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Humoral intestinal immunity against *Encephalitozoon cuniculi* (Microsporidia) infection in mice

Bohumil Sak and **Oleg Ditrich**

Institute of Parasitology, Academy of Sciences of the Czech Republic and Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

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Abstract. Three strains of mice, BALB/c, IL-12 knock-out (KO) and INF-γ knock-out, were chosen as an experimental model for the study of intestinal immunity induction against *Encephalitozoon cuniculi* Levaditi, Nicolau et Schoen, 1923 infection. Mice were infected perorally with 10^7 spores and re-infected with the same dose 70 days after the first infection. The anti-*E. cuniculi* IgA, IgG and IgM responses in sera and extracts of stool samples were determined by ELISA. Results have shown specific antibody production in the sera and intestinal secretions of all three strains of mice induced orally by *E. cuniculi* spores. BALB/c mice developed a stronger humoral immune response than IL-12 KO mice. The lowest antibody response developed in INF-γ KO mice that succumbed to the infection within 28 days post infection.

Most gastrointestinal pathogens are transmitted by food or drink contaminated with a faecal material. The sanitary disposal of waste and vermin, clean drinking water, hand washing and thorough cooking of food can reduce exposure. Where hygiene fails, diarrhoeal disease can become rampant. Infection induces a cascade of immunological events that involve both components of the innate and adaptive immunity. Normal defence against ingested pathogens includes (1) acid gastric juice, (2) the viscous mucus layer covering the gut, (3) lytic pancreatic enzymes including bile detergents, and (4) secreted immunoglobulin A (IgA) antibodies. IgA antibodies are produced by the B cells located in mucosa-associated lymphoid tissues (MALTs) which are covered by a single layer of specialised epithelial cells called M cells. M cells are important for transport of antigen to MALT and for binding or uptake of numerous gut pathogens.

Encephalitozoon cuniculi Levaditi, Nicolau et Schoen, 1923 is an intracellular parasite that infects its host mainly through the oral route. Cell-mediated immunity is currently believed to be main defence of organisms against microsporidial infection. Khan et al. (2001) suggested that a protective immune response against microsporidia is mediated by cytotoxic CD8+ T cells. However, very little is known about the possible protective role of antibodies in the control of microsporidial infection especially in gut.

MATERIALS AND METHODS

Parasites. The spores of *Encephalitozoon cuniculi* strain EC2 were originally isolated from a dexamethasone-treated laboratory mouse (Koudela et al. 1994) and grown *in vitro* in VERO E6 cells (Green monkey kidney cells) maintained in RPMI-1640 medium (SIGMA) supplemented with 2.5% foetal bovine serum (FBS). Spores were isolated and purified from cells by centrifugation over 50% Percoll (SIGMA) at 1,100 *g* for 30 min, and washed three times in deionized water before storing in deionized water supplemented with antibiotics (SIGMA, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin) at 4°C. The spores were washed in PBS before use.

Mice. Adult 6–8 weeks old female BALB/c mice (Charles River Laboratory, Germany), female IL-12 KO mice (strain B6.129-Il12b^{tm1Ts} of the BALB/c background; The Jackson Laboratory, Bar Harbor, Maine, USA) and female INF-γ KO mice (strain C1297S7(B6)-Ifng^{tm1Jm} of the C57BL/6 background; The Jackson Laboratory, Bar Harbor, Maine, USA) were used for the study of intestinal immunity *in vivo*. Mice were housed in plastic cages with wood-chip bedding situated in a mouse room with a constant temperature of 22°C and a relative humidity of 65%. Food and water were provided *ad libitum*.

Enzyme-linked immunosorbent assay (ELISA). The ELISA test was performed according to the method of Hollister and Canning (1987). Briefly, *E. cuniculi* spores were incubated with appropriate undiluted stool supernatants or sera diluted 1:200, and then with 1:10,000 diluted peroxidase-labeled goat anti-mouse IgA (α-chain specific; SIGMA), goat anti-mouse IgG (whole molecule; SIGMA) or goat anti-mouse IgM (µ-chain specific; SIGMA). The colour reaction was developed with 0.1 M acetate substrate solution (pH 5.5) supplemented with 2.5 mM o-phenylendiamine (OPD) and 0.03% H_2O_2. The reaction was then stopped with 2 M H_2SO_4.

