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Prolactin-cytokine network in the defence against *Acanthamoeba castellani* in murine microglia



Abstract

In the central nervous system, cytokine-primed microglia play a pivotal role in host defence against parasite infections. In this study, the effect of recombinant (r) prolactin (rPRL) and rIFN-g on *A. castellani* infection in murine microglia was examined. Priming of microglia with rPRL and rIFN-g synergistically triggered, in a dose-dependent manner, amebastatic activity and the release of endogenous IL-1a, IL-1b, IL-6 and TNF-a. More than 53, 57, 49, 89 or 96 % of amebastatic activity was abrogated by anti-PRLR, IFN-gR, IL-1b (but not IL-1a), IL-6 or TNF-a

antibodies, suggesting that these two receptors and proinflammatory cytokines participate in the anti-microbial function. Inasmuch as DPI and AET, both inhibitors of NO synthase, blocked amebastatic activity only during the priming process, the NO-dependent pathway itself appears not to be directly involved in the anti-parasitic capacity. These data suggest that the PRL/PRLR and IFN-g/IFN-g/R complexes, by the induction of the IFN-gR on microglia, up-regulate the release of endogenous TNF- α , IL-6 and IL-1 β by these cells, which could trigger anti-microbial activity against *A. castellanii* infection in the brain.

Full text

Acanthamoeba castellanii free-living ameba is a biform parasite, which exist in cyst and trophozoite stages in the environment [1, 2]. This protozoan has been recognized as opportunistic pathogen capable to cause a spectrum of infections in human. *A. castellanii* infection generally induces keratitis in health individuals [3, 4]; however, it causes chronic but fatal granulomatous amebic encephalitis (GAE) and extracerebral cutaneous or disseminated acanthamebiasis in immunocompromised patients, including those with the acquired immunodeficiency syndrome (AIDS) [5-10]. Histopathologically, GAE is characterized by focal granulomatous lesions in the central nervous system (CNS), resulting from an immunological response by the host to the presence of trophozoites and cysts. Little is known about defence mechanisms against *A. castellanii* within the brain. Recently, *in vitro* studies, to elucidate the role of microglia in host defence against *Acanthamoeba* species have been carried out using enriched rat or murine microglial cultures challenged with these microorganisms [11-13]. Microglia, the resident macrophages of the CNS [14], have been reportated to manifest a number of macrophage-like functions that may be related to their host defence role, including expression of major histocompatibility complex class II Ag (MHC II Ag) [15], intracellular adhesion molecule 1 (ICAM-1) [16], free radicals (*i.e* nitric oxide (NO)) [16, 17], phagocytosis of microorganisms, such as *A. castellanii* and *T. gondii* [11, 13, 16-19] and production of proinflammatory cytokines, such as TNF- α , IL-1 β , IL-3, IL-6 and IL-8 [16-21] and anti-inflammatory cytokines, such as IL-10 and TGF- β [22, 23] for defence against microorganisms and neuronal injury. In our recent *in vitro* study, we have shown that murine microglia primed with either IL-1 β or IL-6, in the presence or absence of IFN-g, induce amebastatic activity, while microglial cells activated with TNF- α and IFN-g trigger amebicidal capacity [13]. In addition, during the infection process, murine microglia primed by TNF- α and IFN-g appear

to inhibit *A. castellani* growth through an NO-independent mechanism [13], whereas murine microglial cells activated by TNF- α and prolactin are able to partially inhibit *T. gondii* growth in an NO-dependent pathway [16]. Despite microglia have the ability to phagocytose *A. castellani* [11, 13], they may also be induced to apoptosis after challenge with *Acanthamoeba culbertsoni* [12]. It is well known that the immune system influences the neuroendocrine system and vice versa, *via* a bioregulatory feedback loop that permits reciprocal signalling. This signal is carried by cytokines produced in the immune system, which exert bioregulatory actions on the neuroendocrine tissues, and by the hormones produced in the neuroendocrine and peripheral endocrine organs, which biomodulate the activity of immunoeffector cells [24]. Prolactin (PRL), the neuropeptide produced by the pituitary lactotrophic cells and also by others, such as T lymphocytes [24-26], is an implicated hormone in this loop [24]. It is well known that PRL has a lot of potentiality correlated to the quasi-ubiquitous distribution of its receptor [27], which results to be expressed on a number of tissues and cell types, including normal rat and human leukocytes [28, 29]. PRL's capacities are related to lactation, reproduction, behaviour, osmoregulation, proliferation, differentiation, angiogenesis and protection against apoptosis and inflammation. PRL then exerts its actions *via* its receptor (PRLR) [25]. The role of PRL in immunoregulation and normal hemopoiesis is also well known. In particular, PRL is a potent bioregulator and lympho-hemopoietic growth and differentiation factor capable to modulate IFN- γ secretion [30], and to counteract the effects of corticosteroids by enhancing Th1 cellular responses [25]. Our previous *in vitro* findings [16] have shown that PRL and rTNF- α additively up-regulate both the intracellular killing of *T. gondii* and toxoplasmatatic activity. These functions appear correlated with ICAM-1 expression, IL-3 and IL-6 release and NO generation, all markers of macrophage-like activation [16]. Moreover, *in vitro* PRL induces the production of NO and IL-1 in murine peritoneal macrophages [31], and enhances, in additivity with IFN- γ , both the expression of inducible nitric oxide synthase and NO generation in rat C6 glioma cells [32]. Whereas, *in vivo* administration of PRL alone or in combination with rIFN- γ or rTNF- α triggers an immunoprotective effect, increasing survival or delay time to death of mice challenged with a lethal dose of *T. gondii* [33]. Indeed, PRL combined with rTNF- α is implicated in controlling the number of bradyzoite cysts in the brain of surviving mice [33]. In addition, administration of either PRL or an activating anti-PRLR antibody protects mice against *Salmonella typhimurium* infection [34]. Since microglia and proinflammatory mediators could be involved in the immunopathogenesis of GAE, the aim of this study was to assess the role of PRL on *A. castellani* growth in

murine microglia, and to increase the understanding of the effects of IFN- γ or other cytokines in host defence against this parasite infection in the brain.

