Lectin-Induced Mucus Release in the Urn Cell Complex of the Marine Invertebrate *Sipunculus nudus* (Linnaeus)

Abstract. *The mucociliary urn cell complex of the marine coelomate* Sipunculus nudus *secretes mucus 4 to 5 minutes after being exposed to* Lotus tetragonolobus *and* Ricinus communis *I agglutinins. Surface binding of both lectins is confined to the secretory area of the urn cell complex and, like the release of mucus, is inhibited by the specific saccharides* L*-fucose and* D*-galactose or by incubation in* L*-fucosidase and* D*-galactosidase. Mucus secretion may therefore be initiated by the interaction of mucus-releasing stimuli with fucosyl or galactosyl residues of specific membrane receptors.*

Urn cell complexes are mucociliary constituents of the benthic coelomate worm *Sipunculus nudus* (Linnaeus) (*1*, *2*) and consist of an anterior vesicle cell and a posterior basal cell. Mucus is secreted from the basal cell in response to a variety of stimuli, such as foreign cells (*3*), epithelial secretions, and heated autologous and heterologous serums (*4*). Urn cell complexes play an important role in the sipunculid immune response to infectious agents, since the mucus that they secrete can recognize and trap foreign cells, which are then phagocytized and lysed by other coelomic fluid cells (*4*). Bang and Bang (*4*) extensively described the urn cell complex and suggested that mucus secretion in urn cells is controlled by a heat-stable macromolecular factor in serum. Serum-induced mucus release has also been proposed for a variety of mammalian mucous epithelia under certain physiological (*5*) and pathological (*6*) conditions. The mechanisms by which these serum factors stimulate discharge of mucus in invertebrate (and vertebrate) cells are unknown.

