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# INTRACELLULAR *Leishmania amazonensis* KILLING INDUCED BY THE GUANINE NUCLEOSIDE 8-BROMOGUANOSINE

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## SUMMARY

In this study we investigated the effect of 8-Bromoguanosine, an immunostimulatory compound, on the cytotoxicity of macrophages against *Leishmania amazonensis* in an *in vitro* system. The results showed that macrophages treated with 8-Bromoguanosine before or after infection are capable to reduce parasite load, as monitored by the number of amastigotes per macrophage and the percentage of infected cells (*i.e.* phagocytic index). Since 8-Bromoguanosine was not directly toxic to the promastigotes, it was concluded that the ribonucleoside induced macrophage activation. Presumably, 8-Bromoguanosine primed macrophages by inducing interferon alpha and beta which ultimately led to *L. amazonensis* amastigote killing. The results suggest that guanine ribonucleosides may be useful to treat infections with intracellular pathogens.

**KEYWORDS**: *Leishmania amazonensis*; Macrophage; Guanine ribonucleosides; 8-Bromoguanosine; Immunostimulators.

## INTRODUCTION

Leishmaniasis is an endemic parasitosis caused by several species of the genus *Leishmania*. Injected into mammalian hosts by phlebotomus sandflies as extracellular promastigotes, *Leishmania* bind to macrophages and are quickly phagocytosed. All *Leishmania* species are obligate intramacrophage parasites, which live within secondary phagolysosomes. In this manner, the parasite is able to multiply, lyse the host cells and infect surrounding macrophages[20]. The severity of disease varies widely ranging from cutaneous or mucosal to visceral or diffuse cutaneous infection. The former is generally caused by *L. amazonensis*, a species transmitted mainly in the Amazon region, which is associated with localized benign cutaneous lesions[12, 19, 20]. The treatment of leishmaniasis has been based on the use of antimonates and amphotericin B, drugs with several toxic effects[4].

Investigations *in vivo* and *in vitro* suggest that recovery from leishmanial infection involves the destruction of the parasites in their intracellular location, resulting from activation of the macrophages by T cell-derived cytokines. *In vitro*, mouse macrophages stimulated with interferon gamma, tumor necrosis factor or both, and in the presence of lipopolysaccharides are capable of killing the parasite[14,17]. Recently, it was also demonstrated that interferon alpha and lipopolysaccharides induce an *L. major* killing response in macrophages[18].

A pattern of stimulation is observed in macrophages and natural killer cells exposed to certain guanine ribonucleoside-based immunostimulants. For instance, guanine derivates acted on macrophages *in vitro* by increasing phagocytosis[15], interferon alpha and beta production[10, 13] and superoxide anion generation[15]. Based on these studies, recently, we demonstrated that some guanosine derivatives enhance intracellular parasite destruction[5]. This report describes a detailed *in vitro* analysis of the effect of 8-Bromoguanosine (8BrGuo) on the capacity of murine macrophages to destroy *L. amazonensis* amastigotes.

## MATERIALS AND METHODS

**Macrophage cultures.** One to 2 months old mice of C57Bl/6 strain were sacrificed and injected intraperitoneally with sterile saline containing 10 U/ml penicillin and 25 µg/ml streptomycin. The exsudate cells were harvested, counted and distributed (5 x $10^5$ macrophages/well) in 24 well culture plates (Costar, Cambridge, MA, USA) containing 13 mm diameter sterile glass coverslips. The plates were incubated for 2 hours at room temperature, then washed once with saline to remove non-adherent cells, and incubated with Iscove's medium (Sigma, Chemical Co., St.Louis, USA) supplemented with 5% fetal calf serum (Cultilab, Campinas, SP, Brazil), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, 100 mM hepes and 20 mM sodium bicarbonate (Sigma, Chemical Co., St.Louis, USA). The cultures were incubated for 24 hours at 37 °C in 5% $CO_2$ in air in a humidified incubator.

