Glycerol Monolaurate Inhibits the Effects of Gram-Positive Select Agents on Eukaryotic Cells[†]

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ABSTRACT: Many exotoxins of Gram-positive bacteria, such as superantigens [staphylococcal enterotoxins, toxic shock syndrome toxin-1 (TSST-1), and streptococcal pyrogenic exotoxins] and anthrax toxin are bioterrorism agents that cause diseases by immunostimulation or cytotoxicity. Glycerol monolaurate (GML), a fatty acid monoester found naturally in humans, has been reported to prevent synthesis of Gram-positive bacterial exotoxins. This study explored the ability of GML to inhibit the effects of exotoxins on mammalian cells and prevent rabbit lethality from TSS. GML (≥ 10 μg/mL) inhibited superantigen (5 μg/mL) immunoproliferation, as determined by inhibition of ^{3}H-thymidine incorporation into DNA of human peripheral blood mononuclear cells (1×10^6 cells/mL) as well as phospholipase Cγ1, suggesting inhibition of signal transduction. The compound (20 μg/mL) prevented superantigen (100 μg/mL) induced cytokine secretion by human vaginal epithelial cells (HVECs) as measured by ELISA. GML (250 μg) inhibited rabbit lethality as a result of TSST-1 administered vaginally. GML (10 μg/mL) inhibited HVEC and macrophage cytotoxicity by anthrax toxin, prevented erythrocyte lysis by purified hemolysins (staphylococcal α and β) and culture fluids containing streptococcal and *Bacillus anthracis* hemolysins, and was nontoxic to mammalian cells (up to 100 μg/mL) and rabbits (250 μg). GML stabilized mammalian cell membranes, because erythrocyte lysis was reduced in the presence of hypotonic aqueous solutions (0−0.05 M saline) or staphylococcal α- and β-hemolysins when erythrocytes were pretreated with GML. GML may be useful in the management of Gram-positive exotoxin illnesses; its action appears to be membrane stabilization with inhibition of signal transduction.

Many Gram-positive bacterial pathogens produce exotoxins that are potential agents of bioterrorism and contribute to their abilities to cause human diseases (*1−3*). Examples include anthrax toxin made by *Bacillus anthracis* (*2*), superantigens produced by *Staphylococcus aureus*, including staphylococcal enterotoxins (SEs)[1] and toxic shock syndrome toxin-1 (TSST-1) (*1*), and superantigens produced by group A streptococci, including streptococcal pyrogenic exotoxins (SPEs) (*3*). Other exotoxins, such as staphylococcal α- and β-hemolysins and streptolysins, are not agents of bioterrorism but have adjunct roles in virulence and can be used as model toxins to study mechanisms of cellular toxicity.

All of these exotoxins interact with host cell membranes to exert their toxic effects. Some effects are immunostimulatory, such as superantigen exotoxins, which stimulate T lymphocytes and macrophages to cause toxic shock syndrome (*1*, *3*, *4*). Superantigens are defined by their capacities to induce proliferation of T cells as a function of the β-chain variable region of the T cell receptor complex that causes the release of interleukin 2 (IL-2), tumor necrosis factor β (TNF-β), and interferon γ (IFNγ) (*1*, *4*). In concert with the activation of T cells is the release of interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) from macrophages (*1*, *4*).

Alternatively, the interactions of exotoxins with host cell membranes may lead to cytotoxicity, such as the heptamer pore-forming exotoxin staphylococcal α-hemolysin (*5*), streptolysin O (*6*), and anthrax toxin that forms heptamer pores through polymerization of the protective antigen component, which delivers lethal factor protease and edema factor adenylate cyclase into host cells (*2*).

The potential for purified exotoxins to be used as agents of bioterrorism has stimulated research in specific methods to block the actions of exotoxins, such as anthrax toxin and superantigens, on mammalian cells. Dominant mutants of anthrax toxin (protective antigen) have been developed, which block its ability to facilitate translocation of lethal and edema factors into cells (*7*, *8*). In addition, specific compounds have been designed to block the enzymatic functions of both lethal and edema factors (*9−11*). Finally, antibodies obtained from volunteers immunized with protective antigen

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[1] Abbreviations: SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin-1; SPE, streptococcal pyrogenic exotoxin; IL-2, interleukin 2; TNF-β, tumor necrosis factor β; IFNγ, interferon γ; TNF-α, tumor necrosis factor α; GML, glycerol monolaurate; HVEC, human vaginal epithelial cell; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; IP$_3$, inositol 1,4,5-triphosphate; ELISA, enzyme-linked immunosorbent assay; MIP-3α, macrophage inflammatory protein 3α; LD$_{50}$, lethal dose 50% end point.

may have the capacity to neutralize anthrax toxin (*12*). Similarly, polyclonal (commercially available intravenous immunoglobulin) and monoclonal antibody preparations against superantigens have the capacity to neutralize the immunostimulatory activity of superantigens (TSST-1, SEs, and SPEs) (*13−18*).

Glycerol monolaurate (GML), a surfactant compound found naturally in humans with highest concentrations in breast milk, is generally recognized as safe by the Food and Drug Administration for oral consumption in foods and for use in cosmetics (*19−21*). The structure of GML is comprised of a monoester of glycerol and the fatty acid lauric acid, and GML is the most commonly studied member of a class of fatty acid monoesters with antimicrobial properties. GML inhibits the production of Gram-positive exotoxins such as *S. aureus* [hemolysins, superantigens (TSST-1 and SEs), and exfoliative toxins], *β*-hemolytic streptococci [hemolysins and superantigens (SPEs)] (*22, 23*), and *B. anthracis* [anthrax toxin (protective antigen, lethal factor, and edema factor)] at the cellular level of transcription (*24*). GML is considered to inhibit transcription by blocking signal transduction in bacterial cells. Recent studies demonstrated GML-inhibited transcription of *B. anthracis* anthrax toxin and *S. aureus* *msrA1* genes (*24, 25*). GML is hypothesized to interfere with signal transduction by the lauric acid moiety of GML embedding and spanning approximately one half of the lipid bilayer, causing a stabilization of the membranes.

Although GML could be used to prevent exotoxin production by Gram-positive bacteria, a more beneficial use would be to block the actions of exotoxins on human cells (by inhibiting signal transduction and providing mammalian cell membrane stability). The ability of GML to inhibit the immunostimulatory and cytotoxic effects of Gram-positive bacterial exotoxins on eukaryotic cells was explored in this study. Additionally, the ability of GML to prevent TSS in a rabbit model was examined by co-administration of TSST-1 and GML vaginally.

This study demonstrated the ability of GML to inhibit both the immunoproliferative effects of superantigens on human T lymphocytes and immunostimulatory effects of superantigens on human vaginal epithelial cells (HVECs), suggesting an inhibition of signal transduction. GML prevented the cytotoxicity of anthrax toxin for HVECs as well as cytotoxicity of hemolysins for human and rabbit erythrocytes. Finally, GML inhibited TSS and lethality in a rabbit model of TSS, was not toxic to HVECs at concentrations that were effective in preventing exotoxin action on mammalian cells and rabbits, and stabilized erythrocyte membranes to the lytic effects of both hypotonic solutions and hemolysins. We conclude that GML may be a useful compound in preventing the effects of Gram-positive exotoxins, potential agents of bioterrorism, on human cells without significant risk of toxicity.

MATERIALS AND METHODS

All experiments performed in these studies, except those involving animals, were replicated a minimum of 2 times with similar results obtained.

