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Inhibition of Mouse Lymphocyte Proliferative Response by Glycosphingolipids from *Leishmania (L.) amazonensis*

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GIORGIO, S., JASIULIONIS, M. G., STRAUS, A. H., TAKAHASHI, H. K., AND BARBIÉRI, C. L. Inhibition of mouse lymphocyte proliferative response by glycosphingolipids from *Leishmania (L.) amazonensis*. *Experimental Parasitology* 75, 119–125. The effect of a purified preparation of glycosphingolipids (GSLs) extracted from *Leishmania (L.) amazonensis* amastigotes on murine lymphocytes was investigated. GSLs inhibited both concanavalin A (Con A)- or lipopolisaccharide-induced [^{3}H]thymidine uptake by normal and immunized BALB/c mouse lymph node cells. The effect of total GSLs was dose dependent. Total GSLs also suppressed the two-way mixed lymphocyte reaction of BALB/c × C57BL/6 cells and the antigen-specific response of immunized mouse cells. Six pure bands as well as a pool containing a mixture of GSLs with five to seven sugar residues separated by a combination of HPLC and preparative HPTLC were tested and shown to be inhibitors of Con A stimulation. These results suggest that parasite glycosphingolipids may play an immunologically relevant role in leishmaniasis.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Leishmania (L.) amazonensis;* Glycosphingolipids (GSLs); Lymphocytes; Leishmaniasis; High-performance thin-layer chromatography (HPTLC); High-performance liquid chromatography (HPLC); Fetal calf serum (FCS); Concanavalin A (Con A); Lipopolysaccharide (LPS); [*methyl*-^{3}H]thymidine ([^{3}H]thymidine); Mixed lymphocyte reaction (MLR); Ceramide monohexosyl (CMH); Ceramide dihexosyl (CDH); Ceramide trihexosyl (CTH); Ceramide pentahexosyl (CPH); Ceramide tetrahexosyl (globoside); Ceramide-*N*-tetrose-*N*-acetylneuraminic acid (GM1); Ceramide-lactose-*N*-acetylneuraminic acid (GM3); Ceramide-*N*-tetrose-di-acetylneuraminic acid (GD1a); Ceramide-*N*-tetrose-tri-*N*-acetylneuraminic acid (GT1b); Lipid-containing glycoconjugate (LPG).

INTRODUCTION

Leishmania (L.) amazonensis can cause either cutaneous leishmaniasis, a self-healing disease, or diffuse cutaneous leishmaniasis, a chronic disease associated with a specific anergy of the cellular immune system. The latter process was demonstrated in patients and murine models by the absence of delayed-type hypersensitivity reaction to leishmanial antigen and by the inability of lymphocytes to proliferate in response to parasite antigens (Bryceson 1970; Petersen *et al.* 1982; Barral *et al.* 1983).

Two hypotheses have been proposed to explain the nonresponsiveness. The first one suggests that the genetic constitution of the host is the cause for the nonresponsiveness (Bryceson 1970; Convit *et al.* 1972). The other hypothesis proposes that the host immune response is a reflection of parasite-associated determinants (Londner *et al.* 1983; Akuffo *et al.* 1988) which can induce important immunosuppressive effects during the development of leishmaniasis (Farah *et al.* 1976; Akuffo *et al.* 1988; Hviid *et al.* 1990).

The immune suppression has been attributed to some parasite antigens which lead to an exacerbation of cutaneous lesions. Among these it has been determined that

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leishmanial protein antigens can mediate disease aggravation (Scott *et al.* 1988; Bogdan *et al.* 1990). Delipidated water-soluble glycoconjugate derived from *L.* (*L.*) *major* promastigotes is disease promoting, whereas the lipid-containing glycoconjugate (LPG) represents a protective antigen (Handman and Mitchell 1985; Handman and Goding 1985) able to develop delayed hypersensitivity responses in LPG-immunized mice (Moll *et al.* 1989) and proliferation of peripheral blood mononuclear cells from leishmaniasis patients (Jaffe *et al.* 1990; Kemp *et al.* 1991; Mendonça *et al.* 1991). Nevertheless, the immune effects of LPG are still controversial. Recent findings have shown that the stimulation of lymphocytes from mice immunized with *L. donovani* seems to be actually associated with a protein component tightly bound to LPG (Jardim *et al.* 1991).

