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The severe combined immunodeficient mouse as a definitive host for *Sarcocystis muris*

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Abstract Peroral and intraperitoneal inoculation of severe combined immunodeficient (SCID) mice with cystozoites of three coccidia of the genus *Sarcocystis* (Protozoa, Apicomplexa; *S. dispersa*, *Sarcocystis* sp., and *S. muris*) revealed that after peroral administration, only *S. muris* could develop in the immunodeficient mouse host. The cystozoites of *S. muris* transformed into gamonts and, after fertilization, performed sporulation with the production of infectious sporocysts in the small intestine of the SCID mice. Impaired immunity is probably responsible for the unusual behavior of *S. muris* (which is normally the heteroxenous mouse-cat parasite) in the SCID mice. We hypothesize that the phylogenetic distance between the intermediate and final hosts is the reason why cystozoites of the two other *Sarcocystis* species tested (*S. dispersa* with a mouse-owl cycle and *Sarcocystis* sp. with a murine rodent-snake cycle) could not develop when inoculated into SCID mice.

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Introduction

Infections with representatives of the coccidian genus *Sarcocystis* (Protozoa, Apicomplexa) are common in domestic and wild animals. *Sarcocystis* spp. have an obligatory prey-predator, dixenous life cycle. Asexual stages in the form of tissue cysts ("sarcocysts") containing cystozoites develop only in muscles of the intermediate host, which is often a prey animal. The sexual cycle, represented by gamonts, the formation of gametes, their copulation, and the formation of oocysts, occurs only in the intestine of a carnivorous definitive host (a predator). The intermediate host becomes infected by ingesting sporocysts released from oocysts and excreted by the definitive host. Sporozoites excyst from the sporocysts in the small intestine of the intermediate host, and meronts (schizonts) develop in hepatocytes or endothelial cells of arteries. Merozoites issued from the terminal generation of meronts penetrate host muscle cells and initiate sarcocyst formation. The definitive host becomes infected by ingesting tissues containing mature sarcocysts with cystozoites (Dubey et al. 1989).

There are specific intermediate and definitive hosts for each species of *Sarcocystis* (Dubey et al. 1989). However, in some reptilian *Sarcocystis* spp. the life cycle is adapted to the cannibalistic mode of transmission as both the sexual and the asexual development phases take place in different individuals of the same host species (Matuschka and Bannert 1987, 1989; Bannert 1992). Matuschka and Bannert (1989) proposed that these *Sarcocystis* species be called dihomoxenous.

In addition to these reports, Arnastuaskiene and Grikieniene (1989) and Grikieniene and Kutkiene (1998) have provided evidence that the cannibalistic mode of transmission among laboratory rats is also present in *S. rodentifelis*, a rat-cat parasite. However, these results have recently been critically disputed (Odening 1998).

It is known that both cellular and humoral immune responses occur in intermediate hosts infected by *Sarcocystis* (Dubey et al. 1989). In this study, severe

combined immunodeficient (SCID) mice were used to test whether the impaired immunity of the intermediate host could alter the course of development of the parasite in such a definitive host.

Materials and methods

Parasites

Three *Sarcocystis* species (*S. dispersa*, *Sarcocystis* sp., and *S. muris*), all having mice (*Mus musculus*) as intermediate hosts but differing in their final hosts, were used. Sporocysts of *S. dispersa*, involving owls as definitive hosts (Munday 1977; Černá et al. 1978), were obtained from intestinal contents of a dead barn owl, *Tyto alba* (Strigiformes), found in April 1994 in Mikulov, Southern Moravia, Czech Republic. *Sarcocystis* sp. is a newly described species with a murine rodent-snake life cycle. Its sporocysts were isolated from feces of a naturally infected Nitsche's bush viper, *Atheris nitschei* (Serpentes: Viperidae), captured in August 1996 in the Ruwenzori Mountains, Uganda (Šlapeta et al. 1999). Sporocysts of *S. muris*, which has the cat as its final host, were provided by Dr. Rolf Entzeroth, Institute for Zoology, Technical University of Dresden, Germany. The sporocysts of all these species were purified by flotation in Sheather's sugar solution. Skeletal muscles of outbred ICR mice, inoculated with 10^3 sporocysts of all 3 tested *Sarcocystis* species 2 months earlier, were used as the source of cystozoites. The cystozoites were purified from skeletal muscles by tryptic digestion (Tenter et al. 1991), repeatedly washed in sterile phosphate-buffered saline (PBS, pH 7.2), and stored overnight in sterile PBS containing 2,000 units of penicillin/ml and 100 μg of streptomycin/ml.

