Experimental microsporidiosis in immunocompetent and immunodeficient mice and monkeys

E. S. Didier¹, P. W. Varner², P. J. Didier³, A. M. Aldras⁴, N. J. Millichamp⁴, M. Murphey-Corb⁴, R. Bohm⁵ and J. A. Shadduck²

Departments of Microbiology¹, Pathology³, and Veterinary Sciences⁴, Tulane Regional Primate Research Center, 18703 Three Rivers Road, Covington, LA 70433, U.S.A.
Departments of Pathobiology² and Small Animal Medicine and Surgery⁴, College of Veterinary Medicine, Texas A&M University, College Station, TX 77483-4461, U.S.A.

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Abstract. Microsporidia cause opportunistic infections in AIDS patients and commonly infect laboratory animals, as well. Euthymic C57Bl/6 mice experimentally infected with intraperitoneal injections of 1x10⁶ Encephalitozoon cuniculi Levaditi, Nicolau et Schoen, 1923, Encephalitozoon hellem Didier et al., 1991, or Nosema corneum Shadduck et al., 1990 displayed no clinical signs of disease. Athymic mice, however, developed ascites and died 8-16 days after inoculation with N. corneum, 21-25 days after inoculation with E. cuniculi, and 34-37 days after inoculation with E. hellem. All athymic mice displayed hepatomegaly, dilated intestine and accumulation of ascites fluid. Granulomatous lesions were primarily located in the liver, lung, pancreas, spleen, and on serosal surfaces of abdominal organs.

The murine microsporidiosis model also was used to examine immune response that inhibit microsporidia growth in vitro. Recombinant murine interferon-γ (rIFN-γ; 100 u/ml) alone or in combination with lipopolysaccharide (LPS; 10 ng/ml) could activate thiglycollate-induced peritoneal murine macrophages to destroy E. cuniculi. The production of the nitrogen intermediate, NO₃, correlated with parasite destruction. Inhibition of NO₃ generation by addition of the L-arginine analogue, N⁶-monomethyl L-arginine (NMMA), inhibited microsporidia killing, as well.

Since microsporidiosis is becoming an important opportunistic infection in AIDS patients, a microsporidiosis model is being developed using SIV/Delta1070-infected rhesus macaque monkeys (Macaca mulatta). SIV-infected immunocompetent monkeys given E. cuniculi or E. hellem per os developed specific antibodies, and microsporidia could be detected sporadically by calcfluor or antibody fluorescence staining of stool and urine sediment smears. As immunodeficiency progressed, monkeys developed diarrhoea, cachexia, and anorexia, and organisms were detected in urine and stool with greater frequency. Immunodeficient SIV-infected monkeys died approximately 27 days after receiving E. hellem by intravenous inoculation, and approximately 110 days after receiving E. hellem per os. Lesions typical for SIV-infection were observed in both groups of monkeys and microsporidia were detected in kidney and liver of the intravenously-injected monkeys.

The murine microsporidiosis model provides an efficient means for studying protective immune responses to microsporidiosis, and may prove useful for screening immunological and chemotherapeutic agents. The pathogenesis of Encephalitozoon microsporidiosis in SIV-infected monkeys appears to parallel encephalitozoonosis in AIDS patients, suggesting that simian microsporidiosis may provide a useful model for evaluating diagnostic methods and therapeutic strategies during various stages of progressing immunodeficiency.

Opportunistic infections and neoplastic diseases are the major causes of morbidity and mortality in individuals with the acquired immunodeficiency syndrome (AIDS). Microsporidia are obligate intracellular protozoan parasites which commonly infect insects, fish, carnivores, and laboratory animals such as mice and rabbits (Canning and Lóm 1986). Microsporidia now are being reported as causing opportunistic infections in AIDS patients, as well (reviewed by Shadduck 1989, Bryan et al. 1990, Orenstein 1991, Canning and Hollister 1992). Clinical signs of microsporidiosis in AIDS patients include diarrhoea, cholangitis, hepatitis, nephritis, peritonitis, myositis, sinusitis and keratitis.

To better understand microsporidiosis in man, animal models are very useful. Animal models provide a basis for studying immune responses that may protect the host from lethal disease, and may be used to evaluate diagnostic methods, vaccine candidates and therapeutic strategies. The purpose of this paper is to describe studies with experimental murine and simian microsporidiosis. The host-parasite relationship in microsporidiosis can

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readily be studied in genetically identical athymic and euthymic mice while rhesus macaque monkeys with SIV infections provide a model to study pathogenesis and immune responses in the context of progressing immunodeficiency.

