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Modulation of ROS production in human leukocytes by ganglioside micelles

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Abstract

Recent studies have reported that exogenous gangliosides, the sialic acid-containing glycosphingolipids, are able to modulate many cellular functions. We examined the effect of micelles of mono- and trisialoganglioside GM1 and GT1b on the production of reactive oxygen species by stimulated human polymorphonuclear neutrophils using different spectroscopic methods. The results indicated that exogenous gangliosides did not influence extracellular superoxide anion ($O_2^{\cdot -}$) generation by polymorphonuclear neutrophils activated by receptor-dependent formyl-methionyl-leucyl-phenylalanine. However, when neutrophils were stimulated by receptor-bypassing phorbol 12-myristate 13-acetate (PMA), gangliosides above their critical micellar concentrations prolonged the lag time preceding the production in a concentration-dependent way, without affecting total extracellular $O_2^{\cdot -}$ generation detected by superoxide dismutase-inhibitable cytochrome c reduction. The effect of ganglioside GT1b (100 µM) on the increase in lag time was shown to be significant by means of both superoxide dismutase-inhibitable cytochrome c reduction assay and electron paramagnetic resonance spectroscopy ($P < 0.0001$ and $P < 0.005$, respectively). The observed phenomena can be attributed to the ability of ganglioside micelles attached to the cell surface to slow down PMA uptake, thus increasing the diffusion barrier and consequently delaying membrane events responsible for PMA-stimulated $O_2^{\cdot -}$ production.

Key words: Ganglioside micelles; Reactive oxygen species; Human polymorphonuclear neutrophils

Introduction

Gangliosides, the sialic acid-containing glycosphingolipids that constitute the plasma membranes of various cells, regulate many different cellular functions (1). A number of studies have suggested that they appear mainly in specialized membrane microdomains known as the lipid rafts, which are envisaged as lateral assemblies of sphingolipids, cholesterol and a specific set of proteins, proposed to function in many biological processes (2,3). Recent studies have shown that NADPH oxidase activity in neutrophils is dependent on the presence of lipid rafts (4-6). Studies of lipid raft properties in the presence of exogenously supplied gangliosides have demonstrated that ganglioside monomers can be incorporated into the membrane, where they behave as endogenous gangliosides in lipid raft subdomains and possibly disturb multiple raft-dependent signal transduction pathways (7,8). The ability of exogenous ganglioside monomers to enhance oxygen radical production by different cell types and by neuronal cells in particular, has also been reported (9,10). In this context, Avrova et al. (11,12) have shown that monosialoganglioside GM1, supplied in picomolar concentrations, increased the phorbol 12-myristate 13-acetate (PMA)-induced generation of superoxide anion ($O_2^{\cdot -}$) by human neutrophils.

In contrast to ganglioside monomers, which are inserted into cell membrane rafts, the exogenous gangliosides at concentrations above their respective critical micellar concentrations (cmc) in aqueous solution aggregate into micelles of large molecular mass (13). When added to a cell suspension, they either loosely associate with the cell surface, tightly attach to the cell membrane, or fuse with it in such a way that ganglioside monomers are inserted into the cell membrane (14,15).

There is evidence of the ability of gangliosides at micromolar concentrations to protect neuronal cells from the effects of oxidative stress (16). Recently, we have found that ganglioside micelles act as inhibitors of reactive oxygen species (ROS) generation in a cell-free system (17). To the best of our knowledge, no studies have been performed

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to examine the effect of ganglioside micelles on neutrophil production of superoxide anions. Therefore, the present study was designed to investigate how ganglioside micelles influence the kinetics of $O_2^{\cdot -}$ production by human polymorphonuclear neutrophils (PMN) activated by N-formyl-methionyl-leucyl-phenylalanine (fMLP), a receptor-dependent chemoattractant, and PMA, a receptor-bypassing agonist, a protein kinase C (PKC) activator (18,19). In general, these agonists promote NADPH oxidase assembly at the plasma membrane, leading to superoxide release primarily in the extracellular milieu. In this study, we used superoxide dismutase (SOD)-inhibitable ferricytochrome c reduction and electron paramagnetic resonance (EPR) spectroscopy to measure extracellular superoxide production.

Since micellar properties depend on the structural characteristics of gangliosides (20), we compared the effects of monosialoganglioside GM1 and trisialoganglioside GT1b on extracellular superoxide generation by PMN.

