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INHIBITION OF PHAGOCYTE CHEMOTAXIS BY UTEROGLOBIN, AN INHIBITOR OF BLASTOCYST REJECTION

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Abstract—Uteroglobin, a steroid-dependent secretory protein first discovered in the rabbit uterus during early pregnancy, is a potent phospholipase A_2 inhibitor. We found that uteroglobin also inhibited human and rabbit phagocyte chemotaxis in response to formyl peptide attractants in a dose-dependent manner. Half-maximal inhibition was at 1.2 μM. Uteroglobin did not compete with a formyl peptide for its receptor but inhibited internalization of radiolabeled formyl peptide. Uterglobin appears to inhibit chemotaxis by a mechanism different from that of dansylcadaverine, a well studied inhibitor of endocytosis. Unlike dansylcadaverine, uteroglobin did not have any effect upon the synthesis of phosphatidylcholine or phosphatidylinositol. It is suggested that uteroglobin may protect trophoblastic cells from the defense system of the host not only by binding to antigenic determinants of embryonic cells but also by impairing migration of phagocytes, one of the primary components of the immune defense system. These results may explain why embryonic cells do not elicit an inflammatory response in the uterine endometrium during pregnancy.

It is well known that during normal pregnancy the growth of the embryo in the uterus is not counteracted by the immune defense system of the maternal organism, even though it is allogenic to the mother [1, 2]. Thus, for all practical purposes the mammalian pregnancy is nature's successful allograft. Mukherjee *et al.* [3] suggested that the trophoblastic cells may be protected from the host defense by a protein termed blastokinin [4] or uteroglobin [5] which is synthesized in the uterus by the endometrial epithelium during early pregnancy. Their studies suggested that uteroglobin may bind to antigenic determinants of embryonic cells via a transglutaminase reaction [6]. In this way, uteroglobin is suggested to mask the fetus from immune surveillance and subsequent rejection by the host. Immune surveillance involves the detection and subsequent destruction by phagocytic cells of foreign organisms or cells that have invaded the host. Phagocytes may migrate towards their targets in response to chemoattractants generated at the site of invading antigens. Bacteria, for example, produce attractants related to and including synthetic potent chemotatic tripeptides, such as FMet-Leu-Phe (FMLP¶) [7, 8]. Chemotaxis is generally recognized to occur by a receptor-mediated process. After the attractant binds to the receptor, a chemical signal is delivered which may involve turnover of methylated phospholipids [9]. We were interested in determining whether uteroglobin, in addition to its action in masking the antigens in embryonic cells, could directly affect the activity of the host's phagocytes. Therefore, we studied the effect of this protein upon leukocyte chemotaxis.

We found that uteroglobin inhibited the chemoattractant-stimulated motility of phagocytes. Furthermore, we studied its mechanism of inhibition by determining whether uteroglobin affected binding of the attractant, a formylated peptide, to its receptor; whether it altered internalization of the peptide; and whether it caused changes in lipid metabolism. In addition, we studied the effect of uteroglobin on the motility of a different cell type such as the fibroblasts. Finally, we compared the effects of both dansylcadaverine and uteroglobin upon chemotaxis. Dansylcadaverine is a well studied inhibitor of endocytosis in fibroblasts [10] and is a substrate for transglutaminase [11]. Dansylcadaverine also inhibits endocytosis and chemotaxis in the neutrophil [12]. These effects are associated with marked perturbations of phospholipid metabolism. Since uteroglobin may exercise its protective effects upon the trophoblast via transglutaminase, it was important to determine whether the mechanism of inhibition of chemotaxis by uteroglobin was similar to that of dansylcadaverine.

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¶ Abbreviations: FMLP, *N*-formylmethionyl-leucyl-phenylalanine; FNLP, *N*-formylnorleucyl-leucyl-phenylalanine; TPCK, tosyl L-phenylalanyl chloromethyl ketone; Gey's BSS, Gey's balanced salt solution; Gey's BSA, Gey's balanced salt solution containing 2% bovine serum albumin; LTB_4, leukotriene B_4; and C_{5fr}, complement-derived factor.

