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HUMAN UROPOD BEARING LYMPHOCYTES: ISOLATION OF A FACTOR FROM HUMAN MILK THAT ABROGATES THE UROPOD INHIBITORY PROTEIN FROM HUMAN SERUM

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SUMMARY

Previous studies have demonstrated a factor from human serum or plasma that regulates uropod formation in peripheral blood thymus-derived lymphocytes. This study describes the isolation and partial purification of a factor from colostrum and mature human breast milk that abrogates the uropod inhibitory protein (UIP) of serum. The antagonist is protein in nature since trypsin and protease destroy the activity. Thermal inactivation experiments indicate a relatively heat stable protein with complete inactivation occurring only after heating at 75° C. Gel filtration of the milk antagonist on Bio Gel A 15 M demonstrated a peak of biological activity in the void volume indicating a molecular weight of greater than 15 x 10^6 daltons. The milk antagonist is not associated with the cream fraction of milk or with the acid precipitable casein fraction. Finally, the antagonist could be neutralized with anti-whole human milk in a dose dependent fashion. The physiologic role of this antagonist is not known; however, the ability to regulate lymphocyte deformability suggests that the protein may be important in controlling the traffic of lymphocytes to the mammary gland.

INTRODUCTION

Previous studies have demonstrated a plasma-serum protein that inhibited uropod formation in a specific population of peripheral blood lymphocytes, namely T lymphocytes (1,2). The inhibitory protein was pepsin sensitive and had an apparent molecular weight of approximately 1 x 10^6 daltons (1). After heparin precipitation and ultracentrifugation on NaCl gradients, the inhibitory protein was found in the LDL-VLDL fractions; the peak activity was demonstrated in fractions with densities ranging from 1.040-1.050 g/ml (3). The protein appeared to be associated with the major LDL, β-lipoprotein, but

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UIP - Uropod Inhibitory Protein, LDL - Low Density Lipoprotein, VLDL - Very Low Density Lipoprotein

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sera obtained from a-β-lipoproteinemic patients exhibited normal inhibitory levels (3). In addition, the inhibitory effects of this serum protein were negated with the T cell mitogens, phytohemagglutinin, and tetravalent concanavalin A while pokeweed mitogen and sodium periodate were ineffective (5).

Because of the large number of uropod bearing lymphocytes in human milk (4), we hypothesized that milk might contain a factor that antagonizes the serum inhibitory protein and promotes uropod formation. In this communication, a factor from human breast milk is described that induces uropod formation in the presence of the serum inhibitor.

METHODS

Peripheral blood lymphocytes were isolated from normal human adults as previously described (1). Isolated, washed lymphocytes were examined using interference contrast microscopy for the presence of uropods (1). Lymphocytes (100-200) were counted and scored in a blind fashion for the number of rounded (non-motile) and the number of uropod bearing (motile) cells.

Mature milk was collected from normal human volunteers at 4-20 weeks of lactation. All donors were between the ages of 20-35 years and were successfully breast feeding their infants. After collection, milk samples were placed in an ice bath and maintained for 3-6 hours. After low speed centrifugation to remove the cells, samples were pooled, labeled, and stored at -20°C in polypropylene containers (6). Milk samples were thawed at room temperature prior to purification of the milk antagonist. Protein concentration was determined using established techniques (7).

RESULTS

Whole milk was found to antagonize the inhibitory effects of serum upon uropod formation in a dose dependent fashion (Fig. 1). After defatting the milk and removing the acid-insoluble casein, uropod inducing activity comparable to whole milk was present. However, storage of whole milk or decaseinated milk at 4°C for 24 hours rendered the protein inactive.

Since maximal inhibition of uropod formation was achieved with serum concentrations as low as 1% (1), that concentration was chosen for all experiments. Experiments demonstrated that maximum stimulation of peripheral blood lymphocytes in 1% serum occurred when samples were supplemented with 25% milk (Fig. 1).