Material and Methods

Chemicals

Monosialoganglioside GM1, trisialoganglioside GT1b, fMLP, PMA, cytochrome c and dextran (MW = 400,000-500,000) were obtained from Sigma (USA). The spin trap, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO), was purchased from Calbiochem/Merck (Germany). Ficoll-Plaque was purchased from Pharmacia Biotech (Sweden). All other reagents used were laboratory-grade chemicals from Kemika (Croatia).

Isolation and activation of PMN

Leukocytes were isolated from freshly collected EDTA-anticoagulated peripheral blood of 11 healthy volunteers (6 men and 5 women). The study was approved by the Vuk Vrhovac University Clinic's Ethics Committee and written informed consent was obtained from the participating subjects. The cells were separated by sedimenting erythrocytes with 3% dextran in saline at room temperature for 45 min. The leukocyte-rich plasma was collected and centrifuged for 5 min at 300 *g*, and sedimented red cells were removed by hypotonic lysis. PMN were isolated on Ficoll-Paque according to Boyum (21), washed twice with Hank's balanced salt solution (HBSS, pH 7.4) and the pellet was resuspended in HBSS, modified by the deletion of calcium and magnesium ions. Ca-Mg-free buffer provided the best preparation since sample loss due to cell aggregation was markedly reduced (22). Moreover, as Ca^{2+} ions form a complex with the sialic acid from the terminal part of gangliosides (23), they had to be eliminated from this study.

For EPR measurements PMN were resuspended in chelex-pretreated phosphate buffer (PB, 0.1 M, pH 7.4) containing deferroxamine (2 mM/L) (17).

PMA was dissolved in dimethyl sulfoxide (stock solution), fMLP in HBSS (stock solution), and further dilutions to achieve experimental concentrations were prepared in either HBSS or phosphate-buffered saline (PBS) as indicated.

The amount of exogenous gangliosides bound by biological membranes depends on various parameters such as ganglioside concentration, temperature, cell type, divalent cations in the incubation medium, and duration of incubation, as indicated previously (14,15).

In our experiments gangliosides were dissolved in H_2O, and appropriate amounts of stock solution were added to the PMN suspension immediately preceding the addition of respiratory burst stimulators in cytochrome c and EPR measurements.

Measurement of osmolarity denoted that the isotonicity of the cell suspension was not changed by the addition of gangliosides. To establish whether they had an impact on superoxide formation, they were also added to PMN in the absence of the two stimulators, revealing no production of $O_2^{\cdot -}$. Spectroscopic measurements were performed at room temperature.

Cell viability was assessed using a lactate dehydrogenase (LDH) release assay. Treatment of cells was equal to that in the cytochrome c and EPR measurements. Disruption of the cell membrane with Triton X-100 was used as a positive control. No difference in LDH release between the control untreated cells and ganglioside-treated cells after stimulation with PMA was observed.

Assessment of superoxide radical formation by cytochrome c reduction

Extracellular production of superoxide anion was measured by SOD-inhibitable reduction of ferricytochrome c (18,24). Briefly, human peripheral neutrophils (1 x 10^6/mL) were suspended in HBSS containing 80 µM ferricytochrome c, to which the activators fMLP and PMA were added at concentrations of 100 and 162 nM, respectively, to obtain a maximal response of neutrophils (18). The assay was performed in the presence and absence of SOD (90 U/mL). Gangliosides were added to the neutrophil suspension prior to the addition of the activators. The rate of SOD-inhibitable reduction of cytochrome c was measured continuously by recording the increase in absorption at 550 nm using a Pye Unicam SP-8 (UK) spectrophotometer. The amount of superoxide $O_2^{\cdot -}$ was calculated using the molar extinction coefficient of 2.1 x 10^4 cm^{-2} mM^{-1}. Results are reported as nM $O_2^{\cdot -}$ per 10^6 cells.

Assessment of superoxide radical formation by EPR spectroscopy

PMN (5 x 10^6 cells/mL) containing the DEPMPO spin trap (20 mM/L) were exposed to PMA at a concentration of 320 nM to obtain maximally activated PMN (25). The measurements were performed in the glass capillary tubes (inner diameter of 1 mm) using an X-band Varian E-109 spectrometer. Data were collected at room temperature using the software supplied by the manufacturer (26) with the following EPR spectrometer

settings: microwave power, 20 mW, modulation amplitude, 0.1 mT, modulation frequency, 100 kHz. The concentration of superoxide spin adduct formed upon PMN stimulation was estimated from the comparison of spectral intensity with Fremy's salt solution as a standard (27).