Recently a family of glycosphingolipids (GSLs) was identified in the *Leishmania* species (Jasiulionis *et al.* 1990) and high levels of these glycoconjugates have been detected in amastigote form of *L.* (*L.*) *amazonensis* (Jasiulionis, M. G., Straus, A. H., and Takahashi, H. K., manuscript in preparation). Several reports have suggested that glycosphingolipids may play a role in cell signaling and recognition (Hakomori 1981; Yu *et al.* 1984). In particular neutral glycosphingolipids and gangliosides may modulate cell proliferation (Hakomori 1981). Gangliosides have been found to inhibit proliferative responses of immune cells stimulated by mitogens, antigens, and interleukin 2 and to enhance growth of immunogenic tumors (Marcus 1984; Ryan and Shinitzky 1979; Lengle *et al.* 1979; Ladisch *et al.* 1983; Krishnaraj *et al.* 1982; Marcus *et al.* 1987). In view of these results we investigated the effects of glycosphingolipids from *L.* (*L.*) *amazonensis* amastigotes on murine lymphoid cell proliferation.

Materials and Methods

Mice. Female BALB/c (H-2^d) and C57BL/6 (H-2^b) mice 8 weeks old were obtained from the Department of Immunology of the Biomedical Institute, University of Sao Paulo (Sao Paulo, SP, Brazil).

Parasite. L. (*L.*) *amazonensis* (MHOM/BR/73/M2269) was provided by Dr. J. Shaw from Evandro Chagas Institute (Belém, Pará, Brazil). Amastigotes were maintained in Golden hamster footpads. Amastigote suspensions were prepared by homogenization of the excised lesions. The remaining tissue was homogenized in RPMI 1640 medium containing 10% FCS. The suspension was disrupted by four passages through 22-gauge needles and centrifuged at 250*g* for 10 min; the resulting supernatant was centrifuged at 1400*g* for 10 min and the pellet resuspended in RPMI 1640. After agitation for 4 hr at room temperature the suspension was centrifuged at 250*g* for 10 min. The final pellet contained purified amastigotes which were essentially free from contamination by intact host cells and cellular debris.

Immunization. Animals were subcutaneously injected in the base of the tail with 5×10^7 *L.* (*L.*) *amazonensis* amastigotes killed at −20°C and emulsified with complete Freund's adjuvant; the total injected volume was 20 μl.

Glycoshingolipid preparation. Glycolipids were extracted from amastigotes (10^{11} cells) with 10 vol of isopropanol/hexane/water (55/31/14, v/v/v) five times and twice with chloroform/methanol (2/1, v/v). The seven extractions were combined and dried in a rotaevaporator. The dry residue was kept overnight under vacuum in the presence of P_2O_5. Total lipid extract was acetylated as described by Saito and Hakomori (1971), and GSLs were fractionated from other lipid classes in a florisil column. The glycosphingolipid fraction was eluted from the column with 1,2-dichloroethane/acetone (1/1, v/v), dried under reduced pressure, and deacetylated with sodium metoxide (0.5%, w/v). The neutral GSLs were isolated by DEAE-Sephadex ion-exchange chromatography as described by Yu and Ledeen (1972) and fractionated by HPLC in a Iatrobeads 8010 column (0.4 × 30 cm) with a gradient of isopropanol/hexane/water (v/v/v) from 55/43/2 to 55/20/25. GSLs were quantitated by the phenol–sulfuric acid reaction, a colorimetric method for determination of sugar (Dubois *et al.* 1956). The yield of GSLs was 1 to 1.5 mg per 10^{11} amastigotes.

