

## Dimorphism of Spores of *Nosema* spp. in Cultured Cell

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A *Nosema* sp. from the lawn grass cutworm, *Spodoptera depravata*, produced two types of spores in cultured cells of *Antheraea eucalypti*. One was characterized by 3 to 5 coils of the polar tube, thin endospore (35 nm), early development (36 hr postinoculation), and the spontaneous discharge of a short polar tube. The other type characterized by 10 to 12 coils of the polar tube, thick endospore (91 nm), and late development (54 hr postinoculation) was the predominant spore in later culture and was similar to the purified spores harvested from diseased insects, which discharged a long polar tube. *Nosema bombycis* similarly produced two types of spores in cultured cell. © 1991 Academic Press, Inc.

**KEY WORDS:** *Nosema* sp.; *Nosema bombycis*; Microsporidia; cell culture; *Antheraea eucalypti* cell; spore dimorphism; *Spodoptera depravata*; *Bombyx mori*.

### INTRODUCTION

Trager (1937), who examined *Nosema bombycis* development in cell culture, showed that the hemolymph of infected silkworm larvae was infectious to cultured cells and inferred that the infective form "planont" of Stempell (1909) existed in it. Ishihara (1969) observed a sporoplasm-like body, "secondary infective form," inside and outside of cultured cells but did not observe spore germination inside the host cell. Intracellular spore germination was observed in cultured cells infected with *Encephalitozoon cuniculi* (Petri, 1969) and a *Nosema* sp. (Iwano and Ishihara, 1988, 1989) and in mosquito larvae infected with *Nosema algerae* (Vavra and Undeen, 1970; Avery and Anthony, 1983). We herein describe the spore dimorphism of two *Nosema* spp. in cultured cells and present evidence of dissemination of the *Nosema* spp. among culture cells by one type of spore.

### MATERIALS AND METHODS

**Microsporidia.** Two *Nosema* spp. were utilized in this study. One was originally isolated from the lawn grass cutworm, *Spodoptera depravata* (Iwano and Ishihara, 1988). The other was *N. bombycis* obtained from the National Institute of Seri-

culture and Entomological Science, Tsukuba. The two microsporidia were maintained in respective hosts, *S. depravata* and *Bombyx mori*. Spores harvested from the diseased larvae were purified by repeated differential centrifugation (1500 g, 5 min) and Percoll density gradient centrifugation (73,000 g, for 30 min) and stored at 5°C until used. Spores were sensitized to discharge their polar tube either by exposing them to a 3% solution of H<sub>2</sub>O<sub>2</sub> in 0.1 M KCl for 40 min or by changing the pH from alkaline (0.1 M KOH) to near neutral ranges (Grace's cell culture medium, pH 6.4) (Ohshima and Suzuki, 1939; Ishihara and Sohi, 1966).

**Cell line.** An *Antheraea eucalypti* cell line (Grace, 1962) was received from Dr. T. Kawarabata, Kyushu University, Fukuoka. It was maintained at 27°C in Grace's medium supplemented with 5% fetal bovine serum (GIBCO), 5% heat-inactivated *B. mori* hemolymph, 200 units penicillin/ml, and 200 µg streptomycin/ml. To infect the cell culture, 0.2 ml of the spore suspension ( $2.4 \times 10^9/0.2$  ml) was combined with 0.2 ml of 0.1 N KOH, and after incubation at 25°C for 40 min was added to 12 ml of a *A. eucalypti* cell suspension. Aliquots (0.8 ml) of the mixture were placed in culture vials (Corning plastic culture flask, 60 ml), and

after an hour of incubation, the supernatant was replaced with 2 ml of fresh medium.

**Light and electron microscopy.** Every 3 or 6 hr postinoculation (pi), one culture vial of cells was sacrificed to prepare smears for light microscopy and also to process for electron microscopy. Smear slides were fixed with absolute methanol and stained with Giemsa. For electron microscopy, cells were fixed with 2.5% glutaldehyde at 5°C for 90 min, postfixed with 1% osmium tetroxide at 5°C for 50 min, and embedded in Epon 812. Ultrathin sections were stained with uranium acetate and lead citrate and observed with a JEM-1200EX electron microscope.

The polar tube extending from the spores inside the cultured cells was observed by the phase-contrast microscopy as described previously (Iwano and Ishihara, 1989). Those from purified spores were observed by the ordinary microscopy of Giemsa-stained smear slides.

