HYPERSECRETION OF MUCUS INDUCED IN ISOLATED NON-INNERVATED CELLS (1)

by

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Résumé

Le liquide cœlomique (sang) de l'Invertébré marin cœlomate *Sipunculus nudus* contient des milliers d' « urnes » nageantes. Ces éléments d'origine épithéliale sécrètent des queues de mucus grâce auxquelles les débris et les bactéries sont extraits du sang et phagocytés par des amoebocytes. Le processus sécrétoire peut être suivi *in vitro* au microscope, dans le sang.

On a constaté que des urnes vivantes sécrètent des quantités excessives de mucus quand le Siponcle est infecté spontanément ou expérimentalement par certaines bactéries, mais pas par toutes.

Une hypersécrétion nette était provoquée expérimentalement dans les urnes quand les bactéries étaient ajoutées au sang *in vitro* après que l'hôte ait reçu en injection une solution de toxine du *Vibrio cholerae*. Une sécrétion nettement excessive a été provoquée dans les urnes d'un *Sipunculus* en bonne santé quand elles étaient exposées à des bactéries qui avaient été d'abord mélangées à du sérum (plasma) de Siponcle infecté par des bactéries. Quand ces sérums étaient chauffés à 84-98 °C, une rapide excrétion continue de mucus était provoquée immédiatement après l'addition de bactéries. Dans tous les cas, les urnes n'avaient pas auparavant hypersécrété quand elles étaient exposées aux mêmes bactéries.

Des urnes maintenues dans deux préparations stériles de leur propre liquide cœlomique, séparées des autres cellules sanguines pendant sept à quinze jours respectivement, hypersécrétaient du mucus quand elles étaient exposées à certaines bactéries.

Introduction

Sipunculus nudus (fam. Sipunculoidea) is a cigar-shaped marine invertebrate coelomate about 12 centimeters long. Its undivided coelomic cavity contains 20 ml or more of fluid (blood). Among the blood cells is a mucociliated epithelial cell system which is unique in that it functions when completely disjoined from the epithelium: the bicellular « urn cells » which act to remove foreign debris and dead cells from the fluid (Cantacuzène, 1928). Urn cells originate in the lining of the cœlom and pinch off to become free-swimming (Selenski, 1922). Each individual comprises a vesicular anterior cell and a

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ciliated mucus-secreting base or tail cell (Bang & Bang, 1962) Fig. 1, b). Thousands of urns dart about in the blood creating minature ciliary currents which fling dead or foreign particles onto the mucous tail where they become stuck, to be phagocytosed by amebocytes. Normally each load of debris is periodically shed and a new mucous tail is secreted at a slow and regular rate. Certain foreign substances, however, will induce moderate to extensive hypersecretion of mucus in the urn cell population.

Background

Cantacuzène (1928) reported that various degrees of blood cell agglutination in which urns were involved were induced by repeated bleeding of *Sipunculus,* by injection *in vivo* with foreign substances including bacteria, and by spontaneous bacterial infection. He also observed that if whole blood was allowed to stand in a sterile test tube, the heavier blood cells settled to the bottom and left a clear supernatant in which swimming urns were the only cells, and that such urn preparations remained viable and free of bacterial infection for two days. He obtained plasma free of urns, by filtering the supernatant through Chardin paper.

Confirming these observations, we found (1962) that urns stayed alive in their own sterile supernatant for as long as three weeks, that injections of pilocarpine and other foreign substances, including marine bacteria, into a *Sipunculus* induced briefly accelerated mucous secretion in urns. However, if cloudy suspensions of living vibrio (*Limulus* sp.) were injected into sipunculus or added to whole blood *in vitro,* rapid continued mucous secretion was induced in urns (1965).

During two months at Roscoff last summer (1969) we studied means of inducing rapid continuous hypersecretory response in urns isolated from other blood cells and maintained in their own supernatant fluid (supernatant urns).

Materials and methods

Methods of collecting and maintaining *S. nudus,* procedures for injection *in vivo* and for observing urns *in vitro,* separation of urns from other blood cells, and urn morphology have been described previously (Bang & Bang, 1962, 1965).

Sipunculus can be directly injected with foreign substances through a small caudal depression which has a squamous epithelium using a fine (#24) syringe needle; at the same site, using an #18 or #20 needle, whole blood can be withdrawn. Urn secretion can be observed *in vitro,* in either a light or a phase microscope, by placing a few drops of blood on a slide and slip-covering lightly (the slip-cover must be more or less floating) so that urns can swim freely.

