The severe combined immunodeficient mouse as a model for
Encephalitozoon cuniculi microsporidiosis

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Abstract. Microsporidia have been recently recognized as opportunistic pathogens in AIDS patients. In attempt to develop an animal model with features similar to the infections observed in the immunodeficient patients, the adult severe combined immunodeficient mice (SCID) were administered both intraperitoneally and perorally by 2x10^7 spores of the murine isolate of E. cuniculi. The experimental inoculation caused a severe, fatal disease characterized by the dissemination of microsporidia into the host tissues. The dominant route of E. cuniculi dissemination in the SCID mice was continual direct extension from the site of inoculation to adjacent tissues and organs, terminating in hematogenous spread of infection in the host. The different courses of microsporidiosis in SCID mice relative to the mode of inoculation (i. p. vs. p. o.) was observed. The survival time of i. p. infected SCID mice was 3 weeks - vs. 5 weeks in p. o. infected SCID mice. Experimental microsporidiosis in SCID mice should provide a useful model for studies in microsporidial pathogenesis, mechanisms of resistance, immunotherapy, and in evaluating potential antmicrosporidial agents.

Microsporidia are obligate intracellular protozoal parasites that infect a variety of cell types in a broad range of invertebrates and vertebrates. Recently, they are rapidly emerging as important opportunistic pathogens in patients with the acquired immunodeficiency syndrome (AIDS) (Shaduck 1989, Shaduck and Greely 1989, Bryan et al. 1990, Orenstein 1991, Canning and Hollister 1992). Five genera of microsporidia, Encephalitozoon, Pleistophora, Nosema, Enterocytozoon, and Septata, have been reported to infect humans. The first three genera were originally reported from animals and later found in humans (Canning and Lom 1986, Cali 1991). Enterocytozoon and Septata were originally described from human infections (Delportes et al. 1985, Cali et al. 1993).

Encephalitozoon cuniculi Levaditi, Nicolau et Schoen, 1923 is a microsporidian parasite that has a wide range of hosts including rodents, lagomorphs, carnivores and primates (Canning and Lom 1986). Experimental E. cuniculi infections in immunocompetent hosts produced only chronic asymptomatic brain and kidney lesions (Shaduck et al. 1979). In contrast, the inoculation of immunodeficient animals, such as athymic mice, resulted in lethal disease (Gannon 1980, Schmidt and Shaduck 1983).

Severe combined immunodeficient mice (SCID) are deficient in T- and B-lymphocyte functions by virtue of an autosomal recessive mutation (Bosma et al. 1983). The SCID mouse model has proved useful in studies of pathogenesis of other protozoan opportunistic infections associated with AIDS: Cryptosporidium parvum (Mead et al. 1991, Kuhl et al. 1992) and Toxoplasma gondii (Johnson 1992).

The purpose of the study reported here was to examine whether the SCID mouse is a potentially useful animal model for the study of E. cuniculi microsporidiosis.

MATERIALS AND METHODS

Mice. SCID mice breeding pairs were originally obtained from Dr. G. C. Bosma (Fox Chase Cancer Center, Philadelphia, USA). SCID mice were housed in flexible film isolators (BEM, Znojmo, Czech Republic) with high-efficiency particulate air (HEPA) filters. All cages, food, water, and bedding were sterilized before use.

Organisms. A murine isolate of Encephalitozoon cuniculi Levaditi, Nicolau et Schoen, 1923 (Vavra et al. 1972) was grown in E6 cells (Vero green monkey kidney cells) for provision of spores (Fig. 1). The cells were cultivated in modified RPMI 1640 medium supplemented with 5% fetal calf serum. The organisms used for inoculation were freshly collected from the culture supernatants after centrifugation at 400 g for 15 min, washed by centrifugation in sterile phosphate-buffered saline (PBS), counted on a hemocytometer, and adjusted to the desired concentration.

Inoculation. A total of 40 eleven-week-old SCID mice of both sexes were used in two experimental trials throughout this study. Eighteen mice were perorally (p. o.) inoculated with 2x10^7 spores of E. cuniculi in a single 0.5 ml volume. Sixteen mice were given with 2x10^7 spores in a single 0.1 ml volume by intraperitoneal injections (i. p.). Six SCID mice were inoculated with sterile PBS (three i. p. and three p. o.) and served as controls. All mice were monitored for clinical signs of disease and death due to microsporidiosis. On different days post inoculation (DPI) two mice from both infected and control groups were selected randomly and were euthanized with ether and necropsied.

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Isolation and in vitro cultivation of E. cuniculi from SCID mice. At necropsy of infected SCID mice, peritoneal cavities were washed with RPMI 1640 medium. The peritoneal cells were adjusted to approximately 10⁶ macrophages per ml and 200 μl per well were plated in multiwell tissue-culture plates. After incubation for 30–60 min at 37°C, the nonadherent cells were removed and an equal volume of the suspension of E6 cells in RPMI 1640 medium was added.