**MATERIALS AND METHODS**

**Animals.** Six-to-ten week old female BALB/c mice were purchased from Charles River (Wilmington, MA), and six-week old C57Bl/6 euthymic and athymic (nu/nu) mice were purchased from Life Sciences (St. Petersburg, FL). Groups of 5 mice per cage were provided food and water ad libitum. Athymic mice were housed in a micro-isolator environment. Rhesus macaque (Macaca mulatta) monkeys, which were born and bred at the Tulane Regional Primate Research Center (TRPRC) were individually caged and housed in an isolation facility at the TRPRC for use in these studies.

**Organisms.** *Encephalitozoon cuniculi* Levaditi, Nicolau et Schoen, 1923, *Novesma corneum* Shadduck et al., 1989, and *Encephalitozoon hellem* Didier et al., 1991 were grown in Madin Darby Canine Kidney (MDCK) cells and purified by centrifugation over 50% Percoll (Pharmacia) as described (Didier et al. 1991). The Simian Immunodeficiency Virus, SIV/Delta was isolated from co-cultures of lymph node cells from an infected rhesus monkey and phytohemagglutinin-stimulated human peripheral blood lymphocytes as described (Murphy-Corb et al. 1986, Baskin et al. 1988). Virus was quantitated by determining the tissue culture infectivity dose (TCID50).

**Inoculations and monitoring of infections.** Mice were given intraperitoneal injections of 0.5 ml volumes of 10^6 Percoll-purified microsporidia, in Tris-buffered saline (TBS; pH 7.2). Monkeys were inoculated by intravenous injection of 10^6 microsporidia in 1.0 ml volume, or were fed 10^6 microsporidia in 10.0 ml volumes by naso-gastric tube inoculation. Sera were obtained on days 0, 5, 10, 14, 21, 28, and monthly thereafter post-inoculation with microsporidia, and assayed by enzyme-linked immunosorbent assay (ELISA) for antibodies to microsporidia. Stool and urine specimens were examined for the presence of microsporidia by indirect immunofluorescent antibody test (IFAT; A. Dela et al. 1993) and the modified trichrome stain (Weber et al. 1992). In addition, a Uvete 2B stain described by Van Gool et al. (1993) was modified and used to detect microsporidia. Stool and urine sediment smears were allowed to dry, fixed with methanol for 10 min at room temperature, stained with 0.5% fluorescence brightener (Sigma; catalogue number F. 6259) in TBS for one minute at room temperature, rinsed with water, counterstained with Evan’s blue (0.02% in TBS) for one minute at room temperature, and rinsed with water. The slides were examined with a fluorescence microscope under oil (600x) at a wavelength of 396-415 nm. Microsporidia appear white-to-turquoise against the orange-staining background material. Although yeast also stain with calcifluor, microsporidia can be distinguished from yeast because they are concaveated at the anterior end, and display stronger fluorescence in the anterior region with a hollow appearance in the posterior region.

Some of the juvenile rhesus monkeys also were given SIV/Delta (10 TCID50) by intravenous injection in the saphenous veins at approximately 9-16 months of age. SIV p26 serum antigenemia levels were measured at regular intervals using an HIV p24 antigen capture ELISA kit (Coulter; Hialeah, FL). To monitor immunocompetence in the rhesus monkeys, peripheral blood CD4+/CD29+ T lymphocyte levels were measured at monthly intervals as described (Murphy-Corb et al. 1989). Monkeys were monitored daily for appetite, activity, and stool consistency. Physical examinations were performed at biweekly intervals and monkeys were anaesthetized with ketamine for obtaining blood specimens and physical examinations. Antibiotics were administered to monkeys with bacterial infections, and monkeys who became moribund were humanely sacrificed by barbiturate overdose.