**Immunofluorescent microscopy.** Cells from the culture suspension were smeared on clean slides and fixed with formol-acetone (25 ml formalin, 45 ml acetone, 10 ml distilled water buffered with 20 ml of 0.01 M phosphate buffer at pH 4.5). Subse-

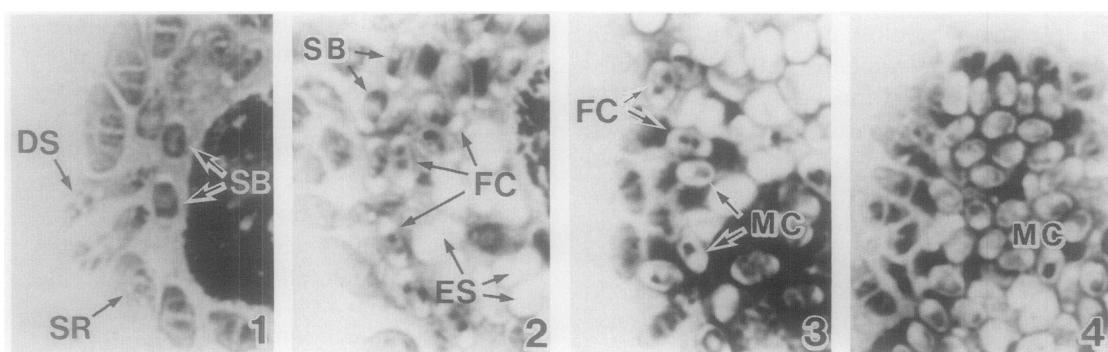
quently, a monoclonal antibody against *N. bombycis* spores BALB/c mouse (1:200) was layered on smears at 37°C for 30 min, followed by rinsing with PBS and indirect staining with FITC-conjugated antiserum against mouse goat (Immunochemicals) (1:100 to 1:500) at 37°C for 30 min. The monoclonal antibody against *N. bombycis* spores was a gift from Dr. A. Mike, Yakult Central Institute for Microbiological Research, Kunitachi, Tokyo (Mike et al. 1988). Slides were mounted with glycerin buffered with 0.05 M Na<sub>2</sub>CO<sub>3</sub>-NaHCO<sub>3</sub> at pH 9.6 and observed with a fluorescent microscope (Olympus BHS-RF).

## RESULTS

### Observation on *Nosema* sp. from *S. depravata*

When *Antheraea eucalypti* cells were inoculated with the cutworm *Nosema*, sporogonial stages were first observed at 30 hr pi. In Figures 1 and 2, sporoblasts from Giemsa-stained preparations are shown.

Sporoblasts were characterized by the presence of unstained areas either at both ends or only at the posterior end. The densely stained cytoplasm obscured the



FIGS. 1-4. Sporogonial stages of *Nosema* sp. from *Spodoptera depravata* in a cultured *Antheraea eucalypti* cell. Giemsa-stained smear preparations.  $\times 500$ .

FIG. 1. Sporont (SR), dividing sporont (DS), and sporoblast (SB) with unstained space at poles; 30 hr postinoculation.

FIG. 2. Sporoblast (SB) and spore (FC) showing two red nuclei against blue cytoplasm with empty space at both ends and empty spore (ES) in a host cell; 36 hr postinoculation.

FIG. 3. FC and another type of spore (MC) with stained core against unstained peripheral region; 54 hr postinoculation.

FIG. 4. Spores (MC) showing uniform contour; 72 hr postinoculation.

site of their nuclei (Fig. 1). In electron microscope preparations, sporoblasts contained a network structure that was associated with lacuna(e). The polar tube coils were produced from the network. Relatively few cross sections of developing polar tube were detected (Fig. 5). Spores were first observed at 36 hr pi and were variable in shape but generally pyriform. The spores had two nuclei and a vacuole at both ends (Fig. 2).

In electron microscopy, two nuclei in the diplokaryotic arrangement were detected in central regions of the spores. Several rows of endoplasmic reticulum surrounded the nuclei. The polar tube coiled three to five times under a relatively thin spore shell. A depression at their posterior pole was evident, leaving a space between the spore shell and host cytoplasm (Fig. 6). The depression can be an artifact of preparation of electron microscopy, but was observed characteristically on spores at this time of culture.

