EGF. We added specific antibodies to human EGF to samples of human milk and tested the samples for their capacity to stimulate DNA synthesis in diploid human fibroblasts in vitro by measuring the incorporation of [^{3}H]thymidine into acid-insoluble material.

In the presence of purified EGF [10 ng/ml, a concentration above that which produces maximal mitogenic activity (*10*)] or milk (5 percent by volume), the incorporation of [^{3}H]thymidine was increased approximately 11-fold over that incorporated by control (unstimulated) cells (Table 1). Adding antibodies to EGF completely blocked the mitogenic

Table 1. Influence of antibody to human EGF on the stimulation of DNA synthesis in human fibroblasts by human milk. Confluent monolayers of foreskin fibroblasts, cultured in Eagle's minimum essential medium (Dulbecco's modification) containing 10 percent calf serum and gentamycin (*10*), were transferred to fresh medium supplemented with 2 percent calf serum and gentamycin. After 2 days the indicated additions were made, and 20 hours later [*methyl*-^{3}H]thymidine was added to each dish. The cells were allowed to incorporate the isotope for 4 hours before the labeling was terminated. The amount of acid-insoluble radioactivity was then determined (*10*). Rabbit antibody to human EGF was prepared (*2*) and the gamma globulin fraction was purified by diethylaminoethane chromatography (*10*). The control gamma globulin, from a rabbit immunized with renin, was prepared in an identical manner. The human milk, donated 5 weeks after the mother gave birth, was centrifuged and sterilized by filtration through a 0.2-μm mesh filter.

Additions	[^{3}H]Thymidine incorporated (count/min per dish)
None	198
EGF (10 ng/ml)	2132
EGF (10 ng/ml) + antibody to EGF (37 μg/ml)	225
Antibody to EGF (37 μg/ml)	169
Control IgG (42 μg/ml)	172
Milk (10 percent)	4582
Milk (5 percent)	2228
Milk (1 percent)	528
Milk (5 percent) + antibody to EGF (37 μg/ml)	343
Milk (5 percent) + control IgG (42 μg/ml)	2001
Calf serum (10 percent)	3023

0036-8075/80/1010-0198$00.50/0

stimulation by purified EGF and reduced the stimulatory effect of milk 93 percent. A similar inhibitory effect of antibody to EGF was observed when milk samples from two other donors were used. Control immunoglobulin G (IgG) had no effect on the mitogenic activity of milk (Table 1). The data suggest, therefore, that the ability of milk to stimulate DNA synthesis, at least in cultured diploid fibroblasts, is due to the presence of EGF.

At a concentration of 10 percent, milk was more potent than purified EGF. This does not necessarily indicate the presence of another mitogen or of a more active form of EGF. The ability of purified human EGF to stimulate DNA synthesis in human fibroblasts is enhanced by certain permissive factors, such as serum proteins (*10, 11*), proteases (*12*), and ascorbic acid (*10, 12*), which themselves do not stimulate DNA synthesis. Since milk is a rich mixture of biochemical molecules including ascorbic acid (*13*), it probably contains molecules that enhance the activity of EGF.

Since measurements of DNA synthesis do not always reflect changes in cell proliferation, we performed an experiment in which cell numbers were determined in a Coulter counter. Human fibroblasts were plated in medium containing 2 percent serum. On the following day, when cell attachment was complete, one of the following was added to the medium: (i) fresh serum to a final concentration of 10 percent, (ii) milk to a final concentration of 10 percent, or (iii) milk (10 percent) plus antibody to human EGF. Control cultures received no additions. After 3 days the cells in each group were counted. The number of cells in cultures grown in added serum or milk was about 50 percent higher than the number of cells in the control cultures, but the number of cells in the cultures that had been incubated with milk plus antibody to human EGF was not increased. Thus antibody to EGF inhibits the capacity of milk to increase cell multiplication.

The capacity of antibody to human EGF to block the mitogenic activity of human milk was also tested with diploid human glia cells, aneuploid mouse 3T3 cells, and a mutant (NR-6) of the 3T3 cell line which has no active EGF receptors (*14*). When the glia cells were tested, the results were very similar to those obtained with the fibroblasts (Table 2). Human EGF or milk stimulated [^{3}H]thymidine incorporation about tenfold, and the addition of antibody to human EGF blocked 90 percent of the stimulatory activity of the milk.

When human milk was used to stimulate the 3T3 cells, approximately 67 percent of the mitogenic activity was blocked by antibody to human EGF. Milk apparently contains non-EGF material that is capable of stimulating 3T3 cells. This observation is substantiated by the results obtained when human milk was employed to stimulate the NR-6 derivative of 3T3 cells. The NR-6 cells do not bind ^{125}I-labeled EGF and are thought to be an EGF receptor-defective 3T3 derivative (*14*). Epidermal growth factor failed to stimulate these cells, and milk produced only a small (threefold) stimulation of DNA synthesis which was not significantly reduced by the addition of antibody to EGF.

Table 2. Effect of antibody to human EGF (37 μg/ml) on the stimulation of DNA synthesis in human glia and mouse 3T3 cells by human milk (5 percent). Confluent monolayers of each cell type were stimulated and assayed for the incorporation of radioactive thymidine as described in the legend to Table 1.

Cell type	Additions	[^{3}H]Thymidine incorporated (count/min per dish)
Human glia	None	183
	EGF (5 ng/ml)	1882
	Milk	1984
	Milk + antibody to EGF	366
	Antibody to EGF	98
Mouse 3T3	None	382
	EGF (5 ng/ml)	971
	Milk	1935
	Milk + antibody to EGF	891
	Antibody to EGF	391
Mouse NR-6	None	214
	EGF (5 ng/ml)	293
	Milk	730
	Milk + antibody to EGF	627
	Antibody to EGF	211

In separate experiments, proprietary formulas (Enfamil, Similac, Isomil, and Prosobee), which are widely used as substitutes for milk, were tested for their capacity to stimulate DNA synthesis in cultured human fibroblasts. At concentrations ranging from 1 to 20 percent, none of the formulas caused measurable stimulation of DNA synthesis.

Radioimmunoassays indicate that the concentration of EGF in human milk is approximately 50 ng/ml (*8*) and in mouse milk is about 300 ng/ml (*15*). The most specific biological assay for EGF is its ability to cause precocious eyelid opening in the newborn mouse by stimulating proliferation and differentiation of epidermoid tissue (*1, 3*). Although this effect usually is assayed by injecting EGF subcutaneously, the response can also be produced by administering EGF orally (*16*). It is possible that ingested EGF is not entirely destroyed in the digestive tract, since both mouse and human EGF are acid-stable (*1, 2*) and since trypsin-treated mouse EGF is as active in the newborn animal as the intact molecule (*17*). Therefore, EGF might act directly on the tissues of the digestive tract and perhaps be absorbed into the circulation to affect other tissues of the neonate.

Klagsburn (*9, 18*) showed that milk from various species enhances the growth of cultured cells. It is likely that much of the mitogenic activity observed in those studies was due to EGF. Colostrum, the first milk secreted at the end of pregnancy, is reported to have a higher mitogenic potency than milk (*18*). It is not known whether EGF is the predominant growth-promoting agent in colostrum.

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29 May 1980