

Chemical Defense by Means of Pigmented Extrusomes in the Ciliate *Blepharisma japonicum*

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Summary

The defensive function of pigment granules in *Blepharisma japonicum* against the predatory ciliate *Dileptus margaritifer* was further investigated by 1) observing the discharge of pigment granules of *Blepharisma* as a response to the attack by the predator and 2) measuring the toxicity of purified blepharismin, the red pigment localized in pigment granules. When a *Blepharisma* was attacked by the toxicysts-bearing proboscis of a *Dileptus*, the *Blepharisma* instantly released a mass of reddish material at the attacked site. The *Dileptus* retreated and the *Blepharisma* swam away. The observation suggested that the *Blepharisma* discharged pigment granules as a response to the attack. This assumption was confirmed by scanning-electronmicroscopic observations; many pigment granules near the attacked site were discharged at the moment of the attack. Purified blepharismin was highly toxic to *Dileptus* and several other ciliates, but was not toxic to *Blepharisma*. We also showed that blepharismin is toxic in the dark. These results strongly support the previously presented hypothesis that the defensive function of pigment granules in *B. japonicum* against *D. margaritifer* is based on the discharge of blepharismin as a response to the attack by the predator. We conclude that pigment granules of *B. japonicum* are extrusomes (extrusive organelles in protists) for chemical defense.

Key words: *Blepharisma*; Pigment granules; Extrusomes; Blepharismin; Chemical defense.

Introduction

Pigment granules of *Blepharisma japonicum* are membrane-bounded spherical organelles of 0.3–0.6 µm in diameter mostly localized in the cortex and attached to the cell membrane [22, 23, 26, 33, see 12 for review].

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They contain the red pigment, blepharismin, and are present in hundreds of thousands between ciliary lines providing the characteristic pink-red coloration to this ciliate [12, 14].

Blepharismin has long been known to be chemically related to hypericin, the photodynamic toxin of *Hypericum*, Saint-John's-wort [12, 14]. Blepharismin itself is a photodynamic toxin; red cells of *B. japonicum* are killed by an exposure to bright light which does little harm to colorless ciliates such as *Paramecium* [8, see 12, 14 for review]. Recently blepharismin derived from *B. japonicum* was found to be a mixture of five similar compounds and chemically identified [2, 28]. In this work, any one of these compounds and their mixtures are called blepharismin.

Pigment granules of *Blepharisma* discharge their content responding to various chemical and physical stimuli [11, 12]. They are, therefore, considered as extrusomes, extrusive organelles in protists, and are classified as a special type of mucocyst along with pigment granules in *Stentor*, *Loxodes* and *Trachelonema* [20].

Three possible functions of pigment granules have been suggested by Giese [12–14] who studied *Blepharisma* extensively; 1) protection against far UV radiation, 2) photoreception, and 3) protection against predators. The protective function against far UV radiation was suggested based on the finding that the blepharismin can protect *Blepharisma* from far UV radiation, e. g. UV light at 265 nm [12]. However, the role of this function in nature is questioned (see Discussion).

The function of photoreception was suggested on the analogy of pigment granules in another heterotrich ciliate *Stentor coeruleus*, in which the blepharismin-like pigment, stentorin, is localized in pigment granules; it was assumed to mediate the photophobic response of this species [12]. Later works showed that stentorin [48,

53, see 47, 49 for review] and then blepharismin [1, 32, 34, 45, see 7, 30 for review] are indeed the photoreceptor in the step-up photophobic response in *S. coeruleus* and *B. japonicum*, respectively. However, the localization of blepharismin in extrusive organelles is puzzling if the main function of this pigment is photoreception.

The protective function against predators was suggested based on the finding that crude extracts of the pigment are toxic to a variety of protozoans and to sea urchin larvae but not to *Blepharisma* [9, see 12 for review]. The suggestion was with reservation [12, 13], however, because the heliozoan *Actinosphaerium eichhorni* ate the deeply pigmented cells avidly [9]. This hypothesis was later verified by two of us (A. M. and T. H.) and collaborators [39] who showed that albino mutant and bleached cells of *B. japonicum* are much more vulnerable than red cells to the predatory ciliate *Dileptus margaritifer*. They also found that red cells, but not albino cells, excrete a toxic substance into the medium. Based on these results, they concluded that pigment granules of *B. japonicum* have a defensive function against *D. margaritifer*. They also assumed that pigment granules of *B. japonicum* carry out the defensive function by discharging the pigment.

In this work we verify this hypothesis on the mechanism of defense by showing that *B. japonicum* discharges pigment granules as a response to the attack by *D. margaritifer* and that purified blepharismin is toxic to *D. margaritifer* but not to *B. japonicum*. The result led us to conclude that pigment granules in *B. japonicum* are extrusomes for chemical defense.

Part of this work has been published in abstract form [19, Miyake A. and Harumoto T., Proc. 4th Asian Conf. Ciliate Biol., Tokyo 1995, pp. 69–71; Harumoto T., Iio H., Zenfuku K. and Miyake A., Programme and Abstr. 8th Eur. Conf. Ciliate Biol., Clermont-Ferrand 1995, p. 54; Harumoto T., Ishikawa N. and Sugibayashi R., Programme and Abstr. 10th Internat. Congr. Protozool., Sydney 1997, p. 99].

Material and Methods

Ciliates: Ciliate and culture methods used in this work are listed in Table 1. Stocks R1072 and A538 of *B. japonicum* are respectively red wild-type and albino mutant [3], both derived from the Bangalore strain [21]. Unless specified, the red stock R1072 was used. Stock SHL1 of *D. margaritifer*, formerly *D. anser* [52], is a hybrid between stocks L and SH, both obtained from Dr. K. Golinska, Nencki Inst. Exp. Biol., Warsaw. Stock D3-I of *D. margaritifer* was obtained from Dr. M. Tavrovskaya, Inst. Cytol., Russ. Acad. Sci., St. Petersburg.

Those grown on the lettuce (L) medium [36] or Wheat-Grass-Powder (WGP) medium [50] were concentrated by centrifugation, washed by and suspended in SMB-III [35], a balanced salt solution (1.5 mM NaCl, 0.05 mM KCl, 0.4 mM CaCl₂, 0.05 mM MgCl₂, 0.05 mM MgSO₄, 2 mM Na-phos-

phate buffer pH 6.8, 2×10⁻³ mM EDTA) (called SMB below) or modified SMB-III (EDTA omitted, called SMB⁻ below). Debris in the culture was removed as described [38] using a nylon net with a mesh size appropriate to the respective ciliate. Unless specified, L-medium and SMB were used for experiments on the toxicity of purified blepharismin, while WGP-medium and SMB⁻ were used for other experiments. Those fed on small ciliates (Table 1) were grown in SMB or SMB⁻ containing the respective food ciliate and used within 1 day after they had consumed most of the food ciliates. Culture, handling of ciliates and experiments were performed at room temperature (23–26 °C) unless specified.