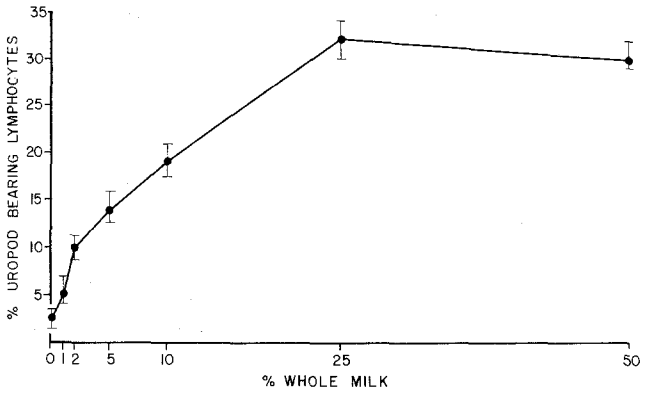

Fig. 1. Antagonist activity of the serum inhibitor of uropod formation to the concentration of whole milk. Serum concentration (1%) was maintained for these experiments.

Differential ammonium sulfate precipitation, Sephadex G-200 chromatography, and DEAE cellulose chromatography yielded greatly purified material while retaining most of the biological activity (Table 1). When milk whey was precipi-

TABLE I

	Protein Concentration mg/ml	% Uropod-Bearing Cells at 25% Milk Equivalent
Whole Human Milk	8.65	36
Defatted Milk	8.3	34
Casein-free Milk	6.46	33
35% $(NH_4)_2SO_4$ (Supernatant)	3.6	33
55% $(NH_4)_2SO_4$ (Pellet)	2.9	30
Sephadex G-200 (pooled void peak)	0.92	24
DEAE Cellulose (pooled peak)	0.05	20

Purification of the Milk Antagonist. All milk samples were collected and stored as described in the materials and methods. Samples were assayed for uropod inducing activity with 25% milk equivalent in the presence of 1% serum. Milk samples past the third step of purification (casein-free milk) were resuspended or subjected to dialysis in 0.15M NaCl buffered with 0.01 M Tris, pH 7.5. Protein concentrations were determined utilizing established techniques (6).

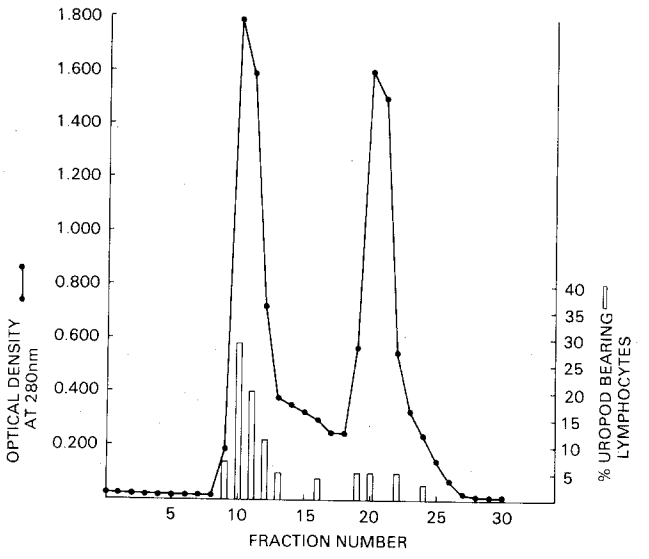

Fig. 2. Sephadex G-200 chromatography of the milk antagonist. The 55% $(NH_4)_2SO_4$ precipitate from 50 ml of pooled milk was applied to a Sephadex G-200 column (2.2 cm x 50 cm) in Tris (0.05M, pH 7.5) buffered 0.15 M NaCl. Fractions (6 ml) were collected and assayed for uropod inducing activity in the presence of 1% serum.

tated with 35% saturated $(NH_4)_2SO_4$, the biological activity remained in the supernatant. By increasing the concentration of the saturated $(NH_4)_2SO_4$ to 55%, the milk antagonist was precipitated with no appreciable loss of activity. Chromatography on Sephadex G-200 demonstrated a large void volume peak which contained the uropod promoting activity (Fig. 2). DEAE cellulose chromatography was effectively employed to further purify the milk antagonist (Fig. 3).

The milk antagonist was rendered inactive by 2x crystallized trypsin (Sigma Chemical Co.)(0.1 mg/55 µg milk antagonist/ml) or by protease (Sigma Chemical Co.)(0.1 mg/55 µg milk antagonist/ml). However, low concentrations (0.1 µg/55 µg milk antagonist/ml) of either proteolytic enzyme had no effect on the milk antagonist.