Statistical analysis

Data are reported as means ± SEM. The effects of different ganglioside concentrations were analyzed by the Student *t*-test. Multiple between-group comparisons were carried out by means of ANOVA, whereas *post hoc* analysis of differences was performed using the Scheffé test. P values <0.05 were considered to be statistically significant.

Results

Extracellular $O_2^{\cdot-}$ production was investigated by two different experimental methods: SOD-inhibitable cytochrome c reduction and EPR spectroscopy. The time course of extracellular superoxide generation by fMLP-stimulated PMN measured by SOD-inhibitable cytochrome c reduction is shown in Figure 1A. The chemotactic peptide fMLP induced a rapid increase in the production of $O_2^{\cdot-}$, which reached its maximum after several minutes. The presence of GM1 or GT1b gangliosides (100 µM) had no influence on any aspect of extracellular $O_2^{\cdot-}$ production. Similarly, in EPR spectroscopy with the DEPMPO spin trap, no significant difference in time course of DEPMPO-OOH signal detection was observed in the presence of GM1 and GT1b gangliosides (Figure 1B).

On the other hand, a short lag time of 0.3-3 min preceding the increase in absorbance, indicative of an extracellular $O_2^{\cdot-}$ production measured by cytochrome c reduction, was observed in PMA-activated PMN, in agreement with literature data (18,24). The concentration-dependent experiments were conducted to investigate the effect of GM1 and GT1b at concentrations below and above their cmc [(2 ± 1) x 10^{-8} M and (1 ± 0.5) x 10^{-5} M, respectively, at pH 7.4 and 20°C] (28) on the lag time of superoxide anion generation measured by cytochrome c reduction. No difference in the onset of superoxide production in the presence of either GM1 or GT1b could be observed compared with the control samples containing no gangliosides at low concentrations (0.01-0.1 and 0.01-5 µM for GM1 and GT1b, respectively). A significant increase in lag time, however, occurred in the presence of ganglioside concentrations higher than 0.5 µM GM1 ($P < 0.01$) and 5 µM GT1b ($P < 0.001$) compared to neutrophils without gangliosides (Figure 2A and B). Further experiments were carried out using the concentration of 100 µM GM1 and GT1b.

Time-dependent superoxide generation by PMA-stimulated PMN (1 x 10^6/mL) in the presence of 100 µM GM1 and GT1b detected by cytochrome c reduction is shown in Figure 3. In the presence of both gangliosides, the onset of $O_2^{\cdot-}$ generation compared to untreated cells

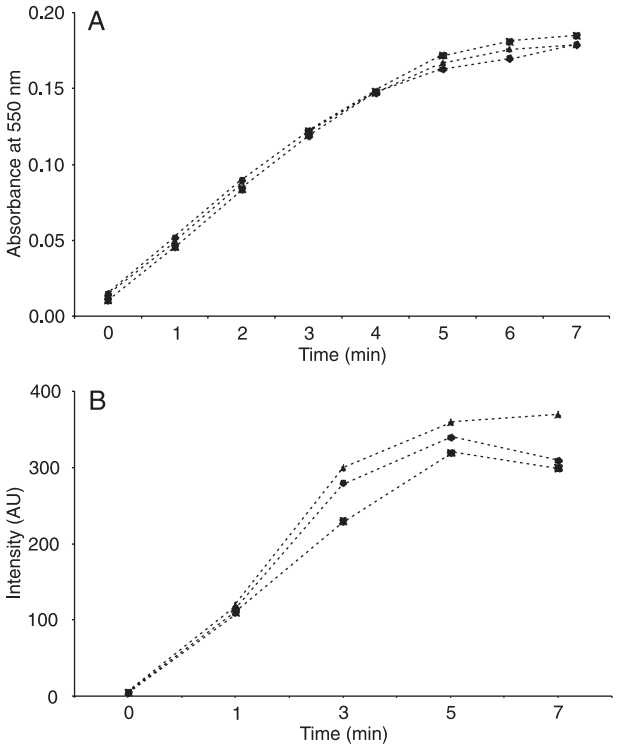

Figure 1. Time course of extracellular superoxide anion generation by PMN stimulated with fMLP in the absence (filled circles) and presence of 100 µM GM1 (filled squares) and GT1b (filled triangles) and detected by (A) cytochrome c reduction assay and (B) EPR spectroscopy in the presence of the DEPMPO spin trap. PMN = polymorphonuclear neutrophils; fMLP = N-formyl-methionyl-leucyl-phenylalanine; EPR = electron paramagnetic resonance; DEPMPO = 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide; AU = arbitrary units.