MATERIALS AND METHODS

Materials. Uteroglobin was purified from New Zealand rabbits primed with human chorionic gonadotropin (HCG) [6]. Goat anti-uteroglobin antisera was a gift from Dr. David Bullock, Baylor University School of Medicine, Houston, TX. *N*-Formylmethionyl-leucyl-phenylalanine (FMLP) was obtained from Peninsula Laboratories, San Carlos, CA, and *N*-formylnorleucyl-leucyl-[^{3}H]-phenylalanine ([^{3}H]FNLP; 13.5 Ci/mmol) was obtained from the New England Nuclear Corp., Boston, MA. Silicone oil was obtained from the General Electric Co., New York. Leukotriene B_4 (LTB_4) was a gift from Dr. Edward J. Goetzl, University of California. Complement-derived factor (C_5fr) was prepared from guinea pig serum by activating it with endotoxin [13].

Preparation of rabbit peritoneal polymorphonuclear leukocytes and human monocytes. A 90% pure population of activated rabbit polymorphonuclear leukocytes was obtained by lavage 14–16 hr after intraperitoneal injection of 0.2% glycogen (Sigma) in normal saline [14]. The cells were suspended in Gey's balanced salt solution (BSS) with 2% bovine serum albumin (BSA). Frozen human monocytes (35% monocytic cells) in RPM1-1640 medium containing 7.5% dimethyl sulfoxide were supplied by Dr. D. M. Strong of the Naval Medical Research Institute, Bethesda, MD. These cells were thawed and prepared according to the established methodology [15].

Neutrophil chemotaxis assay. The assay utilized the modified Boyden chamber procedure [16]. FMLP (10^{-9} M) was added to the lower well, and cells preincubated with uteroglobin were added to the upper well at a concentration of 2.2×10^6 cells/ml in Gey's BSS containing 2% BSA. A Millipore filter (Millipore Corp., Bedford, MA) with an average pore diameter of 5 μm separated the two compartments. After incubation for 2 hr, the filter was removed, washed and stained with haematoxylin solution (Fisher Scientific Co., Pittsburgh, PA). Viability of the cells was tested by the Trypan blue dye exclusion test. The cells that had migrated to the underside of the filter were viewed in a microscope and counted. The results are expressed as the average number of neutrophils within 10 fields at a magnification of 900× for triplicate samples. The SEM did not exceed 10%

Monocyte chemotaxis assay. Human monocyte chemotaxis was performed with a 48 well microchemotaxis assembly (multiwell chamber) according to the method of Harvath *et al.* [17]. FMLP (25 μl of a 10^{-8} M solution) in Gey's BSS containing 2% BSA was added to the lower well. Human monocytes were added to the upper well (50 μl) at a concentration of 3×10^6 cells/ml in Gey's BSS containing 2% BSA. A polycarbonate filter (5 μm pore size, Nuclepore Corp., Pleasanton, CA) separated the two compartments. After incubation for 90 min, cells were wiped off the top layer of the filter, which was then stained with Diff-Quick (Harleco, Gibbstown, NJ). The cells that had migrated to the underside were counted as described above for neutrophil chemotaxis.

Fibroblast chemotaxis assay was performed in modified Boyden chambers as previously described [18].

