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Modulation of Immune Cell Proliferation by Glycerol Monolaurate

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Previous studies have shown that glycerol monolaurate (GML), a surfactant commonly used in a wide variety of food and cosmetic products, inhibits the production of a variety of exotoxins by group A streptococci and staphylococci. Given the highly lipophilic nature of the structure of GML, it is suspected that the surfactant exerts its toxin inhibition effects via interaction with the cell membrane. The present study attempted to characterize some of the potential targets of GML action using the model system of lymphocyte activation. Results from murine splenocytes show that GML stimulates proliferation at concentrations between 10^{-5} and 5 μg/ml/5 × 10^5 splenocytes. At concentrations greater than 5 μg/ml, GML inhibited lymphocyte proliferation and blocked the proliferative effects of the lymphocyte mitogens phorbol myristate acetate and concanavalin A and the potent T-cell mitogen toxic shock syndrome toxin-1. Studies using purified immune cell subsets indicated that GML at a concentration of 0.1 μg/ml optimally induced proliferation of T cells but did not affect B cells. At higher concentrations, GML inhibited the toxic shock syndrome toxin-1 mitogenic effects on T cells, but did not inhibit the lipopolysaccharide-induced stimulation of B cells, suggesting that GML preferentially affects the T-cell population. GML-induced proliferation was blocked by the immunosuppressive drug cyclosporin A, suggesting that GML may be exerting its T-cell-proliferative effects along the calcium-dependent inositol phospholipid signal transduction pathway.

Surface-active agents, or surfactants, are a class of compounds which have served as detergents and wetting agents in common industrial processes and products since the early 1930s. Surfactants act to modulate the surface tension at interfaces such as that between oil and water. Recently, several surfactants have come under investigation for their possible health care applications (5–7, 15). Some surfactants may be useful as delivery systems for bioactive compounds (8, 10, 11, 14, 19) or as antimicrobial agents (3, 4, 16, 21, 22). Skin and eye irritation studies and feeding studies testing the toxicology of some surfactants have been performed in the past (4) and have helped to define a class of surfactants with low physiological toxicity. Glycerol monolaurate (GML) is one of several surfactants which were listed as "generally recognized as safe" in the mid-1950s. GML is commonly used in many household products and in foods for human consumption and can be found in products such as cosmetics, detergents, and insecticides and in foods such as ice cream, margarine, and spaghetti (1, 5).

GML has recently been shown to weakly inhibit the growth of but strongly reduce the production of exotoxins in a variety of gram-positive organisms. GML had effects on the growth of group A, B, F, and G streptococci at concentrations of 10 to 20 μg/ml and *Staphylococcus aureus* strains at concentrations of 100 to 300 μg/ml (16). The ability of these organisms to produce exotoxins, including pyrogenic toxin superantigens and hemolysins, was inhibited by GML concentrations that were not inhibitory to growth (1 to 10 μg/ml). This surfactant has also been shown to inhibit the production of toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins in an in vitro tampon sac method (1, 16) and in an in vivo vaginal rabbit model of TSS (1, 10, 16). GML may therefore be considered for use as an additive to tampons or other surgical materials as a deterrent to staphylococcal and streptococcal infections or development of TSS.

Because of its ability to act at lipid-water interfaces, it was hypothesized that GML inhibited the production of exotoxins via interference with cell signalling events occurring at the plasma membrane. In order to evaluate its effect on cell signalling, the ability of GML to alter well-characterized pathways leading to immune cell activation was investigated. It was shown that GML inhibited superantigen-induced lymphocyte proliferation, but at lower concentrations, GML itself caused significant lymphoproliferation. Initial characterization of specific targets in cell signalling pathways in lymphocytes was begun.

MATERIALS AND METHODS

Surfactants. GML was provided by Personal Products Company (New Brunswick, N.J.). The compound was dissolved in absolute ethanol at a concentration of 100 mg/ml and diluted into medium at 37°C for use.

Mice. Female BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, Maine).