was significantly delayed (12.6 ± 0.9 and 18.2 ± 1.2 min for GM1 and GT1b, respectively, compared to 1.6 ± 0.2 min in the untreated cells; $P < 0.0001$); moreover, a significant difference in the onset of $O_2^{\cdot-}$ production between GM1 and GT1b was observed ($P < 0.01$). However, maximum absorbance, indicating maximal PMA-induced superoxide anion release, was the same in the presence and in the absence of gangliosides.

The data showed that exogenously added gangliosides influenced the first phase of the PMA-induced response, whereas the total extracellular $O_2^{\cdot-}$ production was not affected.

The effect of the washing of PMN after exposure to gangliosides on the onset of PMA-activated PMN response was tested in separate experiments. After washings and adjustment of cells to their original count, a gradual decline of lag time was observed compared to the unwashed cells. The lag time was reduced by 43 ± 3.3 and 73 ± 1.2% (N = 3) after one and two washings, respectively, indicating that loosely adhering ganglioside micelles could be removed (Figure 4).

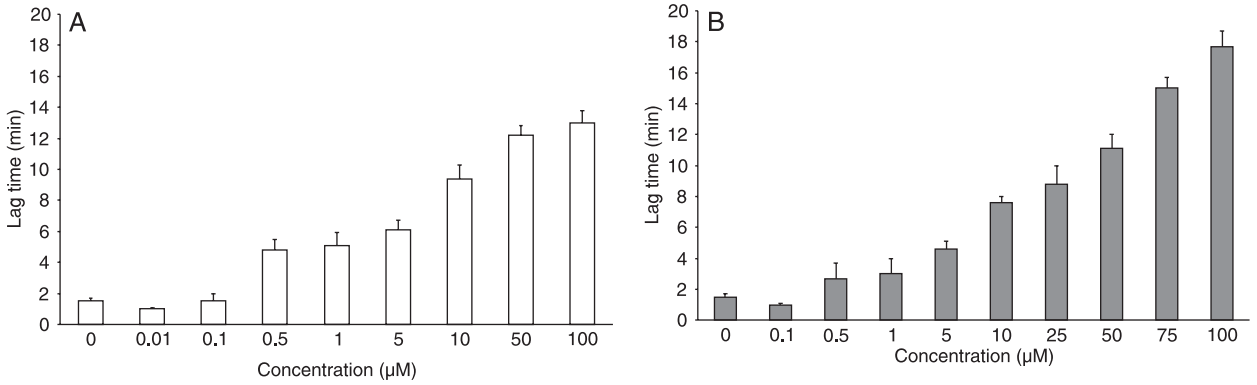

Figure 2. Lag time of the onset of superoxide anion generation by PMN (1 x 10^6/mL) stimulated by PMA in the absence and presence of different concentrations of GM1 (A, open columns) and GT1b (B, filled columns) as detected by the cytochrome c reduction assay. Data are reported as means ± SEM. A significant increase in lag time (min) occurs at the point where gangliosides reach concentrations higher than 0.5 μM GM1 ($P < 0.01$) and 5 μM GT1b ($P < 0.001$) with respect to neutrophils without gangliosides. PMN = polymorphonuclear neutrophils; PMA = phorbol 12-myristate 13-acetate.