In addition, smears from peritoneal cells were prepared and stained with Giemsa stain to determine the percentage of infected macrophages.

Serum examinations. At necropsy of all SCID mice, blood was collected from the heart, and serum was stored frozen at -20°C until used. The phenotypic purity of the SCID mice was verified by the absence of serum IgG on examination using an simple direct ELISA method with peroxidase-conjugated rabbit anti-mouse Ig.

Histopathology. At necropsy, tissue samples of the stomach, duodenum, middle jejunum, ileum, cecum, colon, rectum, mesenteric lymph nodes, heart, lung, liver, kidney, urine bladder, brain, dermal connective tissues from the cheeks, tongue, nose, ears, scrotum from males and vagina from females, and left biceps femoris muscle were fixed in 10% buffered formalin. Fixed tissues were processed for light microscopy using standard methods. Paraffin sections were stained with hematoxylin and eosin (H&E), Giemsa, van Gieson, Masson trichrome, and Gram stains. In addition, tissue smears from liver, spleen, and kidney were prepared and stained with Giemsa stain. Feces and urine samples from necropsied mice were examined for presence of microsporidia by Giemsa and by the modified tri-chrome stain after Weber et al. (1992).

Transmission and scanning electron microscopy. The tissue samples from liver, spleen, and kidney were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 12 hours. Specimens were then washed in cacodylate buffer, postfixed in 1% OsO₄, dehydrated in graded ethanol and embedded in Durcupan. Ultrathin sections were poststained with uranyl acetate and lead citrate and examined at 80 kV with a Philips EM 420 electron microscope.

For scanning electron microscopy (SEM), the peritoneal macrophages from infected SCID mice were plated on polylysine-treated glass cover slips in Petri dishes and maintained at 37°C. After 15–30 min, the macrophages were fixed for 60 min at room temperature in 2.5% glutaraldehyde in 0.1M cacodylate buffer. After fixation, cover slips were rinsed in cacodylate buffer, dehydrated in graded ethanol, critical-point-dried, coated with gold-palladium, and examined with a Jeol JMS 6300 scanning electron microscope.

RESULTS

The SCID mice inoculated i. p. with E. cuniculi were first to develop clinical signs of wasting and lethargy at twelve-to-fourteen days and the first mouse died 17 DPI. The mean survival time of SCID mice inoculated i. p. with E. cuniculi was 20.2 DPI (17–28). The SCID mice inoculated p. o. with E. cuniculi developed the same clinical signs after 23–26 DPI and the mean survival time was 34.4 DPI (30–41).

At necropsy, all SCID mice with clinical signs displayed hepatomegaly, splenomegaly and dilated large intestine. Numerous yellowish nodular lesions and accumulation of ascites fluid in the abdominal cavity were observed.

The first signs of microsporidia replication and coincident histopathological lesions in p. o. inoculated SCID mice were observed 21 DPI. The microsporidia were present in large numbers in focal mucosal necroses, in ulcerative inflammatory lesions in the large intestine (Figs. 3, 4), and were also present in the intestinal content. The focal ulcerations often extended into the submucosa and microsporidia infiltrated adjacent muscularis propria and subserosa. The liver, peritoneum and connective tissue were most often involved in the pathological process. In the parenchymatous organs, lesions were predominantly characterized with scattered miliary necroses and granulomas. These densely packed miliary necroses and granulomas (Figs. 6, 10) were usually in the tissue of the parenchymatous organs adjacent to the infected peritoneum. Numerous microsporidia were identified within those necrotic areas and granulomas (Fig. 10). Diffuse or focal lesions in the peritoneum and connective tissues consisted of inflammatory edema with numerous macrophages containing microsporidia. No fibrotization in pathological lesions was observed in van Gieson and Masson trichrome stains.

On 15 DPI, histological sections from i. p. inoculated SCID mice revealed numerous microsporidia in the visceral and parietal peritoneum as well as in the liver and spleen (Figs. 5, 7, 9). The microsporidial infection directly extended from peritoneum into adjacent liver tissue (Figs. 5, 9) and into muscularis propria of the digestive system (Fig. 2). Predisposition for connective tissue was also observed. Microsporidia were found in the macrophages in edematously inflamed areas scattered predominantly in the subepithelial layers of the skin and throughout the muscles of the body (Fig. 8). Pancreas, brain, lungs and pleura were less frequently involved in infection. Also, renal involvement was relatively low. In some cases, large numbers of spores in the content of the urinary bladder were found.

