

# Different routes of infection with *Encephalitozoon intestinalis* affect the development of immune response in immunocompetent and immunodeficient mice

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## Abstract

The development of an immune response of immunocompetent BALB/c and C57BL/6 mice with different levels of resistance to microsporidial infection and immunodeficient SCID mice was analysed in relation to the route of infection with *Encephalitozoon intestinalis*. Cell-mediated immune responses were characterised by measurement of IFN- $\gamma$  and quantification of CD4<sup>+</sup>, CD8<sup>+</sup> and NK cells. Intraperitoneal (i.p.) infection induced higher levels of IFN- $\gamma$  in *ex vivo* cultures of peritoneal exudate cells or splenocytes than peroral (p.o.) infection. Numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in the spleen increased markedly at later time points (14 and 21 DPI) after i.p. infection of BALB/c mice, whereas in C57BL/6 mice the highest increase was after p.o. infection. Considerable IFN- $\gamma$  production in *ex vivo* cultures of Peyer's patch lymphocytes of both mouse strains was observed after p.o. infection. Intraperitoneal infection of SCID mice induced marked influx of inflammatory cells into the peritoneum with NK cells. The results contribute to the development of an animal model of microsporidiosis caused by *E. intestinalis*.

## Key words

*Encephalitozoon intestinalis*, microsporidia, immunity, BALB/c, C57BL/6, SCID mice

## Introduction

Microsporidia are obligate intracellular protozoan parasites that commonly infect both invertebrate and vertebrate animals. These organisms were rarely reported in humans until the AIDS pandemic development, and since 1985, they have been recognised as causative agents of opportunistic infections of immunocompromised individuals, particularly patients with AIDS or organ transplant recipients. Common clinical signs of microsporidiosis in immunodeficient patients include diarrhoea, cholangitis, hepatitis, nephritis, peritonitis, myositis, sinusitis and keratitis (Franzen and Muller 2001).

*Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are the most common microsporidians observed in AIDS patients (Canning and Hollister 1990). The most frequent clinical manifestations caused by these microsporidian parasites in AIDS patients are diarrhoea, nausea, malabsorption and weight loss (Asmuth *et al.* 1994). Both species mainly develop in enterocytes of the small intestine. *E. intestinalis* can

infect the macrophages of the lamina propria and in this way probably disseminates to other host organs such as the kidneys, hepatobiliary tract and gall bladder (Cali *et al.* 1993). Achbarou *et al.* (1996) developed an experimental model for human intestinal microsporidiosis, where the multiplication of *E. intestinalis* was demonstrated in the duodenum, liver, kidneys and lungs of IFN- $\gamma$  receptor knockout mice. The infection was chronic and characterised by the shedding of spores in the faeces. El Fakhry *et al.* (2001a) demonstrated that resistance to *E. intestinalis* is associated with cellular immune responses.

The immunobiology of microsporidial infections has been studied mainly in the model infection of mice infected with *Encephalitozoon cuniculi* (Schmidt and Shadduck 1984, Koudela *et al.* 1993). This microsporidian commonly infects rodents and carnivores and has been found in humans as well (Canning and Lom 1986). Clinically silent chronic infections generally develop in immunologically competent animals that become infected with *E. cuniculi* (Niederhorn *et al.* 1981). On

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the other hand, immunodeficient hosts (e.g. athymic mice) experimentally infected with *E. cuniculi* develop a lethal disease (Schmidt and Shadduck 1983).

The studies of humoral immunity revealed that transfer of hyperimmune serum failed to protect infected athymic mice from developing a lethal *E. cuniculi* infection. However, specific antibodies probably contribute to resistance as revealed by *in vitro* studies. Antibodies have been shown to opsonise microsporidia to facilitate their killing by macrophages (Enriquez *et al.* 1998). Th1 cytokines like interferon-gamma (IFN- $\gamma$ ) and interleukin-12 (IL-12) are important for protective immunity against intracellular parasites. The cytokine IFN- $\gamma$  seems to be particularly important in resistance to microsporidial infections (Khan and Moretto 1999). Recent studies support the theory of an important role of the cell-mediated immune response in defence against *E. cuniculi* infection. The crucial role of CD8+ cytotoxic T lymphocytes for protective immunity against *E. cuniculi* infection has been shown by Khan *et al.* (1999) using gene knockout mice. In studies with severe combined immunodeficient (SCID) mice, CD8+ T lymphocytes from naive BALB/c mice protected SCID mice from *E. cuniculi* infection (Braunfuchsová *et al.* 2001).

The aim of the present study was to compare the immune response to i.p. and p.o. infection with *E. intestinalis*. The experiments were performed on immunocompetent and immunodeficient hosts to consider the role of particular immune mechanisms in the protective immunity. The results contribute to the development of an animal model of microsporidiosis caused by *E. intestinalis* leading to better understanding of this important microsporidiosis in man.

## Materials and methods

### Mice

BALB/c and C57BL/6 female mice of 7–9 weeks of age were purchased from Charles River Laboratory, Germany. Throughout the experiments, mice were fed a standard rodent diet and drinking water *ad libitum*. They were caged in a mouse room with the temperature kept at 22°C, RH 65%. SCID mice breeding pairs were originally obtained from Dr. G.C. Bosma (Fox Chase Cancer Center, U.S.A.). Their genetic background is of the BALB/c mouse. 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 sterilised before use. Mice were humanely killed by cervical dislocation before harvesting splenocytes or peritoneal exudate cells.