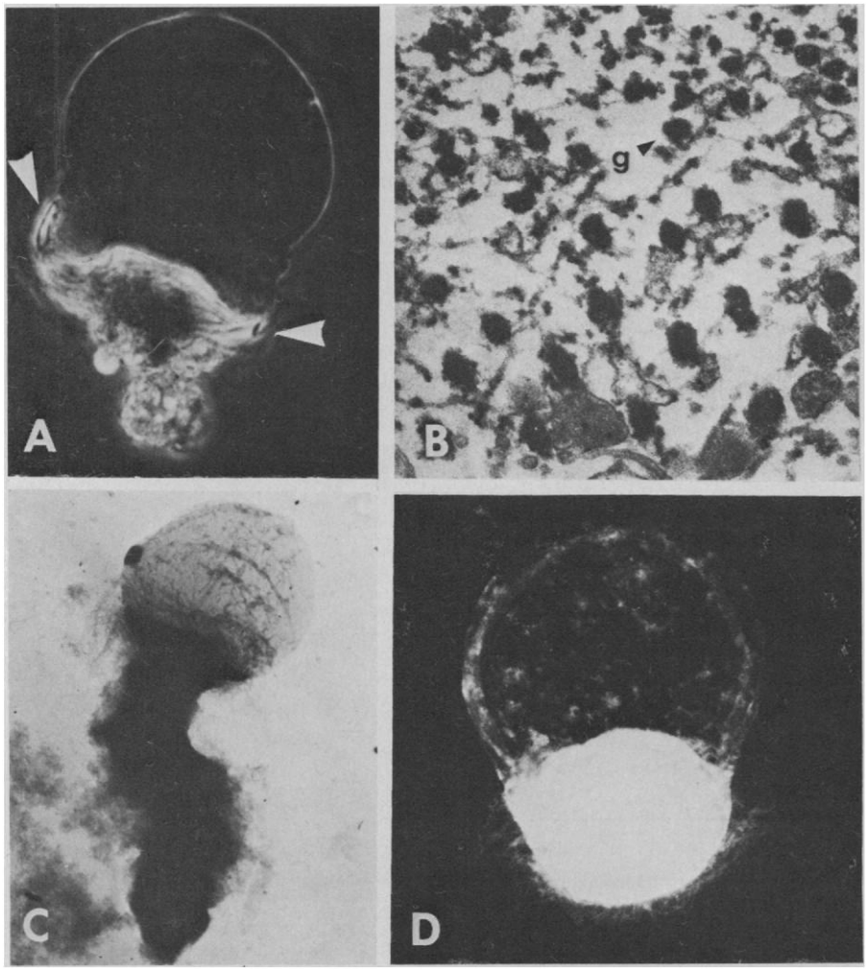

Fig. 1. (A) Phase-contrast micrograph of an unstimulated urn cell complex. This freely swimming constituent of the coelomate fluid of *S. nudus* is composed of a vesicle cell that fits into a basal cell (arrows), much as an acorn fits into its cup. Cellular debris and other coelomic fluid cells (amebocytes) adhere to the inferior aspect of the basal cell (×800). (B) Electron-dense, secretory granules (*g*) in an unstimulated urn cell complex. The secretory function of these granules is suggested by the observations that they display the histochemical properties of mucus glycoproteins and that they decrease in number or disappear after mucus release (*10*) (× 32,000). (C) Longitudinal view of an urn cell complex after 30 minutes of exposure to LTA (200 μg/ml). Mucus originates in the inner aspect of the basal cell and is displaced by new secretion, thus forming the characteristic secretory tail (×250). (D) Immunofluorescence pattern of a formalin-fixed, unstimulated urn cell complex after incubation with fluorescein isothiocyanate-conjugated LTA. The lectin binds uniformly to the surface of the basal cell but not to the vesicle cell (×400).

While testing the influence of various mammalian serums on the secretory activity of urn cell complexes, I noted (*7*) that these cells stop secreting mucus when they are transferred to serum-free, fresh seawater (FSW). This implied that continuous interaction between urn cell complexes and mucus-releasing stimuli may be required for sustained mucus secretion. The present study tested the hypothesis that mucus secretion in urn cell complexes depends on a contact-mediated activation of their plasma membrane. This hypothesis suggests that lectins, which possess specific affinity for membrane components and the ability to send information across membranes (*8*), might cause a detectable secretory response in urn cells.

Urn cell complexes were obtained from fluid samples (1 to 2 ml) microaspirated from the coelomic cavity of adult *S. nudus*. Cells were incubated (30 to 60 minutes) and stored (up to 6 weeks) at 4°C. Cells were incubated in FSW alone (*p*H 7.8) or in FSW containing one of the following lectins or lectin-containing biological fluids: *Lotus tetragonolobus* agglutinin (LTA), *Ricinus communis* I agglutinin (RCA_I), soybean agglutinin (SBA), concanavalin A (Con A), wheat germ agglutinin (WGA), and *Limulus polyphemus* hemolymph. Urn cell complexes reacting to lectins produce characteristic mucus tails whose length is commensurate with the mucus-stimulating effectiveness of the lectin and the duration of stimulation (*4*). The tails were measured under phase-contrast microscopy (×10) through a calibrated cross-line ocular disk. The number of urn cell complexes that were examined in each experimental group varied from 45 to 200, with an average of 90.

Complexes (Fig. 1A) incubated in FSW alone exhibited rapid ciliary motion and retained numerous "secretory" granules (*9*) during a 30- to 60-minute incubation period (Fig. 1B); their secretory