In addition to these spores, another type of spores occurred in infected cells at 54 hr pi. They were smaller than the first type of spore, oval, and more uniform in shape (Figs. 3, 4) and predominated in the later period after inoculation (Fig. 14). The most obvious difference between the two spore types in the cultured cell was the number of coils of the polar tube (Figs. 6, 9).

The second type showed 10 to 12 coils as compared to 3 to 5 of the first type (Table 1). Hence, the two spore types will be designated as few (FC) and many coil (MC), respectively. The MC sporoblast was detected at 54 hr pi in ultrathin sections. There was no lacuna associated with the network structure and there were more cross sectioned coils of the developing polar tube near the network structure (Figs. 7, 8) compared to that of FC. A fine picture of MC is presented in Figure 9. The central region was occupied with two nuclei and a few rows of endoplasmic reticulum. There was no depression at the posterior region while FC often had a depression (Fig. 6).

The endospore of MC was thicker (91 nm) than that of FC (35 nm). Thick sections (1  $\mu\text{m}$ ) adjoining ultrathin sections from the material sampled at 54 hr pi revealed that the bigger and pyriform spores in thick sections corresponded to the FC spores in ultrathin sections. MC appeared in the cell where FC already existed (Figs. 3, 10). At the advanced stages of infection, MC became more numerous, although a few of the FC remained among MC (Fig. 14). Both spores developed apansporoblastically and were binucleated, keeping a diplokaryotic arrangement.

The sporont gave rise to two sporoblasts that indicated that this microsporidium belonged to the genus *Nosema*. However, whether this sequence of development resulted in FC or MC remained to be settled.

Nearly the same numbers of coil as those of MC were observed in stored spores which had been purified from infected larvae or larva-derived, nongerminating spores recovered from the culture medium just after inoculation (Table 1). FC spores inside the infected cell discharged a polar tube, which extended 32.6  $\mu\text{m}$  in length from the cell surface (Table 2). The diameter of the host cell (22.8  $\mu\text{m}$ ) was used as an estimation of length of the polar tube remaining within the host cell. That value, when combined with the polar tube length extended from the host cell surface, was still less than the length of MC's polar tube. Longer tubes were discharged by purified spores from infected larvae and 2-month-old cultures. In as much as the latter spores belonged presumably to MC, the length of the polar tube appeared to correlate with the number of coils.

#### *Observations on N. bombycis*

*N. bombycis*, when inoculated to *A. eu-calypti* cells, gave rise to the same sequence and development of the two types of spores, FC and MC, as the cutworm *Nosema*. Sporoblasts (30 hr pi) and spores of the FC type (36 hr pi) in ultrathin sections are given in Figures 11–13. The num-