The different bands were visualized after staining with orcinol and pooled according to their chromatographic migration. Pure ceramide monohexosyl (CMH), ceramide dihexosyl (CDH), ceramide trihexosyl (CTH), globoside (globo), two ceramide pentahexosyls (CPH1 and CPH2), and a mixture of glycosphingolipids containing five to seven sugar residues (GL5-7) were isolated after preparative HPTLC run in chloroform/methanol/$CaCl_2$ 0.02%, 60/40/9 (v/v/v). GSLs for the cell proliferation assay were dissolved in a mixture of water/RPMI 1640 (15/85; v/v) and sonicated for

5 min and 50 μl per well was added to the cell cultures immediately before addition of mitogens.

In order to check the purity of *L.* (*L.*) *amazonensis* GSLs after HPTLC run, the plate was stained with 0.2% ninhydrin and 5% pyridin in butanol. The purity of unhydrolyzed and hydrolyzed GSLs was assessed by the dabsylisothiocyanate/phenylisothiocyanate (DABITC/PITC) double-coupling method (Yarwood 1989) for detection of amino acid contamination. Acid hydrolysis was carried out for 24 hr at 100°C using 6 *N* HCl.

Proliferation assays. Cell suspensions from periaortic and inguinal lymph nodes or spleens were obtained by homogenization in RPMI 1640 with a loose tissue grinder. After washing, the cells were suspended in RPMI 1640 supplemented with sodium bicarbonate, 10 m*M* Hepes (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid), 3% FCS, 100 U/ml of penicillin, 100 μg/ml of streptomicin, 216 mg/ml L-glutamine, and 5×10^{-5} *M* β-mercaptoethanol. Cells were plated in triplicate at 3×10^5 per well in 96-well flat-bottom plates (Costar, Cambridge, MA, U.S.A.). Different concentrations of GSLs and Con A (2.5 μg/ml) or LPS from *Escherichia coli* (10 μg/ml) (Sigma Chemical Co, St Louis, MO, U.S.A.) were added, and the plates were incubated at 37°C in 5% CO_2, 95% air for 2, 3, or 4 days. Cells were pulsed with 1 μCi of [^{3}H]thymidine per well 18 hr before they were harvested on glass fiber paper. [^{3}H]Thymidine uptake was quantitated by β-scintilator counting. [^{3}H]Thymidine incorporation by each sample of the triplicate did not vary more than 20%. Cell viability was measured by trypan blue exclusion. The percentage of proliferative inhibition is expressed by

$$\% \text{ inhibition} = 100 - \left[\frac{(\text{cells} + \text{GSLs} + \text{Con A}) - (\text{cells} + \text{GSLs})}{(\text{cells} + \text{Con A}) - (\text{cells} + \text{medium})} \times 100 \right].$$

Sugars and lipids. D-(+)-Galactose, *N*-acetyl-D-galactosamine, ceramide type III, L-α-phosphatidyl-DL-glycerol, L-α-phosphatidylcholine, CDH, globoside, GM3, GM1, GD1a, and GT1b were purchased from Sigma, Inc. CMH and CTH were purified from human erythrocytes.

Statistical analysis. Student's *t* test was used to evaluate the significance of the data ($P < 0.02$).

Results

Partial characterization of amastigote L. (L.) amazonensis *glycosphingolipids.* Total neutral GSLs from *L.* (*L.*) *amazonensis* amastigotes were run on HPTLC. Figure 1 shows several bands visualized after staining with orcinol reagent. Arrows indicate the glycosphingolipid bands that were purified by a combination of HPLC and preparative HPTLC and tested in the proliferation assay as described in the legend to Fig. 4. They correspond to CMH, CDH, CTH, globoside, CPH1, and CPH2 with one, two, three, four, and five sugar residues, respectively; GL5-7 is a mixture of GSLs with five to seven sugar residues. No visible bands were detected in plates stained with ninhydrin.