In preparations of whole blood, the secretory process is often obscured by the dense populations of other cells, but if about 5 ml of blood are withdrawn by sterile procedures and placed in a sterile test tube, the heavy cells soon settle and leave a clear supernatant;

this can in turn be subdivided into a series of small sterile tubes. The secreted tails of individual urns are then clearly visible. Supernatant urns can also be derived from smaller amounts of blood by slow centrifugation for two minutes. It is convenient to use such

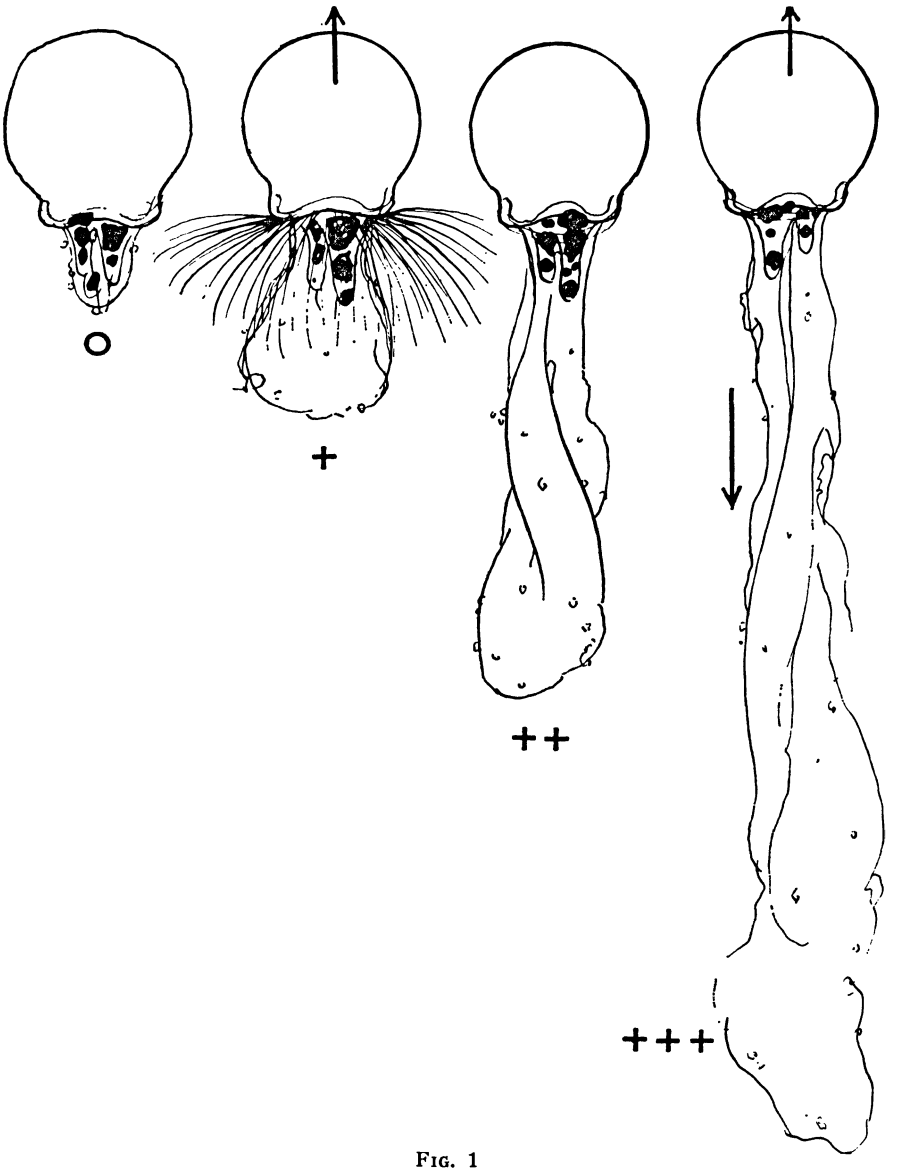

FIG. 1

Diagrammatic representation of amount of excess mucus excreted by urn cells in particular experiments. Cilia are represented only in urn second from left. Black spots indicate secretory areas which stain with neutral red. Arrows on vescicle show direction in which organelle swims; arrow on mucous tail of urn on right shows direction of mucous flow. Degree of secretion + is brief, temporary; ++ may be either a temporary excess which then stops, or the slow, steady increment typical of some responses to infection; +++ represents experimentally induced very rapid, continuing excretion which may persist for several hours or until cell death.

preparations to obtain sera free of urns; the supernatant from 1 ml of whole blood, after being passed through Chardin filter paper, yields about 0.25 ml of cell-free sera.