Bacteria, Exotoxins, and Chemicals. Select agents used in this study were handled in accordance with guidelines established by the University of Minnesota Institutional Biosafety Committee. In addition, the Schlievert laboratory is approved by the Centers for Disease Control for handling such agents. *S. aureus* RN4220 (pCE107) was used as the source of the superantigen TSST-1 (*26*). Staphylococcal strain MNJA was the source of both the superantigen SEB and α-hemolysin (*27*). *S. aureus* strain RN4220 was the source of *β*-hemolysin. *Escherichia coli* containing the gene for streptococcal pyrogenic exotoxin A (*speA*) in a pET28b vector was the source of the superantigen SPE A (*3*). The Sterne strain was the source of culture fluids containing *B. anthracis* hemolysins and anthrax toxin (a mixture of protective antigen, lethal factor, and edema factor) (*28*), and group A streptococcal strain T18P was the source of culture fluids containing the hemolysins streptolysins O and S (*29*). All strains are maintained in the laboratory in the lyophilized state.

Purified superantigen exotoxins were obtained as follows. The producing organisms were grown in a pyrogen-free dialyzable beef heart medium (4−1200 mL flasks) at 37 °C with high aeration (200 revolutions/min) until late stationary phase of growth (24−48 h) (*30*). Briefly, TSST-1, SEB, and SPE A were purified by ethanol precipitation (80% final concentration) from culture fluids, collection of the precipitate by centrifugation (400*g*, 10 min), resolubilization of exoproteins in pyrogen-free water (80 mL), centrifugation (10000*g*, 30 min to remove insoluble material), and thin-layer isoelectric focusing in pH gradients of 3−10 and then 6−8 to purify TSST-1 (pI 7.2) (*31*), 7−9 to purify SEB (pI 8.5) (*32*), and 4−6 to purify SPE A (pI 5.0) (*33*). Samples of all three exotoxins (5 *μ*g of each) were determined to be homogeneous when tested by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and silver-stained. In addition, the three exotoxins, including SPE A produced in the *E. coli* clone, were free of contaminating Gram-negative endotoxin (lipopolysaccharide, LPS) (lower limit of detection, 1 ng of LPS/mg of exotoxin). TSST-1 and SEB were free of detectable hemolysin, protease, and lipase as determined by single-radial diffusion (*34*). Staphylococcal *β*-hemolysin was purified from strain RN4220 comparably as SEB. The exotoxin (5 *μ*g sample) was homogeneous when tested by SDS−PAGE and Coomassie brilliant blue stained. All of these toxins were stored at −20 °C in the laboratory until used.

α-Hemolysin was purified comparably as SEB, except the protein was separated from stationary phase cultures by ammonium sulfate precipitation (75% saturation), resolubilization in pyrogen-free water, dialysis for 2 days to remove residual ammonium sulfate, and isoelectric focusing in pH gradients of 3−10 and then 6−8 (pI 7.5). The protein (5 *μ*g sample) was homogeneous when tested by SDS−PAGE and stained with Coomassie brilliant blue. Other hemolysins were used directly as unpurified proteins in cell-free culture fluids (0.2 *μ*m pore-size filtration) of organisms grown in either dialyzable beef heart medium (streptolysins O and S) or R medium (*B. anthracis* hemolysins). The three *B. anthracis* anthrax toxin components were used as mixtures of unpurified proteins in cell-free culture fluids (0.2 *μ*m pore-size filtration) (*24*) or as purified lethal toxin (purchased as the heptamer pore-forming protective antigen and lethal factor, List Biological Laboratories, Inc., Campbell, CA). We showed that the culture fluids contained all three components of anthrax toxin (protective antigen, lethal factor, and edema

factor) as determined by Western immunoblotting and real-time PCR (*24*). In this study, the combined effects of protective antigen and lethal factor were measured.

GML (monomuls 90-L 12) was a gift from Cognis Corporation, Kankakee, IL. GML analysis determined that the compound was 93.5% monoester, with the remainder being primarily diester (>5.5%) and free glycerin (0.2%). GML was dissolved in absolute ethanol at a concentration of 100 mg/mL and diluted into water, phosphate-buffered saline (PBS; 0.005 M NaPO$_4$ at pH 7.2 and 0.15 M NaCl), or saline (0.15 M NaCl) to a maximum solubility of 100 μg/mL at 37 °C for use in this study.

Inhibition of Superantigen Immunoproliferation. Super-antigens are defined by their abilities to induce nonantigen-specific T lymphocyte proliferation dependent upon the composition of the variable region of the β chain of the T cell receptor (*4*). This stimulatory activity is also dependent upon the presence of antigen-presenting cells (*4*). On day 1, human peripheral blood mononuclear cells containing T lymphocytes and antigen-presenting cells (2 × 10^5 peripheral blood mononuclear cells/well/200 μL volume in quintuplicate in RPMI 1640 medium, supplemented with 2% fetal bovine serum, penicillin/streptomycin, and L-glutamine) were incubated with superantigens (1 μg/well) for 3 days in 96-well microtiter plates (*35*). On day 3, T lymphocyte proliferation was assessed by the addition of 1 μCi/well of ^{3}H-thymidine (20 μL volume) to the wells for an additional 24 h and then harvesting DNA onto glass fiber filters and finally scintillation counting on day 4. In these studies, GML (1, 5, 10, and 20 μg in 5 μL volumes) was added to wells at designated concentrations simultaneously with superantigen on day 1.

An early step in T cell receptor signal transduction leading to immunoproliferation of T lymphocytes by superantigens requires the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into the second messengers inositol 1,4,5-triphosphate (IP$_3$) and diacyl glycerol by phospholipase Cγ1. The ability of GML to inhibit the total cellular IP$_3$ was used as a measure of phospholipase Cγ1 activity on PIP$_2$. Lymphocytes and macrophages (1 × 10^7 cells/mL) were incubated with the superantigen TSST-1 (5.0 μg/mL) with GML (0, 0.1, 0.5, 1.0, 5, and 10.0 μg/mL) overnight at 37 °C, 7% CO$_2$ in PBS supplemented with 1% glucose, and without magnesium. The cellular activity was terminated by the addition of ice-cold trichloroacetic acid (15%) on ice for 20 min, followed by centrifugation (20 min, 2000g, 4 °C). Samples were then treated 3 times with 10 volumes of water-saturated ether to remove trichloracetic acid. Samples were treated with 0.1 M Tris base to bring the samples to neutral pH. Total intracellular IP$_3$ was measured by a comparison to standards in a competition radioactive IP$_3$-receptor assay as described by the manufacturer (NEN Research Products, DuPont, Boston, MA).