Immune cell preparation. Splenocytes were obtained from murine spleens and resuspended in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Gibco). T cells were purified by incubating murine lymph node cells or splenocytes on plastic dishes for 1 h at 37°C with 7% CO_2. Nonadherent cells were passed over a Sephadex G25 column (Sigma, St. Louis, Mo.), and T cells were further purified on a mouse T-cell recovery column (Biotex, Edmonton, Alberta, Canada) with bound monoclonal goat anti-mouse immunoglobulin to remove remaining B cells. B cells were isolated according to the protocol by Mueller et al. (12, 13, 20). Briefly, splenocytes were depleted of T cells by treatment with an anti-T-cell antibody cocktail containing monoclonal antibodies RL172 (anti-CD4), 83-12.5 (anti-CD8), and 30-M12 (anti-Thy 1.2) (generously provided by M. Jenkins, University of Minnesota) for 20 min at 4°C, followed by centrifugation (500 × *g*, 10 min), addition of rabbit complement (1/10 dilution) (Cedarlane Laboratories Ltd., Hornsby, Ontario, Canada) to the pellet, and further incubation for 30 min at 37°C with 7% CO_2. Spleen cell preparations were confirmed to be T cell depleted because they failed to proliferate in the presence of concanavalin A. Accessory-cell preparations were made by depleting murine splenocytes of T cells as described above. Remaining adherent cells were removed from the plastic dishes by scraping and vigorous

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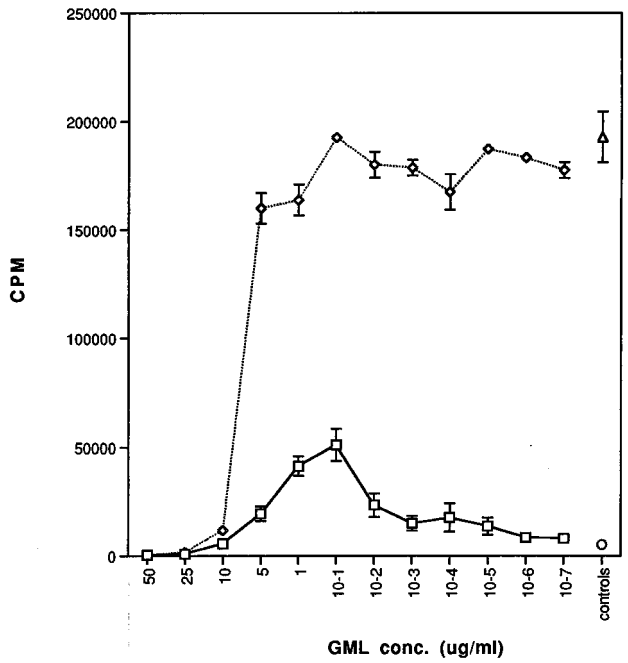

FIG. 1. Effect of GML on the proliferation of murine splenocytes. Splenocytes (2×10^6/ml) were cultured in complete RPMI 1640 medium alone (circle), with 10 μg of TSST-1 per ml (triangle), with indicated concentrations of GML per ml (squares), and with indicated concentrations of GML per ml plus 10 μg of TSST-1 per ml (diamonds). conc., concentration. Error bars indicate standard errors.

pipetting. Adherent cells and T-cell-depleted nonadherent cells were pooled. This cell pool was used directly or after a 3,000-rad irradiation. All experiments were performed in the presence of accessory cells unless otherwise indicated.

Proliferation experiments. After recovery of cells from the spleen or lymph node and subsequent isolation of immune cell subsets as indicated above, cells were screened for purity by fluorescence-activated cell sorter analysis. Cell viability was monitored by trypan blue exclusion. Proliferation assays were performed in quadruplicate in 96-well microtiter plates (Costar, Cambridge, Mass.) in a total volume of 200 μl of RPMI 1640 medium plus 10% fetal calf serum supplemented with glutamine (Sigma) and penicillin-streptomycin antibiotics (Gibco) (complete RPMI). T and B cells (1×10^5 cells per well) and whole splenocyte preparations (5×10^5 cells per well) were cultured with GML, various mitogen controls, and accessory cells (2×10^5 cells per well) at 37°C with 7% CO_2 for 48 h, pulsed with 1 μCi of [^{3}H]thymidine, and harvested 16 h later with a mechanical cell harvester. Thymidine incorporation into DNA was quantified by liquid scintillation counting.