### Parasite

*Encephalitozoon intestinalis* originally isolated from an AIDS patient by Didier *et al.* (1996) was grown in VERO E6 cells for provision of spores. Cells were cultivated in RPMI 1640 medium (Sigma) supplemented with 2.5% foetal calf serum (FCS) (PAA Laboratories). The spores were purified by cen-

trifugation over 50% Percoll (Pharmacia), washed three times in deionised water and stored in deionised water with antibiotics (100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2.5  $\mu$ g/ml amphotericin; Sigma). Before inoculation, the spores were washed in PBS.

### Experimental protocol

Twelve mice of each strain (BALB/c, C57BL/6 and SCID) were injected i.p. with  $10^7$  spores of *E. intestinalis* in 0.1 ml PBS. Another twelve mice of each strain were infected p.o. with the same dose of the organism by intragastric gavage of 0.1 ml suspension of spores in water. On days 3, 7, 14 and 21 post infection (PI), three mice infected i.p. and three mice infected p.o. of each strain were analysed. Groups of three uninfected mice of each strain served as a control.

Mice were killed humanely and bled by a cardiac puncture. Individual sera were collected and stored at  $-75^\circ\text{C}$  until used for detection of IFN- $\gamma$  and specific antibodies to *E. intestinalis*. Peritoneal exudate cells (PEC) were recovered by lavaging the peritoneum with 4 ml of cold RPMI 1640. PEC were washed and counted, and then a portion of PEC was stained by Calcofluor (Sigma) and observed by fluorescent microscope (Olympus IX 70) for detection of *E. intestinalis* spores. A suspension of spleen cells, PEC and Peyer's patch lymphocytes (PPL) were generated in RPMI 1640 medium supplemented with 10% FCS,  $5 \times 10^{-5}$  M 2-mercaptoethanol and antibiotics and seeded into 96-well tissue culture plates (NUNC),  $1 \times 10^6$  cells in 0.2 ml per well. After 24-hour incubation at 37°C and 3.5% CO<sub>2</sub>, supernatants were harvested and stored at  $-75^\circ\text{C}$  for measurement of IFN- $\gamma$  production. A portion of splenocytes and PEC were analysed by flow cytometry.

### Detection of *E. intestinalis* spores in macrophages

PEC were fixed with methanol for 2 min at room temperature and stained with 1% Calcofluor (Sigma) for 10 min. Thereafter, slides with PEC were washed in PBS and covered with 0.5% Evans blue in PBS for 30 sec. The slides were then washed again in PBS and observed by an Olympus epifluorescent microscope with 425 nm and 460 nm exciting and block filters, respectively. Five hundred cells from each mouse were examined.

### Measurement of IFN- $\gamma$ production

Levels of IFN- $\gamma$  in cell culture supernatants and sera were measured by capture enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocol. The following pair of rat anti-mouse cytokine monoclonal antibodies (MAbs) was used in the assay: unconjugated MAb clone R4-6A2 and biotinylated MAb clone XMG1.2 (PharMingen, San Diego, CA). Microtiter plates (flat bottom, high binding) (Costar, Cambridge, MA) were coated with purified MAbs (4  $\mu$ g/ml) diluted with 0.1 M sodium carbonate buffer (pH 8.2). Diluted MAbs (100  $\mu$ l per well) were then incubated overnight at 4°C. After washing three times with 0.05% Tween 20

in PBS (T-PBS), unoccupied sites were blocked with 200  $\mu$ l of 10% newborn calf serum (NBCS) (PAA Laboratories) in PBS for one hour at room temperature. Plates were washed with T-PBS and incubated with cell culture supernatants (100  $\mu$ l/well) overnight at 4°C. Biotinylated antibodies (2  $\mu$ g/ml in PBS, 10% NBCS) were then added at 100  $\mu$ l per well and incubated for 45 min at room temperature. Plates were washed and 100  $\mu$ l streptavidin-peroxidase (Sigma) diluted 1  $\mu$ g/ml in PBS with 10% NBCS was allowed to incubate for 30 min at room temperature. After washing, an enzymatic colour reaction was generated using orthophenylenediamine (OPD) substrate, stopped after 10 min with 100  $\mu$ l of 2 M H<sub>2</sub>SO<sub>4</sub> and measured at 490 nm with an ELISA spectrophotometer (Lab-system Multiskan). Cytokine levels were determined from standard curves generated from known quantities of recombinant IFN- $\gamma$  standard (PharMingen). Results represent the mean of three sample measurements. Each sample was assayed in three wells.

#### Flow cytometry analysis

Splenocytes and PEC were analysed by flow cytometry. CD4-specific monoclonal antibody (MAb) YTS 177.9, CD8-specific MAb KT15 and NK 1.1-specific MAb PK136 (conjugated with fluorescein isothiocyanate or phycoerythrin) (Serotec) were used for the phenotypic analysis. Samples (10<sup>6</sup> cells) were incubated with 100  $\mu$ l of antibody (10  $\mu$ g/ml) in PBS with 1% FCS for 30 min at 4°C. After washing with ice-cold PBS with 1% FCS, the cells were used for flow cytometry analysis.

Cell analysis was performed on an Epics XL Flow Cytometer (Coulter) equipped with a 15-mW argon-ion laser with excitation capabilities at 488 nm. Ten thousand events of viable cells were measured in each suspension. The labelled cell populations were analysed using WinMDI software (Coulter). Results represent the mean measurement of three samples.