Inhibition of HVEC Cytokine Release. Experiments were performed to assess the ability of GML (20 μg/mL) to interfere with the production of chemokines and cytokines by immortalized HVECs (2 × 10^7/flask) through the stimulatory actions of TSST-1 (100 μg/mL), SEB (100 μg/mL), and SPE A (100 μg/mL). These superantigens were previously determined to stimulate chemokine and cytokine production from HVECs (*36*). The 100 μg/mL dose of all superantigens was chosen because our unpublished observations indicate that TSST-1 and SEB-positive *S. aureus* strains

make up to 1000 μg/mL of exotoxin when cultured for 8 h in biofilms (as might be expected to occur in vivo); we used a 10-fold lower dose. HVECs were grown in keratinocyte serum-free medium (Gibco, Invitrogen, Carlsbad, CA) on plastic and passaged with a 1:4 split using trypsin/EDTA. HVECs were immortalized with retroviruses expressing HPV-16 E6/E7 and the reverse transcriptase component of telomerase as described previously (*37, 38*). The HVECs were initially stained with a mixture of IgG monoclonal antibodies AE1 and AE7 (Chemicon International, Temecula, CA) to characterize their cytokeratin production and ensure that the cells were epithelial (*36*). Clone AE1 detects the high-molecular-weight cytokeratins 10, 14, 15, and 16 and the low-molecular-weight cytokeratin 19. Clone AE3 detects the high-molecular-weight cytokeratins 1−6 and the low-molecular-weight cytokeratins 7 and 8.

For all HVEC experiments, confluent monolayers of HVECs in 75 cm^2 flasks (BD Falcon, Bedford, MA) were co-cultured with the superantigens and with and without GML at 37 °C in a 7% CO$_2$ incubator in keratinocyte serum-free medium supplemented with bovine pituitary extract and epidermal growth factor as provided by the manufacturer for 6 h.

Enzyme-linked immunosorbent assays (ELISAs) were performed on cell-culture supernates after the 6 h incubation periods of HVECs with exotoxins ± GML (*36*). ELISA kits were purchased from R and D Systems, Minneapolis, MN, and included assays for chemokine ligand 20 [macrophage inflammatory protein-3α (MIP-3α)], IL-1β, IL-6, IL-8, and TNF-α. All assays including standard curve generation were performed according to the specification of the manufacturer. Absorbance values and a calculated concentration of chemokines and cytokines in the supernates were derived from the linear parts of the standard curves. Controls included the supernates of HVECs incubated without superantigens or with an irrelevant foreign protein (ovalbumin, 100 μg/mL), which were assayed for chemokines and cytokines. Finally, GML (20 μg/mL) was added exogenously to supernates previously determined to contain chemokines and cytokines to demonstrate that GML alone did not interfere with ELISA assays. The lower limits of detection for the ELISA kits were MIP-3α (8 pg/mL), IL-1β (4 pg/mL), IL-6 (4 pg/mL), IL-8 (16 pg/mL), and TNF-α (8 pg/mL),

Inhibition of TSS and Lethality in Rabbits. To evaluate the lethality of TSST-1 in a rabbit TSS model, the synergy between TSST-1 and LPS was exploited. TSST-1 previously has been shown to amplify the lethal effects of Gram-negative LPS by up to 1 000 000-fold (LPS given intravenously 4 h after TSST-1 given intravaginally), as a rabbit model for staphylococcal TSS (death typically occurs in approximately 6 h following the LPS injection) (*39*). [Note that the lethal dose 50% end point (LD$_{50}$) of TSST-1 alone for 2 kg Dutch belted female rabbits is greater than 3 mg/animal in bolus injections, and the LD$_{50}$ of LPS alone intravenously is 1 mg/animal.] Rabbits (3/group, 2 kg female Dutch belted) were treated with TSST-1 (5 μg/rabbit) vaginally in 0.25 mL of PBS with and without GML (250 μg) administered in the same solution. In these experiments, the rabbits were initially anesthetized with ketamine (0.4 mL of 20 mg/mL) and xylazine (1 mL of 20 mg/mL) and then catheters were placed into the vaginas through which the TSST-1-GML samples were administered (*40*). The animals

were allowed to recover from anesthesia for 4 h, then were injected intravenously with LPS from *Salmonella typhimurium* (5 μg in 1 mL of PBS), and were monitored for lethality for 24 h. Rabbits that failed to exhibit escape behavior and right themselves were considered in irreversible shock and were euthanized prematurely in accordance with agreements with the University of Minnesota Institutional Animal Care and Use Committee. Control animals in these experiments received GML alone (250 μg/0.25 mL) vaginally ($n = 2$) and PBS (250 μg/0.25 mL) ($n = 1$) vaginally. Surviving animals were euthanized at 24 h with Beuthanasia D, and vaginal tissue was examined for gross evidence of tissue inflammation.

Inhibition of Cytotoxic Effects of Exotoxins. These experiments used multiple representative cytotoxins, including culture fluids containing lethal toxin (protective antigen and lethal factor) from *B. anthracis*, protective antigen, and lethal factor purchased from List Biological Laboratories, Campbell, CA, purified staphylococcal α- and β-hemolysins, culture fluids containing streptolysins O and S (hereafter referred to as streptolysins), and culture fluids containing *B. anthracis* hemolysins.

Anthrax lethal toxin forms when protective antigen forms heptamer prepores within the plasma membranes of eukaryotic cells and binds to lethal factor (*41*). The internalization of this complex leads to acidification and subsequent formation of mature pores with the insertion of the lethal factor into the cellular cytoplasm where it causes lethality because of protease activity (*42*). Studies were performed to assess the ability of GML (10 μg/mL) to inhibit the toxic effects of lethal toxin from *B. anthracis* culture fluids (2.5 mL/assay) with nonconfluent HVECs (approximately 1 $\times$ 10^4 cell/flask) (*24*). [Note that epithelial cells are thought to have receptors for anthrax toxin protective antigen primarily on their basolateral surface, and for this reason, we investigated nonconfluent HVECs]. The cultures were incubated for 18 h at 37 °C with lethal toxin and with or without GML, and then HVEC viability was determined by Trypan blue dye exclusion. In a second set of studies, murine RAW 264.7 macrophages (1 $\times$ 10^5 cells/well) in quadruplicate in 96-well microtiter plates were incubated for 4 h at 37 °C with 100 ng of protective antigen and 50 ng of lethal factor (List Biological Laboratories, Inc., Campbell, CA) with or without simultaneously added GML (10, 20, and 100 μg/mL). The total incubation volume was 200 μL/well. Percent lethality was determined by Trypan blue dye exclusion. In another identical experiment, we attempted to use the more standard MTT dye assay (*43*), but we discovered that viable macrophages could not accumulate the dye in the presence of GML. We did use this assay to verify that the combination of protective antigen and lethal factor used in our experiments was cytotoxic.

α-Hemolysin was incubated with rabbit red blood cells, as the mammalian cell type most susceptible to the cytolytic effects of the toxin (*5*). Purified α-hemolysin (0.2 and 1 μg/mL) was mixed with rabbit red blood cells (two different concentrations) that upon complete lysis with distilled water would give an absorbance at 410 nm wavelength of approximately 1 and 2, respectively. α-Hemolysin at these concentrations was determined to lyse the rabbit red blood cells completely in less than 15 min. Varying concentrations of GML (0.1, 1.0, and 10 μg/mL) were simultaneously added

to rabbit red blood cells with α-hemolysin in PBS to determine the inhibition of lysis. All samples were incubated at 37 °C and 7% CO_2. Additionally, α-hemolysin (0.1 μg/mL, which completely lysed the rabbit red blood cells in 1 h) was added to rabbit red blood cells in PBS, and the samples were incubated until 50% lysis had occurred (30 min). At that point, GML (1.0 μg/mL) was added to the cultures in an attempt to prevent further lysis. In all of these studies, the samples were centrifuged (400g, 10 min) after incubation periods and the absorbances at 410 nm were determined as a measure of lysis. Controls consisted of rabbit red blood cells added to PBS and incubated without toxin and without GML and with the various concentrations of GML alone.

