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Prophylactic and therapeutic immune reconstitution of SCID mice infected with *Encephalitozoon cuniculi*

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Abstract. Severe combined immunodeficient (SCID) mice develop lethal infections, resembling opportunistic microsporidioses of immunocompromised patients, after intraperitoneal (i.p.) inoculations of spores of *Encephalitozoon cuniculi*. In the present study, SCID mice reconstituted i.p. with $5x10^7$ spleen cells from naive adult BALB/c mice 14 days prior to the i.p. injection of 10^7 spores were completely resistant to the infection, whereas control infected SCID mice developed clinical disease and died within 17 days post infection (DPI). In another experiment, SCID mice infected i.p. with 10^7 spores of *E. cuniculi* and after that (on DPI 7) injected i.p. with $5x10^7$ spleen lymphocytes isolated from immune adult BALB/c mice were partially protected against the parasite (40% of the reconstituted mice survived). In both experiments, high levels of parasite-specific serum antibodies (mostly of the IgG-isotype) were detected in the infected immunocompetent BALB/c mice, whereas virtually no antibodies were found in the infected SCID mice. However, SCID mice reconstituted with either naive spleen cells or immune lymphocytes revealed humoral immune responses comparable with those of immunocompetent mice.

Microsporidia are obligate intracellular parasitic protozoa of invertebrate and vertebrate hosts, which have been recently recognised as the causative agents of microsporidiosis in immunocompromised patients, especially in patients with AIDS (Godfrey-Fausset et al. 1993). Four main genera of microsporidia, *Encephalitozoon*, *Enterocytozoon*, *Septata* and *Nosema*, infecting internal organs, the intestinal epithelium, and the corneal and conjunctival epithelia, have been found in HIV-infected patients (Canning and Lom 1986, Canning and Hollister 1991, 1992).

Encephalitozoon cuniculi Levaditi, Nicolau et Schoen, 1923 is a parasite commonly found in mammals, which resides in a parasitophorous vacuole in a number of cell types (predominantly macrophages). The disease produced in young infected canines or neonatal monkeys is often lethal (Canning and Hollister 1987), but infections in rabbits and mice are usually nonlethal and chronic (Canning and Lom 1986). The susceptibility of mice to *E. cuniculi* varies with the strain, whereby BALB/c mice were shown to be more resistant than C57Bl/6 mice (Niederkorn et al. 1981).

The intact thymus is required for protective immunity, since euthymic BALB/c mice had chronic asymptomatic infections while athymic nude mice of this strain developed acute and lethal infections (Gannon 1980, Schmidt and Shadduck 1984).

Regarding the crucial importance of cell-mediated immunity in the protection against microsporidia, the SCID mice with their lack of any functional T- and B-lymphocytes (Bosma 1989) seem to be an optimal model for the detailed investigation of the role of different subpopulations of lymphoid cells in the development of resistance. It has been shown that SCID mice are extremely susceptible to opportunistic parasitic protozoan infections like cryptosporidiosis (Kuhls et al. 1992, Mead et al. 1991b, McDonald et al. 1992), toxoplasmosis (Johnson 1992), leishmaniasis (Holaday et al. 1991), amoebosis (Cieslak et al 1992). Moreover, SCID mice can be relatively easily reconstituted with haematopoietic cells of the mouse or even human origin (Reiman et al. 1991). Such reconstitution resulted in the increased resistance of SCID mice against *Cryptosporidium* (Mead et al. 1991a, McDonald et al. 1992, Perrymann et al. 1992), *Leishmania* (Holaday et al. 1991) or *Toxoplasma* (Johnson 1992).

In our preliminary experiments, we have demonstrated that SCID mice infected perorally or intraperitoneally with *E. cuniculi* developed lethal infections (Koudela et al. 1993). The main objective of the present study was to investigate whether the reconstitution of SCID mice with either naive or immune lymphoid cells could prevent the development of lethal microsporidiosis in these animals.