#### Histopathology

At necropsy, tissue samples of the spleen, liver, kidney, lung, ileum and colon were fixed in 10% buffered formalin, and then the tissues were examined by light microscope using standard methods. Paraffin sections were stained by haematoxylin and eosin, by Calcofluor and by the Weber stain (Weber *et al.* 1992). Ten sections were examined from each organ.

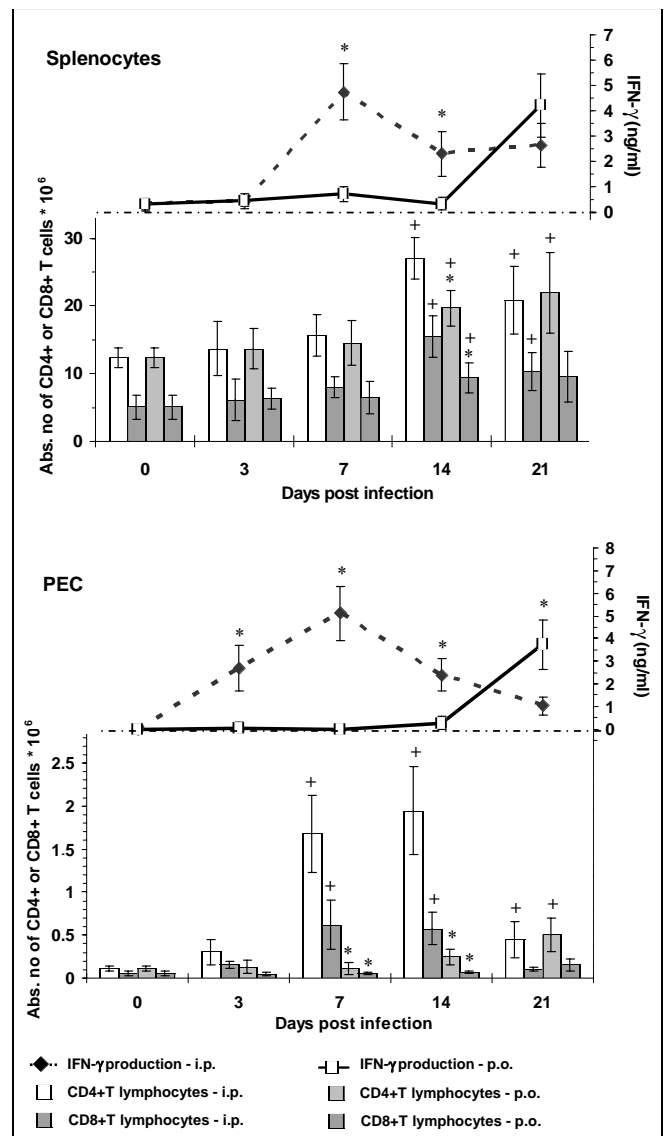
#### Statistical analysis

The significance of any differences between experimental groups was evaluated by Student's t-test. In all figures, error bars represent standard deviations.

## Results

#### Experimental infection of BALB/c mice

*Encephalitozoon intestinalis* organisms were found in the peritoneum of immunocompetent BALB/c mice on days 3 and



**Fig. 1.** Absolute number of CD4+ (CD8+) T lymphocytes after intraperitoneal infection (i.p.) – open bars (open striped bars) and peroral (p.o.) infection – closed bars (closed striped bars) with *Encephalitozoon intestinalis* detected in the spleen or peritoneal exudate cells (PEC) of BALB/c mice. IFN- $\gamma$  production by splenocytes or PEC of infected mice – i.p. infection (dashed line), p.o. infection (full line). Each value represents the mean of three measurements  $\pm$  SD. \*The difference between IFN- $\gamma$  production after i.p. infection versus p.o. infection is significant at  $p < 0.05$ . +The difference in number of cells versus the control group (0 DPI) is significant at  $p < 0.05$ .

7 post i.p. infection. No microsporidia were found at later intervals post i.p. infection of these mice. No microsporidia were found in the peritoneum of BALB/c mice after p.o. infection.

The average absolute number of cells in the spleen of non-infected BALB/c mice was 52.3 million. The absolute number of splenocytes rose after the i.p. inoculation with peak on day

14 PI – 162.7 million cells. Peroral infection similarly resulted in an increase of splenocyte number with the maximum on day 14 PI – 120.5 million splenocytes. The number of CD4+ and CD8+ T lymphocytes corresponded to the increased number of spleen cells. The absolute maximum of CD4+ T lymphocytes (27.0 million) and CD8+ T lymphocytes (15.5 million) were found on day 14 post i.p. infection. The highest number of CD4+ (21.9 million) and CD8+ (9.5 million) were counted on day 21 post p.o. infection (Fig. 1). Monitoring of IFN- $\gamma$  production in the *ex vivo* cultures of spleen cells showed the highest concentration (4.8 ng/ml) on day 7 when the mice were infected i.p. After p.o. infection the peak of IFN- $\gamma$  production (4.2 ng/ml) was observed on day 21 (Fig. 1), when the experiment was terminated.

Intraperitoneal infection resulted in the increase of absolute number of PEC with the maximum of 7.8 million on day 14 PI. The average number of PEC in noninfected control BALB/c mice was 2.7 million. No substantial changes in absolute number of PEC were observed after p.o. infection (the highest number – 3.5 million on day 21). The absolute number of CD4+ and CD8+ T lymphocytes in the peritoneum and the kinetic of IFN- $\gamma$  production in the *ex vivo* cultures of PEC are shown in Figure 1. After i.p. infection, the maximum number of CD4+ T lymphocytes (1.9 million) was counted on day 14, while the maximum of CD8+ T lymphocytes (0.6 million) was found on day 7 PI and the peak of IFN- $\gamma$  production (5.1 ng/ml) was measured on day 7 PI. The highest absolute numbers of CD4+ (0.5 million) and CD8+ T lymphocytes (0.2 million) were counted on day 21 after p.o. infection, and on the same day the highest level of IFN- $\gamma$  production (3.7 ng/ml) was observed.