Material and methods

Reagents

Recombinant (r) human prolactin (rPRL; human prolactin cDNA expressed in C127 cells) was kindly supplied by Genzyme Co. (Framingham, MA). Whereas the mouse anti-rat PRL receptor (PRLR) T6 monoclonal antibody (MAb) was purchased from Affinity Bioreagents (ABR, Italy). Recombinant mouse IFN- γ (rIFN- γ), rabbit anti-mouse TNF- α polyclonal antibody (PAb), rat anti-mouse IL-6 monoclonal antibody (MAb), hamster anti-mouse IL-1 α and IL-1 β MAbs and control antibodies from non-immunised animals were purchased from Genzyme. Whereas the rat anti-mouse IFN- γ receptor (IFN- γ R) MAb was purchased from Pharmingen (Germany). Superoxide dismutase (SOD), lipopolysaccharide (LPS; *E coli* O128: B12), diphenyleneiodonium chloride (DPI; a inhibitor of nitric oxide synthase (NOS)) and 2-aminoethylisothiuronium bromide (AET; an inhibitor of constitutive and inducible NOS) were obtained from Sigma.

The presence of endotoxin in reagents was checked by the *Limulus ameobocyte* lysate test [35] and all results were negative (*i.e.* < 0.05 EU of LPS/ml of sample).

Microglial cell (MGC) cultures

Cell cultures were prepared as previously described by Chao *et al.* [18]. Briefly, cerebral cortical cells from 1-day-old BALB/c mice were dissociated after a 30 min trypsinization (0.25 %), and plated in 75-cm³ Falcon culture flasks (Nunc, Denmark) in RPMI 1640 medium (Seromed), containing 20 % heat-inactivated fetal calf serum (FCS; Seromed), 2 mM L-glutamine (Seromed), 20 mM HEPES (Seromed), 100 U/ml penicillin (Sigma), 100 mg/ml streptomycin (Sigma). The medium was replenished 1 and 4 days after plating. On day 8 of culture, plates were shaken for 20 min, at a speed of 200 rpm in an orbital shaker to remove oligodendrocytes. On day 12 of culture, plates were shaken again for 2 hours at a speed of 180 to 200 rpm. Harvested cells were filtered through a 20 mm nylon mesh, plated onto a 60 mm Petri dish and incubated for 15 min at 37°C. After extensive washing with culture medium, adherent cells (microglia) were collected with a rubber policeman and centrifuged at 200 *g* for 10 min.

Isolated microglia were plated onto glass cover slips previously coated with poly-L-lysine (Sigma) and placed in wells of 24-well plates. Purified microglia were composed of a cell population > 98 % of which stained with MAC-1 antibodies (Boehringer-Mannheim, Biochemical, Milan, Italy) and < 2 % stained with antibodies specific to glial fibrillary acid protein (Sigma), an astrocyte marker.

Preparation of amebas

A. castellanii (ATCC 30010) was obtained from American Type Culture Collection. Parasites were grown as axenic cultures in peptone-yeast-glucose solution in tubes at 35°C as previously described by Benedetto *et al.* [13]. For the infection of MGC, the parasite suspension was removed from a culture of *A. castellanii* in log growth phase and then centrifuged at 250 *g* for 10 min. The pellet containing free trophozoites was then resuspended with RPMI 1640 medium.

Effects of rPRL or rIFN- γ *in MGC infected with A. castellanii*

To determine the effects of rPRL or rIFN- γ on *A. castellanii* growth, in preliminary experiments MGC, at different host cell/parasite ratios, were incubated (37°C, in 5 % CO₂) with medium alone (control) or containing different concentrations of rPRL or rIFN- γ alone or together at various times. Inasmuch as greater anti-parasitic effect was observed when rPRL was combined with rIFN- γ and which was also in agreement with our previous studies [33], we considered that both stimulus were necessary to trigger an effective anti-parasitic activity. The host cell/parasite ratios, concentrations and optimal times turned out to be about 10:1 to 20:1, 25-125 ng/ml and 24-48 hours for rPRL and 5:1 to 20:1, 100-500 U/ml and 24 hours for rIFN- γ . The lower limit concentration of both reagents were then used in our subsequent experiments for microglia activation.

Thereafter, to investigate the dose-effect relationship of rPRL or rIFN- γ on *A. castellanii* growth, after the addition of 100 U/ml SOD (an O₂ - scavenger), MGC cultures (5x10⁴ cells/well) were primed with various concentrations of rPRL in the presence of 100 U/ml rIFN- γ or with different doses of rIFN- γ in the presence of 25 ng/ml of rPRL. After incubation for 24 hours under normal culture conditions, the cells were extensively washed with prewarmed phosphate buffered saline (PBS) and reincubated for an additional 1 hour under the same culture conditions in a medium containing 5x10³ living *A. castellanii* trophozoites, followed by three washes with warm PBS to remove non-ingested parasites. The

cells of one cover slip were fixed in methanol and marked with Diff Quik (Baxter Scientific Products, McGaw Park, IL, USA) stain, for determination of the number ingested trophozoites per 100 MGC at the start (time 0) of the assay. Other replicates were reincubated in the presence of SOD and fresh medium with or without hormone or cytokine alone or together for another 20 hours (time 20), under normal culture conditions, and then fixed and stained as above. The course of intramicroglial infection was assessed microscopically by enumerating the number of amebas/100 cells in Diff Quik stained preparations. If the number of parasites per 100 MGC at time 20 of the assay had increased by more than 7 % relative to the number of parasites per 100 MGC at the start of the assay, the results were discarded. Intramicroglial growth, an index of parasitostatic activity, was expressed as fold increase (FI), *i.e.*, the ratio of the mean number of parasites per 100 MGC after 20 hours (T20) of infection and the mean number of parasites per 100 MGC at 1 hour (T0) of the assay as previously described by Benedetto *et al.* [13].