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activity was minimal or absent, as evidenced by a mucus tail length of only 1.73 ± 0.49 μm (mean length ± standard error). Significant mucus secretion was not caused by WGA, Con A, and *L. polyphemus* hemolymph (Fig. 2A). Their main effect was to agglutinate urn cell complexes and, as in the case of Con A, to stop ciliary motion at concentrations greater than 10 μg/ml. Soybean agglutinin, a lectin specific for *N*-acetyl-D-galactosamine but also displaying some affinity for D-galactose (*8*), elicited a modest secretory response. In contrast, L-fucose (LTA) and D-galactose (RCA_I) binding proteins induced the release of distinct tails of mucus after 30 to 60 minutes of incubation (Figs. 1C and 2A). These tails were dispersed by the mucolytic agent *N*-acetylcysteine (1 mg/ml, 30 minutes). In contrast to serum-induced mucus secretion, which is apparent within 2 minutes of exposure and maximal after just 30 minutes (*7*), lectin-induced mucus release was observed 4 to 5 minutes after incubation and increased gradually during the ensuing 30 minutes and markedly during the next 30 minutes. Stimulation of mucus release by LTA was not observed at concentrations of less than 30 μg/ml; as concentrations were increased above 200 μg/ml, longer and more numerous mucus tails were seen. At the strongest concentration used, 800 μg/ml, many tails were released that measured 160 μm in length or more (Figs. 1C and 2B). These concentrations were not associated with cell agglutination or interference with ciliary motion. The time necessary for initiation of mucus release was not dependent on the dose, since 4 to 5 minutes were needed at all concentrations of LTA. The dose-related effects of RCA_I were not tested. Binding of both LTA and RCA_I was confirmed by experiments in which urn cell complexes were incubated separately with the fluorescein isothiocyanate conjugates of these lectins (*10*), and was restricted to the basal (secretory) cell (Fig. 1D). Surface binding and secretory response were both inhibited by the specific saccharides L-fucose and D-galactose and by first incubating the complexes in L-fucosidase and D-galactosidase (Fig. 2B). In addition, integrity of the intracellular microfilament system was required for lectin activity, since incubation of urn cell complexes with cytochalasin B considerably reduced the stimulatory effect of LTA (Fig. 2B).

While examining the effects of *p*H on mucus secretion by urn cell complexes, I have noted that weak organic acids and bases affect their secretory processes. Such compounds penetrate the cell membrane in their undissociated form and dissociate intracellularly, thereby altering the internal *p*H (*11*). I thus assumed that these compounds similarly affected the internal milieu of urn cell complexes, although no direct measurement was made of their intracellular *p*H. Figure 2C shows the distinct mucus tails formed by urn cell complexes 4 to 5 minutes after exposure to FSW containing sodium acetate (500 m*M*, *p*H 7.5), an agent that should lower the intracellular *p*H. In contrast, when the *p*H within these cells was artificially raised by FSW containing NH_4OH, *p*H 9.0, mucus tails were not formed. Furthermore, the secretory response of NH_4OH-treated urn cell complexes to serum was significantly suppressed (Fig. 2C). Seawater that was rendered acidic or basic with HCl or NaOH did not have the same effect as FSW containing sodium acetate or NH_4OH (Fig. 2C).