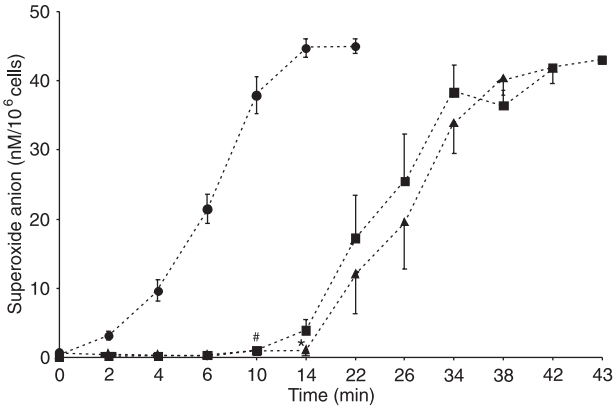

Figure 3. Time course of superoxide anion production by PMA-stimulated PMN (1 x 10^6/mL) in the absence (filled circles) or presence (filled squares) of 100 μM GM1 and GT1b (filled triangles) detected by the cytochrome c reduction assay. Data are reported as means ± SEM of six experiments. PMA = phorbol 12-myristate 13-acetate; PMN = polymorphonuclear neutrophils. #$P < 0.0001$ for GM1 compared to untreated cells; *$P < 0.0001$ for GT1b compared to untreated cells (one-way ANOVA).

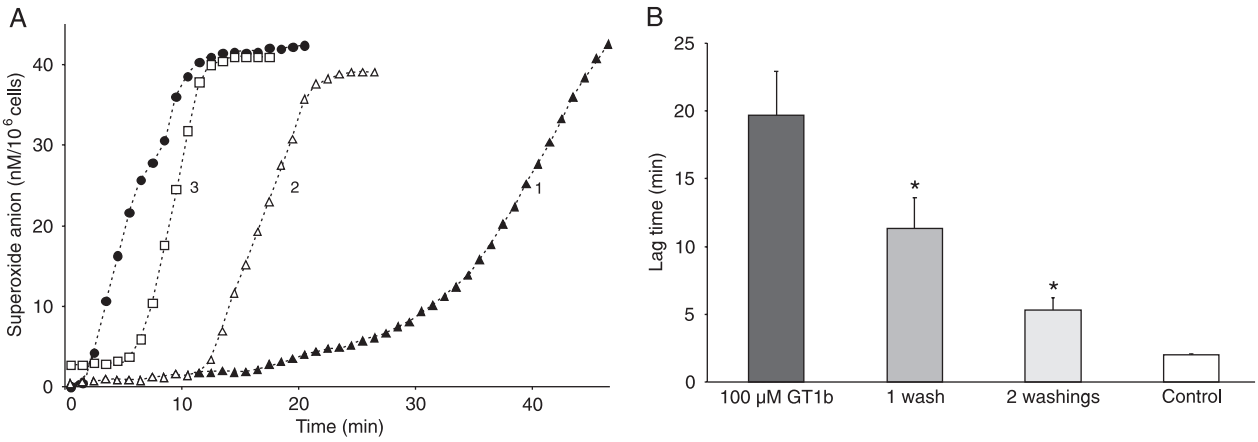

Figure 4. *A*, Time course of superoxide anion production by PMA-stimulated PMN (1 x 10^6/mL) in the presence of 100 μM GT1b (curve 1, filled triangles) and in cell aliquots after one (curve 2, open triangles) and two (curve 3, open squares) washings compared to the control sample without exogenously added gangliosides (filled circles). *B*, Lag time (means ± SEM of three experiments) of superoxide anion production by PMA-stimulated PMN (1 x 10^6/mL) in the presence of 100 μM GT1b, and in cell aliquots after one and two washings compared to control samples without gangliosides. PMA = phorbol 12-myristate 13-acetate; PMN = polymorphonuclear neutrophils. *$P < 0.05$ in washed cells compared to unwashed cells (Student *t*-test).

