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Cytokine response to infection with the microsporidian, *Encephalitozoon cuniculi*

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Abstract. The production of three cytokines, interferon gamma (IFN-γ), interleukin 10 (IL-10) and interleukin 12 (IL-12), was measured after intraperitoneal infection of immunocompetent Balb/c mice and immunodeficient SCID mice with the microsporidian, *Encephalitozoon cuniculi* Levaditi, Nicolau et Schoen, 1923. High levels of IFN-γ were detected in *ex vivo* cultures of peritoneal exudate cells (PEC) of Balb/c mice, a lower, but earlier IFN-γ response was observed in PEC from SCID mice. The early IL-10 response was detected in *ex vivo* cultures of splenocytes from Balb/c but not from SCID mice, explaining a delay in the IFN-γ response in Balb/c mice. IL-12 was detected in PEC cultures from SCID mice, indicating an alternative pathway of IFN-γ production by NK cells stimulated by IL-12 derived from macrophages.

Microsporidia are obligate intracellular protozoan parasites that infect a wide range of invertebrate and vertebrate hosts (Canning and Lom 1986). With the onset of the AIDS pandemic, more attention has been paid to several microsporidians including *Encephalitozoon*, *Enterocytozoon*, *Pleistophora*, *Trachipleistophora*, *Nosema* and *Thelohania* which have been identified causative agents of opportunistic infections in immunocompromised hosts (Orenstein 1991, Weber et al. 1994, Didier et al. 1998). Immunobiology of microsporidial infections has been studied, particularly with the model of *Encephalitozoon cuniculi* Levaditi, Nicolau et Schoen, 1923 infection of Balb/c mice (Schmidt and Shadduck 1983, 1984). *E. cuniculi* is able to persist in its animal host despite an active immune response. Latent infection remains asymptomatic as long as parasite multiplication and host immune response are balanced (Gannon 1980, Schmidt and Shadduck 1983). In immunocompromised hosts, acute, potentially fatal disease can develop.

Humoral immunity may have a role in the control of microsporidial infection. Specific antibodies have been found to have an opsonising effect, increasing phagocytosis of *E. cuniculi* (Niederkorn and Shadduck 1980). An inhibitory effect of monoclonal antibodies directed to potential neutralising-sensitive epitopes on spores on the infection of Vero cells with *Encephalitozoon intestinale* (Hartskeerl et al. 1995) has been demonstrated (Enriquez 1997). However, adoptive transfer with immune sera could not protect athymic mice from a lethal *E. cuniculi* infection (Schmidt and Shadduck 1983).

A central role of cell-mediated immunity in the defence against a microsporidial infection has been demonstrated using athymic mice. They could be protected from the lethal *E. cuniculi* infection by adoptive transfer with sensitised T-enriched spleen cells but not with naive spleen cells (Schmidt and Shadduck 1983). Splenic lymphocytes from *E. cuniculi*-infected mice, when incubated *in vitro* with *E. cuniculi* antigens, released cytokines which activated macrophages to destroy the microsporidia (Schmidt and Shadduck 1984). As it has been shown later (Didier et al. 1994), interferon gamma (IFN-γ) seems to be the most important among these cytokines. It, alone or in combination with lipopolysaccharide (LPS), could activate murine macrophages to destroy microsporidia. The significant role of IFN-γ in the control of *E. intestinale* infection has been confirmed recently using IFN-γ receptor knockout mice (Achbarou et al. 1996). Macrophages kill microsporidia by mechanisms dependent on reactive nitrogen intermediates (Didier 1995).

As mentioned above, infection of immunodeficient mice contributed markedly to the understanding of pathogenesis of microsporidial infections and immune response to microsporidia. In addition to athymic nude mice, mice with the severe combined immunodeficiency (SCID) (Bosma et al. 1983) have been exploited in this research (Koudela et al. 1993, Heřmánek et al. 1993).

As little is known about the cytokine control of the immune response to microsporidial infection, we monitored the cytokine response of immunocompetent Balb/c mice and immunodeficient SCID mice to the infection with *E. cuniculi* spores.

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MATERIALS AND METHODS

Mice. Balb/c female mice of 7-9 weeks of age were purchased from Charles River Laboratory, Germany. 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 sterilised before use.

Organisms. *Encephalitozoon cuniculi*, strain EC2, originally isolated from dexamethasone-treated laboratory mouse (Koudela et al. 1994), was grown in VERO E6 cells for provision of spores. Cells were cultivated in RPMI 1640 medium supplemented with 5% fetal calf serum. The spores were purified using a SEPHACRYL S-300 column (Pharmacia), washed three times in deionised water and stored in deionised water with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin; Sigma). Before inoculation, the spores were washed in PBS three times, counted on a haemocytometer and adjusted to 2×10^7 per ml in PBS.