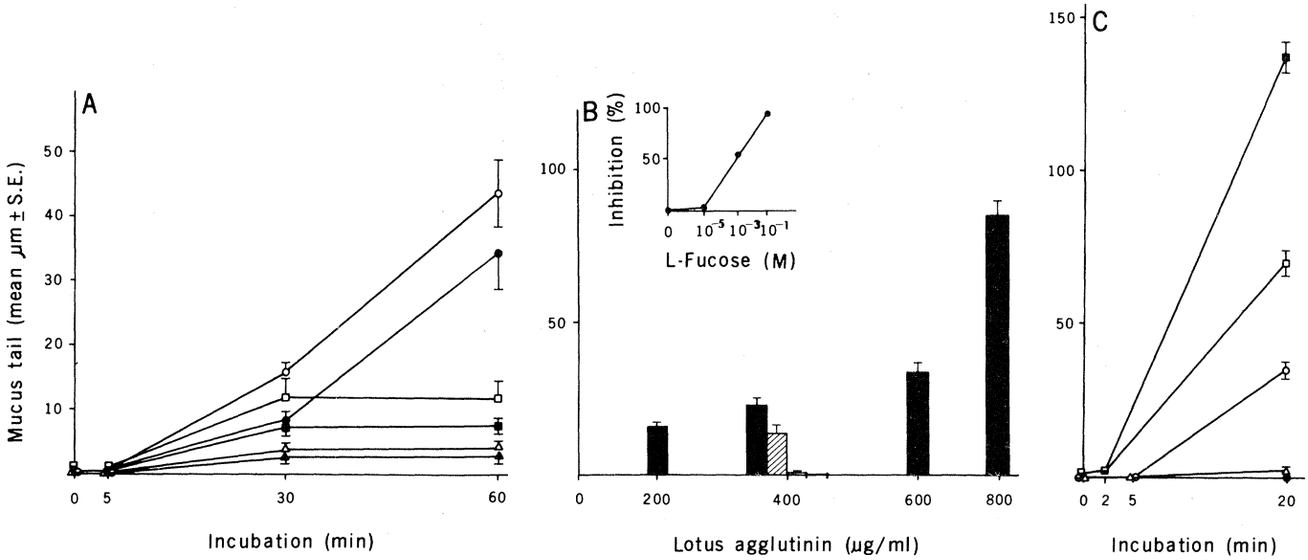

Fig. 2. (A) Influence of lectins on mucus release by incubated urn cell complexes. Lectins [*Lotus tetragonolobus* (○), *Ricinus communis* I (●), soybean (□), and wheat germ (△) agglutinins] were used at a concentration of 200 μg/ml, except for Con A (■), which was used at 10 μg/ml. These doses were selected on the basis of preliminary studies, which had shown only mild stimulation of mucus release with LTA and RCA_I concentrations less than 100 μg/ml, and appreciable cytotoxicity (but no mucus release) with Con A concentrations greater than 30 μg/ml. *Limulus* hemolymph (▲) was used at a dilution ratio of 4:5. All lectin solutions were adjusted to *p*H 7.8. *Lotus* (LTA) and *Ricinus* (RCA_I) lectins are, with the possible exception of SBA, the only lectins that induce the formation of significantly distinct ($P < .001$, Student's *t*-test) mucus tails. *Lotus* agglutinin stimulates mucus release more effectively ($P < .001$) than RCA_I after 15 minutes, but the effects of both lectins are statistically indistinguishable after 60 minutes of incubation. Control urn cell complexes incubated in FSW alone (*p*H 7.8) produce mucus tails 1.73 ± 0.49 μm in length (not plotted here). (B) Dose-response histogram of LTA-induced mucus release (solid bar). The secretory response of urn cell complexes to LTA is nearly abolished by first incubating them for 10 minutes in L-fucosidase (1.0 U/ml, Sigma Chemical Co.) (⊢) and is significantly diminished ($P < .05$) by first incubating them for the same length of time in cytochalasin B (10 μg/ml, Calbiochem) (hatched bar). Extrusion of mucus tails is significantly suppressed ($P < .001$) when the complexes are exposed to LTA with binding sites previously saturated by incubation for 1 hour at 37°C with L-fucose ($10^{-1}M$) (empty bar). Mucus release initiated by a 5-minute exposure to LTA (800 μg/ml) is proportionally reversed by a further 25-minute incubation in increasing concentrations of the specific saccharide (inset). A similar inhibition and reversal of mucus release induced by RCA_I occurs when urn cell complexes are exposed to D-galactose or D-galactosidase (not shown). (C) Mucus release by cultured urn cell complexes after exposure to sodium acetate or NH_4OH. Secretion is significantly stimulated ($P < .001$) by sodium acetate (○) but not by NH_4OH (●) or FSW alone (△), *p*H 5.3. The response of cells first incubated in FSW to a known mucus-stimulating agent (*7*) such as heated rabbit serum (■), is also markedly suppressed ($P < .001$) by a 20-minute incubation in NH_4OH (□).

Previous research on urn cell complexes (*3, 4*) suggests that the mucus they release contributes to the host's protection against heterologous agents, including bacteria and other foreign cells. This report shows that one of the possible controlling mechanisms of mucus release could involve LTA and RCA_1-sensitive fucosyl and galactosyl residues of secretory receptors in the basal cell membrane of urn cell complexes. Internalization of these receptors (*12*) may trigger a cytoplasmic response leading to hypersecretion of mucus. This response may be intracellular acidification, although the possibility (*13*) that an altered *p*H may also induce the release of mucus-stimulating substances by other coelomic cells ("amebocytes") cannot be ruled out. Interestingly, increases in membrane fluidity and ionic permeability are among the earliest molecular events associated with lectin binding (*8*), and theoretically this phenomenon may influence the intracellular *p*H. Connections between intracellular *p*H and the turning on of cellular activities have been described in other systems (*12*). Some of the mucus-stimulating factors described in physiologic and pathologic serums, including poorly characterized macromolecular components (*4, 5*), antigen-antibody complexes, and cationic polypeptides (*6*), could interact with membrane receptors of mucous cells in a manner similar to that of lectins. Thus, lectins used as probes should be helpful in assessing the specificity of mucus-stimulating substances that operate under physiologic and pathologic circumstances.

