ECHINOCOCCUS MULTILOCULARIS INFECTION IN MICE: IN VIVO TREATMENT WITH A LOW DOSE OF IFN- γ DECREASES METACESTODE GROWTH AND LIVER FIBROGENESIS

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Summary :

As no antiparasitic drug is definitively efficient in patients with alveolar echinococcosis, the effects of exogenous IFN- γ on murine *Echinococcus multilocularis* infection were assessed with regards to the parasite burden, parasite-specific immune responses, and the urinary level of the collagen cross-link pyridinolines. They were analyzed after 3-week treatments with 1 or 5 μ g of IFN- γ per day twice a week. The treatment with 1 μ g transiently reduced the liver metacestode load, and the metastase weight as far as 6 weeks after the end of treatment. It slightly increased Th1-type T cell responses and reduced the excretion of pyridinolines. These results should encourage further study to assess whether the decrease in liver fibrosis leads to an improvement of the efficacy of albendazole therapy. In contrast, the treatment with 5 μ g increased the liver metacestode load and was less efficient than that with 1 μ g in decreasing pyridinoline excretion. These results incitate to follow up carefully patients with alveolar echinococcosis who are treated with IFN- γ .

KEY WORDS : *Echinococcus multilocularis*, IFN- γ , liver fibrosis, pyridinolines.

Résumé : INFECTION MURINE À *ECHINOCOCCUS MULTILOCULARIS* : UN TRAITEMENT PAR UNE FAIBLE DOSE D'IFN- γ RÉDUIT LA PROLIFÉRATION PARASITAIRE ET LA FIBROGÈNESE HÉPATIQUE

L'absence de médicament efficace chez les patients atteints d'échinococcose alvéolaire a justifié l'étude des effets de l'apport exogène de l'IFN- γ au cours de l'infection murine à *Echinococcus multilocularis*. Les modifications de charge parasitaire, de réponses immunitaires spécifiques du parasite, et du taux urinaire des pyridinolines, pontages covalents des collagènes, ont été analysés après des traitements de 3 semaines avec des doses bi-hebdomadaires de 1 ou 5 μ g d'IFN- γ . Le traitement par 1 μ g a réduit transitoirement la charge parasitaire hépatique et de façon prolongée le poids des métastases. Il a intensifié les réponses immunitaires de type Th1 et a surtout réduit l'excrétion de pyridinolines. Ces résultats incitent à évaluer l'association IFN- γ -albendazole pour vérifier si la réduction de la fibrose hépatique améliore l'efficacité de l'anti-parasitaire. À l'inverse, le traitement par 5 μ g a augmenté la charge parasitaire et n'a que modérément réduit l'excrétion de pyridinolines. Une surveillance toute particulière des patients atteints d'échinococcose alvéolaire et traités par IFN- γ s'impose donc.

MOTS CLÉS : *Echinococcus multilocularis*, IFN- γ , fibrose hépatique, pyridinolines.

INTRODUCTION

Human alveolar echinococcosis, caused by *Echinococcus multilocularis* metacestode, is a severe liver infection for which no efficient antiparasitic therapy is now available. The key pathogenic event in this disease is the formation of a periparasitic granuloma, mainly composed of macrophages, T cells, and myofibroblasts, embedded in extracellular matrix. This granuloma triggers a dense and irreversible fibrotic process (Miguet *et al.*, 1991). The absence of reversibility of *E. multilocularis*-induced fibrosis is associated with an increase in pyridinoline, an inter-

molecular cross-link specific of mature collagen (Ricard-Blum *et al.*, 1992a; Ricard-Blum *et al.*, 1996). Covalent collagen cross-linking (reviewed by Reiser *et al.*, 1992) leads to a decrease in collagen solubility and confers an increased resistance to proteolytic degradation to collagen (Vater *et al.*, 1979).

In experimental hosts, the metacestode growth can be controlled by both macrophages (Rau & Tanner, 1975; Reuben & Tanner, 1983) and T cells (Ali-Khan 1978; Gottstein *et al.*, 1994; Playford *et al.*, 1992). At the late stage of infection, an increase in parasite burden is associated with a decreased IFN- γ production by spleen cells (Emery *et al.*, 1996). In AKR mice as in humans, a significant increase in pyridinoline occurs in *E. multilocularis*-induced liver fibrosis from week 8 after infection (Ricard-Blum *et al.*, 1995). Hepatic level of pyridinoline is related to the degree of severity of the fibrotic process (Ricard-Blum *et al.*, 1992b; Ricard-Blum *et al.*, 1995). Pyridinoline is excreted in urine as a breakdown product of mature collagen; its urinary excretion has been shown to correlate to the content

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of collagen in granulomas in *Schistosoma mansoni*-infected mice (Grenard *et al.*, 1997). Urinary pyridinoline is thus a sound marker of liver fibrogenesis, especially in parasite-induced processes.

