Nosema cuniculi: In vitro Isolation

Abstract. *A mammalian microsporidian was propagated for the first time in vitro with rabbit choroid plexus monolayer cultures. Tentative classification as* Nosema cuniculi *(Levaditi, 1923) was established by morphologic and tinctorial characteristics and by extrusion of single polar filaments with attached sporoplasm from living spores. The method permits propagation of large numbers of organisms in pure culture.*

Mammalian microsporidia are a ubiquitous group of parasitic protozoa (*1*) frequently encountered as latent contaminants of experimental animals (*2*). The influence of these infections on experimental results is unknown, since there are no methods for detecting latent microsporidian infection in living animals, and in vitro propagation of the organisms has not been reported. An in vitro system for isolating and propagating these parasites would be advantageous, since it could provide the large numbers of purified organisms needed as antigen in the development of diagnostic serologic reactions. Also, present uncertainties about the relationship between these parasites and concomitant diseases (*3*) may be resolved by isolating the organism in vitro, thus avoiding the use of laboratory rodents which may themselves be parasitized.

This report deals with the in vitro isolation and propagation of an intracellular protozoan parasite morphologically identical to the microsporidian *Nosema cuniculi* (Levaditi, 1923) (*4*). The organism was isolated twice, once from a choroid plexus cell culture obtained from a clinically healthy rabbit and once from the brain of a rabbit that had lesions typical of nosematosis (*5*).

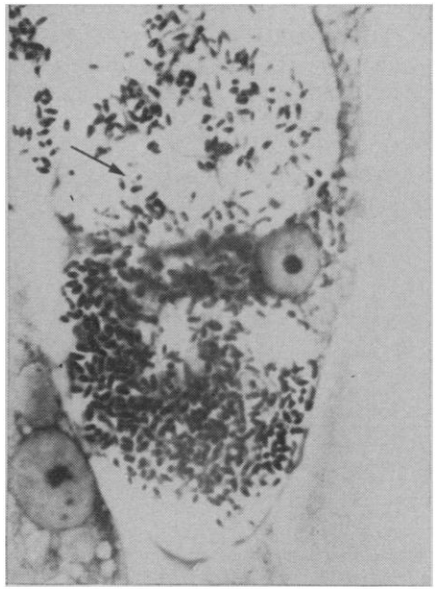

Fig. 1. Cell from a rabbit choroid plexus monolayer culture distended by spores (arrow) and proliferative forms of *Nosema cuniculi* (Goodpasture's; ×890).

Monolayer cultures of rabbit choroid plexus cells (*6*) were used to isolate the parasite. These cells were subpassaged at weekly intervals and maintained for 10 to 15 passages. During the seventh subpassage of one culture many cells were observed to be distended by numerous intracytoplasmic, straight, blunt organisms measuring approximately 1.5 by 2.5 μ, which were morphologically identical to *Nosema cuniculi* (Fig. 1). Sporulating forms had an eccentrically located dark body and vacuoles at one or both poles, while proliferating forms were more homogeneous and lacked vacuoles. The organisms stained intensely blue with Giemsa stain and dark blue to purple by Goodpasture's technique. The spores were gram-positive (*7*).

Infected cultures were subpassaged once or twice before all cells became infected and failed to multiply. Cultures prepared simultaneously from other rabbits were not spontaneously parasitized but could readily be infected by mixing normal and parasitized cells at the time of subculture. By 10 to 14 days after subpassage, nearly every cell in the mixed culture was heavily infected and the cells had to be subcultured again together with normal cells. By this method the parasite has been maintained continuously (4 months).

On the second occasion, normal rabbit choroid plexus monolayer cultures were inoculated with a 20 percent suspension of rabbit brain in which lesions and organisms typical of *Nosema cuniculi* (*5*) had been demonstrated. Seven days after inoculation the first infected cells were observed, and by 14 days almost every cell was infected. The staining properties, morphology, and in vitro behavior of this isolate were identical to those of the first.

Inoculation of both isolates on a variety of acellular media under aerobic and anaerobic conditions at 37°C, 20°C, and 4°C produced no detectable growth.

The identification of these parasites as microsporidia was confirmed by study of living organisms by phase contrast microscopy. They explosively extruded a single polar filament with attached sporoplasm (*8*) when subjected to 0.4 percent H_2O_2, ether, distilled water, and desiccation; H_2O_2 produced the most rapid extrusion from the most organisms. Pressure exerted on the cover slip ruptured infected cells and released organisms, but extrusion of polar filaments rarely occurred.

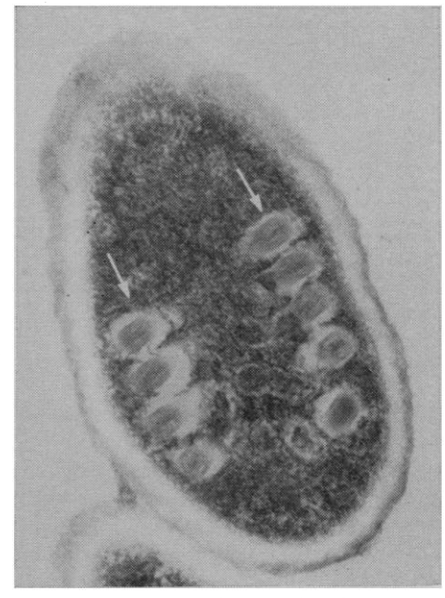

Fig. 2. Electron micrograph of *Nosema cuniculi* spore showing oblique sections of the polar filament (arrows) (lead hydroxide; ×36,800).

