Defense Function of Pigment Granules in the Ciliate Blepharisma japonicum against Two Predatory Protists,Amoeba proteus (Rhizopodea) and Climacostomum virens (Ciliata)

Authors: Terazima, Masayo Noda, and Harumoto, Terue

Source: Zoological Science, 21(8) : 823-828

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.21.823

ZOOLOGICAL SCIENCE **21**: 823–828 (2004)

Defense Function of Pigment Granules in the Ciliate *Blepharisma japonicum* against Two Predatory Protists, *Amoeba proteus* (Rhizopodea) and *Climacostomum virens* (Ciliata)

Masayo Noda Terazima[1]* and Terue Harumoto[2]

[1]*Tokai Women's Junior College, Kagamigahara, Gifu 504-8504, Japan*
[2]*Department of Biology, Faculty of Science, Nara Women's University, Nara 630-8506, Japan*

ABSTRACT—The defense function of pigment granules in the red ciliate *Blepharisma japonicum* against two predatory protists, *Amoeba proteus* and *Climacostomum virens*, was investigated by (1) comparing normally-pigmented and albino mutant cells of *B. japonicum* as the prey of these predators and (2) comparing resistance of the predators to blepharismin, the toxic pigment contained in the pigment granules of *B. japonicum*. Normally pigmented cells which contained more blepharismin than albino cells were less vulnerable to *A. proteus* than albino cells, but not to *C. virens*. *C. virens* was more resistant than *A. proteus* to the lethal effect of blepharismin. The results indicate that pigment granules of *B. japonicum* function as defense organelles against *A. proteus* but not against *C. virens* and suggest that successful defense against a predator depends on the susceptibility of the predator to blepharismin.

Key words: blepharismin, chemical defense, pigment granules, climacostol

INTRODUCTION

The pigment granules of *Blepharisma japonicum* are membrane-bounded spherical organelles 0.3–0.6 μm in diameter that are mostly localized in the cortex and attached to the cell membrane (Inaba *et al*., 1958; Giese, 1973; Jenkins, 1973). They contain the red photodynamic pigment, blepharismin (Checcucci *et al*., 1997; Maeda *et al*., 1997), and the contents of the granules are discharged from the cell in response to various chemical and physical stimuli (Giese, 1973). The granules are, therefore, considered to be extrusomes or extrusive organelles in protists (Hausmann, 1978).

Two functions of pigment granules in *B. japonicum* have been experimentally demonstrated: (1) chemical defense against the predatory ciliate *Dileptus margaritifer* (Miyake *et al*., 1990; Harumoto *et al*, 1998; Terazima *et al*., 1999) and (2) photoreception for photophobic responses (Matsuoka *et al*., 1992; Checcucci *et al*., 1993). In addition, Giese (1973, 1980) showed that pigment granules serve as a protective shield against far UV radiation. The biological significance of this function was questioned, however, because this range of solar radiation scarcely reaches the surface of the present-day earth (Harumoto *et al*., 1998). The chemical basis of these functions is the red pigment, blepharismin.

The evidence for the chemical defense function of the pigment granules is as follows. The albino mutant and bleached cells of *B. japonicum* are much more vulnerable to the predator (Miyake *et al*., 1990). *B. japonicum* discharges pigment granules at and near the attacked site when it is attacked by *D. margaritifer* (Harumoto *et al*., 1998). Purified blepharismin is highly toxic to the predator, in the light as well as in the dark, while the pigment is almost non-toxic to *B. japonicum* (Harumoto *et al*., 1998; Terazima *et al*., 1999). Oxyblepharismin, the photoinduced product of blepharismin, is also toxic to the predator (Terazima *et al*., 1999).

In this work we investigated whether pigment granules in *B. japonicum* are effective in defense against two other predatory protists, *Amoeba proteus*, a rhizopod, and *Climacostomum virens*, a ciliate, by comparing normally-pigmented and albino mutant cells of *B. japonicum* as prey for these predators and by measuring the susceptibility of these predators to purified blepharismin. The result showed that pigment granules of *B. japonicum* are effective in the defense against *A. proteus*, which is sensitive to blepharismin, but that they are not effective against *C. virens*, which is resistant to blepharismin.