Binding of attractant to cells. Neutrophils (4.4×10^6/ml) were incubated with 10 μl of Gey's BSS containing 7.7×10^{-9} M [^{3}H]FNLP (sp. act. 13.2 Ci/mmol) in a final volume of 1 ml of Gey's BSS, pH 7.4, for 1 hr at 4° [19]. In preliminary experiments, we determined that the saturation point of binding was reached at this concentration of the peptide. After incubation, 500 μl of the cell suspension was carefully pipetted into 500 μl of silicone oil in a 1-ml Eppendorf tube and then centrifuged in an Eppendorf centrifuge at 8000 *g* for 30 sec at 4°. The cell pellet was placed in 1 ml of NCS (Amersham Searle) tissue solubilizer containing 34 μl of glacial acetic acid and shaken gently overnight. The solubilized samples were then mixed with 10 ml of Aquasol (New England Nuclear) and counted in a Beckman scintillation counter. The results are expressed as specific binding of labeled peptide where specific binding is defined as the difference between the total binding of labeled peptide at 4° and the non-specific binding, which is defined as the binding of labeled peptide in the presence of a large excess (10^{-5} M) of FMLP. Cells were incubated with labeled peptide at 37° for 30 min in the presence of the protein to determine whether uteroglobin had any effect upon peptide internalization. Then an excess (10^{-5} M) of unlabeled FMLP was added to the incubation mixture. The cells were then processed as described above and counted. The non-displaceable radioactivity is a measure of internalization of the chemotactic peptide.

We employed [^{3}H]FNLP for binding and internalization measurements because use of this ligand gave more reproducible results in our exudate cells than did [^{3}H]FMLP. FNLP is not subject to oxidative degradation as is FMLP [20], and, also, the concentration producing a half-maximal chemotactic response in our system for both ligands was the same (5×10^{-10} M).

RESULTS

Effect of uteroglobin on neutrophil and monocyte chemotaxis. We first determined whether uteroglobin could inhibit the migration of neutrophils and monocytes stimulated with FMLP, a known chemoattractant. Uteroglobin, when preincubated with the cells for 15 min, inhibited FMLP-stimulated migration in a dose-dependent manner (Fig. 1). The concentrations of uteroglobin required to inhibit chemotactic activity by 50% (IC_{50}) were 1.2 and 1.5 μM for neutrophils and monocytes respectively. We have found (not shown) that these concentrations themselves were not chemotactic. We next determined whether antiserum to uteroglobin could overcome its antichemotactic effects. First, antiserum to uteroglobin (1 mg/ml) was incubated at 37° for 15 min with uteroglobin, and then this mixture was incubated with the cells for another 15 min. The cells were assayed for their motile response to FMLP. Preincubation of cells with antiserum alone for 30 min did not have any effect on chemotaxis. Approximately 68% of the inhibitory effect was abolished by the preincubation of uteroglobin with un-

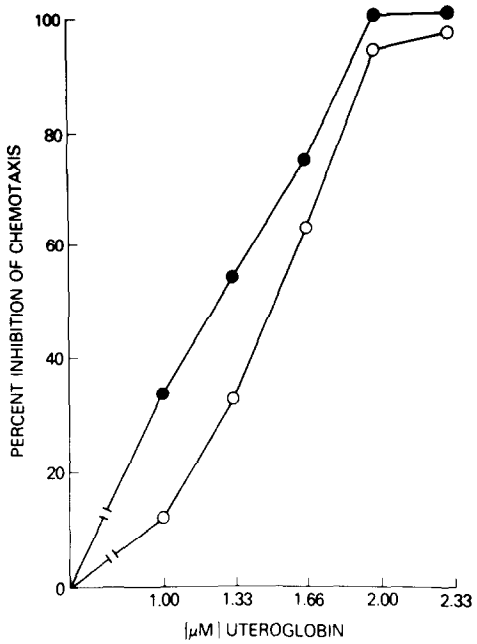

Fig. 1. Inhibition of chemotaxis by uteroglobin. Chemotaxis was assayed as described in Materials and Methods. Each point on the curve is the mean of triplicate values; the SEM did not exceed 10%. One hundred percent chemotaxis was 61 ± 2 cells/field. In the absence of the attractant, 4 ± 1 cells/field were observed. Key: (○) human monocytes, and (●) rabbit neutrophils.

diluted antiserum (Table 1). The antiserum appeared to have some specificity for uteroglobin since antibody fibronectin did not reduce the effect of uteroglobin upon cell motility (not shown). Also, antibodies to the light chain of myosin and to the natriuretic factor did not cross-react with uteroglobin (A. B. M., unpublished data).