Effect of anti-PRLR, IFN-gR, *IL-1a*, *IL-1b*, *IL-6* or *TNF-a* antibodies on *A. castellani* growth in MGC

To investigate the implication of receptor for either PRL or IFN-g or specific cytokines in the induction of anti-amebic capacities, preliminarily, control experiments were carried out by MGC incubated with medium alone or containing monoclonal antibodies (10 mg/ml) specific for either PRLR or IFN-gR or neutralizing antibodies (5 mg/ml) specific for IL-1a, IL-1b, IL-6 and TNF-a alone. Inasmuch as MGC treated with each antibody alone did not shown any blocking effect and did not differ from MGC incubated with medium alone, we decided to utilize MGC and medium alone as control experiment. Therefore, in our subsequent investigations, MGC were treated with medium alone or containing rPRL (25 ng/ml) and rIFN-g (100 U/ml), in the absence or presence of MAbs specific for either PRLR or IFN-gR or neutralizing Abs specific for IL-1a, IL-1b, IL-6 or TNF-a, at the same concentrations as above. The concentration of anti-receptor or anti-cytokine antibodies used for this study was based on our preliminary findings. The 10 mg/ml-antibody concentration specific for either PRLR or IFN-gR inhibited the bioactivity of 0.5 mg/ml of PRL or IFN-g respectively. Whereas the 5 mg/ml-antibody concentration specific for cytokine fully abrogated the bioactivity of 5 ng/ml of each cytokine. Antibodies were added to cell cultures between 30 min before and 24 hours after rPRL and rIFN-g. Anti-

parasitic activity in MGC was then evaluated at T20 of infection by microscopic examination.

Measurement of cytokine release by MGC

To evaluate whether the amebastatic activity of microglia primed with rPRL and rIFN- γ was associated with production of endogenous monokines, supernatant media were removed from cell cultures and analysed for IL-1 α , IL-1 β , IL-6 and TNF- α release, at various times during the 24 hours of the priming process. All determinations were carried out using immunoenzymatic methods. Both IL-1 α and IL-1 β cytokines were measured by hamster anti-mouse IL-1 α and IL-1 β MAbs (enzyme-linked immunosorbent assay [ELISA test]; Genzyme). Whereas IL-6 and TNF- α were determined by rat anti-mouse IL-6 MAb and rabbit anti-mouse TNF- α Pab, respectively (ELISA test; Genzyme).

Effect of DPI *or* AET on *A. castellani* growth in MGC

To determine the implication of NO in the induction of amebastatic activity, MGC were treated with medium alone (negative control (NC)) or containing 25 ng/ml rPRL and 100 U/ml rIFN- γ , in the absence or presence of different concentration (5-100 mM) of DPI or AET. The inhibitors of NOS were added during the 24 hours incubation period of the MGC with rPRL and rIFN- γ , before challenge (priming process), and during the 20 hours infection period with *A. castellani* (infection process). As positive control (PC) of NO-dependent amebastatic activity, MGC were also primed, as in our previous study [13], with LPS (10 ng/ml) and rIFN- γ (100 U/ml).

Statistical analysis

In this paper, each set of results presented is the mean $\pm$ standard deviation (SD) of triplicate independent measurements of four separate repeated experiments. Differences were considered to be significant if $p < 0.05$, determined by the Student's t -test.

Results

Effect of rPRL or rIFN- γ on the intramicroglial growth of *A. castellani*

In preliminary experiments, our data demonstrated that MGC primed with either 0.04 or 5 ng/ml rPRL and with either 0.16 or 20 U/ml rIFN-g alone did not inhibit *A. castellanii* growth; the values of the index of multiplication were ($FI = 6.9 \pm 1.0$ or 7.1 ± 1.5 and 7.0 ± 1.7 or 6.7 ± 1.1 respectively *versus* 7.3 ± 1.8 in control MGC, $p = ns$ each). Whereas MGC (Figure 1A and 1B) activated with either 25 ng/ml rPRL or 100 U/ml rIFN-g alone triggered a low amebastatic activity ($FI = 3.7 \pm 0.4$ or 3.8 ± 0.3 respectively *versus* 7.3 ± 1.8 in control cultures, $p < 0.05$ each). Furthermore, MGC primed with 125 ng/ml rPRL or 500 U/ml rIFN-g alone did not lead to greater amebastatic activity ($p < 0.05$ each *versus* control). These findings suggest that the subsequent lack of dose-response for higher concentrations is probably linked to the blocking of either PRL or IFN-g receptor activation. As a result of this, we determined the dose-effect relationship of rPRL or rIFN-g on inhibition of *A. castellanii* growth, hence, MGC were primed with different concentration of rPRL in the presence of 100 U/ml rIFN-g or with various doses of rIFN-g in the presence of 25 ng/ml rPRL. In the presence of 100 U/ml rIFN-g, 25 ng/ml rPRL markedly triggered an effective amebastatic activity ($FI = 2.2 \pm 0.3$ *versus* 7.3 ± 1.8 in control MGC, $p < 0.01$). The anti-parasitic effect of rPRL was synergistic, in a dose-dependent manner, in the presence of a fixed concentration of rIFN-g and vice versa. Furthermore, the presence of polymyxin B added to bind free endotoxin did not reverse amebastatic activity. The growth of *A. castellanii* in microglia primed with rPRL (25 ng/ml) and rIFN-g (100 U/ml) in the presence of 30 mg/ml of polymyxin B was similar (2.5 ± 0.5) to that in microglia primed with rPRL (25 ng/ml) and (100 U/ml) rIFN-g alone. Visual observation of the cultures of microglia using an inverted light microscope did not reveal cyst forms but only phagocytosed trophozoites, which appeared round with punctate lesions rather than ameboid forms.