The measurement of IFN- $\gamma$  production in sera showed the maximum (1 ng/ml) on day 7 after i.p. infection. A high concentration of IFN- $\gamma$  (3.5 ng/ml) was measured on day 21 after p.o. infection (Fig. 4).

The maximal production of IFN- $\gamma$  (1.9 ng/ml) in the *ex vivo* cultures of PPL was detected on day 7 after p.o. infection. Intraperitoneal infection did not result in IFN- $\gamma$  production by PPL throughout the experiment (Fig. 5).

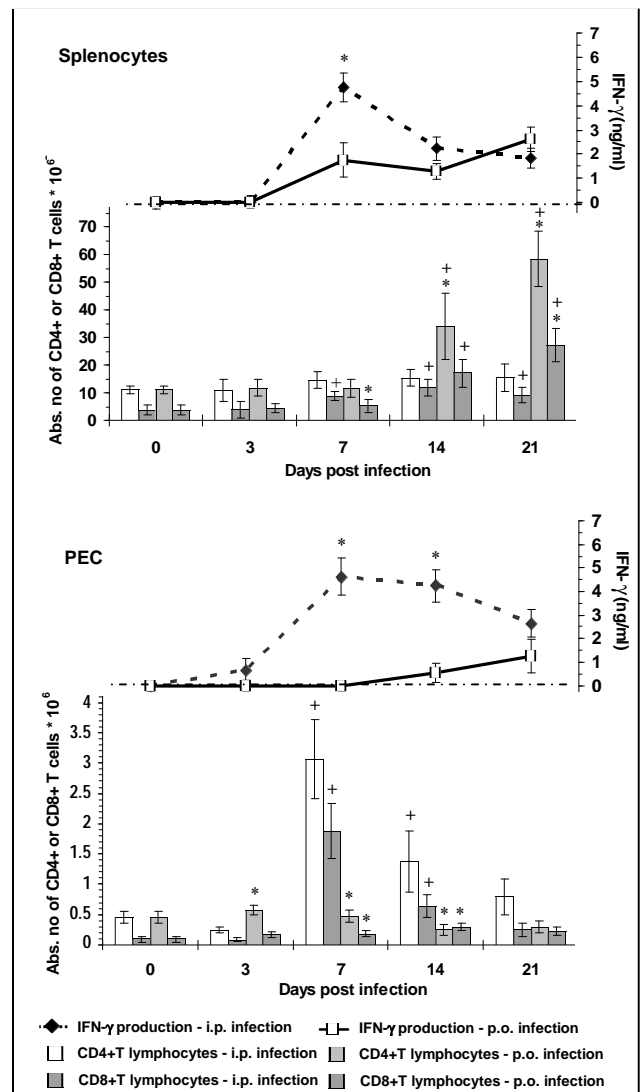
Production of specific antibodies to *E. intestinalis* was detected in sera of infected BALB/c mice from day 7 post i.p. infection (from day 14 after p.o. infection), gradually increasing to the end of the experiment (data not shown).

Histological examination did not reveal any pathological changes or microsporidia presence in any selected organs of infected BALB/c mice at any time during the experiment.

#### Experimental infection of C57BL/6 mice

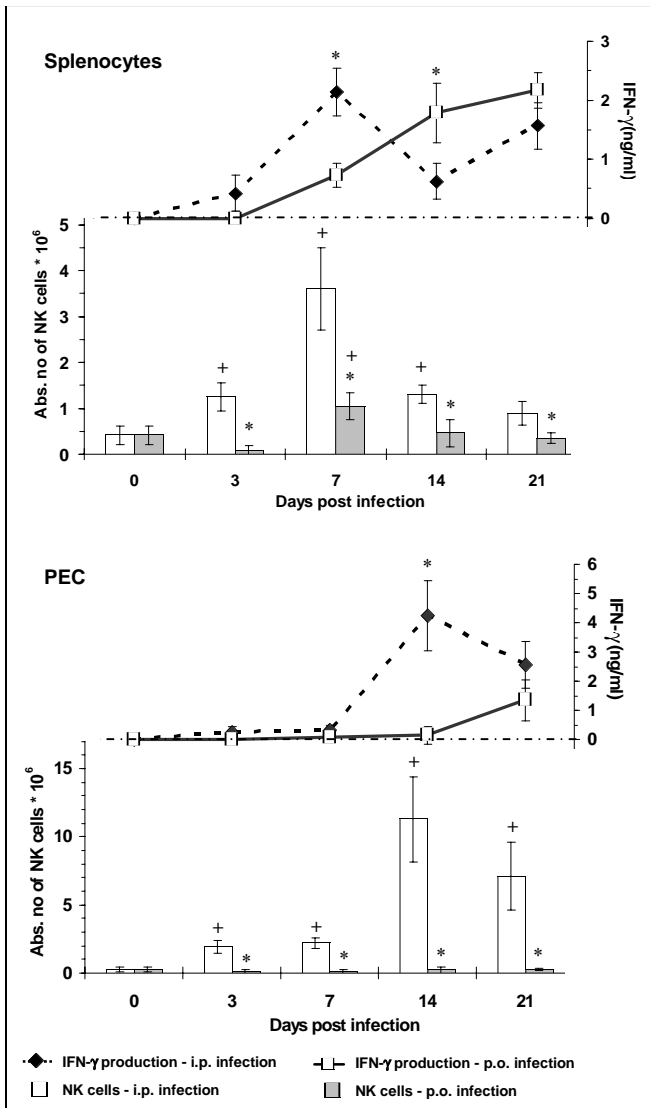
Spores of *E. intestinalis* were found in the peritoneum of immunocompetent C57BL/6 mice on days 3 and 7 post i.p. infection. No microsporidia were found at later intervals and no parasites were detected in the peritoneum of C57BL/6 mice after peroral infection.