We tested whether the effect of uteroglobin was specific to FMLP-stimulated cell migration and whether it affected the motility of a cell type other than the phagocytes. We found that uteroglobin inhibited the chemotactic response to other classes of chemoattractants such as leukotriene B_4 (LTB_4) [21] and a complement-derived factor (C_{5fr}) [22, 23]. At a concentration of 2 μM uteroglobin which produced 100% inhibition of chemotaxis to FMLP, the chemotactic response of neutrophils towards LTB_4 and C_{5fr} was also inhibited markedly (Table 2). The results suggest that uteroglobin acts against the cell and not against its attractants. Uteroglobin did not have any effect (not shown) on migration of fibroblasts toward a potent tumor derived factor from ZR-75-1 cell conditioned medium [24]. Thus, it is likely that uteroglobin specifically affects phagocyte migration.

Reversible effect of uteroglobin upon neutrophil and monocyte chemotaxis. The effect of uteroglobin upon inhibition of cell migration was reversible. When neutrophils and monocytes were pretreated for 15 min with 2.2 μM uteroglobin and washed, the chemotactic response was restored to 96 ± 4% of the control cells. Furthermore, gel electrophoresis (not shown) of the solubilized cell membranes from monocytes exposed to ^{125}I-labeled uteroglobin in the presence and absence of attractant did not indicate

Table 1. Effects of uteroglobin and its antiserum upon neutrophil migration to FMLP

Additions to cells*	Percent inhibition of chemotaxis†
Antiserum	9 ± 1
Uteroglobin (2 μM)	95 ± 3
Uteroglobin + antiserum (1:4)	65 ± 3
Uteroglobin + antiserum (1:2)	43 ± 2
Uteroglobin + antiserum (1:0)	32 ± 2

* Dilutions of antiserum were preincubated at 37° for 15 min with 2 μM uteroglobin after which this mixture was incubated with the cells for another 15 min. Antiserum was diluted with buffer at the ratio of (antiserum:buffer) 1:4, 1:2 and 1:0 and incubated with a constant amount of uteroglobin (2 μM). Undiluted antiserum contained 1 mg protein/ml.

† Chemotaxis was determined in the presence of FMLP (1×10^{-9} M) and in its absence. Maximal chemotaxis was established as 82 cells/field; the control value was 5 cells/field. Results are the mean (±SEM) of triplicate values.

Table 2. Effect of uteroglobin upon neutrophil migration to different attractants*

Attractant	Percent inhibition of chemotaxis†
Leukotriene (10^{-8} M)	86 ± 2
C_{5fr} (1:10 dilution)	100 ± 3
FMLP (10^{-9} M)	100 ± 4

* Cells were preincubated at 37° for 30 min with uteroglobin (2 μM), and chemotaxis was determined in the presence of leukotriene on C_{5fr}. Values are mean ± SEM, N = 3.

† One hundred percent chemotaxis vs FMLP was established as 99 cells/field, and the value using BSA was established as 5 cells/field. One hundred percent chemotaxis vs leukotriene was established as 358 cells/field, and the control value vs using BSA was established as 5 cells/field. One hundred percent chemotaxis vs C_{5fr} was established as 261 cells/field, and the control vs using BSA was established as 5 cells/field.

Table 3. Binding of formylnorleucyl-leucyl-[^{3}H]-phenylalanine to rabbit neutrophils in the presence of uteroglobin*

Concentration of uteroglobin in incubation mixture (μM)	Percent inhibition of binding†
0	0
1.25	0
1.50	13 ± 1
2.00	28 ± 6

* Cells (4.4×10^6/ml) were incubated at 4° for 1 hr with [^{3}H]FNLP (10,000 cpm/10 μl) in the presence (and absence) of uteroglobin.