The study of immune response. Mice, eight of each strain, were infected perorally with 10^7 spores and re-infected with the same dose 70 days after the first infection. Negative control groups consisted of eight animals of each strain as well. The anti-*E. cuniculi* IgA, IgG and IgM responses in sera

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Address for correspondence: B. Sak, Institute of Parasitology, Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic. Phone: ++420 387 775 419; Fax: ++420 385 310 388; E-mail: casio@paru.cas.cz

and extracts of stool samples were determined by ELISA and the absolute IgA, IgG and IgM concentrations were calculated on the basis of optical density of positive controls containing known amount of antibodies.

The stool samples were obtained daily from each mouse separately. They were weighted, mixed in 1 ml of PBS containing 1% FBS, centrifuged at 1,100 *g* for 10 min and the supernatants stored at –20°C until used. Blood samples were taken daily from the retroorbital sinus of each mouse and the sera stored at –20°C until used.

RESULTS

BALB/c mice developed the strongest humoral immune response (Fig. 1A). First IgM antibody appeared in sera and reached the highest concentration at day 16 post infection (p.i.). At day 8 p.i. the IgG isotype appeared and increased till day 24 p.i. The increase of IgA antibodies was recorded at day 17 p.i. and culminated at day 22 p.i. After re-infection the reaction was faster and stronger, the concentration of IgG reaching 4.2 times of the negative control concentration (NCC). In stool extracts (Fig. 2A) both IgA and IgG antibodies appeared at the same time and reached the highest concentration at day 21 p.i. (IgA) or 25 p.i. (IgG). Again, after re-infection the reaction was faster and stronger and the IgA concentration rose up to 10.5 times of NCC. No significant increase in IgM antibodies was observed. The BALB/c mice expressed no clinical symptoms.

The IL-12 KO mice developed weaker immune response than BALB/c mice both in sera and in stool extracts. The increase of IgM and IgA concentration in sera at the beginning of the infection was only 25% of NCC or 47% of NCC, respectively. The IgG reached the maximum point of 2.7 times of NCC at day 27 p.i. After re-infection, the concentration of IgG in sera rose to 3.4 times of NCC (Fig. 1B). The intestinal response was slower than in BALB/c mice, the IgA concentration reached the maximum (5.6 times of NCC) at day 28 p.i., and IgG at day 32 p.i. Again, after re-infection the reaction was faster and stronger and the IgA concentration increased to 8.6 times of NCC. No significant increase in IgM antibody was observed (Fig. 2B). The IL-12 KO mice suffered the infection without any clinical signs like the BALB/c mice.

INF-γ KO mice developed the lowest response. The observed increase of all three antibodies concentration in sera at the beginning of the infection was very weak (Fig. 1C). IgG in sera reached the maximum 2.2 times of NCC at day 19 p.i. The IgA intestinal response was stronger in comparison with IL-12 KO mice and the IgA concentration reached the maximum (6.1 times of NCC) at day 19 p.i. No significant increase in IgM antibody was observed in stool extracts (Fig. 2C). These mice revealed severe clinical symptoms including diarrhoea, bristled hair and general illness from day 14 p.i. and they succumbed to the infection within 28 days p.i.

DISCUSSION

Very little is known about the possible preventive role of antibodies in the control of microsporidial infection, especially in the gut. Induction of strong gut immunity in response to oral *Encephalitozoon cuniculi* infection, characterized by a significant and rapid increase in the intraepithelial lymphocyte (IEL) population, was studied by Moretto at al. (2004). The immune IELs exhibited significant Ag-specific *ex vivo* cytotoxicity and elevated cytokine messages during infection. It is generally agreed that protective immune response against microsporidia is mediated by cytotoxic CD8+ T cells (Braunfuchsová et al. 2001, Khan et al. 2001). However, the results of Braunfuchsová et al. (2002) indicate that whereas CD8+ T cells are critical for the protection against an intraperitoneal *E. cuniculi* infection, both CD4+ and CD8+ T lymphocyte subpopulations play a substantive protective role in a peroral infection, i.e. natural route of infection.