Effect of anti-PRLR, IFN-g *R*, *IL-1a*, *IL-1b*, *IL-6* or *TNF-a* antibodies on *A. castellanii* growth in MGC

The involvement of receptors for either PRL or IFN-g or specific cytokines in the triggering parasitostatic capacity was assessed using either anti-PRLR or IFN-gR MAb or anti-IL-1a, IL-1b, IL-6 and TNF-a neutralizing Ab. Priming (Figure 2) of MGC with rPRL (25 ng/ml) plus (100 U/ml) rIFN-g, in the presence of anti-PRLR, IFN-gR, IL-1b (but not IL-1a), IL-6 or TNF-a antibodies, blocked amebastatic activity by more than 53, 57, 49, 89 or 96 % ($p < 0.05$, $p < 0.05$, $p < 0.05$, $p = ns$ or $p < 0.01$ or $p = ns$ or $p < 0.01$ each *versus* control or rPRL + rIFN-g-primed cells, respectively). Anti-PRLR MAb combined with anti-rIFN-gR MAb then,

synergistically, blocked amebastatic activity by more than 80 % ($p = \text{ns}$ or $p < 0.01$ *versus* control or rPRL + rIFN-g cells); while, anti-TNF- α PAb in combination with anti-IL-6 MAb inhibited neither synergistically nor additively anti-*Acanthamoeba* activity as compared with that anti-TNF- α or anti-IL-6 antibody alone ($p < 0.05$ *versus* control or rPRL + rIFN-g-primed cells). Besides, anti-TNF- α PAb together with anti-IL-1 β MAb as well as anti-IL-6 MAb combined with anti-IL-1 β MAb blocked, in a neither synergistic nor additive manner, anti-parasitic activity as compared with that anti-cytokine antibody alone (data not shown). In addition, either anti-PRLR or anti-IFN-gR MAb alone or together combined with each anti-cytokine neutralizing Ab inhibited neither synergistically nor additively anti-amebic activity as compared with that anti-receptor antibody alone (data not shown). Antibodies specific to receptors had maximal blocking effect when added 30 min before addition of activation signals, i.e., rPRL and rIFN-g, while those specific to cytokines had maximal inhibitory effect when added at 3 hours after priming signals. These data indicate that during these periods both activators stimulate the intracellular signal transduction pathways involved in the cytokine/cytokine receptor and hormone/hormone receptor network.

Release of IL-1 α , IL-1 β , IL-6 and TNF- α by MGC

These results suggested that endogenous release of IL-1 β , IL-6 and TNF- α during the priming process, before infection with trophozoites, contributes to the subsequent inhibition of *A. castellanii* growth. We, therefore, checked the levels of all four IL-1 α , IL-1 β , IL-6 and TNF- α monokines in culture supernatants, taken every 3 hours during the priming process of MGC with hormone and cytokine. Priming (Figure 3) of MGC with rPRL (25 ng/ml) and rIFN-g (100 U/ml) for 24 hours elicited significant levels of all four cytokines. TNF- α peaked early in the course of the priming process with significant levels already detectable after 3 hours of the incubation period ($p < 0.05$ *versus* control). Maximal amounts for this cytokine were then elicited at 6 hours of the incubation period ($p < 0.001$ *versus* control) and undetectable levels by 12 hours. IL-6 also peaked soon in the course of the incubation period with significant levels at 9 hours ($p < 0.05$ *versus* control) and maximal amounts at 24 hours ($p < 0.001$ *versus* control). Both IL-1 α and IL-1 β showed similar kinetic patterns and were undetectable in the course of the priming process, although, small but significant amounts were measurable at 24 hours ($p < 0.05$ each *versus* control).

Effect of DPI or AET on the amebastatic activity of primed-MGC

To determine the implication of NO in the induction of amebastatic activity, MGC were treated with rPRL (25 ng/ml) and rIFN-g (100 U/ml rIFN-g), in the absence or presence of different concentrations of DPI or AET, added during the priming process, before challenge with *A. castellanii*, and during the infection process. We found that the presence of 10 mM DPI or 20 mM AET added during the first hour of the priming process of MGC, before infection, blocked amebastatic activity ($FI = 7.0 \pm 1.4$ or 7.2 ± 1.0 , $p = ns$ or $p < 0.01$ each *versus* negative control or positive control, respectively); while, the presence of DPI or AET during the infection process did not affect amebastatic capacity ($FI = 2.4 \pm 0.4$ or 2.2 ± 0.2 , $p < 0.01$ or $p = ns$ each *versus* NC or PC, respectively) (Figure 4). These data confirm that NO-dependent pathway itself is not directly involved in the anti-amebic function.

Discussion

IFN-g, TNF- α , IL-1 α , IL-1 β , IL-6 cytokines and PRL hormone, all proinflammatory mediators, play a crucial role in host defence against parasitic infections. The importance of cytokines and microglia, in regulating protective immunity to *A. castellanii* in the brain, has recently been reported by us and others [13, 11]. Microglia have the ability to ingest *A. castellanii*, and elicit IL-1 α , IL-1 β and TNF- α in response to the parasite [11]. Moreover, murine microglia primed with either IL-1 β or IL-6, in the presence or absence of IFN-g, induce amebastatic activity, while microglial cells activated with TNF- α and IFN-g additively trigger amebicidal activity [13]. The pleiotropic effects of PRL on the immune system have well been established; nevertheless, the effect of PRL on *A. castellanii* growth in microglia is not yet known. Prolactin is a potent immunomodulator capable to regulate both cell and humoral-based immunity, although, appears immunostimulatory in malignancies and autoimmune rheumatic diseases [36, 37, 38]. In this context, it is of immunopathogenetic interest the correlation between hyperprolactinaemia and disease activity [38]. Based on these assumptions as well as on our previous *in vitro* studies of *T. gondii* replication in murine microglia [16], we considered that these glial cells together with PRL and IFN-g or other cytokines would be involved in the inhibition of *A. castellanii* within the brain. From the findings of this study, it can be concluded that both rPRL and rIFN-g, alone or together, can directly prime murine microglia to trigger anti-parasital functions in these cells. This conclusion is founded on the following