Immunosuppressants. Immunosuppressive agents with well-characterized effects on the immune system, notably cyclosporin A (CsA) and rapamycin, were used in an attempt to block the activity of GML and thus localize the possible molecular targets of the surfactant. CsA and rapamycin were generously provided by M. Jenkins (University of Minnesota).

RESULTS

Effects of GML on murine splenocytes. The effects of GML on immune cell proliferation were assessed in the presence and absence of lymphocyte mitogens. The mitogens used in these experiments included 10 ng of phorbol 12-myristate 13-acetate per ml in combination with either 0.67 μM ionomycin or the ionophore A23187 (PMA); 1 μg of concanavalin A per ml; 10 μg of TSST-1 (a potent T-cell mitogen) per ml; and 10 μg of lipopolysaccharide (LPS) (a stimulator of B-cell proliferation) per ml. GML alone stimulated murine splenocyte proliferation at low concentrations, optimally between 0.001 and 1 μg/ml. At concentrations greater than 5 μg/ml, GML inhibited lymphocyte proliferation and also blocked any proliferation induced by TSST-1 (Fig. 1). Similar results were obtained when PMA and concanavalin A were comparably tested in place of TSST-1 (data not shown).

The lymphocyte-mitogenic effect of GML was further characterized according to whether the surfactant affected T-cell or B-cell populations or both. At a concentration of 0.1 μg/ml (Fig. 2), GML stimulated T-cell proliferation but not B-cell proliferation. The T-cell-mitogenic effect was comparable to that seen with unfractionated splenocytes. No proliferation of T cells was observed in the absence of accessory cells at any GML concentration, indicating the need for costimulatory signals (data not shown). B cells appeared to be completely unaffected by GML at any lymphocyte- and T-cell-mitogenic concentrations. When tested at concentrations that blocked mitogenicity (e.g., 10 μg/ml) (Fig. 3), GML inhibited both T-cell and B-cell function. Since the higher concentration of GML blocked both T-cell and B-cell activity, it is likely that the surfactant's inhibitory effects resulted from a general poisoning of all cellular function.

Effects of immunosuppressants on GML mitogenic activity. The sensitivity of GML T-cell-mitogenic activity to well-characterized immunosuppressive agents such as CsA was monitored in an attempt to characterize potential molecular mechanisms of action of the surfactant. As shown in Fig. 4, CsA at concentrations as low as 20 ng/ml blocked GML proliferative activity at all of the surfactant's putative mitogenic concentrations. GML at concentrations previously shown to block mitogenicity (i.e., 10 μg/ml) appeared to have an additive inhibitory effect on splenocyte proliferation when tested in conjunction with CsA. The mitogenic activity of GML was also sensitive to the immunosuppressive agent rapamycin. The GML mitogenic response was inhibited by rapamycin concentrations as low as 2 ng/ml (Fig. 5). Unlike with CsA, GML at concentrations shown to inhibit mitogenicity did not have an additive antiproliferative effect on splenocytes when tested in conjunction with rapamycin (data not shown).

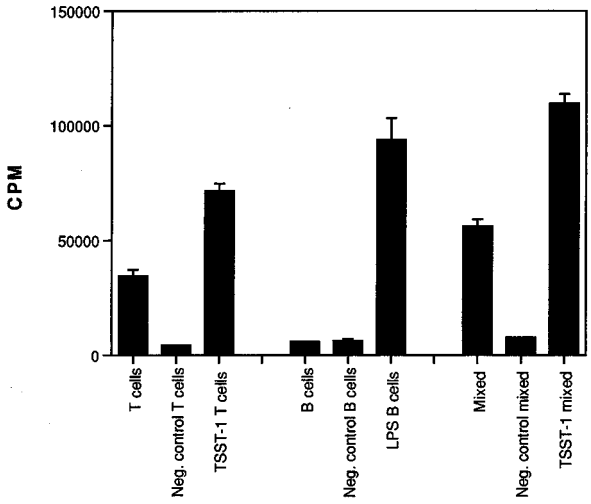

FIG. 2. Effect of GML on the proliferation of purified immune cell subsets. Purified T cells (5×10^5/ml), purified B cells (5×10^5/ml), and a mixed population of T and B cells (mixed) were incubated in complete RPMI 1640 medium containing 0.1 μg of GML per ml and γ-irradiated accessory cells (10^6 cells per ml). Positive controls included 10 μg of TSST-1 per ml and 10 μg of LPS per ml as indicated. Negative controls (Neg. control) were cells incubated in the absence of GML. Error bars indicate standard errors.