Both IL-12 KO mice and INF-γ KO mice have a defect in cytokine production that leads to immunosuppression. Genetically engineered knock-out mice are the most important type of model animals for investigating the progression of opportunistic infections in the absence of effective immune mediator. IL-12 is an important regulatory cytokine that has a function central to the initiation and regulation of cellular immune responses. It has the capacity to regulate the differentiation of naive T cells into Th1 cells that are crucial in determining the resistance and type of response elicited to a particular pathogen.

IL-12 is a natural killer cell activator which stimulates the growth and function of T cells, induces T and NK-cells to produce INF-γ and alters the normal cycle of apoptotic cell death. Monocytes, B-cells and other accessory cells can produce IL-12 (Trinchieri et al. 1992, Trinchieri 1993). In our experiments, the absence of functional IL-12 surprisingly led to a decrease in antibody production while the Th2 response was not intensely inhibited due to Th1 cytokines. IL-12 KO mice, however, survived the infection without any clinical symptoms, in contrast to IL-12 KO mice infected i.p. with 10^7 *E. cuniculi* spores, which died by day 29 p.i. in another experiment (Khan and Moretto 1999). The survival of our mice could be explained by the route of infection, where we infected the mice perorally, whereas Khan and Moretto (1999) chose the intraperitoneal route.

INF-γ, the main immune messenger, is produced by Th1 cells and especially by NK cells. INF-γ enhances IL-12 production by mononuclear cells whereas IL-10 and IL-4 that are products of Th2 cells efficiently inhibit it (Trinchieri et al. 1992). In our study, a defect in INF-γ production led to the weakest immune response and consequently death of the mice within 28 days p.i. Also

