A species of *Encephalitozoon* isolated from an AIDS patient: criteria for species differentiation

W. S. Hollister, E. U. Canning and N. I. Colbourn

Department of Biology, Imperial College of Science, Technology and Medicine, London, SW7 2AZ, U. K.

Key words: microsporidia, *Encephalitozoon*, species differentiation, AIDS

Abstract. A microsporidium isolated *in vitro* from urine of an AIDS patient was identified as a species of *Encephalitozoon*. Investigation of the isolate by electron microscopy, amplification of DNA by RAPD PCR, protein analysis by SDS-PAGE and Western blotting and infectivity to athymic mice revealed that it differs from established species of the genus.

Microsporidia were isolated into monolayers of MDCK cells from a 26-year-old homosexual man who had been admitted to hospital with intermittent, severe abdominal pain, anorexia, nausea, vomiting, fever and a productive cough, only 5 months after he had been diagnosed HIV-positive. At the time of admission he had no diarrhoea and no indication of renal disease. No pathogens were found in blood, stool or jejunal aspirate and microsporidia were not found in jejunal or colonic biopsies. However, serum urea and creatinine quickly rose from 9.8 mmol/l and 130 μmol/l on admission to 37.6 mmol/l and 487 μmol/l respectively indicating renal dysfunction. Ziehl Neelsen staining of urine sediment still revealed no microsporidia but gallium scanning showed marked concentration of radioactivity in both kidneys. A renal biopsy was performed which revealed a heavy infection of microsporidia and by this time spores were detectable in urine.

Cultures, established from the spores, have been used to examine the parasite by light and electron microscopy, protein profiles and by random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR). The isolate has been identified as a species of *Encephalitozoon* but differs from established species in a number of characters.

MATERIALS AND METHODS

Urine from the patient was centrifuged at 1500 g for 30 min. The sediment was resuspended for 4 h in MEM with 200 units/ml penicillin and 200 μg/ml streptomycin and 2.5 μg/ml fungizone. The spores were then washed, resuspended in MEM with 10% FCS. Approximately 5x10^5 spores per well were added to monolayers of MDCK cells on coverslips in 24-well plates and 1.5x10^6 spores to monolayers in 25 cm base flasks.

When heavy infections were established the isolate was compared with cultures of *E. cuniculi* which had been established from a mouse and a dog and with *E. hellem* isolated from nasal polyps of an AIDS patient (Hollister et al. 1993). The complete history of the murine isolate is unknown. It was established from spores provided by Dr. Dawn Owen who had previously maintained the species in mice. The canine isolate was provided by Professor C. G. Stewart directly after isolation from the kidney of a dog (Stewart et al. 1979). Spores were purified from the supernatant and analyzed by SDS-PAGE as described by Hollister et al. (1993). The protein profiles were immunoblotted with a 1:8,000 dilution of serum from a child who was strongly positive for antibodies to *Encephalitozoon* (McInnes and Stewart 1991). The conjugate was alkaline phosphatase linked goat α human IgG (Sigma) diluted 1:1000. The substrate was NBT.

For electron microscopy cells were removed from the coverslips, pelleted and fixed in Karnovsky's fixative in 0.1M sodium cacodylate buffer pH 7.2. Subsequent processing was as described by Hollister et al. (1993). For RAPD PCR 5x10^6 spores, purified on a Percoll gradient, were disrupted in lysis buffer containing Proteinase K and the DNA was amplified with BAM primers.

RESULTS

Light microscopy revealed stages developing in vacuoles in host cells and hypertrophic cells containing masses of spores after 7 days. Previous amplifications of microsporidian DNA by RAPD PCR had indicated that BAM primers gave amplification products with clear differences between species (Canning et al. 1993). Of special note were bands of approximately 2.0 Kb and 2.4 Kb, which were amplified from the DNA of a murine isolate of *E. cuniculi* but not from 2 isolates of *E. hellem*. These products were amplified from the human urine isolate but there were differences in the smaller fragments. In particular there were bands of about 1.1 Kb, 1.0 Kb and 500 bp in the human urine isolate. Of these, the 1.0 Kb and 500 bp products were not amplified from the murine *E. cuniculi*. However, when the urine isolate was compared with a canine isolate of *E. cuniculi* the amplification products were virtually identical.

Presented at the International workshop "Microsporidiosis and Cryptosporidiosis in Immunodeficient Patients", September 28 – October 1, 1993, České Budějovice, Czech Republic.
In order to examine further whether the patient had become infected with *E. cuniculi* by contact with a dog, the human urine isolate was compared by SDS-PAGE with the canine *E. cuniculi* and with the Wainwright isolate of *E. hellem* which we had established in culture from nasal polyp tissue of an AIDS patient (Hollister et al. 1993). Significant antigenic differences were detected between all three isolates. Of particular note were proteins at about 55 kD and 70 kD in the urine isolate compared with a 60 kD protein of the canine *E. cuniculi*. In *E. hellem* there was a prominent band at 50 kD which was absent in the others. When profiles were blotted with human anti-serum to *E. cuniculi*, the binding profiles were similar for canine and murine isolates of *E. cuniculi* but the human urine isolate differed from these. There appeared to be strong binding to proteins in the region of 60 kD (*E. cuniculi*) and 55 kD and 70 kD (urine isolate) that may have corresponded to proteins of similar Mr separated by SDS-PAGE.