**Enzyme-linked immunosorbent assay (ELISA).** To measure the relative levels of microsporidia-specific serum antibodies, a modified ELISA method described by Hollister and Canning (1987) was employed. Briefly, tissue culture-derived microsporidia were adjusted to 1x10^7/ml in bicarbonate buffer and 100µl was added to each well of 96-well ELISA plates (Corning, Marietta, GA). The plates were allowed to dry and were fixed with a 1:1 mixture of acetone and methanol for 10 minutes at room temperature. After blocking the unbound sites with bovine serum albumin (BSA; 3% w/v) in TBS for 30 min at room temperature, the plates were washed with TBS containing 0.3% Tween 20 (TBS-Tween) and incubated with serial dilutions of test sera for one hr at 37°C. The plates were then washed with TBS-Tween, and incubated with alkaline phosphatase-conjugated goat anti-human IgG or IgM (which also bind to rhesus Ig) for one hr at 37°C. The plates were washed again with TBS-Tween and incubated with 100 µl/well of p-nitrophenyl phosphate [1.0 mg/ml in 10 mM diethanolamine (pH 9.5) and 0.5 mM MgCl2]. Absorbance values were measured using an ELISA spectrophotometer (Dynatek, Chantilly, VA) at a wavelength of 405 nm.

**Macrophage infectivity assays.** Peritoneal exudate cells were recovered by peritoneal lavage three or four days after intraperitoneal injection with 2.0 ml of 3% dextrose/bovine serum. Peritoneal cells were collected in ice-cold RPMI 1640 containing antibiotics (penicillin, 100 U/ml; streptomycin, 100 µg/ml) and washed, followed by resuspending the cells in complete RPMI 1640 containing 10% fetal bovine serum, 2mM L-glutamine, and antibiotics. The cells were adjusted to approximately 1x10^6 macrophages/ml and 200µl was added per well in 8-well chamber slides. After incubation at 37°C for two hours, the non-adherent cells were washed off and fresh medium containing recombinant murine interferon-γ (mIFN-γ; Genzyme, Cambridge, MA) or lipopolysaccharide (LPS; Escherichia coli serotype 055:B5; Sigma, St. Louis, MO). The competitive inhibitor of L-arginine, Nω-monomethyl L-arginine (NMMA; Chem Biochem Research, Inc., Salt Lake City, UT) was added to some cultures, as well. Twenty-four hours later, 6x10^6 microsporidia were added to each well giving a ratio of parasites:macrophages of 3:1. After three hours incubation at 37°C, the non-internalized microsporidia were washed off and fresh medium containing the treatments, was added to each culture well. To determine the percent of infected macrophages and the number of microsporidia per 100 macrophages, the calceofluor stain was used as described above.

**Measurement of nitrite (NO) production.** Macrophage supernatants (100 µl each in four replicates) were mixed with 100 µl of Griess reagent (1 part 0.1% naphthylendiamine dihydrochloride to 1 part 1% sulfanilamide in 5% phosphoric acid) (Green et al. 1982). Absorbance at a wavelength of 550 nm was read on an ELISA spectrophotometer (Dynatek, Chantilly, VA) and values were quantitated from a standard curve using sodium nitrite.

**Statistics.** Two-tailed Student’s t-test was used to compare results from each treatment culture with results in the medium control cultures.
RESULTS

Previous studies demonstrated that C57Bl/6 (nu/nu) and BALB/c (nu/nu) athymic mice die from infection with *E. cuniculi* approximately three-to-five weeks post inoculation (Gannon 1980, Schmidt and Shadwick 1983). At that time, *E. cuniculi* was the only mammalian microsporidian that could be grown in tissue culture. In recent years, *N. corneum* (Shadwick et al. 1990) and *E. hellem* (Didier et al. 1991) were isolated from humans and cultured in vitro. Therefore, experiments were performed to compare pathogenesis of these three mammalian microsporidia in athymic and euthymic C57Bl/6 mice. Mice, in groups of five or six animals, were each inoculated with 1x10^6 organisms by intraperitoneal injection and monitored for clinical signs of disease and survival times. Four experimental trials were performed and necropsies were performed at regular intervals on one mouse per group, or at death due to microsporidiosis.

Athymic mice infected with *N. corneum* were first to develop clinical signs of lethargy and ascites at approximately seven-to-ten days and the mice died between 8 and 16 days post inoculation. The *E. cuniculi*-inoculated athymic mice developed the same clinical signs after 11-to-15 days and died between 21 and 25 days post inoculation. The athymic mice given *E. hellem* were slowest to develop ascites at approximately 28-to-30 days and died approximately 34-to-37 days post inoculation. None of the euthymic mice developed clinical signs of disease.

