

Effects of interferon gamma and specific polyclonal antibody on the infection of murine peritoneal macrophages and murine macrophage cell line PMJ2-R with *Encephalitozoon cuniculi*

Jiří Jelínek^{1,2}, Jiří Salát^{1,2}, Bohumil Sak¹ and Jan Kopecký^{1,2}

¹Institute of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic;

²Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

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Abstract. Experimental activation of peritoneal macrophages by interferon gamma (IFN- γ) resulted in the inhibition of *Encephalitozoon cuniculi* replication. However, *E. cuniculi* could replicate either in a non-activated cell line of murine macrophages PMJ2-R or in IFN- γ -activated PMJ2-R cells. Moreover, activation with IFN- γ led to faster replication of *E. cuniculi* in these cells. Opsonisation of *E. cuniculi* spores with anti-*E. cuniculi* polyclonal antibody did not affect *E. cuniculi* replication in both, non-activated and activated murine macrophages. In contrast, opsonisation of *E. cuniculi* spores caused the most effective replication of *E. cuniculi* in activated PMJ2-R cells. However, production of nitric oxide by these cells was significantly more intensive than that in non-activated, infected cells, where the parasite replicated to a much lesser extent. Our results support the hypothesis that *E. cuniculi* uses phagocytosis for the infection of host cells. They also indicate that the mechanism by which spores of *E. cuniculi* are killed by macrophages is not dependent on nitric oxide and they reveal that PMJ2-R cells cannot substitute peritoneal murine macrophages in immunological studies on *E. cuniculi*.

The microsporidium *Encephalitozoon cuniculi* Levaditi, Nicolau et Schoen, 1923 (Fungi: Microsporidia) is a single-cell intracellular parasite of mammals, that causes mainly opportunistic infections in humans (Didier 2005). It can infect the host cell by penetration of the plasma membrane, using extrusion of the polar tube and passing of the infectious sporoplasm through the polar tube into the host cell. Another way by which *E. cuniculi* infects the host cell is phagocytosis of its spores. Spores are rapidly digested in the phagolysosome. However, some spores discharge the polar tube from the phagolysosome to the surrounding cytoplasm and infect the host cell by the sporoplasm injected through the polar tube (Franzen 2005). The entry of *E. cuniculi* spores into the host cell could be inhibited by cytochalasin D. These observations suggest that the entry of spores to the host cell is mediated by directed, actin-dependent phagocytosis (Franzen 2005).

Although *E. cuniculi* can replicate in non-activated murine macrophages, the replication is stopped in murine macrophages activated with a dose of 100 U of interferon gamma (IFN- γ) per ml of culture medium (Didier 1995). The way by which macrophages prevent *E. cuniculi* replication is still obscure. It was proposed that nitric oxide is implicated in the killing of *E. cuniculi* spores in murine macrophages (Didier 1995). This hypothesis was rejected using mice with functionless nitric oxide synthetase, as these mice withstood high doses of pathogen challenge similarly to normal wild-type animals (Khan and Moretto 1999).

Very little is known about the role of antibodies in *E. cuniculi* infection. Although *E. cuniculi* induces strong humoral immune response against various antigens of its spores and the opsonisation of *E. cuniculi* spores by antibody decreases the infectivity of its spores in non-professional phagocytes *in vitro* (Schmidt and Shadduck 1984, Enriquez et al. 1998, Sak et al. 2004), anti-*E. cuniculi* antibodies do not have a protective effect (Schmidt and Shadduck 1983, Salát et al. 2004).

PMJ2-R cell line used in our study originated from peritoneal macrophages of C57BL 6J mice and was derived using infection by J2 retrovirus (*v-raf*, *v-myc*) *in vivo*. PMJ2-R cells possess surface antigens of peritoneal macrophages (including Fc receptor), they show anti-tumour activity, produce interleukin 6 (IL-6) after stimulation with lipopolysaccharide (LPS), but do not produce interleukin 1 (IL-1) and tumour-necrosis factor- α (TNF- α) (Adami et al. 1993).

