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Photodynamic Action of the Pigment in Ciliated Protozoan *Blepharisma*

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ABSTRACT

The quinone pigment blepharismin isolated from *Blepharisma* killed other ciliates *Paramecium caudatum* when it was activated by light. We demonstrated by using electron spin resonance (ESR) spectrometry that light-activated blepharismin generated hydroxyl radicals (-OH). The result suggests that photodynamic action of the pigment may be attributed to hydroxyl radicals.

INTRODUCTION

Recently, pharmacologically important actions of quinoid pigments have been reported. One of the actions of the pigments is an inactivation of mature and assembled retroviruses such as HIV (Lavie et al. 1989). Another important action is an inhibition of protein kinase C activity (Kobayashi et al. 1989; Watanabe et al. 1995) which plays important roles in signal transduction (Nishizuka 1986). The merit for clinical application of the quinoid pigments is that the activity of the pigments can be controlled by light (Hudson et al. 1993; Watanabe et al. 1995) such as laser beam.

Ciliated protozoan *Blepharisma* has numerous pigment granules containing quinoid pigment (Sevenants 1965) called blepharismin (Giese 1973) that are located just beneath of plasma membrane (Kennedy 1965; Matsuoka et al. 1994). The blepharismin pigment has been suggested to be a photoreceptor pigment controlling the photobehavior of the cells (Scevoli et al. 1987; Matsuoka et al. 1992). On the other hand, the pigment is known to kill other protozoans such as *Paramecium* when it is activated by light (Giese 1973). Therefore, another function of blepharismin might be defence against predators (Miyake et al. 1990). However, the mechanism of such a photodynamic action has not been elucidated.

In the present study, we demonstrated that light-activated blepharismin generated hydroxyl radicals which might be related to photodynamic action. The blepharismin pigment contained in the cells cultured under dark condition is pink-colored (pink form), while it is converted to blue-colored form *in vivo* when the cells were exposed to light in the presence of O_2 (Giese 1973; Matsuoka et al. 1992). Generation of hydroxyl radicals was also examined in blue form of blepharismin.

MATERIALS AND METHODS

Blepharisma japonicum or Paramecium *caudatum* was cultured under dark condition in 0.1% cereal leaves infusion containing bacteria (*Enterobacter aerogenes*) as food. The bacteria were cultured on 1.5% agar plates containing 0.5% polypeptone, 1% meat extract and 0.5% NaCl. The cultured cells of *Paramecium* were transferred into a saline solution containing 5 mM Tris-HCl (pH7.4), 1 mM $CaCl_2$ and l mM KCl prior to assays.

In order to extract blepharismin, the packed cells obtained by centrifugation (150 *g*, 1 min) were suspended for l min in acetone. After sedimentation (150 *g*, 1 min) of the cells, the supernatant containing pigment was decanted, and concentrated with a rotary evaporator (Rotavapor, Sibata) for applying on a plate (silica gel 60 F_{254}, Merck) for thin-layer chromatography (TLC). Pigment was developed with a mixture of ethyl acetate and acetone (4:1, v/v). All procedures were carried out under dim red light. For stock solutions, the pigment was dissolved in ethanol.

Electron spin resonance (ESR) spectra were measured at room temperature with a JES-RE3X spectrometer(JEOL) with 100-kHz field modulation. For a spin trapping reagent for superoxides (Rosen and Rauckman 1981), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was used.

RESULTS AND DISCUSSION

The cells of *Paramecium* were killed when TLC-purified blepharismin (pink form) was added in the cell suspension and it was exposed to white light. The effect of the pigment was dependent upon its concentration (Fig. 1a). In much higher concentration of the pigment, the cells were killed, although the pigment was not light-activated (Fig. 1a). The photokilling effect (photodynamic action) of the pigment was dependent upon light intensity (Fig. 1b).

The fact that light irradiation of blelpharismin causes an increase in O_2 consumption (Giese and Zeuthen 1949) implies that certain species of superoxide may be involved in such a photodynamic action. Therefore, we tried to detect superoxides produced by light irradiation of the pigment. As shown in Fig. 2A, a

prominent signal corresponding to hydroxyl radical adduct (DMPO-·OH) was detected when blepharismin (pink form) preparation was exposed to light.