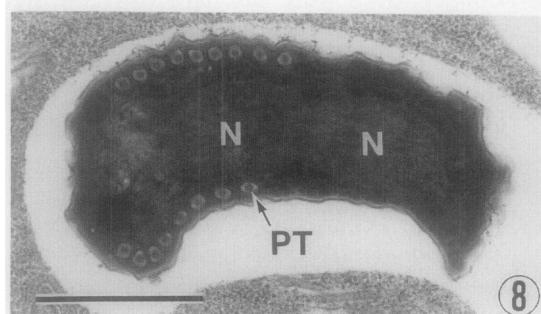
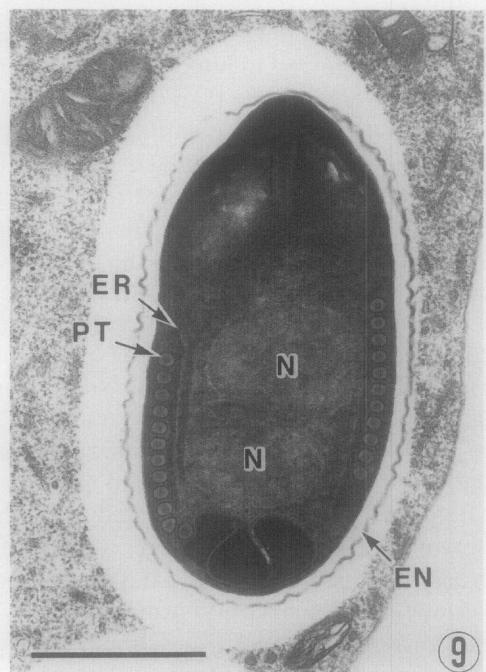
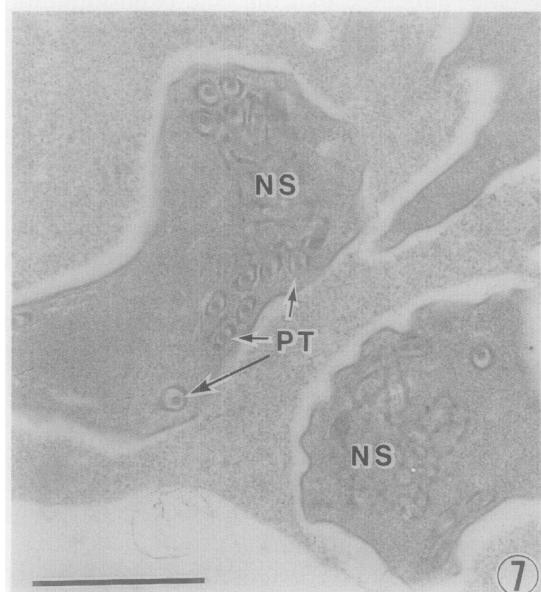
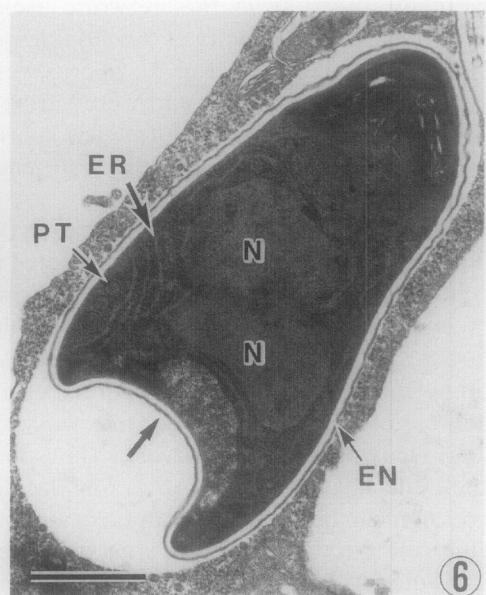
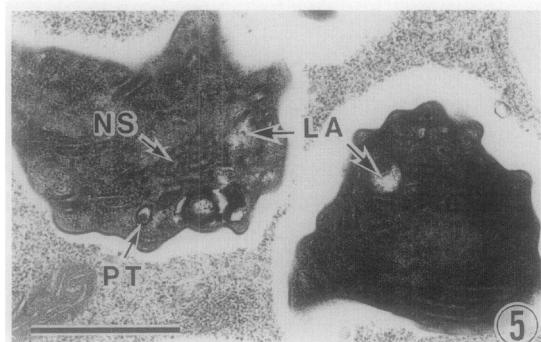


TABLE I  
NUMBER OF COILS OF THE POLAR TUBE

Microsporidia	Source of spore examined	Number of spore	Number of coils Mean ± SD
<i>Nosema</i> sp. from			
<i>Spodoptera depravata</i>	Spore from cultured cell 36–48 hr pi	22	3.8 ± 0.5
	Spore from cultured cell 54–72 hr pi <sup>a</sup>	24	11.4 ± 0.8
	Nongerminated spore recovered from culture medium	50	11.4 ± 0.8
<i>Nosema bombycis</i>	Spore from infected larvae	60	11.8 ± 0.9
	Spore from cultured cell 36 hr pi	52	4.3 ± 0.7
	Spore from infected larvae	60	11.4 ± 0.8

<sup>a</sup> The many coils type was selected.

ber of sections of polar tubes was not numerous in the sporoblast, which had two nuclei of a diplokaryotic arrangement. The network structure at the posterior pole had lacuna and an isthmus was at the central region of sporoblast. The FC type spore, in ultrathin section, was pyriform, had thin endospore, lacuna at posterior pole, a depression of spore shell, two nuclei of a diplokaryotic arrangement, and a few sections of polar tube coils (mean = 4.3 coils).

The MC type of spore appeared at 54 hr pi or later and was oval and thick in endospore, having diplokaryotic nuclei, and many coils (more than 10) of the polar tube lacking lacuna. Its fine structure was similar to that of spores purified from infected insects observed by Sato et al. (1982). The sequence of spore developments of both types was apansporoblastic. As was the case of the cutworm *Nosema*, spores purified from silkworm larva had many coils of

the polar tube (Table 1) and discharged a long polar tube (Table 2).