Animals

SCID mice were housed in groups of two to four animals per cage in flexible isolators (BEM, Znojmo, Czech Republic) equipped with high-efficiency particulate air (HEPA) filters. All cages, food, water, and bedding were sterilized before use. A total of 49 SCID mice of both sexes aged 8–11 weeks were used in experimental trials throughout this study. Immunocompetent mice used were inbred BALB/c and outbred ICR animals obtained from a commercial supplier (AnLab, Czech Republic). Mice (females weighing 20–25 g) were housed in groups in plastic box cages and were provided rodent chow and water ad libitum. A total of 18 BALB/c mice and 6 ICR mice were used in experimental trials in this study.

Examination of mice for *Sarcocystis* infection

Mice were euthanized and necropsied. For the presence of sarcocysts, squashed smears of the diaphragm and gluteal skeletal muscles were examined and photographed using Nomarski interference contrast (NIC) microscopy. Mice were considered infected when sarcocysts containing cystozoites were found in their skeletal muscles.

Histological examination

A total of 28 tissue samples (Table 1) from each mouse were excised and fixed in 10% neutral buffered formalin. Tissue samples were embedded in paraffin, sectioned and stained with hematoxylin/eosin (HE), and examined using light microscopy.

Design of experiments

Experiment 1: are Sarcocystis spp. cystozoites infective for the intermediate host?

Cystozoites of each of the 3 *Sarcocystis* species used were given to an experimental group of animals (3 SCID and 3 BALB/c mice, respectively) in 2 ways: 25×10^6 cystozoites were inoculated perorally (p.o.) into one group of animals, and the same amount of cystozoites was injected intraperitoneally (i.p.) into another group of experimental animals. Mice were monitored daily for development of clinical signs. Between 76 and 92 days postinfection (DPI), mice from all groups were randomly selected, euthanized, and necropsied.

Table 1 Tissues examined in SCID mice infected with *Sarcocystis muris* cystozoites

Liver	Brain
Gallbladder	Pancreas
Upper cheek	Spleen
Eyelid	Lung
Nose	Kidney
Urinary bladder	Foot pads
Tongue	Jowl
Scrotum (external genitalia of females)	Stomach
Gluteal skeletal muscles	Duodenum
Triceps brachii	Middle jejunum
Diaphragm	Ileum
Cardiac muscle	Cecum
Mesenteric lymph node	Colon
Inguinal lymph node	Rectum

Experiment 2: verification of the infectivity of S. muris cystozoites for SCID mice

In this experiment, of SCID mice were inoculated p.o. with 5×10^7 *S. muris* cystozoites obtained from the SCID mouse that had been found to be infected in experiment 1. Animals were euthanized at 61, 62, 83, and 85 DPI and their tissues were histologically examined as described above.

Experiment 3: study of the development of S. muris sarcocysts in SCID mice after feeding of cystozoites

In all, 7 SCID mice were inoculated p.o. with 5×10^7 *S. muris* cystozoites isolated from SCID mice infected during experiment 2. Animals were euthanized at 7, 14, 21, 28, 35, 50, and 83 DPI and their tissues were histologically examined as described above.

Experiment 4: study of the development of S. muris oocysts in the intestines of SCID mice after peroral infection with cystozoites

In this study, 10 SCID mice were inoculated p.o. with 1×10^8 *S. muris* cystozoites isolated from SCID mice infected during experiment 3. Animals were euthanized at 1, 2, 5, 7, 15, 27, 29, 35, 50, and 62 DPI. Squashed smears of each of the four equal segments of the small intestine, large intestine, diaphragm, and skeletal musculature, respectively, were tested and all tissues were also studied histologically. Feces of experimental mice were examined daily for sporocyst shedding from 1 to 7 DPI and then twice per week until 50 DPI.

