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ISOLATION OF COLONY STIMULATING FACTOR FROM HUMAN MILK

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Human milk contains colony stimulating factor (CSF), a polypeptide growth factor, which stimulates in in vitro bone marrow culture proliferation and differentiation of colony forming granulocytic macrophage progenitor cells (CFU-GM) to form colonies. This activity was not found in either bovine milk or colostrum when assayed in human or mouse bone marrow cells. The human milk CSF activity is destroyed by treatment with proteases. However, neither 6M urea, 4M guanidine hydrochloride, 5 mM dithiothreitol, nor exposure to pH 2 will inactivate the milk derived CSF. Gel filtration and isoelectric focusing indicate that human milk CSF differs biochemically from the other CSFs isolated from various sources and has a molecular weight between 250,000 and 240,000 and an isoelectric point between 4.4 and 4.9.

Milk is a body secretion which is involved in nutrition, growth and development. Breast milk is important to developing infants not only as a source of gross nutrients such as proteins, carbohydrates and fat and micronutrients like minerals and vitamins, but also of biologically active macro molecules essential for specialized roles. The human milk IgA and other antibodies are known to confer passive immunity on the infant (1,2). An important activity found in human milk by Klagsburn (3) was its ability to stimulate DNA synthesis and proliferation. Recently, Beerns et al. (4) have reported that human milk contains growth promoting factors for bifidobacterium in the infant's intestine, and due to this, breast fed infants have a natural defense against E. coli, bacteroides, and clostridium.

In this report we demonstrate that human milk contains colony stimulating factor (CSF), a polypeptide bone marrow growth factor, capable of inducing in in vitro bone marrow culture, proliferation and differentiation of granulocytic macrophage progenitor cells (CFU-GM) to form colonies composed of granulocyte and macrophages. Some preliminary characterization of the biological and biochemical properties of milk-derived CSF is described.

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MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DME), fetal calf serum, and horse serum were obtained from Grand Island Biological Co. (New York, N.Y.). Ultrogels (AcA 34 and AcA 44), Ultrodex and preparative flat bed isoelectrofocusing apparatus were from LKB (Rockville, MD). Ampholine (4 - 6.5) was from Pharmacia (Sweden). Human milk samples were donated by normal nursing mothers at various times after giving birth. In all, five samples were tested; most of the isolation reported in this paper was done from milk obtained from a donor three months after childbirth. The human milk samples were stored frozen at -20°. All other chemicals were of reagent grade.

Assay of CSF. The method developed by Bradley and Metcalf (5) was used with slight modifications (6). The milk samples were dialyzed against distilled water, centrifuged and filter sterilized prior to the assay. Aggregates of 50 cells or more were scored as colonies. A unit is arbitrarily defined as the amount of CSF which stimulates the formation of one colony under the specified condition of the assay.

Morphological Analysis. The study of colony morphology in assay plates were done by a simplified procedure developed in this laboratory (7). The soft agar plates were fixed with 30% acetic acid in absolute ethanol and stained with alum hematoxyline for the analysis.

Preparative Isoelectric Focusing. Human milk (30 ml) was dialyzed, centrifuged and concentrated to approximately 4 ml by ultrafiltration using YM 5 membrane. The samples were then subjected to preparative flatbed isoelectrofocusing for 8 hr in granulated gel (Ultrodex) containing 2.5% ampholine (pH 4-6.5) as described in LKB application note 198 (8). Fractions were eluted with 6 ml of .02 M tris, pH 7.4, containing 0.15 M NaCl, 0.02% Tween 20 and 0.02% Sodium azide (buffer A).

Gel Filtration. Concentrated pooled fractions from the previous step were subjected to successive gel filtration on an Utrogel AcA 44 column (1.1 x 92 cms) and then on an Ulrogel AcA 34 column (1.1 x 96 cms). Both columns were equilibrated and eluted with buffer A and 1.9 ml fractions were collected.

Characterization. Unfractionated human milk after removal of fat and other precipitates by centrifugation at 40,000 g for 20 min was filtered through a 0.45 micron filter and used for characterization experiments. Activity assays were done using mouse marrow. The stability of human milk CSF after exposure to various reagents and conditions were determined by the procedure described earlier (9).

RESULTS

Presence of Colony Stimulating Factor in Human Milk. Samples of human milk were tested for their ability to stimulate colony formation in both mouse bone marrow and human bone marrow. Fig. 1 shows that human milk stimulated colony formation in both mouse marrow (day 6 of incubation) and in human marrow (day 14 of incubation) in a concentration dependent manner. In mouse marrow large colonies, some consisting of more than 200 cells, were observed. The unfractionated dialyzed human milk had an average activity of approximately 500 U/ml. The protein concentration, estimated by Bio-Rad dye assay was

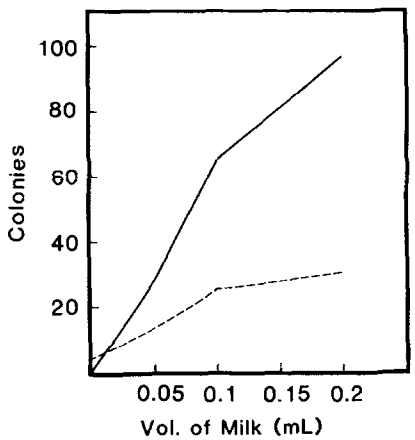

Figure 1. Colony stimulating activity of human milk in mouse marrow (day 6) and in human marrow (day 13)

approximately 9 mg/ml. Similar experiments with either bovine milk or bovine colostrum failed to stimulate any colony formation either in mouse bone marrow or in human marrow.