Studies were performed in quadruplicate to assess whether GML would prevent α-hemolysin insertion into rabbit red blood cell membranes. Prior observations determined that α-hemolysin heptamerizes in solution, and the heptamers then form pores in rabbit red blood cell membranes, causing lysis (*44*). Fresh heparinized blood (10 mL) was drawn from a rabbit. The rabbit red blood cells were washed 3 times with 15 mL of PBS (centrifugation at 400g, 10 min). Finally, the packed cell volume (obtained by centrifugation at 400g, 10 min) was diluted $^1/_2$ with PBS, and 0.5 mL volumes were transferred into separate tubes. α-Hemolysin (100 μg) alone and with GML (100 μg) was mixed with rabbit red blood cells (to create final volumes of 1 mL) at 37 °C until complete lysis occurred (less that 1 h) in the samples with α-hemolysin and rabbit red blood cells alone. This step was performed with the hypothesis that α-hemolysin would be removed from the solution by embedding into the membranes of rabbit red blood cells in the absence of GML but would remain in solution if GML prevented its insertion into the rabbit red blood cell membranes. Supernate samples were collected by centrifugation (10000g, 5 min) and were treated with 4 volumes of absolute ethanol at 4 °C to precipitate α-hemolysin and retain GML in solution. The precipitates were resuspended in the same volume of PBS, and 20 μL volumes were added to agarose slides containing rabbit red blood cells. Briefly, slides were made as follows: agarose (0.75%) was melted in PBS and cooled to 50 °C, and rabbit red blood cells (rRBCs) were added (0.1% final concentration) with 4 mL volumes of the mixture used to coat the surface of microscope slides. After the agarose slides solidified, 4 mm wells were cut in the agarose and samples (20 μL) applied. The slides were then incubated for 6 h at 37 °C in a humidified 7% CO_2 incubator. Zones of lysis were determined as measures of α-hemolysin present in the supernates (not absorbed out by prior treatment with rabbit red blood cells), and the concentration of α-hemolysin was determined by a comparison to lysis zones with known amounts of purified α-hemolysin treated comparably. The concentration of α-hemolysin was proportional to the area of lysis (*34*). Control samples contained rabbit red blood cells (1.0 mL final volumes) and rabbit red blood cells with GML (1.0 mL final volumes) only.

The ability of GML (1, 5, and 10 μg/mL) to inhibit the lytic effects of streptolysins and *B. anthracis* hemolysins was performed on rabbit red blood cells with culture fluids (100 μL/mL red blood cells) containing hemolysins, using essentially the same procedures as for α-hemolysin. The only differences in the assays were that culture fluids containing

hemolysins were used rather than purified toxins and the streptococcal culture fluids were reduced with dithiothreitol (5 mM) to ensure that streptolysin O was active. In addition, the lytic effects of *B. anthracis* hemolysins were tested similarly to those of α-hemolysin; however, both rabbit and human red blood cells were studied. (Note that *B. anthracis* hemolysins in sterile culture fluids were self-inactivated 48 h after production and secretion. Experiments using these culture fluids were performed on the same day as the culture fluids were obtained.)

Staphylococcal β-hemolysin is a sphingomyelinase that prepares red blood cells for lysis by its enzymatic activity at 37 °C (*45*). At 37 °C, the red blood cells do not lyse, but upon shifting the temperature to 4 °C, the red blood cells lyse, making the β-hemolysin a hot−cold hemolysin. The activity of β-hemolysin was utilized to evaluate the mechanism of action of GML on red blood cell membranes. Rabbit red blood cells (absorbance at 600 nm wavelength = 2.4) in PBS were incubated with purified β-hemolysin (3, 7.5, and 15 μg/mL) at 37 °C for 1 h. At that time, the red blood cell samples were centrifuged (400*g*, 10 min) and the supernates were discarded. Subsequently, the samples were restored to the original volumes (5 mL) with PBS, and each sample was split in half. One half received 10 μg/mL of GML (final concentration), and the other half received PBS. The samples were then immediately incubated at 4 °C for 4 h, and absorbance at 600 nm wavelength was determined. All assays were performed in duplicate.

GML Stabilization of Cells in Hypotonic Aqueous Solutions. Human red blood cells were obtained from heparinized venous blood and processed by dilution 10-fold in normal saline and then washing 3 times by centrifugation (400*g*, 10 min). The human red blood cells (5 μL) were then diluted with 2 mL of saline to give an absorbance at 600 nm wavelength of approximately 0.8. Human red blood cell stability measurements were conducted by adding diluted human red blood cells (5 μL into 2 mL) to solutions of GML (20, 50, and 100 μg/mL) in distilled water, 0.025 M NaCl, and 0.05 M NaCl, 0.075 M NaCl, and 0.15 M NaCl and then measuring absorbance at 600 nm wavelength continuously for 300 s (5 min). At room temperature, where measurements were made, GML (50 and 100 μg/mL) solutions were slightly turbid, indicating incomplete solubility at that temperature.

Lack of GML Cytotoxicity for HVECs. GML (20 and 100 μg/mL) was added to partially confluent HVECs (approximately 5×10^6/flask) growing in 75 cm^2 flasks (BD Falcon) and incubated for 2 weeks with changes of keratinocyte serum-free medium and GML daily. Each day, flasks from GML-treated and untreated cells were examined by Trypan blue dye exclusion for direct HVEC toxicity because of GML. On the last experimental day (day 14), the media from one flask of HVECs from each treatment group (treated or not treated with GML) were changed to fresh keratinocyte serum-free medium without added GML, and the HVECs were monitored for 1 week for the ability to grow normally.

HVEC Microarray Studies with GML. Affymetrix Gene-Chip Human Genome U133A (Santa Clara, CA) was used to analyze HVEC gene up- or downregulation in response to incubation in keratinocyte serum-free medium (without antibiotics) with GML (20 μg/mL) for 6 h (*36*). Control HVEC flasks contained keratinocyte serum-free medium
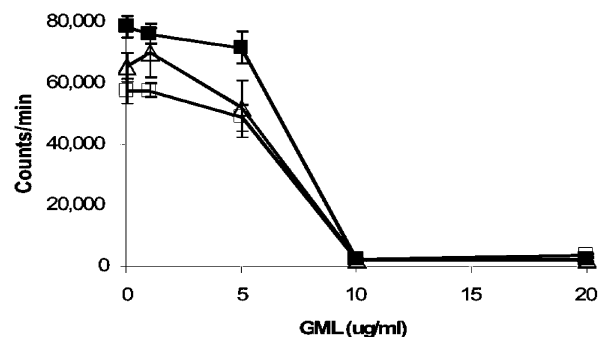

FIGURE 1: Effect of GML on superantigenicity of TSST-1 (1 μg/mL, □), SPE A (1 μg/mL, ■), and SEB (1 μg/mL, △). Superantigens plus GML (1, 5, 10, and 20 μg) in 5 μL volumes were added simultaneously to 1×10^6/mL human peripheral blood mononuclear cells in 200 μL total volumes in replicates of five in 96-well microtiter plates. The plates were incubated at 37 °C in the presence of 7% CO$_2$ for 3 days, and then, the wells were pulsed for 24 h with 1 μCi of ^{3}H-thymidine for incorporation into DNA. DNA was harvested on day 4 with use of a mechanical sample harvester onto glass fiber filters, and total counts were determined by scintillation counting.