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**Parasite and Infection of macrophage cultures**. *Leishmania amazonensis* (MHOM/BR/73/M2269) amastigotes were prepared from infected BALB/c footpad lesions as previously described[1]. Promastigotes used in the experiments were grown from BALB/c mice footpad lesions in Iscove's medium supplemented with 5% fetal calf serum and antibiotics. After washing by centrifugation, the parasite suspensions were added to the macrophage cultures. The parasite to cell ratio was 3 for amastigotes or 10 for promastigotes. The cultures were maintained in room temperature for one hour to allow the phagocytosis of the parasites. The infected cultures were then washed to remove free parasites and incubated for twenty four hours at 37 °C in 5% $CO_2$ in air in a humidified incubator.

**Macrophage Treatment**. Macrophages cultured on plates were incubated in the presence of different concentrations of 8BrGuo (Sigma, Chemical Co., St.Louis, USA). 8BrGuo was dissolved in 0.5 N NaOH and diluted with Iscove's medium. As control, an equivalent volume of 0.5 N NaOH was diluted with Iscove's medium. At the final concentration of 0.01 N NaOH in culture wells the pH of the medium was 7.5. The cells were exposed to 8BrGuo, before or after parasite infection. After the indicated periods of treatment, coverslip cultures were fixed in methanol, stained with Giemsa and examined under the light microscope.

**Measurement of intracellular parasite killing**. Parasites were assessed by counting at least 200 macrophages, and the number of infected cells and amastigotes per cell for each coverslip. All experiments were repeated at least three times in duplicate wells. The results were also expressed as phagocytic index, which is the product of the percentage of infected macrophages times the average number of amastigotes per macrophage[1,3].

**Promastigote Treatment**. Parasites growing in 25 $cm^2$ plastic flasks were treated with different doses of 8BrGuo, diluent or glucantime (Rhodia, SP, BR), and the number and the morphological characteristics of the parasites were visualized in a Neubauer chamber for five days.

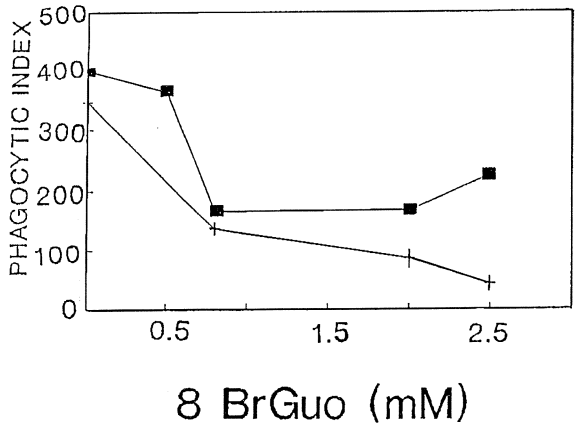


Fig. 1 - Effect of 8BrGuo treatment on macrophages infected with *L. amazonensis* amastigotes. Macrophages from C57Bl/6 mice were treated for 24 hours with different concentrations of 8BrGuo and then infected with amastigotes. After 24 hours, cell cultures on glass coverslips were stained with Giemsa and examined microscopically. (■). Alternatively, macrophage cultures were infected with amastigotes and then treated with different concentrations of 8BrGuo for 24 hours (✚). The results are from one experiment, representative of a total of six.

## RESULTS

Macrophages from C57Bl/6 mice were exposed for 24 hours to increasing concentrations of 8BrGuo. The cultures were then washed and infected with amastigotes, and the phagocytic index was calculated 24 hours later. As shown in Fig. 1, 8BrGuo induced intracellular killing in a concentration-dependent manner. A similar effect and of the same order of potency was observed in macrophage cultures infected with amastigotes and then treated with 8BrGuo for 24 hours (Fig. 1). Pre treatment with 8BrGuo for 24 hours was also effective in protecting macrophages from infection with promastigotes (Fig. 2). The phagocytic indexes obtained during promastigote (Fig. 2) and amastigote infection (Fig. 1) were correlated with the infectivity of the forms; promastigotes were less virulent than amastigotes[1].