MATERIALS AND METHODS

Mice. SCID mice of the BALB/c background (originally obtained from Dr. Bosma - Fox Chase Cancer Center, Philadelphia, USA)

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were housed in flexible film isolators (BEM Znojmo, Czech Republic) with high-efficiency particulate air (HEPA) filters and supplied by sterilised diet and water ad libitum. Mice aged 8 weeks at the moment of infection were used throughout the experiments. Naive or immune age-matched BALB/c mice (Charles River WIGA, Sulzfeld, Germany) were used as donors of transferred cells.

Parasite. The experimental mice were infected by intraperitoneal (i.p.) injections of 10^7 *E. cuniculi* spores originated from the *in vitro* cultivation of our murine isolate in E6 cells (Vero green monkey kidney cells) on DPI (day post infection) 0.

Experimental protocol and cell transfer. The experimental protocol is summarised in Table 1. In experiment 1, twelve SCID mice (SCID+Spl group) were injected i.p. with $5x10^7$ spleen cells (prepared from ten naive BALB/c mice) 14 days prior to the infection with 10^7 spores of *E. cuniculi*, and twelve control mice (SCID group) were injected with the same volume of medium only. Six mice of the SCID+Spl group and three remaining mice of the control group (because of the high mortality in this group) were killed on DPI 16. The remaining six reconstituted mice were killed on DPI 35. Three naive (BALB-0) and three infected (BALB-inf) BALB/c mice were killed on the same days.

In experiment 2, twenty SCID mice were infected with 10^7 spores of *E. cuniculi* on DPI 0. On DPI 7, ten mice (SCID+Ly-group) were injected i.p. with $5x10^7$ Ficoll-isolated lymphocytes from spleens of twelve BALB/c mice immunised by the repeated i.p. infections with 10^7 spores (35, 28 and 21 days prior to the cell transfer). Ten control mice (SCID-group) were injected with the same volume of medium only. Four mice of both groups, together with four non-infected (BALB-0), four infected (BALB-inf) and four immunised (BALB-im) BALB/c mice, were killed on DPI 14. The remaining five mice (one mouse of both groups died on DPI 8 due to the i.p. injections) were left for the analysis of survival.

Table 1. The experimental protocol (time schedule is expressed in DPI related to the moment of infections of SCID mice).

Group/ number of mice	Infection (10^7 *E. cuniculi* spores i.p.)	Lymphocyte transfer ($5x10^7$ cells i.p.)	Killing
Experiment 1			
SCID /12	0	–	16[c]
SCID+Spl /12	0	-14[a]	16,35[d]
BALB-0 /16	–	–	-14,16,35
BALB-inf / 6	0	–	16,35
Experiment 2			
SCID /10	0	–	14[c]
SCID+Ly /10	0	7[b]	14,35[e]
BALB-0 / 4	–	–	14,35
BALB-inf / 4	0	–	14,35
BALB-im /16	-28,-21,-14	–	7,14,35

[a] non-separated naive spleen cells
[b] Ficoll-isolated immune spleen lymphocytes
[c] all control infected SCID mice died before the termination of experiment (DPI 35)
[d] all reconstituted mice survived to the end of experiment (DPI 35)
[e] 40% of reconstituted mice survived to the end of experiment (DPI 35)

Histopathology. At necropsy, tissue samples were fixed in 10% buffered formalin, embedded in paraffin, processed for routine histological sectioning and staining with hematoxylin and eosin, Giemsa, Gram and azur eosin stains as described elsewhere (Koudela et al. 1993). In addition, peritoneal exudate cell (PEC) smears were prepared, stained with Giemsa stain and examined microscopically for the presence of microsporidia.

