LIGHT AND ELECTRON MICROSCOPE STUDY OF THE PHAGOCYTOSIS OF *PLEISTOPHORA OVARIAE* (PROTOZOA, MICROSPORIDA) SPORES

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CHAPTER I

INTRODUCTION

The intracellular parasites belonging to the Order Microsporida are believed to enter cells under natural conditions by the injection of the sporoplasm through the polar filament (Weidner, 1972). Development of in vitro culturing techniques of microsporidans has demonstrated that entry may also occur by phagocytosis (Kurtti and Brooks, 1971). Phagocytosis has also been shown to be a primary mechanism of infection for some sporozoan parasites, including Plasmodium and Toxoplasma (Ladda et al., 1969; Jones et al., 1972).

Phagocytosis is also theorized to be of major importance in other aspects of the host-parasite relationships of microsporidans and their hosts. The transport of the ejected sporoplasm from the gut epithelium to the site of establishment has been hypothesized to occur via host cell macrophage containing phagocytized microsporidans (Canning, 1977). Host responses to a microsporidan infection include dispersal and removal of the spores by phagocytosis. Further study was needed to evaluate the effects of phagocytosis on the microsporidan spore since phagocytosis is involved in several aspects of the host-parasite relations.

Pleistophora ovariae is a microsporidan ovary parasite of the golden shiner, *Notemigonus crysoleucas*. In this fish host, macrophages eliminate spores during certain seasons of the year; therefore, this host-parasite complex makes a good model for studying the process of phagocytosis of microsporidan spores.

Established cell line cultures from a variety of sources and electron microscopy were utilized to study the stages of spore phagoctyosis. Several techniques for isolating *P. ovariae* spores from golden shiner ovaries were also examined to identify a method for obtaining a clean spore isolation for use in the cell culture phase of the research. The specific objectives of this research were

1) preparation of a clean spore suspension
2) determination of the mode of entry of *P. ovariae* spores into cultured cells
3) description of the spore morphology and staining reactions of spores isolated from ovary tissue, and
4) determination of changes in spore morphology and staining reactions after phagocytosis.

CHAPTER II

LITERATURE REVIEW

Microsporida

Microsporidan parasites are obligate intracellular protozoans. To date nearly 700 species have been described (Canning, 1977), most of which are reported to be parasites of arthropods and fish. Bony fish harbor more microsporidan species than any other vertebrate class, but little research has been done with fish microsporidans even though "their effects upon their hosts are among the most severe of any parasite group" (Sinderman, 1970).

Life Cycle

The majority of known cycles for microsporidans do not include an intermediate host or vector (Kudo, 1966); however, Glugea stephani and G. anomala have been shown to utilize invertebrates as carriers (Putz and McLaughlin, 1970).

The most common method of transmission for this group of parasites is thought to be by ingestion of spores by the host animal, followed by invasion of the host tissue (Kudo, 1966). Invasion is accomplished in the digestive tract when the polar filament is extruded and the

sporoplasm emerges as an amoebula which penetrates the gut epithelium and enters either the blood or body cavity to reach the site of establishment (Canning, 1977). Alternatively, the polar filament may inject the sporoplasm directly into the gut epithelium. The mechanism of extrusion is not clear, but it is believed to involve eversion of the polar filament due to changes in osmotic pressure (Weidner, 1972). The route from the gut epithelium to the site of establishment has not yet been determined, but probably involves a passive transfer in the blood or in a host macrophage to the site of localization (Cannning, 1977).

Microsporidan parasites typically show host and tissue specificity, but some are able to infect a wide range of hosts or tissue types. For example, Perezia pyraustae invades the Malpighian tubules, silk glands, and the ovary of both larvae and adult European corn borers (Kudo, 1966). Other species have flourished when experimentally transferred to abnormal hosts and have established infections in new tissue locations in the new hosts. Nosema algerae, a mosquito microsporidan, has produced spores in eleven other insect species of six orders and in one decapod species after experimental infection (Undeen and Maddox, 1973).

Once the parasite reaches a suitable site in the host, it grows and reproduces asexually (Kudo, 1966). The first stage in asexual reproduction recognizeable with the light

microscope is called a transitional fusiform cell. The transitional cell undergoes multiple fission resulting in schizonts which in turn produce sporonts. During sporogony the sporont matures into a sporoblast; various spore organelles are formed from endoplasmic reticulum and the extensive Golgi apparatus, and finally the spore wall is deposited (Canning, 1977). Developing stages may be free in the cytoplasm of the host cell, or they may be isolated within a host derived membrane (Canning, 1977). Microsporidans do not have mitochondria (Lom and Corliss, 1967).

Not all microsporidans follow the previously described pattern of transmission. Some are ovary parasites and may be transmitted transovarianly to the host progeny (Kudo, 1924; Kellen et al., 1965). At least one species is transferred by the male host during mating (Manwell, 1961). Autoinfection also occurs with the movement of secondary infective forms into neighboring noninfected cells in the same host tissue (Ishihara, 1969).

Host-Parasite Relationships

The majority of microsporidans are not known to be harmful to their hosts (Manwell, 1961), but some species cause severe pathological changes, including *Nosema apis* in honey bees, *Pleistophora salmonae* in salmonids, *P. cepedianum* in gizzard shad, *Thelohania opacita* in mosquito larvae, and *T. contejeani* in river crayfish (Manwell, 1961; Putz et al., 1965). Considerable economic losses occur

because of the infection of some host species (Putz and McLaughlin, 1970). However, in some cases the effect on the hosts can be beneficial to man since Perezia pyraustae and P. fumiferanae serve as a biological control of the European corn borer and the spruce budworm respectively, thereby assisting in preventing severe economic damage to cultivated plants (Kramer, 1959; Kudo, 1966). N. locustae has demonstrated some potential in biological control of its grasshopper host (Henry and Oma, 1974). Retardation of transplantable tumors in mice has also been attributed to microsporidan infection (Putz and McLaughlin, 1970).

The mechanism of injury to the host varies, but microsporidans (especially Glugea spp.) often cause cellular hypertrophy and hypertrophy of nuclei and chromosomes of infected cells resembling cancer overgrowth (Sprague and Vernick, 1968a). Other effects of infection by microsporidans include disruption of the endoplasmic reticulum, depletion of ribosomal particles, irregularities in mitosis, and enhanced pinocytic activity at the host cell's basal membrane (Pavan et al., 1969).

Spore Morphology

Microsporidan spores are among the smallest of animal cells; but in spite of their small size, species identity of microsporidan parasites is determined by spore size, shape, and number of spores produced per sporont (Kudo, 1966). Each ovate spore contains a polar cap, a

polaroplast, a tightly coiled polar filament, and one or two nuclei (Canning, 1977).

Electron microscopy has revealed many details about the structure of the microsporidan spore. The wall consists of two layers: the outer electron dense layer, the exospore (50 nm), and the inner electron translucent layer, the endospore (200 nm) (Vavra, 1968; Canning, 1977). A membrane-bound polar cap seals the base of the polar filament at the anterior (smaller) end of the spore in a dome-shaped cavity within the endospore (Canning, 1977).

The polar filament descends centrally to the middle or posterior (larger) end of the spore where it is coiled about the nucleus (Huger, 1960). The coils vary in number from 4 to 34 and are arranged in single, double, or triple layers at the periphery of the spore (Canning, 1977). The details of filament structure remain in question. First described as a cylinder of fibrils with two central fibrils resembling cilia and flagella (Huger, 1960), the polar filament has more recently been reported to consist of a simple double-walled tube filled with an electron-dense core (Stanier et al., 1968; Weidner, 1970; Walker and Hinsch, 1972; Sanders and Poinar, 1976; and Canning, 1977). It has also been portrayed as a double-walled tube containing rings or microtubules in one of the walls (Canning and Nicholas, 1974; Sprague and Vernick, 1968a), or as a three-layered tube with one layer possessing longitudinal ridges which give the appearance of fibrils (Lom and Corliss,

1967; Colley et al., 1975). The derivation of the polar filament is similarly confused. Various authors hypothesize that it is derived from the nucleus (Sprague and Vernick, 1968a), the endoplasmic reticulum (Sanders and Poinar, 1976; Lom and Corliss, 1967), an aggregation of ribosomes (Milner, 1972), the Golgi appratus (Walker and Hinsch, 1972), the Golgi apparatus and the endoplasmic reticulum (Weidner, 1970), or the Golgi apparatus and the nuclear isthmus (Sprague and Vernick, 1969).

Anterior to the coiled portion of the polar filament lies the polaroplast, a series of membranes or saccules which is believed to function in filament extrusion (Weidner, 1972). A posterior vacuole filled with an amorphous material has been reported in some species (Lom and Corliss, 1967; Vernick et al., 1969). The remainder of the spore is filled with undifferentiated cytoplasm, a few ribosomes, and some endoplasmic reticulum (Canning, 1977).

Cytochemistry

Microsporidans commonly contain mucopolysaccharides and proteins with numerous sulfhydryl groups, but they lack glycogen and fats (Maurand and Loubes, 1973; Vivares et al., 1976). Biochemical analysis of intact spores has determined the presence of cysteic acid, aspargic acid, glutamic acid, serine, threonine, allanine, proline, tyrosine, valine, fenyl-alanine, isoleucine, leucine, and arginine (Vavra, 1966), but the locations of these substances

in the spore have not been identified. The specific nature of some of the organelles of the spore have, however, been determined by specific stains. For example, the polar cap stains positively with the Periodic acid schiff method (Lom and Corliss, 1967; Sprague and Vernick, 1968a, 1969), and the exospore is composed of protein while the endospore is chitinous (Canning, 1977). Young spores react positively to protein tests, but mature spores are generally negative due to protein complexing with chitin in the spore wall during maturation (Maurand, 1975).

