

INTERFERON GAMMA INCREASES SURVIVAL IN MURINE EXPERIMENTAL CRYPTOCOCCOSIS

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SUMMARY

Systemic disease by *Cryptococcus neoformans* (*C. neoformans*) is a common opportunistic infection in immunodeficient patients. Cellular immunity seems to be the most important determinant of resistance. The aim of this study was to assess the effect of recombinant rat interferon gamma (IFN- γ) in murine cryptococcosis (Balb/c mice infected by IP route with the Rivas strain of *C. neoformans*), evaluating survival time, macroscopic and microscopic examination of the organs, and massive seeding of brain homogenate. IFN- γ treatment, at a daily dose of 10,000 IU, did not modify significantly these variables when mice were challenged with a high inoculum (10^7 yeasts) and treatment was delayed to 5 days after infection (median survival 21 days in control mice vs. 23 days in IFN-treated). Another set of experiments suggested that IFN- γ treatment, at a dose of 10,000 IU/day, begun at the moment of infection could be useful (it prolonged survival from 20 to 28 days, although the difference did not achieve statistical significance). When used simultaneously with infection by 3.5×10^5 yeasts, IFN- γ at 10,000 IU/day for 15 days significantly prolonged survival of mice ($p = 0.004$). These results suggest that, depending on the experimental conditions, IFN- γ can improve survival of mice infected with a lethal dose of *C. neoformans*.

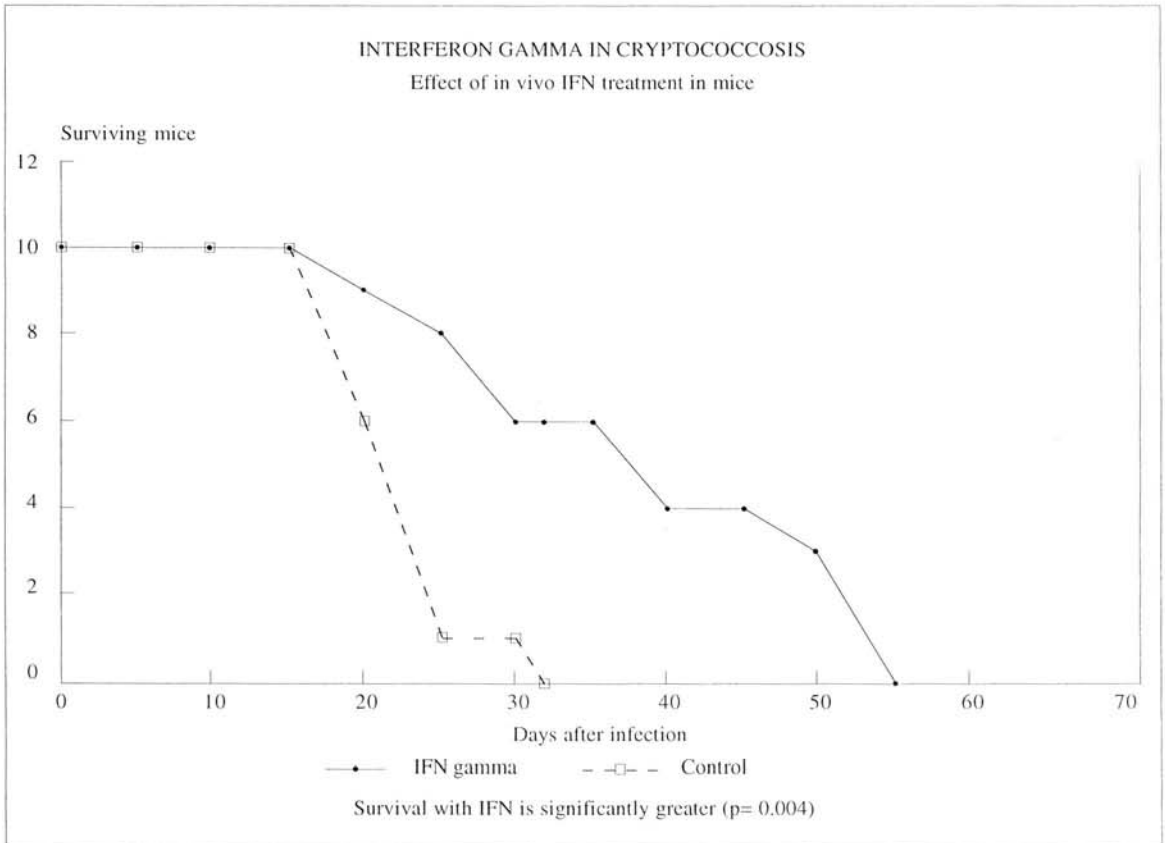
KEYWORDS: Cryptococcosis; Interferon gamma; Experimental treatment.