* Corresponding author: Tel. +81-583-82-1148;
FAX. +81-583-83-5455.
E-mail: idea21@tokai-wjc.ac.jp

MATERIALS AND METHODS

Cells

Blepharisma japonicum, stocks R1072 (wild-type) and A538 (mutant albino), *Climacostomum virens*, clone W-24, isolated by Dr. M. Tavrovskaya, Inst. Cytol., Russ. Acad. Sci., St. Petersburg, and *Amoeba proteus*, stock G, provided by Dr. Y. Tsukii, Hosei Univ., Tokyo, were used.

The mutant albino of *B. japonicum* appeared spontaneously in the laboratory among wild-type red cells (Chunosoff *et al*., 1965). It contains only a minute amount of the red pigment blepharismin and looks white (Chunosoff *et al*., 1965; Giese and Grainger, 1970). For more details about stocks of *Blepharisma*, see Harumoto *et al*., 1998. *C. virens* is usually green because of the presence of symbiotic *Chlorella*. A permanently white subclone (clone W) was isolated from the clone W-24 and used in this work.

Blepharisma was grown in the dark at 25°C in the wheat-grass-powder medium (Takagi *et al*., 1993) inoculated with *Enterobacter aerogenes* 2 days before use. Cells were collected by centrifugation (100×g, 3 min), washed with SMB- (1.5 mM NaCl, 0.05 mM KCl, 0.4 mM $CaCl_2$, 0.05 mM $MgCl_2$, 0.05 mM $MgSO_4$, 2.0 mM Na-phosphate buffer, pH 6.8), a modified medium of SMB, which is a synthetic medium for *Blepharisma* (Miyake *et al*., 1981), filtered through a nylon net to remove debris, resuspended in SMB-, and used after 1 day. *Climacostomum* and *Amoeba* were grown on SMB- suspension of *Sathrophilus sp*., a small ciliate, that was grown, washed and suspended in SMB- as *Blepharisma*. Only slightly starved cells in the stationary phase were used for the experiments. Handling of cells and experiments were performed at room temperature (23±5°C). Experimental cells were kept in dark, moist chambers except during handling and observation.

Blepharisma – predator interaction

Five predators (*Climacostomum* or *Amoeba*) were placed in 200 μl SMB- with 10, 20, and 40 blepharismas. These mixtures were made for albino and red blepharismas. In each mixture, the numbers of living blepharismas and predators were counted each day for 4 days. Data are the means of nine experiments.