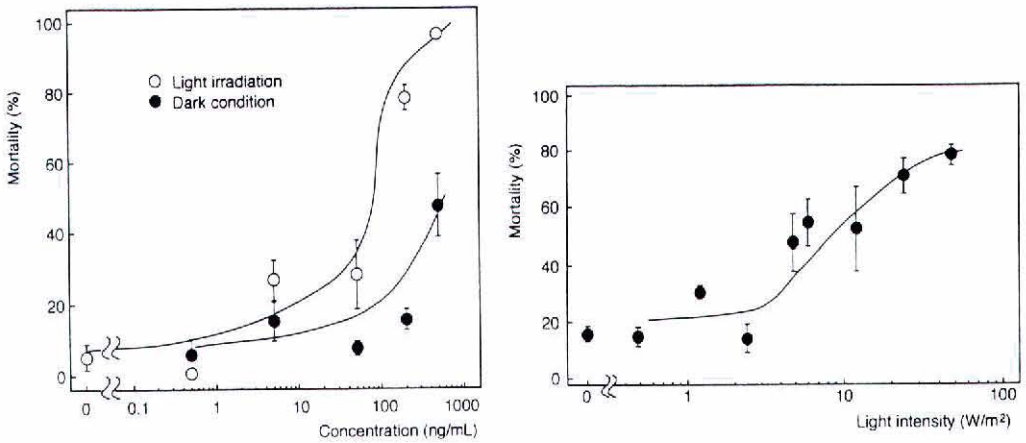

Figure 1. Action of blepharismin (pink form) on *Paramecium*.. 1a: Relationship of blepharismin concentration and mortality of *Paramecium*. Open circles, photodynamic action of the pigment induced by light irradiation. (50 W/m², 20 min); closed circles, effect of blepharismin on *Paramecium* in dark (treated for 20 min). 1b: Relationship of light intensity and motarity of *Paramecium*. Pigment concentration was adjusted to 200 ng/ml. Small amounts of stock ethanol solutions containing blepharismin were added into 5 cm culture dishes filled with *Paramecium* suspension. In this case, final concentrations of ethanol was less than 0.1%. Motality (%) of *Paramecium* is expressed as percentage of killed cells of total number (50-100 cells). Circles and bars correspond to the means and SE obtained from identical measurements (n=3).

Crude blue-colored blepharismin which was extracted from the cells after exposure to light in the presence of excess O_2 was separated into two components (blue forms I and II) by TLC. The blue forms of the pigment still generated hydroxyl radicals, although the amplitude of the signals were decreased (Fig. 2B, C). The photodynamic action of blue forms was extremely weak (Data not shown). The result may imply that the photodynamic action is attributable to

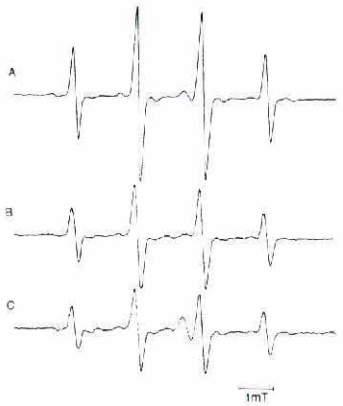

Fig.2. ESR spectra of adducts spin-trapped with DMPO which were generated by light irradiation of TLC-purified blepharismin. (A), pink form of blepharismin; (B), blue form 1 (obtained from an upper spot on TLC); (C), blue form II (obtained from a lower spot on TLC). Pigment preparations (0.14 mg/ml) were exposed to white light (400 W/m^2) for 30sec.The pigment concentration was adjusted to 0.14 mg/ml. The pigment was dissolved in 1% (v/v) Triton X-100, 5mM Tris-HCl (pH7.4), 0.92M DMPO solution.

hydroxylradicals which are involved in lipid peroxidation (Czapski 1984) or fragmentation of double-stranded DNA (Rowley and Halliwell 1983). Even the cells of *Blepharisma* (pink form) are partially killed by photodynamic action when strong light is applied, whereas blue-colored *Blepharisma* cells do not (Giese 1973; Ghetti et a1. 1992). This might be consistent with the present results.

Quinoid pigments are known to produce singlet oxygen (Thomas et al.1992). Therefore, not only hydroxyl radicals but also singlet oxygen ($^1 O_2$) is possibly involved in the photodynamic action on *Paramecium.*

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