IFN- γ is a major mediator of resistance against a wide range of pathogens through macrophage activation (reviewed by Murray, 1988). On the other hand, IFN- γ *in vitro* inhibits the synthesis of type I collagen by human fibroblasts (Jimenez *et al.*, 1984) and by human myofibroblastic Ito cells (Mallat *et al.*, 1995). Moreover, an *in vivo* treatment with IFN- γ decreases collagen deposition and types I and III procollagen mRNA levels in the liver of *S.mansoni*-infected mice (Czaja *et al.*, 1989).

We therefore analyzed the effects of exogenous IFN- γ on an established *E. multilocularis* infection in AKR mice in order to mimic a treatment that could be performed in humans. We examined these effects with regards to changes induced in parasite-specific immune responses and in the urinary level of pyridinoline.

MATERIAL AND METHOD

ANIMALS AND PARASITES

The same *E. multilocularis* isolate used in previous studies (Emery *et al.*, 1996; Ricard-Blum *et al.*, 1995) was maintained in the gerbil, *Meriones unguiculatus*. For mice infection, 5 g of metacystode recovered from *M. unguiculatus* were aseptically minced in 20 ml of saline. A crude *E. multilocularis* antigen (EmAg) was obtained from the $40\,000 \times g$ supernatant fraction of a homogenate of metacystodes recovered from *M. unguiculatus*, as previously described (Emery *et al.*, 1996). AKR mice, purchased from Bonmice (Bomholtjev, Denmark) and then housed under standard conditions, were used for both their susceptibility to *E. multilocularis* (Liance *et al.*, 1990; Gottstein *et al.*, 1994), and their ability to develop an extensive liver fibrosis after metacystode injection (Ricard-Blum *et al.*, 1995). Six-week-old female mice received 0.1 ml of the parasite suspension by a direct intrahepatic injection (Liance *et al.*, 1990). Uninfected control mice received 0.1 ml of saline in the same way.

EXPERIMENTAL DESIGN

A rat recombinant IFN- γ , which exhibits a homology of 87 % with murine IFN- γ (Banchereau & Wijdenes, 1986), was provided by Roussel Uclaf (Romainville, France). It displayed specific antiviral activities of 1×10^7 units/mg, was diluted in saline and frozen in ali-

quots at -80°C until use. Treatments started six weeks after infection. IFN- γ -treated mice, either infected or not, received intramuscular injections of either 1 or 5 μg of IFN- γ in 0.1 ml of saline, twice a week for three weeks (total doses of 6×10^4 and 3×10^5 antiviral units, respectively). Control mice, either infected or not, received saline in the same way. The effects of IFN- γ administration were evaluated at a period of active hepatic collagen synthesis (Vuitton *et al.*, 1986; Ricard-Blum *et al.*, 1995) and for two follow-up periods. Groups of six infected IFN- γ - and saline-treated mice were randomly selected and examined at two or six weeks after the end of treatment (i.e. 11 or 15 weeks after infection, respectively).

PARASITOLOGICAL MONITORING

At the end of experiments, mice were sacrificed by bleeding and serum samples were aliquoted and kept at -80°C until use. The size of superficial hepatic parasites was measured and their surface was recorded (mm^2). The liver parasite burden was also assessed by liver weight since inter-individual differences in hepatic metacystode surface were usually observed (Emery *et al.*, 1997). Metastases were weighed.

IMMUNOLOGICAL MONITORING

EmAg-specific delayed-type hypersensitivity (DTH) was assessed *in vivo* the day before autopsies by the foot-pad swelling reaction, as previously described (Liance *et al.*, 1990). Serum EmAg-specific IgG₁, and IgG_{2a} responses were analysed by ELISA using biotinylated anti-mouse and streptavidin-peroxidase conjugate according to Emery *et al.* (1997).

