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Defensive Function of Pigment Granules in *Blepharisma japonicum*

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SUMMARY

The defensive function of pigment granules in the red heterotrichous ciliate *Blepharisma japonicum* was studied by comparing normally-pigmented red cells, albino mutant cells and light-bleached cells (a phenocopy of the albino mutant) as prey for a carnivorous ciliate *Dileptus margaritifer*. Albino and bleached cells were eaten more readily than red cells by dilepti. Under certain conditions, dilepti were killed by red cells, but not by albino and light-bleached cells. The cell-free fluid of red cells, but not of albino and bleached cells, was toxic to dilepti. We conclude that one of the functions of pigment granules in *B. japonicum* is defense against predators. The offense-defense interaction between *D. margaritifer* and *B. japonicum* is, therefore, mediated by their extrusomes, toxicysts in the former and pigment granules in the latter. Other extrusomes of unknown function might also participate in offense-defense cell-cell interaction in protists.

Introduction

Blepharisma japonicum is a red, fresh-water, heterotrichous ciliate. The red coloration of *Blepharisma* is due to the pigment, blepharismin (formerly zoopurpurin), contained in extrusive pigment granules of 0.3–0.5 μm in diameter [14, 16–18, see 7 for review]. The pigment granule of *Blepharisma* is tentatively classified by Hausmann [12] as a mucocyst-type extrusome together with similar organelles in *Stentor, Loxodes* and *Trachelonema*.

The pigment, which appears to be related to hypericin [7, 27, 29], is photosensitizing and blepharismas are killed by an exposure to visible and near UV light of high intensity which does little harm to colorless cells such as *Paramecium* [5, 7]. Proposed functions of the pigment are photoreception [7, 19, 22, 27, 28], protection against far-UV radiation [7] and defense against predators [7].

In this work we experimentally tested the hypothesis of the defensive function of pigment granules of *Blepharisma* using a carnivorous ciliate *Dileptus margaritifer* as the predator. *Dileptus* has a long flexible proboscis equipped with toxicysts. The prey touched with the proboscis is paralyzed or lysed by discharged toxicysts and is engulfed through the mouth located at the base of the proboscis [10, 32]. Specifically, we compared normally-pigmented red cells, light-bleached cells and albino mutant cells of *B. japonicum* as prey for *D. margaritifer* and also examined the effect of cell-free fluids of these three types of cells on *D. margaritifer*. The results strongly support the hypothesis and led us to speculate that extrusomes might be organelles for the offense-defense interaction in free-living protists.

Part of this work has been published in abstract form [26].

Material and Methods

Cells. Clones A53, R18 and R107 of the Bangalore strain of *Blepharisma japonicum* [13], formerly *B. intermedium* [1], and stock L of *Dileptus margaritifer* were used. Clone A53 (mating type I) is a subculture of the albino strain derived from the Bangalore strain [2, 13]. Clones R18 (mating type II) and R107 (mating type I) are normally pigmented wild-type clones. Stock L was previously referred to as *Dileptus anser*, but it is now classified as *D. margaritifer* [9] based on a recent revision [33].

D. margaritifer used is about 500 μm long. About two fifths of its length is occupied by a tapering proboscis. The rest of the cell, or the trunk, can dilate to accommodate a massive prey such as an albino cell of *B. japonicum* which is about 300 μm long and is usually wider than *D. margaritifer*.

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Blepharismas were grown on the lettuce-juice culture medium [24] inoculated with *Enterobacter aerogenes* 1–2 days before use. Cultures were kept under strictly monoxenic condition using aseptic techniques until harvesting, which was carried out as follows. Cells were concentrated by mild centrifugation, washed by and suspended in SMB-III [23] (called SMB below), a balanced salt solution (1.5 mM NaCl, 0.05 mM KCl, 0.4 mM $CaCl_2$, 0.05 mM $MgCl_2$, 0.05 mM $MgSO_4$, 2 mM Na-phosphate buffer pH 6.8, 2×10^{-3} mM EDTA) at the density of $4–6 \times 10^3$ cells/ml. Debris in the culture was removed as described in [25]. Cells were used 1–2 days after the suspension in SMB. Dilepti were grown on a SMB suspension of *Saprophilus* sp., a small ciliate, which was grown, washed and suspended in SMB in the same way as blepharismas. Culture, handling of cells and experiments were performed at 24 ± 1 °C. Unless specified, experimental cells were kept in dark moist chambers except at the time of observation.