In this report, the relative amount of mucus secreted by urns will sometimes be expressed symbolically : 0, +, ++. and +++, as illustrated in Fig. 1. Only continuous, rapid secretion is designated +++. It is necessary to recognize that while these degrees of secretion are quite sharp in urns from freshly collected healthy *Sipunculus*, they are less incisive in animals maintained for weeks or months in the laboratory. The 0 and +++ responses, however, are distinct.

Cholera toxin solutions for experiment #4 were freshly prepared on the day of use, from commercially obtained crude dried toxin known to contain less than 0.5 p. 100 endotoxin; 8 p. 100 solutions were prepared in boiled cooled seawater.

There were two sources of bacteria. One, used as the stock suspension, comprised a mixture of unidentified Gram-negative marine bacteria grown on agar plates and prepared as cloudy suspensions in boiled cooled sea water. The other was a Gram-negative bacterial population which developed as a contaminant in an 11 day old refrigerated solution of 8 p. 100 cholera toxin. These bacteria induced ++ to +++ hypersecretion in urns *in vitro* from almost any source animal.

Preliminary

Bacteria are known to stimulate mucous secretion in epithelial cells in many animal systems. The cholera vibrio, for example, induces rapid hypersecretion of mucus in the gut of man and experimental animals. We first explored the comparative effects of bacterial suspensions and of solutions of crude cholera toxin on stimulating mucous secretion in urns *in vitro*.

A series of tests in which cloudy suspensions of the stock Gram-negative marine bacteria were added to several drops of whole blood, (whole coelomic fluid) or to supernatant urns from many individual animals, failed to show significant effects. On similar *in vitro* preparations, an 8 p. 100 solution of crude dried cholera toxin was equally ineffective, as were combinations of toxin and bacteria. Quite suddenly, when one drop of the toxin solution which had been refrigerated for 11 days was added to 4 drops of whole blood, urns began rapid continuous hypersecretion. The preparation was found to be grossly contaminated with bacteria. For the remainder of the summer, urns from nearly any source animal, whether in whole blood or in their own supernatant serum, consistently responded to this toxin-bacterial mixture. Cloudy suspensions of derivatives of these bacteria grown on agar plates did not evoke the response.

Results

1) *Hypersecretion in long-maintained supernatant urns.*

The toxin-bacterial mixture was used to determine whether urns, maintained for some time in their own supernatant serum, would still

to, respectively, the 7th and 15th days. The percentage of responsive urns decreased steadily after the 4th day, and the diameter of the stream of excreted mucus progressively diminished after the 6th day, but bacteria were trapped in the secretion throughout. It should be be able to hypersecrete. Supernatant urns from two *Sipunculus* were subdivided into a series of 0.5 ml sterile preparations. At intervals up to 7 and 15 days respectively, one drop of the toxin-bacterial mixture was added to an urn preparation from each animal. As shown in Table I, the urns showed ++ to +++ hypersecretion up emphasized that in these preparations the normal cellular components other than urns were absent from serum. Breakdown products of urns were probably present.

TABLE I

Secretory responses of urns maintained in supernatant sera

Days after prep.	*Sipunculus* # 3	*Sipunculus* # 4
1		+
2		++
3	++	
4	++	++
5	++	
6	++	
7	++	++
8		++
9		++
11		++
13		+++
14		++
15		+++

Supernatant urns from *Sipunculus* # 3 and # 4 were subdivided into 5 and 10 small sterile test tubes respectively. Into each 0.3 ml preparation, 1 drop of toxin-bacterial mix (using a # 24 needle) was added on the successive days indicated in the left hand column. Positive responses of urns up to 7 and 15 days are shown symbolically.

It was puzzling that this toxin-bacterial mix could induce, in supernatant urns, an effect similar to acute bacterial infection in nature, while neither freshly prepared toxin nor stock bacteria alone could do so. This suggested that the toxin-bacterial interaction substituted for a stage in the natural infection in which a stimulus to hypersecretion was released into the coelomic fluid in response to pathogenic bacteria. The idea was tested in supernatant urns of normal animals.