Experimental protocol. Twelve ten-week-old SCID mice were injected i.p. with 10^7 spores of *E. cuniculi*. On days 3, 7, 11 and 15 post infection, three mice for each time interval were killed. Twenty one Balb/c mice were infected the same way and groups of three mice were killed on days 3, 7, 11, 15, 21, 28 and 35 post infection. Groups of three uninfected mice of both strains served as a control.

Mice were killed humanly and bled by a cardiac puncture. Individual sera were collected and stored at -70°C until used for cytokine enzyme-linked immunosorbent assay (ELISA). Peritoneal exudate cells (PEC) were recovered by lavaging the peritoneum with 2 ml of cold RPMI 1640. A suspension of spleen cells was generated in RPMI 1640 medium supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol and antibiotics and seeded into 24-well tissue culture plates (NUNC), 10^7 cells in 1 ml. PEC were washed in RPMI 1640 medium and plated at 10^6/ml in 24-well culture plates. After 24 h incubation at 37°C and 3.5% CO_2, supernatants from both splenocyte and PEC cultures were harvested and stored at -70°C for cytokine ELISA.

Measurement of cytokine production. Cytokine levels in serum and cell culture supernatants were measured by capture ELISA, following the manufacturer's protocol, using the following pairs of rat anti-mouse interleukin monoclonal antibodies (Mabs) (PharMingen, San Diego, CA): interferon gamma (IFN-γ), unconjugated Mab clone R4-6A2 and biotinylated Mab clone XMG1.2; IL-10, unconjugated Mab JES5-2A5 and biotinylated SXC-1; IL-12, unconjugated Red-T/G297-289 monoclonal antibody cocktail and biotinylated Mab clone C17.8.

Micro E.I.A./R.I.A. plates, flat bottom, high binding (Costar, Cambridge, MA) were coated with purified Mab (4 μg/ml) diluted with 0.1 M sodium carbonate buffer (pH 8.2). One hundred μl of diluted Mab, per well, were incubated overnight at 4 °C. After washing two times with 0.05% Tween 20 in PBS (T-PBS), unoccupied sites were blocked with 200 μl of 10% newborn calf serum (NBCS) in PBS for one hr at room temperature. Plates were washed four times with T-PBS and incubated with undiluted sera or cell culture supernatants overnight at 4°C. Biotinylated antibody (2 μg/ml in PBS 10% NBCS) was then added at 100 μl per well and incubated 45 min at room temperature. Plates were washed six times and 100 μl streptavidin-peroxidase (Sigma) diluted 2400-fold in PBS 10% NBCS was allowed to incubate for 30 min at room temperature. After washing eight times, an enzymatic colour reaction was generated using 2,2'-azino-bis-(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS) substrate (Sigma), stopped after 10 min with 100 μl of 1% sodium dodecylsulphate in water and measured at 405 nm with an ELISA spectrophotometer (Labsystems Multiskan). Cytokine levels were determined from standard graphs generated from known quantities of recombinant interleukin standards (PharMingen). Results show the mean of triplicate wells.

RESULTS

The levels of three cytokines, IFN-γ, IL-10 and IL-12, were determined in sera and in *ex vivo* cultures of PEC and splenocytes from Balb/c and SCID mice after intraperitoneal injection of *E. cuniculi* spores. In comparison with sera, much higher cytokine levels were found in PEC cultures.

The dynamics of IFN-γ in the supernatant of 24 hr PEC cultures from Balb/c mice is shown in Fig. 1. The steep rise of this cytokine started after day 11 post infection with the maximum 3.5 ng/ml on day 15. The cytokine level then decreased and the second maximum was recorded on day 28, followed by a decline to the level of 0.5 ng/ml on day 35. Similar course of the levels of IFN-γ was recorded in serum from Balb/c mice, while the cytokine concentrations were about 20× lower (Fig. 2). The dynamics of IFN-γ in cultures of Balb/c splenocytes showed only one maximum on day 15 (Fig. 3). The maximum cytokine concentration was similar to that in the serum.

In the supernatant of Balb/c splenocyte cultures, IL-10 was detected reaching high levels as early as 3 days post infection (Fig. 4). The maximum was recorded on day 7 post infection, followed by a drop to the lowest level on day 11. The levels then slightly increased, keeping the value about 250 pg/ml until the end of the experiment. We were unable to detect IL-10 either in cultures of PEC or in sera of infected Balb/c mice. IL-12 was not detected in any of the material tested from Balb/c mice.