observations. First, low but activating concentrations of either rPRL or rIFN- γ alone slightly induce amebastatic activity, accompanied by a decrease of *A. castellanii* growth in microglia, whereas combinations of both reagents synergistically prime microglia, in a dose dependent fashion, resulting an effective amebastatic activity and the release of IL-1 α , IL-1 β , IL-6 and TNF- α . Second, neutralization of either PRLR or IFN- γ R as well as endogenous IL-1 β , IL-6 or TNF- α by specific antibodies, partially or completely, blocks the induction of amebastatic capacity. It is well known that hormones or cytokines exert their influence on different cell types through a signal transduction pathway, which begins with the binding of the hormone or cytokine to a transmembrane receptor. Thus, primed cells can display receptors for the hormone or cytokine that stimulate or regulate these cells. Because either rPRL or rIFN- γ alone slightly prime microglia, and both agents are required for an effective amebastatic activity, it is conceivable that the binding of either hormone or cytokine to its receptor alone is not sufficient to trigger the intracellular signal transduction pathways implicated in the induction of anti-amebic capabilities. Hence, a second stimulus (*e.g.*, low concentrations of rIFN- γ or rPRL vice versa) is needed for induction of intracellular signalling events that convert the initial interaction of the hormone or cytokine with its receptor into pathways leading to the biological functions. There is emerging evidence that rPRL and rIFN- γ operate as two key signals in the triggering of microglial amebastatic activity. It is likely that *in vivo* circulating levels of these two immunomodulatory molecules are sufficient to trigger the anti-amebic capacity. Thus, PRL in synergy with IFN- γ could reduce the progression and after-effects of *A. castellanii* infection in the brain. Because concentrations of 25 ng/ml rPRL and 100 U/ml for rIFN- γ are required to prime microglia in order to get a protective effect, and higher concentrations do not lead to greater effect, it is likely that the subsequent lack of dose-response for higher concentrations is linked to the blocking of either PRL or IFN- γ receptor activation. Besides, evidence supports that the anti-parasitic effect of rPRL and rIFN- γ on microglia is not due to contamination with LPS as confirmed by the inability to reverse anti-parasitic activity by treatment with polymyxin B. Analogously, in our previous *in vivo* reports on murine toxoplasmosis, we have shown that mice administrated with PRL and rIFN- γ have greater survival, accompanied by a lower number of cysts in the brain, than do untreated mice [33]. In addition, other studies have reportated that administration of either PRL or an activating anti-PRLR antibody increases both phagocytosis and the intracellular killing of peritoneal macrophages to *Staphylococcus epidermidis*, and induces protection in mice challenged with *Salmonella typhimurium* [34]. This protective effect appears be associated to

the PRL receptor activation [34]. Although IFN-g results to be the most active regulatory factor in controlling the growth of *A. castellanii* and *T. gondii* in microglia [13, 18, 19], our data also suggest that IFN-g is not the sole regulatory factor in the triggering of anti-parasitic activity, since PRL can act in a manner similar to IFN-g in the induction of anti-microbial functions too. In this context, PRL acts as a cytokine and appears as an essential component for an effective immunoprotection. Since anti-parasitic activity is abolished by more than 57 % with the anti-IFN-gR MAb, it is clear that IFN-gR is crucial for the activation of microglia and their associated anti-parasitic functions. Indeed, it has also been reported that IFN-gR, but not TNF receptor signalling, is the major pathway for the activation of microglia in murine *Toxoplasma* encephalitis [39]. Given that amebastatic activity is abrogated by more than 53 % with the anti-PRLR MAb, it is evident that PRL receptor signalling is essential for the activation of microglia and their associated anti-microbial activity too. Probably, *in vivo* PRLR and IFN-gR in concert exert an immunoregulatory activity on the anti-microbial function by transporting hormone and cytokine signalling into cells. This hypothesis is supported on the ability of anti-PRLR and anti-IFN-gR antibodies to synergistically block by more than 80 % the anti-parasitic activity of rPRL + rIFN-g-primed microglia. Our data also suggested that, in the triggering of anti-amebic activity, other cytokines endogenously produced, such as IL-1b (but not IL-1a), IL-6 and TNF-a could, through a complex network, participate in an autocrine or paracrine manner in this function. This hypothesis is supported by the abrogation by more than 49, 89, 96 or 50 % of the amebastatic activity by anti-IL-1b, IL-6, TNF-a or TNF-a plus anti-IL-6 antibodies when added to rPRL + rIFN-g-primed microglia. The results with anti-IL-6 or anti-TNF-a antibody alone are in agreement with our *in vitro* previous findings and others on the inhibition of *T. gondii* growth in microglia [16, 18, 19]; furthermore, they show that anti-TNF-a and anti-IL-6 Abs do not act either synergistically or additively in the inhibition of amebastatic activity. The low blocking effect observed in the presence of anti-TNF-a and anti-IL-6 Abs as compared with that observed with each antibody alone could be linked to the formation of immune complexes, with subsequent elevation of TNF-a or IL-6 levels in the glial microenvironment. On the other hand, the lack of blocking effect by anti-IL-1a MAb suggests that this cytokine may drive a local inflammatory reaction, so contributing, probably, to focal lesions. Consistent with our findings, we found that murine microglia, primed with rPRL and rIFN-g, elicited remarkable levels of either TNF-a or IL-6 but small levels of IL-1 (both a-and-b-isoforms). Kinetic analyses of the response revealed that TNF-a peaked early and then fell to undetectable levels by 12 hours, whereas IL-6, IL-1a and IL-1b peaked later and continued to accumulate