Blepharismin

Blepharismin was extracted and purified as indicated else-

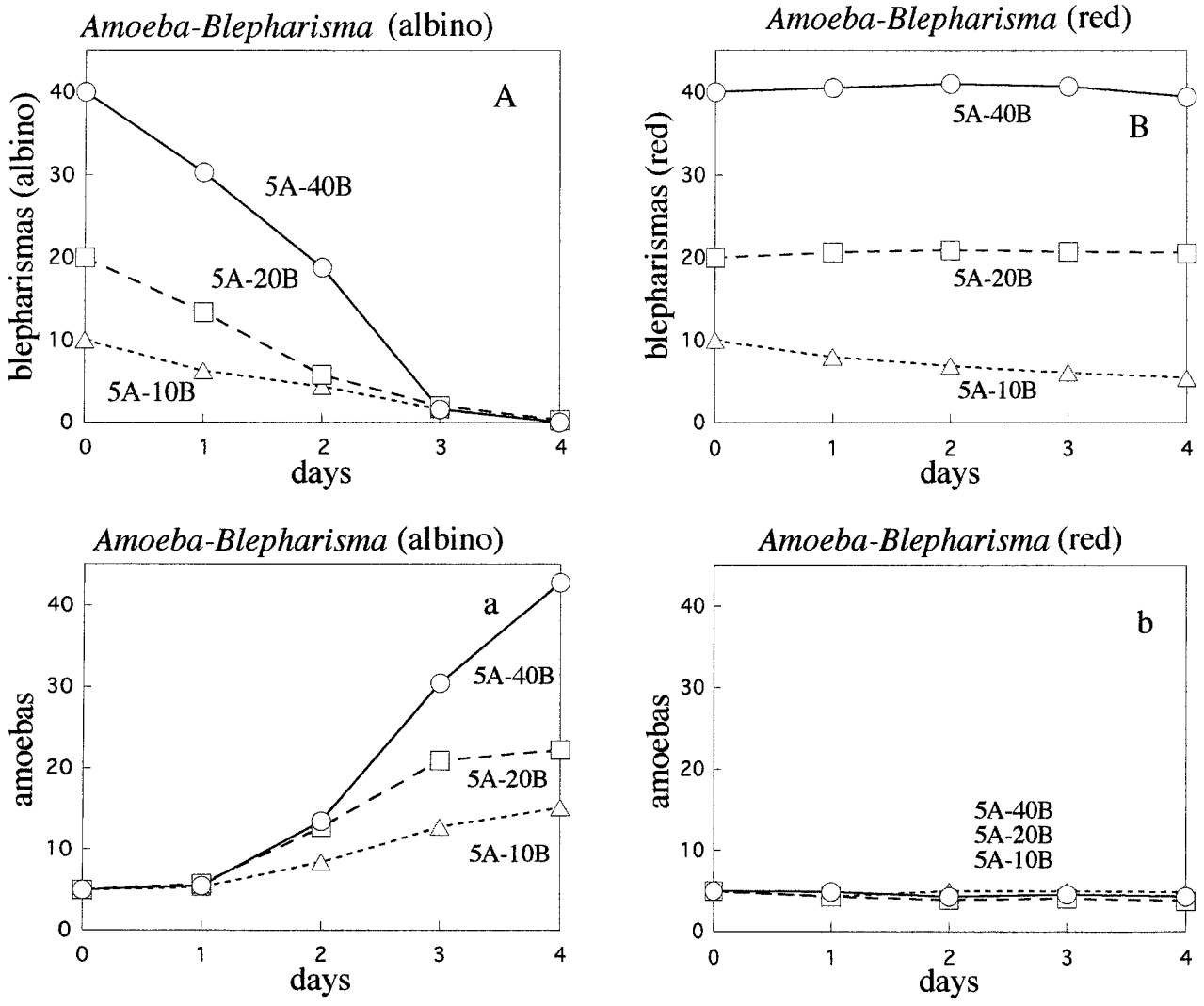

Fig. 1. Effect of pigmentation and cell density of *Blepharisma japonicum* on the offense-defense interaction between *Amoeba proteus* and *B. japonicum*. The numbers of blepharismas and amoebas are plotted in separate graphs (A, B and a,b, respectively) against the time after the mixing of 5 amoebas with 10(5A-10B, △), 20(5A-20B, □) and 40(5A-40B, ○) blepharismas in 200 μl SMB-. The blepharismas used are albino (mutant) in A and a, and red (wild type) in B and b.

where (Terazima *et al.*, 1999). Blepharismin was dissolved in 99.5% ethanol and further diluted with SMB- so that the final concentration of ethanol was less than 2% (vol/vol). The concentration of blepharismin was calculated based on the molar extinction coefficient of blepharismin in ethanol, 3.75×10^4 $M^{-1}cm^{-1}$ (Checcucci *et al.*, 1997).

Lethal effect of blepharismin

The lethal effect of blepharismin on a protist was tested by placing ten cells of the protist in 200 μl of SMB- containing various concentrations of blepharismin in a slide depression and by counting the number of living cells after 1 day. The lethal dose 50% (LD_{50}) of blepharismin was obtained using the concentration-survival curve as described elsewhere (Harumoto *et al.*, 1998).

RESULTS

Amoeba-Blepharisma interaction

To test the defensive function of pigment granules in red *Blepharisma*, we compared normally pigmented and albino mutant cells of *B. japonicum* in the interaction with amoebas. In the mixtures containing albino blepharismas, the number of blepharismas decreased (Fig. 1, A), while the number of amoebas increased (Fig. 1, a). Amoebas multiplied more intensively in the mixture in which more blepharismas disappeared, indicating that albino blepharismas were consumed as food by amoebas. On the contrary, normally-pigmented red blepharismas survived. In the mixtures containing red blepharismas, the number of blepharismas decreased only slightly in 5A-10B and did not decrease at all in 5A-20B and 5A-40B (Fig. 1, B). The number of amoebas did not change (Fig. 1, b). They were often floating just under the surface of the medium, and the phenomenon was not observed in the mixture containing albino blepharismas, suggesting that amoebas were affected by red blepharismas but not by albino blepharismas.