In SCID mice, only IFN-γ and IL-12 were recorded. In PEC cultures, IFN-γ started to rise on day 7 post infection, that is 4 days earlier than in Balb/c mice (Fig. 5). Similarly, the maximum of this cytokine was on day 11, compared with day 15 for Balb/c mice. At the end of the experiment, the cytokine concentration was about half of the maximum value. In the supernatant of PEC cultures from SCID mice, increasing levels of IL-12 were detected from day 3 post infection to the end of the experiment (Fig. 6). None of the tested cytokines was detected either in sera or in splenocyte cultures of

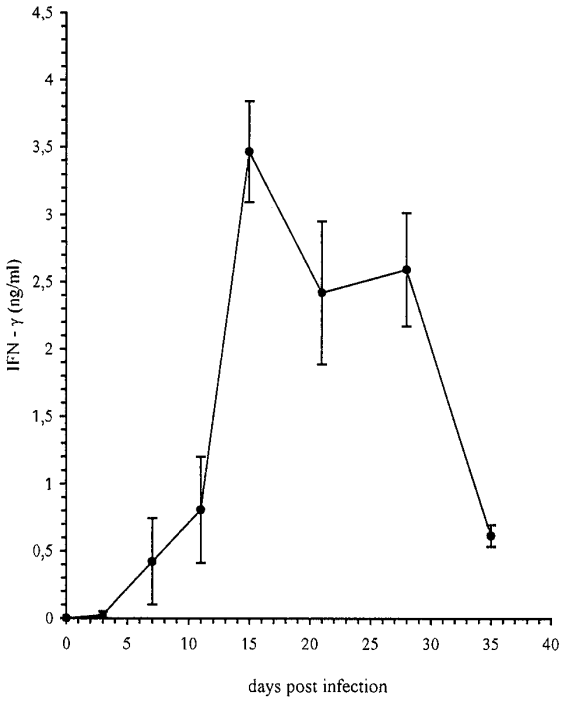

Fig. 1. Levels of IFN-γ in the supernatants of PEC cultures from Balb/c mice intraperitoneally infected with *Encephalitozoon cuniculi* spores. Mean of three measurements with SD.

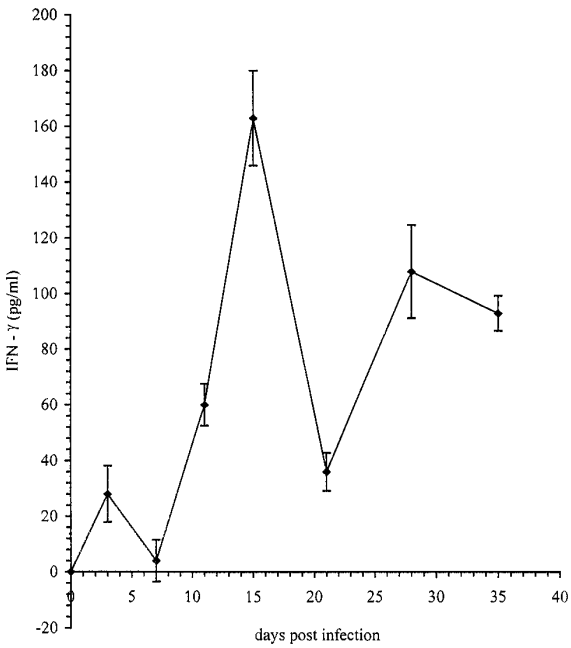

Fig. 2. Levels of IFN-γ in sera from Balb/c mice intraperitoneally infected with *Encephalitozoon cuniculi* spores. Mean of three measurements with SD.