† Values are the means of triplicate samples ± SEM. Total binding was 5470 ± 320 cpm/2.2×10^6 cells and nonspecific binding was 190 ± 130 cpm/2.2×10^6 cells.

the formation of a covalent cross-link between uteroglobin and monocyte membrane proteins. If transglutaminase were involved in the effect of uteroglobin in inhibiting phagocyte chemotaxis, one might expect a protein species with a larger molecular weight than uteroglobin to have been produced upon incubation of the cells with this protein. These results suggest that uteroglobin does not exert its inhibitory effect upon chemotaxis in our assay by covalently binding to the phagocyte.

Effect of uteroglobin upon binding of chemoattractant to neutrophils. Since uteroglobin inhibited the motility of the cells to FMLP, we determined whether this protein competed with the binding of a formyl peptide chemoattractant to specific cell surface receptors. These studies were carried out at 4° in order to minimize endocytosis. Uteroglobin at a concentration of 1.25 μM, which causes half-maximal inhibition of neutrophil chemotaxis, did not alter the binding of [^{3}H]FNLP (Table 3). Uteroglobin at a concentration of 2 μM, which completely inhibits chemotaxis, inhibited the binding of FNLP by only 28%. Therefore, the inhibition of peptide binding to neutrophil receptors by uteroglobin was not correlated with its effect upon chemotaxis, suggesting that its action is not through competition with the formyl peptide for its receptor. These results on binding also indicate that the principal action of uteroglobin is directed against the cell rather than against the attractant.

Effect of uteroglobin on internalization of peptide by neutrophils. We next tested whether uteroglobin affected the internalization of [^{3}H]FNLP by neutrophils. After incubating the cells at 37° with increasing concentrations of uteroglobin and a saturating amount of radioactive [^{3}H]FNLP, the amount of internalized peptide was measured. Uteroglobin inhibited the internalization of radioactive peptide in a dose-dependent manner (Fig. 2). Furthermore, the inhibitory effects of various concentrations of uteroglobin on internalization paralleled its inhibitory effect upon chemotaxis. The results suggest that uteroglobin may act by inhibiting a step in receptor processing that is required for neutrophil chemotaxis.

Effect of a transglutaminase substrate on neutrophils. Since uteroglobin [25] and dansylcadaverine [11] are substrates for transglutaminase, and since dansylcadaverine has been shown to inhibit endocytosis [26], we compared the effects of uteroglobin and of dansylcadaverine on the migration of rabbit neutrophils to a formylated peptide and upon the internalization of a labeled attractant. Like uteroglobin, dansylcadaverine reduced both the migration of rabbit neutrophils and the internalization of labeled attractant (Fig. 3) and had no