Experiment 5: test of the infectivity of S. muris sporocysts isolated from the small intestines of SCID mice

Small intestines of SCID mice from experiment 4 were homogenized in a blender in 2.5% potassium dichromate and then filtered through gauze, and the filtrate was centrifuged at 1,500 *g* for 10 min. The sediment was stored in 2.5% potassium dichromate at 4 °C until used. Before the inoculation, individual homogenates were washed three times by centrifugation in PBS and the sporocyst content was determined using a hemocytometer. In all, 6 SCID

mice and 6 outbred ICR mice aged 8 weeks were inoculated p.o. with 10–10^3 sporocysts that had been stored for 6 weeks. Surviving mice were euthanized at 2 months after infection. All tissues of euthanized SCID mice and portions of the liver, spleen, diaphragm, and gluteal skeletal muscles of outbred ICR mice were histologically examined.

Experiment 6: does the hypothesized excretion of sporocysts of S. muris by SCID mice occur? Is the infection that spreads among SCID mice mediated by sporocysts?

In all, 2 SCID mice were inoculated p.o. with 1×10^8 *S. muris* cystozoites isolated from SCID mice from experiment 4. These SCID mice were housed in the same cage with two other, noninoculated SCID mice ("housed-together" SCID mice). At 58 DPI, all animals were euthanized and squashed smears of the small intestine, large intestine, diaphragm, and skeletal musculature were examined.

Results

Experiment 1

No clinical sign was observed in any mouse used in this experiment and euthanized at between 76 and 92 DPI. No sarcocyst or coccidian stage was detected in the tissues of any SCID or BALB/c mouse infected p.o. or i.p. with *Sarcocystis dispersa* or *Sarcocystis* sp. cystozoites. Similarly, no coccidian stage was found in tissues of the SCID mice inoculated i.p. with *S. muris* cystozoites or in the BALB/c mice inoculated both i.p. and p.o. with *S. muris* cystozoites. However, one of the three SCID mice inoculated p.o. with *S. muris* cystozoites showed swollen and anemic skeletal muscles when necropsied at 92 DPI. Squashed smears and histological examination revealed abundant sarcocysts in skeletal muscles. Mature sarcocysts containing banana-shaped cystozoites as well as immature cysts filled with round metrocytes were found. Focal inflammatory infiltrates into hepatic parenchyma and sarcocysts in cardiac muscles were also observed.

Experiment 2

Mature and immature sarcocysts as well as histopathological changes in the liver were observed in three of the four inoculated SCID mice necropsied at 62, 83, and 85 DPI when the higher dose of *S. muris* cystozoites (5×10^7) had been used as the inoculum.