QUANTITATION OF THE COLLAGEN CROSS-LINK PYRIDINOLINES

To assess IFN- γ treatment-induced changes in liver extracellular matrix, the urinary excretion of pyridinolines was measured at different time-points from the beginning of IFN- γ treatments. For this purpose, pooled urine samples were collected from five IFN- γ - or saline-treated *E. multilocularis*-infected mice and from three IFN- γ - or saline-treated uninfected mice over 24 hr in metabolic housings. They were filtered through $0.45\ \mu\text{m}$ filters and stored at -20°C until analysis. Total pyridinolines were measured by a competitive immunoassay (PyrilinksTM assay, Metra Biosystems Inc, Palo Alto, CA, USA). Assays were performed according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation were below 8 %, as reported by Gomez *et al.* (1996). Urinary creatinine was quantified by the Jaffé procedure using a reagent kit (Sigma Diagnostics, St Louis, MO, USA).

STATISTICAL ANALYSIS

Data were presented as means $\pm$ SD except for urinary pyridinolines which levels were determined from pooled samples. Comparison between two groups was performed using Student's *t* test. Pearson's correlation test was used to test the relationships between parasitological and immunological data. Values with $P < 0.05$ were defined as significant.

RESULTS

TIME COURSE OF MICE SURVIVAL FOLLOWING INFECTION AND TREATMENTS

There was no sign of toxicity after IFN- γ injections and none of the animals died during the experiments. At autopsy, the mean body weight of infected IFN- γ -treated mice was similar to that of infected saline-treated mice. All mice, either saline- or IFN- γ -treated, harboured hepatic parasites and peritoneal metastases.

EFFECTS OF IFN- γ TREATMENTS ON METACESTODE BURDEN

In mice treated with 1 μ g of IFN- γ per day, there was a trend towards a reduction of both the mean superficial surface of hepatic metacestodes and the mean liver weight, as compared to saline-treated mice, two weeks after the end of treatment (i.e. 11 weeks p.i.) (Table I). At that time, the mean metastatic burden was significantly decreased ($P = 0.01$). Six weeks after the end of treatment (i.e. 15 weeks p.i.), the means of both surface of hepatic metacestodes and liver weight were increased while the mean metastatic burden was slightly decreased ($P = 0.05$), as compared to those of saline-treated mice. In mice treated with 5 μ g of IFN- γ per day, the means of superficial surface of hepatic

metacestodes and liver weight slightly increased at both examination times, as compared to those of saline-treated mice. There was no difference in the metastatic burden between these IFN- γ -treated mice and their controls.

EFFECTS OF IFN- γ TREATMENTS ON PARASITE-SPECIFIC IMMUNE RESPONSES

Table II shows changes in parasite-specific immune responses that occurred in IFN- γ -treated mice according to the dose of IFN- γ used and the examination time. The mean values of foot-pad responses measured in uninfected mice, either treated or not, remained below 1.75 mm^{-1} at both examination times. In mice treated with 1 μ g of IFN- γ per day, there was a trend towards an increase in the mean intensity of *EmAg*-specific DTH responses at both examination times. In these mice, mean levels of *EmAg*-specific IgG_{2a} significantly

IFN-g: μ g a week	Weeks after the end of treatment	DTH	IgG ₁	IgG _{2a}
-	2	6.14 $\pm$ 1.21	0.35 $\pm$ 0.09	0.21 $\pm$ 0.01
1	2	7.16 $\pm$ 2.40	0.37 $\pm$ 0.07	0.36 $\pm$ 0.02 *
5	2	5.20 $\pm$ 0.83	0.71 $\pm$ 0.19	0.33 $\pm$ 0.03 *
-	6	4.44 $\pm$ 1.33	0.86 $\pm$ 0.18	0.33 $\pm$ 0.02
1	6	5.50 $\pm$ 1.3	0.36 $\pm$ 0.04 **	0.35 $\pm$ 0.04
5	6	3.66 $\pm$ 0.83	0.84 $\pm$ 0.26	0.32 $\pm$ 0.03

Results of DTH are expressed as the difference (mm^{-1}) in footpad thickness measured before and 24 hr after *E. multilocularis* antigen challenge, those of antibody levels as the *A* minus the mean $\pm$ 2 SD values obtained from uninfected mouse sera. Mean values $\pm$ SD of 6 mice per group are shown.

* Significantly different from the results of the corresponding saline-treated group (* $P < 0.003$ and ** $P = 0.04$, respectively, Student's *t* test).

Table II. – Parasite-specific delayed-type hypersensitivity and serum antibody levels in *E. multilocularis*-infected mice after a 3-week treatment with 1 or 5 μ g of IFN- γ per day twice a week.