On necropsy, all athymic mice displayed hepatomegaly, dilated intestine, and occasional splenomegaly. Ascites volumes ranged from one-to-ten ml of straw-coloured fluid. The microscopic lesions varied according to the microsporidia species used to inoculate the mice. Lesions in the *N. corneum*-infected athymic mice were primarily seen in the liver parenchyma and serosal surfaces of abdominal organs. Lesions were composed of infected cells that were randomly located, and which often formed multinucleated syncytia. Granulomatous lesions also were noted in the liver, and were composed of polymorphonuclear and mononuclear cells, pyknotic nuclei, and necrotic debris. In some mice, the granulomatous lesions were detected in the lung, as well. Lesions in the *E. cuniculi* and *E. hellem*-inoculated athymic mice were similar to each other except that they developed more slowly in the *E. hellem*-inoculated mice. Focal lesions of infected macrophages and histiocytes, with fewer lymphocytes and neutrophils were observed predominantly in the parenchyma of the pancreas, as well as in the liver, lung and spleen. No clinical signs developed in euthymic mice inoculated with microsporidia but histological lesions included mononuclear inflammatory foci in liver and lung.

The murine model also was used to examine host responses against microsporidia. In previous studies, we showed that cytokines generated by incubating *E. cuniculi*-sensitized spleen cells with *E. cuniculi* organisms activated macrophages to kill *E. cuniculi* in vitro (Schmidt and Shadwick 1984). In studies presented here, thioglycollate-induced peritoneal macrophages from BALB/c euthymic mice were treated with various doses of LPS, mIFN-γ, or *E. cuniculi*-induced spleen cell cytokines for 24 hrs prior to addition of *E. cuniculi*. After three hrs incubation, parasites were washed off and one set of cultures was stained to determine the initial percent of macrophages infected and the number of parasites per 100 macrophages. Among all cultures, an average of 55% of the macrophages were infected with *E. cuniculi*, ranging from 49% in the medium control group to 64% in cultures treated with LPS (10 ng/ml) + mIFN-γ (100 u/ml). On average, 126 microsporidia were detected per 100 macrophages in all treatment groups with no statistically significant differences between treatment groups. The percent of infected macrophages and the number of organisms per 100 macrophages were similar for all treatments 24 hrs after adding *E. cuniculi* to the cultures (data not shown). The fluorescence intensity of the parasites in macrophages treated with LPS + mIFN-γ, cytokine supernatant, or mIFN-γ(100 u/ml) decreased, suggesting microsporidial degradation. The percent of infected macrophages and the number of microsporidia per 100 macrophages had decreased significantly in the cultures treated with LPS + mIFN-γ, mIFN-γ (100 u/ml), or cytokine treatment groups 48 hrs after addition of *E. cuniculi* (Fig. 1a,b). By 72 hrs after addition of *E. cuniculi*, replication of organisms within parasitophorous vacuoles of macrophages treated with medium progressed to where it became too difficult to count the organisms in many of the infected macrophages (not shown).

Concentration of the nitrogen intermediate, NO₃⁻, was measured in the supernatants of each macrophage culture to determine if nitric oxide intermediates play a role in destruction of microsporidia. Fig. 1c shows that cultures treated with IFN-γ, or LPS + IFN-γ, produced significantly higher levels of NO₃⁻ than macrophages treated with medium. These were the same cultures in which the number of microsporidia and the percent of infected macrophages decreased relative to the control cultures. Conversely, cultures treated with the L-arginine analogue, NMMA, at a dose of 100 uM or higher, produced NO₃⁻ at the same level as the medium controls. Competitive inhibition of NO₃⁻ generation using NMMA restored the percent of infected macrophages and the number of microsporidia per 100 macrophages to control levels.

Because microsporidial infections are of concern in AIDS patients, and because a simian AIDS model has been established at the Tulane Regional Primate Research
Center (M. Murphy-Corb et al. 1986), attempts were made to develop a simian microsporidiosis model to study pathogenesis in the context of progressing immunodeficiency. Juvenile rhesus macaque monkeys were infected with SIV/Delta870 (10 TCID50) and 10^9 E. cuniculi as described in Table 1. Healthy monkeys given E. cuniculi or E. hellem by naso-gastric tube inoculation (Group A) displayed no clinical signs of disease and only rarely shed microsporidia in the urine or stool. Specific IgM was detected between days 10 and 21 (not shown). The IgG ELISA titers in Fig. 2 are shown for two monkeys of Group A; J224 and J348 who were inoculated per os with E. cuniculi and E. hellem, respectively. In all the monkeys of Group A, IgG ELISA titers to microsporidia increased beginning approximately 42 after inoculation with microsporidia, and all monkeys continued expressing relatively high levels of specific IgG for at least one and one-half years post inoculation. All monkeys in Group A are still alive so necropsy and histology data are unavailable.