Preliminary ultrastructural studies have shown that the urine isolate develops in parasitophorous vacuoles with meronts adherent to the vacuolar membrane and sporogonic stages free in the lumen. The surface coat of the sporonts was laid down initially in strips. Early sporonts appeared to be multinucleate and of highly irregular shapes but, once the sporont coat was complete, disporoblastic division was observed. There was a well developed granular matrix in early vacuoles which dispersed as spores were formed. The endospore layer of the spore wall often appeared finely granular with an inner dense border adjacent to the plasma membrane. The number of coils of the polar tube varied from 3 to 4.5.

Infectivity to athymic mice was investigated after intraperitoneal inoculation of 10⁷ spores per mouse. Two weeks post infection, spores were detected with Cacofluor M2R (Vávra et al. 1993) in spleen, pancreas, liver, kidney, heart, abdominal muscle and intestinal wall. The most heavily infected organs were spleen and pancreas. No infections were detected at this stage in lung, brain or eyes.

**DISCUSSION**

A microsporidium isolated into culture from the urine of a patient with AIDS had ultrastructural characters in common with the genus *Encephalitozoon*, namely development in parasitophorous vacuoles with peripherally-located meronts, sporonts free in the vacuole lumen and unpaired nuclei. Although multinucleate stages were observed, which showed patchy deposition of sporont surface coat, it is possible that division occurred before complete maturation into sporonts, as disporoblastic divisions were observed in sporonts with uniform surface coats. Sporogony is typically disporoblastic in *Encephalitozoon*.

*Encephalitozoon cuniculi* Levaditi, Nicolau et Schoen, 1923 has been reported from a wide range of mammals and, as no morphological differences were observed between parasites from rodents, rabbits, carnivores and primates, it has been accepted that these all belong to one species. However, a second species, *Encephalitozoon hellem*, was established for parasites isolated from the cornea of AIDS patients on the basis of antigenic differences from *E. cuniculi* (Didier et al. 1991a). No ultrastructural differences were observed between the two species (Didier et al. 1991b) but sequence data for rRNA genes have confirmed that they are distinct (Vossbrinck et al. 1993). In the present study we have demonstrated unique antigenic and immunogenic profiles for isolates of *E. cuniculi* of murine and canine origin, the Wainwright isolate of *E. hellem* of human origin (Hollister et al. 1993) and the new isolate from the urine of an AIDS patient. However, the human isolate appeared closer to the *E. cuniculi* isolates than to *E. hellem* on the PCR amplification products and antigenic profiles.

These findings raise the question of speciation in the genus *Encephalitozoon*. Although antigenic differences might be interpreted as representative of differences between species, we believe that a greater range of isolates of *Encephalitozoon*, from different hosts, should be examined before conclusions are drawn. At this time we do not propose to establish a new species. *Encephalitozoon* spp. have also been reported from a lizard (Canning 1981), from lovebirds (Kemp and Kluge 1975, Novilla and Kwapien 1978, Lowenstein and Petrak 1980, Powell et al. 1989) and from a parrot (Poonacha et al. 1985). Unfortunately in vitro isolates of *Encephalitozoon* species from non-mammalian hosts and from two AIDS patients (Terada et al. 1987, Zender et al. 1989) have not been established for antigenic analysis, gene sequencing or cross infectivity studies.

Cross infectivity studies of *Encephalitozoon* spp. have been limited. *E. cuniculi* and another human isolate, *Noema corneum*, were both found to cause ascites and death in athymic C57Bl/6 mice, whereas no deaths were recorded in this strain of mouse during an 80-day observation period post-inoculation with *E. hellem* (Shaduck et al. 1991). Although we have infected athymic mice with the human urine isolate, the infections were light at two weeks and no mortalities have occurred. In this respect the new isolate is more like *E. hellem* than *E. cuniculi* but it was not determined whether the low infectivity to mice was due to long-term propagation in vitro after its isolation from the human host. Clearly interesting information could be gained by a series of infectivity studies using *Encephalitozoon* isolates in strains of mice with known genetic background.

**Acknowledgements.** We are grateful to Dr. Emma Aarons and colleagues at the Chelsea and Westminster Hospital, London for provision of clinical information and samples. The work was supported by the Wellcome Trust.
REFERENCES


Received 11 October 1993

Accepted 24 November 1993