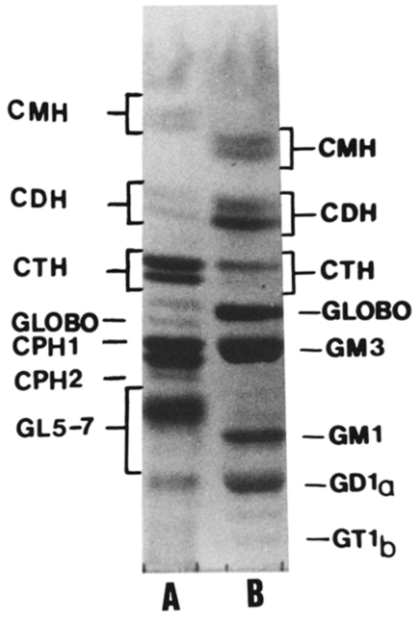

FIG. 1. HPTLC pattern of glycosphingolipids extracted from *L.* (*L.*) *amazonensis* amastigotes. GSLs from *L.* (*L.*) *amazonensis* (Lane A) isolated as described under Materials and Methods and standard glycosphingolipids (Lane B) were separated on HPTLC using a solvent system (chloroform/methanol/$CaCl_2$ 0.02%, 60/40/9, v/v/v) and stained with orcinol. Arrows indicate the GSL bands tested in experiments illustrated in Fig. 4.

No free amino acids were present in unhydrolyzed and hydrolyzed samples of GSLs as determined by the DABIT/PITC double-coupling method sensitive to nanomole levels (0.5–5 nmole).

Effects of L. (L.) amazonensis *amastigote glycosphingolipids on lymphocyte activation.* The effects of GSLs from *L.* (*L.*)

amazonensis amastigotes upon the proliferative responses of BALB/c lymphocytes induced by Con A were monitored by [^{3}H]thymidine incorporation as described under Materials and Methods.

The inhibitory effect of GSLs was dose dependent as demonstrated by the representative experiment shown in Fig. 2. The unspecific response elicited by Con A on lymphocytes from normal or immunized mice was equally inhibited by GSLs (Fig. 2). The GSLs' inhibitory effect was not reverted by increasing concentrations of Con A as verified by the similar degree of inhibition obtained with Con A at 1.25, 5, and 10 μg/ml (data not shown).

In order to examine whether the marked inhibition of Con A responsiveness by GSLs might represent a particular effect, lymph node cells from immunized BALB/c mice were incubated with GSLs and LPS (a B cell mitogen). The results presented in Fig. 3 demonstrate that GSLs inhibited the LPS proliferative response at 2μg/well. Spleen and lymph node cells from normal

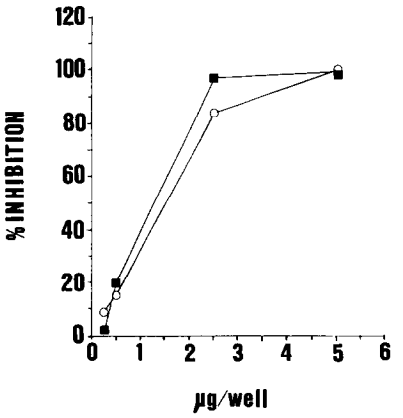

FIG. 2. Inhibition of Con A induced BALB/c mouse lymphoproliferative response by GSLs from *L.* (*L.*) *amazonensis* amastigotes. Lymph node cells (3 × 10^5) from normal mice (○—○) and immunized mice (■—■) were incubated with Con A (2.5 μg/ml) in presence of the indicated concentration of GSLs for 48 hr. The inhibition percentage refers to reduction of mitogenic response induced by GSLs compared to that from controls without GSLs. In general the cpm achieved with Con A was 90.000–120.000.