The monkeys in Group B received SIV/Delta870 6-18 months prior to oral inoculation with microsporidia, and were still immunocompetent at the time of microsporidia inoculation based upon the absence of recurrent serum SIV p26 antigenemia, normal peripheral blood CD4+/CD29+ T lymphocyte levels (15-30%), and the absence of clinical signs relating to immunodeficiency. IgG ELISA titers are shown for monkeys I117 and J326 who were inoculated per os with E. cuniculi or E. hellem, respectively (Fig. 2). All the monkeys in Group B initially produced microsporidia-specific IgG and IgM antibodies at a similar rate as the non-SIV-infected monkeys of Group A. As the monkeys began to show signs of progressing immunodeficiency (weight loss, splenomegaly, hepatomegaly, recurrent SIV p26 antigenemia and CD4+/CD29+ peripheral blood T cell levels falling to below 10%), the microsporidia-specific antibody levels fell, as well (Fig. 2). Microsporidia were rarely detected in stool and urine early after inoculation with microsporidia, but were observed more frequently and in greater numbers as immunodeficiency progressed, and as SIV p26 antigenemia occurred. On average, monkeys fed E. cuniculi died earlier after inoculation than monkeys fed E. hellem. At necropsy, lesions typical for SIV-infection were present (Baskin et al. 1988), and several monkeys had secondary infections due to Mycobacterium avium-intracellulare. Microsporidial organisms could not be identified in any of the lesions, however.

SIV-infected monkeys in Group C were immunodefi-

<table>
<thead>
<tr>
<th>Monkey Group:</th>
<th>Number of monkeys inoculated with:</th>
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<tr>
<td></td>
<td>E. cuniculi</td>
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<td></td>
<td>iv</td>
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<tr>
<td>A. Healthy (not infected with SIV)</td>
<td>n.d.</td>
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<tr>
<td>Mean survival time in days</td>
<td>&gt;550</td>
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<td>B. SIV-infected:</td>
<td>n.d.</td>
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<tr>
<td>Mean survival time in days (range of survival times)</td>
<td>182 (121-219)</td>
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<tr>
<td>C. SAIDS:</td>
<td>n.d.</td>
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<tr>
<td>Mean survival time in days (range of survival times)</td>
<td>26.7 (14-45)</td>
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* Juvenile rhesus macaque monkeys were inoculated with 1x10^9 microsporidia by iv injection or by naso-gastric tube inoculation (per os).
* Survival time after inoculation with microsporidia.
* n.d.; not done
* Monkeys are still alive.
* Juvenile rhesus macaques were inoculated by iv injection with SIV/Delta870 (10 TCID50) 6-18 months earlier. Monkeys were immunocompetent at the time of microsporidia inoculation as determined by absence of clinical signs for SAIDS, absence of detectable SIV p26 serum antigenemia, and normal levels of CD4+/CD29+ peripheral blood T lymphocytes.
* One monkey in this group was still alive 550 days after inoculation with microsporidia.
* Juvenile rhesus macaques, inoculated by iv injection with SIV/Delta870 (10 TCID50) 6-9 months earlier, were expressing signs of immunodeficiency at the time of microsporidia inoculations.
* One monkey was alive 225 days after inoculation with E. hellem.

SIV-infected monkeys in Group C were immunodeficient at the time of microsporidia inoculation (recurrent SIV p26 antigenemia, CD4+/CD29+ peripheral blood T cell levels that were below 10%, and weight loss), and received E. hellem by intravenous injection, or per os by naso-gastric tube inoculation. The monkeys died more rapidly after iv inoculation than per os inoculation, and neither group produced detectable specific IgM or IgG by ELISA. Organisms were readily detected in the stool and urine of monkeys within one week of infection. As described for Group B, microsporidial organisms were not identified in tissues of Group C monkeys inoculated per os. However, monkeys in Groups C who received E. hellem intravenously had focal to extensive lesions in the liver and kidney with few to numerous intrallesional