(without antibiotics) alone and were comparably incubated for 6 h. The protocol entailed the culture of the HVECs in 75 cm^2 flasks (BD Falcon, Bedford, MA) until confluent (determined to be approximately 2×10^7 HVECs/flask). Prior to initiation of the experiments, the media was then changed to include keratinocyte serum-free medium alone (without antibiotics) or keratinocyte serum-free medium (without antibiotics) plus GML.

The HVECs were removed from the flasks by trypsinization (5−8 min at 37 °C) and collected by centrifugation (250*g* for 5 min). The cell pellets were used for RNA extraction, which was accomplished using an RNeasy Mini kit from QIAGEN (Valencia, CA). RNA samples were processed for hybridization assays as described by Affymetrix, Inc. (Santa Clara, CA). Hybridization analyses were performed at the University of Minnesota Biomedical Genomics Center. The data obtained were analyzed by programs provided by both Affymetrix Microarray Suite 4.0 and Microsoft (Excel). Only those genes that were definitively present and statistically determined to be up- or downregulated by 2-fold or more were considered.

RESULTS

GML Inhibits T Lymphocyte Immunoproliferation. Superantigens are defined by their capacities to induce proliferation of T cells after cross-linking with antigen-presenting cells, as a function of the β-chain variable region of the T cell receptor complex that causes the release of IL-2, TNF-β, and IFNγ (*1, 4*). In concert with the activation of T cells is the release of IL-1β and TNF-α from macrophages (*1, 4*). GML (10 and 20 μg/mL) effectively interfered with the T cell proliferative effect induced by three superantigens, TSST-1, SEB, and SPE A (Figure 1). Partial inhibition of T cell proliferation was observed with GML doses of 5 μg/mL.

GML at concentrations of 0.5−10 μg/mL caused significant and dose-dependent inhibition of the formation of IP$_3$ from PIP$_2$ in lymphocytes incubated overnight with TSST-1 (5 μg/mL) compared to the positive control consisting of lymphocytes incubated with TSST-1 in the absence of GML

Table 1: GML Inhibition of the Generation of IP$_3$ by Lymphocytes Stimulated with TSST-1

treatment	IP$_3$ generated[a] (pmol/0.1 mL $\pm$ SE)
untreated cells	0.1 $\pm$ 0.05
TSST-1[b]	8.5 $\pm$ 0.6
TSST-1 + GML (0.1 μg/mL)	8.0 $\pm$ 0.5
TSST-1 + GML (0.5 μg/mL)	5.0 $\pm$ 0.6
TSST-1 + GML (1.0 μg/mL)	2.0 $\pm$ 0.1
TSST-1 + GML (10.0 μg/mL)	1.0 $\pm$ 0.05

[a] IP$_3$ generated by phospholipase Cγ1 cleavage of PIP$_2$. [b] TSST-1 concentration (5 μg/mL).

(Table 1). The reduction in generation of IP$_3$ in these assays is a reflection of reduced activity of phospholipase Cγ1 in the presence of GML and, consequently, early events in T cell receptor signal transduction.

GML Inhibits Cytokine Release by HVECs. HVECs have been determined previously to upregulate production of chemokines and cytokines in response to the stimulation with the superantigens TSST-1, SEB, and SPE A for 6 h (*36*). We explored whether GML could inhibit chemokine and cytokine release by HVECs in response to stimulation by TSST-1, SEB, and SPE A.

Representative cytokines (IL-1β and TNF-α) and chemokines (MIP-3α, IL-6, and IL-8) were released by HVECs in response to the stimulation with TSST-1, SEB, and SPE A as determined by ELISA on culture supernates (Table 2). All superantigens stimulated release of approximately the same amounts of cytokines and chemokines, except SEB caused statistically significant release of more MIP-3α and IL-8 than the other two superantigens. GML (20 μg/mL) completely prevented superantigen-induced cytokine and chemokine release from HVECs (Table 2). GML (20 μg/mL) also inhibited a constitutive low-level release of cytokines and chemokines when HVECs were incubated alone in keratinocyte serum-free medium (Table 2). Ovalbumin (100 μg/mL), which caused only a low-level release of cytokines and chemokines from HVECs, was included as a control to indicate that the cytokines and chemokines stimulated by the three superantigens were the result of unique superantigen activity, rather than simply the presence of any foreign protein.

GML Inhibits TSS and Lethality in a Rabbit Model. The ability of GML to protect rabbits from TSS by administration of GML and TSST-1 vaginally was also determined. GML (250 μg/0.25 mL) when co-administered vaginally to rabbits with TSST-1, completely prevented TSST-1-induced TSS

after 24 h (^{0}/$_3$ animals succumbed compared to ^{2}/$_3$ animals treated with TSST-1 alone). Minor visible edema and angiogenesis were observed in the vaginal tissue of surviving rabbits that had been treated with TSST-1 plus GML (data not shown). The one surviving rabbit that had been treated with TSST-1 alone had small amounts of pus present vaginally, and there was significant edema, angiogenesis, neutrophil influx, apoptotic cells present, and epithelial sloughing (data not shown). GML alone (250 μg/0.25 mL administered volume) was not toxic (^{0}/$_2$) when administered vaginally to rabbits, with a lack of observable pathologic effects on vaginal mucosal tissue.

GML Inhibits the Effects of Cytotoxins. Many heptamer pore-forming exotoxins have been described, including staphylococcal α-hemolysin and group A streptolysin O (*6*, *46*, *47*). In addition, the protective antigen component of anthrax toxin is a heptamer pore-forming toxin that delivers lethal factor and edema factor into susceptible mammalian cells, leading to anthrax disease (*2*). Finally, *B. anthracis* also makes hemolysins (*48−50*), and group A streptococci make another hemolysin, streptolysin S (*51*).

GML (1 and 10 μg/mL) effectively prevented the lytic activity of staphylococcal α-hemolysin for rabbit red blood cells in duplicate experiments containing two different α-hemolysin (0.2 and 1 μg/mL) and two different rabbit red blood cell concentrations (complete lysis with distilled water yielded absorbances at 410 nm wavelength of 0.97 and 2.2) (Table 3). GML partially prevented hemolysis at a concentration of 0.1 μg/mL. In addition, GML (1 μg/mL) immediately stopped additional lysis because of staphylococcal α-hemolysin (0.1 μg/mL) when administered approximately 30 min into the 1 h total incubation period (Table 3).

GML also blocked the cytolytic effect of streptolysin O and S and anthrax hemolysins (Table 4). GML (1, 5, and 10 μg/mL) completely inhibited rabbit red blood cell lysis by 100 μL of group A streptococcal supernate. Interestingly, a higher concentration of GML (10 μg/mL) was required to inhibit the lytic effects of anthrax supernate hemolysins than either α-hemolysin (1 μg/mL) or the streptococcal hemolysins (1 μg/mL) (Table 4). Finally, GML (10 μg/mL) prevented anthrax hemolysin lysis of human red blood cells (data not shown).

