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NOTES

Effect of Glycosphingolipids Purified from *Leishmania (Leishmania) amazonensis* Amastigotes on Human Peripheral Lymphocytes

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The effect of purified glycosphingolipids from *Leishmania* (*Leishmania*) *amazonensis* on human lymphoproliferation, on expression of human lymphocyte and monocyte markers (CD3, CD4, CD8, CD14, CD19, and CD45), and on lymphocyte protein kinase C activity was analyzed.

Parasites belonging to the genus *Leishmania* present two forms in their life cycle: promastigotes, which multiply in the midgut of the sandfly vector, and amastigotes, the obligate intracellular forms which live within macrophage phagolysosomes in the vertebrate host. *Leishmania* (*Leishmania*) *amazonensis* is responsible for most cases of human cutaneous leishmaniasis in the Amazon region of Brazil.

A family of glycosphingolipids (GSLs) was identified in amastigotes of *L.* (*L.*) *amazonensis* (1, 18, 19). In an earlier report we demonstrated that GSLs purified from *L.* (*L.*) *amazonensis* amastigotes inhibited the proliferation of murine T and B cells (2), suggesting that parasite glycosphingolipids may play a relevant role in leishmaniasis. Data from our studies were in agreement with those of several reports showing that GSLs isolated from brain or tumor cells inhibit proliferation of lymphoid cells (10–12, 16) and that gangliosides inhibit the expression of the immunoregulatory antigen CD4 (15). Glycosphingolipids can also modify signal transduction through receptors associated with protein kinase C (PKC) (4–6, 9). These data prompted us to investigate the effect of these glycoconjugates on human lymphoproliferation and on the expression of lymphocyte and monocyte markers, as well as their possible role in human lymphocyte PKC activity.

L. (*L.*) *amazonensis* (MHOM/BR/1973/M2269) amastigotes were isolated from foot lesions of infected hamsters, as described by Barbiéri et al. (1). Glycolipids were extracted with a mixture of isopropanol-hexane-water (55:20:25), and the GSLs were purified as described previously (18). Acetylated GSLs were fractionated from other lipids and phospholipids by Florisil chromatography, where lipids and cholesterol are eluted with 1,2-dichloroethane, glycosphingolipids are eluted with a mixture of 1,2-dichloroethane–acetone (1:1), and phospholipids are eluted with a mixture of 1,2-dichloroethane–methanol-water (4:8:2) (17). The GSL fraction was deacetylated with 0.5% sodium methoxide in methanol, neutralized with Dowex 50 (H^+ form), and subjected to DEAE-Sephadex ion exchange chromatography in chloroform-methanol-water (30:60:8) to separate neutral from acidic GSLs. The neutral GSL fraction purity was analyzed by high-performance thin-layer chromatography using staining with orcinol-H_2SO_4 and Dittmer-Lester reagent, which detect carbohydrates and phosphodiester linkages, respectively. No contamination with phospholipids or peptides was detected, as described previously (2, 18, 19). The effect of amastigote GSLs on T-cell responses induced by mitogen was analyzed in lymphocytes purified from human peripheral blood by Ficoll-Hypaque density gradient centrifugation. The human procedures were approved by the Ethical Committee for Human Care from Escola Paulista de Medicina, Universidade Federal de São Paulo. The cells were cultured into 96-well plates (10^5 cells/well) in RPMI 1640 medium with 10% fetal calf serum and stimulated with 5 μg of phytohemagglutinin (PHA) in the presence of different amounts of purified GSLs. After 72 h at 37°C in 5% CO_2, the cells were pulsed with 0.5 μCi of [^{3}H]thymidine/well for 6 h, and the [^{3}H]thymidine uptake was determined after filtration on glass fiber filters. A dose-dependent inhibition of the lymphocyte proliferation was observed (Fig. 1). GSLs at concentrations of 10, 25, and 50 μg/ml induced 45, 55, and 76% inhibition of [^{3}H]thymidine uptake, respectively. GSLs did not cause any toxic effect on lymphocytes as measured by trypan blue exclusion (viability, >95%).