On a morphological basis, 8BrGuo proved to be nontoxic to macrophage at concentrations (0.5- 2.5 mM) that induced *Leishmania* killing (Fig. 3). At higher concentrations (more than 3 mM), 8BrGuo damaged macrophages (data not shown). Similarly, the compound did not alter promastigote viability at concentrations up to 2.6 mM under conditions where a toxic effect of the clinically used agent glucantime was observed (Fig. 4).

8BrGuo is likely to induce macrophage activation because it was not directly toxic to promastigotes (Fig. 4), or macrophage cultures (Fig. 3). Interestingly, 8BrGuo reduced the number of infected cells as compared with control (NaOH), (around 20% and 50%, respectively); and a synergistic action was observed with interferon alpha and beta (around 10% of infected macrophages) (Fig. 5A). The cytokines alone were unable to induce efficient parasite killing (around 30% of infected macrophages, but no reduction in the number of amastigotes per cell) (Figs. 5A and 5B). Similar results were obtained with macrophages from another mouse strain (BALB/c) (data not shown).

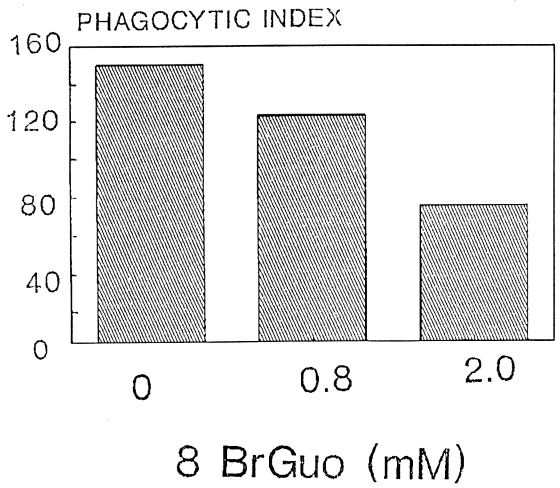


Fig. 2 - Effect of 8BrGuo treatment on macrophages infected with *L. amazonensis* promastigotes. Macrophages from C57Bl/6 mice were treated with different concentrations of 8BrGuo for 24 hours and then infected with promastigotes. After 24 hours, cell cultures on glass coverslips were stained with Giemsa and examined microscopically. The results are from one experiment, representative of a total of three.

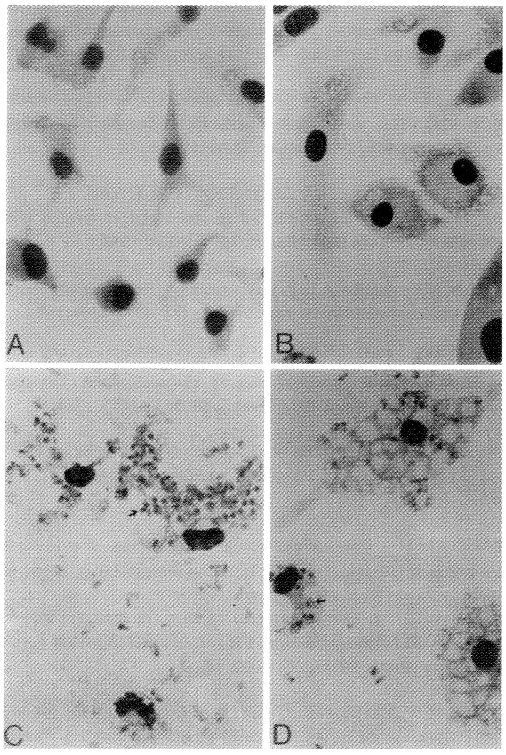


Fig. 3 - Morphological aspect of macrophage cultures. A: control culture of C57Bl/6 mouse peritoneal macrophages. B: same culture as in A after exposure for 24 hours to 0.8 mM 8BrGuo. C: same culture as in A after 24 hours infection with *L. amazonensis* amastigotes. D: same culture as in C, pretreated with 0.8 mM 8BrGuo. Note the disappearance of the parasites in D. Cell cultures on glass coverslips were stained with Giemsa. 1000 X.