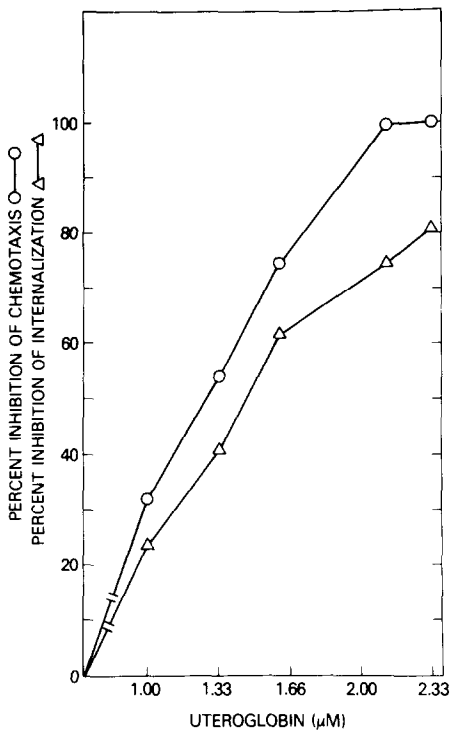

Fig. 2. Effect of various concentrations of uteroglobin upon internalization of [^{3}H]FNLP. Rabbit neutrophils (4.4 × 10^6 cells/ml) were incubated at 37° for 30 min with [^{3}H]FNLP in the presence of uteroglobin. Values are the means of triplicate values; the SEM did not exceed 10%. One hundred percent chemotaxis was 59 ± 5 cells/field. In the absence of a chemoattractant, 7 ± 2 cells/field were observed. Internalized radioactivity in the absence of uteroglobin was 4700 ± 320 cpm/2.2 × 10^6 cells and non-specifically internalized radioactivity was 180 ± 10 cpm/2.2 × 10^6 cells. Key: (○——○) inhibition of neutrophil chemotaxis, and (△——△) inhibition of internalization of [^{3}H]FNLP.

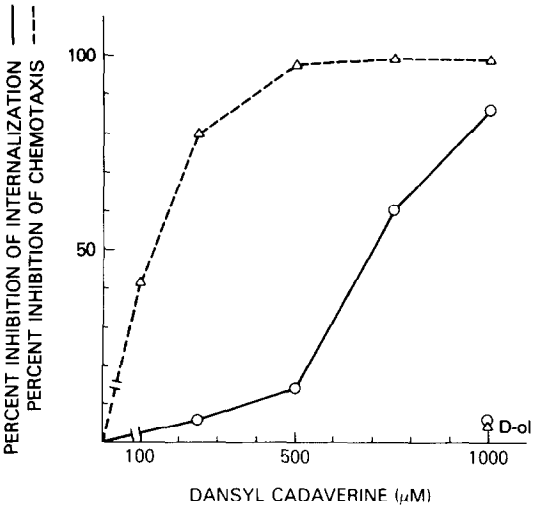

Fig. 3. Effect of dansylcadaverine on neutrophil chemotaxis (△---△) and internalization of the radioactive peptide (○——○). Dansylcadaverol (D-ol) was a control for dansylcadaverine. D-ol would be expected to have the same membrane solubility as the amine, but it did *not* affect cell function. Results are the mean of at least three independent values which varied less than 10%. Internalized radioactivity in the absence of dansylcadaverine was 5030 ± 160 cpm/2.2 × 10^6 cells and non-specifically internalized radioactivity was 280 ± 60 cpm/2.2 × 10^6 cells. One hundred percent chemotaxis was 71 ± 3 cells/field. In the absence of a chemoattractant, 5 ± 2 cells/field were observed.

effect on its binding to cell surface receptors (not shown). Unlike the effects of uteroglobin, those of dansylcadaverine upon chemotaxis and internalization of attractant were not well correlated (Fig. 3). A concentration of 500 μM produced almost a 100% inhibition of chemotaxis but reduced internalization by only 15%. This suggested that uteroglobin and dansylcadaverine may act by different mechanisms.

Another approach to the problem of whether these agents inhibit chemotaxis by similar or different mechanisms was to test the effect of a mixture of the two components on cell migration. We found that addition of concentrations of dansylcadaverine that by themselves were not inhibitory actually reduced the inhibition of cell migration by uteroglobin in a concentration-dependent manner by as much as 52% (Table 4). This result would not be expected if uteroglobin and dansylcadaverine inhibited chemotaxis by the same mechanism. In that case, one would expect to observe an additional inhibition of chemotaxis in the presence of both compounds.

We tested the effect of uteroglobin upon the incorporation of choline into neutrophil phospholipids, since it has been shown previously that dansylcadavarine markedly reduces this reaction [12, 27]. Uteroglobin at concentrations which completely inhibited chemotaxis did not affect the incorporation of choline (data not shown). These results again suggest that uteroglobin and dansylcadavarine inhibit chemotaxis by different mechanisms.