Culture

Microsporidans cannot be cultured without living cells since they are intracellular parasites. However, several microsporidan species have been grown in primary cell cultures prepared from their normal hosts (Ishihara and Sohi, 1966; Kurtti and Brooks, 1971), and some have also been propagated in established cell lines (Undeen 1975).

The first infection of a cell culture by a microsporidan was recorded when vegetative stages of Nosema bombycis were observed 24 hours after inoculation of spores into primary cultures from the natural host, the silkworm Bombyx mori. The sporoblasts appeared at 72 hours and spores filled host cells by 21 days after inoculation (Ishihara and Sohi, 1966). Successful infection of non-host cell culture was first reported in 1968 when primary cultures of mammalian and chicken embryos were infected

with N. bombycis (Ishihara, 1968). Other microsporidans have since been grown in a variety of cell culture types: N. cuniculi in primary cultures of rabbit choroid plexus cells (Shadduck, 1969), N. disstriae in primary cultures of tent caterpillars (Kurtti and Brooks, 1971), N. algerae in an established culture of pig kidney cells (Undeen, 1975), and N. michaelis on duck red blood cells (Weidner and Trager, 1973).

In order to stimulate the entry of microsporidans into cells, spores are often primed to enhance polar filament extrusion before they are introduced into cell culture medium. The most commonly used primer is an alkaline pretreatment consisting of incubation of spores in 0.1 M KOH (Ishihara and Sohi, 1966; Kurtti and Brooks, 1971). Stimulation with a veronal acetate buffer at pH 10 has also been utilized (Weidner, 1972). Spore germination has also been reported to occur without prior stimulation when spores from a marine host were introduced into culture medium (Undeen, 1975). Infection of culture cells is generally indicated by penetration of the host cell by the everted polar filament (Canning, 1977). However, in one study the majority of spores failed to evert their polar filaments, but spores still entered cells as a result of active phagocytosis by the cultured cells (Kurtti and Brooks, 1971).

Role of Phagocytosis

Phagocytosis may be involved at several stages in the life cycle of microsporidan parasites (Canning, 1977). Theoretically phagocytes transport the sporoplasm after its entry into the host body, i.e. injection into the gut epithelium, to the site of localization (Canning, 1977), and function in autoinfection of other host cells. Additionally, the invasion of uninfected culture cells by one of the microsporidan vegetative stages has also been reported (Ishihara and Sohi, 1969; Sohi and Wilson, 1976). These secondary infective forms apparently infect healthy host cells after random contact (Ishihara, 1969). Finally the host response to microsporidan infections can include dispersal and removal of spores by phagocytic cells (Kudo, 1924; Canning, 1977).

Several other types of intracellular sporozoans have been observed to utilize phagocytosis as an entry mechanism during infection of host cells. Examples include Toxoplasma gondii (Jones et al., 1972), Plasmodium species (Ladda et al., 1969), and Eimeria magna (Jensen and Hammond, 1975).

Phagocytosis is a common biological process, occurring on all levels in the animal kingdom. The four stages involved are attraction of the particle or chemotaxis, adhesion of the particle to the phagocytic cell, ingestion of the particle by movement of pseudopodic processes around

the particle, and finally disposal of the particle (Carr, 1972).

The destruction of the particle in a phagocytic vacuole occurs after the fusion of such a vacuole with a primary lysosome and subsequent digestion (Joklik and Willett, 1976). The mechanism of digestion makes phagocytosis a powerful defense against invaders. However, not all particles are digested after phagocytosis (Carr, 1972). The fate of the ingested particle depends on the type of phagocyte and its interaction with the ingested particle. A polymorphic nucleocyte generally dies after ingesting a number of particles, releasing undigested particles to the environment. However, a macrophage usually survives to synthesize more enzymes and ingest more particles. A particle may thus be digested and disappear, or fragments may remain in the phagocytic cell to form a residual body, or the cell and particle may die and form pus. If an ingested microorganism survives within a phagocyte, it may multiply within the cell as do the bacteria which cause leprosy. The organism may even kill the phagocytic cell and be released (Carr, 1972).

Pleistophora ovariae

Range

Pleistophora ovariae (Microsporida, Pleistophoridae) was first observed in golden shiner ovaries (Summerfelt,

1964). This organism is widespread among cultured populations of golden shiners as it was reported in 45 of 49 commercial sources sampled from 12 states with a total prevalence of 48% (Summerfelt and Warner, 1970a). Since the golden shiner is the most widely cultured bait minnow in the United States, P. ovariae poses a serious economic problem to minnow producers (Summerfelt and Warner, 1970b). P. ovariae has also been found in ovary tissue from the fathead minnow (Pimephales promelas), another commonly cultured bain minnow in the United States (Nagel and Hoffman, 1977). Although goldfish (Carassius auratus) are often raised together with golden shiners and fathead minnows and are thus exposed to P. ovariae, no infection by P. ovariae in goldfish has been reported (Nagel and Summerfelt, 1977a).

Host-Parasite Relationship

The complete life cycle of P. ovariae is unknown, but it has been shown to live and reproduce within the oocytes of the golden shiner, proliferating to the extent that egg production and fecundity are drastically reduced (Summerfelt and Warner, 1970a). In 1967 half of the spawning failures on 60 fish farms in the United States were attributed to infections of P. ovariae (Meyer, 1967). This parasite probably costs the minnow industry more than any other single problem (Malone, 1970).

Fish farmers compensate for the progressive increase

of infection by P. ovariae with increasing age of fish by using more and younger brood stock (Warner, 1972). However, the younger brood fish produce fewer eggs and are less reliable in their spawning than older fish. Neither of the two drugs tested (fumagillin and nitrofurazone) has accomplished a complete cure (Tonguthai, 1972; Nagel and Summerfelt, 1977b).

Both transovarian and oral transmission have been demonstrated (Summerfelt, personal communication), and the multiplicative stages of the parasite have shown close synchrony with ovary maturation (Warner, 1972). Maximum spore development coincides approximately with the shiner's spawning season. The abundance of specific parasitic stages thus appears to be seasonal and dependant upon a particular stage of ovary development (Warner, 1972). Since P. ovariae is latent in young-of-the-year shiners, infection by P. ovariae cannot be detected until the fish approaches sexual maturity. Thereafter, stages of the parasite can be found almost any time of the year. The developmental stage of the parasite observed depends upon the degree of egg maturity in the ovary. Schizogony occurs in stage III and IV oocytes and sporogony, in stages V, VI, and VII. After spawning is completed, unspawned ova break down and are resorbed by the fish, and spores are phagocytized by the host macrophages (Warner, 1972).

CHAPTER III

METHODS AND MATERIALS

Spore Isolation

P. ovariae spores were extracted from ovaries of naturally infected golden shiners that were one year of age or older. The fish were obtained from the Oklahoma Cooperative Fishery Research Unit,[1] or from Ozark Fisheries, Inc.[2] Approximately 50 ml of naturally infected ovary tissue was used for each isolation procedure.

The first method used for spore isolation involved maceration of ovaries with a mortar and pestle. The homogenate was strained through several layers of cheesecloth using a sterile physiological saline rinse, and centrifuged at 2500 rpm (840 g) for 10 minutes. The pellet was resuspended in 25 ml sterile physiological saline for storage at 4°C until microscopic examination.

The second method of spore isolation was a modification of the Cole triangulation technique (Cole, 1970). Ovaries were macerated in a mortar and pestle, strained

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[2]Stoutland, Missouri 65567

through cheesecloth and rinsed with sterile physiological saline. Spores were then concentrated by centrifugation at 2500 rpm (840 g) for 30 minutes, and the centrifugation process was repeated. Following the rinse, the triangulation procedure described by Cole (1970) was done.

The third method of spore isolation was a modification of the sucrose density gradient technique described for the myxosporidan parasite, Myxosoma cerebralis, eliminating the enzyme digestions. Ovaries were macerated with a mortar and pestle, centrifuged at 3650 rpm (1790 g) for 5 minutes, and resuspended in sterile physiological saline. This suspension was poured onto an equal amount of 55% aqueous sucrose and centrifuged at 3000 rpm (1200 g) for 30 minutes. The pellet was resuspended in 25 ml sterile physiological saline and stored at 4°C for later microscopic examination.

The fourth method of isolation of P. ovariae spores was a modification of Landolt's (1973) technique for isolation of M. cerebralis, omitting enzyme digestions. Ovaries were crushed with a mortar and pestle, homogenized in a tissue grinder, and mixed with 25 ml 0.1 N HCl solution, and added to an equal amount of ethyl ether. After this suspension separated into two layers (about 15 minutes), the aqueous portion was removed and centrifuged at 3650 rpm (1790 g) for 15 minutes. The supernatant fluid was discarded, and the pellet was resuspended in 8 ml sterile physiological saline. The centrifugation procedure

was repeated three times to thoroughly wash the spores, which were then resuspended in 25 ml sterile distilled water and stored at 4°C for later microscopic examination.

Cell Culture

Four established cell lines were utilized. An established cell line of mosquito (Aedes albopictus) cells (MOS) was procured from Dr. Lane Corley.[3] Madin-Darby bovine kidney cells (MDBK) and Vero M monkey kidney cells (VERO) were obtained from the Oklahoma Animal Disease Diagnostic Laboratory.[4] A fathead minnow cell line (FHM) was purchased from American Tissue Type Collection.[5] All cell culture work was carried out in the tissue culture laboratory of the Oklahoma Animal Disease Diagnostic Laboratory.