Scanning electron microscopy: *Blepharisma*, *Dileptus* and their mixtures were fixed with the Parducz' fixative [41] for 20 min, washed with double distilled water, dehydrated, dried with a critical point dryer (Hitachi HCP-2), coated with gold with an Ion coater (Eiko IB-3) and observed in a scanning electron microscope (Hitachi S-430).

Purification of blepharismin: A concentrated cell suspension of *B. japonicum* (stock R1072 grown on the L-medium) in SMB (60 ml) was mixed with acetone (60 ml) and ethylacetate (70 ml). The mixture was sonicated for 10 min and filtered through a Celite bed. The red pigment in the filtrate was extracted with ethylacetate and concentrated in vacuo to dryness. Thin-layer chromatography of the residue (Silica gel 60F₂₅₄ 0.5 mm, Merck; eluting solvent, 3 methanol : 17 dichloromethane) yielded two red bands (Rf 0.2 and 0.25). They were separately extracted with a 1:5 mixture of methanol and dichloromethane as fractions I (Rf 0.2) and II (Rf 0.25) of blepharismin, rechromatographed and dried in vacuo. The weight ratio of the two fractions (I/II) was about 4. Fraction I was a mixture of blepharismins; ¹H NMR (500 MHz, DMSO-d₆) showed that the major isomer (about 80%) was identical to blepharismin, 2, 4, 5, 7, 2', 4', 5', 7'-octahydroxy-6, 6'-diisopropyl-1, 1'-(p-hydroxybenzylidene)-naphthodianthrone, reported in [2] or BL-3 in [28]. Fractions I and II of blepharismin were dissolved in 75% ethanol at concentrations of 1.8 mg/ml and 4.0 mg/ml respectively, stored in the dark at 2–6 °C and diluted with SMB for use.

Table 1. Ciliates and culture methods used in this work.

Species (stock)	Culture methods ¹
<i>Blepharisma japonicum</i> (R1072, A538)	L, WGP
<i>Colpidium</i> sp. (War1)	L
<i>Didinium nasutum</i> (Waseda)	C (<i>P. tetrarella</i>)
<i>Dileptus margaritifer</i> (SHL1, D3-I)	C (<i>Sathrophilus</i> sp.)
<i>Euplotes octocarinatus</i> (3–58)	C (<i>T. thermophila</i>)
<i>Lembadion bullinum</i> (3A)	C (<i>Colpidium</i> sp.)
<i>Paramecium caudatum</i> (Kyk402)	L
<i>Paramecium tetrarella</i> (51)	L
<i>Stentor coeruleus</i> (Mün1)	C (<i>Sathrophilus</i> sp.)
<i>Stentor polymorphus</i> (Mün32)	C (<i>Sathrophilus</i> sp.)
<i>Sathrophilus</i> ² sp. (Pisa7) ³	L, WGP
<i>Tetrahymena thermophila</i> (210) ³	L

¹ L, grown on the lettuce medium inoculated with *Enterobacter aerogenes* [36]; WGP, grown on the Wheat-Grass-Powder medium [50] inoculated with *E. aerogenes*; C, grown on the ciliate shown in parentheses.

² Formerly *Saprophilus* [4].

³ Used only as food for other ciliates.

Lethal effect of blepharismin: The lethal effect of blepharismin to a ciliate was examined by placing 10 cells of the ciliate in 250 µl (100 µl for *Colpidium* sp.) blepharismin solution in a slide depression and by counting the number of cells after 1 day. Three sets of experiments were carried out for

each test. The lethal dose 50% (LD_{50}) of blepharismin for a ciliate was obtained using the concentration-survival curve of blepharismin for the ciliate as shown in Fig. 3–5. Unless specified, cells were kept in the dark in metal moist chambers throughout the experiment except during preparation at the