Cell-free fluid of blepharismas. The SMB suspension of blepharismas described above was centrifuged after one day for 5 min at 200 g in pear-shaped centrifuge tubes to precipitate blepharismas. The supernatant was filtered through two sheets of filter paper and used as "cell-free fluid" of blepharismas.

Bleaching of cells. Red blepharismas can be temporarily bleached by light [4, 7]. In this work the bleaching was performed by illuminating blepharismas in a crystallizing dish ($4–6 \times 10^3$ cells/ml) for 1 day or longer by a 40 W fluorescent lamp (120 cm long) fixed 30 cm above the dish, which was covered with a larger inverted crtystallizing dish to avoid evaporation. Immediately after the treatment, cells were slightly pink or almost as colorless as albino cells depending on the length of illumination. They appeared normal otherwise. These bleached cells gradually recovered the red pigmentation in the dark, but the recovery was not noticeable at least for several hours after the treatment.

Results

Observations on the Encounter between a Dileptus and a Blepharisma

A dileptus was placed among about 100 blepharismas suspended in 500 µl SMB in a slide depression and the outcome of the encounter between the *Dileptus* and a *Blepharisma* was immediately observed under a stereomicroscope. A characteristic interaction was evoked only when the proboscis of the *Dileptus* touched the *Blepharisma.*

When the proboscis hits a normally-pigmented red *Blepharisma,* the latter is locally lysed where it was hit. The *Dileptus* usually pulls back with or without the lysed fragment, which is sometimes engulfed by the *Dileptus.* The remaining part of the *Blepharisma,* which is usually much larger than the lysed part, swims away. Thus the first encounter with a *Dileptus* is seldom fatal for a red *Blepharisma,* though it may be more or less wounded. However, a red *Blepharisma* can completely disintegrate, if it is repeatedly hit by the proboscis. This often occurs when a *Blepharisma* is confined in a small space with dilepti, e.g., in 200 µl SMB in a slide depression with 10 dilepti.

When a *Dileptus* hits an albino *Blepharisma* with its proboscis, local lysis occurs as described above. However, the *Dileptus* seldom pulls back remaining close to the prey, the lysis extends and the *Dileptus* immediately starts engulfing the lysed portion. A large portion of the *Blepharisma,* sometimes even nearly the whole cell, is engulfed. Thus for an albino *Blepharisma,* the first encounter with a *Dileptus* is often fatal. Even if it survives the attack, it is more severely wounded than the red counterpart.

For a light-bleached wild-type *Blepharisma,* the outcome of the encounter with a *Dileptus* depends on the extent of the bleaching. For an intensively bleached colorless-looking cell, the outcome is similar to that of an albino cell. If, however, the bleaching is partial and a cell is still slightly pink, the *Dileptus* often pulls back after hitting the cell. But the pulling back is less extensive than the one which occurs when a dileptus hits a red cell. The extent of lysis, the frequency of engulfing of the lysed part by the *Dileptus* and the chance of survival of the *Blepharisma* are generally between those of an albino and red cells.

These observations indicate that dilepti prey more easily on less pigmented cells and suggest a defensive role of the red pigmentation against dilepti. The effect of the pigmentation on the *Dileptus-Blepharisma* interaction was examined more quantitatively in the following experiments.