The MDBK, VERO, and FHM cell lines were grown in a CO_2 incubator at 37°C. These three types of cells were propagated in Eagle's minimum essential medium (MEM) (I.S.I. Biologicals)[6] with 10% viral and mycoplasma screened fetal bovine serum (FBS). Eagle's MEM with 2% FBS was used for maintenance of cell monolayers. MOS cells

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[4]Oklahoma State University, Stillwater, Oklahoma 74074

[5]1230 Parklawn Drive, Rockville, Maryland 20852

[6]Mention of a brand or company name does not constitute an endorsement or imply approval to the exclusion of of other suitable products. Addresses for companies mentioned appear in the appendix.

were cultured at room temperature and grown in prepared mosquito culture medium (Gibco) with 20% FBS.

All cell cultures were subdivided by the addition of a trypsin mixture (Difco), followed by centrifugation of the resulting cell suspension at 1000 rpm (225 g) and resuspension in fresh medium. Cultures were maintained in disposable 30 ml Falcon cell culture flasks (Division Beston, Dickinson, and Co.); experimental monolayers were grown in either 30 ml Falcon flasks, Lab-Ted champer slides (Scientific Products), or on sterile coverslips in disposable 35 mm Corning cell culture dishes (Scientific Products).

Spore Inoculation

Only spores isolated by the ether separation method previously described were used. Spores were treated with antibiotics (0.5 ml fungizone, 1.0 ml penicillin, and 0.25 ml streptomycin per 10 ml spore suspension) 24 hours prior to introduction into cell culture (Undeen and Maddox, 1973). After antibiotic treatment, spores were centrifuged at 1000 rpm (225 g) for 10 minutes, the antibiotic solution was discarded, and the spores were resuspended in 0.1 M KOH and incubated for 30 minutes. Following incubation, the spores were centrifuged and resuspended in MEM with 2% FBS and 0.1% gentamycin (Schering). This calibrated spore solution was introduced into cell culture flasks, dishes, or chamber slides containing a monolayer of cells. The

number of spores per ml of medium was determined with the use of a hemacytometer (American Optical Bright-line). Cell monolayers, with and without spores, were observed daily using an inverted microscope (Leitz).

Light Microscopy

Monolayers of cells were fixed *in situ* with 10% neutral buffered formalin, stained, dehydrated with graded alcohols, cleared in xylene, and mounted in Permount (Fisher Scientific Co.). Controls for cell culture samples included stained sections (5 μm) of golden shiner ovaries (collected in early summer) and spore smears of *P. ovariae* isolated by the ether separation method described above. Mallory's trichrome stain, hematoxylin, Giemsa, Periodic acid schiff (PAS), and toluidine blue were utilized for light microscopic examination, according to standard procedures (Humason, 1967; McManus and Mowry, 1960).

Comparison of the different established cell lines inoculated with the same number of *P. ovariae* spores (1×10^5) included counts of both the number of cells and the number of engulfed spores. The average number of phagocytized spores per cell was computed for each of five random fields (X 1500) for each monolayer sampled. The student's t-test was used to examine significant differences between groups (Beyer, 1968). A difference was judged significant if the probability of the calculated t-value was less than 0.05.

Electron Microscopy

Cell monolayers were removed from flasks with a trypsin solution, concentrated by centrifugation at 1000 rpm (225 g), and fixed with 2% glutaraldehyde in 0.27 M cacodylate buffer containing 1500 ppm ruthenium red (Sigma Chemical Company). Post-fixation with OsO_4 in cacodylate buffer containing ruthenium red followed (Luft, 1971a, 1971b). The cultured cells were embedded in Spurr embedding medium (Spurr, 1969) after dehydration through a graded series of ethanols and passage through a transition solution of propylene oxide.

Thick (1 μm) and thin (500 A $\pm$ 100 A) sections were prepared with an ultramicrotome (Sorvall MT-2 Porter-Blum), using glass knives. Thick sections were stained with Richardson's blue (Richardson et al., 1960); thin sections were mounted on copper grids and stained with uranly acetate and lead citrate (Venable and Coggeshall, 1965). Examination of thin sections was made with a transmission electron microscope (Philips 200). Measurements were estimated by comparing photographs of 0.312 μm latex beads (Polysciencs, Inc.) and photographs of spores taken at the same magnification.

CHAPTER IV

SPORE ISOLATION

Experimental Design

The first spore isolation technique utilized naturally infected golden shiners secured in early spring from stock ponds belonging to the Oklahoma Cooperative Fishery Research Unit. Two repetitions of the second spore isolation technique were performed. Naturally infected golden shiner ovaries obtained from Oklahoma Cooperative Fishery Research Unit in early spring were utilized for the first trial with a centrifugation speed of 550 rpm (40 g) for 5 minutes. Naturally infected golden shiner ovaries obtained in early spring from Ozark Fisheries, Inc. were used for the second trial with a centrifugation speed of 150 rpm (3 g) for 5 minutes. The sucrose density gradient centrifugation technique was performed with ovaries from naturally infected golden shiners obtained from Ozark Fisheries, Inc. in early summer. Naturally infected golden shiners obtained from the Oklahoma Cooperative Fishery Research Unit in early summer were used to perform the ether separation technique.

Quantification of the spore yield produced by the

various methods was not attempted because of variation in time of year when fish were acquired and because of the difference in sources for the naturally infected golden shiners. Incidence and intensity of infection of P. ovariae is seasonal and varies among populations of golden shiners (Warner, 1972). Spore suspensions were, however, examined microscopically to determine the relative purity of each spore preparation.

Results

All four methods of spore isolation yielded clean preparations of apparently viable spores. Spores were judged viable by their characteristically refractive appearance under the microscope (Kurtti and Brooks, 1977) and by ability to extrude the polar filament following mechanical extrusion (Kudo, 1924).

The first isolation method furnished a large number of spores, but they were often mixed with considerable debris, including large yolk droplets and some particles of ovary tissue. This method required the least effort and time of the methods used.

The modification of Cole's triangulation method of spore isolation also yielded numerous spores. In the first trial (550 rpm or 40 g), spores were found mainly in the pellet in the second tube, although spores were also seen in the supernatant fluid and pellet of the first tube and in the pellet of the third tube. All tubes contained

tissue debris of various sizes. The largest particles were seen in the first tubes while the finest particles were found in the last tubes of the series. The second repetition, which utilized a centrifugation speed of 150 rpm (3 g), resulted in the transfer of spores to all centrifugation tubes. Large numbers of spores were seen in the pellets in tubes one through six, and smaller numbers were found in tubes seven and eight. In all cases, considerable debris was located with the spores.

The sucrose density gradient centrifugation procedure yielded spores in two separate bands in the preparation. The band in the upper phase of the preparation contained a few spores and small particles of tissue debris, while the band in the lower phase contained a large number of spores along with the larger debris particles.

The ether separation produced large numbers of spores with very little extraneous tissue debris. The debris which remained in the spore preparation were very small particles, and no yolk material or proteinaceous globules were present in the final spore preparation.

Discussion

All the methods of spore isolation yielded spores. All spore preparations were clean enough to be suitable for some routine purposes, such as preparation of stained smears. However, most of the spore suspensions contained too much contaminating debris for analytical work.

The first spore isolation technique, the simple maceration technique, quickly yielded large numbers of P. ovariae spores. However, spore preparations contained considerable ovary debris, which made these preparations undesireable for analytical or cell culture work.

Application of the modification of Cole's triangulation technique to ovaries containing P. ovariae resulted in good spore yields, but spore suspensions were contaminated with tissue debris. At the completion of the triangulation process, P. ovariae spores were located primarily in the first and second tubes, but some were seen in all the other tubes also. In all tubes, considerable tissue debris was located in the same area with the spores. Cole (1970), however, was able to utilize the triangulation technique to prepare a 95% pure spore suspension of Nosema heliothidus at a centrifugation speed of 600 rpm for 5 minutes. Spores were concentrated in tubes 3 through 6 while debris particles were found largely in tubes 1, 2, 7, and 8. Spores of N. apis were similarly isolated at 300 rpm for 5 minutes. Fowler and Reeves (1974) also used the triangulation procedure with a centrifugal speed of 125 g for 30 seconds to harvest spores from several insect hosts: N. necatrix, N. plodiae, N. sphingidis, N. whitei, N. trichoplusia, Thelohania diazoma, T. legeri, and Pleistophora species. Since P. ovariae infects a vertebrate host, the nature of the tissue debris differs from that of insect host tissues and may have inhibited spore

isolation by the triangulation method. Another speed and time combination for centrifugation might, however, improve the purification of P. ovariae spores by the triangulation technique.

The sucrose density centrifugation method concentrated spores in two bands of the sucrose preparation; however, ovary tissue debris was also found in both of these locations. Although Markiw and Wolf (1974) successfully isolated Myxosoma cerebralis spores using the sucrose density gradient centrifugation technique following a trypsin and pepsin digestion with debris remaining in a dense band at the interface of the digestion and sugar solutions, no such spore separation from debris resulted when the technique was applied to ovary tissue containing P. ovariae spores. The difference in the nature of the host tissue--ovary for P. ovariae and cartilage for M. cerebralis--was probably responsible for the difference in the nature of the results. Application of enzyme digestion to tissues containing P. ovariae spores prior to execution of the sucrose density gradient centrifugation technique may also have been helpful in degrading ovary debris particles and thereby giving a better separation of spores from debris.

The ether separation method modified from Landolt (1973) produced a relatively pure suspension of P. ovariae spores and is the best method among those examined in this study. Landolt (1973) was also able to prepare a clean preparation of M. cerebralis spores using this technique,

including an enzyme digestion procedure prior to implementing the ether separation tehcnique to release *M. cerebralis* spores from the cartilage where they are located. Enzyme digestion was not needed in this study because maceration and homogenization sufficiently released spores of *P. ovariae* from ovary tissue.