Experiment 3

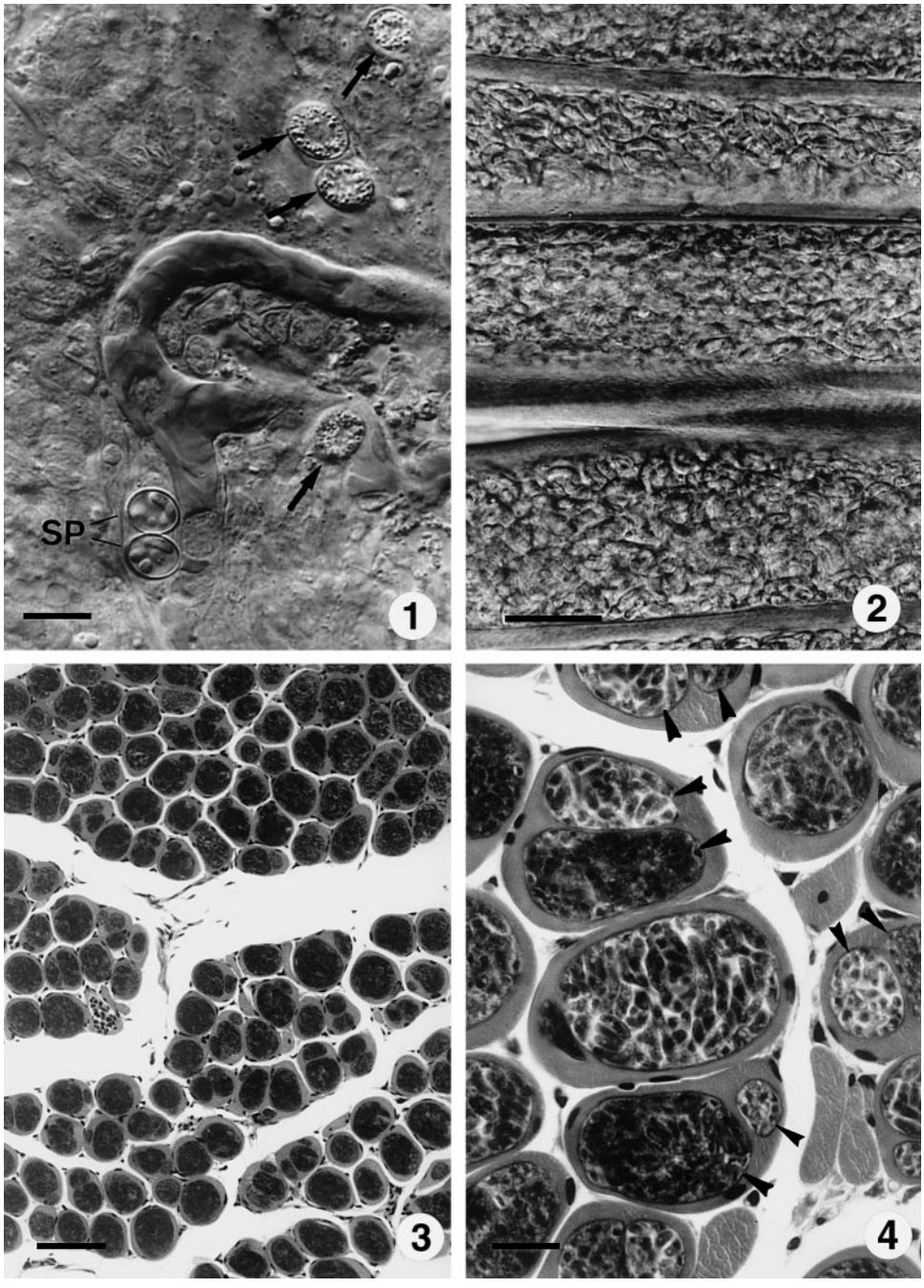
No sarcocyst was found in the SCID mice euthanized at 7, 14, 21, 28, or 35 DPI. Sarcocysts were first detected in an SCID mouse necropsied at 50 DPI. The remaining SCID mouse used in this experiment was euthanized when it became severely lethargic at 83 DPI. At necropsy it displayed swollen and edematous skeletal muscles, accumulations of ascites in the abdominal cavity, and hemorrhages in the anterior jejunum. Squashed smears of this part of the small intestine revealed numerous unsporulated and sporulated *Sarcocystis*-like oocysts (Fig. 1). Thin-walled unsporulated oocysts found in the intestinal lamina propria measured 12.0–15.0 × 10.0–12.0 μm. Sporulated oocysts contained 2 elliptical sporocysts that measured 8.5–12.0 × 7.5–9.0 μm, and each contained 4 sporozoites. The thin oocyst wall was often ruptured and the released sporocysts were frequently found in the intestinal lamina propria. Numerous sarcocysts were simultaneously present in skeletal muscles (Fig. 2). Histological examination confirmed the massive occurrence of sarcocysts in skeletal muscles and of oocysts in the lamina propria of the small intestine.