Effects of the Pigmentation and Cell density of Blepharismas on the Dileptus-Blepharisma Interaction

Five slightly starved dilepti were placed in 200 µl SMB with 0, 5, 10, 20 and 40 blepharismas which had been passed through SMB to remove previously excreted materials. These mixtures, which were called 5 D–0 B, 5 D–5 B, 5 D–10 B, 5 D–20 B and 5 D–40 B mixtures, respectively, were made for albino, red and light-bleached blepharismas in quadruplicate and in each mixture the numbers of blepharismas and dilepti were counted for 3 days.

Albino blepharismas disappeared within 1 day in every mixture (Fig. 1A). Dilepti multiplied roughly in proportion to the number of disappeared blepharismas (Fig. 1a) indicating that albino cells were consumed as food by dilepti.

On the contrary, normally-pigmented red blepharismas (R107) survived. A larger proportion of cells survived in mixtures with more blepharismas (Fig. 1B). A slight increase in the number of blepharismas was seen in the 5 D–40 B mixture. On the other hand, dilepti decreased in number and the decrease was more extensive in mixtures in which more blepharismas were present (Fig. 1b). In the 5 D–40 B mixture more than 80% of dilepti disappeared in 3 days and the surviving ones were abnormally round. Some of the dilepti were probably eaten by blepharismas after becoming abnormal, because in mixtures in which many dilepti disappeared a *Blepharisma* with a food vacuole containing a round black object of the size of a *Dileptus* was sometimes seen. The slight increase in the number of blepharismas in the 5 D–40 B mixture (Fig. 1B) might be partly due to this feeding, but it can also be due to the feeding on the bacteria which grew on the material released in the medium during the offense-defense interaction between dilepti and blepharismas. There was no sign