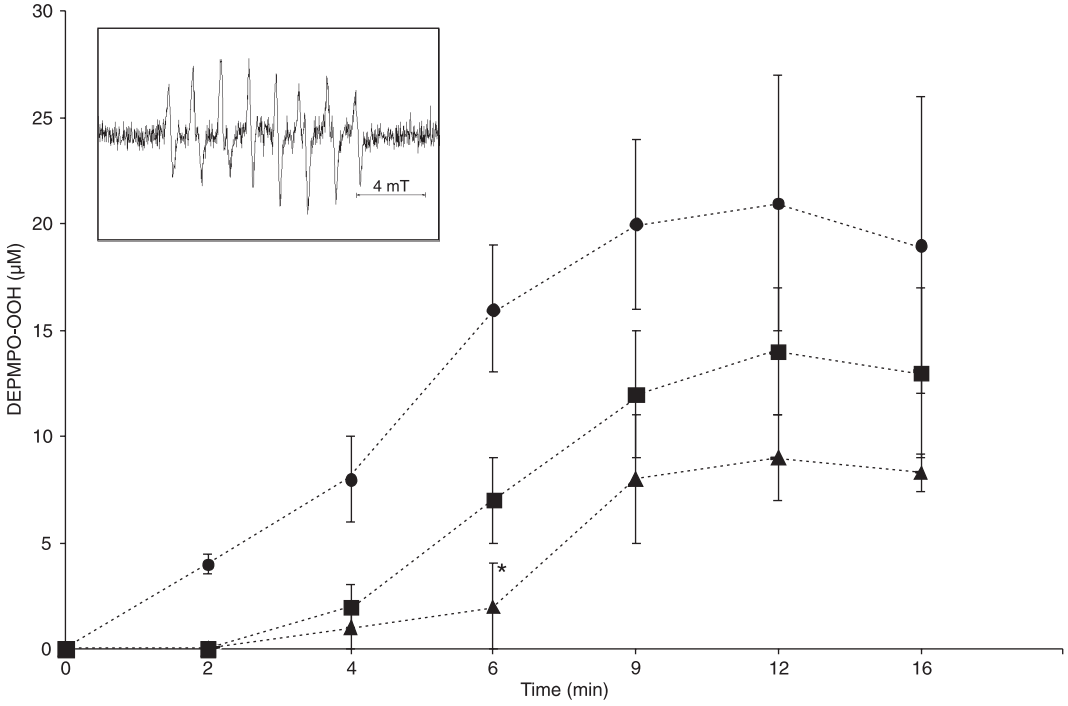

Figure 5. Influence of GT1b and GM1 micelles on the onset of superoxide production by PMA-activated PMN (5 x 10^6/mL) detected by EPR spectroscopy in the presence of the DEPMPO spin trap. The time-dependent superoxide anion generation by PMA-stimulated PMN in the absence (filled circles) or presence (filled squares) of 100 μM GM1 and GT1b (filled triangles). Data represent means ± SEM of five experiments. The typical experimental spectrum of the DEPMPO-OOH spin adduct is shown in the inset. PMA = phorbol 12-myristate 13-acetate; PMN = polymorphonuclear neutrophils; DEPMPO = 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide; DEPMPO-OOH = DEPMPO superoxide adduct. *P < 0.005 with GT1b compared to untreated cells (one-way ANOVA).

EPR spectroscopy

In order to demonstrate the presence of extracellular superoxide radicals in stimulated neutrophils, EPR spectroscopy with the DEPMPO spin trap was applied. The experimental spectra of PMN stimulated by PMA are typical of DEPMPO-OOH adduct formation (25) (Figure 5, inset). Since in the presence of SOD, which cannot penetrate the cell membrane (29), no EPR spectra could be measured, it can be assumed that the observed DEPMPO-OOH was due to the trapping of extracellular superoxide anion (25). The time course of superoxide spin adduct formation indicated again a short delay in the onset of superoxide generation when PMN were activated by PMA. However, when PMN were exposed to 100 μM GM1 or GT1b the formation of DEPMPO-OOH was additionally delayed with respect to the samples without gangliosides (Figure 5). This phenomenon was statistically significant in the presence of 100 and 200 μM GT1b (5.4 ± 1.2 and 11.5 ± 1.7 min, respectively; P < 0.005), while in the presence of 200 μM GM1 a tendency towards an increase in lag time (7.7 ± 1.9 min; P < 0.52) was observed in comparison with 0.7 ± 0.4 min in the untreated cells. In contrast to data obtained by cytochrome c reduction, the effect of gangliosides on the total ROS generation could not be quantitatively evaluated in EPR experiments due to the complex decay of spin adduct within the time course of the measurements (25). Therefore, only the delay in the onset of superoxide production in the presence of gangliosides was considered to be relevant.

Discussion

Exogenously added gangliosides have been involved in a variety of cell properties and biological events. In this study, we investigated the impact of micelles of two different types of gangliosides on extracellular superoxide production by activated human PMN detected by cytochrome c reduction assay and EPR spectroscopy. To activate PMN we used fMLP and PMA, each with different pathways but both resulting in the activation of NADPH oxidase. The peptide fMLP exerts its effect via receptor-dependent multiple signaling pathways, including lipid kinases, production of second messengers by various phospholipases, and the activation of PKC. This activation usually results in rapid $O_2^{\cdot-}$ production, whereas activation by PMA, a receptor-independent activator, causes prolonged generation of $O_2^{\cdot-}$ until necessary substrates and cofactors are depleted (30).