CHAPTER V

ENTRY OF *PLEISTOPHORA OVARIAE* INTO CULTURED CELLS

Experimental Design

Monolayers of MDBK cells, VERO cells, MOS cells, and FHM cells were grown in chamber slides or in 35 mm Corning cell culture dishes containing sterile coverslips in an experiment designed to examine the mode of entry of *P. ovariae* spores into selected culture cells at the light microscope level. After pretreatment with antibiotics, *P. ovariae* spores were introduced onto monolayers at a rate of 1×10^5 spores per chamber slide or cell culture dish. Experimental monolayers were periodically observed with an inverted microscope. Slides or coverslips were fixed at intervals of 0.25, 0.50, 0.75, 1, 2, 4, 24, 48, or 72 hours and 6, 7, or 13 days after exposure to spores and stained Mallory's triple stain. Evaluation included counting the number of cells and number of spores per field (X 1500). The average number of spores per cell was estimated by averaging counts from five fields and dividing the average number of spores per field by the average number of cells per field. Analysis by the student's t-test followed.

P. ovariae spores which had been pretreated with

antibiotics were also introduced onto MOS monolayers grown in 30 ml cell culture flasks and fixed for electron microscopy at intervals of 0.50, 2, 3, 4, 12, or 24 hours after exposure and processed for electron microscopy.

Results

Light Microscopy

Observations using the light microscope revealed that more spores per cell were located inside MOS cells than in the other established cell line types (Table I). By four hours after exposure, an average of 1.183 spores per cell had been engulfed by MOS cells, more than double the average number of spores engulfed by the VERO cells (0.529). No significant difference in the average number of spores phagocytized by MOS cells at 1, 2, and 3 hours after exposure was observed, but the average number of spores phagocytized by 4 hours after exposure (1.183) was significantly greater than at either 1, 2, or 3 hours after exposure (0.166, 0.191, and 0.230 respectively). A greater number of spores per cell was observed in MOS cells than in the other types of cultured cells at 4, 24, 48, and 72 hours after exposure to spores. The average number of spores engulfed by MOS cells was significantly greater at 24 hours (3.487) than at 48 hours (1.834) or 72 hours (2.025) after exposure; there was no significant difference between the average number of spores within MOS cells at 24 and 48

TABLE I. Average number of spores per cell with the average number of cells per field in parentheses

Time	Cell Type		
	VERO	MDBK	MOS
0.25 hour	0.038 (28.6)	0.029 (35.2)	ND[a]
0.50 hour	0.064 (30.8)	0.023 (34.5)	ND
0.75 hour	0.062 (30.0)	0.025 (34.8)	ND
1 hour	0.077 (31.4)	0.147 (39.6)	0.166 (17.2)
2 hours	0.588 (31.8)	0.071 (53.6)	0.191 (16.0)
3 hours	ND	ND	0.230 (50.4)
4 hours	0.529 (41.8)	ND	1.183 (11.8)
24 hours	0.469 (76.6)	0.015 (67.2)	3.487 (17.0)
48 hours	0.409 (88.8)	0.016 (95.4)	1.834 (23.8)
72 hours	0.270 (87.6)	0.020 (98.6)	2.025 (28.8)

[a]ND = No Data

hours after exposure. The largest average number of spores phagocytized by MOS cells (3.487) was observed at 24 hours after exposure.

Some spores were seen adhering to the surface of VERO cells, and some spores were seen inside cultured cells at 0.25 hour after exposure (0.038 per cell). There was no significant difference in the average number of spores engulfed by VERO cells at 0.25, 0.50, 0.75, or 1 hour after exposure; however, a significantly greater average number of spores was phagocytized by VERO cells at 2 hours (0.588) and 4 hours (0.529) than at 0.25, 0.50, 0.75, or 1 hour after exposure (0.038, 0.064, 0.062, and 0.077 respectively). No significant difference was observed in the average number of spores per cell between 2 and 4 hours after exposure, and no significant changes in the average number of spores engulfed at 24, 48, or 72 hours after exposure were observed. The greatest average number of spores phagocytized by VERO cells (0.588)was observed at 2 hours post exposure. The average number of spores engulfed by VERO cells at 24 hours (0.469), 48 hours (0.409), and 72 hours (0.270) was significantly less than the average number phagocytized by MOS cells (3.487, 1.834, and 2.025 respectively) but significantly greater than the average number engulfed by MDBK cells at the same time periods (0.015, 0.016, and 0.020 respectively). Spores were still visible in VERO monolayers stained with Mallory's triple stain at 7 and 13 days after exposure, but spores were no longer

visible in monolayers 12 to 14 days after exposure to spores.

MDBK cells were observed to contain some spores 0.25 hour after exposure. Although there was no significant difference in the average number of spores engulfed by MDBK cells at 0.25, 0.50, and 0.75 hours, some MDBK cells had engulfed a significantly greater average number of spores at 1 hour (0.147) than at 0.25 (0.029), 0.50 (0.023), 0.75 (0.025), or 2 hours (0.071) after exposure. No significant difference was observed between the average number of spores engulfed by MDBK cells at 24, 48, or 72 hours after exposure. The average number of spores engulfed by MDBK cells was less than the average number engulfed by other cell types at every time period sampled except at 1 hour post exposure when the average number of spores engulfed by MDBK cells (0.147) was greater than the average number engulfed by VERO cells (0.077) and less than the average number engulfed by MOS cells (0.166). However, these differences were not significant. Spores were visible 7 and 13 days after exposure in MDBK cells stained with Mallory's triple stain, but as in VERO monolayers, spores were no longer visible 12 to 14 days after exposure.

Fathead minnow cells, a fibroblast cell line, engulfed few spores in comparison to the other cell lines. Some spores were phagocytized within 24 hours after exposure, and could be seen within the cells 4 days after exposure. These cells usually degenerated quickly after addition of

the spore suspension to the cell culture, possibly due to a toxic reaction to some substance in the spore suspension. Therefore, no comparative counts were attempted.

Electron Microscopy

Ruthenium red, a membrane specific stain used to determine membrane integrity, was used as an aid in determining the sequence of events during spore entry. Ruthenium red is a tracer stain which stains only cell membranes of intact cells when applied at the time of fixation. It will, however, stain the cytoplasm and organelles of broken or degenerating cells (Jensen and Hammond, 1975). Since the cell membrane integrity would have to be compromised to allow the ruthenium red stain entry into the cell cytoplasm and since most cells containing spores did not show cytoplasmic staining, it does not appear that spore entrance disrupts the membrane integrity of the cultured cell.

All stages of phagocytosis were observed in samples of MOS cells fixed 4 hours after exposure to spores, but entire spores were observed inside cells 0.50 hour after exposure. Spores were observed to settle randomly when introduced into cell culture medium and were observed both on and between cells in the culture. Once spores had settled in the culture medium, cells were observed with pseudopodia extending toward and touching free spores (Fig. 1A). Spores were also seen partially within cells

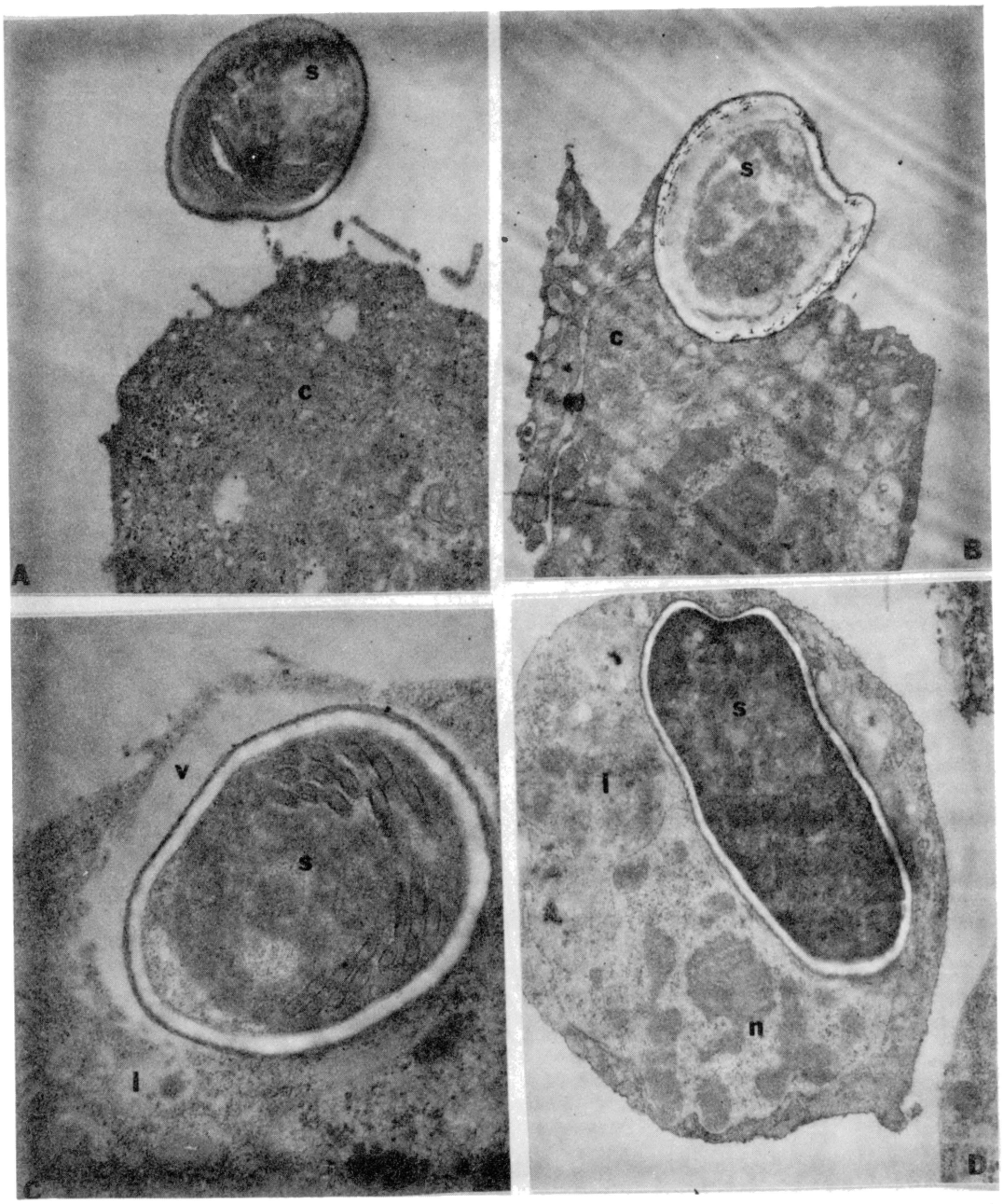

FIGURE 1. The entry of P. ovariae into cultured mosquito cells by active phagocytosis. A. Pseudopods from the cell (c) reach out toward a spore (s), X 12,000. B. Adhesion of the spore to the cultured cell, X 12,000. C. Spore closed in a heterophagocytic vacuole (v) with an attaching lysosome (l), X 23,000. D. Spore free in the cytoplasm of a cell. The nucleus (n) and a large lysosome are prominent structures, X 12,000.