The severity of infection appeared to be related to the route of inoculation. SCID mice inoculated intraperitoneally developed a disease of greater severity than mice inoculated perorally. The principal routes by which microsporidial infection spread in the organism were direct extension and hematogenous spread of infection. In p. o. infected mice the spread of infection was mainly via the portal vein.

spores are visualized. 21 DPI. Gram stain. Scale bar 100 μm. Fig. 5. Section demonstrating the continual direct extension (arrow) of E. cuniculi in the liver of SCID after i. p. inoculation. 15 DPI. H&E. Scale bar 100 μm. Fig. 6. Microsporidial granuloma in the liver (arrow). Peroral inoculation. 23 DPI. H&E. Scale bar 100 μm.
Fig. 1. Numerous *E. cuniculi* spores in Vero green monkeys kidney (E6) cell culture. SEM. Scale bar 10 μm. Fig. 2. Stomach of intraperitoneally infected SCID mice with accumulation of macrophages and clusters of spores (arrows) in the visceral peritoneum. 21 DPI. H&E. Scale bar 100 μm. Fig. 3. Ulcerative inflammatory lesion in the large intestine after peroral inoculation of *E. cuniculi*. 21 DPI. H&E. Scale bar 100 μm. Fig. 4. Extent of *E. cuniculi* load in the ulcer of the large intestine after peroral inoculation of *E. cuniculi*. Only micro
Fig. 7. Semithin section of the spleen with spores of *E. cuniculi* present within parasitophorous vacuole (arrows). Peroral inoculation. 15 DPI. Toluidine blue. Scale bar 10 μm. Fig. 8. Abdominal surface of the diaphragm showing attached macrophages (M) with *E. cuniculi* spores and microsporidia in connective tissues of the diaphragm. Intraperitoneal inoculation. 23 DPI. Semithin section. Toluidine blue. Scale bar 10 μm. Fig. 9. Numerous spores of *E. cuniculi* situated on the visceral peritoneum (arrow) and in the liver granuloma.
Among all smears prepared from peritoneal cells from SCID mice with clinical microsporidiosis, an average of 60% of the macrophages were infected with *E. cuniculi*. Many of those were destroyed and free spores with a fully extruded polar tube were observed. Extruded polar tubes measured 12–16 μm in length.

Spores of *E. cuniculi* were sporadically detected in feces specimens from necropsied mice with clinical microsporidiosis. Great numbers of microsporidia also were found in urine specimens stained by Giemsa and by the modified trichrome stain after Weber et al. (1992).

The development of *E. cuniculi* in SCID mice hepatocytes, splenocytes and macrophages has been studied by transmission and scanning electron microscopy. The earliest developmental stage seen, the sporoplasm (not figured), had a distinct round nucleus and delicate outer membrane in contact with host-cell cytoplasm. Further development occurred within a parasitophorous vacuole (PV) that was located near the nucleus of the host cell and was surrounded by numerous mitochondria (Fig. 13). Meronts of *E. cuniculi* were found attached to the membrane of the PV and they had spherical nuclei and scattered cisternae of endoplasmatic reticulum. The meronts divided by binary fission and resultant daughter cells were often seen in pairs (Fig. 14). After detaching from the membrane of the PV, the daughter cells transformed into sporonts with thickened and wrinkled outer walls (Figs. 14, 16). Sporoblasts resulted from division of sporonts (Fig. 15) and had a thick outer wall, nucleus, ribosomes, cisternae of rough ER, rudimentary polar tube, and membranous Golgi apparatus-like motor; the latter appeared to contribute to the formation of the polar tube (Fig. 18). Different stages of the formation of the polar tube were seen in developing spores. Multiple cross sections of the polar tube were observed with a range of five to six coils per spore (Fig. 18). Spores with an extruded polar tube were seen seldom within the PV in hepatocytes or macrophages. Those spores that already ejected their contents were seen as empty, lacking the polar tube and sporoplasm. The center of these spores was electron lucent, and there was amorphous, moderately electron-dense material marginated along the endospore wall (Fig. 17).

Most of peritoneal macrophages from infected SCID mice were enlarged and contained different developmental stages of *E. cuniculi*. SEM of these peritoneal macrophages illustrated smooth, ellipsoidal mature spores, wrinkled sporonts and a smooth-walled sporoblast (Figs. 11, 12). Many of spores were discharged with a fully extruded polar tube.