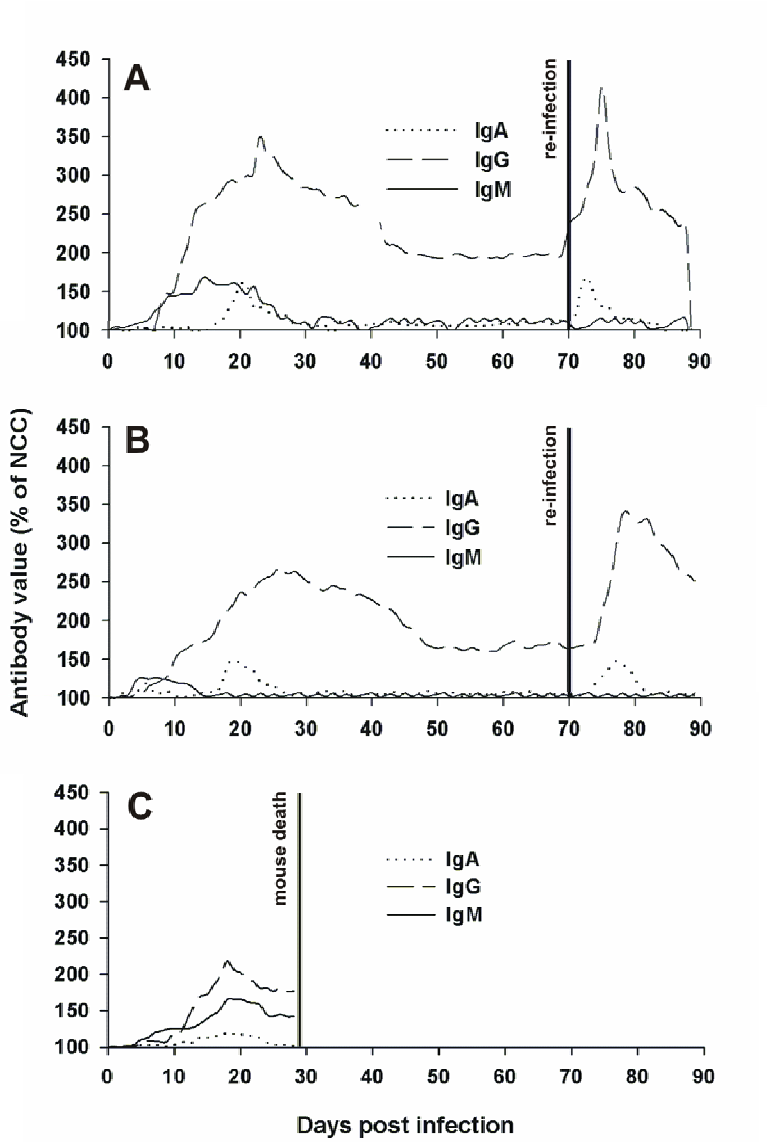

Fig. 1. Specific antibody concentrations in sera of mice infected with *Encephalitozoon cuniculi* (mean values of eight mice). **A** – BALB/c mice; **B** – IL-12 KO mice; **C** – INF-γ KO mice. NCC – negative control concentration.

Salát et al. (2004) obtained similar results; the mean survival time of their INF-γ KO mice perorally infected with *E. cuniculi* was 33.2 ± 5.4 days. These results differ from those obtained by El Fakhry et al. (1998). They infected mice defective in INF-γ receptor (INF-γ $R^{0/0}$) with *Encephalitozoon intestinalis* and these mice survived 60 days without any visible clinical signs. However, splenomegaly, enlargement of the biliary tract and the occurrence of numerous nodules in the liver and small intestine wall were observed in infected INF-γ $R^{0/0}$ mice autopsied 4 weeks p.i. Achbarou et al. (1996) also described the development of chronic infection in INF-γ $R^{0/0}$ mice infected with *E. intestinalis*. The infection was not lethal over a period of 6 months. In another study the dissemination of *E. intestinalis* was described using the PCR technique and a low morbidity of the infection in INF-γ $R^{0/0}$ mice was emphasised (El Fakhry et al. 2001). The differences in the course of infection could be caused by different level of specialisation of these two microsporidians compared. Whereas *E. intestinalis* is more specialised for the intestinal mucosa, *E. cuniculi* has higher tendencies to disseminate via blood stream into all organs. Thus, the infection can expand faster and damages the inner organs, which leads to clinical symptoms and death.

It is well known that IgG isotype predominate in serum, but more surprising was the high level of IgG antibodies in gut secretions since IgG levels in intestinal secretions of mice are normally very low (Haneberg et al. 1994). O'Neal et al. (2000) described that IgG levels in intestinal secretions of normal mice that were rotavirus immunized and protected were low as expected, but