DISCUSSION

Our data suggest that uteroglobin reversibly inhibits the motility of rabbit neutrophils and human monocytes towards a formylated peptide attractant. A rather narrow range of concentrations from no effect (<1 μM) to maximal inhibition (2 μM) occurs for this protein. It is possible that more complex kinetics are involved with the interaction of a protein with the cell than that occurring with a low molecular weight inhibitor. Antibody to uteroglobin blocked the inhibitory effect of uteroglobin. Uteroglobin did not appreciably inhibit formyl peptide binding to its cell surface receptor, but it did inhibit its internalization at concentrations comparable to those required to inhibit chemotaxis. The data also suggest that uteroglobin may be a general inhibitor of leukocyte chemotaxis, since it prevented a number of chemically different attractants from stimulating migration in neutrophils.

Table 4. Effect of dansylcadavarine on the inhibition of neutrophil chemotaxis by uteroglobin

Additives*		
Uteroglobin (μM)	Dansylcadaverine (μM)	Percent inhibition of chemotaxis†
2		100
	25	0
2	5	87 ± 3
2	10	61 ± 1
2	25	52 ± 4

* Rabbit neutrophils (2.2×10^6 cells/ml) were first incubated with dansylcadaverine at 37° for 15 min and then with uteroglobin for another 15 min.

† Chemotaxis was performed in the presence of FMLP (10^{-9} M) and in its absence. Results are the means of three values ± SEM. Maximal chemotaxis was established as 82 cells/field. The control value for chemotaxis was 5 cells/field.

Uteroglobin was 250 times more potent in inhibiting chemotaxis than dansylcadaverine, an inhibitor of endocytosis. It has been reported that dansylcadaverine [11] as well as uteroglobin [25] are substrates of transglutaminase. Both substrates did not have any effect on the binding of the chemoattractant to cell receptors, but they did inhibit the internalization of the chemoattractant. Recently, it has been shown that dansylcadaverine inhibits phospholipid synthesis by both the transcholination and transmethylation pathways but stimulates the synthesis of phosphatidylinositol in rabbit neutrophils [12]. These effects could well perturb membrane lipid turnover and thus affect chemotaxis. Uteroglobin, however, did not have any effect on phosphatidylcholine synthesis. It, therefore, may inhibit cell behavior by a different mechanism from that of dansylcadaverine. This suggestion is supported by the fact that addition of dansylcadaverine to uteroglobin-treated cells reduced the inhibition of cell migration by uteroglobin in a concentration-dependent manner. This antagonism was markedly different from the additive effects that one might expect if the two agents inhibited chemotaxis by the same mechanism.

Uteroglobin does not appear to depend upon the action of transglutaminase for its inhibitory activity since we could not demonstrate the formation of a covalent link between uteroglobin and cells. In the case of the trophoblasts, on the other hand, uteroglobin may exert its protective effects on these cells via transglutaminase [3, 6, 28].

These studies have identified uteroglobin as another protein such as migration inhibitory factor (MIF) and leukocyte inhibitory factor (LIF) which have anti-chemotactic effects [29, 30]. The action of uteroglobin may be specific for phagocytes since it did not affect fibroblast motility. In addition, a unique feature of uteroglobin appears to be that it not only masks the antigenic determinants of cells such as trophoblasts from recognition by the host's defense system but it also impairs directly the phagocyte chemotactic response.

A protein having immunologically identical features to uteroglobin has been found in human endometrium, tracheobronchial epithelium, and the prostate [31, 32]. Also a rat seminal vesicle protein is immunologically cross-reactive to uteroglobin [33]. Recently, it has been demonstrated that uteroglobin is a potent phospholipase A_2 inhibitor [34]. Thus, the protein may reduce cellular prostanoid levels by reducing the level of arachidonate which is the substrate for prostanoid synthesis. It has been reported that intracellular phospholipase A_2 (PLA_2) functions as a mediator of various cellular functions including chemotaxis of leucocytes [9] and mitogenesis [35]. Therefore, it is reasonable to expect that uteroglobin, being a potent PLA_2 inhibitor, will

affect the chemotaxis by neutrophils and monocytes as shown in the present study. Further investigations on the mechanism of inhibition of chemotaxis by uteroglobin may elucidate some of the processes responsible for the survival of the implanting embryo in an allogenic environment of the maternal organism.

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