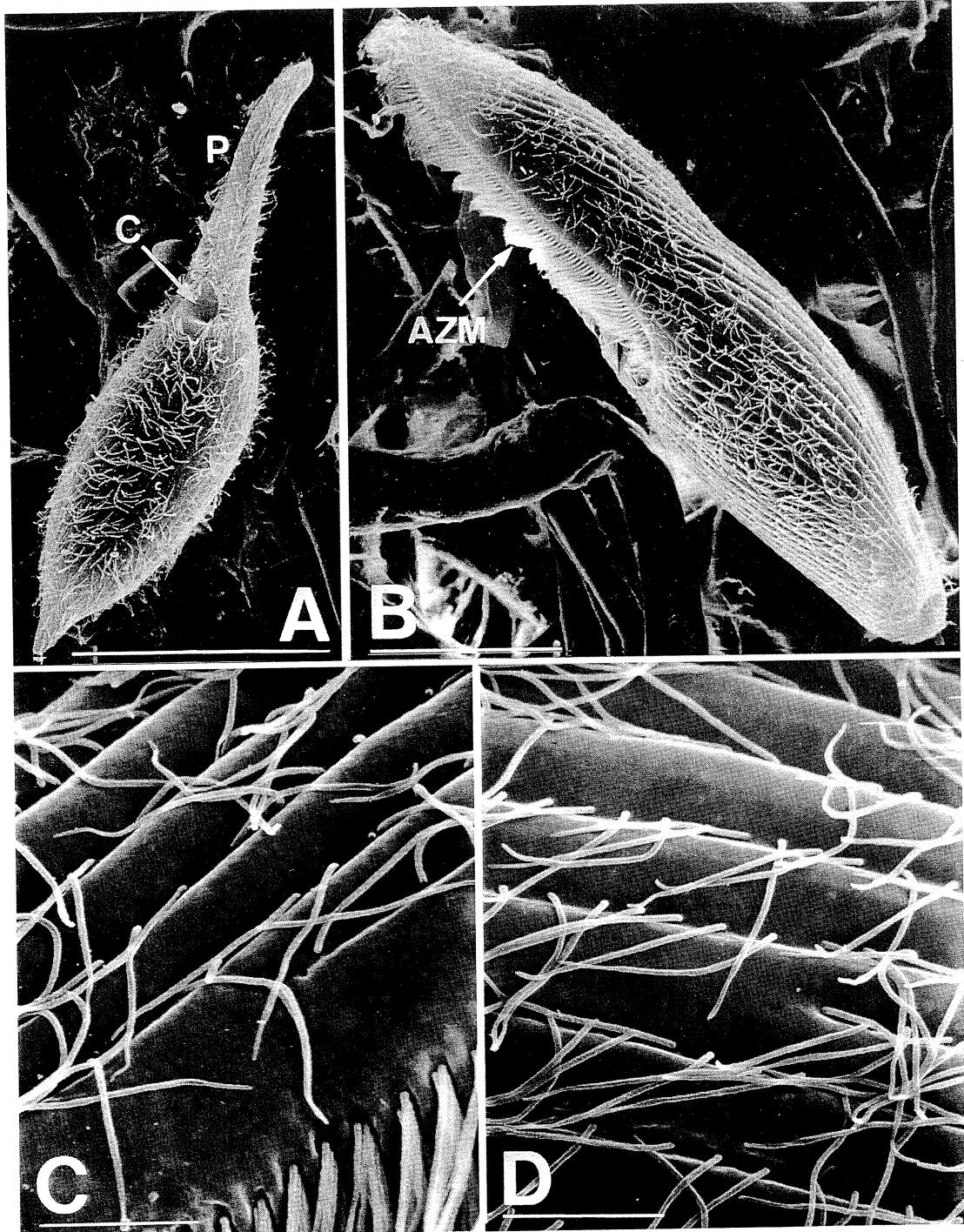


Fig. 1. Scanning electron micrographs of *Dileptus margaritifer* (stock SHL1) and *Blepharisma japonicum* (stock R1072). A: *D. margaritifer* (slightly contracted on fixation). Bar; 50 µm. B: *B. japonicum*. Bar; 50 µm. C: A part of the surface near the adoral zone of membranelle (shown at the bottom) of *B. japonicum*. Bar; 5 µm. D: A part of the ventral surface of *B. japonicum*. Bar; 5 µm. AZM: Adoral zone of membranelle. C: Cystotome. P: Proboscis.

beginning (15–30 min) and during observation at the end of the experiment.

Preparation of the crude extract of blepharismin: Cells of *Blepharisma* (R1072) grown on a 500 ml WGP-medium were suspended in about 2 ml of SMB⁻ and mixed with 25 ml SMB⁻ of 4 °C to induce a massive discharge of pigment granules. The mixture was filtered first through a nylon net (mesh size, 60 µm), then through a filter paper (Toyo No. 2) and centrifuged (15,800 g, 7 min, 4 °C) to obtain a red supernatant with the absorbance of 0.189 at 580 nm. The supernatant was the crude extract of blepharismin and used after dilution with SMB⁻. The relative concentration of the pigment in a diluted extract was shown by the absorbance at 580 nm, at which blepharismin has the highest absorption peak in visible light [12].

Red light for observations in the "experiment in the dark": Blepharismin scarcely absorbs radiation longer than 630 nm [12]. Red light longer than 640 nm, obtained by placing a filter (Sharp-cut filter R-66, Hoya optics) in front of a 21W-electric bulb (Hitachi LS1601), was, therefore, used to observe cells in the "dark" in the experiment in which the effect of light on a blepharismin-participating phenomenon was examined.

Results

Observations on the interaction between a *Blepharisma* and a *Dileptus*

A few cells of *Dileptus margaritifer* (Fig. 1A) were placed among 100–200 red cells of *Blepharisma japonicum* (Fig. 1B) suspended in 200–500 µl SMB or SMB⁻ in a slide depression and the first encounter between a *Blepharisma* and a *Dileptus* was observed using a stereomicroscope.

D. margaritifer attacks prey with a long flexible toxicysts-bearing proboscis. When the proboscis of stocks D3-I or SHL1 touched a small ciliate (e. g. *Sathrophilus* sp.), the cell immediately started disintegrating. When the proboscis hit a large slender ciliate (e. g. *Spirostomum ambiguum*) at the middle part of the cell, the cell instantly changed the shape into a L-figure form bending at the hit site. In cannibalistic attack of D3-I on the stock Mü1 of *D. margaritifer* (collected in Münster, Germany), it was often observed that the proboscis of D3-I severed a Mü1 cell in two parts by a single hit at the middle part of the cell (A. M. and T. H., unpublished). These observations indicate that the proboscis of *D. margaritifer* can instantly inflict a lesion on the surface of various ciliates.

When the proboscis of *Dileptus* touched *Blepharisma*, a mass of reddish material appeared, within a second, between the *Blepharisma* and the proboscis. The *Dileptus* went backward and the *Blepharisma* swam away. Sometimes a reddish cloud-like mass was seen rising from the touched area of the *Blepharisma*. That the reddish material was derived mainly from the *Blepharisma* was deduced from the fact that the *Blepharisma*, but not the *Dileptus*, was red colored.

If the retreat of *Dileptus* occurred instantly, *Blepharisma* usually did not show any change in the shape in spite of the material loss described above. If the retreat occurred after several seconds, however, more material came out of the *Blepharisma*, which was visibly distorted indicating a severe infliction.

The retreated *Dileptus* sometimes had a shorter proboscis, and this sign of infliction was particularly evident when the retreat was delayed. The retreated *Dileptus* often ate the material released from *Blepharisma*. If the *Dileptus* was not removed from the suspension of *Blepharisma*, the shortening of the proboscis continued, *Dileptus* lost the entire proboscis, became spherical and died.

These observations are essentially the same with the ones previously made in [39], but we paid a particular attention to the phenomenon not explicitly described in [39], the coming out of the reddish material from the attacked site of a *Blepharisma*. The phenomenon indicates that the pigment is released from the *Blepharisma* as a response to the attack. To examine how it occurs more in detail, we observed the moment of the attack with a scanning electron microscope.

Blepharisma and *Dileptus* were mixed. Sixty seconds later, they were fixed, prepared for and observed in a scanning electron microscope (Fig. 2). Unmixed cells were also similarly observed (Fig. 1).

Fig. 2 shows what appears at the moment when a *Dileptus* attacks a *Blepharisma*. A *Dileptus* diagonally overlays a *Blepharisma* (Fig. 2A). Close to the proboscis of the *Dileptus* there is a crack on the *Blepharisma* (Fig. 2A, B), indicating that the crack is most probably a wound inflicted by the proboscis or a break produced during manipulation at the site damaged by the proboscis.

On both sides of the crack, many spherical bodies of 0.2–0.6 µm in diameter are seen on or over the surface of the *Blepharisma* (Fig. 2B–D). They are particularly numerous at the region close to the crack and are fewer or absent in the region farther from the crack (Fig. 2B). On the other hand, such spherules are seldom seen on the surface of *Blepharisma* not mixed with *Dileptus* (Fig. 1C, D). These spherules are, therefore, something arising as a result of the *Blepharisma*–*Dileptus* interaction, most probably as a result of the attack on *Blepharisma* by the proboscis of *Dileptus*.