in the medium through 48 hours, suggesting different modes of cytokine regulation. Recently, it has been reported that IFN- γ and PRL both affect the expression of the IFN- γ R gene by increasing IFN- γ R mRNA levels and IL-6 release in monoblastic cells [40]; furthermore, it is well known that in normal leukocytes, PRL signals through PRLR associated Janus tyrosine kinase 2 (JAK2, a kinase involved in IFN- γ signalling), and signal transducers and activators of transcription (Stat). Indeed, PRL, at physiological concentrations, stimulates the expression of the interferon regulator factor-1 (IRF-1) gene in both human granulocytes and rat lympho-hemopoietic cells [28, 29]. Consequently, it is likely that both PRL/PRLR and IFN- γ /IFN- γ R complexes, *via* common JAK-Stat pathways, up-regulate the release of endogenous TNF- α , IL-6 and IL-1 β by an overexpression of the IFN- γ R on microglia. In this context, it is very interesting that TNF- α (but not IL-1- β , IL-10, IL-12 p40 and IL-15) mRNA production of microglia appears dependent of the IFN- γ R signalling and, hence, impaired in IFN- γ R-deficient mice [39]. Accordingly, IFN- γ R knockout (KO) mice show reduced TNF- α but normal IL-6 and L-1a serum levels upon stimulation with LPS [41]. On the other hand, PRLR KO mice show numerous deficiencies, including pituitary hyperplasia, lack normal mammary development and progesterone production, sterility and hyperprolactinemia [42, 43, 44, 45]. In addition, mice overexpressing PRL manifest several autoimmune perturbations that resemble human systemic lupus erythematosus (SLE), including cytokine aberrations and autoantibody formation [37]. In particular, prolactin accelerates disease expression and activity in the both NZB x NZWF1 mouse lupus model and SLE patients [30, 38]. The up-regulation of TNF- α , IL-6 and IL-1 β or other proinflammatory cytokines represent a key event in the initiation of cerebral immunity to *A. castellanii*. Since during infection within the brain, microglia produce both proinflammatory [16, 18, 19, 20, 21] and anti-inflammatory cytokines [22, 23], it is clear that the up-regulated production of TNF- α , IL-6 and IL-1 β or other proinflammatory and anti-inflammatory cytokines serves to trigger anti-amebic functions and limit inflammation associated to brain injury. In cultured microglia, PRL and rTNF- α up-regulate the production of endogenous IL-1 β , IL-3 and IL-6, which also participate in the toxoplasmastatic activity [16]. Whereas in cultured astrocytes, PRL induces the expression of TNF- α , IL-1 α and TGF- α cytokines, which in turn can synergize with PRL in promoting astrocyte proliferation and secretory activity [46]. Anyway, the mechanism whereby hormone-cytokine priming triggers anti-amebic activities in microglia is not yet well understood. Since DPI and AET, both inhibitors of NO synthase, completely block the anti-parasitic activity only during the priming process, it is evident that NO molecules itself does not directly affect the anti-amebic activities. These

data coupled with those of our previous study [13] reconfirm, thus, that during the infectious process could intervene other NO-independent anti-parasitic mechanisms for defence against the parasite. In conclusion, the findings of this study may be more directly pertinent to understanding the immunopathogenesis of GAE. There is exciting evidence that perturbations of PRL or cytokine physiology, could have serious immunologic repercussions. In patients with hypoprolactinaemia or AIDS, for example, a shift from Th1 to Th2 cytokines, which is accompanied by a down-regulation of IFN-g, TNF- α , IL-1 β and IL-6, could represent a dramatic event in the development of GAE. Such a role for both PRL/PRLR and IFN-g /IFN-gR complexes in regulating microglial anti-parasitic function *in vivo* appears particularly relevant to the control of GAE in the immunocompromised subjects. Future studies on the relationship of the PRL/PRLR or IFN-g/IFN-gR complex and the immune system will provide further insights into the potential therapeutic utility of these complexes in patients at risk of *Acanthamoeba* encephalitis.