(Fig. 1B), with pseudopodic processes extending around the spore. No particular orientation of the spores to the cell was necessary for engulfment. The contact between the spore and the cell was generally quite close. Where the cell membrane was visible, it appeared to be against the spore wall with no intervening space.

Spores which had entirely entered intact cells were often surrounded by a heterophagocytic vacuole (Fig. 1C). Occassionally the remains of a membrane was distinguishable on one end of the spore. Lysosomes were sometimes seen fusing with this membrane in an apparently normal process of digestion. On the other end of the same vacuole, however, the spore often appeared to be in contact with the cell cytoplasm without an intervening membrane (Fig. 1c). Some spores were also observed in vacuoles surrounded by a clear halo, yet the plasma membrane appeared to be absent. Other spores appeared to be surrounded by the remains of a vacuole, with only portions of a membrane present. Spores were also frequently seen within cells without any sign of limiting membranes or phagocytic vacuoles around them (Fig. 1D).

Discussion

Light Microscopy

Although all cell types phagocytized spores, no developmental stages of *P. ovariae* were seen in cell culture.

MOS cells engulfed the largest average number of spores (3.487) at 24 hours post exposure. The average number of spores engulfed by MOS cells was significantly greater than that engulfed by VERO or MDBK cells at 4, 24, 48, and 72 hours after exposure. Since the MOS culture consisted of both macrophage and fibroblast cell types, it had greater potential for engulfing foreign particles than the VERO or MDBK cell lines which consist of epithelial cell types. The higher spore per cell ratio in MOS cells was also probably influenced by the lower number of MOS cells per field. For example, at 24 hours post exposure, there was an average number of 17.0 MOS cells per field in contrast to 67.2 MDBK cells and 76.6 VERO cells per field. If all cell cultures had phagocytized the same number of spores, the average number of spores per cell would still have been larger for the MOS group because it had the smallest population of cells per field (lower divisor in the ratio).

VERO cells generally engulfed an average number of spores intermediate to the number engulfed by MOS and MDBK cells. The decrease in the average number of spores engulfed by VERO cells after 2 hours after exposure was probably due to the increasing number of VERO cells. The reason for the decreased phagocytic rate exhibited by the MDBK cells is unknown.

Electron Microscopy

In the process of phagocytosis of P. ovariae spores,

the following steps were observed. Spores appeared to settle randomly when placed into cell culture. Adherence of the spore was accomplished by the reaction of cellular pseudopodia. As the pseudopodia flowed around the spore, it was ingested and became located in a phagocytic vacuole. At this point the parasite was membrane-bound and subject to destruction due to fusion of primary lysosomes with the membrane. In some cases the plasma membrane, however, appeared to break down, leaving the spore in direct contact with the cytoplasm.

Entry of P. ovariae spores into cultured MOS cells was, therefore, observed to occur by active phagocytosis, similar to that described for Toxoplasma gondii in HeLa cell cultures (Jones et al., 1972). Other modes of entry which have been described for sporozoan parasites include active invasion by Eimeria magna sporozoites (Jensen and Hammond, 1975) and passive engulfmemt of Plasmodium species (Ladda et al., 1969).

T. gondii has been reported to enter cells with random orientation; whatever part first contacts the cell initiates phagocytosis (Jones et al., 1972). P. ovariae spores, like T. gondii tachyzoites, are phagocytized following contact with a cell's pseudoposia, regardless of the orientation of the infective stage. Whichever process of parasite entry is involved, none of these sporozoans penetrates or ruptures the plasma membrane of the cell upon entering (Ladda et al., 1969).

T. gondii remains within the cytoplasmic vacuole following entry into a cell and reproduces in a generation time of 5 to 10 hours. There is no evidence of the formation of a second membrane (Jones and Hirsch, 1972). Plasmodium also lives within a vacuole membrane produced by the host, but this membrane is antigenically different from the initial phagocytic vacuole membrane, indicating removal of the first membrane and replacement with a second (Ladda et al., 1969). The phagocytic vacuole membrane formed around E. magna sporozoites also breaks down and is replaced by a second membrane of host origin (Jensen and Hammond, 1975). Similar breakdown of the first membrane was observed in this study of P. ovariae, but the formation of a second membrane was not observed. Although not totally substantiated by this study, the breakdown of the first membrane and the intracytoplasmic localization of the parasite may indicate that at least part of the microsporidan development occurs directly in the cytoplasm of the host cell.

The destruction of the first vacuole, formed by the invagination of the plasma membrane, may be one factor which allows the parasite to survive in its intracellular location. It is not only protected from host defenses such as antibodies and macrophages within the host cell, but but it is also protected against digestion by the host cell components. During the period when it is not surrounded by any membrane, lysosomes cannot attach and fuse

with the vacuole membrane to destroy it. The second membrane formed around E. magna and probably Plasmodium protects them from host digestion.

T. gondii which remains in the first cytoplasmic vacuole is believed to secrete a substance which alters the nature of the enclosing membrane so that lysosomes can no longer attach to it (Jones et al., 1972). Mycobacterium tuberculosis and Chlamydia are also able to prevent transfer of lysosomal material into their vacuoles (Jones and Hirsch, 1972). Another possibility for survival of intracellular parasites is the ability to withstand the toxic substances and enzymes delivered by the lysosomes, as in the case of Mycobacterium lepraemurium (Jones and Hirsch, 1972).

From observations of P. ovariae, it appears that there is still another pattern for intracellular survival. P. ovariae spores escape from the vacuole membrane of the host cell before encountering lysosomal products in the vacuole. Their intracytoplasmic existance thus insures a minimal amount of contact with host defense mechanisms and maximizes the opportunities for survival and multiplication.

CHAPTER VI

SPORE MORPHOLOGY AND STAINING REACTIONS

Experimental Design

Spore smears were prepared and stained with Giemsa, hematoxylin, PAS, and toluidine blue in order to determine staining reactions of P. ovariae spores. Paraffin sections of infected golden shiner ovaries were also processed and stained as described earlier. All slides were dehydrated, cleared, and mounted according to standard procedures. Spore morphology was determined by electron microscopy from a centrifuged sample of P. ovariae spores isolated from infected ovary tissue collected in early spring by the ether separation technique.

Results

Light Microscopy

Spore smears stained well with one polysaccharide stain (toluidine blue) but only faintly with the other (PAS). Toluidine blue stained P. ovariae spores in smear preparations a dark blue-purple in the region of the polar cap and the polar filament less intensely blue. PAS stained both the polar cap and polar filament only faintly

or not at all. P. ovariae spores in sections of ovary tissue stained more variably than spores in smears, possibly due to the thickness of the sections. When stained with toluidine blue, some spores appeared to have a dark blue band in the middle of the spores; others displayed one dark blue end; and other spores were stained entirely light blue. Spores were not stained when PAS was applied to ovary sections, but the secondary oocyte membranes on the same slides were strongly positive. Control slides of mouse intestine displayed the characteristic positive PAS reaction. Spores in smear preparations were also PAS negative.

Variable results were also obtained with the use of nuclear stains. Spores in smears treated with hematoxylin were predominantly pale with a dark blue or purple longitudinal band medially or with a dark nuclear spot in the posterior region of the spore. Many spores, however, stained homogenously pale or displayed a light vertical band. Ovary sections containing spores showed most spores staining negatively when treated with hematoxylin, but some displayed a dark dot either centrally or at the posterior end.

Giemsa applied to spore smears stained the polar cap intensely blue and the nucleus in the posterior end of the spore less intensely purple. The polar filament sometimes stained faintly and sometimes a longitudinal stripe was displayed. Spores in ovary sections stained in the same

manner as the spores in smears. Many spores displayed both a dark polar cap and nucleus, but some spores remained unstained.

Electron Microscopy

Observations using electron microscopy showed that the P. ovariae spores are bounded by a thin electron-dense exospore (40 nm) and a thick electron-translucent endospore (Fig. 2). The endospore measured approximately 120 nm except at the anterior end (40 nm) where a cone-shaped cavity, the polar sac, surrounded the site of polar filament attachment. The base of the polar filament was surrounded by an electron-opaque polar cap (Fig. 2B). The polar filament descended medially to the middle of the spore, then coiled next to the spore wall in peripheral rows. Coils were arranged in single, double, or triple layers, as the single strand coils inside the first row of coils. These coils extended to the larger end of the spore. The maximum number of coils observed in any one section was 34. The diameter of the polar filament in the descending portion was estimated at 120 nm while the diameter of the polar filament in the descending portion was estimated at approximately 100 nm.