The effect of amastigote GSLs on the expression of lymphocyte and monocyte markers was analyzed by fluorescence-activated cell sorter (FACS) with a human blood mononuclear suspension by using monoclonal antibodies conjugated to phycoerythrin or fluorescein isothiocyanate directed to Th cells and to other lymphocyte and monocyte markers (Beckton Dickinson). The mononuclear cells were washed with phosphate saline buffer, fixed in formalin, and gated on the basis of forward-angle and right-angle scatter, and the fluorescence

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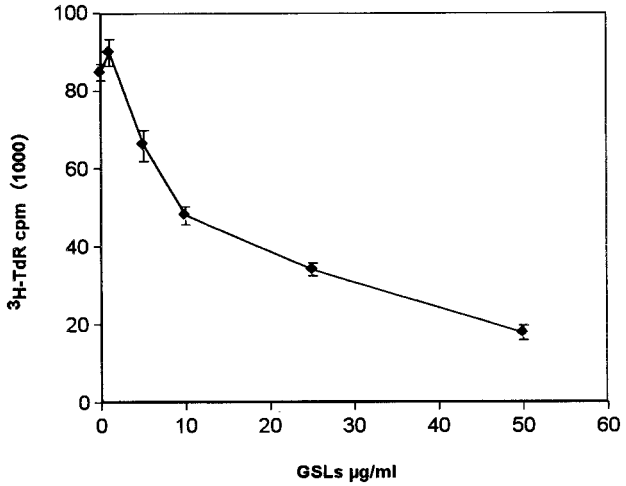

FIG. 1. Inhibition of the PHA-induced human lymphoproliferative response by GSLs from *L.* (*L.*) *amazonensis* amastigotes. Mononuclear cells were incubated with PHA for 72 h in the presence of different neutral GSL concentrations. Five different experiments were carried out, and results represent the average for triplicate cultures ± standard deviations.

intensity was analyzed by FACS (Beckton Dickinson). As shown in Fig. 2, amastigote GSLs did not alter the expression of surface markers (CD3, CD4, CD8, CD14, CD19, and CD45). Variable concentrations of GSLs (1, 10, and 50 μg/ml) did not change the expression of these markers over a period of 48 h (data not shown). On the other hand, a mixture of gangliosides (1 μg/ml) quickly induced a selective loss of CD4 (about 90%), confirming previous observations that gangliosides modulate CD4 expression on human T cells (15). These results indicate that inhibition of lymphoproliferation by *Leishmania* GSLs is not correlated to modifications in the expression of immunoregulatory surface determinants. In order to investigate if amastigote GSLs could modulate PKC activity from human lymphocytes, this enzyme was partially purified from human peripheral blood (8). About 5×10^7 cells were homogenized with a solution containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, and 25-μg/ml aprotinin and leupeptin, incubated on ice for 30 min, and centrifuged at 12,000 × *g* for 1 min. The supernatant was applied to a DEAE-cellulose column, and the PKC was eluted