CONCLUSION

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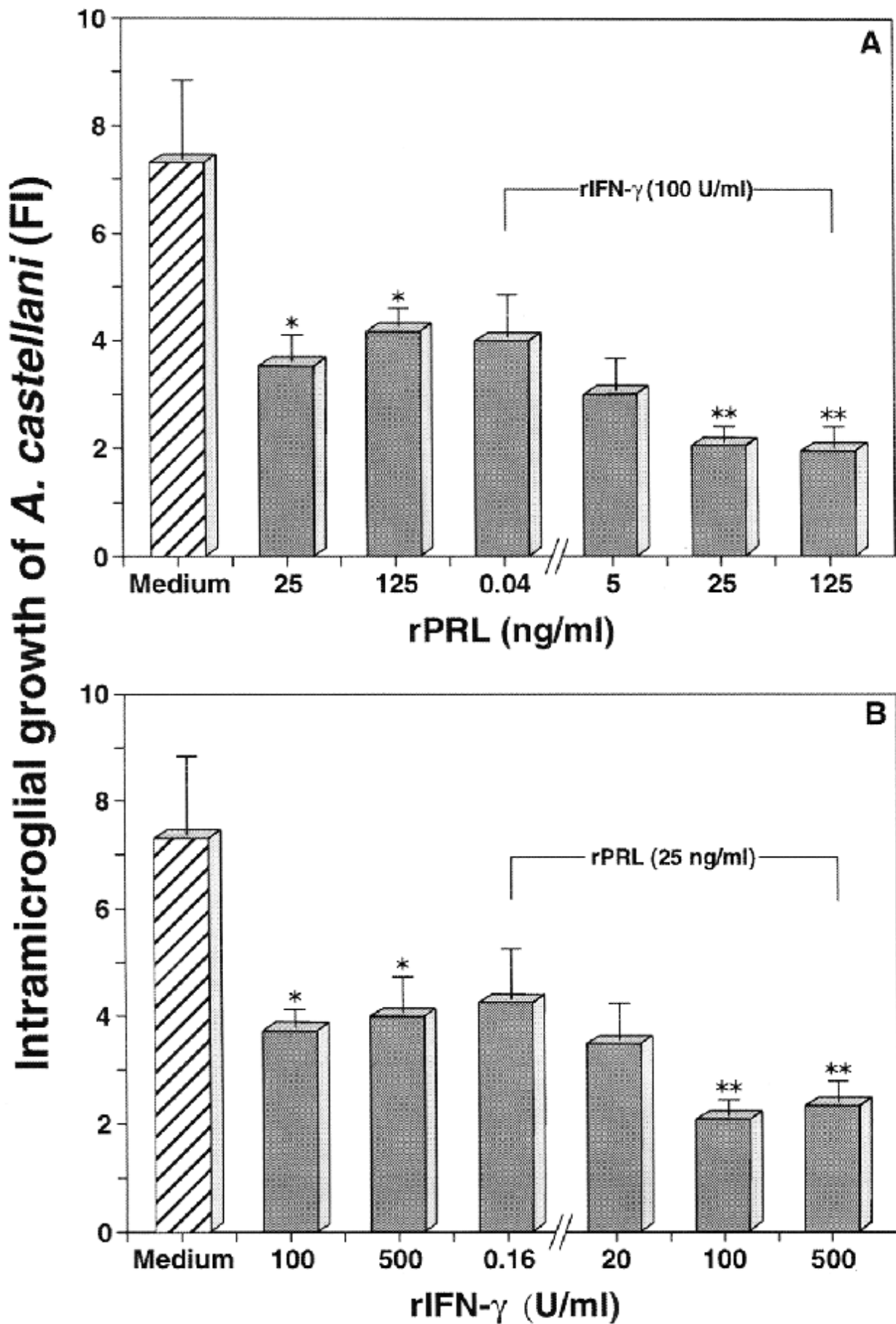
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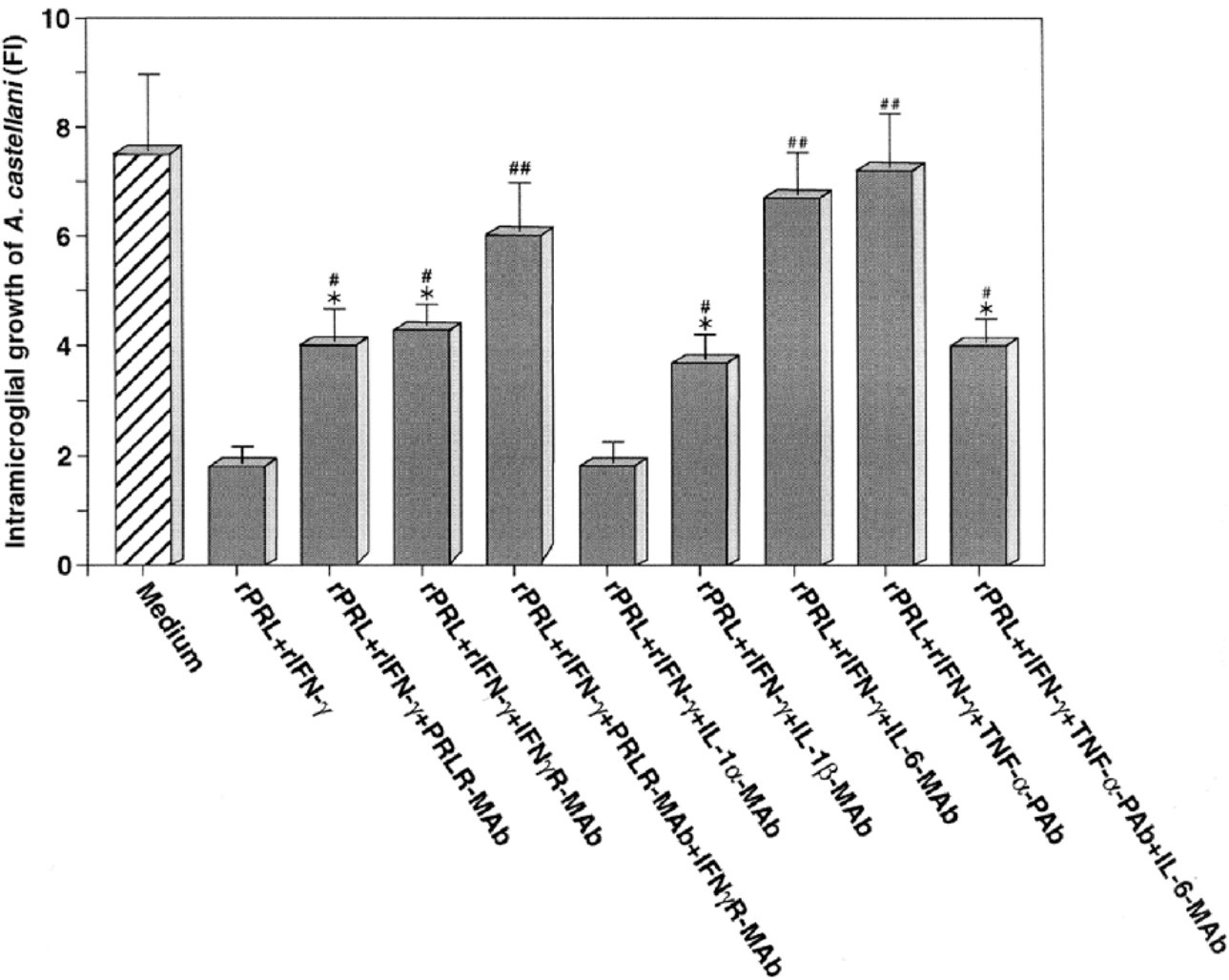
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Figures