INTRODUCTION

Cryptococcus neoformans (*C. neoformans*) is a capsulated yeast that causes severe systemic disease in immunocompromised hosts⁶. In the last years, since the advent of AIDS pandemic, the rate of human cryptococcosis has greatly and progressively increased⁶. Despite considerable efforts, the current treatment of cryptococcosis is limited to amphotericin B alone or in association with flucytosine. Triazoles such as itraconazole and fluconazole are also used but they seem to be less effective, so the optimal treatment remains to be determined²¹.

In previous reports^{3,4}, we have adapted a murine model of cryptococcosis which is highly reproducible

and allowed us to study different antifungal drugs in short times. Since the immune system is crucial for both natural and experimental cryptococcosis, as dramatically demonstrated by the susceptibility of AIDS patients, we tried to explore the effect of IFN- γ in our experimental model. IFN- γ is a cytokine produced by T lymphocytes and large granular lymphocytes² and its production is greatly decreased in AIDS¹⁵, along with depletion of CD4+ T lymphocytes¹⁶. Previous studies suggested that in vitro treatment with IFN- γ increased the anti-cryptococcal ability of murine macrophages^{7,12}. The availability of recombinant IFN- γ provided us with amounts of this cytokine great enough to evaluate its effect in vivo. Depending on the inoculum size and the moment of admin-



istration, our results show that IFN- γ improves the survival of infected mice.

MATERIAL AND METHODS

Mice:

• Male Balb/c mice (n=65), between 18 and 25 g of weight, were inbred in the facilities of the Argentine National Academy of Medicine. Animals were housed in groups according to the treatment and allowed food and water ad libitum. Four different experiments were done (see below): with 2 groups (control vs. treated) in three of them and with 3 groups in the other (control vs. two different schedules of treatment).

C. neoformans inoculum:

We used the Rivas strain of *C. neoformans* var. *neoformans*, which was isolated in 1984 from a white, 35 years old, HIV-infected male with meningoencephalitis, as previously reported¹⁹. It is a well-capsulated strain, highly adapted to in vitro culture and reproducibly lethal for mice (Bava et al. manuscript in preparation). Yeasts

were cultured in Sabouraud honey agar slants, at 37°C for 48 h. Fungal cells were suspended in saline and inoculated by intraperitoneal (IP) route. Inoculum was 10^7 yeasts in experiments # 1 and 2 and 3.5×10^5 yeasts in experiments # 3 and 4. Viability of the inocula was controlled by culture in dextrose broth agar at 37°C.

Beginning of treatment:

In experiments # 1 and 2, treatment began 5 days after infection. In experiment # 3 two schedules were compared: in one, treatment began 5 days before infection and ended 15 days after infection (20 days of duration); in the other, treatment began simultaneously with infection and ended 15 days thereafter (15 days of duration). In experiment # 4, treatment began simultaneously with infection and ended 15 days later.

Treatment:

Recombinant rat IFN- γ with an extra methionine residue at the N-terminus of the natural sequence (RU 53561) was kindly provided by Roussel-Uclaf (Romainville, France). The specific activity of the prod-

uct was 1×10^7 units per mg of protein (batch CB22821-23). Lyophilised product (0.25 mg) was stored at -20°C up to the moment of study, reconstituted with 1 ml of sterile apyrogenic water and used in the following 15 days. Further dilution were done with saline to achieve the dose (as indicated in the text). IFN- γ was administered by IP route. As control, mice received similar volumes of saline by IP route. Preliminary studies showed no direct effect of IFN- γ over *C. neoformans*.

Treatment evaluation:

After infection, either treated and control mice were observed until death, and then subjected to necropsy. The following variables were recorded and analyzed:

- i) Survival time of the different groups;
- ii) Macroscopic aspect, including presence of hepato

and/or splenomegaly, softening of the brain, and lung nodules.