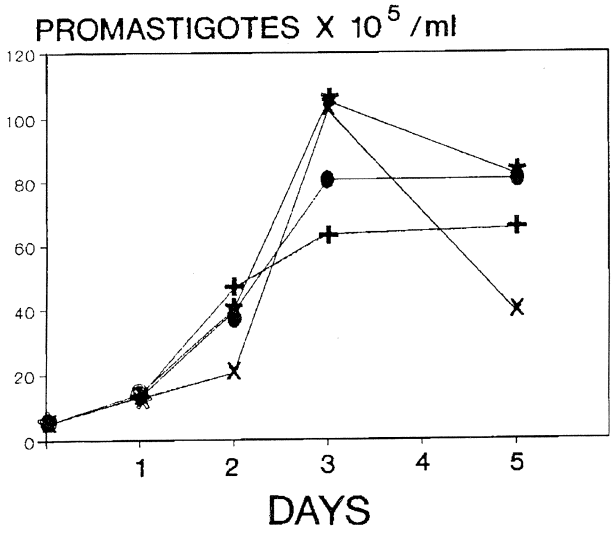


Fig. 4 - Toxicity 8BrGuo for extracellular parasites. Free promastigotes of *L. amazonensis* were exposed to different concentrations of 8BrGuo (✚) 0.5 mM; (●) 2.6 mM ; (★) diluent and (✕) 2.6 mM glucantime for five days at 28° C. Parasite viability was assessed by microscopic examination. The results are from one experiment, representative of a total of three.

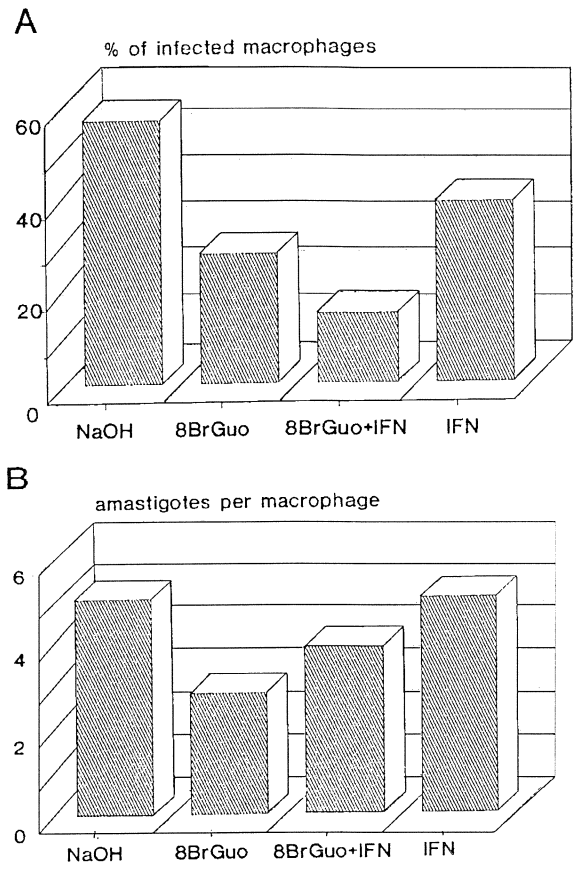


Fig.5 - Effect of 8BrGuo and interferon alpha and beta on *L. amazonensis* infection in murine macrophages. Cells were obtained as decribed in the Materials and Methods section. The cell cultures were pretreated for 24 hours with medium containing 0.01 NaOH; 0.8 mM 8BrGuo; 0.8 mM 8BrGuo plus 100 U/ml murine interferon alpha and beta (Sigma, Chemical Co., St.Louis, USA) and, 100 U/ml interferon alpha and beta. After infection with amastigotes and 24 hours incubation, cell cultures on glass coverslips were stained with Giemsa and examined microscopically. A: percentage of infected macrophages; B: number of amastigotes per macrophage. The results are from one experiment, representative of a total of four.