Experiment 4

Oocysts were first detected in the lamina propria of the small intestine of an SCID mouse examined at 24 h postinoculation. The oocysts were distributed focally in all examined parts of the small intestine but not in the large intestine. The oocysts were also found in SCID mice euthanized at 2, 5, 7, 15, 27, 29, and 35 DPI but not in those euthanized at 50 DPI, although they were again detected at 62 DPI. No sporocyst was found in the feces of any SCID mouse examined. Two SCID mice became moribund and lethargic and were necropsied at 27 and 29 DPI, respectively. Histopathological lesions consisted of multifocal inflammatory infiltrates in the liver associated with numerous rosette-like meronts in hepatocytes. Sarcocysts were not found in the skeletal muscles of these SCID mice. Developing immature sarcocysts with round or ovoid metrocytes were first detected in an SCID mouse examined at 50 DPI. Mature sarcocysts containing cystozoites and peripherally arranged metrocytes were found in an SCID mouse examined at 62 DPI. In histological sections, almost all muscle fibers contained sarcocysts and some muscle fibers were filled with two sarcocysts (Figs. 3, 4).

Experiment 5

Sporocysts isolated from the small-intestinal tissue of SCID mice were infective for other mice. All SCID mice inoculated with 10^2–10^3 sporocysts were euthanized when they became severely lethargic at 17 and 19 DPI, respectively. At necropsy these SCID mice had hepatomegaly and accumulation of ascites in the abdominal cavity. Histologically, there were multifocal inflammatory infiltrates in the liver and foci of necrosis associated with rosette-like meronts in hepatocytes. Two SCID mice inoculated with ten sporocysts and examined at 59 DPI had minimal hepatic inflammatory lesions, although numerous sarcocysts were found in their skeletal muscles. No oocyst was detected in the small intestine of this SCID mouse. Oral inoculation of 4 immunocompetent outbred ICR mice with 10^2 or 10^3



Figs. 1–4 *Sarcocystis muris* in SCID mice. **Fig. 1** Squashed smear of the lamina propria of the jejunum, showing unsporulated (*arrows*) and completely sporulated oocysts (*SP*) of *S. muris* at 83 DPI (Nomarski interference contrast, NIC). *Bar* 10 μm. **Fig. 2** Squashed smears of the diaphragm, demonstrating massive infection of muscles with sarcocysts at 83 DPI (NIC). *Bar* 20 μm. **Fig. 3** Histology section of skeletal musculature containing numerous sarcocysts at 62 DPI (HE). *Bar* 100 μm. **Fig. 4** Histology section showing some muscle fibers containing two sarcocysts (*arrowheads*) at 62 DPI (HE). *Bar* 20 μm

sporocysts obtained from SCID mice also led to the development of sarcocysts in skeletal muscles.

Experiment 6

Sarcocysts were detected in skeletal muscles of the inoculated as well as the noninoculated (''housed-together'') SCID mice. Oocysts were detected at necropsy only in the small intestine of SCID mice that had been inoculated with *S. muris* cystozoites. The ''housed-together'' SCID mice had no parasite in their intestines.

Discussion

Coccidia of the genus *Sarcocystis* have a complex life cycle involving two different hosts. Only the cystozoite stage in muscles of the intermediate host is capable of initiating sexual development in the gut of a definitive host. Intermediate hosts are infected either by oral ingestion of sporocysts excreted by the definitive host or by transplacental transmission (Dubey et al. 1989). However, in contrast to this generally accepted course of the life cycle, our results demonstrate that cystozoites of

S. muris, for which the mouse is the intermediate host, transform into gamonts and perform sporulation, ending with the production of infectious oocysts in the small intestine of SCID mice. Thus, the feline part of the *S. muris* developmental cycle occurs in mice, provided that the immune reactions of the latter are suppressed, as is the case in SCID mice. The indication that conversion of *S. muris* cystozoites is somehow controlled by immune reactions in the intermediate host is challenging and should be further investigated. Another aspect of the results presented herein is the indication that the cannibalistic mode of transmission, known to occur in some *Sarcocystis* species in lizards (Matuschka and Bannert 1987, 1989; Bannert 1992), may also occur in *Sarcocystis* species parasitizing mammals (see also Arnastuaskiene and Grikieniene 1989; Grikieniene and Kutkiene 1998).