2) *A serum factor plus bacteria.*

Although the blood of newly collected *Sipunculus* is usually free of bacteria, the animals may develop spontaneous bacterial infections in the laboratory, as they presumably do in nature. While +++ amounts of urn hypersecretion were not encountered in spontaneous infections, very occasionally an infected animal's blood was slippery with mucus, and urns were found to be producing new ++ tails at a steady, if not rapid, rate, indicating that some factor was present

in the blood which continued to evoke significant hypersecretion. Whether this factor might be able to induce hypersecretion in the urns of an uninfected animal in the presence of stock bacteria was tested in freshly prepared supernatant urns from nine healthy animals. First, the supernatant urns of each of the nine animals were tested for response to stock bacteria. None showed any increased mucus secretion. Then from six animals which currently had urns producing ++ hypersecretion, filtered sera were obtained from the supernatant fluid of 1 ml of centrifuged blood. Filtered sera of three apparently normal animals were prepared as controls. To each .25 ml of sera, one drop of stock bacterial suspension was added, and about 20 minutes

TABLE II

Responses of supernatant urns
to sera of normal and infected *Sipunculus* plus bacteria

Serum source animals : secretory state of urns in whole blood	To bacteria alone	To bacteria in sera, and added bacteria
0	0	0
0	0	0
0	0	0
++	0	+
++	0	++
++	0	++
++	0	++
++	0	++
++	0	+++

The left hand column shows the amount of excess secretion in urns in whole blood samples of 3 healthy control animals (0), and 6 bacterially infected animals (++). 0.25 ml of filtered sera were prepared from each and 1 drop of stock bacteria was added to each filtrate. Then supernatant urns from each of 9 other healthy animals were tested for response to 1 drop of stock bacteria; the center column shows nil responses of these urns. The right hand column shows responses of these 9 urn preparations when they were added to the supernatant sera of control and infected animals plus bacteria, after 1 more drop of stock bacterial suspension was added.

later, .25 ml of supernatant urns from the nine healthy animals. By about 15 minutes, the urns added to the test sera showed insignificant response. But when an additional drop of bacterial suspension was added, the urns in the test preparations showed +, ++, and in one case a +++ response within 10 to 20 minutes (Table II). Urns in the control preparations (normal sera + twice-added bacteria) accumulated bacteria on the tails, but no hypersecretion was forthcoming. Thus, urns derived from healthy animals did not hypersecrete when exposed to stock bacteria, but did so when these bacteria were mixed with filtered sera from actively bacteremic animals, plus a second inoculum of the stock bacteria.

3) *Heated "acute" sera plus bacteria.*

Only one aspect of this apparent factor in the sera of infected animals could be explored in the time that remained. Heat stability was selected because it had been found that a factor in *S. nudus* blood which rapidly lysed a protozoon ciliate, *Anophrys magii*, was destroyed by heating to 45° C for five minutes (Bang & Bang, 1962).

From five apparently healthy laboratory-maintained *Sipunculus*, and three *Sipunculus* freshly collected from the off-shore sand, preparations of urn supernatant were prepared. In all cases, urns showed no response to one drop of stock bacterial suspension ; bacteria simply piled up and formed shimmering masses on the tails. These eight preparations of unresponsive supernatant urns were used to test the effects of heated sera from acutely infected animals.

Sources of sera were three spontaneously bacteremic animals whose blood was slippery and whose urns were producing ++ amounts of mucus. As much blood was obtained from each as could be managed

TABLE III

Responses of supernatant urns to heated sera of infected *Sipunculus* plus bacteria

Serum source animal	Secretory state of urns in source animal	Source animals for supernatant urns	Responses of supernatant urns to bacteria alone	Responses of supernatant urns to sera and bacteria, sera heated to : 20° C	63° C	84° C	98° C	Responses of supernatant urns to sera heated to 84° C
A	++	L 1	0	+	++	+++		
B	++	L 2	0	+	++	+++		
		L 3	0			+++	+++	+
		L 4	0			+++	+++	
		L 5	0	+		+++	+++	
C	++	N 1	0	0		+++	+++	+
		N 2	0	0		+++	+++	+
		N 3	0	+		+++	+++	

The two left-hand columns show the hypersecretory state of 3 bacterially infected *Sipunculus*, from which filtered sera were prepared, and one drop of stock bacterial suspension added to each. The next two columns show the 8 healthy *Sipunculus* from which supernatant urns were derived ("L" are laboratory and "N" are newly collected animals) and nil responses of their urns to the stock bacteria. The next column shows responses of those same urns to the same bacteria after the bacteria had been added to the "acute" sera prepared at room temperature (20 °C). The next 3 columns show responses of urns after the sera were heated for 5 minutes at 63 °C, 84 °C, and 98 °C. The last column shows responses of urns to sera heated to 84 °C to which bacteria had not been added.