## DISCUSSION

The results reported here demonstrate that the nucleoside 8BrGuo was able to reduce *in vitro L. amazonensis* infection of mouse peritoneal macrophages. These cells treated before or after the infection were equally protected by 8BrGuo. The cytotoxic effect of the compound on the intracellular amastigotes was critically dependent on the concentration added to the culture medium (Figs. 1, 2 and 3). A basic question concerns the mode of action of the nucleoside. Since 8BrGuo was not toxic to promastigote (Fig. 4) or macrophage cultures (Fig.3), we may be conclude that the ribonucleoside induced macrophage activation. It has been shown that ribonucleosides substituted at the C8 position activate a broad range of immunological functions, including B cell proliferation, antibody response and NK cell and macrophage-mediated

cytotoxicity [2, 6, 7, 8, 13, 15]. In B cells, nucleoside activity appears to be mediated by specific cytoplasmic nucleoside-binding proteins that rapidly undergo nuclear translocation upon ligand binding[9]. The increase in macrophage activity is likely to involve different processes such as cytokine production (interferon alpha and beta)[11, 13] and respiratory burst (generation of oxygen metabolites)[15] . *Leishmania* killing could result from a direct action of 8BrGuo on the parasites within vacuolar spaces of the infected macrophages. Alternatively, the compound may act by stimulating the microbicidal process in the phagocytes. The experiments described here favor the latter possibility. Indeed, the compound did not inhibit the growth of free living parasites at concentrations near those required for intracellular destruction (Fig. 4), and acted synergistically with interferon alpha and beta on macrophage activation against *L. amazonensis* (Fig. 5). Other investigators have defined 8BrGuo as an inductor of interferon alpha and beta[11, 13]. Also, it has been recently reported that interferon alpha plus lipopolisaccharides activate macrophages to kill *L. major*[18]. Consequently, it is reasonable to assume that at least part of the leishmanicidal effects of 8BrGuo are due to macrophage activation. On the basis of the intracellular killing of *L. amazonensis* promoted by 8BrGuo *in vitro* and other guanosine derivatives[5] it would be interesting to determine whether these compounds are also capable of killing parasite during murine leishmaniasis.

## RESUMO

### Morte intracelular de *Leishmania amazonensis* induzida pelo nucleosídeo de guanina 8-Bromoguanosina

Neste trabalho, nós investigamos o efeito da 8-Bromoguanosina, um composto imunoestimulador, na citotoxicidade de macrófagos infectados com *Leishmania amazonensis* em um sistema *in vitro*. Os resultados mostraram que macrófagos tratados com 8-Bromoguanosina pré- ou pós- infecção foram capazes de reduzir a carga parasitária, monitorada pelo número de amastigotas por macrófago e a percentagem de células infectadas (*i.e.* índice fagocítico). Sendo a 8-Bromoguanosina inócua para promastigotas, concluímos que o composto induz ativação celular. Os macrófagos produziriam interferon alfa e beta e teriam seus mecanismos leishmanicidas estimulados. Esses resultados sugerem que compostos como a 8-Bromoguanosina (ribonucleosideos de guanina) podem auxiliar no tratamento contra patógenos intracelulares.

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## REFERENCES

1. BARBIÉRI, C. L.; GIORGIO, S.; MERJAN, A. J. & FIGUEIREDO, E. N. - Glycosphingolipid antigens from *Leishmania (Leishmania) amazonensis* amastigotes identified by use of a monoclonal antibody. **Infect. Immun., 61**: 2131-2137, 1993.

2. BONNET, P.A. & ROBINS, R.K. - Modulation of leukocyte genetic expression by novel purine nucleoside analogues. A new approach to antitumor and antiviral agents. **J. med. Chem., 36**: 635-653, 1993.

3. CANTOS, G.; BARBIÉRI, C. L.; IACOMINI, M.; GORIN, P.A. J. & TRAVASSOS, L.R. -Synthesis of antimony complexes of yeast mannan and mannan derivatives and their effect on *Leishmania*-infected macrophage. **Biochem. J., 289**: 155-160, 1993.