The number of splenocytes increased after the i.p. infection with the peak on day 14 – 111.9 million cells. Peroral



**Fig. 2.** Absolute number of CD4+ (CD8+) T lymphocytes after intraperitoneal (i.p.) infection – open bars (open striped bars) and peroral (p.o.) infection – closed bars (closed striped bars) with *E. intestinalis* detected in the spleen or PEC of C57BL/6 mice. IFN- $\gamma$  production by splenocytes or PEC of infected mice – i.p. infection (dashed line), p.o. infection (full line). Each value represents the mean of three measurements  $\pm$  SD. \*The difference between IFN- $\gamma$  production after i.p. infection versus p.o. infection is significant at  $p < 0.05$ . +The difference in number of cells versus the control group (0 DPI) is significant at  $p < 0.05$ .

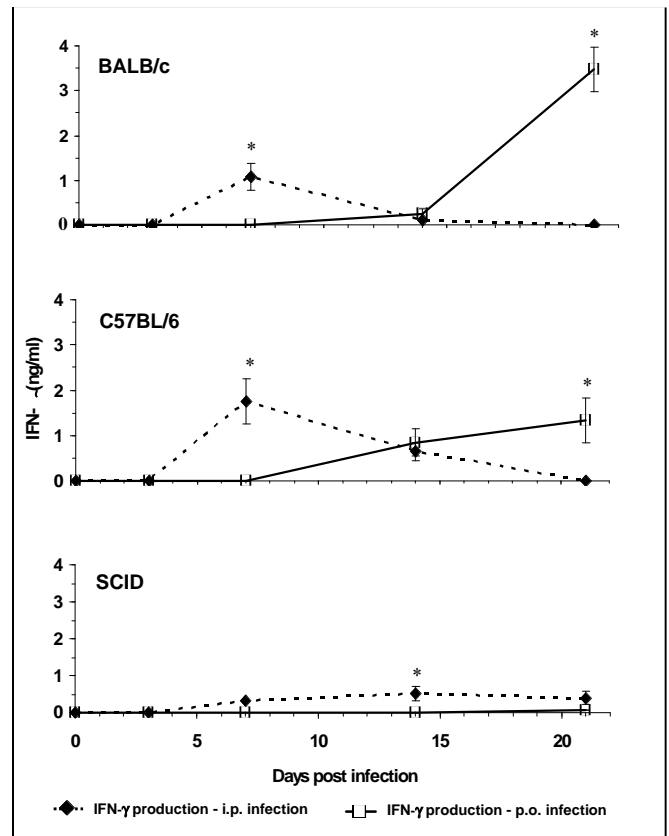
infection led to the rise of spleen cells with the maximum on day 21 – 308.4 million splenocytes. The total number of cells in the spleen of noninfected C57BL/6 mice was 50.3 million. In an i.p. infection the absolute maximum number of CD4+ T lymphocytes (15.5 million) was observed on day 21. The maximum number of CD8+ T lymphocytes (12 million) was counted on day 14 post i.p. infection. The highest numbers of CD4+ (58.3 million) and CD8+ T cells (27.1 million) were found on day 21 after p.o. infection. Measurement of IFN- $\gamma$  production in the *ex vivo* cultures of spleen cells showed the



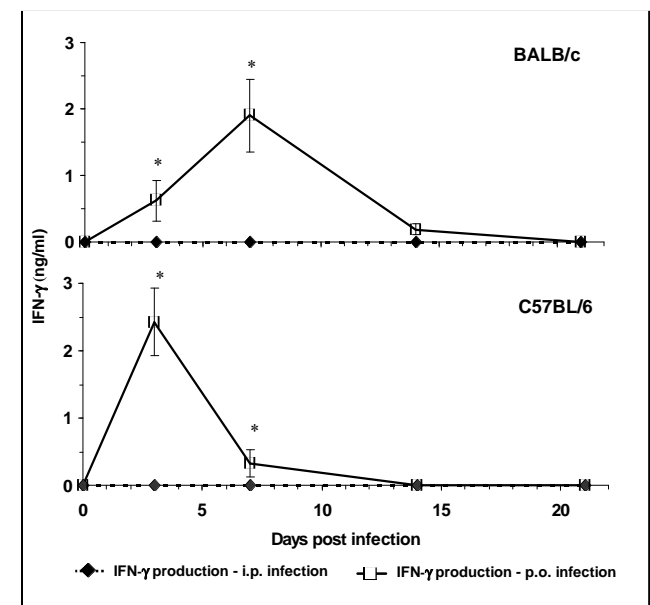
**Fig. 3.** Absolute number of NK cells after intraperitoneal (i.p.) infection (open bars) and peroral (p.o.) infection (closed bars) with *E. intestinalis* detected in the spleen or PEC of SCID mice. IFN- $\gamma$  production by splenocytes or PEC of infected mice – i.p. infection (dashed line), p.o. infection (full line). Each value represents the mean of three measurements  $\pm$  SD. \*The difference between IFN- $\gamma$  production or number of NK cells after i.p. infection versus p.o. infection is significant at  $p < 0.05$ . +The difference in number of NK cells versus the control group (0 DPI) is significant at  $p < 0.05$

highest concentration (4.8 ng/ml) on day 7 after i.p infection. When the mice were infected p.o. the peak of IFN- $\gamma$  production (2.6 ng/ml) was found on day 21 (Fig. 2).