The size of the majority of spherules (0.2–0.3 µm in diameter) is close to the size of pigment granules in this species previously reported (0.3–0.6 µm) [22, 23, 26]. Each of these spherules is above or near a round depression of 0.3–0.5 µm in diameter on the cell surface, while such a close association with the surface depression is seldom seen for larger spherules (Fig. 2C, D). Such surface depressions are not seen on the area of the cell surface where spherules are not present. These re-

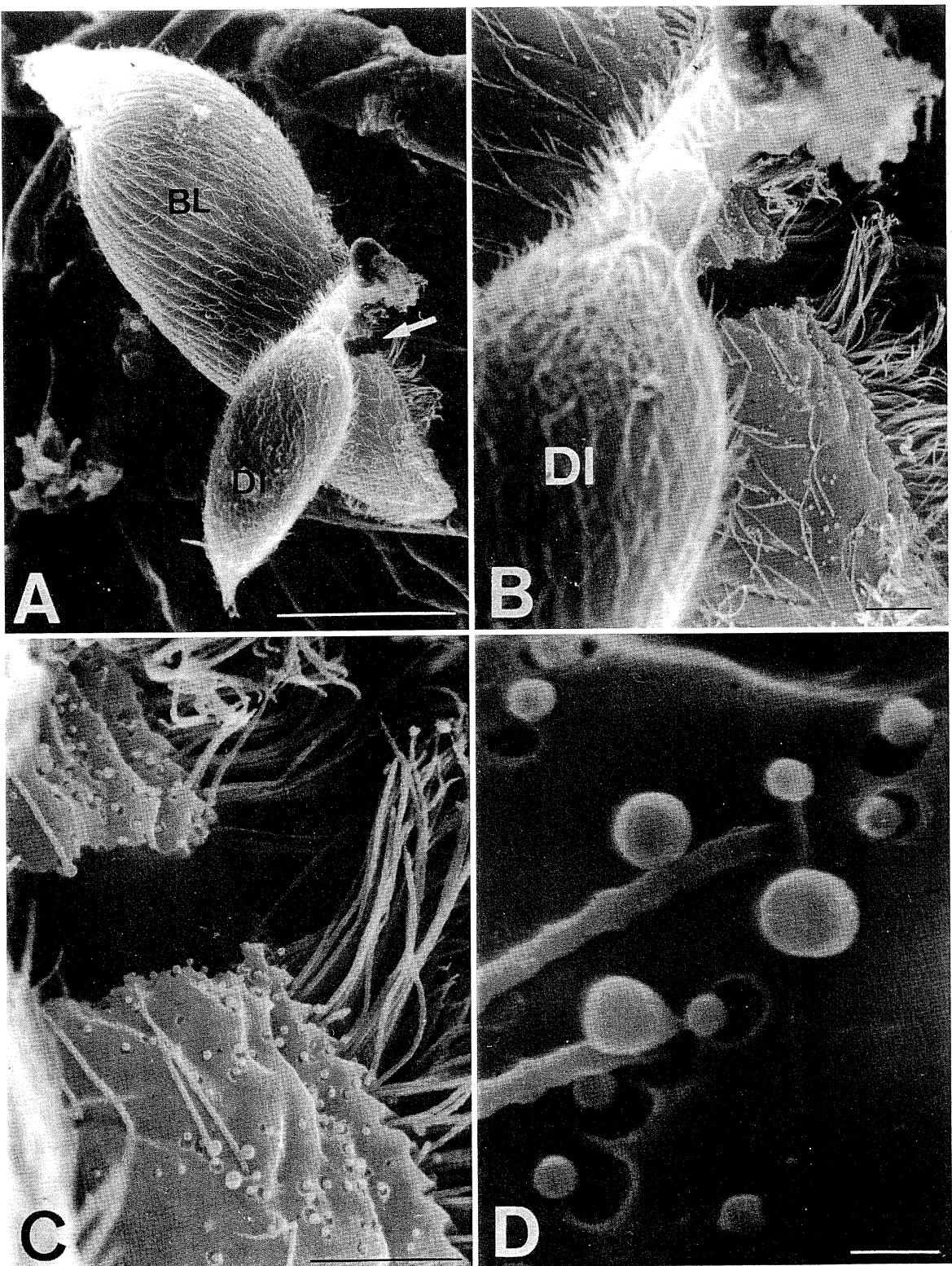


Fig. 2. Scanning electron micrographs of the *Blepharisma-Dileptus* interaction. **A:** *Blepharisma* being attacked by *Dileptus*. Arrow indicates the crack, the site of the wound inflicted by the proboscis of the *Dileptus*. The crack runs across the adoral zone of membranelles of *Blepharisma*. Bar; 50 µm. **B:** Enlargement of the region near the crack in A. Bar; 5 µm. **C:** Enlargement of the vicinity of the crack in B, showing the surface of *Blepharisma* peppered with spherules discharged from pigment granules. The surface is also pitted with small depressions presumably formed at the spots where the spherules have passed through the cell membrane. Bar; 5 µm. **D:** Enlargement of a part of C. Bar; 0.5 µm. BL: *B. japonicum* (stock R1072). DI: *D. margaritifer* (stock SHL1).

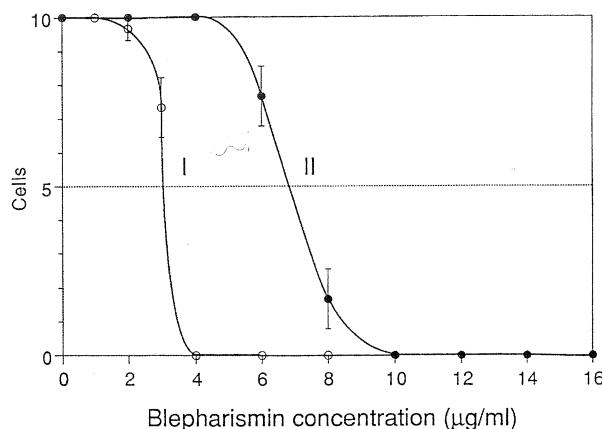


Fig. 3. Lethal effect of fractions I and II of purified blepharismin on *Dileptus margaritifer* (stock D3-I). ○—○ I: Fraction I. ●—● II: Fraction II. Means of 3 experiments with SE.

sults indicate that a spherule is the content of a pigment granule just discharged and that it has been discharged at the spot marked by a depression on the cell surface below the spherule. It is also likely that the discharged content swells into a larger spherule, while the surface depression disappears.

As described above, we observed that a *Blepharisma* attacked by the proboscis of a *Dileptus* locally released the pigment at the attacked site. The electronmicroscopic observations indicate that the pigment release is due to the discharge of pigment granules.

Toxicity of purified blepharismin

1. Behavioral responses of *Dileptus*: Several cells of *Dileptus* (SHL1) were placed in different concentrations of fraction II of purified blepharismin and their behavior was observed using a stereomicroscope. At concentrations higher than 5 μg/ml, some cells immediately started swimming backward. At 10 μg/ml, nearly all cells reacted in this way. Cells then became sluggish and their proboscis started shortening in a few minutes. At higher concentrations, the proboscis degenerated more quickly. The degeneration of the proboscis was reversible; the *Dileptus* whose proboscis was completely lost regenerated a new proboscis, if the cell was removed of blepharismin. The responses of *Dileptus* (SHL1) to fraction I of purified blepharismin were essentially the same except that they occurred at lower concentrations.

These responses of *Dileptus* to blepharismin are quite similar to those behavioral reactions of *Dileptus* in the *Blepharisma-Dileptus* interaction, supporting the assumption that the latter is evoked by blepharismin re-

leased by *Blepharisma*. More quantitative studies on the toxicity of blepharismin were carried out on the lethal effect as described below.