In immunofluorescent microscopy, two spores exhibited affinity to the monoclonal antibody prepared against larva-derived purified spores, which indicated that the two types of spores shared a common antigen(s).

## DISCUSSION

We demonstrated the hitherto unreported cases of spore dimorphism in two *Nosema* spp., including the type species of the genus. Our finding that two *Nosema* species gave an almost identical sequence of spore development resulting in FC and MC spores indicated that the dimorphism was not caused by separate microsporidian strains or species. Moreover, the two types of spores of *N. bombycis* in cultured cell were serologically related. A serological test to *Nosema* sp. from *S. depravata* was

FIGS. 5–10. Ultrathin sections of sporogonial stages of *Nosema* sp. from *Spodoptera depravata* in cultured *Antheraea eucalypti* cell. Bar = 1 μm.

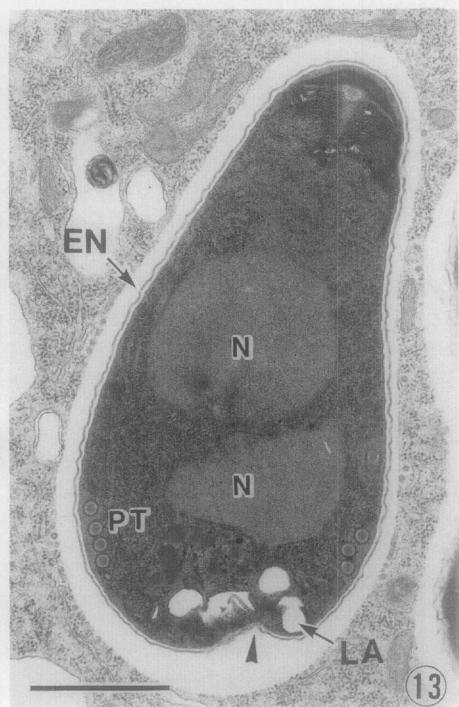
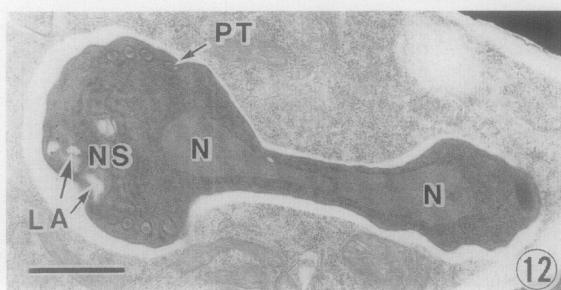
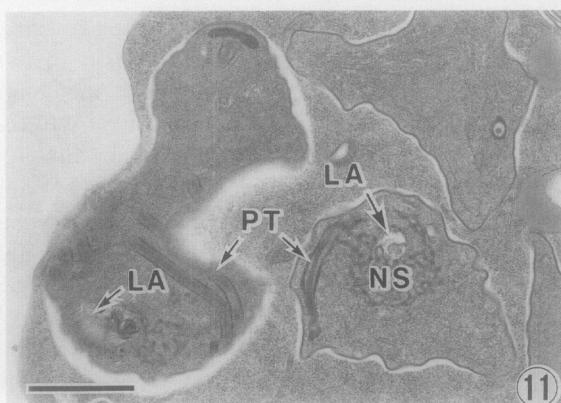
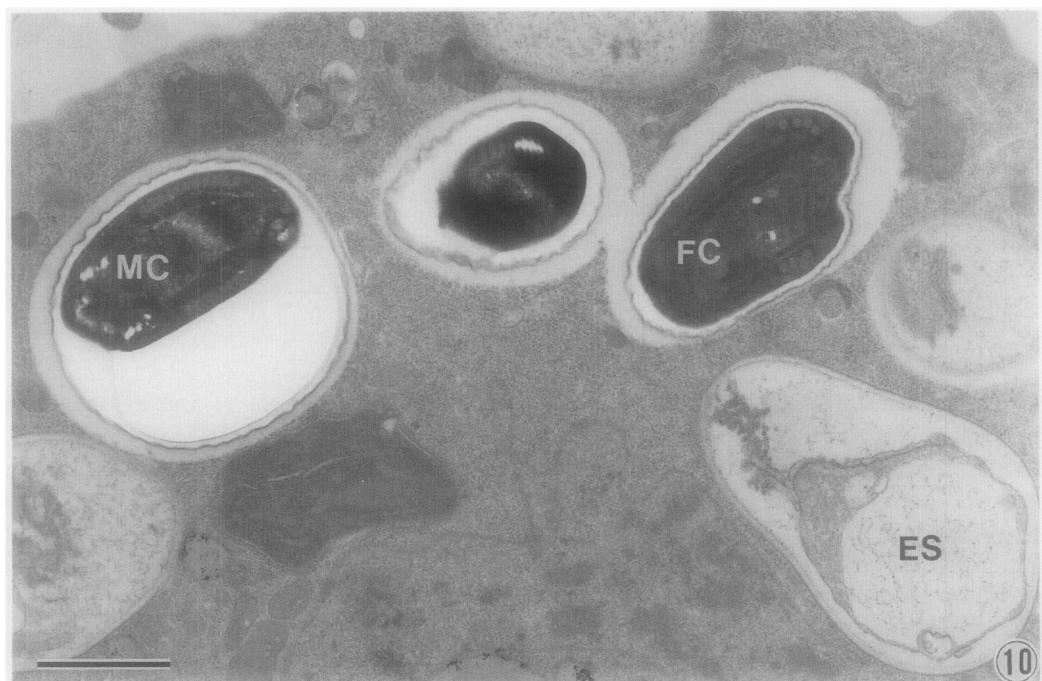
FIG. 5. Sporoblast with a few coils of the polar tube (PT). Network structure (NS) with lacuna (LA); 36 hr postinoculation.