Parasite-specific serum antibodies. Mice killed by ether were bled by a cardiac puncture, individual sera were collected and stored at -20°C until used. Levels of parasite-specific IgGAM, IgG, IgA, IgG1 and IgG2a antibodies were determined using an indirect ELISA method. Microplates (GAMA, České Budějovice, Czech Republic) were coated with antigen prepared from spores disrupted by 15 cycles of freezing (liquid nitrogen) and consequent thawing (56°C water bath). Individual sera were diluted to 1:100 in PBS-Tween (0.05% Tween-20 in PBS) and tested in triplicate. Peroxidase-conjugated goat anti-mouse-IgGAM, -IgA and -IgG (Sigma, USA), all diluted 1:1,000, were used for the detection of bound antibodies. Biotinylated rat anti-mouse antibodies (Binding Site, UK) followed by a streptavidin-peroxidase conjugate (Boehringer Manheim, Germany) were used for the detection of IgG1- and IgG2a-antibodies. The results are expressed as the mean differences between optical densities (dOD) of the experimental and control (naive non-infected BALB/c mice) samples.

***In vitro* lymphocyte blast-transformation assay (LBTA).** Spleens were aseptically removed, pooled within the same group (3-6 mice per group) and single cell suspensions were prepared in complete RPMI-1640 (Sigma, USA). Mononuclear cells (i.e. lymphocytes) were isolated by gradient centrifugation on Ficoll-Paque (Pharmacia, Sweden) and, after 2 washings, the cells were resuspended in medium containing 10% of fetal calf serum (FCS, Flow, UK) up to the final concentration of 10^6 cells/ml. Volumes of 200 µl of the final cell suspensions (i.e. 10^5 cells) were placed into the wells of 96-well flat-bottomed microplates (Nunc, Denmark). Mitogens, concanavalin A (ConA, Sigma, USA) and lipopolysaccharide (LPS, Sigma, USA), were added in the volume of 20 µl/well so the final concentration was 2.5 µg/ml and 5 µg/ml respectively. Spores of *E. cuniculi*, disrupted by 15 cycles of freezing in liquid nitrogen and consequent thawing (on 56°C water bath) were used as an antigenic preparation. The final concentrations used were 10^4, 10^5 and 10^6 spores/ml. The plates were incubated for 5 days at 37°C and 5% CO_2 and then the level of blast-transformation was evaluated by a modified MTT-assay (Page et al. 1988).

Statistical evaluation of experimental data. The significance of the differences between experimental groups was evaluated by the ANOVA test. The level lower than 0.05 was considered as significant.

RESULTS

In both experiments, an i.p. inoculation of 10^7 spores of *E. cuniculi* into SCID mice resulted in the development of acute microsporidiosis with characteristic clinical and histopathological signs within 2nd week after infection. All these control non-treated animals (SCID group) died within 3 weeks of infection. The mean survival times were 15.3 days (14-17) for experiment 1 and 17.4 days (17-18) for experiment 2. In contrast, all infected immunocompetent mice (BALB-inf group) did not develop any noticeable signs of disease.