Ultrastructural examination of infected cells which had been fixed in osmium tetroxide and stained with lead hydroxide revealed large cytoplasmic vacuoles containing numerous organisms resembling *Nosema cuniculi* (*9*). The spores had a coiled polar filament within a polaroplast at one end of the cell (Fig. 2). The other end was occupied by a large vacuole, and the remainder of the space was filled with coarsely granular material.

The morphologic characteristics and the demonstration of polar filament extrusion placed this protozoan parasite among the microsporidia. Since it so closely resembled *Nosema cuniculi* as described by others (*4*, *9*), it is suggested that this name be tentatively assigned, until the taxonomic classification of these parasites is further clarified (*10*).

Note added in proof: Using the methods described above I have succeeded in isolating and propagating *Nosema* organisms from infected mice (obtained from J. B. Nelson) and hamsters (obtained from V. Kinzel).

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References and Notes

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11. Part of this work was performed while I was a guest at the Institut für Mikrobiologie und Infektionskrankheiten der Tiere, Munich, Germany. Dr. A. Koestner provided the electron photomicrograph. Supported in part by grants FR05463 and 1F 10 NB 1802 from NIH.

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Antithrombin III: Protection against Death after Injection of Thromboplastin

Abstract. *Intravenous injection of autologous lipoprotein (thromboplastin) or thrombin produced a lethal, hemorrhagic syndrome in chicken embryos. The embryos could be protected from this fatal result by injection of antithrombin III, an alpha$_2$-globulin (molecular weight 60,000 to 80,000) purified from human, bovine, and guinea pig blood. Heparin also protected the embryos, but other inhibitors were less protective.*

It is well known among researchers in several branches of experimental biology that the intravenous administration of autologous cell homogenates or even of improperly washed cell suspensions may be fatal to the recipient animal. This fact may be viewed as an experimental model for the study of that part of homeostasis that deals with something as basic as the defense against the breakdown products of the individual's own cells.

Because thromboplastic material (of poorly defined lipoprotein nature) may be extracted from almost any kind of tissue and because the pathological findings in animals injected with toxic cell preparations are usually indicative of hemorrhagic disorders, it is natural to turn to experimental hematology for further elucidation. As early as 1947 Schneider (*1*) used the lethality that results from intravenous injection of mice as a bioassay of the thromboplastin content of homogenates of placenta and other tissues. Schneider (*1*) and Thomas (*2*) demonstrated clearly that the same toxic syndrome in mice could be circumvented by preincubating the thromboplastic material in vitro with normal mouse serum and concluded that the latter must contain a natural antithromboplastin; however, they did not succeed in defining this substance much further. Although later workers have investigated the changes in the blood level of various coagulation factors after injection of thromboplastic material, there is surprisingly little known about the nature of the inhibitor, or inhibitors, that hold in check the presumably never-ceasing flow of thromboplastins from decay of natural or pathological tissues (*3*). Our own work shows that a progressive antithrombin with the mobility of an alpha$_2$-globulin (in agarose) is one of these inhibitors. (The word "progressive" refers to the observed time-dependence of the inactivation of thrombin.)

We used chicken embryos as recipients of lipoproteins (LP), the thromboplastic material being prepared from allantoic fluid or the homogenized lungs from 17-day chicken embryos. The allantoic fluid was collected carefully in order to avoid contamination with blood or tissue fluid. Lipoproteins, partly purified by dialysis against phosphate-buffered saline followed by differential centrifugations (1,500 and 150,000*g*), contained 35 to 40 percent protein, 50 to 60 percent lipid, and 2 to 5 percent RNA.

The intravenous injection of LP from either of these sources produced a fatal hemorrhagic syndrome in chicken embryos (Fig. 1). The amount of LP required to produce the reaction was roughly proportional to the body weight of the 13-day embryos that were used in the present experiments; 12 μg of LP produced a fatal reaction in nearly all embryos. Death was ascertained by transillumination of the eggs and usually occurred within 1 to 4 hours after the injection. The following findings strongly suggest that the syndrome is caused by the thromboplastic effect of the injected LP. (i) Heparin could prevent death when administered before or simultaneously with the LP; (ii) there was a close correlation between different preparations with respect to their in vivo toxicity and their thromboplastic effect on chicken plasma in vitro; (iii) the injection of chicken and bovine thrombin produced similar lesions; and (iv) a marked thrombocytopenia developed before death.

The hemorrhagic syndrome could be prevented by preinjections of plasma or serum from chickens as well as by preincubation of LP with serum and plasma from various mammals. Chromatography of guinea pig serum or human plasma on diethylaminoethyl-cellulose (*4*) separated two fractions that had a protective effect in vivo. One of these had no anticoagulant effect in vitro,

Table 1. Comparison of in vivo protective effect of antithrombin III. Each dose was tested on eight to ten eggs. Controls (not shown) ensured that a lack of protection was not due to toxicity of the test substance. Lethality in 24 hours is recorded as follows: −, no significant difference from controls treated with lipoprotein; +, 25 to 50 percent of group protected; and ++, 50 to 100 percent protected; I.U., international unit.

Test substance	Dose (micrograms per egg)	Protection
Guinea pig antithrombin III	100	++
Guinea pig antithrombin III	25	+
Human antithrombin III		
From Copenhagen	35	++
From Oslo	22	++
Bovine antithrombin III	70	++
Human alpha$_2$-macroglobulin	660	−
Heparin* (1.0 I.U.)	7.1	++
Heparin (0.2 I.U.)	1.5	+
Lima bean trypsin inhibitor†	2000	++
	500	+
Ovomucoid trypsin inhibitor‡	2000	−
Pancreatic trypsin inhibitor‡	200	−
ϵ-Amino caproic acid (EACA)§	20,000	−

* Leo Pharmaceutical, Copenhagen. † Worthington Chemical. ‡ Sigma Chemical. § Kabi, Stockholm.