FIG. 6. FC showing a depression at posterior pole (arrow). Thinner endospore (EN), diplokaryotic nuclei (N), several rows of endoplasmic reticulum (ER), and relatively few coils of the polar tube (PT); 36 hr postinoculation.

FIG. 7. New type of sporoblast showing many coils of the polar tube. Network structure (NS) without lacuna; 72 hr postinoculation.

FIG. 8. Advanced stage of the new type of sporoblast; 72 hr postinoculation.

FIG. 9. MC showing 12 coils of the polar tube, two nuclei (N) in diplokaryotic arrangement, thick endospore, two rows of endoplasmic reticulum, and no depression at the posterior part of the spore; 72 hr postinoculation.



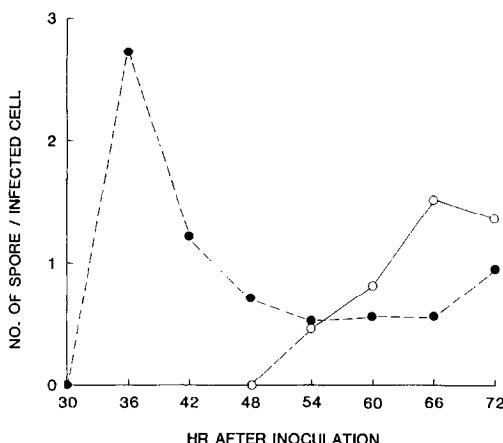


FIG. 14. Number of spores of *Nosema* sp. from *Spodoptera depravata* having a few coils of the polar tube (●) and those having many coils of the polar tube (○), found in cultured cell of *Antheraea eucalypti*. Note. Identification of those two types of spores was based on their morphological features in Giemsa-stained slides.

left to be done. We are expanding our survey of spore dimorphism presented herein to other less related microsporidia.

The sequence of development of both types of spores occurred in the same cultured cell. They were apansporoblastic and diplokaryotic. One type of spore (FC) was characterized by its few coils of the polar tube, discharge of a short polar tube, thin endospore, early development, and spontaneous germination, while the other type (MC) showed many coils of the polar tube, discharge of a long polar tube, thick endospore, late development, and germination induced by  $H_2O_2$  or the change of pH.

These features were quite different from those of spore dimorphism of other families

of microsporidia in its sequence of spore development and karyology. According to Sprague (1982), microsporidia in which the sporulation process exhibits dimorphism, e.g., Amblyosporidae, Burenellidae, Culicosporidae (genus *Hazardia*) and Spraginiidae, produce both binucleate and uninucleate spores. All binucleate spores develop without membrane (sporocyst), while the development sequences of uninucleate spores are varied. Spores of Amblyosporidae and Burenellidae are pansporoblastic but the uninucleate spore developments of *Hazardia* and Spraginiidae are apansporoblastic.