Supernatant urns were all prepared less than three days prior to use; the new animals' urns were prepared the day of use.

(the thickened blood tended to clog the needle) and the centrifuged supernatants were filtered to remove urns. To each .25 ml of filtered "acute" sera, one drop of stock bacterial suspension was added, allowed to stand for 15 minutes, then the mixture was added to .25 ml of urn supernatant from each of the eight test animals. Additional bacteria were not introduced. With the sera at room temperature, as shown in Table III, urns in half the test preparations showed no response, in the others minor response. Sera of two of the infected animals were then heated to 63° for 5 minutes, cooled, bacteria were added, and supernatant urns from 2 of the laboratory animals were added 10 minutes later. As seen in Table III, urns in both preparations showed++responses.

Sera were then heated to 84° C for fives minutes, cooled, bacteria were added, and supernatant urns were added 10 minutes later ; supernatant urns in both preparations then produced vigorous, continuing+++responses (Table III). Similar responses were evoked from each of the 6 supernatant urn preparations from the other animals.

Sera were then heated to 98°C for fives minutes, and the procedure was repeated in six of the urn cultures. Again a nearly explosive, somewhat more untidy, outpouring of mucus was produced by all visible urns, an activity which continued until the urns died, in about half an hour. Bacteria were enmeshed in the secretion throughout. The deposits of bacteria on the distal 1/3 to 1/2 of the secretory mass were lysed, and showed as ghosts, in both 84°C and 98°C preparations.

To test the effect of the heated sera alone, the remaining urns in three of the supernatant preparations were added to equal amounts of the sera which had been heated to 84°. As Table III shows, there was brief mild response of urns to the heated sera in each case, but no sustained hypersecretion. Unfortunately the effect of heating normal filtered sera was not tested, and it may well be that breakdown products of normal sera can stimulate degrees of excess secretion in urns.

4) *Cholera toxin* in vivo, *bacteria* in vitro.

The effect of the sera of acutely infected animals suggested that some factor in the host's coelom may have somehow altered the urns'

TABLE IV

Effect of cholera toxin injected into *Sipunculus* on urn response to bacteria *in vitro*

Sipunculus #	Urn response to bacteria alone	Urn response to toxin alone	Urn response to toxin and bacteria	
1	0	+	++	++
2	0	0	++	
3	0	+	++	
4	0	+	++	++
5	0	+	++	
6	0	+	+	
7	0	0	+	
8	0	0	+	
9	0	+	++	
10	0	+	++	

The two left hand columns show that urns in whole blood preparations of 10 *Sipunculus* had nil response to stock bacteria. The next column shows responses of urns, seen in whole blood preparations, 20 minutes after 1 ml of 8 p. 100 cholera toxin was injected into each *Sipunculus*. The right hand column shows responses of urns in the same blood preparations after 1 drop of stock bacterial suspension was added.

response to new bacteria. To test whether toxin of the cholera vibrio might have a similar effect, ten sipunculus whose urns showed no response to a drop of stock bacterial suspension in whole blood samples *in vitro*, were each injected with 1 ml of freshly prepared 8 p. 100 cholera toxin solution. Examined in about 20 minutes in whole blood *in vitro*, the urns of some animals showed minor responses (Table IV). One drop of stock bacterial suspension was then added to each whole blood preparation. Within about 15 minutes, urns in three of the preparations showed+response, those in seven preparations clear++ responses to the same bacteria to which they had previously failed

to respond. (Table IV). The procedure thus evoked moderate excess secretion but did not induce rapid continuous hypersecretion. Heating the toxin to 63°C did not alter the results. The effect was not produced if cholera toxin and bacteria were successively introduced into whole blood *in vitro*.

Discussion

Since rapid continous hypersecretion of mucus can be consistently produced in supernatant urns, a method is now available for studying the secretory process in isolated mucous cells or in a community of such cells. The taxonomy of marine bacteria is still in progress (Wood, 1967) yet the fact that a unique population which consistently evoked the hypersecretory response developed as a contaminant in a solution of crude cholera toxin indicates that a standard stimulus for evoking excess secretion in isolated urns can be developed.