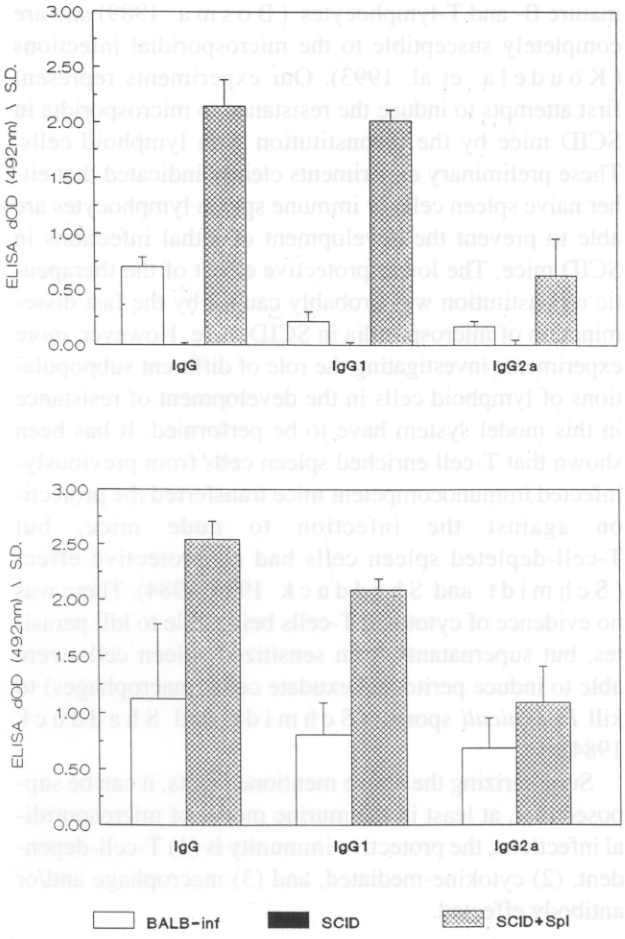

Fig. 1. The level of parasite-specific serum IgG antibodies in experiment 1 assessed on DPI 16 (a, above) and 35 (b, below). Immunocompetent BALB/c mice (group BALB-inf), control SCID mice (SCID group) and SCID mice reconstituted with spleen cells from naive BALB/c donors (SCID+Spl group) were infected i.p. with 10^7 spores of *Encephalitozoon cuniculi* on DPI 0. The results are expressed as mean differences in optical densities (dOD) of experimental samples and naive non-infected BALB/c measured in an indirect ELISA assay.

SCID mice reconstituted i.p. with $5x10^7$ spleen cells from naive adult BALB/c mice 14 days prior to the infection (experiment 1) were completely resistant to the parasite and survived up to the end of experiment (DPI 35) without any clinical symptoms. Moreover, no microsporidia were found in the PEC smears or in histological sections of different organs under an optical microscope.

In experiment 2, the SCID mice (SCID+Ly group) were infected i.p. with 10^7 spores of *E. cuniculi* and after that (on DPI 7) injected i.p. with $5x10^7$ Ficoll-isolated lymphocytes from spleens of the immunized adult BALB/c mice (given 3 consecutive i.p. infections of *E. cuniculi* 2 weeks apart). All mice of the control infected group died within 3 weeks after infection and 40% of the reconstituted mice survived to the end of experiment (DPI 35).

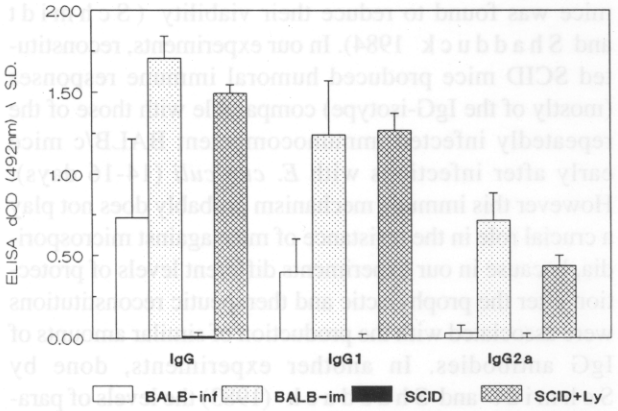

Fig. 2. Parasite-specific serum IgG antibody level in experiment 2 assessed by an indirect ELISA method. Immunocompetent BALB/c mice (group BALB-inf), control SCID mice (SCID group) and SCID mice reconstituted with Ficoll-isolated spleen lymphocytes (SCID+Ly group) from immunised BALB/c donors (group BALB-im) were infected i.p. with 10^7 spores of *Encephalitozoon cuniculi* on DPI 0 and bled on DPI 14.