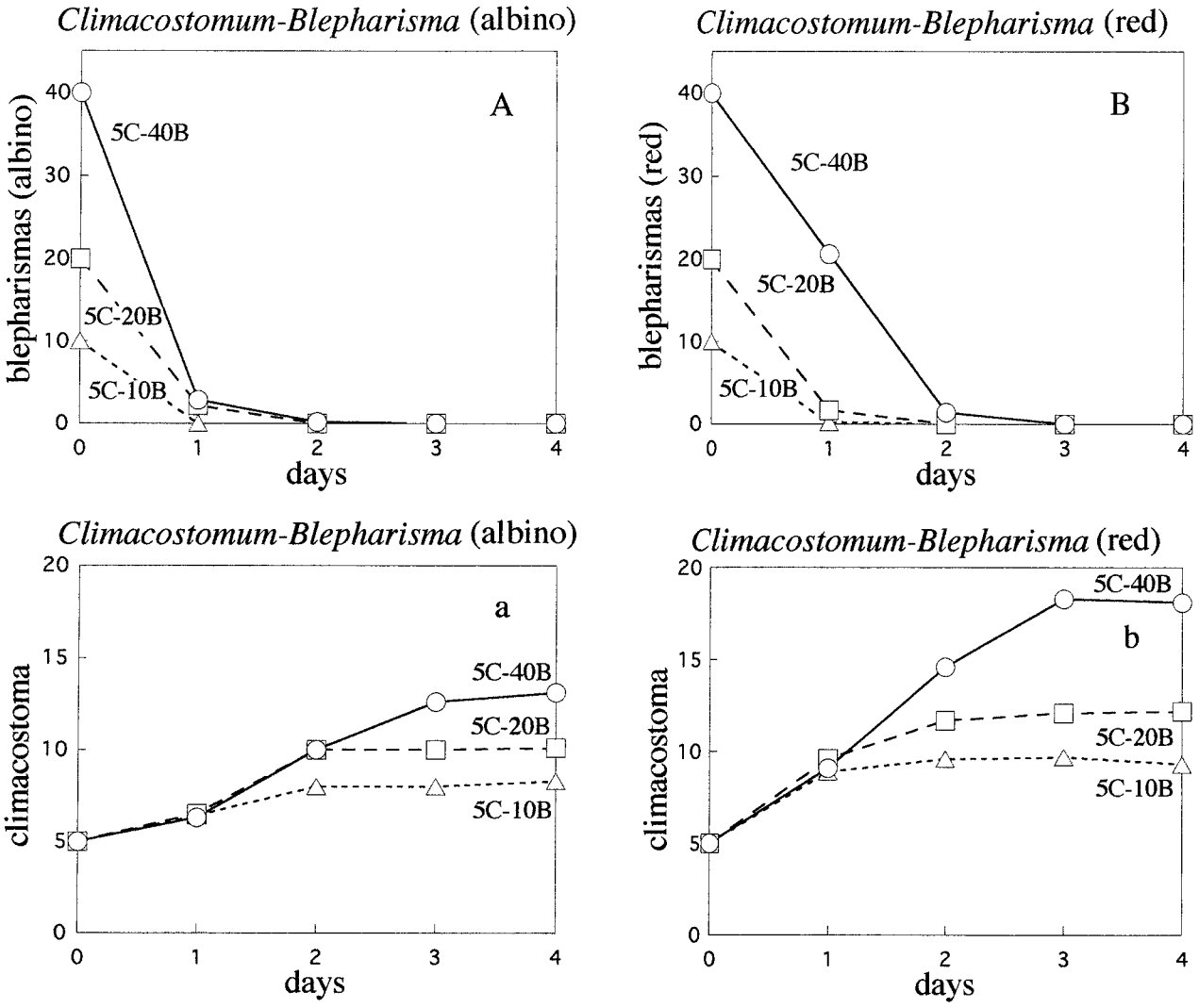

Fig. 2. Effect of pigmentation and cell density of *Blepharisma japonicum* on the offense-defense interaction between *B. japonicum* and *Climacostomum virens*. The numbers of blepharismas and climacostoma are plotted in separate graphs (A, B and a, b, respectively) against the time after the mixing of 5 climacostoma with 10(5C-10B, △), 20(5C-20B, □) and 40(5C-40B, ○) blepharismas in 200 μl SMB-. Blepharismas used were albino (mutant) in A and a, and red (wild type) in B and b.