IFN- γ : μ g twice a week	Weeks after the end of treatment	Liver parasites (mm ²)	Variation (%)	Liver weight (g)	Variation (%)	Metastase weight (g)	Variation (%)
-	2	70.50 $\pm$ 41.71	-	1.82 $\pm$ 0.15	-	1.46 $\pm$ 0.33	-
1	2	54.16 $\pm$ 62.92	- 23	1.57 $\pm$ 0.32	- 14	0.47 $\pm$ 0.35 *	- 68 *
5	2	175.16 $\pm$ 118.50	+ 60	2.25 $\pm$ 0.78	+ 19	1.30 $\pm$ 0.58	- 11
-	6	71.76 $\pm$ 68.0	-	1.61 $\pm$ 0.35	-	1.83 $\pm$ 0.23	-
1	6	134.0 $\pm$ 61.42	+ 86	2.02 $\pm$ 0.36	+ 20	1.31 $\pm$ 0.39	- 28
5	6	204.83 $\pm$ 70.83	+ 185	2.65 $\pm$ 1.54	+ 39	1.78 $\pm$ 1.17	- 3

Variations in the parasite burden of IFN- γ -treated mice were calculated with regard to parasite burden of the corresponding saline-treated mice. Mean values $\pm$ SD of 6 mice per group are shown.

* Significantly different from the results of the corresponding saline-treated group ($P = 0.01$, Student's *t* test).

Table I – Parasitological findings in *E. multilocularis*-infected mice after a 3-week treatment with 1 or 5 mg of IFN-g per day twice a week.

increased two weeks after the end of treatment. IgG₁ levels significantly decreased six weeks after the end of treatment. In mice treated with 5 µg of IFN-γ, DTH responses were slightly decreased at both examination times. The mean IgG₁ and IgG_{2a} antibody levels increased transiently two weeks after the end of treatment. There was no individual correlation between the parasite burden and the intensity of immune changes, whatever the dose of IFN-γ used and the examination time.

EFFECTS OF IFN-γ TREATMENTS ON THE URINARY EXCRETION OF PYRIDINOLINES

The effects of treatment with IFN-γ on pyridinoline excretion was first assessed in uninfected mice. The urinary excretion of pyridinolines was lower during and after treatments in uninfected mice injected with 1 or 5 µg of IFN-γ than in uninfected saline-treated mice (Fig. 1). The *E. multilocularis* infection induced an increase in the urinary excretion of pyridinolines, starting from week 10 p.i. and reaching seven-fold the control values at week 13 p.i. (Fig. 2). Unfortunately, no urine sample from infected saline-treated mice was collected after the 13th week p.i. The urinary excretion of pyridinolines was lower in infected mice treated with 1 µg of IFN-γ than in infected saline-treated mice (Fig. 3). This excretion did not change significantly, neither during, nor after the treatment. By contrast, the mice treated with 5 µg of IFN-γ showed a sharp but transient increase in the urinary excretion of pyridi-

nolines at the end of the treatment, followed by a decrease (Fig. 3). At the end of the follow-up period, the excretion of pyridinolines was similar in the two treated groups and was strongly lower than in the untreated one.

DISCUSSION

Since IFN-γ has antifibrogenic properties and is an important activating macrophage factor, it represents a potential drug for the treatment of non-healing cases of human alveolar echinococcosis. We therefore analyzed the effects of exogenous IFN-γ on secondary *E. multilocularis* infection in AKR mice, in order to mimic a treatment that could be performed in human patients.

We first showed that a three week treatment with 1 µg of IFN-γ twice a week decreases the hepatic and peritoneal *E. multilocularis* metacestode growth shortly after cessation of immunotherapy. At the same examination time, there was a trend towards an increase in parasite-specific Th1 cell-dependent immune responses, as observed in mice when the metacestode grows slowly (Ali-Khan, 1978; Emery *et al.*, 1997). The present study also shows that the infection by *E. multilocularis* leads to an increase in the excretion of pyridinolines in urine, in agreement with the results obtained in other fibrotic disorders such as murine

Fig. 1. – IFN-γ treatment of uninfected AKR mice decreases urinary levels of pyridinolines.

Mice were either treated with 1 or 5 µg of IFN-γ or injected with saline twice a week, from week 6 to week 9, during the experimental follow-up period. The urinary excretion of the free pyridinolines was measured in pooled urine samples from 3 uninfected mice during and after the end of treatment. Results are expressed as nM pyridinolines/mM creatinine.