The main task of this study was to compare the effect of macrophage activation with IFN- γ and opsonisation of spores by specific polyclonal antibody on the replication of *E. cuniculi* in murine peritoneal macrophages and murine macrophage cell line PMJ2-R *in vitro*.

MATERIALS AND METHODS

***Encephalitozoon cuniculi* spores.** *Encephalitozoon cuniculi* strain EC2, originally isolated from a dexamethasone-treated laboratory mouse (Koudela et al. 1994), was 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 by centrifugation over 50% Percoll (Sigma) at 1,100 g for 30 minutes. They were then washed three times in deionized water and stored in deionized water supplemented with antibiotics (Sigma, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin) at 4°C.

Mice. BALB/c mice, 7–9 weeks old, were purchased from Charles River Laboratory. ICR mice, 7–9 weeks old, originated from Bio Test s.r.o., Konárovice, Czech Republic. During the experiments, mice were fed standard rodent diet and drinking water *ad libitum*. They were caged in a mouse room with the temperature kept at 22°C and relative humidity of 65%. Before harvesting of peritoneal exudate cells, the mice were killed by cervical dislocation.

Production of anti-*E. cuniculi* serum. ICR mice were immunized intraperitoneally with a dose of 10^7 *E. cuniculi* live spores three times in 15-day intervals. Mice were exsanguinated 8 days after the last immunisation. After 12 hours at room temperature the blood was centrifuged at 160 g for 10 minutes. The serum was obtained and stored at –25°C.

Murine macrophage cultures. Peritoneal exudate cells (PEC) were recovered from BALB/c mice by lavage with RPMI 1640 (Sigma), washed once by centrifugation at 400 g for 10 minutes and resuspended in RPMI 1640 medium supplemented with 5% FBS, 1% antibiotics (Sigma Cell Culture Antibiotic Antimycotic), 1% gentamicin and 1% L-glutamine. The proportion of macrophages was established on the basis of flow cytometry analysis as 20% of all leukocytes. After incubation for 24 hours at 37°C with 5% CO₂, non-adherent cells were washed out.

Cell line PMJ2-R cultures. The cell line PMJ2-R was cultivated in DMEM medium (Sigma) supplemented with 5% FBS, 1% antibiotics (Sigma Cell Culture Antibiotic Antimycotic), 1% L-glutamine. The same culture conditions as for murine macrophage cultures were used.

Experimental design. Macrophages and PMJ2-R were cultured in 0.2 ml of culture medium per well of 96-well tissue culture testplate (TPP, Switzerland). The wells were seeded with 40×10^3 cells per well for counting of total number of *E. cuniculi* spores and with 80×10^3 cells per well for the measurement of nitric oxide production. Cells were infected with *E. cuniculi* spores at the ratio 1:3. Spores were left in the culture for the whole period of experiment to infect macrophages and PMJ2-R cells. Activation of cells was performed with 100 U of IFN-γ (R&D-System) per ml of culture medium at the time of infection. Opsonisation of spores was performed by addition of anti-*E. cuniculi* serum (of final dilution 1:800) to culture medium. Naive mouse serum diluted 1:800 was used as a control for elimination of effects of other components of the serum.

Counting of total number of *E. cuniculi* spores. The infected cells were lysed by 50 µl of 2M KOH in H₂O and 50 µl of 0.1% sodium-dodecyl-sulfate (SDS) (Osserman and Lawlor 1966) at 24, 48 and 72 hours post infection (p.i.). The total number of *E. cuniculi* spores was counted using a Bürker chamber.

Measurement of nitric oxide (NO) production. An indirect method (Green et al. 1982) for measurement of nitrogen intermediates (NO² and NO³) in supernatants was used. Sam-

ples of culture medium (100 µl) of each category were mixed with 100 µl of Griess reagent prepared just before use from solutions A (1% sulfonamide in 30% acetic acid) and B (0.1% N-(naphthyl)-ethylenediaminedihydrochloride in 60% acetic acid), mixed 1:1. Absorbances were measured at 540 nm with a spectrophotometer and plotted against the corresponding absorbance resulting from a standard curve generated using 1M NaNO₂. Measurements were performed at 24, 48 and 72 hours p.i.