Anthrax toxin heptamerizes upon insertion into susceptible plasma membranes, forming what is referred to as the prepore (*41*). The heptamerized protective antigen has the capacity to bind the protease lethal factor to form lethal toxin (*52*). Endocytosis and acidification leads to the formation of a

Table 2: Cytokines and Chemokines Produced by HVECs in Response to TSST-1, SEB, and SPE A and Inhibition by GML

protein tested (100 μg/mL) (N = 3)	GML[a] (20 μg/mL)	IL-1β (pg/mL $\pm$ SE)	TNF-α (pg/mL $\pm$ SE)	MIP-3α (pg/mL $\pm$ SE)	IL-6 (pg/mL $\pm$ SE)	IL-8 (pg/mL $\pm$ SE)
none	−	8 $\pm$ 1	16 $\pm$ 1.5	9.0 $\pm$ 1.5	8 $\pm$ 1.5	16 $\pm$ 2
none	+	<4[b]	<8	<8	<4	<8
ovalbumin	−	11 $\pm$ 1.5	16 $\pm$ 2	36 $\pm$ 1.5	10 $\pm$ 1.5	12 $\pm$ 2
TSST-1	−	16 $\pm$ 3	62 $\pm$ 8	320 $\pm$ 4	18 $\pm$ 2	500 $\pm$ 33
TSST-1	+	<4	<8	<8	<4	<8
SEB	−	18 $\pm$ 4	56 $\pm$ 6	470 $\pm$ 7	19 $\pm$ 4	620 $\pm$ 7
SEB	+	<4	<8	<8	<4	<8
SPEA	−	15 $\pm$ 2	97 $\pm$ 4	396 $\pm$ 8	19 $\pm$ 2	470 $\pm$ 10
SPEA	+	<4	<8	<8	<4	<8

[a] GML did not interfere with ELISAs because the compound did not affect cytokine and chemokine detection when added to supernates that already contained chemokines. [b] < refers to samples where quantities of chemokines and cytokines were below detection by the ELISA kits.

Table 3: GML Inhibits Staphylococcal α-Hemolysin Lysis of rRBCs

experiment number (incubation time)	treatment group[a]	absorbance at 410 nm[a]
1 (1 h)	rRBCs in distilled water[b]	0.97
1 (1 h)	rRBCs in PBS	0
1 (1 h)	rRBCs + GML (10 μg/mL)	0
1 (1 h)	rRBCs + 0.2 μg of α-hemolysin	0.97
1 (1 h)	rRBCs + 0.2 μg of α-hemolysin + GML (0.1 μg/mL)	0.44
1 (1 h)	rRBCs + 0.2 μg of α-hemolysin + GML (1.0 μg/mL)	0
1 (1 h)	rRBCs + 0.2 μg of α-hemolysin + GML (10 μg/mL)	0
2 (1 h)	rRBCs in distilled water	2.2
2 (1 h)	rRBCs + 1.0 μg of α-hemolysin	2.2
2 (1 h)	rRBCs + 1.0 μg of α-hemolysin + GML (1.0 μg/mL)	0
2 (1 h)	rRBCs + 1.0 μg of α-hemolysin + GML (10 μg/mL)	0
2 (1 h)	rRBCs in distilled water	2.2
2 (1 h)	rRBCs + 0.1 μg of α-hemolysin	2.2
2 (30 min of incubation)	rRBCs + 0.1 μg of α-hemolysin	0.9
2 (30 min of incubation with α-hemolysin and then GML added; total incubation of 1 h)	rRBCs + 0.1 μg of α-hemolysin + GML (1.0 μg/mL)	1.2

[a] Rabbit red blood cells were incubated with the designated agents in PBS for the indicated time periods at 37 °C and then centrifuged at 400*g* for 10 min to pellet red blood cells. The absorbances of the supernates at 410 nm were determined. *[b]* All experiments were conducted in PBS except where noted (in distilled water).

Table 4: GML Prevents Group A Streptococcal and *B. anthracis* Culture Fluid Lysis of rRBCs

treatment group	GML (μg/mL)	absorbance at 410 nm (1 h)[a]
rRBCs in distilled water[b]	0	2.4
rRBCs in PBS	0	0
rRBCs in PBS + GML	10	0
rRBCs + 100 μL of streptococcal supernate[a]	0	2.4
rRBCs + 100 μL of streptococcal supernate	1	0
rRBCs + 100 μL of streptococcal supernate	5	0
rRBCs + 100 μL of streptococcal supernate	10	0
rRBCs + 100 μL of *B. anthracis* supernate	0	2.4
rRBCs + 100 μL of *B. anthracis* supernate	1	2.4
rRBCs + 100 μL of *B. anthracis* supernate	5	1.4
rRBCs + 100 μL of *B. anthracis* supernate	10	0

[a] Rabbit red blood cells were incubated with the designated agents in PBS at 37 °C and then centrifuged at 400*g* for 10 min to pellet red blood cells. The absorbance of the supernates at 410 nm were determined. Streptococcal and *B. anthracis* supernates were filter-sterilized (0.2 μm pore size) prior to use. *[b]* All experiments were conducted in PBS except where noted (in distilled water).

Table 5: GML Prevents Cytotoxic Activities of *B. anthracis* Supernates and Purified Lethal Toxin [Mixture of Protective Antigen (PA) and Lethal Factor (LF)]

treatment group[a]	percent killing of HVECs $\pm$ SE
HVECs + 2.5 mL of supernate	45 $\pm$ 4
HVECs + 2.5 supernate + 10 μg/mL GML	0
macrophages + lethal toxin	85 $\pm$ 2
macrophages + lethal toxin + 10 μg/mL GML	30 $\pm$ 8
macrophages + lethal toxin + 20 μg/mL GML	10 $\pm$ 5
macrophages + lethal toxin + 100 μg/mL GML	0

[a] HVECs (1×10^4) were treated with 2.5 mL of *B. anthracis* supernate and lethality determined by Trypan blue dye exclusion after incubation for 18 h at 37 °C. *B. anthracis* supernates were filter-sterilized (0.2 μm pore size) prior to use. RAW 264.7 macrophages (1×10^5) were treated with PA (100 ng) and LF (50 ng) purchased from List Biological Laboratories, Inc. for 4 h. This combination of proteins comprises lethal toxin. Cell viability was determined by Trypan blue dye exclusion.

Table 6: GML Prevents Staphylococcal α-Hemolysin Insertion into rRBC Membranes

treatment group	α-hemolysin remaining in the supernate (μg)[a]
rRBCs alone	0
rRBCs + GML (100 μg)	0
rRBCs + α-hemolysin (100 μg)	5 $\pm$ 1
rRBCs + α-hemolysin (100 μg) + GML (100 μg)	90 $\pm$ 1

[a] α-Hemolysin (100 μg) alone and with GML (100 μg) were mixed with rabbit red blood cells in final volumes of 1 mL of PBS at 37°C until complete lysis occurred in the samples with α-hemolysin and rabbit red blood cells alone. Supernate samples were collected by centrifugation, treated with 4 volumes of absolute ethanol, and resuspended in 1 mL amounts of PBS, and 20 μL volumes were added to agarose slides containing rabbit red blood cells. The slides were incubated for 6 h at 37 °C in a humidified 7% CO_2 incubator. Zones of lysis were determined as compared to lysis zones with known amounts of purified α-hemolysin treated comparably. The concentration of α-hemolysin was proportional to the area of lysis.

mature pore that inserts lethal factor into the cells (*41*). GML (10 μg/mL) completely prevented the lethal action of *B. anthracis* culture fluids containing lethal toxin for HVECs (Table 5). These data suggest that the effect of GML was either to interfere with protective antigen insertion into HVEC membranes or to interfere with the endocytic process. In a separate experiment, GML (100 μg/mL) was highly effective in preventing purified anthrax lethal toxin killing of RAW 264.7 macrophages (Table 5) compared to control macrophages treated only with lethal toxin. Lower concentrations of GML provided partial protection from lethality.