Fig. 1. Macrophage-mediated destruction of E. cuniculi in vitro. Thioglycolate-induced BALB/c peritoneal macrophages were incubated with different doses of LPS, mIFN-γ, or LPS (10 ng/ml) + mIFN-γ (100 μg/ml) with or without NMMA for 24 hrs prior to adding E. cuniculi at a 3:1 ratio of parasites to macrophages. The percent of infected macrophages (A), and the number of microsporidia per 100 macrophages (B) were determined by calcifluor staining, and the release of NO (C) was measured by Griess reaction. Data are shown for cultures incubated 48 hrs with microsporidia, and 72 hrs with treatments. Error bars represent standard deviations. Levels of statistically significant differences using Student's t-test are *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.
microsporidia. In the kidney, microsporidia were mixed with neutrophils and inflammatory cell debris within lumens of collecting ducts surrounded and infiltrated by mononuclear cells (Fig. 3a,b). Microsporidia also were found encysted in epithelial cells of collecting ducts and pelvic epithelium, and rarely in cortical stroma, near or in vascular endothelium. In the liver, microsporidia were mixed with necrotic debris and neutrophils in multifocal confluent necrotic zones and encysted in residual hepatocytes bordering necrotic foci (Fig. 3c,d).

**DISCUSSION**

Microsporidia are common parasites of laboratory animals and recently are being recognized more frequently in humans, particularly in AIDS patients. As such, information gleaned from natural and experimental infections of laboratory animals provide a basis for understanding the host-parasite relationship of microsporidiosis in humans. The usefulness of an animal model, depends upon similarities with the human disease. Such comparisons are difficult at present, however, because relatively little is known about the pathogenesis and clinical spectrum of disease in humans infected with microsporidia. To date, *Enterocytozoon bieneusi* has been found to infect humans only and no animal models currently exist. Conversely, *Encephalitozoon* and *Nosema* are commonly reported in laboratory animals and insects, and are only recently being reported with greater frequency in humans. Information from experimental microsporidial infections of mice and monkeys should increase our understanding of the pathogenesis and clinical course of microsporidiosis in humans.
Fig. 3. Lesions in SIV-infected immunodeficient rhesus monkeys from Group C inoculated intravenously with *E. hellem*. A. Scattered collecting ducts in the renal pelvis contain plugs of neutrophils and mononuclear cells. (Hematoxylin and eosin stain, 800x) B. Collecting ducts contain spores of *E. hellem* within the lumen and in the epithelium. (Brown and Brenn stain, 800x) C. Multifocal zones of acute necrosis and inflammation in the liver are sharply defined. Intrahepatic cysts containing microsporidia are present at the edge of necrotic zones (arrow). (Hematoxylin and eosin stain, 800x) D. Liver with microsporidia which are freely scattered within necrotic zones (small arrows) and clustered within intrahepatic cysts (large arrow). (Brown and Brenn stain, 1200x)
Microsporidiosis in mice and rhesus monkeys appears to parallel what we do know about human microsporidiosis. Immunocompetent euthymic mice and rhesus monkeys inoculated with E. cuniculi or E. hellem infections rarely, if ever express clinical signs of disease. The animals sporadically shed organism in the urine and stool, and expressed relatively high levels of specific antibody which have been useful for antemortem diagnosis of microsporidiosis in laboratory animals (Shad d u c k and Pak e s 1971, C a n n i n g and L o m 1986). Speculations have been raised as to whether humans also may carry subclinical microsporidial infections. Serological studies with homosexuals or persons who travelled to the tropics demonstrated the presence of antibodies specific for E. cuniculi (Bergquist et al. 1984a,b; Hollister and Canning 1987), and relatively high ELISA antibody titers for E. cuniculi, E. hellem, and N. corneum have been detected in HIV-seropositive and HIV-seronegative homosexuals and heterosexuals, as well (D i d i e r et al. 1993). Antisera raised against E. hellem in mice and rabbits do bind to E. cuniculi better than to N. corneum by western blot immunodetection and ELISA (D i d i e r et al. 1991a, 1993) indicating that these two species of microsporidia are immunologically related. In the earlier human serological studies, however, no experiments were performed to actually detect parasites in urine, stool or tissues. As sensitive methods for detecting microsporidia become available (eg. polymerase chain reaction), it may become possible to correlate the presence of microsporidia-specific antibody with true infection versus casual exposure to microsporidial antigens in the absence of infection. A recent retrospective study testing sera from an AIDS patient with E. hellem infection indicated that microsporidia-specific antibodies were present four years prior to clinical diagnosis of microsporidiosis strongly suggesting that humans may carry subclinical microsporidial infections (Hollister et al. 1993a). The SAIDS model may prove useful for determining if subclinically-infected individuals who are seropositive for microsporidiosis and then become infected with SIV, will subsequently develop clinically-significant microsporidiosis.