Statistical analysis. Statistically significant differences were calculated using ANOVA (Statistika® 6, Czech Republic). All experiments were performed at least in triplicate. Experimental data were compared to corresponding controls and P values of 0.05 or less were considered significant.

RESULTS

Replication of *E. cuniculi* in murine macrophages and PMJ2-R cells

Encephalitozoon cuniculi replicated in non-activated murine peritoneal macrophages during 72 hours of the experiment (the number of spores increased from inoculated 120×10^3 spores per well to 690×10^3 spores per well at 72 hours p.i.). In contrast, the parasite could replicate in activated macrophages until 24th hour p.i., when the number of spores increased to 384×10^3 per well. Thereafter the number of spores did not change significantly. The number of spores in cultures of activated macrophages (400×10^3 per well) was significantly different from that in non-activated macrophages (690×10^3 per well) at 72 hours p.i. The influence of antibodies on the replication of *E. cuniculi* was not observed. The number of spores per well in non-activated macrophages (690×10^3) was not significantly different from that obtained in non-activated macrophage cultures treated with anti-*E. cuniculi* serum (670×10^3) at 72 hours p.i. Similarly, no significant difference was observed at 72 hours p.i. between the number of spores in activated macrophages (400×10^3) and those in activated, anti-*E. cuniculi* serum-treated cells (367×10^3) (Fig. 1A).

In contrast to the results obtained in murine macrophages, *E. cuniculi* replicated in both non-activated (423×10^3 spores per well at 72 hours p.i.) and activated PMJ2-R cells (762×10^3 spores per well at 72 hours p.i.). Moreover, *E. cuniculi* replicated faster in activated cells than in non-activated ones. The significant differences between the number of spores per well in non-activated cells and that in activated cells were observed at 72 hours p.i. The effect of opsonisation with polyclonal anti-*E. cuniculi* antibody was observed only in activated PMJ2-R cells, where *E. cuniculi* replicated faster and the number of spores per well reached $1,082 \times 10^3$ at 72 hours p.i. compared to 762×10^3 spores per well in activated cells only or 937×10^3 spores per well in activated cells treated with naive mouse serum, respectively. The effect of opsonisation with polyclonal anti-*E. cuniculi* antibody was not observed in non acti-