Isoelectric Focusing. The preparative isoelectric-focusing profile of concentrated and dialyzed human milk is shown in Fig. 2. The results indicate that milk CSF exhibits heterogeneity and has a pI of 4.4 to 4.9

Gel Filtration. The pooled fractions from PIEF (fractions 5 to 15) was concentrated to 1.5 ml and chromatographed on Ultrogel AcA 44 column. Almost all the CSF activity as assayed on mouse marrow was found in the void volume of the column (data not shown). The fractions having CSF activity were pooled, concentrated to 1 ml and resubjected to gel filtration on Ultrogel AcA 34. The activity profile presented in Fig. 3 indicates that the human milk CSF elutes in the region close to catalase (MW 240,000) indicating an approximate molecular weight in the range of 250,000-240,000. The specific activity after this step was 3700 U/mg indicating approximately 51 fold purification over crude milk.

Colony Morphology. The mouse marrow culture plates from AcA 34 column fractions 23, 25, 27, 29 and 31 (Fig. 3) were examined for colony morphology. Three kinds of colonies - macrophagic, granulocytic and mixed were observed. In all the plates more than 90% of the colonies were found to be macrophagic.

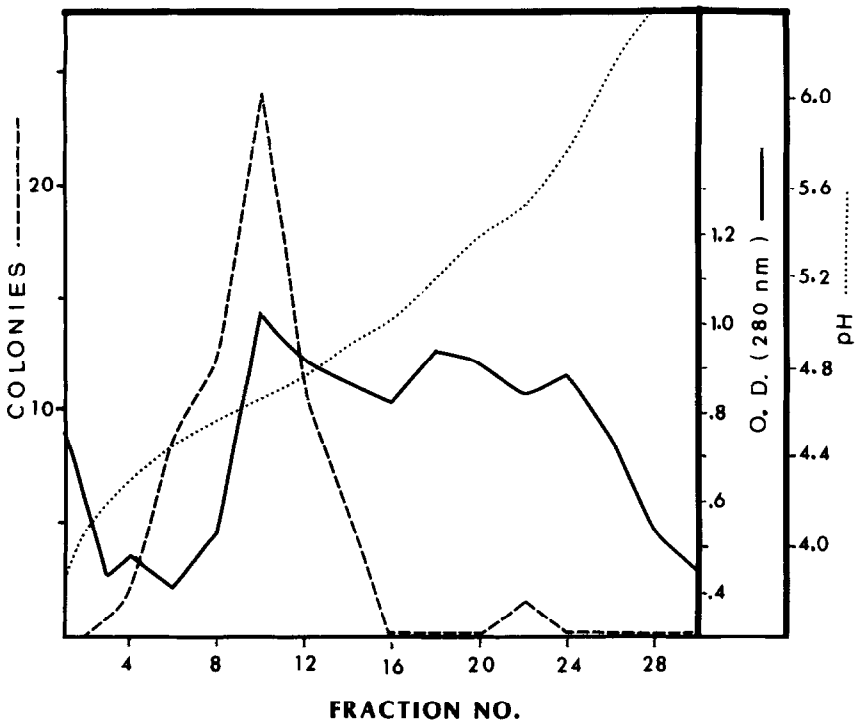

Figure 2. Isoelectric focusing profile of human milk CSF. Aliquotes of 0.2 ml were assayed in mouse marrow.

Only few granulocytic colonies (5%) were observed with the rest being mixed colonies.

Stability of Human Milk CSF. The CSF in human milk appeared to be extremely stable even after exposure to harsh denaturing conditions. Incubation for 2

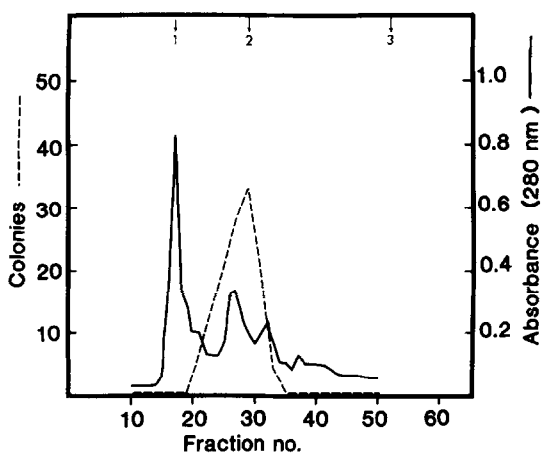

Figure 3. Gel filtration on Ultrogel AcA 34 column. Fractions of 1.9 ml were collected and 0.05 ml aliquots were assayed for activity in mouse marrow. 1. Ferritin (450,000 D); 2. Catalase (240,000 D); 3. Thymidine (VR).