That microsporidia can cause clinical disease in immunodeficient hosts has been documented in experimentally- and naturally-infected mice and humans, respectively (C a n n i n g and L o m 1986). Difference in pathogenesis resulting from the various species of microsporidia infections occur, as well. N. corneum appears to be most pathogenic in athymic mice, and was isolated and cultured from an HIV-seronegative individual (Shadduck et al. 1990). Nosema sp. have only been reported in HIV-seronegative individuals but infect the corneal stroma which is immunologically privileged (Cal i 1991). E. hellem, on the other hand, appears to be the least pathogenic in the athymic mice, yet is more commonly reported in AIDS patients than are N. corneum or E. cuniculi (Didier et al. 1991a,Schwartz et al. 1993, Weber et al. 1993). E. cuniculi, the mammalian microsporidia prototype, displayed intermediate levels of pathogenicity in the athymic mice, and has been isolated and cultured from rabbits, dogs, mice and hamsters (Canning and Lom 1986), although one human isolate close to E. cuniculi has recently been reported (Hollister et al. 1993b). Although E. cuniculi and E. hellem are morphologically identical (Didier et al. 1991b), they can be distinguished on the basis of SDS-PAGE and western blot immunodetection profiles (Didier et al. 1991a,b). Until recently, however, E. cuniculi was the only mammalian microsporidian which was available for use as antigen in serological studies (Bergquist et al. 1984a;b; Hollister and Canning 1987). As such, it is difficult to determine if earlier cases of encephalitozoonosis in humans were due to E. cuniculi or E. hellem.

The differences in species pathogenicity in athymic mice may reflect host specificities since E. hellem and N. corneum were isolated from humans and E. cuniculi used in this study was isolated from a rabbit. The pathogenicity differences also may reflect replication times for each organism. N. corneum and E. cuniculi replicate more rapidly in tissue culture than do E. hellem (unpublished observation). In previous studies where athymic mice were given various doses of E. cuniculi, the mice given higher doses died earlier than did mice given lower doses, suggesting that when a threshold number of organisms is reached, the athymic mice succumb (Schmidt and Shadduck 1983).

The lesions that developed in athymic mice and immunodeficient monkeys with SAlDS, were similar to lesions that were reported in AIDS patients with Encephalitozoon infections. Liver and kidney are two preferential sites of infection by microsporidia in mice and monkeys. Lesions also were observed in the pancreas, lung and spleen of athymic mice, and ascites was common, as well. These tissue sites of infection parallel several reports of microsporidiosis due to Encephalitozoon - associated hepatitis, peritonitis, sinusitis, and nephritis in AIDS patients (Tera d a et al. 1987, Zender et al. 1989, Schwartz et al. 1992, Metcalfe et al. 1992). E. cuniculi and E. hellem were consistently detected in the pancreas of athymic mice, yet have not been detected in the pancreas of humans. This may represent a weakness or inconsistency of the murine model, or may indicate that too few autopsy studies have been performed in AIDS patients with encephalitozoonosis to ascertain if the pancreas may serve as a site of parasite infection. Additionally, the observation that the liver was infected may explain why microsporidia were detected in the stool. Encephalitozoon organisms
have not been identified in stool specimens from humans although the Encephalitozoon-like microsporidian, *Septata intestinalis* was detected in stool and urine (Orenstein et al. 1992, Cali et al. 1993). Encephalitozoon-associated hepatitis was described in an AIDS patient which could have resulted in parasite shedding in stool. Although this patient’s stained stool smears were classified as negative for ova, cysts and parasites, it is possible that microsporidia may have been present in the stool because methods for specifically identifying microsporidia were not then available (Tera d a et al. 1987).

Whereas *E. hellem* was first identified in corneal/conjunctival tissue (Friedberg et al. 1989, Yee et al. 1990, Didier et al. 1991), *Encephalitozoon* organisms now are being detected in the urine, nasal sinuses, and respiratory secretions of AIDS patients with keratitis indicating that these organisms cause more generalized infections in humans which were also seen in laboratory animals (Visvesvara et al. 1991; Schwartz et al. 1992, 1993; Weber et al. 1993; Hollister et al. 1993b). Numerous inoculation routes have been used for establishing systemic microsporidial infections in laboratory animals, including intravenous, intranasal, oral, intraperitoneal, intrarecal, and intracerebral inoculations, although the lengths of time required for inducing systemic infections and the pathogenicity for these routes varied (Canning and Lom 1986). Because autopsies are rarely permitted in AIDS patients, however, it is difficult to fully appreciate the clinical spectrum of *Encephalitozoon*-associated microsporidiosis and to ascertain which routes of infection for microsporidiosis primarily occur in AIDS patients.