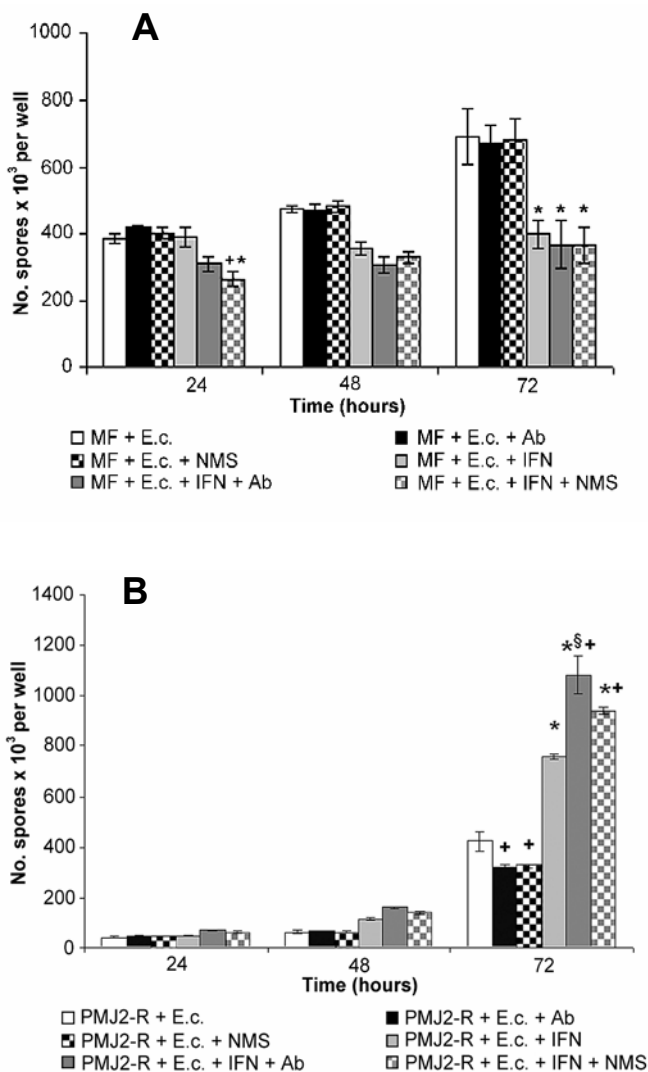


Fig. 1. Effect of interferon gamma and the treatment with specific polyclonal antibodies on the replication of *Encephalitozoon cuniculi* in murine peritoneal macrophages (A) and PMJ2-R cells (B) (mean values of triplicate cultures \pm SD). MF – peritoneal macrophages from BALB/c mice (40×10^3 macrophages per well); PMJ2-R – macrophage cell line (40×10^3 macrophages per well); IFN – 100 U IFN- γ /ml of culture medium; E.c. – *E. cuniculi* infection (120×10^3 spores/well); Ab – anti-*E. cuniculi* serum (dilution 1:800); NMS – naive mouse serum (dilution 1:800); * – the difference versus appropriate non-activated (without IFN- γ) group is significant at $P < 0.05$; + – the difference versus appropriate non-treated (without Ab or NMS) group is significant at $P < 0.05$; § – the difference between activated (with IFN- γ) group treated with Ab and that treated with NMS is significant at $P < 0.05$.

vated cells. The number of spores per well was significantly lower in non-activated PMJ2-R cells treated with anti-*E. cuniculi* serum (320×10^3 spores) than in untreated cells (423×10^3 spores), but it did not differ from the number of spores per well in non-activated cells treated with naive mouse serum (329×10^3 spores) (Fig. 1B).

Influence of activation, infection with *E. cuniculi* and opsonisation by antibody on nitric oxide production in murine macrophages and PMJ2-R cell line

The production of nitric oxide (NO) was significantly higher in non-activated peritoneal macrophages infected with *E. cuniculi* or activated cells compared to non-activated ones at all time points tested. Infected macrophages activated with IFN- γ produced the highest amount of NO. The concentration of NO in culture medium from this group was $58.75 \mu\text{mol}$ per litre in contrast to $8.34 \mu\text{mol}$ per litre in activated, non-infected macrophages and $10.14 \mu\text{mol}$ per litre in non-activated, infected macrophages at 72 hours p.i. The influence of polyclonal anti-*E. cuniculi* antibody was not recorded. Non-infected, non-activated macrophages treated with anti-*E. cuniculi* serum did not produce significantly different amount of NO than macrophage cultures treated with naive mouse serum during 72 hours of the experiment. Concentration of NO in culture medium of infected, non-activated macrophages or of infected, non-activated macrophages treated with anti-*E. cuniculi* serum was also not significantly different at 72 hours p.i. Activated infected macrophages treated with anti-*E. cuniculi* serum did not produce significantly more NO than activated infected macrophages without anti-*E. cuniculi* serum (Table 1).

PMJ2-R cells responded to *E. cuniculi* infection and IFN- γ stimulation in a similar manner as murine peritoneal macrophages. Significant differences in NO production between non-activated and activated cells or infected, non-activated cells were measured at 48 hours. Activated cells infected with *E. cuniculi* produced the highest amount of NO. Concentration of NO in this group was $34.3 \mu\text{mol}$ per litre compared to $20.4 \mu\text{mol}$ per litre in activated, non-infected cells and $22.5 \mu\text{mol}$ per litre in infected, non-activated cells at 72 hours p.i. Effect of antibody on NO production was not observed in PMJ2-R cells, too (Table 2).

DISCUSSION

The results of Didier (1995) show that IFN- γ stops replication of *Encephalitozoon cuniculi* spores in peritoneal murine macrophages. Our results provide further evidence that IFN- γ has a negative impact on the replication of *E. cuniculi* in peritoneal murine macrophages. The total number of spores did not increase after 24 hours p.i. in macrophages activated with IFN- γ . However, *E. cuniculi* could replicate in PMJ2-R activated with the same dose of IFN- γ . Moreover, *E. cuniculi* replicated faster in activated cells than in non-activated ones. This indicates that PMJ2-R cells cannot substitute peritoneal murine macrophages in immunoparasitological studies.