2. Lethal effect: The toxicity of fractions I and II of purified blepharismin to stock D3-I of *Dileptus* was tested as described in Material and Methods (Fig. 3). LD₅₀ values of the two fractions obtained from Fig. 3 are listed in Table 2 together with those for other ciliates. As shown, fraction I is about twice as toxic as fraction II.

Neither fraction was toxic to *Blepharisma*. In both stocks R1072 (red) and A538 (albino), no cell was killed up to the highest concentration tested, i.e. 50 and 80 μg/ml for fractions I and II, respectively (Fig. 4J–K for fraction II).

The toxicity of fraction II tested on various ciliates is shown in Fig. 4 and LD₅₀ values obtained from these results are listed in Table 2. LD₅₀ values for 7 of the 10 species tested cluster between 1.3 and 6.8 μg/ml, while those values for *Stentor coeruleus* (52.7 μg/ml) and *S. polymorphus* (14.9 μg/ml) are much higher. *S. coeruleus* has, in pigment granules, stentorin, the pigment closely related to blepharismin, while *S. polymorphus* is colorless. For possible explanation of the high resistance of these two species, see Discussion. The difference in LD₅₀ values between stocks D3-I and SHL1 of *D. margaritifer* (Table 2) suggests that the sensitivity to blepharismin is subject to considerable intraspecific variation.

Table 2. Lethal dose 50% (LD₅₀) of fractions I and II of purified blepharismin for various ciliates.

Species and stocks	LD ₅₀ of blepharismin (μg/ml)	
	Fraction I	Fraction II
<i>Lembadion bullinum</i>	0.7	1.3 ⁺⁺ 1.3 ⁺
<i>Didinium nasutum</i>		2.6 ⁺
<i>Paramecium caudatum</i>		2.7 ⁺
<i>Paramecium tetraurelia</i>		3.0 ⁺
<i>Dileptus margaritifer</i> , SHL1	1.5	3.0 ⁺
<i>Colpidium</i> sp.		4.7 ⁺
<i>Euplotes octocarinatus</i>		4.7 ⁺
<i>Dileptus margaritifer</i> , D3-I	3.1 [*] 4.4 ^{**}	6.8 [*]
<i>Stentor polymorphus</i>		14.9 ⁺
<i>Stentor coeruleus</i>	35.0	52.7 ⁺
<i>Blepharisma japonicum</i> , A538 (albino)	>50	>80 ⁺
<i>Blepharisma japonicum</i> , R1072 (red)	>50	>80 ⁺

* Based on data in Fig. 3. ⁺ Based on data in Fig. 4. ⁺⁺ Based on data in Fig. 5A. ^{**} Based on data in Fig. 5B.

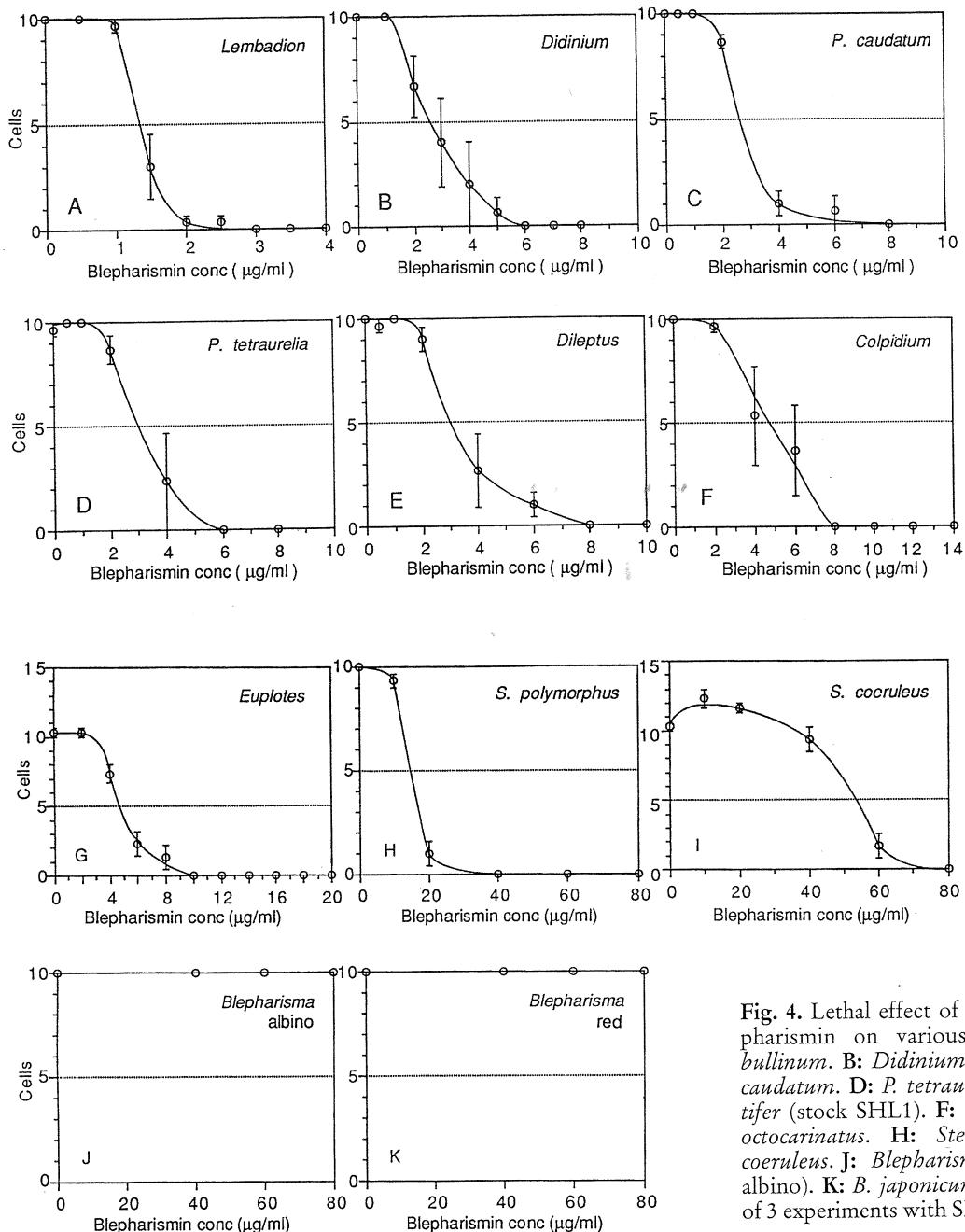


Fig. 4. Lethal effect of fraction II of purified blepharismin on various ciliates. A: *Lembadion bullinum*. B: *Didinium nasutum*. C: *Paramecium caudatum*. D: *P. tetraurelia*. E: *Dileptus marginifer* (stock SHL1). F: *Colpidium* sp. G: *Euplotes octocarinatus*. H: *Stentor polymorphus*. I: *S. coeruleus*. J: *Blepharisma japonicum* (stock A538, albino). K: *B. japonicum* (stock 1072, red). Means of 3 experiments with SE.