The production of parasite-specific serum IgG antibodies in different groups of experimental mice is shown in Figs. 1 and 2. In both experiments, high levels of parasite-specific antibodies of the IgG-isotype were detected in the infected immunocompetent BALB/c mice, whereas virtually no antibodies were found in the infected SCID mice. However, SCID mice reconstituted either with naive spleen cells (SCID+Spl group, experiment 1) or immune lymphocytes (SCID+Ly group, experiment 2) revealed humoral immune responses comparable with those of immunocompetent mice (BALB-inf group) or even of immunised mice (BALB-im group). The levels of specific IgA antibodies in the sera of infected mice were low and did not differ significantly between the experimental groups (data not shown).

In contrast, only minor differences in the cellular immune responses (mitogen- and antigen-induced *in vitro* proliferation of spleen lymphocytes) were noticed between control infected and reconstituted SCID mice (data not shown).

DISCUSSION

At the present time, little is known about the immune response elicited by microsporidial infections. About 20-30% of HIV-infected European homosexuals were seropositive (Canning and Hollister 1987), however, no antibodies against *E. cuniculi* were found in blood donors in England. An unexpectedly high incidence of antibodies to *E. cuniculi* has been found in patients with some tropical diseases which cause immunodepression (Hollister and Canning 1987).

Antibodies may be involved in immunity to *E. cuniculi*, since pretreatment of spores with serum from immune

mice was found to reduce their viability (Schmidt and Shadduck 1984). In our experiments, reconstituted SCID mice produced humoral immune responses (mostly of the IgG-isotype) comparable with those of the repeatedly infected immunocompetent BALB/c mice early after infections with *E. cuniculi* (14-16 days). However this immune mechanism probably does not play a crucial role in the resistance of mice against microsporidia, because in our experiments different levels of protection after the prophylactic and therapeutic reconstitutions were associated with the production of similar amounts of IgG antibodies. In another experiments, done by Schmidt and Shadduck (1983) the levels of parasite-specific antibody in infected mice correlated with their immune status, but passively transferred serum from immune mice failed to protect nude mice from infection.

An increase in the natural killer cell activity of the spleen in both BALB/c and C57Bl/6 mice during the infection with *E. cuniculi* has been described, however, an increased activity was also observed in nude mice, indicating that this nonspecific mechanism may not play an important role in the resistance (Niederkorn et al. 1983). Moreover, SCID mice, being absolutely susceptible to microsporidial infections (Koudela et al. 1993), at the same time, have similar numbers of functionally normal NK cells like immunocompetent mice (Dorshkind et al. 1985).

The intact thymus-dependent immunity is clearly required for protection, since euthymic BALB/c mice had chronic nonlethal infections while athymic nude mice of this strain developed acute and lethal infections (Gannon 1980, Schmidt and Shadduck 1984). SCID mice, due to the autosomal recessive mutation in a gene essential for the Ig- and T-cell-receptor gene rearrangement (chromosome 16), lack any functional mature B- and T-lymphocytes (Bosma 1989) and are completely susceptible to the microsporidial infections (Koudela et al. 1993). Our experiments represent first attempts to induce the resistance to microsporidia in SCID mice by the reconstitution with lymphoid cells. These preliminary experiments clearly indicated that either naive spleen cells or immune spleen lymphocytes are able to prevent the development of lethal infections in SCID mice. The lower protective effect of the therapeutic reconstitution was probably caused by the fast dissemination of microsporidia in SCID mice. However, more experiments investigating the role of different subpopulations of lymphoid cells in the development of resistance in this model system have to be performed. It has been shown that T-cell enriched spleen cells from previously-infected immunocompetent mice transferred the protection against the infection to nude mice, but T-cell-depleted spleen cells had no protective effect (Schmidt and Shadduck 1983, 1984). There was no evidence of cytotoxic T-cells being able to kill parasites, but supernatants from sensitized spleen cells were able to induce peritoneal exudate cells (macrophages) to kill *E. cuniculi* spores (Schmidt and Shadduck 1984).

Summarizing the above mentioned facts, it can be supposed that, at least in the murine model of microsporidial infections, the protective immunity is (1) T-cell-dependent, (2) cytokine-mediated, and (3) macrophage and/or antibody effected.

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