The effect of heat upon the biologic activity was tested over a broad range of temperatures. Milk samples (1 ml) from the 55% $(NH_4)_2SO_4$ step were kept at 4^oC prior to heating in a water bath for 10 minutes. After heating, samples were placed in an ice bath and later assayed for uropod inducing ac-

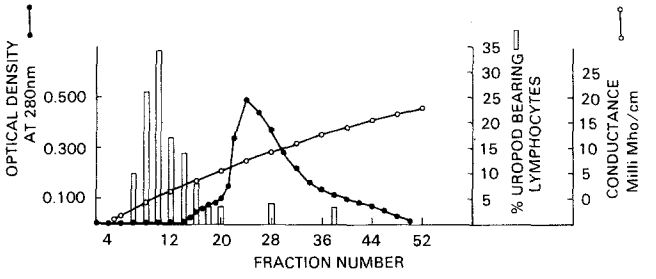

Fig. 3. DE-52 chromatography of the milk antagonist. Sephadex G-200 void volume fractions were pooled and dialyzed against 0.005 M Tris, pH 8.3. Dialyzed milk antagonist was pumped onto a DE-52 column (1.2 cm x 20 cm). The protein was eluted from the column by a linear gradient produced by mixing 250 ml of 0.05 M Tris, pH 8.3, 0 M NaCl with 250 ml of 0.05 M Tris, pH 8.3, 0.45 M NaCl. Fractions (3 ml) were collected and assayed for uropod inducing activity in the presence of 1% serum.

tivity. Biologic activity was virtually unaffected up to 45°C, whereas, heating for 10 minutes at 75°C completely destroyed the activity. This thermal stability profile and the enzyme inactivation data are evidence that the biologically active moiety is a protein(s).

Gel filtration chromatography of the 55% $(NH_4)_2SO_4$ step material was performed on Bio Gel A 15 M to estimate the molecular weight of the milk antagonist. Uropod inducing activity was consistently obtained in the void volume fraction of the column indicating a molecular weight in excess of 15×10^6 daltons (Fig. 4).

Rabbit anti-whole human milk antisera (Cappel Laboratories) was employed for neutralization experiments. The anti-whole human milk antisera was washed on DEAE cellulose to remove the serum inhibitor prior to assaying for neutralization of the milk antagonist. Titration of the milk antagonist with the anti-whole human milk demonstrated a dose response curve with a decrease in biological activity as the antisera concentration increased.

As shown in Figure 5, 1 µl of antisera removed approximately 50% of the activity while 25 µl rendered the milk fraction completely inactive.

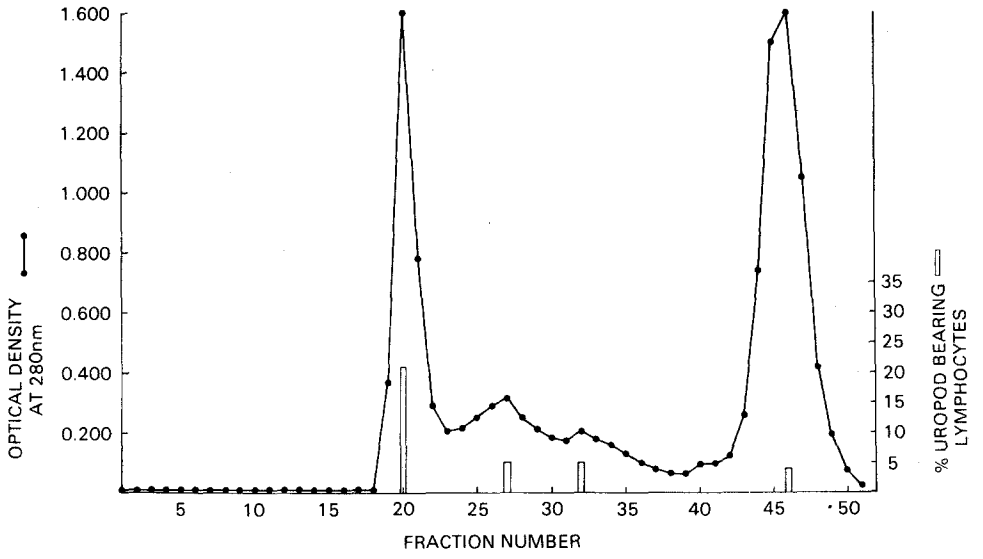

Fig. 4. Bio Gel A 15M chromatography of the milk antagonist. The 55% $(NH_4)_2SO_4$ precipitate from 50 ml of pooled milk was applied to a Bio Gel A 15M agarose column (2.2 cm x 50 cm) in Tris (0.05M, pH 8.3) buffered 0.15 M NaCl. Fractions (6 ml) were collected and assayed in the presence of 1% serum.