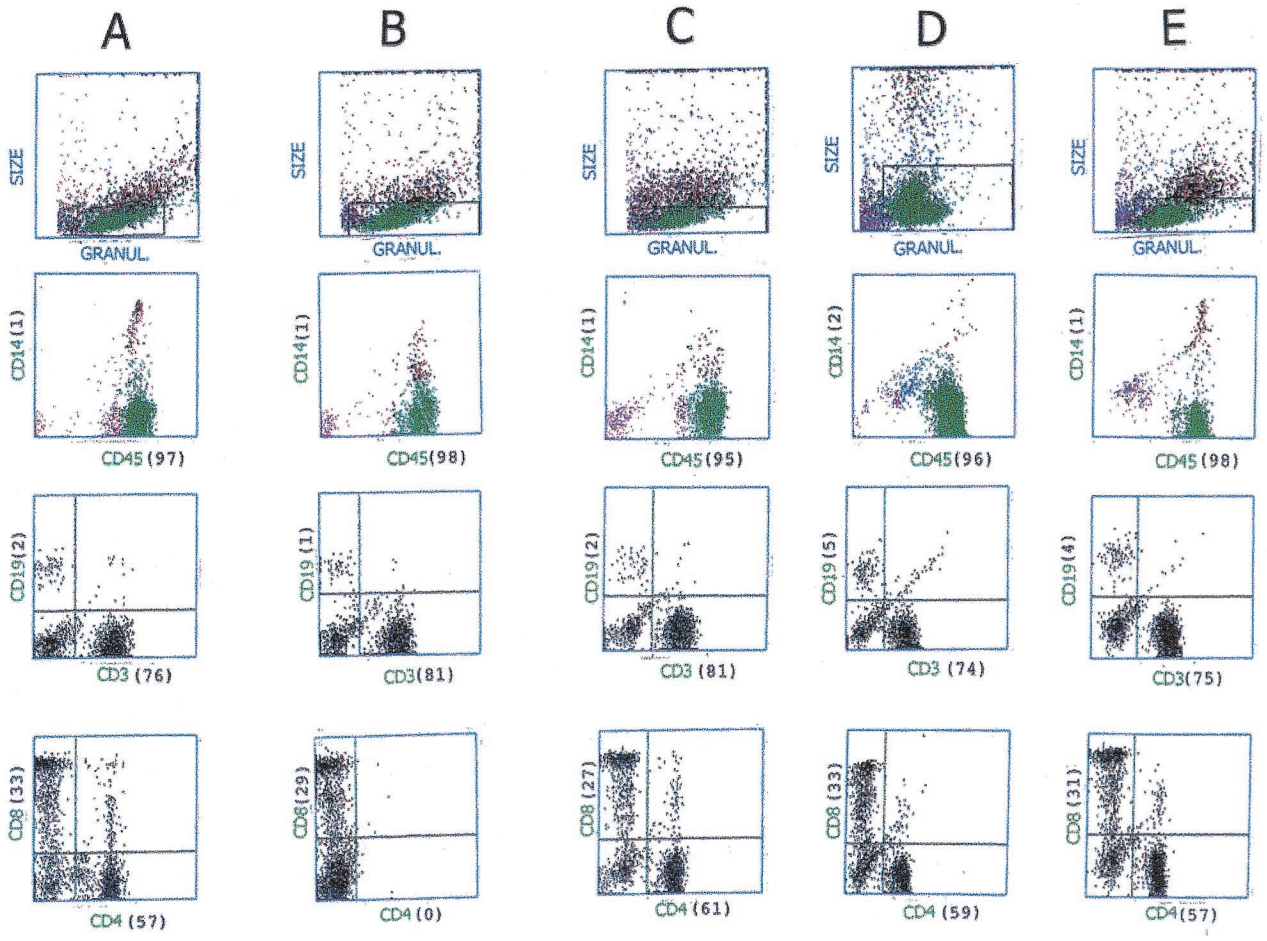

FIG. 2. Effect of different concentrations of *L.* (*L.*) *amazonensis* GSLs and gangliosides on lymphocyte and monocyte surface marker expression. Human mononuclear cells were treated for 48 h with RPMI medium (A), brain bovine gangliosides at a concentration of 1 μg/ml (B), or neutral GSLs at a concentration of 1 μg/ml (C), 10 μg/ml (D), or 50 μg/ml (E) and then analyzed by FACS. Numbers inside parentheses represent the percentage of cells positively stained for the specified cell surface marker. The lymphocyte population is indicated in green at top rows. For all analysis performed, the relationship between the mean of fluorescence intensities and size surface counting was very similar, and more than 95% of the cell populations analyzed were lymphocytes. Results are representative of five experiments.

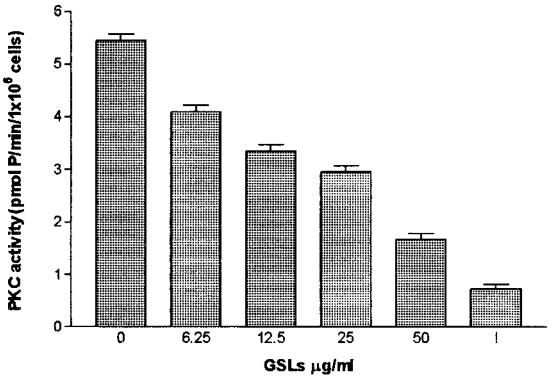

FIG. 3. Effect of GSLs from *L.* (*L.*) *amazonensis* amastigotes on the PKC activity of human lymphocytes. PKC activity of lymphocyte extracts from human peripheral blood preincubated for 20 min with the indicated neutral GSL concentrations or with the specific PKC inhibitor (I), peptide PKC(19-36). The PKC activity was expressed in picomoles of ^{32}P incorporated to Ac-MBP(4-14)/min/10^6 cells. Four different experiments were carried out, and results represent the averages of triplicate determinations ± standard deviations.