- iii) Standard histopathological examination of the tissues.
- iv) Presence of capsulated yeasts in imprints of lung, brain, liver and spleen, under phase contrast microscopy.
- v) Growth of *C. neoformans* after massive seeding of brain homogenate (100 mg/ml) in Sabouraud honey agar tubes for a week at 37°C .

Statistics:

Survival times were analyzed by the Mann-Whitney rank sum test. Macroscopic and microscopic findings were analyzed with contingency tables (X²). All calculations were done with the Stat Primer program⁸.

TABLE 1

Effect of intraperitoneal (I.P.) IFN- γ (10,000 IU) on experimental murine cryptococcosis. Abbreviations: exp. = experiment; N = number of animals; Li = Liver; Sp = spleen; Lu = lung; Br = brain; IFN- γ = interferon γ .

Exp.	Inoculum	Treatment	N	Microscopy ¹				Survival ² time	Massive ³ seeding
				Li	Sp	Lu	Br		
1	10^7 cells	control	8	8	8	8	8	21.0 (17-28)	8
		IFN- γ	10	10	10	10	10	22.9 (19-28)	10
2	10^7 cells	control	6	6	6	6	6	20.7 (18-24)	6
		IFN- γ	6	5	6	5	6	23.2 (18-30)	6
3	3.5×10^5	control	4	4	4	4	4	19.6 (13-25)	4
		IFN- γ^4	5	5	5	5	5	18.6 (14-21)	5
		IFN- γ^5	6	6	6	6	6	27.5 (14-37)	6

¹ Positive microscopy: detection of cryptococci in necropsy specimens of mice, in wet preparations and histopathological sections staining with hematoxylin-eosin and with Meyer's mucicarmín.

² Survival time (expressed in days post infection) is presented with mean (range).

³ Number of animals with positive culture of massive seeding of brain homogenate

⁴ IFN- γ was administered for 20 days, beginning 5 days before infection and up to day 15 post-infection.

⁵ IFN- γ was administered for 15 days, beginning at the moment of infection.

RESULTS

High inoculum with delayed treatment:

The classical model of murine cryptococcosis involves a high inoculum (10^7 *C. neoformans* yeasts) by I.P. route, followed by a five days period before antifungal treatment. This lag period allows systemic cryptococcosis to develop, even with CNS involvement. When assayed under these conditions, IFN- γ did not increase anti-cryptococcal resistance of mice (Table 1, experiments # 1 and 2). To different extent, all mice (either treated or not) presented with all macroscopic and histopathological features of systemic cryptococcosis. Capsulated yeast invading Virchow Robin's space were observed in the brain. The weak inflammatory response surrounding the microorganisms was remarkable; only mononuclear cells (lymphocytes and macrophages) were frequently observed. The subarachnoid space showed a large number of *C. neoformans* as well as weak or absent inflammatory response. The liver and spleen showed pseudocystic lesions with great amount of capsulated yeasts surrounded by a weak inflammatory reaction with mononuclear cells. Few epithelioid granulomas were observed in the lungs and all of them contained capsulated yeasts. In experiment # 2, despite the lack of statistically significant differences, treated animals seemed to have less fungi in microscopical examination by two trained observers, under blind conditions. This prompted us to assess the effect of IFN- γ under less exigent conditions, i.e. with a lower inoculum and without lag time.

Effect of the moment of treatment:

To evaluate whether the moment of beginning IFN treatment was important to determine the course of the infection, we also analyzed treatment either previous or simultaneous with a very low inoculum infection (Table 1, experiment # 3). As shown, treatment since 5 days before *Cryptococcus* inoculum did not modify the variables under study. On the other hand, treatment begun simultaneously with the infection resulted in a trend to longer survival ($p = 0.103$). We concluded that there was no benefit in beginning treatment before inoculum, but that simultaneous treatment deserved further attention.

Low inoculum with simultaneous treatment:

When mice were inoculated with 3.5×10^5 *C. neoformans* cells and treated with 10,000 IU/day for 15 days since the moment of infection, the protective effect of IFN- γ achieved statistical signification, as shown in Fig. 1. Survival of treated mice was 75% greater than that of untreated controls ($p=0.004$). However all mice lately died due to *C. neoformans* systemic infection, without difference in the yield of yeasts in fresh imprints.