DISCUSSION

Little information is available about the factors controlling translational movement of lymphocytes. A high molecular weight protein has been isolated from plasma that inhibits uropod formation in peripheral blood T lymphocytes (1-3,5). We have hypothesized that regulatory factors in blood, tissues, and secretions exist that modulate adherence and deformability of these cells and thus effect their traffic. Since the mammary gland has highly specialized cells including lymphocytes (8,9) that enter from the mother's peripheral circulation and ultimately exit to the baby, a likely place to search for an antagonist to the serum inhibitor would be in breast milk.

This study describes a factor from human breast milk that abrogates the serum inhibition of uropod formation by peripheral blood T lymphocytes. The antagonist was found in the whey fraction but not in the cream or acid precipitable casein fractions. Since the antagonist was inactivated by trypsin,

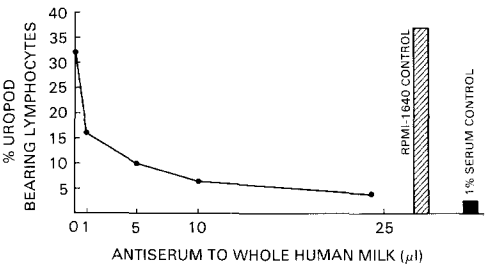

Fig. 5. Antibody neutralization of the milk antagonist. Milk samples 55% $(NH_4)_2SO_4$ step were incubated at room temperatures for 5 minutes with increasing concentrations of anti-whole human milk. Antiserum was washed on DEAE cellulose prior to incubation with milk samples. Uropod bearing cells were determined in the standard assay.

protease, heating at 65-75°C, or treatment with antisera to whole human milk (Fig. 4), it is most likely that the factor is a protein or is complexed to a protein in milk. Using column chromatography, the molecular weight of the antagonist was estimated to be greater than 15 x 10^6 daltons (Fig. 3). This high molecular weight protein or protein complex may be nonacid precipitable casein (10). It is unknown whether the antagonist is related to the acid precipitable casein that is chemotactic for human T lymphocytes (11).

In conclusion, a high molecular weight protein or protein containing complex has been isolated from human milk that abrogates the serum inhibitory protein of uropod formation.

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REFERENCES

1. Schmalstieg, F.C., Rudloff, H.B., Goldblum, R.M. and Goldman, A.S. (1976) J. Reticuloendothel. Soc. 20, 331-339.
2. Schmalstieg, F.C., Rudloff, H.B. and Goldman, A.S. (1980) J. Reticuloendothel. Soc. 28, 67-75.
3. Schmalstieg, F.C., Dickey, W.D., Rudloff, H.B. and Goldman, A.S. (1980) Submitted for Publication.

4. Smith, C.W., Goldman, A.S. (1970) J. Reticuloendothel. Soc. 8, 91-104.
5. Schmalstieg, F.C., Rudloff, H.B. and Goldman, A.S. (1980) J. Reticuloendothel. Soc. 28, 77-87.
6. Goldblum, R.M., Garza, C., Johnson, C.A., Nichols, B.L. and Goldman, A.S. (1980) Submitted for Publication.
7. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
8. Diaz-Jouanen, E. and Williams, Jr., R.C. (1974) J. Clin. Imm. Immunopath. 3, 248-255.
9. Parmely, M.J., Beer, A.E. and Billingham, R.E. (1976) J. Exp. Med. 144, 358-370.
10. O'Neill, G.J. and Parrott, G.J. (1977) Cell. Immunol. 33, 257-267.
11. Parrott, D.M.V., Good, R.A., O'Neill, G.J. and Gupta, S. (1978) Proc Natl. Acad. Sci. U.S.A. 75, 2392-2395.