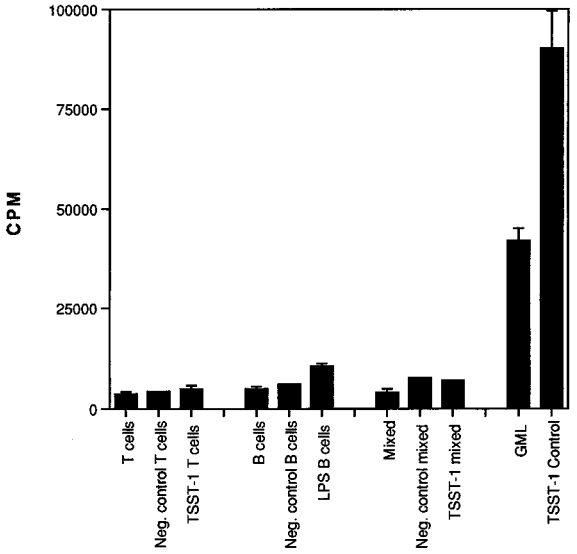

FIG. 3. Effect of GML (10 μg/ml) on the proliferation of purified murine immune-cell subsets. Purified T cells (5×10^5/ml), purified B cells (5×10^5/ml), and a mixed population of T and B cells were incubated in complete RPMI 1640 medium in the presence of γ-irradiated accessory cells (10^6 cells per ml). Positive controls included 0.1 μg of GML per ml and 10 μg of TSST-1 per ml. Negative controls (Neg. control) were cells incubated in the absence of GML. Error bars indicate standard errors.

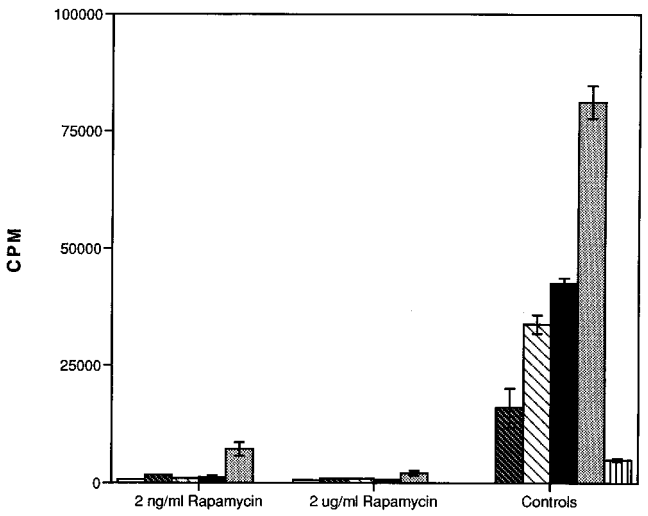

FIG. 5. Effect of rapamycin on GML activity. Murine splenocytes (2.5×10^6 cells per ml) were cultured in the presence of rapamycin at concentrations of 2 ng/ml and 2 μg/ml. Open bars, rapamycin alone; white hatched bars, 5 μg of GML per ml; black hatched bars, 1 μg of GML per ml; closed bars, 0.1 μg of GML per ml; stippled bars, 10 μg of TSST-1 per ml; vertically striped bars (positive value for controls only), murine splenocytes alone. Error bars indicate standard errors. The controls are cells treated as described above in the absence of rapamycin. In all cases, 50 U of IL-2 per ml was given exogenously.