The polar filament was composed of a bilayer wall and an inner core (50 nm) of electron dense material. The outer wall of the polar filament (10 nm) was denser than the inner wall (15 nm); the inner wall contained about 20

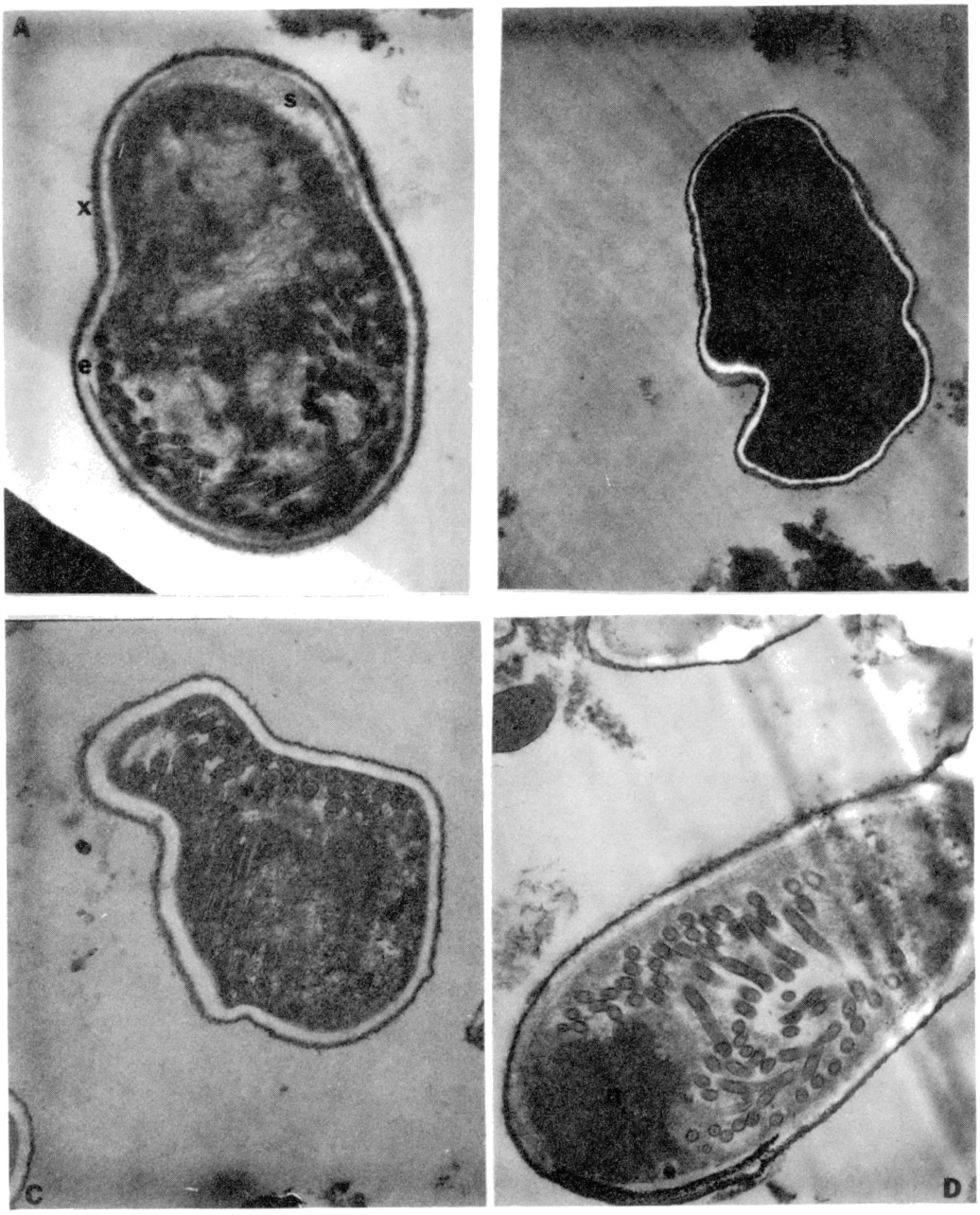

FIGURE 2. Ultrastructural morphology of P. ovariae spores. A. The exospore (x), endospore (e), polar space (s), and polar filament (f) are prominent structures illustrated, X 17,000. B. Oblique section through the polar cap, X 12,000. C. Details of polar filament structure, X 23,000. D. The nucleus (n) of a P. ovariae spore.

electron dense fibrils which spiraled along the length of the polar filament (Fig. 2C).

A single nucleus was observed enclosed by the coils of the polar filament (Fig. 2D). Undifferentiated cytoplasm, a few ribosomes, and smooth endoplasmic reticulum filled the rest of the space within the coils of the polar filament. Between the coiled region of the polar filament and the polar cap, the space between the spore walls was filled with a delicate polaroplast structure. No mitochondria were observed. The plasmalemma was located just within the endospore. A diagrammatic representation showing the spore morphology of P. ovariae as observed with the electron microscope is shown in Figure 3.

Discussion

Light Microscopy

Both hematoxylin and Giemsa stains colored the nuclei in the larger ends of P. ovariae spores. The spores which were not colored by nuclear stains or which stained aberrantly were probably degenerate or dead or, in the case of ovary sections, sectioned through a region not containing the nucleus of the spore. All other Pleistophora species so far examined had only one nucleus (Weiser, 1976). The metachromatic reaction to toluidine blue revealed the polysaccharide nature of the polar cap and, less prominently, the polar filament. Many other workers have reported the

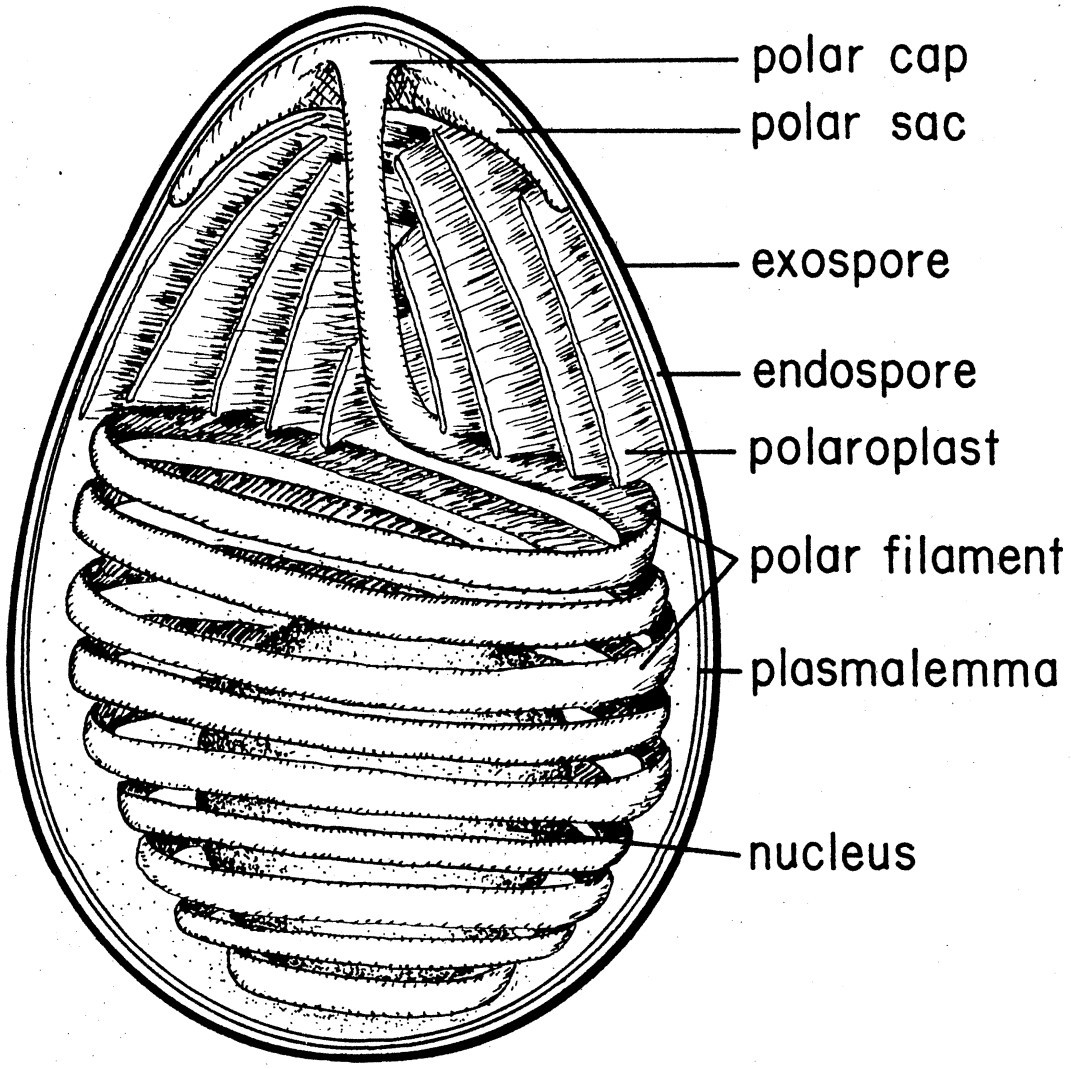

FIGURE 3. Schematic representation of the internal structures of a P. ovariae spore.

polysaccharide nature of these two organelles using PAS, but P. ovariae spores stained only faintly or not at all with PAS.

Electron Microscopy

Electron microscopy revealed that the ultrastructural morphology of P. ovariae corresponds well with that of other microsporidans. All microsporidans so far investigated possessed a thick spore wall divided into exospore (50 nm) and endospore (200 nm), as well as a coiled polar filament and one or more nuclei (Canning, 1977). Diversity among species occured in the number, arrangement, and angle of the polar filament, presence of a polar cap, and the shape and location of the posterior vacuole.