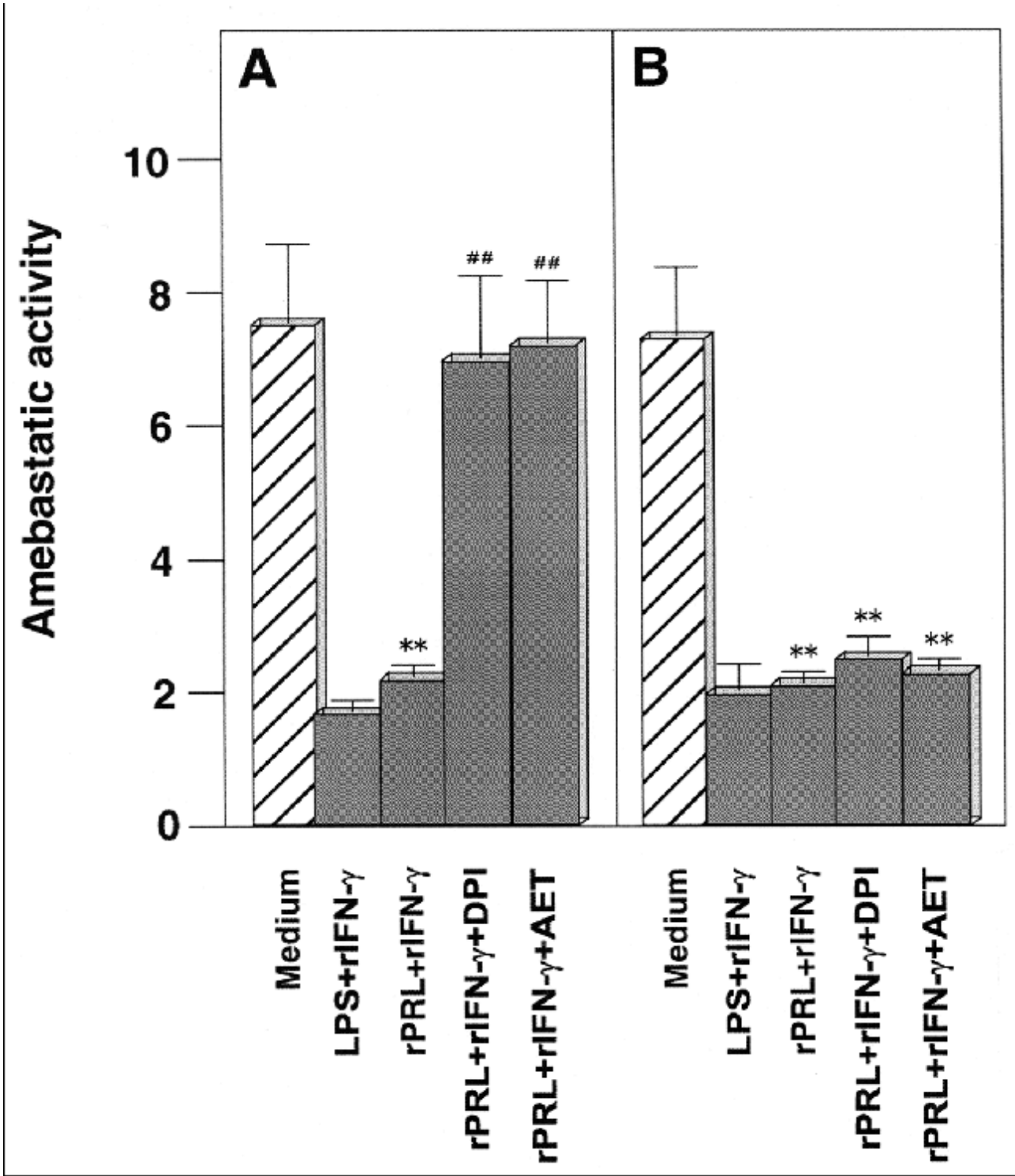
Dose-effect relationship of rPRL or rIFN-g on the inhibition of *A. castellani* growth.

Microglial cell (MGC) cultures, at a concentration of (5×10^4 cells/well), were treated with medium alone (control) or containing the indicated concentrations of (A) PRL in the absence or presence of 100 U/ml rIFN- γ or (B) rIFN- γ in the absence or presence of 25 ng/ml PRL for 24 hours and then challenged for 1 hour and 20 hours with *A. castellani* trophozoites under normal condition cultures. Intramicroglial growth of *A. castellani*, an index of amebastatic activity, was expressed as fold increase (FI) in the number of trophozoites per 100 MGC at 20 hours of infection. Values are the means $\pm$ SD of triplicate counts of four separate experiments. Significant differences *versus* control (* $p < 0.05$; ** $p < 0.01$).



Effect of antibodies against PRLR, IFN-gR, IL-1a, IL-1b, IL-6 or TNF-a on the amebastatic activity in MGC.

MGC cultures (5×10^4 cells/well) were incubated with medium alone or containing PRL (25 ng/ml) and rIFN-g (100 U/ml) for 24 hours. At 3 hours of incubation, monoclonal antibodies (10 mg/ml) specific for either PRLR or IFN-gR or neutralizing antibodies (5 mg/ml) specific for IL-1a, IL-1b, IL-6 or TNF-a were added under normal condition cultures. The cells were then challenged with trophozoites. Intramicroglial growth of *A. castellanii* was determined after 20 hours of infection as above. Data are the means $\pm$ SD of triplicate counts of four experiments. *: significant difference *versus* control; #: significant difference *versus* rPRL + rIFN-g cells (*, # $p < 0.05$; **, ** $p < 0.01$).



Course of the release of IL-a, IL-1b, IL-6 and TNF-a by MGC during incubation with rPRL and rIFN-g.

Cell cultures (5×10^4 cells/well) were incubated with medium alone or containing rPRL (25 ng/ml) and rIFN-g (100 U/ml). At various time points during the priming process, the culture supernatant was collected and the levels of IL-a, IL-1b, IL-6 or TNF-a were determined by ELISA

methods. At 72 hours no TNF- α , IL-1 α or IL-1 β could be detected; nd: not detected. Values are the means $\pm$ SD of three determinations of four performed experiments. Significant difference *versus* control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Effect of DPI or AET on the amebastatic activity in MGC.

Cell cultures were primed with medium alone (negative control) or containing either LPS (10 ng/ml) and rIFN- γ (100 U/ml) (positive control) or rPRL (25 ng/ml) and rIFN- γ (100 U/ml), in the absence or presence of 10 mM of DPTA or 20 mM of AET added (A) during the 24 hours priming process, before infection with *A. castellanii* trophozoites, and (B) during the 20 hours infection process with trophozoites. Anti-parasitic activity was expressed as fold increase in the number of amebas per 100 MGC at T20 of infection as above. Values are the means $\pm$ SD of triplicate determinations of four performed experiments. *: significant difference *versus* negative control; #: significant difference *versus* positive control (*, # $p < 0.05$; **, # # $p < 0.01$; ***, # # # $p < 0.001$).

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