DISCUSSION

GML is a common industrial and commercial surfactant with the demonstrated ability to inhibit the exotoxin production of a variety of gram-positive organisms. The inhibitory effect of GML on bacterial exotoxin production makes the surfactant potentially useful in applications involving the treatment or prevention of toxin-mediated illnesses. Consideration of GML use in health care has led to the investigation of possible biological effects that the surfactant may have on cells of the immune system. These studies were undertaken to begin characterization of the potential molecular targets and mechanisms of action of GML. Results from studies using murine and rabbit lymphocytes (unpublished data) indicated that GML is able to modulate lymphocyte proliferation, stimulating proliferation at concentrations between 10^{-4} and 5 μg/ml in standard in vitro mitogenicity assays. At concentrations greater than 5 μg/ml, GML inhibited lymphocyte proliferation and blocked the proliferative effects of cell mitogens such as TSST-1, concanavalin A, PMA, and LPS. Further in vitro studies using purified immune cell subsets indicated that GML at low levels (i.e., mitogenic concentrations) was a nonspecific T-cell but not B-cell mitogen but at higher concentrations (i.e., >5 μg/ml) was a general inhibitor of T-cell and B-cell function. There is an apparent correlation between the concentrations of GML which can modulate lymphoproliferation and those which can inhibit bacterial toxin production, suggesting that GML biological activity is directly related to the concentration of the surfactant in the surrounding medium. It appears that GML at concentrations above a threshold level (e.g., 10 μg/ml) nonspecifically inhibits cellular function, as evidenced by the suppression of the proliferation of lymphocytes and the growth of microorganisms, while GML at concentrations below this threshold level has more subtle effects, causing lymphoproliferation and toxin inhibition. The lipophilic nature of GML makes it likely that the observed biological activity involves the interaction of the surfactant with the cell membrane.

Because its structure is composed of glycerol and lauric acid, it was postulated that GML interacted with the cell membrane and, most likely, affected cell signalling. Because GML appeared to be mitogenic at low concentrations, the signalling pathway controlling the up regulation of interleukin-2 (IL-2), a lymphokine necessary for T-cell proliferation, was used as a model system for the surfactant's activity. Rapamycin and CsA, immunosuppressive drugs with well-characterized effects on the signalling pathways involving IL-2, were used in an attempt to localize possible GML activity. Rapamycin acts at the level

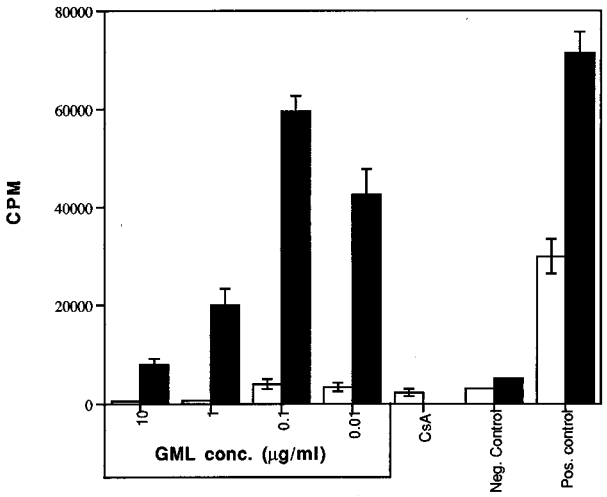

FIG. 4. Effect of CsA on GML activity. Murine splenocytes (2.5×10^6 cells per ml) were cultured in complete RPMI 1640 medium with various concentrations of GML (0.01 to 10 μg/ml) in the presence of 20 ng of CsA per ml (open bars) or absence of CsA (closed bars). Controls included cells incubated alone (Neg. control) with or without 20 ng of CsA per ml and cells incubated with 10 μg of TSST-1 per ml (Pos. control) with or without 20 ng of CsA per ml. Error bars indicate standard errors.

of T-cell response to IL-2 by inhibiting IL-2 receptor-mediated signalling and entry into the S phase of the cell growth cycle (2, 17, 18). CsA is an anti-inflammatory drug that selectively blocks the transcription of IL-2 mRNA by inhibiting activation of the nuclear factor of activated T cells, a necessary transcriptional regulator of IL-2 mRNA (9, 17, 18). GML mitogenic activity was blocked by rapamycin, suggesting that GML was not rendering the cells more sensitive to IL-2. CsA also blocked GML-induced T-cell proliferation. CsA sensitivity suggested that GML may target early cell signalling along the calcium-dependent inositol phospholipid signal transduction pathway.

In summary, GML appears to modulate T-cell proliferation by affecting the cell signalling cascade leading to the regulation of IL-2 production. Further studies are being conducted in an attempt to elucidate the mechanism of action and the molecular targets of GML in the inositol phospholipid signalling pathway. The calcium-dependent inositol phospholipid pathway is a well-characterized cell signalling pathway linking membrane effects to IL-2 regulation and appears to be a viable candidate as a target for GML action.

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