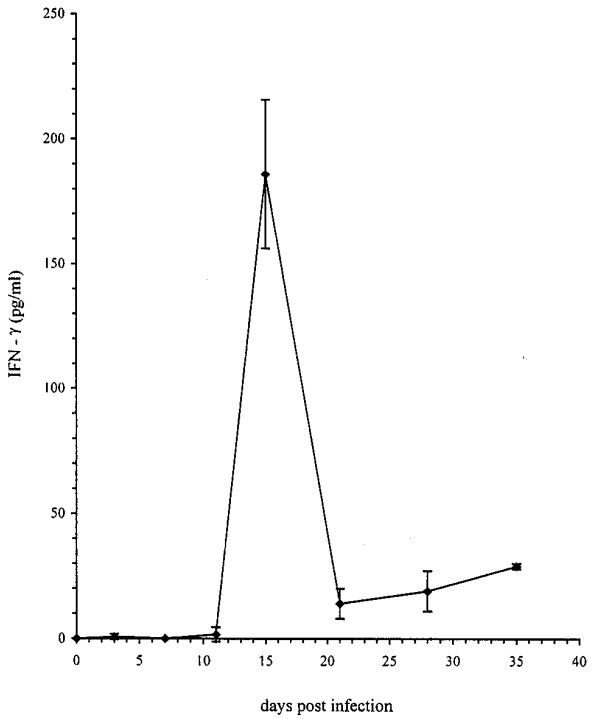

Fig. 3. Levels of IFN-γ in the supernatants of splenocyte cultures from Balb/c mice intraperitoneally infected with *Encephalitozoon cuniculi* spores. Mean of three measurements with SD.

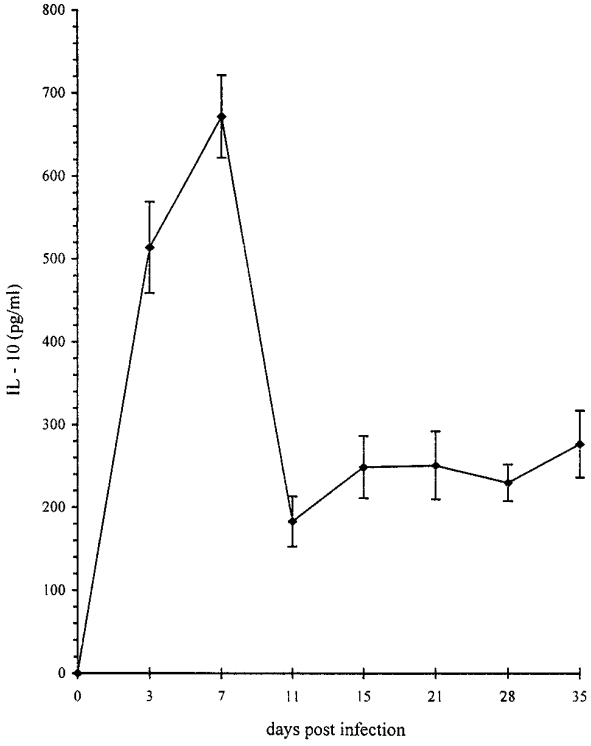

Fig. 4. Levels of IL-10 in the supernatants of splenocyte cultures from Balb/c mice intraperitoneally infected with *Encephalitozoon cuniculi* spores. Mean of three measurements with SD.

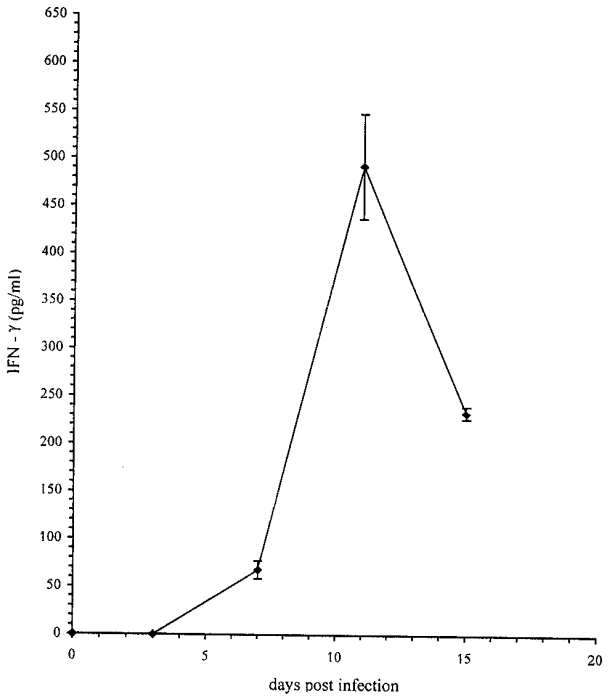

Fig. 5. Levels of IFN-γ in the supernatants of PEC cultures from SCID mice intraperitoneally infected with *Encephalitozoon cuniculi* spores. Mean of three measurements with SD.