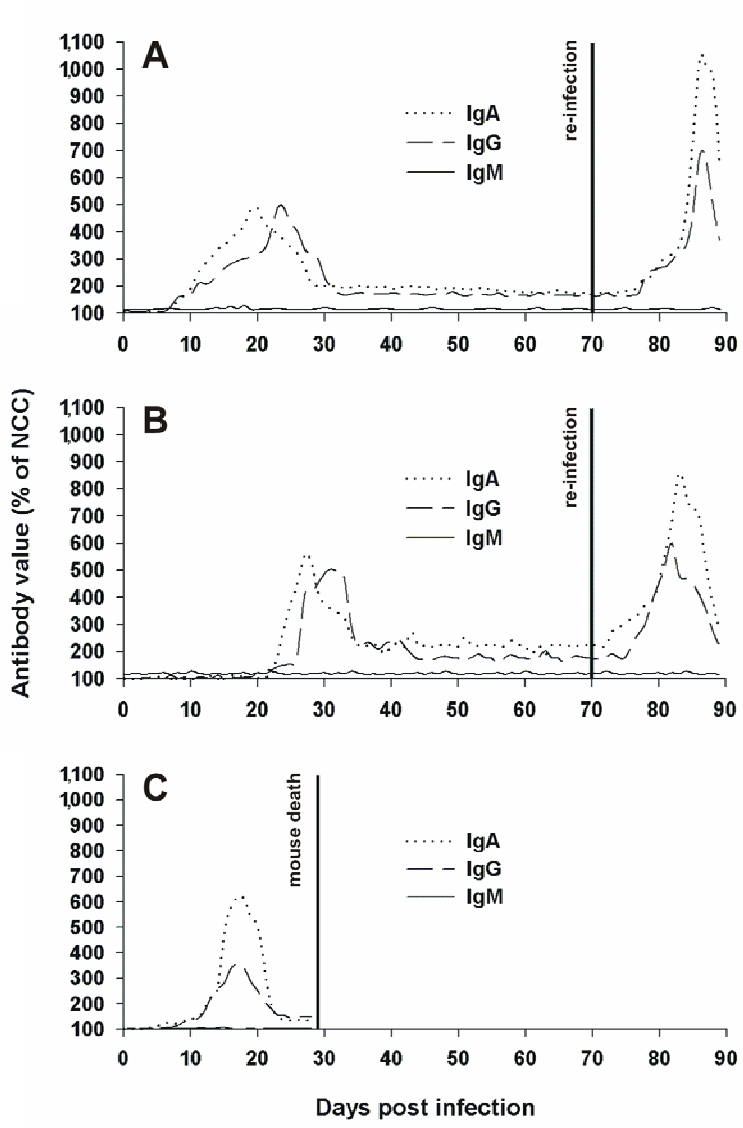

Fig. 2. Specific antibody concentrations in stool extracts of mice infected with *Encephalitozoon cuniculi* (mean values of eight mice). **A** – BALB/c mice; **B** – IL-12 KO mice; **C** – INF-γ KO mice. NCC – negative control concentration.

the immunized IgA knock-out mice (which were also protected) had elevated antiviral IgG levels in intestinal secretions. It seems that in the case of rotavirus infection, IgG can supply the IgA production. IgG can be sufficient to protect mucosal surfaces where significant amounts of IgG, transudated from serum or produced locally, are normally present in secretions (Murphy 1999). In contrast, the reovirus-immunized IgA knock-out mice did not have elevated antiviral IgG in intestinal secretions and were not protected against re-infection despite high levels of antireovirus IgG in serum (Silvey et al. 2001).

We obtained results completely different from those of El Fakhry et al. (1998). Both of their mouse groups, wild-type and INF-γ $R^{0/0}$ mice, at day 15 p.i. displayed the same low reactivity in all three anti-*E. intestinalis* isotypes (IgM, IgG, IgA) in sera. IgA isotype remained low in all studied mice throughout the 60-day study period. IgM titres increased slowly from day 15 to day 30 p.i. in infected INF-γ $R^{0/0}$ mice and then decreased slowly from day 30 p.i. to the end of the study. IgM titres remained low in wild-type mice and were similar to those of IgA. The major difference observed between both groups concerned IgG titres. In mutant mice they increased continuously from 1:1,000 at day 15 p.i. to 1:16,000 at day 45 p.i. and then they reached 1:19,000 at day 60 p.i. In wild-type mice IgG titres remained low. They increased from 1:1,000 at day 15 p.i. to 1:2,500 at day 45 p.i. and this value did not vary until the end of the study. We also obtained a higher production of IgG than the IgA isotype in sera. However, our INF-γ KO mice developed lower immune response than immunocompetent BALB/c mice.

On the basis of our results obtained in immunodeficient mouse models, we could conclude that both IgA and IgG antibodies were produced in intestine, however they were not able to prevent perorally acquired microsporidial infection. The humoral immunity is part of complex defence mechanisms against microsporidiosis. Main important barrier for multiplication of microsporidia probably is intraepithelial lymphocytes (Moretto et al. 2004) and humoral antibodies produced in intestine could enhance its effect.

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REFERENCES

ACHBAROU A., OMBROUCK C., GNERAGBE T., CHARLOTTE F., RENIA L., DESPORTES-LIVAGE I., MAZIER D. 1996: Experimental model for human intestinal microsporidiosis in interferon gamma receptor knock-out mice infected by *Encephalitozoon intestinalis*. Parasite Immunol. 18: 387–392.