An increase in the absolute number of PEC with the maximum of 16.6 million on day 7 was observed after i.p. infection. The average number of PEC in noninfected control C57BL/6 mice was 3 million. The maximum absolute number of PEC (5.3 million) was observed on day 7 after p.o. infection. After i.p. infection, the maximum number of both CD4+ T lymphocytes (3.1 million) and CD8+ T lymphocytes (1.9 million), together with the peak of IFN- $\gamma$  production (4.6

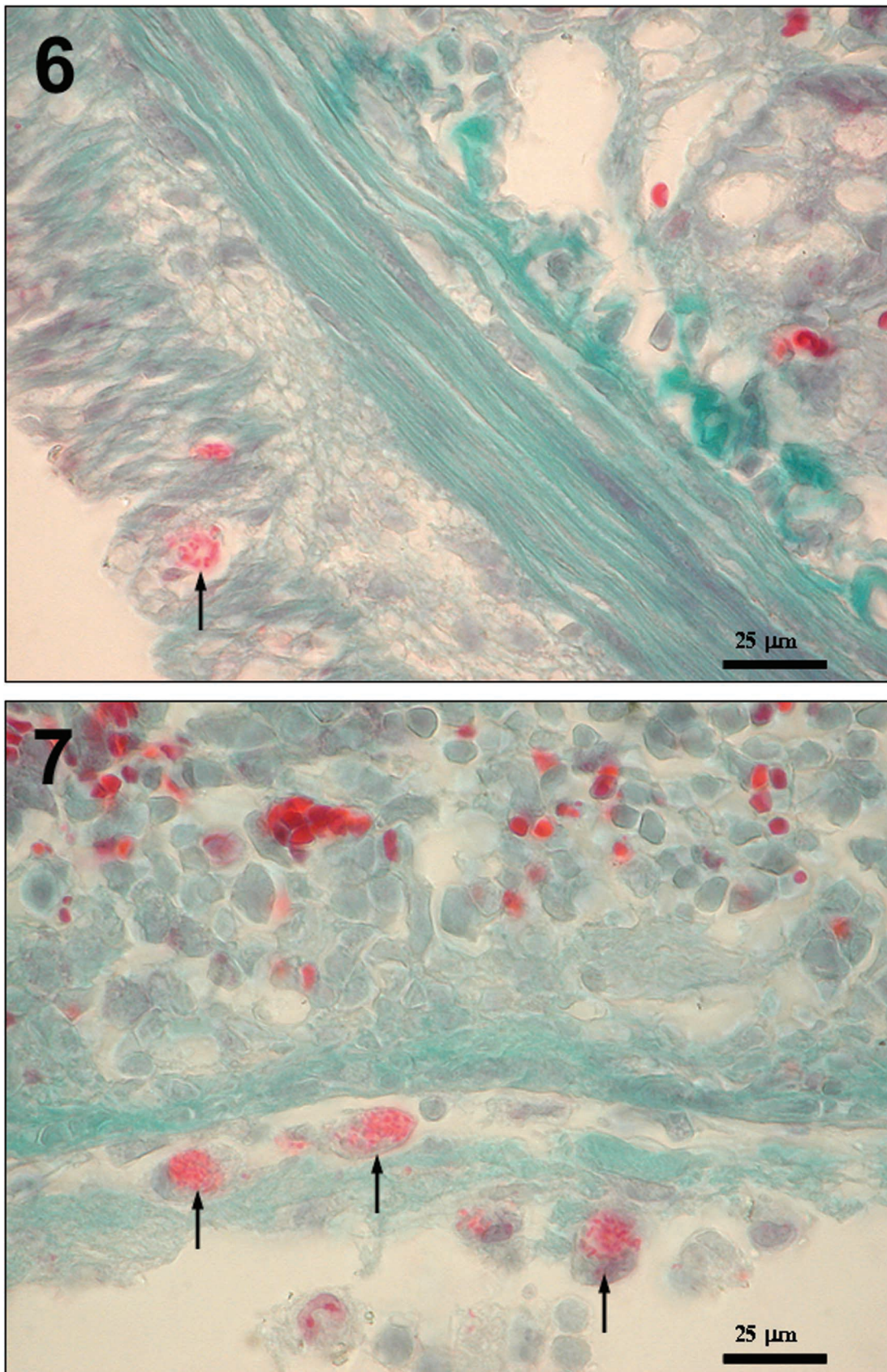


**Fig. 4.** Levels of IFN- $\gamma$  in sera of BALB/c, C57BL/6 and SCID mice after intraperitoneal (i.p.) infection (dashed line) and peroral (p.o.) infection (full line) with *E. intestinalis*. Each value represents the mean of three measurements  $\pm$  SD. \*The difference between IFN- $\gamma$  production after i.p. infection versus p.o. infection is significant at  $p < 0.05$