Marked similarities were observed between the ultrastructure of P. ovariae and P. hyphessobryconis, a muscle parasite of the fish, the neon tetra (Lom and Corliss, 1967). This species is the only other Pleistophora species from a fish whose ultrastructure has been described in detail. Both P. hyphessobryconis and P. ovariae have thick spore walls which are typical of microsporidan parasites. The exospore of P. ovariae (40 nm) is thicker than that of P. hyphessobryconis (7 nm), while the endospore of P. ovariae (120 nm) is thinner than that of P. hyphessobryconis (150 nm), but the entire thickness of the spore walls of P. ovariae (160 nm) and P. hyphessobryconis (157 nm) are remarkably similar.

Both P. ovariae and P. hyphessobryconis possessed polar caps and coarse granular nuclei. The polaroplast membranes were present in both species, but more prominent in P. hyphessobryconis. The one major difference between P. ovariae and P. hyphessobryconis was the lack of a posterior vacuole in P. ovariae at the electron microscope level. Although a large posterior vacuole had been reported from P. ovariae at the light microscope level, no corresponding structure could be seen with the electron microscope. Perhaps the posterior vacuole observed with the light microscope was produced by the refraction of light by the coiled polar filament.

The polar filament of P. ovariae was arranged in 34 single, double, and triple coils of a single strand, one within another in the posterior half of the spore. This arrangement is very similar to those of P. hyphessobryconis and P. gigantea, another fish microsporidan (Sprague and Vernick, 1968b), but a smaller number of coils has usually been observed in other microsporidan genera (Canning, 1977). The details of the polar filament structure in P. ovariae were similar to those of Unikaryon legeri, a hyperparasite of a trematode metacercaria (Canning and Nicholas, 1974). The polar filament consisted of a double wall with the inside wall containing approximately 20 fibrils (described as rings by Canning and Nicholas, 1974). Since the polar filament everts and expands to facilitate extrusion of the nuclear material

(Weidner, 1972), the circular structures observed in one layer of the polar filament of P. ovariae were described as fibrils. Proteolytic digestion has also revealed the fibril nature of these substructures within the polar filament wall (Vavra, 1972).

The structure of the polar filament of P. ovariae was also similar to that of P. hyphessobryconis which has been described as a bilayered wall, whose central cylinder was reported to include ridges extending into the inner layer of the wall to give the appearance of fibrils (Lom and Corliss, 1967). However, the fibrils in the wall of P. ovariae's polar filament did not connect with either the electron dense material in the lumen of the tube or with the electron dense material in the outer wall. The diameter of the polar filament of P. ovariae (100-120 nm) was slightly less than that of P. hyphessobryconis (110-130 nm) (Lom and Corliss, 1967). The vast diversity of observations regarding polar filament structure may be due to inter- and intrageneric differences, but such discrepancies could also result from differences in tissue processing for electron microscopy. Good fixation of microsporidan spores is very difficult due to their thick spore wall.

CHAPTER VII

CHANGES ASSOCIATED WITH PHAGOCYTOSIS

Experimental Design

P. ovariae spores (1×10^3 per ml) were obtained by the ether separation method and were introduced onto monolayers of cultured cells (MDBK, VERO, and MOS) as described in the methods section. Samples of these monolayers were fixed *in situ* at 24, 48, and 72 hours and 6 or 7 days after exposure to spores. They were subsequently stained with the same solutions used to stain spore smears: Giemsa, hematoxylin, PAS, toluidine blue, and Mallory's triple stain. The cell monolayers on slides or coverslips were then dehydrated, cleared, and mounted according to standard procedures, and cells examined with a light microscope.

Pieces of ovaries from naturally infected golden shiners collected in the fall were fixed for electron microscopy. These post-spawning ovaries were in the process of resorbing unspawned ova and therefore were used so that the effect of the host macrophages on *P. ovariae* spores could be observed and described.

Results

Light Microscopy

Results and discussion of cell culture monolayers containing *P. ovariae* spores will be limited to observations of VERO cells because MOS cells containing spores were generally too rounded to allow observation of staining reactions within the spores, and because MDBK cells did not engulf enough spores for adequate observation.

One day after exposure, VERO monolayers contained *P. ovariae* spores that stained approximately the same as smear preparations of spores (Table II). Toluidine blue stained the polar cap darkly and the polar filament more lightly. Hematoxylin staining revealed dark blue nuclei in *P. ovariae* spores. Giemsa stained the entire spore blue with dark blue nuclei in the larger ends of the spores. Mallory's triple stain colored mature spores yellow-gold, but immature spores were red and dead spores were light blue.

Three days after exposure, spores phagocytized by cultured cells no longer stained like spores in smear preparations. The entire spore appeared a pale homogenous grey-purple when stained with Giemsa. Similarly hematoxylin treatment resulted in pale blue spores with no neuclear differentiation, and toluidine blue staining resulted in a non-specific pale purple color with a few dark dots in some spore walls. However, spores phagocytized by culture cells still stained with Mallory's triple stain in the same manner

TABLE II. Staining reactions of P. ovariae spores engulfed by VERO cells compared to spores in spore smear preparation

	Time		
Stain	1 day[a]	3 days	6-7 days
Toluidine blue	S[b]	D[c]	D
Hematoxylin	S	D	D
Giemsa	S	D	D
Mallory's	S	S	S

[a]day(s) = day(s) after exposure of spores to cultured cells

[b]S = Same staining reaction for spores phagocytized by cultured cells as for spores in smear preparation

[c]D = Different staining reactions for spores phagocytized by cultured cells as for spores in smear preparation

as spore smear preparations. The cultured cells stained the same at 3 days after exposure to spores as they had at 1 day after exposure.

Seven days after exposure to spores, the VERO cells were still staining like they had 1 day after exposure to spores with all stains. However, spores phagocytized by cultured cells stained with toluidine blue appeared pale blue and wrinkled, and spores stained with hematoxylin were homogenously light gray. VERO cells stained with Giemsa 6 days after spore exposure had very faintly blue staining spores. Mallory's triple stain still colored spores in the same way as it did in spore smear preparations.

Electron Microscopy

Fish ovaries collected in the fall contained large numbers of spores which had been engulfed by macrophages (Fig. 4A). Many stages of spore digestion were present, but no spores exhibiting morphology similar to that of unphagocytized spores were observed. Intermediate developmental stages of P. ovariae were also not observed.

The first change induced by phagocytosis which was observed was the disappearance of the details of the polar filament structure and substitution by empty spaces (Fig. 4B). Internal structures then became homogenous or condensed (Fig. 4C). The spore wall was either intact or the endospore was permeated by an electron dense material, followed by the collapse of the spore walls to give the

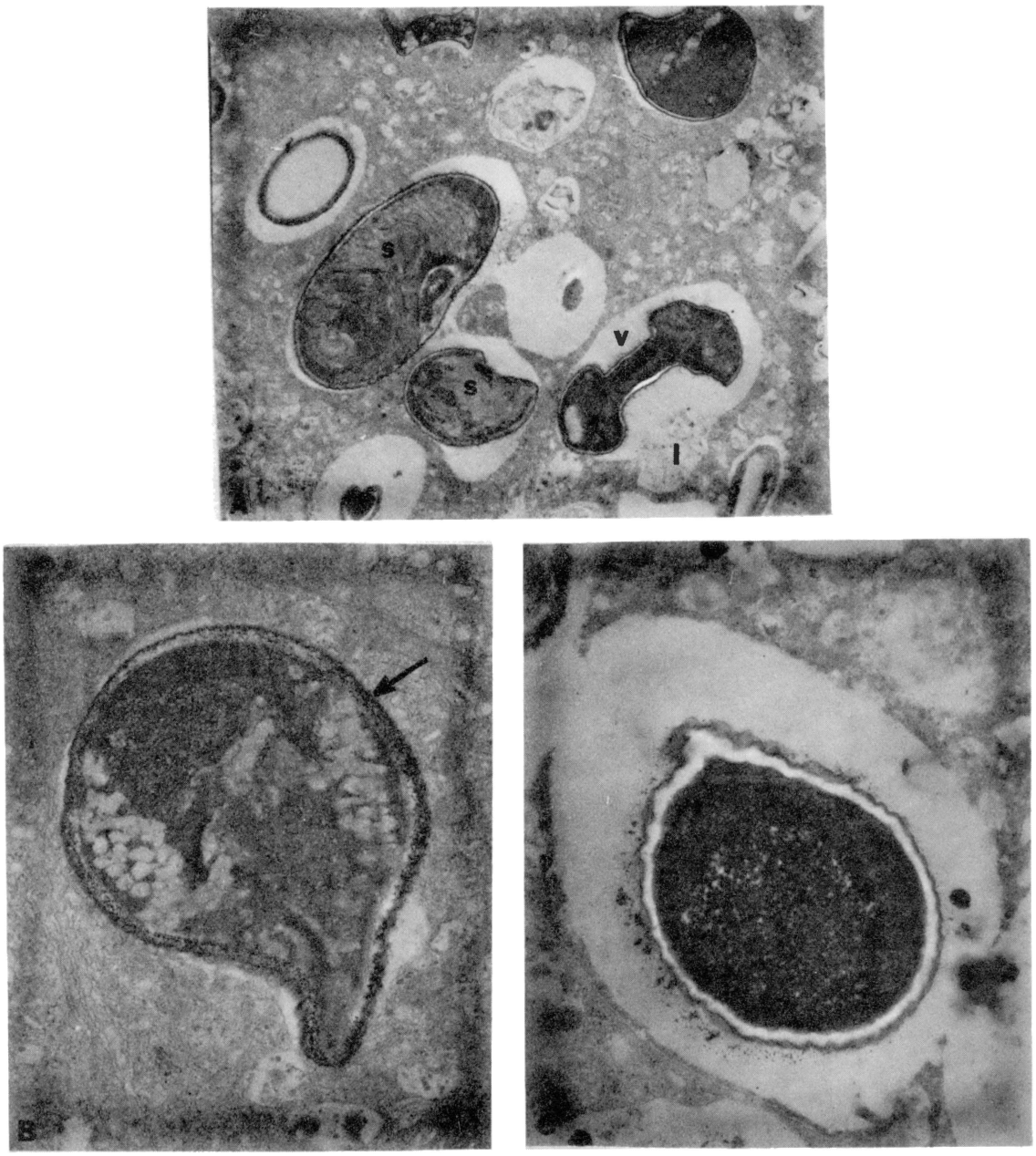

FIGURE 4. Early stages of spore digestion in golden shiner ovary tissue. A. Numerous spores (s) in various stages of digestion. Lysosomes (l) are attached to some heterophagoctyic vacuoles (v), X 6,000. B. Empty spaces have been substituted for polar filament structures and the integrity of the spore wall is disrupted, as shown by an arrow, X 17,000. C. Condensation of internal structures within the spore wall, X 17,000.

spores a wrinkled or crumpled appearance (Fig. 5A). The electron dense material inside the spore walls then disappeared, leaving empty spore shells (Fig. 5B). However, the plasmalemma and some debris were also seen inside some of these latter structures. The remnants of the spore wall also disappeared (Fig. 5C), leaving only debris which resembled pieces of membranes in the phagosomes.