The role of specific antibodies in the immune response against *E. cuniculi* infection is still not fully understood. Antibodies probably slow down *E. cuniculi* infection in nonprofessional phagocytes (Schmidt and

Table 1. Nitric oxide production ($\mu\text{mol/litre}$ of culture medium) in cultures of murine peritoneal macrophages (mean values of triplicate cultures \pm SD); MF – peritoneal macrophages from BALB/c mice (80×10^3 per well); IFN – 100 U IFN- γ /ml of culture medium; infected – *Encephalitozoon cuniculi* infection (240×10^3 spores per well); Ab – anti-*E. cuniculi* serum (dilution 1:800); NMS – naive mouse serum (dilution 1:800); ^P – the difference versus appropriate non-activated (without IFN- γ) group is significant at $P < 0.05$; ^R – the difference versus appropriate non-infected group is significant at $P < 0.05$; ^S – the difference versus appropriate non-treated group (without NMS or Ab) is significant at $P < 0.05$.

	Time (hours)	MF	MF+NMS	MF+Ab	MF+IFN	MF+IFN+NMS	MF+IFN+Ab
Infected	24	2.37 ± 0.22^R	0.35 ± 0.04^S	0.36 ± 0.06^S	13.60 ± 0.88^{PR}	11.00 ± 0.82^{PR}	13.21 ± 2.10^{PR}
	48	4.44 ± 1.00^R	2.61 ± 0.19^R	3.85 ± 0.62^R	31.35 ± 2.27^{PR}	24.96 ± 1.84^{PR}	28.94 ± 1.40^{PR}
	72	10.14 ± 3.64^R	5.65 ± 0.65^R	7.20 ± 1.16^R	58.75 ± 4.30^{PR}	54.40 ± 1.62^{PR}	58.34 ± 5.45^{PR}
Non-infected	24	0.37 ± 0.02	0.32 ± 0.01	0.35 ± 0.05	3.46 ± 0.72^P	2.49 ± 0.47^P	2.41 ± 0.33^P
	48	0.38 ± 0.03	0.36 ± 0.01	0.37 ± 0.02	4.91 ± 1.50^P	4.62 ± 0.89^P	4.70 ± 0.95^P
	72	1.50 ± 0.71	0.37 ± 0.04^S	0.37 ± 0.03^S	8.34 ± 1.86^P	7.35 ± 1.10^P	10.30 ± 1.24^P

Table 2. Nitric oxide production ($\mu\text{mol/litre}$ of culture medium) in cultures of PMJ2-R cells (mean values of triplicate cultures \pm SD); PMJ2-R cells were seeded at 80×10^3 cells per well. Abbreviations as in Table 1.

	Time (hours)	PMJ2-R	PMJ2-R+NMS	PMJ2-R+Ab	PMJ2-R + IFN	PMJ2-R + IFN+NMS	PMJ2-R+IFN+Ab
Infected	24	0.39 ± 0.02	0.39 ± 0.03	0.39 ± 0.02	8.93 ± 0.46^{PR}	10.40 ± 0.70^{PR}	9.17 ± 0.15^{PR}
	48	14.40 ± 0.76^R	12.90 ± 0.14^R	10.20 ± 0.30^R	29.20 ± 1.23^{PR}	35.10 ± 1.83^{PR}	33.20 ± 0.77^{PR}
	72	22.00 ± 1.51^R	21.50 ± 1.23^R	19.50 ± 0.87^R	34.30 ± 1.72^{PR}	43.30 ± 1.85^{PRS}	40.20 ± 0.20^{PRS}
Non-infected	24	0.39 ± 0.02	0.39 ± 0.01	0.39 ± 0.01	0.39 ± 0.03	0.39 ± 0.01	0.39 ± 0.01
	48	0.38 ± 0.01	0.39 ± 0.02	0.39 ± 0.03	4.81 ± 0.03^P	12.60 ± 0.20^{PS}	9.40 ± 0.29^{PS}
	72	0.39 ± 0.01	0.39 ± 0.02	0.39 ± 0.02	20.40 ± 1.70^P	29.80 ± 1.60^{PS}	28.80 ± 1.11^{PS}