**Fig. 5.** Levels of IFN- $\gamma$  in *ex vivo* cultures of Peyer's patch lymphocytes of BALB/c and C57BL/6 mice after intraperitoneal (i.p.) infection (dashed line) and peroral (p.o.) infection (full line) with *E. intestinalis*. Each value represents the mean of three measurements  $\pm$  SD. \*The difference between IFN- $\gamma$  production after i.p. infection versus p.o. infection is significant at  $p < 0.05$





**Fig. 6.** Small intestine of SCID mouse infected i.p. with *E. intestinalis* spores. A vacuole with microsporidia (arrow) in the cell of serous layer; 21 days after infection. Weber staining. **Fig. 7.** Foci with spores (arrows) on the spleen surface of SCID mouse infected i.p. with *E. intestinalis*; 21 days after infection. Weber staining

ng/ml), were detected on day 7. The highest absolute number of CD4<sup>+</sup> T lymphocytes (0.6 million) was counted on day 3 and CD8<sup>+</sup> T lymphocytes (0.3 million) on day 14 after p.o. infection. On day 21 after p.o. infection the highest IFN- $\gamma$  production (1.2 ng/ml) was observed (Fig. 2).

The maximum concentration of IFN- $\gamma$  in sera (1.7 ng/ml) was detected on day 7 after i.p. infection. The highest concentration of IFN- $\gamma$  (1.3 ng/ml) was measured in sera on day 21 after p.o. infection (Fig. 4).

The maximum concentration of IFN- $\gamma$  (2.4 ng/ml) in the *ex vivo* cultures of PPL was detected 3 days after p.o. infection. No IFN- $\gamma$  production by PPL was detected throughout the experiment after i.p. infection (Fig. 5).

The kinetics of production of specific antibodies to *E. intestinalis* was similar to that described in BALB/c mice.

Histological examination did not reveal any pathological changes or microsporidia presence in any selected organs of infected C57BL/6 mice at any time during the experiment.

#### *Experimental infection of SCID mice*

Spores of *E. intestinalis* were found in PEC of SCID mice after i.p. infection on days 7, 14 and 21. No spores were found in peritoneum of SCID mice after p.o. infection throughout the experiment.

The average number of spleen cells in noninfected SCID mice was 8 million. The absolute number of splenocytes increased during the i.p. infection with the maximum on day 7 – 23.3 million cells. Peroral infection did not result in a marked increase of splenocyte number (maximum on day 3 PI – 11.3 million splenocytes). The number of NK cells correlated with the increase of splenocyte number. The maximum absolute number of NK cells (3.6 million) was found 7 days after i.p. infection. The highest number of NK cells (1 million) was counted on day 7 after p.o. infection. The peak of IFN- $\gamma$  production in the *ex vivo* cultures of spleen cells (2.1 ng/ml) was measured on day 7 when the mice were infected i.p. After p.o. infection, the peak of IFN- $\gamma$  production (2.2 ng/ml) was observed on day 21 (Fig. 3).

The average absolute number of PEC in noninfected control SCID mice was 1 million. Intraperitoneal infection markedly increased the absolute number of PEC (maximum 25.6 million on day 14 PI). The highest absolute number of PEC (2.3 million) was observed 21 days after p.o. infection. After an i.p. infection the maximum number of NK cells (11.3 million) and the peak of IFN- $\gamma$  production (4.2 ng/ml) were measured on day 14. The highest absolute number of NK cells (0.3 million) was counted on day 21 after p.o. infection. At the same time the highest IFN- $\gamma$  production in *ex vivo* cultures of PEC (1.4 ng/ml) was observed (Fig. 3).

The maximum IFN- $\gamma$  production in sera was measured on day 7 after i.p. infection (0.5 ng/ml) and on day 21 after p.o. infection (0.1 ng/ml) (Fig. 4).

Peyer's patches were not found on the intestine of SCID mice and therefore PPL were not analysed for IFN- $\gamma$  production.

Specific anti-*E. intestinalis* antibodies were not present in the sera of infected SCID mice.

Histological examination revealed miliary necroses in the liver, spleen, small intestine and large intestine on days 14 and 21 after i.p. infection. Numerous microsporidia were present within these necrotic areas and granulomas. Developmental stages of microsporidia could be found in cells of small intestine serous layer or on the spleen surface (Figs 6 and 7). However, neither histopathological changes nor microsporidia presence were found in SCID mice infected perorally until the termination of the experiment on 21 day post infection.

## Discussion

This study deals with the development of immune response of immunocompetent BALB/c and C57BL/6 mice and immunodeficient SCID mice in dependence on the route of infection with *E. intestinalis*. Two strains of immunocompetent mice were chosen to compare the development of immune response in hosts with different level of resistance to microsporidia infection. The natural resistance to microsporidial infection based on genetic background was studied by Niederkorn *et al.* (1981) who infected several strains of mice with *E. cuniculi* i.p. Susceptibility to the infection was compared on the basis of percentage of infected peritoneal macrophages two weeks after infection. BALB/c mice were relatively resistant with less than 1% of infected peritoneal macrophages, but C57BL/6 mice were relatively susceptible with more than 15% of infected peritoneal macrophages. Liu *et al.* (1989) suggested that the relative susceptibility of C57BL/6 mice to encephalitozoonosis may be due to defective accessory cell function, depressed lymphoproliferation against spore fragments, and a delay in lymphokine production. To elucidate the role of specific adaptive immunity against *E. intestinalis* infection, the SCID mice, which lack T and B lymphocytes, were chosen as a model immunodeficient host (Bosma and Carroll 1991). Both routes of *E. intestinalis* infection induced an increase in the number of spleen cells. Similar results were obtained in analysis of the immune response of IFN- $\gamma$  receptor knockout mice to *E. intestinalis* infection (El Fakhry *et al.* 2001b).