Effect of light on the toxicity of blepharismin and on the *Blepharisma-Dileptus* interaction

Since blepharismin is a photodynamic pigment [12, 14], we examined how photodynamic action participates in the toxic action of blepharismin and in the *Blepharisma-Dileptus* interaction by observing these phenomena in the light and in the condition in which photodynamic action hardly takes place.

1. Toxicity of blepharismin

- Lethal effect: The lethal effect of fractions I and II of purified blepharismin were tested, respectively on *Dileptus* (stock D3-I) and *Lembadion*, in the light and in the dark. The test in the dark (D) was the same as the test for the lethal effect of blepharismin described above. The test in the light (L) was carried out in the same way except that cells were kept in a glass moist chamber placed under a 40W cylindrical fluorescence

bulb at a distance of 40 cm until the time of observation.

The toxicity of both fractions was distinctly higher in L (Fig. 5). LD₅₀ values of fraction II for *Lembadion* in D and L were 1.3 and 0.4 respectively (Fig. 5A); LD₅₀ values of fraction I for *Dileptus* (D3-I) in D and L were 4.4 and 1.1 µg/ml, respectively (Fig. 5B). These results indicate that photodynamic action participates in the toxic action of both fractions of blepharismin in the light.

- Inhibition of feeding: A starved *Dileptus* (SHL1) was isolated in 200 µl of the crude blepharismin extract (see Material and Methods) in a slide depression. The isolate was made in 42 duplicates for each concentration of the extract under the red light described in Material and Methods. These isolates were equally divided in two groups, D and L. Group D was kept under the red light, while group L was placed under a 60W elec-

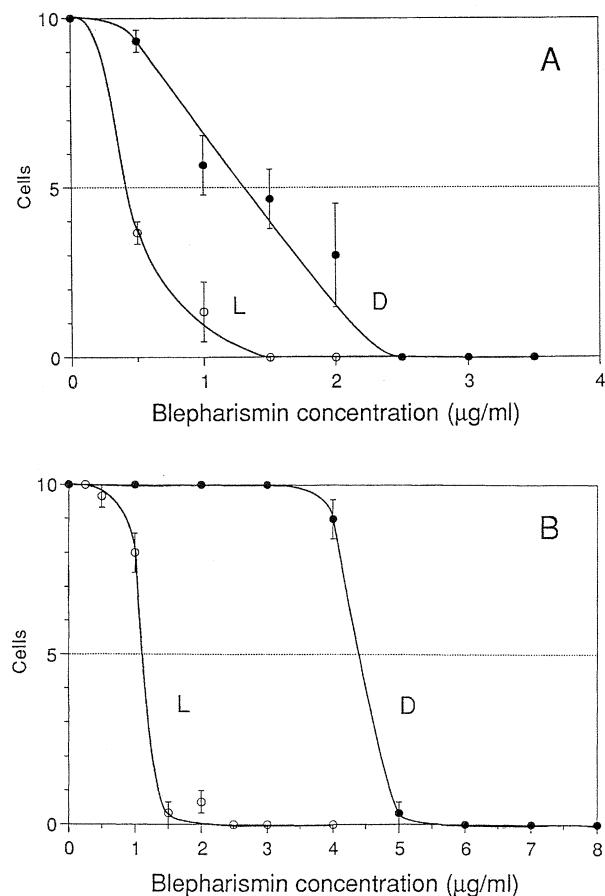


Fig. 5. Enhancement of the lethal effect of blepharismin by light. A: Toxicity of fraction II of purified blepharismin on *Lembadion bullinum*. B: Toxicity of fraction I of purified blepharismin on *Dileptus marginifer* (stock D3-I). ○—○, L: Experiment in the light. ●—●, D: Experiment in the dark. Means of 3 experiments with SE.

tric bulb (National) placed at a distance of 35 cm. After 1 h, a 200 µl suspension of *Sathrophilus* sp. (65,000–145,000 cells/ml) was added to each *Dileptus* and then both groups were placed in complete darkness. After 10 min, the *Dileptus* was examined for the presence of food vacuoles under the red light.

In both groups, fewer cells formed food vacuoles as the concentration of the extract increased (Fig. 6), indicating that feeding was inhibited by the previous 1 hour treatment by the extract. At a given concentration of the extract, the inhibition was stronger in group L (Fig. 6), indicating that photodynamic action participated in the inhibition.

2. *Blepharisma-Dileptus* interaction

The observation on the interaction between *Blepharisma* and *Dileptus* described above was carried out under the red light. No difference was detected in the behavioral reaction of *Dileptus* including the proboscis degeneration. The *Blepharisma-Dileptus* interaction described above is, therefore, likely to occur almost unchanged also in the dark.

A *Dileptus* was isolated in 50 µl of SMB⁻ in 21 duplicates under the red light. To each isolate, 150 µl of cell suspension of *Blepharisma* (4000 cells/ml) were added. After 1 hour of incubation in the complete darkness, *Dileptus* were observed under the red light. In all *Dileptus*, the proboscis had completely degenerated, confirming that blepharismin induces degeneration of the proboscis in the *Dileptus* without any participation of photodynamic action.

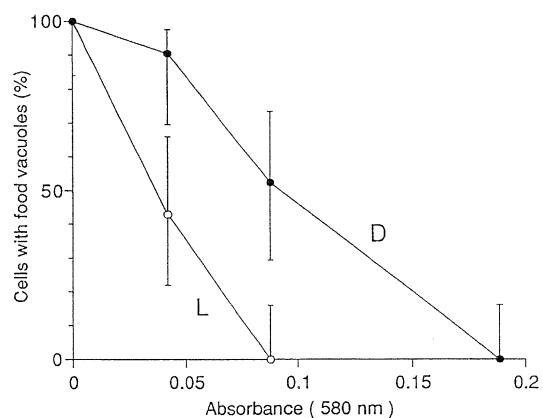


Fig. 6. Effect of light on the inhibition of feeding of *Dileptus marginifer* (SHL1) by blepharismin. Abscissa: Concentration of the crude extract of blepharismin indicated by absorbance. Ordinate: Percentage of *Dileptus* with food vacuoles. ○—○, L: Group L (*Dileptus* was kept under the white light for 1 h before feeding). ●—●, D: Group D (*Dileptus* was kept under the red light for 1 h before feeding). See text for further explanation. Means of 21 experiments with 95% confidence limits.