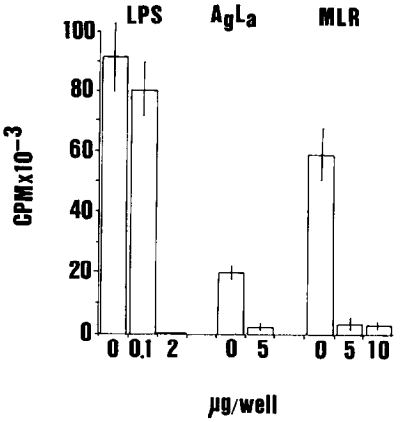

FIG. 3. Inhibition of mouse lymphoproliferative response by GSLs from amastigotes. Lymph node cells from immunized BALB/c mice were incubated with LPS (10 μg/ml) or 10^6 killed *L.*(*L.*) *amazonensis* amastigotes (AgLa) for 48 and 96 hr, respectively, in the presence of the indicated GSL concentrations. MLR was performed by incubating 1.5 × 10^5 spleen lymphocytes from normal BALB/c mice plus 1.5 × 10^5 spleen lymphocytes from normal C57Bl/6 mice in the presence of the indicated GSL concentrations for 72 hr. The results represent the average of triplicate cultures ± standard deviation.

BALB/c mice incubated with GSLs and LPS were also not able to proliferate (data not shown).

The effect of GSLs on lymphoproliferative response to allogeneic cells (MLR) was also studied to determine whether inhibition was restricted to proliferative responses induced by mitogens. Proliferative responses in MLR (BALB/c × C57BL/6) were markedly inhibited by GSLs (Fig. 3).

In addition, the antigen-specific response in lymph node cell cultures obtained from BALB/c mice immunized to amastigote antigens was inhibited by GSLs (Fig. 3).

Therefore, inhibition of proliferation by GSLs was not dependent on the nature of the stimulation, reflecting a general inhibitory effect on lymphoproliferation.

It should be noted that in all the cell cultures examined the antiproliferative activity of GSL was not associated with any cytotoxic effects. At 48, 72, and 96 hr the viability of untreated cells and GSL-treated

cells was about 90% as measured by trypan blue exclusion.

Effects of purified glycosphingolipids on lymphocyte activation. GSLs were fractionated by HPLC and preparative HPTLC, and six pure bands and a pool containing a mixture of GSLs (Fig. 1, lane A) were tested in the proliferative assay: CMH, CDH, CTH, globoside, CPH1, CPH2, and GL5-7. Significant inhibition of Con A-induced mitogenesis is seen with CMH (3.6 μ*M*/well), CDH (2.9 μ*M*/well), CTH (2.4 μ*M*/well), and globoside (4.1 μ*M*/well). On the other hand, CPH1, CPH2 and GL5-7 (2.9–3.6 μ*M*/well) were less effective as inhibitors of [^{3}H]thymidine incorporation in lymphocytes than CMH, CDH, and CTH at similar concentrations (Fig. 4).

Table I shows that individual components of GSLs do not appear to affect Con A-induced lymphoproliferation. Specifically, ceramide, galactose, and acetylgalactosamine do not cause this effect. In addition, other lipids did not exert any inhibitory activity upon lymphoproliferation. As expected, a ganglioside, GD1a, demonstrated inhibitory activity (60%, 1.24 μ*M*/well) on the Con A response.

TABLE I
Effect of Glycolipid Components on Con A Mitogenesis Activity[a]

Compounds	cpm	Inhibition (%)
Medium	107,159 ± 2,789	
Ceramide	85,487 ± 448	20
Phosphatidylcholine	91,799 ± 10,822	14
Phosphatidylglycerol	96,570 ± 1,600	10
Galactose	105,455 ± 5,593	1.6
Acetylgalactosamine	96,971 ± 4,342	9.5
GD1a[b]	45,307 ± 5,580	58

[a] Spleen cells were incubated with 2.5 μg/ml Con A and the indicated compounds (10 μ*M*/well) for 48 hr. Cells were harvested and counted as described under Materials and Methods. Compounds' concentrations from 1 to 20 μ*M*/well could not inhibit Con A mitogenesis activity.
[b] GD1a was used at 1.24 μ*M*/well.