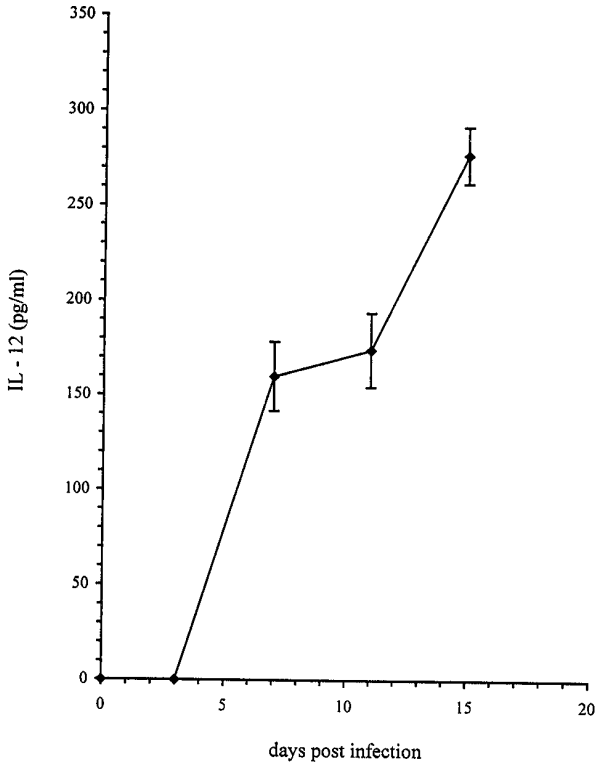

Fig. 6. Levels of IL-12 in the supernatants of PEC cultures from SCID mice intraperitoneally infected with *Encephalitozoon cuniculi* spores. Mean of three measurements with SD.

SCID mice. IL-10 was not found in any of the material tested from SCID mice.

DISCUSSION

In the immunobiology of microsporidial infections the role of both humoral and cellular immunity has been demonstrated. As microsporidia are intracellular parasites, the role of cell-mediated immunity is probably more significant (Schmidt and Shadduck 1983, 1984). The cytokine control of the immune response to microsporidia has not been studied in detail.

In this study, we demonstrated the production of IFN-γ, the macrophage-activating cytokine, in both immuno-competent Balb/c mice and mice with severe combined immunodeficiency. The significance of IFN-γ in the control of microsporidial infections has been recently demonstrated (Didier et al. 1994, Achbarou et al. 1996). Macrophages activated by IFN-γ kill microsporidia by the nitric oxide-dependent mechanism (Didier 1995). A much lower production of IFN-γ by SCID mice, compared with their immunocompetent counterparts can, in part, explain a higher sensitivity of SCID mice to *E. cuniculi* infection, which is lethal for immunodeficient mice. As the SCID mice lack any functional T- and B-lymphocytes (Bosma 1989), the source of IFN-γ is different from that of immunocompetent mice. While various subsets of T lymphocytes are the main source of IFN-γ in immunocompetent mice, in SCID mice this cytokine is produced by NK cells activated by IL-12 derived from macrophages (Bancroft et al. 1991, Bancroft 1993). IL-12 was demonstrated in gradually increasing levels in the PEC supernatant from SCID mice. The rapid rise of this cytokine preceded the elevation of IFN-γ.

IFN-γ was found in much higher concentrations in supernatants from 24 hr cultures of PEC from Balb/c mice than in similar cultures of splenocytes or in blood serum. In SCID mice, PEC cultures were the only source of this cytokine among the tested material. The reason could be the local inflammation and migration of immunocompetent cells into the peritoneal cavity.

In cultures of splenocytes from Balb/c mice, another important cytokine, IL-10, was detected. This cytokine is produced by the Th2 subset of CD4+ T lymphocytes, by activated B lymphocytes and activated macrophages (Abbas et al. 1994). Formerly called cytokine synthesis inhibitory factor, IL-10 suppresses cytokine production by the Th1 subset, including IFN-γ (Mosmann and Moore 1991). The early appearance of IL-10 could explain the delayed rise of IFN-γ in the PEC super-

natant of Balb/c mice in comparison with SCID mice, where no measurable IL-10 was detected. The early induction of IL-10 can also explain the transient immunosuppressive effect of *E. cuniculi* infection, which was selectively restricted to the T-cell-mediated immunity (Didier and Shadduck 1988).

In conclusion, the first information about the cytokine response to *in vivo* infection with the microsporidian *E. cuniculi* was obtained. The production of macrophage-activating cytokine – IFN-γ, playing the central role in the anti-microsporidial immunity, was confirmed, and an involvement of IL-10 in the immunosuppression accompanying microsporidial infections was suggested. Further investigations should be focused on the polarisation of the immune response and the role of various subpopulations of lymphocytes in the elimination of microsporidia from a host body. For these studies, immune reconstitution of SCID mice can be exploited.

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