DISCUSSION

*Encephalitozoon cuniculi* is a parasite with large host range among mammals (Canning and Lom 1986). Human infections with *E. cuniculi* have been reported to cause hepatitis (Terada et al. 1987) and peritonitis (Zender et al. 1989) in individual AIDS cases, but no human in vitro isolates of *E. cuniculi* have been established till now. Recently, *in vitro* culture of human *E. cuniculi* from an AIDS patient was isolated and species determination was proved by molecular methods (Pieczynski, personal communication). In addition, a growing number of AIDS patients have been identified with infection due to *Encephalitozoon hellem*, observed in conjunctival, corneal and sinonasal tissues, urinary and respiratory tracts (Didier et al. 1991, Schwartz et al. 1992). Recently, Orenstein et al. (1992) described *Encephalitozoon*-like intestinal microsporidian, which cause chronic diarrhea in AIDS patients and severe tubulointerstitial nephritis.

The aim of our study was to develop an animal model with features similar to the infections observed in immunodeficient human patients. The disseminated lesions and tissue sites of infection that developed in SCID mice were similar to lesions that were reported in AIDS patients with *Encephalitozoon* infections.

Gannon (1980) and Schmidt and Shad duck (1983, 1984) used athymic mice as a model to study the host-parasite interactions during *E. cuniculi* infection. Their results in athymic mice were similar to results obtained in our study in SCID mice. The liver and spleen were two preferential sites of infection by *E. cuniculi* in athymic mice (Gannon 1980) and mean survival time of athymic mice inoculated i.p. 10^7 spores of *E. cuniculi* was also three weeks (Schmidt and Shad duck 1983).

The dominant route of *E. cuniculi* dissemination in the SCID mice was continual direct extension from the site of infection to adjacent tissues and organs, terminating in hematogenous spread in the host. In perorally inoculated SCID mice the large intestine lesions preceded the dissemination to other tissues. We suppose that the character of *E. cuniculi* spreading in the host is dependent on the mode of infection. Numerous routes of inoculation have been used in experimental infections with *E. cuniculi* in laboratory animals and the lengths of time required for disseminated infections for these routes also varied (Canning and Lom 1986). Schmidt and Shad duck (1983) transferred T-enriched spleen cells from previously infected BALB/c mice to athymic BALB/c mice to determine when the ability

Intraperitoneal inoculation, 15 DPI. Semithin section. Toluidine blue. Scale bar 10 μm. Fig. 10. Semithin section of the liver showing microsporidia PV (arrow) in a granuloma after peroral inoculation. Toluidine blue. 23 DPI. Scale bar 10 μm. Fig. 11. Peritoneal macrophage with *E. cuniculi* spores. SEM. Fig. 12. Residuum of the destroyed peritoneal macrophage illustrating an elongate and segmented sporont (arrow) and mature spores (s). SEM.
Fig. 13. Meront of *E. cuniculi* in the parasitophorous vacuole (PV), n - nucleus, m - host cell mitochondria. TEM. Scale bar 1 μm.

Fig. 14. Mononucleate and binucleate meronts (arrow) attached to the membrane of the PV and detached sporonts (Sp). TEM. Scale bar 2 μm.

Fig. 15. Sporont dividing into 2 uninucleate sporoblasts and sporoblast with anchoring disc (arrow). TEM. Scale bar 0.5 μm.

Fig. 16. Developed meronts with large nuclei (n), detached sporont (Sp) and sporoblast (Sb) of *E. cuniculi*. TEM. Scale bar 1 μm.

Fig. 17. Empty *E. cuniculi* spore (E) after extrusion of the polar tube. TEM. Scale bar 0.5 μm.

Fig. 18. Various stages of development of *E. cuniculi*, including sporont (Sp), sporoblasts (Sb) with transected polar tubes and mature spore (S). TEM. Scale bar 1 μm.
to transfer resistance to infected athymic mice was acquired. The SCID mouse is available for an adoptive transfer model as an alternative to the conventional athymic mouse. The reconstitution of the SCID mice with congenic mouse cells eliminate the problem of graft versus host reactions common in other murine models, including athymic mice (McCune 1991). The SCID mice could be used as a novel experimental system to specify agents for immunotherapy of microsporidiosis.

There have been a number of TEM studies of E. cuniculi but until this study, no material showing spores with an extruded polar tube in the PV localized in degenerate host cytoplasm was published. Empty spores which had already ejected their contents were observed in hepatocytes of SCID mice. This was documented only in degenerating epithelial cells with Encephalitozoon-like microsporidia from urine in an AIDS patient (Orenstein et al. 1992) and in disseminated infection with E. hellem in an AIDS patient (Schwartz et al. 1992). The ultrastructural features of E. cuniculi in hepatocytes, splenocytes or in macrophages of SCID mice was similar to E. cuniculi studied in tissue culture (Pakes et al. 1975, Hamilton et al. 1977) or in mouse macrophages (Sprague and Vernick 1971, Barker 1975).

Microsporidiosis in SCID mice provides a useful model for studies of the microsporidial pathogenesis, mechanisms of resistance, immunotherapy, and in evaluating potential antimicrosporidial agents.

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