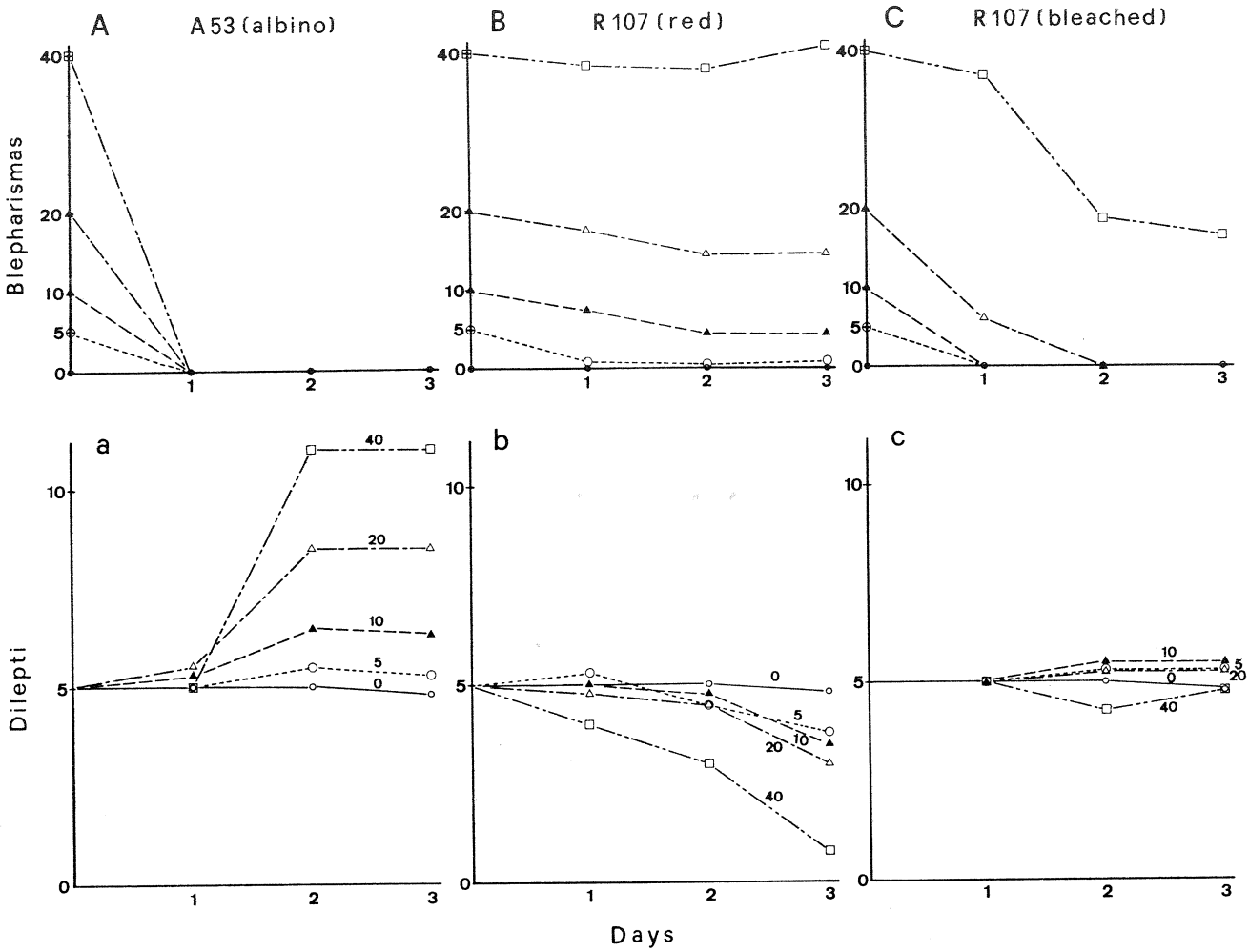

Fig. 1. Effects of pigmentation and cell density of *Blepharisma japonicum* on the offense-defense interaction between *B. japonicum* and *Dileptus margaritifer*. The number of cells of *B. japonicum* and that of *D. margaritifer* are plotted in separate graphs (A, B, C and a, b, c, respectively) against the time in days after mixing 5 dilepti with 0 (o—o), 5 (○-----○), 10 (▲————▲), 20 (△———△) or 40 (□————□) blepharismas in 200 µl SMB in a slide depression. Blepharismas used were albino mutant (A53) in A and a, red R107 in B and b, and 1-day-bleached pale pink R107 in C and c. The numeral beside each curve in a, b and c indicates the number of blepharismas placed at the beginning of experiment. Data are means of 4 experiments carried out in parallel.

of multiplication of dilepti except that a few dilepti divided in the 5 D–5 B mixture on the first day (Fig. 1b). Even in this mixture, some dilepti disappeared after killing many blepharismas. Essentially the same results were obtained in the experiment in which another red clone R18 was used: In 5 D–0 B, 5 D–5 B, 5 D–10 B, 5 D–20 B, 5 D–40 B mixtures, 0, 0, 0.3, 20.5, 41.3 blepharismas and 5, 5, 3.8, 0.8, 0.3 dilepti were respectively counted after 3 days (means of 4 experiments).

Therefore, red blepharismas are not edible for dilepti under these conditions. Dilepti can kill some blepharismas, but are also killed.

The R107 cells used in the above experiment were kept in the dark for 1 day after suspension in SMB (see Material and Methods). Another aliquot of R107 cells was placed under light for bleaching for 1 day starting at the time of the suspension in SMB. These bleached cells, which still looked slightly pink, were then mixed with dilepti. The decrease in the number of blepharismas (Fig. 1C) was between that of albino cells and that of red cells (Fig. 1A, B). Dilepti multiplied a little, except in the 5 D–40 B mixture (Fig. 1c). However, the multiplication is far less than that in mixtures with albino cells (Fig. 1a), in spite of the fact that the comparable number of blepharismas disappeared in 5 D–5 B, 5 D–10 B and 5 D–20 B mixtures (compare Fig. 1A and 1C). This suggests that dilepti engulfed a smaller portion of a cell when they preyed on 1-day-bleached cells than when they preyed on albino cells.