Discussion

Pigment granules of *B. japonicum* as extrusomes

Previous works showed that *Blepharisma* releases blepharismin, the red pigment localized in pigment granules, responding to various physical and chemical agents, such as low temperature, strichynine sulfate and sodium chloride [11, 12]. These results strongly suggest that pigment granules are extrusomes, but they hardly suggest how pigment granules function as extrusomes in nature. Giese, who found that crude extracts of blepharismin are toxic to various types of other organisms [9], thought that pigment granules might discharge their toxic content to repel predators [12]. However, he was skeptical over this hypothesis [13], because no more evidence was available and *Blepharisma* are readily eaten by predators, such as *Actinosphaerium eichhorni* [9] and small crustaceans [13].

More recently, two of us (A. M. and T. H.) and collaborators showed that pigment granules of *B. japonicum* function as organelles for defense against the predatory ciliate *Dileptus margaritifer* and hypothesized that *Blepharisma* releases the pigment to repel the predator [39]. They assumed two possible ways for the pigment release; 1) the local cytoplasmic lysis inflicted by *Dileptus*, and 2) the discharge of pigment granules as a response to the attack by *Dileptus*.

The present work supports the second possibility, though it does not exclude the first one. In our observation on the *Blepharisma-Dileptus* interaction, a *Blepharisma* attacked by a *Dileptus* instantly releases a mass of reddish material, sometimes without any distinct morphological changes in the cell. This mode of extrusion of cell material is characteristic to extrusomes, suggesting that the pigment release is due to the discharge of pigment granules rather than to the cytoplasmic lysis.

A stronger support for this hypothesis comes from our electronmicroscopical study on the *Blepharisma-Dileptus* interaction. In Fig. 2, which we think to show the moment of the attack on a *Blepharisma* by a *Dileptus*, the cell surface of the *Blepharisma* near the attacked region is peppered with many spherules of the size of pigment granules. In addition, the spherule characteristically lies over a round depression on the surface of *Blepharisma* (Fig. 2B-C) suggesting that the spherule has just been extruded from the interior of the cell through the spot marked by the depression.

We, therefore, conclude that a *Blepharisma* attacked by a *Dileptus* discharges pigment granules at and near the attacked region as a response to the attack. This confirms the assumption that pigment granules in *Blepharisma* are extrusomes and also provides an example

of the circumstances in which pigment granules of *B. japonicum* function as extrusomes in nature.

The stimulus that evokes the discharge of pigment granules in the *Blepharisma-Dileptus* interaction is unknown. Although toxicysts of *Dileptus* appear to contain acid phosphatase [5], little is known about the biologically active substance in these offensive extrusomes.

Pigment granules in *Blepharisma* are classified by Hausmann [20] as a special type of mucocyst together with pigment granules in *Stentor*, another heterotrich, and in *Loxodes* and *Trachelonema*, both karyorelictids. This classification has phylogenetic implications, in view of recent suggestions that extrusomes are important in phylogenetic and taxonomic studies of protists [6, 25, 43]. Since karyorelictids and heterotrichs are now considered to be most primitive ciliates [46], their pigment granules might be primitive forms of mucocysts in ciliates.

Mucocysts are characterized by 1) the paracrystalline structure in their content, 2) multiplication in length and diameter upon discharge with an extended paracrystalline structure and 3) the relatively slow discharge occurring in several seconds [20]. In pigment granules of *Blepharisma*, granulated [22], tubular [26] and honeycomb [26, 33] structures were seen, but not much is known about their paracrystalline structure. The content of pigment granules is discharged as a spherule and appears to increase the diameter as described in Results. On the other hand, we observed that pigment granules are discharged within a second. In this respect, they do not behave as typical mucocysts. It should be noted, however, that not much is known about many of the extrusomes so far classified as mucocysts. Moreover, the definition of mucocysts is not firmly established; Kugrens and collaborators [25] regard subsurface vesicles containing unstructured material as mucocysts; in their classification, mucocysts include rhoptries of Apicomplexa and spermatial vesicles of red algae, both of which were excluded from mucocysts by Hausmann [20]. The validity to classify pigment granules as mucocysts and phylogenetic problems of pigment granules are, therefore, largely left for future studies.

Mechanism of the defensive function of pigment granules

Observations on the *Blepharisma-Dileptus* interaction in this and the previous work [39] show how *Blepharisma* repels *Dileptus*. As soon as a *Dileptus* attacks a *Blepharisma* with its toxicysts-bearing proboscis, the *Blepharisma* releases a mass of pigmented material at and near the attacked region. The *Dileptus* swims backward and then becomes sluggish. These reactions of the *Dileptus* allow the *Blepharisma* to swim away from the

predator. In addition, after attacking *Blepharisma*, the proboscis of *Dileptus* often shortens. That an injury is involved in this shortening; this is indicated by the observation that the shortening continues until the whole proboscis is lost, if the *Dileptus* remains in the suspension of *Blepharisma*. If the proboscis is injured by an attack, it probably makes the next attack less effective.

These reactions of *Dileptus* suggest that the mechanism of repelling is essentially chemical. Since *Blepharisma* releases the red pigment as soon as it is attacked by *Dileptus*, it is assumed that blepharismin is the chemical basis of the repelling. This assumption is supported by the previous finding that a crude extract of blepharismin is toxic to various ciliates, but not to *Blepharisma* [9]. The present work showed that purified blepharismin is highly toxic to various ciliates including *Dileptus* but not to *Blepharisma*, thus strengthening the support. Blepharismin's participation in the repelling is further supported by the finding that purified blepharismin induces all major reactions of the *Dileptus* at the *Blepharisma-Dileptus* interaction, i.e. backward swimming, sluggishness, degeneration of proboscis. We, therefore, conclude that the repelling of *Dileptus* by *Blepharisma* is due to blepharismin released from *Blepharisma*.

As shown above, the *Blepharisma* attacked by a *Dileptus* immediately discharges pigment granules at and near the attacked region. We conclude that *B. japonicum* defends itself against the attack of *D. margaritifer* by discharging pigment granules which contains the repellent, blepharismin. Pigment granules of *B. japonicum* are, therefore, organelles for chemical defense.

Since blepharismin is a photodynamic pigment [12, 14], photodynamic action, i.e. oxidation by photoactivated pigment under the presence of molecular oxygen, participates in any toxic effect of blepharismin in the light. Indeed, the toxic effect of blepharismin measured in the light is distinctly higher than that measured in the dark (Fig. 5, 6). It is likely, therefore, that photodynamic action participates in the defensive function of blepharismin.

We think, however, that the role of photodynamic action in the chemical defense of *Blepharisma* is minor, if any, because of the following reasons. 1) The photophobic response of *Blepharisma* [24, 28, 45] should keep this ciliate mainly in shady or dark places. Indeed *Blepharisma* is known as a bottom dweller [14], living in the detritus where it is dimly lit or dark even in the daytime. 2) Blepharismin is highly toxic to *Dileptus* and other ciliates in the dark, while *Blepharisma* is immune against the toxicity (Fig. 3, 4, Table 2). 3) Repelling of *Dileptus* by *Blepharisma* observed under the red light, under the condition in which photodynamic action of blepharismin does not occur, is as strong as the one observed under the white light.