Albino blepharismas are, therefore, much more vulnerable to amoebas than red blepharismas, indicating that blepharismin, and hence also pigment granules which contain the pigment, have a defense function against *Amoeba proteus*.

Climacostomum-Blepharisma interaction

Climacostomum virens has a large mouth opening. Preys of various sizes are sucked into the buccal cavity and ingested through the cytostome. Blepharismas, albino and red, were mixed with climacostoma in 200 μl SMB- in a slide depression, and the interaction between the two protists was observed in a stereomicroscope. Climacostoma ingested both albino and red blepharismas apparently indiscriminately. Albino and red blepharismas were compared more quantitatively as prey for climacostoma (Fig. 2). In all of the interactions in any of the mixtures, the number of blepharismas decreased (Fig. 2, A, B) while the number of climacostoma increased (Fig. 2, a, b). In 5C-10B and 5C-20B, albino and red blepharismas decreased in number in nearly the same way. In 5C-40B, more red cells survived after 1 day, but the difference largely disappeared after 2 days. The slightly higher increase in the number of climacostoma in the mixtures with red blepharismas is probably due to the fact that red blepharismas are slightly larger than albino blepharismas. The result indicates that albino and red blepharismas were similarly consumed as food by climacostoma. Albino and red blepharismas are, therefore, equally vulnerable to climacostoma, indicating that pigment granules of *B. japonicum* are not effective for the defense against *C. virens*.

Resistance to blepharismin

To investigate further the role of blepharismin in the interactions between the protists used in this work, the resistance of these protists to blepharismin expressed by LD_{50} was compared, the concentration of blepharismin required for 50% lethality (Table 1). The resistance of *Blepharisma* (red, albino) was so high that LD_{50} was not measurable at the concentrations tested. *Climacostomum*, against which blepharismin was not effective in the defense, was 29 times more resistant than *Dileptus*, against which blepharismin is known to be effective in the defense (Miyake *et al*., 1990). *Amoeba*, against which blepharismin was effective in the defense, was only twice as resistant as *Dileptus*. These results suggest that the defense function of blepharismin against predatory protists is based on the susceptibility of these predators.

Table 1. Lethal dose 50% (LD_{50}) of blepharismin in the dark for the four species of protists used in this work.

Protists	LD_{50} (M)*	LD_{50}/LD_{50}(*Dileptus*)**
Blepharisma japonicum (red)	$>>2.0 \times 10^{-4}$	>>59
Blepharisma japonicum (albino)	$>>2.0 \times 10^{-4}$	>>59
Climacostomum virens	1.0×10^{-4}	29
Amoeba proteus	7.6×10^{-6}	2.2
Dileptus margaritifer	3.4×10^{-6}	1

* Molar concentration (M) was calculated based on the molar extinction coefficient of blepharismin in ethanol at 580 nm, 3.75×10^4 M^{-1} cm^{-1} (Checcucci *et al*.,1997).

** The relative resistance to the LD_{50} for *Dileptus margaritifer* .

DISCUSSION

The purified blepharismin was highly toxic to various ciliates (Harumoto *et al*. 1998), suggesting that the defensive function of red *B. japonicum* is widely effective against various predatory ciliates. This finding was not verified in the cell-cell interaction, however, except in the case of the *Blepharisma-Dileptus* interaction (Miyake *et al*., 1990). So we studied the defensive function of the red *B. japonicum* against heterotrophic heterotrich *C. virens*, which is different from *D. margaritifer* in its mode of feeding.

On the other hand, the observation that rhizopod *Actinosphaerium eichhorni* ingests many red blepharismas suggests that pigment granules have no protective function against this predator (Giese, 1973). We examined the defensive function of pigment granules in the red *B. japonicum* against the predatory protist *Amoeba proteus*, which belongs to the same phylum Sarcomastigophora as *Actinosphaerium eichhorni*. *Amoeba* is different in its mode of feeding from *D. margaritifer* and *C. virens*. Whether the defensive function of the pigment granules of *B. japonicum* is effective against the other predatory protists *A. proteus* and *C. virens* or not is a question that attracts much interest.

Our results show that normally pigmented albino cells of *B. japonicum* are more vulnerable than red cells to the predatory rhizopod *A. proteus* but not to the predatory ciliate *C. virens*. Since the red coloration of *B. japonicum* is due to the pigment, blepharismin, localized in pigment granules (Inaba *et al*., 1958; Giese, 1973), the result indicates that pigment granules of *B. japonicum* function as defense organelles against *A. proteus* but not against *C. virens*. These facts suggest that the pigment granules in *B. japonicum* are effective not only against *D. margaritifer* but also other predatory protists, and are not effective against some others.