Prior to the AIDS epidemic, microsporidia were rarely described in humans, and often occurred in individuals with immunodeficiency or resulted from infections in immunoprivileged sites (Canning and Lom 1986; Canning and Hollister 1991, 1992). Studies using euthymic and athymic mice were particularly important for demonstrating that resistance to lethal disease is dependent on a functioning immune system which is consistent with the increased reports of disease associated with microsporidiosis in AIDS patients who are immunodeficient. Athymic mice could be protected from lethal *E. cuniculi* infection by adoptive transfer with sensitized T-enriched spleen cells but not with naive spleen cells (Schmidt and Shad d uck 1983). In vitro studies showed that sensitized splenic lymphocytes release cytokines after incubation with *E. cuniculi*, which in turn, could activate macrophages to destroy microsporidia (Schmidt and Shad d uck 1984). With the availability of recombinant cytokines, experiments were performed using INF-γ + LPS or INF-γ alone, to activate murine macrophages to destroy microsporidia. A correlation was found between the production of the nitrogen intermediate, NO₂, and reduction in infected macrophages. Conversely, addition of the L-arginine analogue, NMMA, correlated with increased numbers of infected macrophages. Addition of L-arginine to macrophages treated with IFN-γ + LPS + NMMA reversed the effects of NMMA resulting in microsporidia destruction (unpublished). Incubating murine peritoneal macrophages with murine tumour necrosis factor-α, however, failed to induce microsporidia destruction and NO₂ levels in the supernatants were low, as well (unpublished). As immune responses that mediate microsporidia destruction are delineated, it may be possible to apply immunological strategies toward controlling microsporidiosis in AIDS patients.

The murine and simian microsporidiosis models offer different advantages for studying microsporidiosis. The murine model is particularly suited for defining immune responses which protect against lethal disease. Genetically identical euthymic and athymic strains of mice are available for adoptive transfer models which can be used to define relevant microsporidial antigens that induce synthesis and release of T cell cytokines. These antigens could subsequently be utilized as vaccine candidates for high risk individuals. In addition, the adoptive transfer model could be used for defining and evaluating chemotherapeutic and immunologic agents that prolong survival in infected athymic mice.

The simian microsporidiosis model offers several advantages, as well. The SAIDS model is probably the best-characterized model for AIDS to date, and we have been able to establish *Encephalitozoon* infections in monkeys by iv inoculation. Additional studies are required to determine if monkeys who express high antibody levels to microsporidia after oral inoculation are truly infected by utilizing transmission electron microscopy (TEM) on stool or urine specimens. It is difficult to find organisms by TEM when the microsporidia are sporadically shed in relatively low numbers, and polymerase chain reaction methods may prove to be more feasible. The simian model primarily lends itself to evaluating diagnostic and chemotherapeutic methods in the context of progressing immunodeficiency. Presently, antibodies are considered diagnostic for microsporidiosis in immunocompetent animals and parasite detection is required for diagnosing microsporidiosis in immunodeficient individuals. The period of time between microsporidial infection and progressing immunodeficiency is still a mystery regarding microsporidiosis. For example, we do not know if chronic or latent microsporidial infections can develop into clinically significant disease as immunodeficiency progresses, or whether a person must be immunodeficient before becoming susceptible to microsporidial infection and disease. Additional studies are needed for comparing intravenous and oral inoculations in SIV-infected immunocompetent monkeys with different species of
microsporidia. Although it is believed that most natural infections in humans occur by ingestion of organisms, inhalation has been reported as a likely route of infection (Schwartz et al. 1992) and trauma may lead to infection. Furthermore, a model for enteric microsporidiosis utilizing Enterocytozoon bieneusi (Desportes et al. 1985) has not yet been developed. Continued studies eventually may help determine if progressing immunodeficiency leads to re-expression of microsporidiosis, which could have implications regarding which diagnostic methods are used at various stages of HIV infection, and when therapeutic strategies would be applied.

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REFERENCES


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