FC and MC were discrete in the number of coils of the polar tube, which appear to be a distinctive character and can be used for microsporidian classification and typing.

Avery and Anthony (1983) argued that "first generation" spores germinated intracellularly without presenting convincing data. We showed that spores which developed first germinated spontaneously and were different in structure from those produced in the more advanced stage of infection.

Purified spores of both *Nosema* spp. used in this experiment were of the MC type. We assume that purified spores were probably MC which developed in host insects. They presumably survive outside the host, being protected by a thick spore shell, and germinate when sensitized by proper stimulants, e.g., host insect gut juice. However, Sato and Watanabe (1980, 1986) reported spontaneous germination of *N. bombycis*.

FIG. 10. Spore having fewer coils of the polar tube (FC), spore having many coils of the polar tube (MC), and empty spore shell (ES) within the same cell; 72 hr postinoculation.

FIGS. 11-13. Ultrathin sections of sporogonial stages of *Nosema bombycis* in cultured *Antheraea eucalypti* cell. Bar = 1  $\mu m$ .

FIG. 11. Sporoblast (early developing type) showing a few coils of the polar tube (PT) and network structure (NS) with lacuna (LA); 33 hr postinoculation.

FIG. 12. Sporoblast in advanced stage having isthmus; 33 hr postinoculation.

FIG. 13. Spore corresponding to FC of *Nosema* sp. from *S. depravata*, spore having thinner endospore, lacuna at posterior pole, depression of spore shell (arrowhead), and a few coils of the polar tube; 36 hr postinoculation.

TABLE 2  
LENGTH OF THE POLAR TUBE EXTENDING FROM THE SURFACE OF THE HOST CELL OR FROM THE SPORE

Source of spore examined	The way of the polar tube discharged	Number of spore examined	Length of polar tube Mean ± SD (μm)
Spores developed in cultured cell 36 to 48 hr pi	Spontaneous	24	32.6 ± 23.2
Spores purified from 2-month-old infected culture	H <sub>2</sub> O <sub>2</sub> + KCl	30	83.7 ± 10.1
Spores purified from infected larvae	pH reduction after exposed to KOH	30	83.4 ± 10.6
	H <sub>2</sub> O <sub>2</sub> + KCl	43	96.7 ± 12.3
	pH reduction after exposed to KOH	30	88.5 ± 10

Note. The diameter of host cells was 22.8 ± 3.8 μm (n = 20).

bycis spores when they were layered on a sucrose solution for density gradient centrifugation or when they had been purified by Percoll density gradient centrifugation. Some fractions of purified spores may be sensitized to germinate by the change of environmental conditions. Some of the MC developed in cultured cell germinated by a change in the physicochemical nature of the culture medium. Petri (1969) reported that germination of *Nosema* (= *Encephalitozoon*) *cuniculi* was intensified by rinsing the host tumor with physiological saline.

FC spores play an important role in spreading the infection by ejecting the polar tube into the other cultured cells by spontaneous germination inside the host cell (Iwano and Ishihara, 1989). We assume that the sporoplasm injected into culture cells from FC gives rise to the same sequence of spore development as the sporoplasm from insect-derived spores.

Ishihara (1969) found a sporoplasm-like body inside and outside of cultured cells in smeared slides stained with Giemsa, but considered that it was different from the sporoplasm because of its stronger affinity to Giemsa stain and its earlier appearance that was reported later by Kawarabata and Ishihara (1984) and designated the body as a secondary infective form. In light of the present investigation, we conclude that the secondary infective form is the sporoplasm of FC, which developed much earlier than MC. Sporoplasm discharged from FC may not be taken up by the host cell as assumed

by Ishihara (1969), but may be injected to other host cell through the polar tube.

The posterior end of FC's shell occasionally had a depression which might have been caused by relatively thin endospore. FC had lacuna(e) near the network structure from which the polar tube is produced. That lacuna(e) render(s) the shell of posterior region weakness against mechanical pressure, although their thin wall is not disadvantageous to FC which germinates intracellularly without being exposed to the harsh environment outside.

A few FC occurred among many MC in the advanced stages of infection. Whether they represented spores which persisted without germination or produced just before sampling needs to be clarified.

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