In the other experiment, an aliquot of R107 cells were illuminated for 2 days after suspension in SMB. They were almost as colorless as albino cells. These cells were compared with the other aliquot of unbleached cells kept

in the dark for 2 days after harvesting. In mixtures with unbleached blepharismas, 0, 0.3, 0, 17.4, 37.6 blepharismas and 5, 4.8, 4.8, 1.5, 2.5 dilepti were counted in 5 D–0 B, 5 D–5 B, 5 D–10 B, 5 D–20 B, 5 D–40 B mixtures, respectively, after 3 days (means of 4 experiments). In mixtures with 2-days-bleached blepharismas, 0, 0, 0, 0, 10.4 blepharismas and 5, 6.3, 7.8, 8.3, 6.3 dilepti were counted in 5 D–0 B, 5 D–5 B, 5 D–10 B, 5 D–20 B, 5 D–40 B mixtures, respectively (means of 4 experiments). Thus the multiplication of dilepti is between those in the two experiments described above in which 1-days-bleached cells and albino cells were respectively fed.

Therefore, under these conditions, blepharismas are edible for dilepti in this order: albino cells > 2-days-bleached cells > 1-day-bleached cells > red cells. That is, blepharismas are more edible for dilepti when they are less pigmented. It should be noted that 2-days-bleached cells were less edible than albino mutant cells in spite of the fact that both are almost equally colorless. This result was probably due to the capacity of bleached cells to recover pigmentation. Since the *Dileptus-Blepharisma* mixtures were kept in the dark during the 3-days of experimentation except during observations, bleached cells, which were probably as palatable as albino cells at the beginning, should have become less and less edible as they recovered their pigmentation. Indeed, surviving blepharismas in these mixtures were pale pink when the experiment ended after 3 days.

In addition, these results indicate that the pigmented blepharismas can kill dilepti under certain conditions. Whether the killing is mediated by a secreted factor was examined in the following experiments.

Effects of Cell-free Fluids of Blepharismas on Dilepti

Five dilepti were placed in a 200 µl cell-free fluid of blepharismas in a slide depression and dilepti were counted after 1 day. Cell-free fluids of normally-pigmented red cells killed all dilepti, but those of albino and 2-days-bleached R107 cells did not kill them at all (Table 1).

In the other experiment cell-free fluids were serially diluted (Table 2). The killing effect of the cell-free fluid of red cells disappeared, if it was diluted about 30 times. The cell-free fluid of 1-day-bleached cells was several times less toxic than that of red cells. Again any toxic effect was not detected in the cell-free fluid of albino cells.

It is evident, therefore, that red cells excrete in the medium a toxic factor which can kill dilepti. Bleached cells excrete it in a smaller quantity, and albino cells excrete it in a still smaller quantity or do not excrete it at all. The toxic factor is probably the red pigment, because the toxicity of the cell-free fluid is positively correlated with the red coloration of cells from which the cell-free fluid was obtained, and because the cell-free fluid of red cells, but not of albino and bleached cells, was slightly red-colored indicating that the pigment was really excreted under these conditions. However, this assumption is still to be confirmed by using isolated pigment.

Table 1. Toxic effect of cell-free fluids of *Blepharisma japonicum* on *Dileptus margaritifer*[a]

Source of cell-free fluid of blepharismas	Dilepti
Red R18 cells	0
Red R107 cells	0
Bleached R107 cells[b]	5
Albino cells (A53)	5
SMB (control)	5

[a] Five slightly-starved dilepti were placed in 200 µl of cell-free fluid and the number of surviving dilepti was counted after 1 day.
[b] Light-bleached for 2 days.
Data are means of 4 experiments.