Fig. 2. – *E. multilocularis* infection increases the urinary excretion of free pyridinolines. The urinary excretion of the free pyridinolines was measured in pooled urine samples from 5 infected and 3 uninfected mice treated with saline from week 6 to week 9 after infection. Results are expressed as nM pyridinolines/mM creatinine.

Fig. 3. – A 3-week treatment with 1 µg of IFN- γ reduces the *E. multilocularis*-induced increase of pyridinoline urinary excretion at the late stage of infection.

Infected mice were either treated with 1 or 5 µg of IFN- γ or injected with saline twice a week, from week 6 to week 9 p.i. The urinary excretion of the free pyridinolines was measured in pooled urine samples from 5 mice at designated times p.i. Results are expressed as nM pyridinolines/mM creatinine.

schistosomiasis (Grenard *et al.*, 1997), human viral hepatitis and cirrhosis (Ricard-Blum *et al.*, 1997). However, this increase was slightly delayed, as compared to that of hepatic pyridinoline level in *E. multilocularis*-infected AKR mice (Ricard-Blum *et al.*, 1995). The treatment with 1 µg of IFN-γ led to a marked decrease in the urinary excretion of pyridinolines of *E. multilocularis*-infected mice, although it was insufficient to normalize it. It should be mentioned that the total dose of IFN-γ used in the present study is markedly lower than that promoting a decrease in collagen deposition in the liver of *S. mansoni*-infected mice (Czaja *et al.*, 1989). These data therefore encourage an assessment of IFN-γ immunotherapy for a longer period of time during murine alveolar echinococcosis. From the decrease in urinary pyridinoline it can be expected that the efficacy of albendazole, the most efficient antiparasitic drug against *E. multilocularis* metacystode, would be improved by its combination with IFN-γ. Wen *et al.* (1994) have suggested that human patients with echinococcosis harbouring large and old parasitic lesions, probably associated with severe fibrosis, respond less well to albendazole than patients with smaller and/or younger lesions. However, the long-term effects of IFN-γ treatment should be evaluated more accurately. Indeed, six weeks after cessation of immunotherapy, the hepatic metacystode development increased. This exacerbation could be directly related to IFN-γ administration. In fact, relapses of infectious diseases may occur after the cessation of treatments with cytokines, as in murine toxoplasmic encephalitis (Suzuki *et al.*, 1990), or in human chronic hepatitis (Davis *et al.*, 1989). Since the delayed increase in hepatic metacystode development was associated with a long-lasting reduction of urinary pyridinolines, it could also be related to a reduced liver fibrogenesis. The decrease in the peritoneal parasite burden, maintained at the same time, argues for this hypothesis. In our knowledge, two patients with advanced alveolar echinococcosis have been previously treated over 15 months with IFN-γ alone (Jenne *et al.*, 1997) or in combination with mebendazole (Schmid *et al.*, 1995). The results obtained in the present study after a three-week treatment with 1 µg of IFN-γ twice a week in mice with secondary alveolar echinococcosis are in agreement with the hypothesis that the stabilization of the parasitic lesions, and even some improvement, of these patients were favoured by IFN-γ. They also show that the safety and efficacy of a combined therapy with IFN-γ plus albendazole should be analyzed in terms of both parasitic load and liver fibrosis.

In contrast, the three-week treatment with 5 µg of IFN-γ twice a week had no significant inhibitory, but in many instances a rather exacerbating effect on *E. multilocularis* metacystode growth. Indeed, it induced an

early and long-lasting increase of hepatic larval growth. We observed neither significant changes in parasite-specific immune responses nor a more pronounced decrease in urinary excretion of pyridinolines than that induced by the treatment with 1 µg of IFN-γ. This negative effect could be due to a downregulation of macrophage activities. Indeed, high doses of exogenous IFN-γ have been shown to reduce monocyte-mediated antitumor cytotoxicity or to decrease oxidative activity of blood monocytes (reviewed by Murray, 1988). Whereas the effects of *in vivo* IFN-γ treatments against parasites are usually dose-dependent (Suzuki *et al.*, 1990; Watier *et al.* 1993) and collagen synthesis is dose-dependently decreased by IFN-γ, *in vitro* (Mallat *et al.*, 1995) and *in vivo* (Czaja *et al.*, 1989), our results underline the necessity for a careful follow-up in patients with alveolar echinococcosis treated with high doses of immunomodulatory compounds which could be harmful rather than beneficial. They also should encourage an assessment of the effects of treatments with other anti-fibrotic agents for comparison with IFN-γ immunotherapy.

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