4. CHANCE, M.L. - New developments in the chemotherapy of leishmaniasis. **Ann. trop. Med. Parasit., 89 (suppl.1)**: 37-43, 1995.

5. GIORGIO, S.; BARÃO, S.C.; AUGUSTO, O. & KWEE, J.K. – *Leishmania amazonensis* infection is reduced in macrophages treated with guanine ribonucleosides. **Acta trop. (Basel), 70**: 119-122, 1998.

6. GOODMAN, M.G. & WEIGLE, W.O. - Induction of immunoglobulin secretion by a simple nucleoside derivative. **J. Immunol., 128**: 2399-2404, 1982.

7. GOODMAN, M.G. & WEIGLE, W.O. - Manifold amplification of in vivo immunity in normal and immunodeficient mice by ribonucleosides derivatized at C8 of guanine. **Proc. nat. Acad. Sci. (Wash.), 80**: 3452-3455, 1983.

8. GOODMAN, M.G. & WEIGLE, W. O. - Derivatized guanine nucleosides: a new class of adjuvant for in vitro antibody response. **J. Immunol., 130**: 2580-2585, 1983.

9. GOODMAN, M.G. - Cellular and biochemical studies of substituted guanine ribonucleoside immunostimulants. **Immunopharmacology, 21**: 51-68, 1991.

10. GOODMAN, M.G. - A new approach to vaccine adjuvants. In: POWEL, M.F & NEWMAN, M.J. **Vaccine design: the subunit and adjuvant approach**. New York, Plenum Press, 1995. p. 581-609.

11. GOODMAN, M.G.; REITZ, A.B.; CHEN, R. et al.- Selective modulation of elements of the immune system by low molecular weight nucleosides. **J. Pharmacol. exp. Ther., 274**: 1552-1557,1995.

12. GRIMALDI JR., G.; TESH, R. B. & MACMAHON-PRATT, D. - A review of the geographic distribution and epidemiology of leishmaniasis in the New World. **Amer. J. trop. Med. Hyg., 41**: 687-725, 1989.

13. KOO, G.C.; JEWELL, M.E.; MANYAK, C.L.; SIGAL, N.H. & WICKER, L.S. - Activation of murine natural killer cells and macrophages by 8-bromoguanosine. **J. Immunol., 140**: 3249-3252, 1988.

14. MILON, G.; DEL GIUDICE, G. & LOUIS, J. A. - Immunobiology of experimental cutaneous leishmaniasis. **Parasit. today, 11**: 244-247, 1995.

15. OJO-AMAIZE, E. A.; RUBALCAVA, B.; AVERY, T. et al. - Activation of the respiratory burst in murine phagocytes by certain guanine ribonucleosides modified at the 7 and 8 positions: possible involvement of a pertussis toxin-sensitive G-protein. **Immunol. Lett., 23**: 173-178, 1990.

16. POPE, L.P.; MACINTYRE, P.; KIMBALL, E. et al. - The immunostimulatory compound 7-allyl-8-oxoguanosine (loxoribine) induces a distinct subset of murine cytokines. **Cell. Immunol., 162**: 333-339, 1995.

17. REINER, S. L. & LOCKSLEY, R. M. - The regulation of immunity to *Leishmania major*. **Ann. Rev. Immunol., 13**: 151-177, 1995.

18. SHANKAR, A.H.; MORIN, P. & TITUS, R.G. - *Leishmania major*; differential resistance to infection in C57BL/6 (high interferon-α/ß) and congenic B6.C-H-28c (low interferon-α/ß) mice. **Exp. Parasit., 84**: 136-143, 1996.

19. WALTON, B.C. - American cutaneous and mucocutaneous leishmaniasis. In: PETERS, W. & KILLICK-KENDRICH, R. **The leishmaniases in biology and medicine.** London, Academic Press, 1987. v.1, p. 637-664.

20. WHO - Control of the leishmaniasis. **Wld. Hlth. Org. techn. Rep. Ser., 793, 1990**.

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