Table 2. Effect of dilution on the toxicity of cell-free fluids of *Blepharisma japonicum* against *Dileptus margaritifer*[a]

Source of cell-free fluids of blepharismas	Dilepti Dilution of cell-free fluids[b]						
	1	2	4	8	16	32	64
Red R18 cells	0	0	0	3.3	3.5	4.8	5
Bleached R18 cells	0	0	5	5	5	5	4.8
Albino cells (A53)	5	5	5	5	5	5	5

[a] Experiments were performed in the same way as indicated in Table 1 except that blepharismas were bleached for 1 day.
[b] Cell-free fluids were diluted indicated times with SMB. Data are means of 4 experiments.

Conclusion

Based on these results we conclude that pigment granules of *B. japonicum* have a defensive function against *D. margaritifer* and that *B. japonicum* can excrete a factor, probably the pigment in these granules, which is toxic to *D. margaritifer*. Combining these conclusions, we present a working hypothesis that pigment granules of *B. japonicum* carry out the defensive function by discharging the pigment.

Discussion

The function of the red pigment of *Blepharisma*, and hence also the function of pigment granules in which the pigment is localized, can be studied by comparing red cells with colorless cells such as albino mutants and their phenocopies.

The albino strain of red species of *Blepharisma* was first obtained by Inaba *et al.* [14] as a spontaneously-arising mutant in the Nara strain which was later classified as *B. stoltei* v. *narai* [15]. The albino strain of *B. japonicum*, which was used in this work, also arose spontaneously in the laboratory [2]. Both of these mutants had apparently colorless pigment granules [14, 20]. Although the albino strain of *B. japonicum* looks white, it contains a minute amount of pigment which resembles the pigment of red

strains [2]. This albino strain appeared to be as viable as any of the red strains [7]. A subculture of this strain, received through Inaba in 1967, is still alive in our laboratory. Another vigorous albino strain was obtained in *B. stoltei* [7, 13]. These results indicate that the pigmentation is not necessary for supporting the life of *Blepharisma* in the laboratory. Phenocopies of albino mutants, *i.e.* temporarily colorless-looking cells, can be obtained by treating red cells by various agents including visible light, low temperatures and chemicals [7 for review].

Albino and light-bleached blepharismas are less susceptible to visible and near UV light than red cells which are killed by intensive illumination [7]. In spite of this advantage, albino strains of the pigmented species of *Blepharisma* have not been collected in the field [7, 8], suggesting that the pigmentation is more advantageous than albinism in nature.

This work has shown that a distinct advantage of pigmented cells is their capacity to repel their predator. Since blepharismas can avoid visible and near UV light by staying in layers of sedimented detritus, which appears to be their natural habitat [3, 7], the selective advantage of the defensive capacity is probably more than enough to offset the disadvantage of the susceptibility to light. It is interesting to study whether extrusomes of colorless species of *Blepharisma,* such as *B. hyalinum* [21], contain a colorless toxic substance for defense.

The tentative conclusion of this work that pigment granules carry out the defensive function by discharging their pigment is supported by the previous finding that blepharismin is toxic to many cells [6, 7] and that pigment granules discharge the pigment outside the cell responding to various physical and chemical stimuli [7]. Indeed, these findings led Giese to suggest a defensive function for the pigment [7]. However, his hypothesis was questioned [7] and even judged to be unlikely [8] by Giese himself based on the following observations; blepharismas in a mixed culture with other protists, such as *Colpidium, Paramecium, Stentor, Didinium,* and *Actinosphaerium,* do not appear to discharge the pigment [7]; blepharismas are engulfed by *Actinosphaerium eichhorni* without discharging the pigment [7]; blepharismas are readily eaten by small crustaceans [8]. Also *Bursaria truncatella* can multiply on red blepharismas engulfing them without evoking the discharge of their pigment granules.