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10. Fluorescein isothiocyanate-conjugated LTA and RCA_1 were obtained from Sigma Chemical Co. and used at a concentration of 200 μg/ml in FSW adjusted to *p*H 7.8. Formalin-fixed urn cell complexes were incubated for 10 minutes, rinsed three times in FSW, and observed under a Leitz Ortholux microscope, with the appropriate filters being used for fluorescein fluorescence. Controls contained $10^{-1}M$ L-fucose or D-galactose in staining and washing solutions. Photomicrographs were taken with Kodak Tri-X (ASA 400).
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Calcium Dependence of Toxic Cell Death: A Final Common Pathway

Abstract. *Primary cultures of adult rat hepatocytes were treated in the presence or absence of extracellular calcium with ten different membrane-active toxins. In all cases more than half the cells were killed in 1 to 6 hours in the presence but not in the absence of extracellular calcium. An effect of calcium on the primary mechanism of membrane injury by any of the agents cannot be implicated. Viability, as determined by trypan blue exclusion correlated well with other indices of viability such as plating efficiency and the hydrolysis of fluorescein diacetate. It is concluded that the cells are killed by processes that involve at least two steps. In each type of injury, disruption of the integrity of the plasma membrane by widely differing mechanisms is followed by a common functional consequence involving extracellular calcium, and most likely representing an influx of calcium across the damaged plasma membrane and down a steep concentration gradient. This later step represents, or at least initiates, a final common pathway for the toxic death of these cells.*

All cells in the body are bathed in a fluid very rich in Ca^{2+} ($10^{-3}M$), while intracellular Ca^{2+} concentrations are much lower, on the order of $10^{-6}M$ (*1*). The electrical potential across the plasma membrane of these cells tends to drive Ca^{2+} into them. Such a large electrochemical gradient is maintained by the relative impermeability of the plasma membrane to Ca^{2+} and by active extrusion. Damage to the plasma membrane by any one of a number of different mechanisms would disrupt this permeability barrier with a consequent influx of Ca^{2+}. Calcium ions are biologically very active, being capable of considerable disruption of metabolic order. Liver cells lethally injured by several quite different chemical toxins exhibit marked alterations in intracellular Ca^{2+} homeostasis with large accumulations of Ca^{2+} (*2*). Although consistent with an active role of Ca^{2+} in toxic liver cell death, such accumulations of Ca^{2+} could also be explained as simply the passive equilibration of Ca^{2+} concentrations in cells lethally injured by different and as yet unexplained mechanisms.

To explore more directly the role of Ca^{2+} in toxic cell death, we evaluated the dependence on extracellular Ca^{2+} of the toxic cell death of primary cultures of adult rat hepatocytes. The results (Table 1) show an absolute requirement for extracellular Ca^{2+} in the killing of these cells by ten different toxins which, although varying widely in chemical composition, share two characteristics. First, none of the agents seems to need metabolic activation to a proximate or ultimate toxin. Second, all of the ten compounds are capable of interacting with cell membranes; indeed, many are known hemolysins. The compound A23187 is a Ca^{2+} ionophore whose specific biological activity is to create Ca^{2+} channels and thereby overcome the permeability barrier represented by plasma membranes (*3*). Lysolecithin is known to react strongly with membranes (*4*). It is a hemolysin (*5*) whose mechanism of action is related to its ability to solubilize the lipid components and thus induce a change in the molecular organization of membranes (*6*). Amphotericin B is a polyene macrolide antibiotic that interacts specifically with sterols in cell membranes (*7*). Complexes of amphotericin B and sterol molecules are believed to form hydrophilic pores in the membranes, allowing increased ion permeability. Melittin is the main cytolytic component of bee venom (*8*). The first 20 amino acids of this peptide are mostly apolar, whereas the C-terminal hexapeptide is polar and highly basic (*9*). Melittin interacts with phospholipids both in liposomes and in biomembranes (*10*), causing a decrease in the mobility of the fatty acid chains and a rearrangement of the phospholipid head groups. Phalloidin is a bicyclic heptapeptide isolated from the toxic mushroom *Amanita phalloides* (*11*). It is a very specific hepatotoxin

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