Of the three *Sarcocystis* species tested in this study, only *S. muris* cystozoites produced infection in the SCID mice. The different behavior of the *Sarcocystis* spp. in the SCID mouse may have been due to the phylogenetic distance between the respective definitive and intermediate hosts. The definitive and intermediate hosts of *S. muris* are more closely phylogenetically related (mouse-cat) than are those of *S. dispersa* (mouse-owl) or *Sarcocystis* sp. (murine rodent-snake). The assumption that the phylogenetic relationship is the factor deciding that the sexual part of the *Sarcocystis* life cycle can take place in the intermediate host is corroborated by observations made on *S. rodentifelis* (rodent-cat parasite; Arnastuaskiene and Grikieniene 1989).

Among the 3 SCID mice inoculated with 25×10^6 *S. muris* cystozoites in experiment 1, sarcocysts formed in the skeletal muscles of only 1 animal. Further experiments, however, indicated that the infection rate depended on the number of cystozoites inoculated. For example, in experiment 2, of the 4 SCID mice inoculated with 5×10^7 *S. muris* cystozoites, 3 animals became infected.

In our experiments, immature sarcocysts were first detected in the skeletal muscles of SCID mice at 50 DPI. This finding differs from the data of Rommel et al. (1981), who detected the formation of sarcocysts at from 28 to 50 DPI in mice inoculated p.o. with 50 sporocysts of *S. muris*. This difference might be attributable to the irregular shedding of *S. muris* sporocysts, which served as the source of spontaneous infection in SCID mice in our experiments.

In the present investigation the entire process of gametogony was observed in the SCID mouse within 24 h. The earliest oocysts were detected in the intestinal lamina propria at the same time and were present until 62 DPI. No oocyst was seen at 50 DPI, but this was probably caused by the low potential of the detection technique used. Grikieniene and Kutkiene (1998) found sporulated oocysts of *S. rodentifelis* in mucosal scrapings of the small-intestinal tissue of laboratory rats, inoculated with cystozoites, at from 8 to 47 DPI.

Despite the detection of oocysts and sporocysts in intestinal tissues of SCID mice, neither oocysts nor sporocysts were detected in fecal samples using Sheather's sugar-solution flotation method. We believe that this was due to sporocysts being discharged irregularly and in low numbers only. Sporocysts found in the intestinal tissues of SCID mice corresponded in morphology and size to *S. muris* sporocysts as characterized by Ruiz and Frenkel (1976).

In the present study the precise course of infection of SCID mice after inoculation with *S. muris* cystozoites was not determined. However, the comparison of the development of sarcocysts in SCID mice after p.o. inoculation of cystozoites (experiment 3) with the course of infection after p.o. inoculation with sporocysts (experiment 5) indicates that SCID mice were spontaneously infected with *S. muris* sporocysts at around 10 DPI. However, our results also demonstrated that the sporocysts were probably discharged repeatedly and, hence, SCID mice could also have been infected several times.

Our data also indicate that the course and pathogenesis of *S. muris* infection in SCID mice is dose-dependent. SCID mice receiving 10 *S. muris* sporocysts per mouse had numerous sarcocysts in their skeletal muscles and survived for more than 8 weeks postinoculation. In contrast, the SCID mice given a higher dose of sporocysts were moribund at 17 and 19 DPI. Histopathological examination revealed severe hepatitis and numerous merogonial stages within hepatocytes in these animals.

The last experiment of this study proved our assumption concerning the way of dissemination of *S. muris* in SCID mice. Results of the experiment confirmed the peroral route of infection, as the only way in which noninoculated SCID mice could acquire infection was via the ingestion of sporocysts excreted in feces of inoculated SCID mice.

In conclusion, *S. muris* seems to be more flexible in its biological adaptability than has been assumed. It is attractive to speculate that after peroral inoculation the bradyzoites of *Toxoplasma gondii* or *Neospora caninum* might behave similarly in SCID mice.

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