BRAUNFUCHSOVÁ P., SALÁT J., KOPECKÝ J. 2001: CD8+ T lymphocytes protect SCID mice against *Encephalitozoon cuniculi* infection. Int. J. Parasitol. 31: 681–686.

BRAUNFUCHSOVÁ P., SALÁT J., KOPECKÝ J. 2002: Comparison of the significance of CD4+ and CD8+ T lymphocytes in the protection of mice against *Encephalitozoon cuniculi* infection. J. Parasitol. 88: 797–799.

El FAKHRY Y., ACHBAROU A., DESPORTES-LIVAGE I., MAZIER D. 1998: *Encephalitozoon intestinalis*: humoral responses in interferon-γ receptor knock-out mice infected with a microsporidium pathogenic in AIDS. Exp. Parasitol. 89: 113–121.

El FAKHRY Y., ACHBAROU A., FRANETICH J.F., DESPORTES-LIVAGE I., MAZIER D. 2001: Dissemination of *Encephalitozoon intestinalis*, a causative agent of human microsporidiosis, in IFN-gamma receptor knock-out mice. Parasite Immunol. 23: 19–25.

HANEBERG B., KENDAL D., AMERONGEN H.M., APTER F.M., KRAEHENBUHL J.P., NEUTRA M.R. 1994: Induction of specific immunoglobulin A in the small intestine, colon-rectum, and vagina measured by a new method for collection of secretions from local mucosal surfaces. Infect. Immun. 62: 15–23.

HOLLISTER W.S., CANNING E.U. 1987: An enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to *Encephalitozoon cuniculi* and its use in determination of infections in man. Parasitology 94: 209–219.

KHAN I.A., MORETTO M. 1999: Role of gamma interferon in cellular immune response against murine *Encephalitozoon cuniculi* infection. Infect. Immun. 4: 1887–1893.

KHAN I.A., MORETTO M., WEISS L.M. 2001: Immune response to *Encephalitozoon cuniculi* infection. Microbes Infect. 3: 401–405.

KOUDELA B., LOM J., VÍTOVEC J., KUČEROVÁ Z., DITRICH O., TRÁVNÍČEK J. 1994: *In vivo* efficacy of albendazole against *Encephalitozoon cuniculi* in SCID mice. J. Eukaryot. Microbiol. 41: 49–50.

MORETTO M., WEISS L.M., KHAN I.M. 2004: Induction of a rapid and strong antigen-specific intraepithelial lymphocyte response during oral *Encephalitozoon cuniculi* infection. J. Immunol. 172: 4402–4409.

MURPHY B.R. 1999: Mucosal immunity to viruses. In: P.L. Ogra, J. Mestecky, M.E. Lamm, W. Strober, J. Bienenstock and J.R. McGhee (Eds.), Mucosal Immunology. Academic Press, San Diego, California, pp. 695–707.

O'NEAL C., HARRIMAN G.R., CONNER M.E. 2000: Protection of the villus epithelial cells of the small intestine from rotavirus infection does not require immunoglobulin Am. J. Virol. 74: 4102–4109.

SALÁT J., SAK B., LE T., KOPECKÝ J. 2004: Susceptibility of IFN-γ or IL-12 knock-out and SCID mice to infection with two microsporidian species, *Encephalitozoon cuniculi* and *E. intestinalis*. Folia Parasitol. 51: 275–282.

SILVEY K.J., HUTCHINGS A.B., VAJDY M., PETZKE M.M., NEUTRA M.R. 2001: Role of immunoglobulin A in protection against reovirus entry into murine Peyer's patches. J. Virol. 75: 10870–10879.

TRINCHIERI G. 1993: Interleukin-12 and its role in the generation of Th1 cells. Immunol. Today 14: 335–338.

TRINCHIERI G., WYSOCKA M., D'ANDREA A., RENGARAJU M., ASTE-AMEZAGA M., KUBIN M., VALIANTE N.M., CHEHIMI J. 1992: Natural killer cell stimulatory factor (NKSF) or interleukin 12 is a key regulator of immune response in inflammation. Progress in Growth Factor Research 4: 355–368.

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