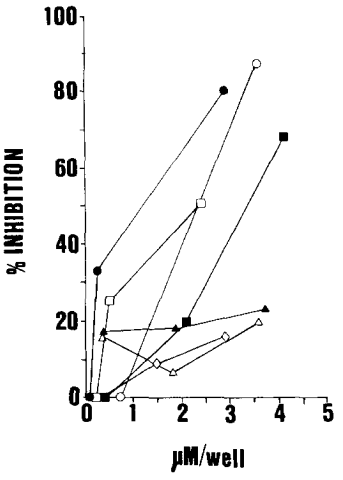

FIG. 4. Inhibition of Con A-induced BALB/c mouse lymphoproliferative response by GSLs from *L.* (*L.*) *amazonensis* amastigotes. Lymph node cells (3×10^5) from normal mice were incubated with Con A (2.5 μg/ml) in the presence of the indicated concentrations of CMH (○), CDH (●), CTH (□), globoside (■), CPH1 (▲), CPH2 (△), and GL5-7 (◇). The percentage of inhibition refers to the mitogenic response reduction induced by each GSL compared to that from controls without GSL. Student's *t* test ($P < 0.02$) was applied to the data.

DISCUSSION

Our study shows that a highly purified preparation of neutral GSLs extracted from *L.* (*L.*) *amazonensis* amastigotes (devoid of protein contamination) was a potent inhibitor of Con A, LPS, MLR, and specific-antigen responses of lymphocytes from BALB/c mice (Figs. 2–4). Consequently, the results provide a demonstration of the inhibitory activity of parasite cell-derived glycosphingolipids.

Our findings extend the results previously reported by Londner *et al.* (1983) on inhibition of human lymphocyte blast transformation by *L.* (*L.*) *major* promastigote excreted factor (a carbohydrate-rich substance released by the parasite in the culture medium).

Inhibition of the lymphoproliferative response of lepromatous patients was documented by Mehra *et al.* (1984) using methylated glycolipids of *Mycobacterium leprae*. In addition, a number of studies indicated that gangliosides isolated from brain or tumor cells inhibit proliferation of

lymphoid cells (Ryan and Shinitzky 1979; Ladisch *et al.* 1983; Krishnaraj *et al.* 1982; Marcus *et al.* 1987); gangliosides from normal tissue can also modulate expression of immunoregulatory antigen CD4 (Offner *et al.* 1987). Taken together, these studies suggest that glycolipids independently of their cell origin may inhibit lymphoproliferation by a common mechanism. Some of the possibilities are changes in the Ca^{2+} influx, a change in the intracellular pH through Na^{+}/H^{+} antiporter, or a modification of signal transduction through receptor-or transducer-associated kinase (Hakomori 1990).

Despite the similar inhibitory activities reported *in vitro* for glycosphingolipids extracted from brain, parasite, and tumor cells, an important point should be considered in *in vivo* studies. While brain cells are normally found in a restricted area, tumor and parasite cells can spread widely, making it more feasible for glycosphingolipids of tumor or parasite cells to make contact with, and significantly affect, cells involved in the immune system.

The immune inhibitory properties of GSLs seem to be modulated by their carbohydrate moieties. The individual GSLs vary in the composition and arrangement of sugars. On a molar basis CMH, CDH, CTH, and globoside (1, 2, 3, and 4 sugar residues, respectively) presented higher inhibitory effects when compared to CPH1, CPH2, and GL 5–7 (5, 5, and 5–7 sugar residues, respectively) (Fig. 4). As seen in Table I Con A mitogenic activity was not inhibited by ceramide or some monosaccharides. The inhibitory properties of GSLs not only may be defined by the primary chemical structure of their carbohydrate moieties but also may be influenced by the organization of GSLs at cell surface membranes modulated by the ceramide composition.

In conclusion the lymphoproliferative inhibition activity of *Leishmania* glycosphingolipids represents a new approach to be considered in the cellular immune mechanisms in leishmaniasis.

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