Thus the defensive mechanism of blepharismas, which can effectively ward off the attack of dilepti, is apparently not mobilized against such predators as *Actinosphaerium* and *Bursaria,* suggesting that some predators can evade the recognition mechanism of *Blepharisma.*

Based on the results of this work and the above discussion, the following outline of the *Dileptus-Blepharisma* interaction may be constructed. When the proboscis of a *Dileptus* touches a *Blepharisma,* the *Dileptus* discharges toxicysts recognizing the *Blepharisma* as a prey. The *Blepharisma* is partly lysed, but it responds by discharging pigment granules. The *Dileptus* retreats sensing the toxic effect of the discharged pigment. *Blepharisma* swims away leaving behind the lysed portion which may or may not be engulfed by *Dileptus.* It is not known, however, whether the discharge of pigment granules is triggered by blepharismas which recognize the presence of predators or the discharge is the outcome of the partial lysis of blepharismas.

Since *Blepharisma* eats various small protists in addition to bacteria, the finding that the cell-free fluid of red blepharismas was toxic to *D. margaritifer* can be interpreted as an indication that pigment granules function also as offensive organelles. However, the cell-free fluid in this work was taken after suspending a high density of blepharismas for one day and the toxic effect of the cell-free fluid disappeared when it was diluted only 30 times (Table 2). It is possible that the observed toxicity of the cell-free fluid was due to the discharge of some of the pigment granules induced under artificial conditions such as centrifugation and suspension in SMB. Also albino mutants and photo-bleached cells easily eat *Saprophilus* sp., a small ciliate, indicating that pigment granules are not needed for preying on *Saprophilus.* Further study is needed to confirm the offensive role of pigment granules in nature.

It may be asked whether pigment-containing extrusomes in other heterotrichous ciliates, like *Stentor* and *Fabrea* also have a defensive function against predators. Visscher [32] observed how *D. gigas* ate, little by little, a single cell of *Stentor coeruleus* attached at the bottom of a container. When a *Dileptus* touched the *Stentor* with its proboscis, the *Stentor* was partially lysed. The *Dileptus* frequently ate the lysed part and apparently swam away. A dozen or more dilepti, which were placed with the *Stentor,* took a nibble in turn. The *Stentor* died unless it detached from the bottom and escaped before it was too badly injured. The fact that dilepti did not remain by the *Stentor* to finish it and gave it a chance to escape might be an indication that the *Dileptus* was repelled by the *Stentor* just as it is repelled by the red *Blepharisma.* If so, pigment granules of *Stentor* might also have a defensive function against predators.

Extrusomes [10], or extrusive organelles in protists, are widely present in free-living protists [12]. Some extrusomes, such as toxicysts, haptocysts and pexicysts, are offensive organelles, but many extrusomes are still organelles of unknown function [12]. Although a defensive function has been proposed for various extrusomes, it has been far less convincing than the offensive function of extrusomes. This is partly due to the fact that mutants in extrusomal characters or their phenocopies are lacking in most species. In addition, appropriate predators should be chosen for the experiment, because any defensive device cannot be equally effective against different offenders. Recently, the putative defensive function of trichocysts was experimentally verified in *Paramecium* [11, 26] using trichocyst-non-discharge mutants, their phenocopies and, by choosing, as the predator, *Dileptus* rather than *Didinium* which had been commonly used in previous studies of offense-defense interactions between *Paramecium* and predators. It is still to be studied whether pigment granules of the hypotrichous ciliate *Pseudokeronopsis rubra* have defensive function against predators by comparing wild-

type cells, which produces a toxic pigment keronopsin [31], and albino cells [30] as prey. More examples of defensive function of extrusomes may be found in similar ways.

These discussions leads us to speculate a unifying view that extrusomes in free-living protists are primarily organelles for the offense-defense interaction. These protists live in a complex network of offense-defense interactions resulting from a long evolutionary history of the interaction between predators and preys. The wide distribution of extrusomes, their bewildering diversity in morphological and physiological characters and a considerable number of examples of offensive extrusomes are consistent with the assumption that extrusomes are organelles for this vital interaction in protists.

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Key words: Pigment granules – Extrusomes – *Blepharisma* – *Dileptus* – Predator-prey interaction

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