That blepharismin is toxic in the dark was first suggested by Giese who showed that a crude extract of blepharismin is toxic in the dark [10, 14]. The present work confirms it by using purified blepharismin. The recent report that blepharismin is toxic to bacteria and fungus in the dark as well as in the light [40] suggests that blepharismin is toxic in the dark to many kinds of cells.

The immunity of *B. japonicum* against the toxicity of blepharismin in the dark is seen in red cells as well as in albino cells (Fig. 4) suggesting that the immunity is a genetic trait of this species. *Stentor coeruleus*, another heterotrich, who uses stentorin for chemical defense against *Dileptus* [18, Miyake A. and Harumoto T. in preparation], is more resistant to blepharismin than all other ciliates tested except *Blepharisma* (Fig. 4, Table 2). Stentorin is a hypericin derivative similar to blepharismin [51] suggesting that the high resistance to blepharismin of *S. coeruleus* is correlated with the possession of stentorin. If so, the relatively high resistance of *S. polymorphus* to blepharismin (Fig. 4, Table 2) and the repelling of *Dileptus* by this colorless *Stentor* [Miyake A. and Harumoto T., unpublished] might be due to a toxin related, to some extent, to blepharismin. Whether the target molecule of blepharismin has a lower affinity to the toxin in these resistant species is worthy of examination.

Blepharismin in pigment granules is thought to associate weakly with a protein of 35–39 kD [15] and a protein of 200 kD [31, 33]. It is suggested that these proteins play a role in photoreception [15, 31, 33]. Alternatively, or in addition, these proteins may serve, together with other materials in pigment granules, to maintain the integrity of the discharged content of pigment granules while increasing the volume of the content, as it occurs at the discharge of other extrusomes such as trichocysts of *Paramecium* [20] and mucocysts of *Tetrahymena* [20]. Thus the discharged content of a pigment granule forms a spherule observed in this work (Fig. 2) which delivers concentrated blepharismin to a target cell, provoking a devastating effect on the cell surface.

Function of extrusomes

Extrusomes are widely distributed in protists [20, 25], but relatively little is known about their function. Offensive function of extrusomes in predatory ciliates, such as toxicysts and haptocysts, are long known [20], but it was only recent that the century old hypothesis for the defensive function of trichocysts in *Paramecium* was experimentally confirmed [16, 17, 37]. Concurrently, the defensive function of pigment granules in *B. japonicum* was demonstrated [39] and the hypothesis on its mechanism [39] has been confirmed by the present work.

Pigment granules in *S. coeruleus* [18] and trichocysts in *Pseudomicrothorax dubius* [R. K. Peck, personal communication] also function as organelles for defense against *Dileptus*. It is now more likely that pigment granules in other heterotrichs and trichocysts in other protists have the function of defense. Some of the other extrusomes of unknown function might turn out to be organelles for defense.

On the other hand, pigment granules in *B. japonicum* have been studied as photoreceptors for decades. Red cells of *Blepharisma* are killed if exposed to visible and near UV light of high intensity in the presence of molecular oxygen [8, see 12, 14 for review]. The killing is due to photodynamic pigment, blepharismin, which is localized in pigment granules [12, 14]. Pigment granules of *Blepharisma* are, therefore, photoreceptors for this photodynamic killing. This was the first time any function of pigment granules was discovered, although few regarded it as a function.

Meanwhile, it was demonstrated in *Stentor coeruleus* that pigment granules function as photoreceptors for the step-up photophobic response [48, 53, see 47, 49 for review] and the mechanism of this photoreception has been actively investigated [47, 49, 54]. Later, pigment granules in *B. japonicum* were also found to have the same function [1, 32, 34, 45] and since then these organelles have been investigated as photoreceptors for the photophobic response [7, 30].

It was suggested that the photophobic response of *Blepharisma* is important to avoid the lethal effect of light [29]. However, since photophobic response and the lethal effect of light (photodynamic killing) are both due to the same pigment, blepharismin, it is evident that *Blepharisma* does not possess blepharismin only to avoid the lethal effect of light which is caused by blepharismin itself. Blepharismin, therefore, must have an important function other than to avoid photodynamic action. What is this function?

So far four functions have been experimentally shown for blepharismin; 1) protection against far UV radiation [12, 14], 2) photoreception for photodynamic killing [12, 14], 3) photoreception for photophobic response [1, 32, 34, 45], and 4) chemical defense against *D. margaritifer* [39, this work].

Blepharismin in pigment granules protects *Blepharisma* from far UV radiation, e. g. UV light at 265 nm [12, 14]. However, this range of solar radiation scarcely reaches on the surface of the present-day earth and, in spite of the protection from far UV radiation, *Blepharisma* is highly sensitive to sunlight because of photodynamic action caused by visible and near UV light as described above. Moreover, *Blepharisma* is a bottom dweller, living in detritus [14] where strong light is not expected. It is, therefore, unlikely that the protection from far UV radiation is the main function of blepharismin.

For photodynamic killing, it is difficult to imagine a beneficial aspect of this function. Indeed, Giese, who intensively studied this photosensitization by blepharismin [8, see 12, 14 for review], did not regard it as a function.

Although the molecular mechanism of the photophobic response of *Blepharisma* is unknown, the response requires molecular oxygen [32]. That both the photodynamic killing and the photophobic response are based on the same pigment blepharismin and that both requires molecular oxygen suggest that these two photoresponses are based on the same mechanism. The photodynamic effect might stimulate cells to induce a reversal in ciliary beating resulting in a light-induced backward swimming, a photophobic response which keeps *Blepharisma* in dark or shady places. Irrespective of the validity of this assumption, the role of photophobic response in the life of *Blepharisma* is not known except that it serves to avoid the lethal effect of light caused by the presence of blepharismin. It is conceivable that photophobic response also serves for other roles, but the assumption that the main function of blepharismin is photoreception is not consistent with the fact that the pigment is localized in extrusomes.

On the other hand, there is no doubt about the importance of the defense against the common bottom-dwelling predator *Dileptus*. The localization of blepharismin in extrusomes is consistent with its function of chemical defense. Because the toxicity of blepharismin is not restricted to *Dileptus*, pigment granules should be effective in defense against other predators whenever their attack induces the discharge of these organelles.

Based on the above discussion, we propose a working hypothesis that the primary function of blepharismin and pigment granules is chemical defense. The role of photophobic response is then understood. Even if the only role of the photophobic response is to avoid the lethal effect of light, the response is important in that it enables *Blepharisma* to continue producing blepharismin for chemical defense in spite of the potential hazard of photodynamic action.

The hypothesis can be examined, for example, by investigating the function of cortical vesicles in colorless or apparently colorless heterotrichs such as *Blepharisma hyalinum* [27], *Stentor polymorphus* [44] and *Climacostomum virens* [42], which are morphologically similar to pigment granules in red species of *Blepharisma*.

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