Discussion

Both light and electron microscopy showed that P. ovariae spores are susceptible to phagocytosis. Although spores phagocytized by cultured cells stain like spore smear preparations 1 day after exposure, they lost their characteristic staining with most stains after 3 days. With Mallory's triple stain, spores at 7 days after exposure reacted the same way as did spores in spore smear preparations; however, other stains revealed that the spores were empty and only the spore wall of the spore remained. Since Mallory's triple stain colors primarily the spore wall, yellow-gold or red staining is a misleading indication of spore viablity. Eventually spores disappeared entirely from the culture system.

Electron microscopy supported the light microscopy observations in that the first changes induced by phagocytosis included destruction of cytoplasmic organelles and the nucleus, followed by collapse of the spore walls. Eventually the entire spore was digested.

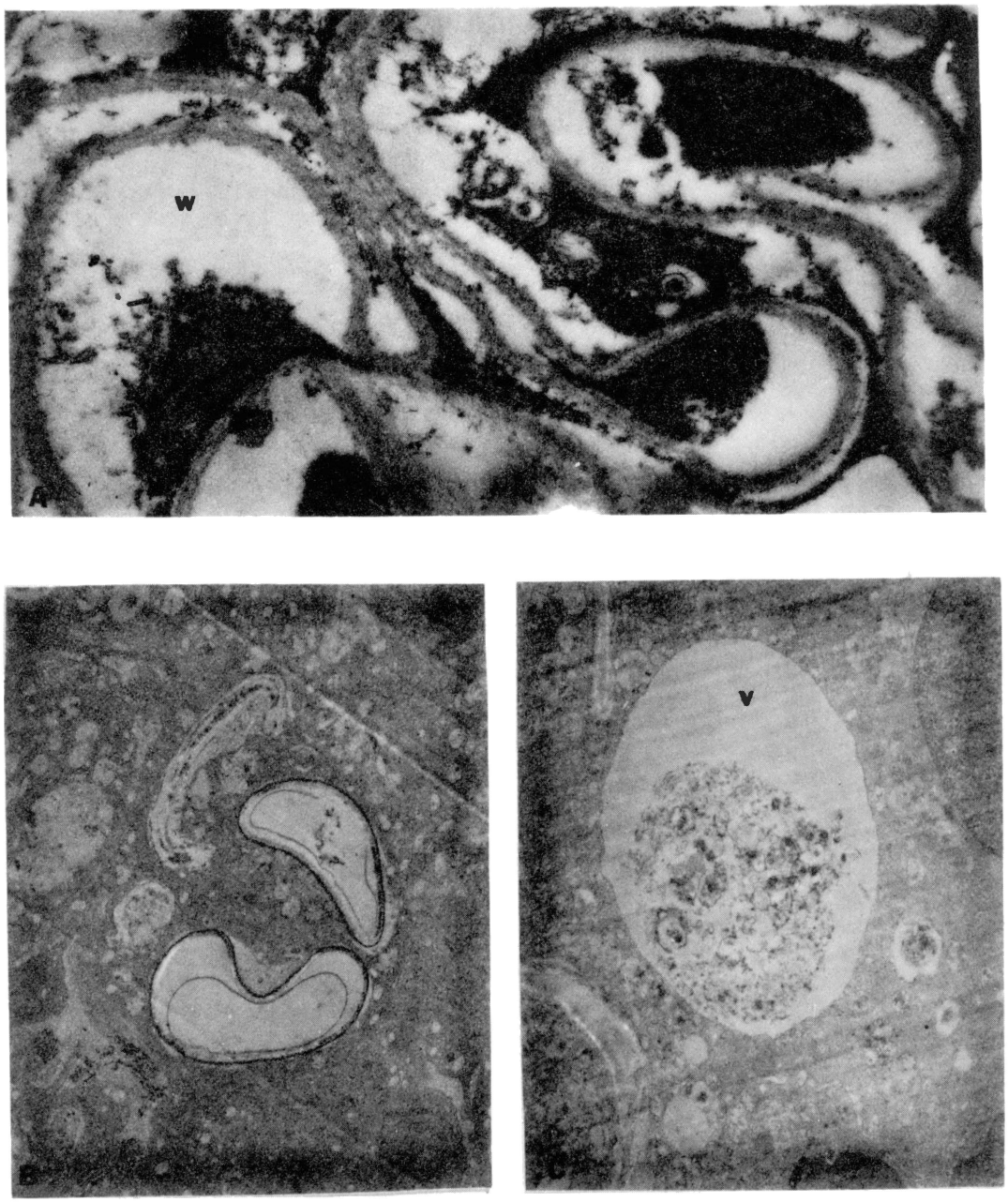

FIGURE 5. Later stages of spore digestion in golden shiner ovary tissue. A. Collapsed spore walls (w) filled with a homogenous electron-dense substance, X 36,000. B. Further digestion of the spore contents, leaving only empty spore shells, X 7,000. C. Remnant debris after spore digestion in a heterophagocytic vacuole (v), X 9,000.

Viable spores and developmental stages of P. ovariae were not observed in the fall ovary sample, but very few immature oocytes, which generally contain developmental stages of P. ovariae, were seen in the sample. Since no viable spores were observed in the fall sample, the stage which overwinters in the ovary is probably not the spore. It is possible that an intermediate or latent stage survives in an immature oocyte. Fish may also be reinfected by ingestion of a spore from the environment in early spring. The infection level of P. ovariae in golden shiners would appear to be the lowest in the fall and winter seasons as the spore stage is eliminated from the ovary. The large number of P. ovariae spores produced may not pose as great a threat of environmental contamination as might be imagined since only the spores which are voided from the host with the ova have a chance of spreading the infection to new hosts. All mature spores which remain in the host in unspawned eggs or in the supporting tissues of the ovary are probably destroyed by host macrophages and do not appear to contribute appreciably to the continued infection or reinfection of the host.

CHAPTER VIII

CONCLUSIONS

A relatively pure spore preparation of P. ovariae can be obtained by the ether separation method. Analytical research, including protein analysis, in vitro culture, and immunologic studies can be facilitated using this technique due to the large number of spore produced, purity of the preparation, and the ease in handling.

The entry of P. ovariae spores into cultured cells can best be described as active phagocytosis. The similarity of this entry mechanism to those of other sporozoans suggests that the process of phagocytosis is an alternative method to sporoplasm extrusion for entry of microsporidans into host cells. The observation that spores appeared free in the cytoplasm of phagocytic cells supported the theory of phagocytic entry for P. ovariae infection since P. ovariae would be protected from digestion in this location. These observations offer one explanation for the survival of the parasite in transit from entry at the host's gut epithelium to establishment at the site of localization.

Some spores were identified as foreign objects by the cells since lysosomes were observed attaching to the vacuole membrane. However, spores which did not contact

lysosomes were relatively protected from host cell defenses. Digestion of the spore seemed to be initiated by the presence or persistence of the phagosome membrane rather than any characteristic of the engulfed spore, since spores exhibiting viable and nonviable morphology were observed within vacuoles with attaching lysosomes.

Characters of viable P. ovariae spores have been described in this study, including ultrastructural morphology and selected staining reactions. In general the characteristics of P. ovariae correspond with those of the other two Pleistophora parasites of fish, which have been described in detail. This description provides good information for later taxonomic studies, as well as a comparative standard for studying phagocytosis. Since microsporidan spores are so small and appear so similar by light microscopy, more detailed study is necessary to properly distinguish between species. P. ovariae differs from other Pleistophora species infecting fish primarily in its lack of a posterior vacuole and in the details of polar filament structure and arrangement. In addition, P. ovariae stains only faintly with PAS in contrast to the strong PAS positive reaction of the polar cap in numerous other microsporidans. However, staining reactions typical for other microsporidans were observed also in P. ovariae spores treated with Giemsa, hematoxylin, and toluidine blue.

Changes associated with phagocytosis were observed in samples of fish ovary tissue obtained in the fall.

Observation of spore digestion in ovary tissue by host macrophages illustrates part of the host's defense mechanism against P. ovariae. Although enormous numbers of spores are produced in the fish ovary, only those spores which leave the host, either inside ova or being carried out with ova at the time of spawning, are capable of transmitting the infection to new hosts. Mature spores which remain in the ovary are destroyed or inactivated and do not appear to be a major factor in infection or environmental contamination. The parasite apparently overwinters in low numbers in an immature stage in the ovary, thus resulting in continued asexual development the following spring. However, reinfection may occur by ingestion or by some yet to be determined means.

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APPENDIX

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