Intraperitoneal infection of mice caused an increase in the absolute number of peritoneal exudate cells. Intraperitoneal infection of SCID mice especially induced a marked influx of cells into the peritoneum on day 14 and 21 PI, followed by an intensive replication of the parasite in these cells. Similar results were obtained in the experimental study performed with immunocompetent and immunodeficient mice infected intraperitoneally with *E. cuniculi* (Salát *et al.* 2001). In contrast, p.o. infection with *E. intestinalis* did not change the absolute number of PEC significantly.

Resistance to lethal microsporidiosis was found to be dependent on functional T cell response. Athymic mice, that lack functional mature T cells, and SCID mice, lacking T and

B lymphocytes, died after inoculation with *E. cuniculi* spores (Koudela *et al.* 1993, Didier *et al.* 1994). It has been shown that the protection against i.p. infection with *E. cuniculi* is mediated by cytotoxic T lymphocytes. In addition, IFN- $\gamma$  is necessary for the recovery from the infection (Khan *et al.* 1999). Based on this fact, we tried to characterise cell-mediated immune response to the infection by the measurement of IFN- $\gamma$  production and quantification of CD4+, CD8+ and NK cells.

Intraperitoneal infection of both immunocompetent BALB/c and C57BL/6 mice leads to the increase of numbers of CD4+ and CD8+ T lymphocytes in the spleen. This increase was correlated with the production of IFN- $\gamma$  in *ex vivo* cultures of splenocytes (Figs 1 and 2). The maximum number of CD8+ T lymphocytes in the peritoneum was observed on day 7 post infection in both mouse strains. These findings correlate with the parasite disappearance from the peritoneum of infected mice. A significant amount of IFN- $\gamma$  was detected earlier in cultures of BALB/c PEC – 3 days PI, than in cultures of C57BL/6 PEC – 7 days PI (Figs 1 and 2). These results can be correlated with the higher sensitivity of C57BL/6 mice to microsporidia infection as shown by Niederkorn *et al.* (1981).

The considerable IFN- $\gamma$  production and increase of CD4+ and CD8+ T lymphocyte numbers in the spleen of C57BL/6 and BALB/c mice indicate that T-cell mediated immunity is activated after p.o. infection with *E. intestinalis*. IFN- $\gamma$  production in *ex vivo* cultures of PPL and splenocytes of immunocompetent mice was recorded. No considerable changes in the number of PEC and the absence of the parasite in peritoneum of immunocompetent mice after p.o. infection imply that immunocompetent mice resolved the p.o. infection locally in the intestine. It has been shown that both CD4+ and CD8+ T lymphocyte subpopulations play an important role in the protection against the lethal outcome of p.o. infection with either *E. cuniculi* or *E. intestinalis* (Braunfuchsová *et al.* 2002, Salát *et al.* 2002).

It is well known that interferons activate NK cells that can then produce IFN- $\gamma$ . The NK cells in SCID mice are probably activated by IL-12 produced by infected macrophages (Braunfuchsová *et al.* 1999). Our study showed that the main source of IFN- $\gamma$  in SCID mice are apparently NK cells, because a correlation exists between the production of IFN- $\gamma$  and the rise of NK cell number in the spleen and peritoneum. Our data show lower IFN- $\gamma$  production in *ex vivo* cultures of splenocytes from SCID mice compared with that in immunocompetent mice. The peak of IFN- $\gamma$  production by PEC of SCID mice was detected significantly later than in BALB/c and C57BL/6 mice (Fig. 3). Furthermore, the IFN- $\gamma$  blood level of SCID mice was lower compared to immunocompetent mice (Fig. 4). Since SCID mice succumb to the infection, the role of NK cells in the protection of the host against *E. intestinalis* infection does not seem to be crucial. The cytotoxic activity of these cells in SCID mice is highest soon before death of the mice (Salát *et al.* 2001). A similar observation was reported by Niederkorn *et al.* (1983) who recorded increased NK activity

in athymic nu/nu mice infected with *E. cuniculi*, but the mice succumbed to the infection.

The role of humoral immunity in microsporidiosis is not clear. In our experiments, anti-*E. intestinalis* antibodies detected by ELISA, appeared in sera of immunocompetent mice on day 7 post i.p. infection, and on day 14 post p.o. infection, respectively. This occurrence corresponds with the disappearance of the parasite from peritoneum of BALB/c and C57BL/6 mice. On the other hand, experimental infections of IFN- $\gamma$  knockout mice with *E. intestinalis* showed that these mice died in spite of high anti-*E. intestinalis* antibody titres in sera (Salát *et al.* 2004). Based on these results, we can speculate that specific antibodies can contribute to the protection of the host, but antibodies alone cannot protect the host against a lethal microsporidiosis.

It can be concluded from the present data that infection with *E. intestinalis* activates both T-cell mediated and humoral immunity in both strains of immunocompetent mice. Intraperitoneal infection leads to the migration of CD4+ and CD8+ T lymphocytes into the peritoneum followed by the production of IFN- $\gamma$  by PEC and splenocytes. Peroral infection initiates early IFN- $\gamma$  production by PPL of infected immunocompetent mice. Missing T-cell mediated immunity in SCID mice is responsible for the development of a lethal disease. Particularly, peroral infection of SCID mice can serve as a model of human infection, on which immunomodulatory treatment or chemotherapy of *E. intestinalis* infection can be tested.

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