Shadduck 1984, Sak et al. 2004). However, antibodies have no protective effect against development of a lethal infection (Schmidt and Shadduck 1983, Salát et al. 2004). Polyclonal antibodies were used in our study, because they cover the variability of *E. cuniculi* spore antigens better than monoclonal antibodies. Another reason for using polyclonal antibodies was a closer similarity to the situation in the infected host. The cultures were treated with an anti-*E. cuniculi* serum diluted 1:800 in culture medium, due to its highest specific reactivity with *E. cuniculi* spores in ELISA at this dilution. For elimination of the effect of other components of anti-*E. cuniculi* serum, treatment with naive mouse serum in the same dilution was used. Our study did not confirm the influence of specific polyclonal antibody on *E. cuniculi* replication in murine macrophages. Although the addition of anti-*E. cuniculi* serum decreased significantly the number of spores per well in non-activated macrophages compared to the control without anti-*E. cuniculi* serum, addition of naive mouse serum had the same effect. Thus the difference was not caused by the antibody, but by other components of the serum. Schmidt and Shadduck (1984) observed that the treatment with anti-*E. cuniculi* serum slowed down parasite replication in thioglycolate-induced adherent peritoneal exudate cells. Macrophages after thioglycolate treatment could be partially activated and thus different from these used in our experiments. We did not detect the effect of antibodies because we harvested non-activated peritoneal macrophages without thioglycolate treatment and activation by IFN- γ could cover possible impact of antibodies on parasite replication. Another reason for

our different result can be the fact that macrophages were exposed to the influence of other serum components in contrast to the study by Schmidt and Shadduck (1984).

However, the effect of opsonisation by antibodies was observed in PMJ2-R cells. The presence of polyclonal antibodies in activated cell cultures resulted in the fastest replication of *E. cuniculi* compared to other experimental cultures. This result and the fact that activation with IFN- γ increased the number of spores per well in PMJ2-R cells too, support the hypothesis that *E. cuniculi* enters the host cell by phagocytosis and subsequent infection of the host cell from the phagolysosome (Franzen 2005). Activation with IFN- γ and opsonisation of the spores make phagocytosis more effective. Therefore more *E. cuniculi* spores can enter host cells and start infection. Moreover, *E. cuniculi* spores replicated very slowly in non-activated PMJ2-R cells and the impulse for pathogen replication was the activation by IFN- γ and opsonisation of spores by antibodies. Our observations support the idea that this way of infection with *E. cuniculi* is the main way for infection of phagocytic cells (Franzen 2005).

Although activated PMJ2-R cells infected with *E. cuniculi* produce significantly more NO than non-activated infected cells, *E. cuniculi* can successfully replicate in both non-activated and activated cells. This supports conclusion by Khan and Moretto (1999) that IFN- γ -mediated protection against *E. cuniculi* is independent of NO production.

PMJ2-R cell line originated from C57BL 6J mice (Adami et al. 1993). Comparison of the susceptibility or

resistance level of some inbred mouse strains revealed that C57BL 6J mice are more susceptible to the infection with *E. cuniculi* than BALB/c mice (Niederkorn et al. 1981). The comparison was based on the percentage of infected peritoneal macrophages 2 weeks after infection. The sensitivity of activated PMJ2-R cell line to the infection with *E. cuniculi* can be related to the susceptibility of C57BL 6J mice to *E. cuniculi* infection. Moreover, PMJ2-R cells do not produce TNF- α after stimulation with lipopolysaccharide, which is in contrast to murine macrophages. It was shown that stimulation of TNF- α inhibited the replication of *E. cuniculi* in macrophages from BALB/c mice *in vitro* (Didier and Shaduck 1994). Thus another reason for the sensitivity of

PMJ2-R to *E. cuniculi* infection can originate from the inability to produce TNF- α . Other comparative studies between BALB/c and C57BL 6J murine macrophages and PMJ2-R cells can help to find the mechanism of the killing of *E. cuniculi* spores in murine macrophages.

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