Our finding that *C. virens* is much more resistant than *D. margaritifer* and *A. proteus* to the lethal effect of blepharismin (Table 1) suggests that the successful defense by pigment granules against a predatory protist is due to a high susceptibility of the predator to blepharismin.

On the other hand, in the defense of *B. japonicum* against *D. margaritifer*, pigment granules discharge blepharismin in response to the predator's attack (Harumoto *et al*., 1998), while the pigment is not discharged when blepharismas are ingested by *Actinosphaerium* (Giese, 1973), suggesting that the successful defense by pigment granules against a predatory protist depends on the capacity of blepharismas to discharge pigment granules as a response to the predator's attack. To confirm these assumptions, further studies, particularly the study of the discharge of pigment

granules of *B. japonicum* at the time of attack by *A. proteus* and *C. virens*, and the study of the susceptibility of *A. eichhorni* to blepharismin, are needed.

Blepharismin is a photodynamic pigment. Even a dilute solution of blepharismin photosensitizes colorless cells (Giese, 1953; Giese, 1973; Harumoto *et al*., 1998). The toxicity of blepharismin to *D. margaritifer* is much higher under illuminated conditions (Harumoto *et al*., 1998; Terazima *et al*., 1999). When *Blepharisma* is exposed to strong light in the presence of oxygen, it is killed by photodynamic action due to its own pigment blepharismin (Giese and Zeuthen, 1948; Giese, 1953). Up to now, the demonstration of the defense function of blepharismin was mostly based on experiments carried out in the dark (Miyake *et al*., 1990; Harumoto *et al*., 1998; this work). The intrinsic toxicity (the toxicity in the dark) of blepharismin is, therefore, sufficient for the use of this pigment as a chemical defense, but to understand fully the mechanism of defense by means of blepharismin, it is necessary to consider also the toxicity due to the photodynamic action against both *Blepharisma* and its predator.

The mechanism of the defense function of pigment granules in the light is made more complicated by the fact that light changes blepharismin to oxyblepharismin (Giese and Zeuthen, 1948; Giese and Grainger, 1970; Ghetti *et al*., 1992; Watanabe *et al*., 1995: Spitzner *et al*., 1998). We found that oxyblepharismin also has intrinsic toxicity and phototoxicity and that it also plays a role in the defense function of the pigment granules in *B. japonicum* (Terazima *et al*., 1999). The phototoxicity of blepharismin is thought to be due to the generation of short-lived oxygen species, such as singlet oxygen ($^1O_2$)(Jardon *et al*., 1987; Checcucci *et al*., 1991) and hydroxyl radical (OH •)(Kato *et al*., 1996). Very little is known about the mechanism of the intrinsic toxicity of blepharismin (Pant *et al*., 1997).

Some heterotrich ciliates other than *B. japonicum* have pigment granules. Of these, *Stentor coeruleus* is known to exhibit chemical defense against *D. margaritifer* by means of the pigment stentorin, which is localized in the pigment granules of this ciliate (Harumoto and Miyake, 1996; Miyake *et al*., 2001).

Many other heterotrichs, such as *C. virens* (Peck *et al*., 1975) and *Blepharisma hyalinum* (Larsen and Nilsson, 1983), have colorless cortical vesicles which are morphologically similar to pigment granules. The cortical vesicles in *C. virens* were recently shown to be extrusomes that have a defensive function against *D. margaritifer* (Miyake *et al*., 2003). That research also showed that the chemical basis of this defense is climacostol discharged from cortical vesicles. Climacostol is the colorless toxin isolated from the whole extract of *C. virens* by Masaki *et al*. (1999), who suggested that climacostol is metabolically related to stentorin and hypericin from plants, and hence is probably also related to blepharismin. These findings suggest that the study of the chemical evolution of extrusomal defense toxins in ciliates has interesting possibilities.

ACKNOWLEDGMENTS

We are grateful to Dr.Y.Takagi of Nara Women's Univ. and Dr.H.Iio of Osaka City Univ. for helpful discussion.

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(Received January 5, 2004 / Accepted May 17, 2004)