GML Prevents Insertion of Staphylococcal α-Hemolysin into Rabbit Red Blood Membranes. GML effectively prevented the insertion of heptameric α-toxin into the eukaryotic membrane (Table 6). Prior incubation of rabbit red blood cells with GML (100 μg/mL) and staphylococcal α-hemolysin (100 μg/mL) prevented α-hemolysin embedding into rabbit red blood cell membranes and consequently removal from supernates. In contrast, prior incubation of rabbit red blood cells alone with α-hemolysin (100 μg/mL) resulted in nearly the complete removal of the exotoxin from the supernates.

GML Prevents the Lytic Effects of Staphylococcal β-Hemolysin on Rabbit Red Blood Cells. The ability of GML to
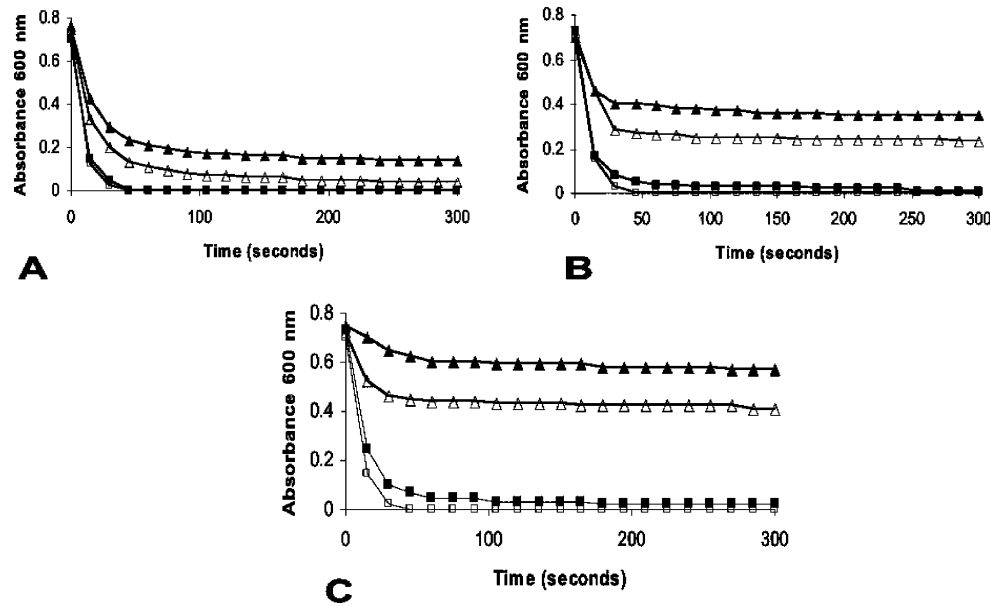
FIGURE 2: GML added to hypotonic solutions stabilizes human red blood cells. (A) Absorbance changes at 600 nm wavelength with GML (control, 0 μg/mL, □; 20 μg/mL, ■; 50 μg/mL, △; and 100 μg/mL, ▲) added to human red blood cells in distilled water. (B) Absorbance changes at 600 nm wavelength with the same GML concentrations added to human red blood cells in 0.025 M NaCl. (C) Absorbance changes at 600 nm wavelength with the same GML concentrations added to human red blood cells in 0.05 M NaCl. Human red blood cells added to 0.075 and 0.15 M NaCl did not lead to red blood cell lysis. GML (10−100 μg/mL) added to human red blood cells in 0.075 and 0.15 M saline did not lead to red blood cell lysis.

Table 7: GML Inhibits the Hemolytic Effects of Staphylococcal β-Hemolysin

β-hemolysin[a] (μg/mL)	GML (μg/mL)	absorbance (600 nm)
none	0	2.4
none	10	2.4
3	0	1.0
3	10	2.2
7.5	0	0
7.5	10	2.2
15	0	0
15	10	0.5

[a] β-Hemolysin was added to rabbit red blood cells in PBS at time 0 h. The samples were incubated at 37 °C for 1 h and centrifuged (400g, 10 min), and supernates were discarded. The samples were restored to the original volumes with PBS, and each sample was split in half. One half received 10 μg/mL GML (final concentration), and the other half received PBS. The samples were then immediately incubated for 4 h at 4°C and absorbance at 600 nm wavelength determined.

prevent the hot/cold hemolytic activity of β-hemolysin was determined. β-Hemolysin is a sphingomyelinase that sensitizes red blood cells at 37 °C to subsequent lysis at 4 °C (45). Preincubation of rabbit red blood cells with β-hemolysin resulted in partial (3 μg/mL β-hemolysin) or complete (7.5 and 15 μg/mL β-hemolysin) lysis when the red blood cells were subsequently shifted to a temperature of 4 °C in the absence of additional β-hemolysin (Table 7). In contrast, the same pretreatment and temperature shift to 4 °C in the presence of GML (10 μg/mL) completely (3 and 7.5 μg/mL β-hemolysin) or partially (15 μg/mL β-hemolysin) protected the red blood cells from lysis. These data suggest that GML (10 μg/mL) stabilized the red blood cell membranes to the β-hemolysin-induced membrane lipid conformational change that leads to lysis of red blood cells at 4 °C.

GML Stabilizes Human Red Blood Cells to Lysis by Hypotonic Aqueous Solutions. From the above experiments, we hypothesized that GML may exert its effects by stabilizing membranes and preventing signal transduction. GML was tested for its ability to stabilize human red blood cells against lysis by distilled water (Figure 2A) and hypotonic solutions (0.025 M NaCl in Figure 2B and 0.05 M NaCl in Figure 2C) at concentrations of GML (20, 50, and 100 μg/mL). Interestingly, GML stabilized and prevented lysis of human red blood cells minimally by distilled water (Figure 2A) but significantly by hypotonic saline solutions (parts B and C of Figure 2), in a concentration-dependent manner, with GML (100 μg/mL) providing the greatest stabilization. Hypotonic saline solutions containing 0.075 M NaCl and normal saline containing 0.15 M NaCl did not result on the lysis of human red blood cells in the presence or absence of GML (20, 50, and 100 μg/mL).

GML Is Not Toxic to Eukaryotic Cells. Initial studies examined the toxicity of GML for HVECs over a 2 week period. HVECs at approximately 50% confluence (5 × 10⁶/ flask) were incubated with 10 mL of keratinocyte serum-free medium containing 20 and 100 μg/mL amounts of GML for 2 weeks with daily exchanges of GML. From Trypan blue dye exclusion, no toxicity was observed at the end of 2 weeks in GML-treated groups versus controls (data not shown). However, HVECs treated with GML (100 μg/mL) were observed to grow more slowly compared to control non-GML-treated HVECs. On day 14, the remaining GML in the supernates was removed from one flask of each treatment group by changing the media to KSFM without GML and cell growth was monitored. Cells in all three flasks (untreated control; GML, 20 μg/mL; and GML, 100 μg/mL) grew normally, indicating that even though GML delayed the growth of HVECs treated with GML (100 μg/mL), the delayed growth effect was not permanent. After the media was changed to KSFM without GML and the cells were allowed to grow to confluence, the HVECs could be restimulated with superantigens to release cytokines at the expected levels.