TABLE I. Stability of Human Milk CSF After Exposure to Various Conditions

Reagents	Percent[a] activity	Temperature (^{o}C)	Percent[b] activity	pH	Percent[c] activity
No addition	100	4^{o}	100	2.0	88
4 M guanidine-HCl	116	37^{o}	85	3.1	98
6 M Urea	115	47^{o}	90	5.6	94
5 mM DTT	82	60^{o}	77	7.5	100
		100^{o}C (5 min)	16	9.2	90

Samples were assayed in mouse marrow. Each value represents an average of two independent experiments assayed in duplicate. (a) Samples of human milk were exposed for 2 hr at room temperature to various conditions, dialysed and then assayed. (b) Milk samples were incubated at various temperatures for 30 min., dialysed and centrifuged before assay. (c) Samples of human milk were mixed with various buffers and were incubated for 24 hr at 4^{o}C by dialysing against the same buffer. The pH of the samples were adjusted to pH 7.4 by dialysis against 0.1 M Tris-HCl buffer, pH 7.4. The samples were then dialysed against distilled water and assayed.

hr at room temperature with 6M urea, 4M guanidine hydrochloride, or 5 mM dithiothreitol had no appreciable effect on activity (Table I). The human milk CSF activity was also found to be stable with no appreciable loss in activity after incubating for 30 min at temperatures up to 60^{o}C (Table I). Even after boiling for 5 min, there remained a residual activity of approximately 16%; however, prolonged boiling for 15 min removed all of the CSF activity. Table 1 also demonstrates the stability of human milk CSF at various pH conditions. It is stable over the pH range of 2.0 - 9.2. At pH 2.0, 88% of the activity remains after incubation for 24 hr at 4^{o}C. The effects of proteolytic enzymes (trypsin and chymotrypsin) are summarized in Table II. The CSF activity was destroyed by incubation with chymotrypsin for 3 hr at 37^{o}C, but is relatively less susceptible to trypsin.

DISCUSSION

The human milk CSF appears to differ biochemically from the CSF that has been isolated from various human or murine sources. It is a well known fact that CSF isolated from different species and tissues exhibits significant

TABLE II. Stability of Human Milk CSF to Protease Digestion

Treatment	Percent Activity
control[(a)]	100
trypsin	61
heat inactivated trypsin	106
chymotrypsin	15
heat inactivated chymotrypsin	97

The samples (1 ml) of human milk were incubated for 3 hr at 37°C with enzymes. In each case enzyme to protein ratio was 1:10. The reaction was terminated by lowering the temperature to 5°C and the addition of 100 µl of fetal calf serum. (a) Two controls were included: (1) CSF incubated without enzyme; (2) CSF and enzyme added simultaneously to the culture plate prior to the addition of bone marrow cells in agar mixture. The results are average of two experiments.

size and charge variations (10). Although the pattern of human CSF appears to be complex, generally a similar pattern of two types of CSF has been encountered (11). Type I CSF has higher molecular weight reported in the range of 40,000-150,000 and exhibits activity primarily in mouse marrow, with little or no activity in unfractionated human marrow on day seven of culture. Human urinary CSF (12) and Type I CSF from placenta (7,13), lung (9,14), and MIA PaCa-2 (a human pancreatic carcinoma cell line) conditioned medium (15) all share similar properties. The second type of CSF from human sources is Type II CSF which is smaller in size (20,000-30,000 D) and has a much higher activity in human marrow than in mouse marrow after seven days of culture (11). Recent studies suggest that the activity of Type I CSF is directed specifically to a subpopulation of CFU-GM which form colonies after 13 days of culture. The targeted CFU-GM is an earlier, less differentiated CFU-GM which is probably the immediate precursor of the seven day colony forming CFU-GM (11). Thus, it appears possible that human Type I CSF may function *in vivo* as a bone marrow growth factor. It is of interest to note that human milk CSF is of Type I in nature. However, it is considerably larger than any of the other human CSF. The bigger size may represent a relatively unprocessed form than

those obtained from conditioned medium of various tissues and urine. It is possible that only a small segment of the protein is required for biological activity and the larger size is to impart greater stability. Neonatal intestine is much more permeable than adult intestine and has the ability to transport intact proteins in much larger amounts (16-19). Several proteins such as IgG, bovine serum albumin, and α-lactalbumin have previously been shown to be absorbed intact (19). The stability of human milk CSF makes it possible for either the whole molecule or an active fragment of it to be absorbed from the gastrointestinal tract of the newborn and to play a specific physiological role. However, the in vivo significance of milk CSF is speculative at the present and requires further study.

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