In all experiments, no differences were observed between the histopathological aspect of studied organs in treated and untreated mice.

DISCUSSION

This report shows that in vivo treatment with IFN- γ prolongs survival of mice infected with *C. neoformans*, without modification in any of the other parameters under study. The effect of IFN- γ treatment depends upon several variables, such as inoculum size and time of treatment, but it provides a rationale for further searching the best way to treat this disease.

The resistance against *C. neoformans* depends upon several ill-characterized factors. Humoral immunity does not seem to be protective, although under experimental conditions, *Cryptococcus* - specific monoclonal antibodies prolong survival¹⁴. This fungus can be killed by different cell types, including natural killer effectors and macrophages^{5,11}. Macrophages can kill either phagocytosed or extracellular cryptococci⁹. Depletion of CD4+ T lymphocytes results in an impairment in host defense against the yeast, with decreased survival, earlier dissemination and greater cryptococcal burden¹³. One of the key products of CD4+ T lymphocytes is IFN- γ which is the main activator of macrophages¹⁸. In several in vitro systems it activates microbicidal activities of macrophages, including murine macrophages, against fungi⁵, even *C. neoformans*⁷.

The possible mechanism(s) involved in antifungal activity triggered by IFN- γ are ill-defined. The spectrum of fungi susceptible is broad (reviewed in ref. 22) and only in some cases mechanistic studies have been undertaken. In the case of *Histoplasma capsulatum*, the limitation in iron availability has been suggested as the mechanism, for the inhibitory effect of IFN- γ in murine macrophages¹⁰. In marrow-derived macrophages FLESH et al.⁷ found that IFN- γ induced the secretion of a protein able to inhibit *C. neoformans* growth.

The survival of mice treated with IFN- γ is strikingly similar to that of mice treated with flucytosine or triazolic compounds such as fluconazole, itraconazole and Sch 39304⁴. Treatment with any of these agents prolongs survival, but lately mice die with all the characteristics of disseminated cryptococcosis. Since neither triazoles nor IFN- γ actually cured infected mice, it would be worthy to assay combined treatment with IFN- γ and one of the triazolic chemotherapeutic agents. This approach has been suggested for treatment of several microbial diseases²⁰ based on the effect of IFN- γ on visceral leishmaniasis¹ and lepra¹⁷.

In summary, in some condition IFN- γ is able to increase the survival of mice to the lethal dose of *C. neoformans*. Further studies will be addressed to determine the cell(s) and mechanism (s) involved in the inhibitory effect of IFN- γ .

Since the cross-reactivity of rat IFN- γ in murine cells is mainly derived of "in vitro" experiments, it would be also interesting to determine whether murine IFN- γ is more effective than rat IFN- γ in murine cryptococcosis.

RESUMEN

El Interferon Gamma incrementa la sobrevivida de un modelo experimental murino de Criptococosis

Se evaluó la efectividad del interferon- γ (IFN- γ) recombinante de rata en un modelo experimental de criptococosis desarrollado en ratones Balb/C inoculados por vía intraperitoneal con la cepa Rivas de *Cryptococcus neoformans* (*C. neoformans*).

Se tuvieron en cuenta el tiempo de sobrevivida de los animales, el aspecto macroscópico de los órganos en la autopsia, la presencia de levaduras capsuladas en los tejidos y la siembra masiva de un homogenato de cerebro.

El tratamiento con IFN- γ , en dosis diarias de 10.000 UI, no modificó estos parámetros cuando la dosis infectante fue de 10^7 levaduras y el tratamiento se retardó 5 días post-infección (media de sobrevivida de 21 vs. 23 días en los grupos de control y tratados con IFN- γ , respectivamente).

En otros experimentos observamos una prolongación del tiempo de sobrevivida (aunque no significativo) de 20 a 28 días, cuando el tratamiento con 10.000 UI/día de IFN- γ comenzó en el momento de la infección experimental. Cuando el IFN- γ , a razón de 10.000 UI/día durante 15 días, fue administrado simultáneamente con una dosis infectante de $3,5 \times 10^5$ levaduras, el tiempo de sobrevivida de los animales aumentó significativamente ($p=0.004$).

Los resultados obtenidos sugieren que, dependiendo de las condiciones experimentales, IFN- γ prolonga el tiempo de sobrevivida de ratones infectados con una dosis letal de *C. neoformans*.

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