Additionally, GML (20 μg/mL) was evaluated for effects on the gene expression of HVECs following 6 h of incubation compared to untreated controls for 6 h. Significant alteration of normal gene expression was observed in a small number of genes [160 compared to 2386 for TSST-1 (100 μg/mL)] (36), consistent with the low toxicity of the compound [a complete listing of genes that were affected by GML (20 μg/mL) can be found at http://www.micab.umn.edu/faculty/Schlievert.html]. Examples of genes that were significantly upregulated by GML (20 μg/mL) included insulin-induced gene (13-fold), DNaJ (Hsp40) homologue, subfamily B, member 9 (13-fold), and corneodesmosin (12-fold). Only 21 genes were significantly downregulated, with all less than 3-fold (the most downregulated was the gene for carbonic anhydrase XII by 2.8-fold). GML was not proinflammatory to HVECs, as evidenced by the lack of induction of proinflammatory cytokines and chemokine genes.

DISCUSSION

Gram-positive bacteria produce a large number of human diseases, with TSS and necrotizing pneumonia caused by *S. aureus* (1, 53), pharyngitis and TSS with necrotizing fasciitis induced by group A streptococci (3, 54, 55), and cutaneous, gastrointestinal, and inhalation anthrax caused by *B. anthracis* (56). Many of these diseases initiate at mucosal surfaces and occur because of the action of exotoxins on the cells of human hosts. Because many of the diseases are associated with high morbidity and mortality, specific exotoxins, such as the superantigen exotoxins SEs, TSST-1, and SPEs, and anthrax toxin have been classified as select agents of bioterrorism.

This study was designed to investigate the prevention of toxicity of exotoxins on host cells by GML, a molecule from a class of fatty acid monoesters that are likely to affect globally cell membranes and signal transduction in both bacteria and eukaryotes. GML is generally recognized as safe by the Food and Drug Administration for oral use because of past safety experience and is used as an additive to foods and cosmetics (19-21). GML is a surfactant comprised of glycerol and a 12-carbon fatty acid, which is found naturally in human breast milk. GML in aqueous solutions has a solubility limit of approximately 100 μg/mL at 37 °C, and for this reason, we investigated the inhibitory effects of GML up to that concentration. However, the concentrations of GML that are used in foods may reach 10 mg/mL (20) or 100 times the concentrations used in this study. Other members of this general class of compounds include mono- and diglycerides with longer or shorter fatty acid chain length. We chose GML as the representative compound to study because previous research suggested that GML inhibited exotoxin production by Gram-positive bacteria more effectively than other members of the class with longer or shorter fatty acid chain length (23).

In general, the most significant findings of this study were the abilities of GML to interfere with the toxic effects of Gram-positive exotoxins on mammalian cells. Exotoxins typically have two types of effects on human cells: (1) direct and indirect cytotoxic capabilities through the formation of pores, functioning as surfactants, or delivering enzymes or factors that disrupt normal cellular functions; and (2) immunostimulatory, particularly as superantigens or other exotoxins stimulating cytokine and chemokine release from immune cells. Typically, patients infected with Gram-positive organisms present with clinical signs that are due in part or completely to the effects of exotoxins on human cells (54-60). Our studies suggest that GML may be able to inhibit both the initiation of the interaction of exotoxins with human cells and also block further toxicity even when cytotoxicity has begun. Thus, GML inhibited the immunostimulatory effects of superantigens on mammalian cells in vitro and in vivo, including TSS-inducing and lethal effects of TSST-1 vaginally in rabbits. In addition, GML blocked the cytotoxic effects of several hemolysins for red blood cells and anthrax toxin for HVECs and macrophages. Notably, different concentrations of GML were required to interfere with the effects of hemolysins, with lower concentrations (1 μg/mL) inhibiting α-hemolysin activity but higher concentrations (10 μg/mL) required to inhibit *B. anthracis* hemolysins. The capacity of GML and related compounds to interfere with exotoxin effects may also depend upon fatty acid chain lengths and whether the molecule is a mono- or diester; these effects were not tested in this study.

Previous research suggested that GML interfered with signal transduction in Gram-positive bacteria, leading to inhibition of transcription of exotoxins (22, 24, 25). Prior research has also suggested that GML inhibits T lymphocyte proliferation stimulated with nonspecific mitogenic agents (61). In this study, we demonstrated that GML inhibits the lymphocyte proliferative effects of three representative superantigens, TSST-1, SEB, and SPE A. TSST-1 is the principal cause of menstrual TSS and one half of nonmenstrual TSS (62); SEB is a classical SE that causes food poisoning, is a significant cause of nonmenstrual TSS, and is associated with necrotizing pneumonia caused by *S. aureus* (53, 62, 63); and SPE A is a major superantigen associated with streptococcal TSS with necrotizing fasciitis (55). The effects of GML appear to be to interfere with the early T cell receptor signal transduction events as evidenced by GML inhibition of IP$_3$ formation from membrane PIP$_2$, as catalyzed by phospholipase Cγ1.

Our prior studies suggested that TSS is initiated by low-level inflammation produced by *S. aureus* and exotoxin activation of HVECs to release chemokines and cytokines through inflammation, which facilitates superantigen transport across the mucosal barrier (36). GML was highly effective in preventing the release of chemokines and cytokines by HVECs in response to stimulation with TSST-1, SEB, and SPE A, probably through inhibition of signal transduction through an unknown HVEC receptor.

The cytotoxic exotoxins studied included hemolysins that function as heptamer pore-forming agents, including staphylococcal α-hemolysin, streptolysin O, and the protective antigen component of anthrax toxin. Staphylococcal α-hemolysin forms heptamers in solution with the oligomeric forms embedding in the host red blood cell membrane to cause lysis (5). Our studies demonstrated that α-hemolysin was unable to bind to red blood cells in the presence of GML, suggesting that the primary effect of GML was to stabilize red blood cell membranes and block the binding of α-hemolysin to red blood cell membranes. This likely also explains the ability of GML to block toxicity associated with other heptamer pore-forming exotoxins. Our observation was confirmed by studies that showed that staphylococcal

β-hemolysin, a sphingomyelinase and hot/cold hemolysin (*45*), was unable to lyse red blood cells when the temperature was shifted to 4 °C in the presence of GML. These data indicate that GML prevented the conformational change in sphingolipids that appear to mediate β-hemolysin lytic activity at 4 °C. Finally, GML also stabilized red blood cells to the lytic effects of hypotonic aqueous solutions. The predominant effect of GML inserted into the membrane may be to "freeze" signal transduction mechanisms, such as host cell receptor signaling systems usurped by exotoxins.

Since anthrax spores were sent via post in 2001, there has been considerable effort in the United States to develop ways to interfere with the toxic effects of exotoxins of Gram-positive bacteria. Many of the studies have attempted specifically to interfere with their actions by competitive inhibition with small molecules or antibody neutralization of function (*9−12, 18*). The potential significance of the present studies is that GML has the ability to block signal transduction and stabilize membranes of a wide range of host cells without significant host cell or rabbit toxicity as applied vaginally. GML is both inexpensive and has already been used in products for human consumption. It is important to note, however, that GML inhibition of signal transduction is dose-dependent, and doses higher than tested in this study may interfere with normal host functions. We have not tested GML effects when administered intravenously, but significant inhibition of signal transduction may preclude its use systemically. Finally, the inhibition of inflammatory cytokine and chemokine production as was observed in the case of inhibition of superantigen effects on T cells, macrophages, and vaginal epithelial cells suggests that GML may function also generally as a topical anti-inflammatory agent (*36*).

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