with a solution containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM 2-mercaptoethanol, and 0.2 M NaCl. The PKC activity was determined in the presence of 50 μM acetylated myelin basic protein [Ac-MBP(4-14)], 20 μM ATP, 1 mM $CaCl_2$, 20 mM $MgCl_2$, 4 mM Tris-HCl (pH 7.5), 20- to 25-Ci/ml [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham), and a lipid mixture containing 10 μM phorbol 1,2-myristate 13-acetate and 0.28 mg of phosphatidyl serine/ml according to recommendations of the PKC assay kit (Gibco-BRL) (21). GSLs were preincubated with the partial purified PKC for 20 min at room temperature before the PKC assay. After incubation at 30°C for 10 min, aliquots of 25 μl (each) were spotted onto a phosphocellulose disk, which was immersed in 1% H_3PO_4 for 5 min and washed with distilled water, and radioactivity was measured in a β-scintillator counter. As shown in Fig. 3, a dose-dependent inhibition of PKC activity by GSLs was observed. Concentrations of 12.5, 25, and 50 μg/ml were able to inhibit the phosphorylation of the Ac-MBP(4-14) substrate in 48, 54, and 71%, respectively. The PKC inhibitor used, peptide PKC(19-36), is based on the pseudosubstrate region common to the α-, β-, and γ-isozymes of PKC and acts as a potent and specific inhibitor for the Ac-MBP(4-14) substrate (7). This inhibitor at a concentration of 25 μM reduced the PKC activity 77%.

The PKC activity could not be recovered when higher concentrations of phorbol 1,2-myristate 13-acetate and phosphatidyl serine were added, indicating that GSLs do not compete with these activators. Thus, amastigote GSLs apparently down modulate the PKC activity. Overall analyses of the results presented here allow us to hypothesize that the remarkable inhibition of lymphoproliferation induced by GSLs purified from *Leishmania* would be related to the PKC activity inhibition.

Inhibition of the oxidative burst in *Leishmania*-infected macrophages was shown to be due to the effect of lipophosphoglycan (LPG) on PKC activity (3, 13, 14, 20). This inhibition may represent a critical step for successful establishment of *Leishmania* promastigotes within the host's macrophages. The results described here differ from those reported earlier by McNeely et al. (14), who studied the inhibition of PKC activity using intact LPG or fragments of LPG of *Leishmania* (*L.*) *donovani* and intact glycophosphatylinositols (GIPLs) of *Leishmania* (*L.*) *major*. LPG and GIPLs, although classified as glycolipids, do not present any structural analogy with neutral GSLs: (i) LPG and GIPLs present lipid moiety composed by 1-*O*-alkylglycerol and alkylacylglycerol, respectively, whereas GSLs present ceramide (composed by sphingosine and fatty acid) as their lipid moiety; (ii) LPGs and GIPLs are phosphorylated glycolipids, whereas GSLs are neutral glycosphingolipids; and (iii) the carbohydrate sequences of LPGs, GIPLs, and GSLs are distinct. Furthermore, neutral GSLs used in this study were completely free of LPG or GIPL contamination as assessed by ^1H-nuclear magnetic resonance and immunochemical methods (18). Our results and those of McNeely et al. (14) may complement each other, demonstrating that different glycolipids may have a key role in macrophage infection. Thus, amastigote GSLs could act as immunomodulator molecules during the progress of cutaneous leishmaniasis, whereas the early stages of macrophage infection could possibly be modulated by promastigote LPGs.

Currently, we are performing studies aiming to evaluate whether other T-cell signaling pathways are also altered by *Leishmania* GSLs.

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