It is clear that certain bacteria, or bacteria pre-treated in a certain way, have this capacity. Whether bacteria change the sera (or evoke release of substances from blood cells into the sera) or vice versa is not clear. If one considers together the circumstances in which degrees of hypersecretion were induced:

1) cholera toxin-bacteria mixture+any *in vitro* urn system=rapid continuous secretion;

2) toxin solution *in vivo*+stock bacteria+urns *in vitro*=moderate excess secretion;

3) "acute" sera+bacteria, +supernatant urns+bacteria=moderate excess secretion;

4) heated "acute" sera+bacteria+supernatant urns=rapid continuous secretion.

The single common thread is response of urns to a living vibrio or toxin of a vibrio under particular conditions.

In nature, *Sipunculus* is often subjected to minor invasions of bacteria, including vibrios (Cantacuzène, 1928). Presumably some of these are pathogenic and some are not. Reactions to the pathogenic and non-pathogenic forms mays be mediated through quite different chains of events in the coelomic fluid. Pathogens may induce the rapid secretion needed for quick immobilization and lysis, while harmless bacteria are treated much in the manner of other foreign particles. Our stock bacteria were evidently non-pathogenic, yet in experiments #2 and #4 we may have altered the coelomic fluid in ways which simulated part of the reaction to pathogens. These interpretations are subject to testing.

In the case of heated sera a different interaction is suggested. It may be that in nature two factors function to keep in balance the capacity to produce excess mucus: one which stimulates enhanced secretion and one which limits the degree of enhancement. Heating the sera may have selectively destroyed the inhibitor, so that enhanced secretion was unchecked.

Summary

The coelomic fluid (blood) of the marine invertebrate coelomate *Sipunculus nudus* contains thousands of free-swimming "urn cells". These organelles, of epithelial origin, secrete mucous tails by means of which debris and bacteria are scavenged from the blood and phagocytosed by amebocytes. The secretory process can be monitored microscopically in blood *in vitro*.

Living urns were observed to secrete excess amount of mucus when *Sipunculus* were spontaneously or experimentally infected with some, but not all, bacteria. Significant excess secretion was induced in urns experimentally when bacteria were added to blood *in vitro* after the host animal was first injected with a solution of toxin of *Vibrio cholerae*. Significant excess secretion was induced in urns from a healthy *Sipunculus* when the urns were exposed to bacteria which had first been mixed with sera (plasma) from a bacterially infected *Sipunculus*. When the latter sera were heated to 84 °C or 98 °C, a rapid continuing excretion of mucus was induced immediately after the addition of the bacteria. In all cases, the urns had previously failed to hypersecrete when exposed to the same bacteria.

Urns, maintained in two sterile preparations of their own coelomic fluid, separated from other blood cells for seven and fifteen days respectively, hypersecreted mucus when exposed to particular bacteria.

Riassunto

Il liquido celomatico (sangue) dell'invertebrato celomato marino *Sipunculus nudus* contiene migliari di "cellule ad urna", liberamente natanti. Questi organelli, d'origine epiteliale, secernon o code mucose per mezzo delle quali essi spazzano dal sangue detriti e batteri che vengono fagocitati poi dagli amebociti. Il processo secretorio si prò seguire microscopicamente nel sangue *in vitro*.

Si è osservato che le "urne" viventi secernono una quantita di muco in eccesso, quando i Sipunculi s'infettano — spontaneamente o sperimentalmente, con diversi batteri ; ma non con tutti i batteri. Un eccesso significativo di secrezione si induce sperimentalmente nelle urne quando si addizionano batteri al sangue *in vitro* dopo che l'animale à stato iniettato con una soluzione di tossina di *Vibrio cholerae*. Un eccesso significativo di secrezione si è indotto nelle urne di un Sipunculo sano, quando le cellule sono state esposte a batteri previamente mescolati con siero (plasma) di un Sipunculo batteriologicamente infetto. Quando i sieri si riscaldati ad 84 °C o 98 °C, un escrezione rapida e continua di muco è indotta immediatamente dopo l'addizione dei batteri. In ogni caso, le urne, esposte agli stessi batteri, erano state in precedenza incapaciti ipersecernere.

Urne mantenute in due preparazioni sterili del lore stesso fluido celomatico, separate da altre cellule sanguigne per sette e per quindici giorni ipersecernono muco quando si esponga a particolari batteri.

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