The delay in the generation of $O_2^{\cdot -}$ that we observed only in PMA-activated PMN in the presence of gangliosides, as opposed to the unchanged $O_2^{\cdot -}$ generation using fMLP, was probably due to different mechanisms of the activator used. It may be presumed that ganglioside micelles do not interfere with fMLP binding to its receptors. This indicates the importance of membrane involvement in the PMN activation by PMA, as the activator bypassing receptor-mediated signal pathways diffuses through the cell membrane to activate a PKC-mediated cascade of events leading to the production of superoxide. Therefore, it cannot be excluded that ganglioside micelles sterically interfere with PMA stimulation across the cell membrane. In this context, by adhering to the cell surface, gangliosides might effectively increase the diffusion barrier for a stimulant responsible for triggering membrane events, and thus induce a delay in superoxide production. It should be emphasized that, despite this delay, the total ROS generating capacity of PMA-activated PMN was not influenced by the presence of ganglioside micelles as measured by cytochrome c reduction.

Exogenously supplied ganglioside micelles have mainly been described to bind loosely to the cultured cell surface (13,14,31), in contrast to ganglioside monomers that fuse with the membrane (8,10). Our experimental data also point to a loose association, as we were able to almost completely remove exogenously added ganglioside micelles by repeated washings of PMN.

In the presence of higher concentrations of ganglioside micelles at the cell surface the delay of superoxide generation was found to be more pronounced, with trisialoganglioside GT1b at a concentration of 100 µM exhibiting a more significant effect than GM1.

The structural properties of ganglioside micelles, which are mainly governed by the respective hydrophilic parts of these amphiphilic compounds, could provide an explanation for the observed phenomena. GM1 has five sugar rings and only one carboxylic group, whereas GT1b has seven sugar rings and three sialic acid residues. As a consequence, GT1b micelles exhibit a higher negative charge due to a higher sialic acid content compared to GM1. Because of a greater hydrophilic repulsive contribution and a larger number of hydrogen-bonding groups, GT1b molecules are prone to form smaller and more spherical aggregates that exhibit a two times lower aggregation number (176) than GM1 (301) (3). The difference in their respective surface curvatures has been evidenced in their mean hydrodynamic diameters (13), which we have found to be lower in GT1b micelles (9 nm) than in GM1 micelles (11 nm) (17). Based on the reports from the literature pointing to the importance of GM1 and GT1b headgroups in conformational properties of aggregate surfaces (32), the more pronounced modulation of superoxide production observed in the presence of GT1b micelles compared to those of GM1 can be ascribed to the better steric shielding from PMA uptake by PMN when smaller GT1b aggregates adhere to the cell surface.

The idea that ganglioside micelles could be responsible for the increased diffusion barrier across the cell membrane is in agreement with our previous findings that exogenous GT1b acts as an inhibitor of hydrogen peroxide diffusion across the sperm membrane (33).

Gangliosides are known to be present in increased concentrations in different diseases, especially neoplasms, due to their overexpression and shedding from the cell surface (34-36). Evidence suggests that tumor-derived gangliosides influence specific aspects of the immune response (34). In comparison with the physiological levels of gangliosides in healthy individuals (10 nM), the levels of serum-shed gangliosides, such as those in neuroblastomas, have been found to be 10-50 times higher (37,38). In our study, the effect of exogenous gangliosides on PMN production of oxygen radicals occurred at ganglioside concentrations higher than the cmc. Tumor-derived gangliosides form micelles depending on their structure; the greater the hydrophobicity, the more shed gangliosides in micellar form (38). Although gangliosides in their natural states are known to mainly exist in the form of micelles (31,38), some studies have reported on their association with serum lipoproteins (39,40). Our results on the effect of ganglioside micelles on the *in vitro* oxygen radical production by PMN point to their possible relevance in *in vivo* conditions. Further studies are required to determine whether gangliosides, either in their free micellar form or bound to lipoproteins, are able to provide micellar coverage of leukocytes, thus modulating their superoxide anion production.

The attachment of ganglioside micelles to the surface of the cell increases the membrane diffusion barrier for the signal translocation to the interior of the cell. This study shows that exogenous GM1 and GT1b micelles, depending on their concentrations and structure, delay the onset but not the total extracellular production of superoxide radicals in PMA-activated PMN detected by the cytochrome c reduction method.

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