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C_2-ceramide and C_6-ceramide inhibited priming for enhanced release of superoxide in monocytes, but had no effect on the killing of leukaemic cells by monocytes

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SUMMARY

Ceramide acts as an intracellular second messenger in cellular signal transduction. We examined the effects of two cell-permeable ceramides, C_2-ceramide and C_6-ceramide, on human monocyte functions. After monocytes were primed with lipopolysaccharide (LPS) or interferon-γ (IFN-γ) for 18 hr in suspension culture, they produced a high amount of superoxide (O_2^-) when triggered by phorbol myristate acetate. C_2- or C_6-ceramide inhibited O_2^- release from monocytes primed with LPS (1 ng/ml) or IFN-γ (100 U/ml), but did not affect unprimed monocytes. An analogue, C_2-dihydroceramide, was inactive. C_2-ceramide was most effective at 6 μM, and C_6-ceramide at 60 μM. C_2- or C_6-ceramide at these concentrations was not toxic for monocytes, as assessed by trypan blue exclusion and by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay which measures the ability of live cells to produce formazan. C_2-ceramide (20 μM) had no effect on the killing of leukaemic cells (HL-60 and K562 cells) by monocytes treated with IFN-γ, LPS, or both for 18 hr, with killing assessed by an ^{111}Indium-releasing assay. C_2-ceramide (20 μM) induced secretion of low amounts of tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) from the monocytes. But C_2-ceramide did not alter the higher secretion of TNF-α or IL-1β from monocytes treated with IFN-γ or LPS. Thus, the cell-permeable ceramides acted like antagonists of LPS, rather than analogues of LPS, as has been proposed. The results here showed that the signal transduction pathway for O_2^- release by monocytes differed from that for the cytolysis of leukaemic cells, and confirmed that oxygen radicals are not involved in cytolysis.

INTRODUCTION

Macrophages play an important role in host defence against cancer[1] and infections.[2,3] Blood monocytes or tissue macrophages can be activated by lymphokines such as interferon-γ (IFN-γ), by bacterial products like lipopolysaccharide (LPS), or by both, to become tumoricidal.[1] Several substances, including IFN-γ,[4] bacterial products,[5] tumour necrosis factor-α (TNF-α),[6] and platelet–activating factor (PAF)[7] prime monocytes or neutrophils for increased release of microbicidal oxygen radicals like superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Primed monocytes release increased amounts of oxygen radicals when they are triggered by phorbol myristate acetate or the bacterial peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). These priming agents elicit a variety of biochemical reactions in the cells, including Ca^{2+} mobilization,[8] alteration of arachidonic acid metabolism,[9] activation of protein kinase C,[10] and protein tyrosine phosphorylation.[11,12] But the biochemical nature of priming or activation is not completely understood. We recently demonstrated in human monocyte-derived macrophages that the serine protease inhibitor, 4-(2-aminoethyl)-benzene-sulphonyl fluoride (AEBSF), inhibited both priming for enhanced O_2^- release[13] and killing of leukaemic cells.[14] These results suggest that a serine protease is involved in priming or activation of human monocytes/macrophages for both microbicidal activity and tumoricidal activity.

Recently, ceramide has been appreciated as a second messenger in signal transduction of TNF-α, interleukin-1β (IL-1β), and IFN-γ. Ceramide is made from membrane sphingomyelin by sphingomyelinase.[15] There is a structural similarity between a portion of the lipid A region of LPS and ceramide.[16] Cell-permeable ceramide enhanced expression of LPS-inducible genes in murine LPS-responsive macrophages but not in LPS-hyporesponsive macrophages.[17] Therefore, ceramide might activate murine macrophages via a signal transduction pathway shared with LPS. But in other studies, cell-permeable ceramide inhibited oxygen radical release from human neutrophils,[18,19] an effect opposite to that of LPS. These results prompted us to examine the effects of ceramides on three human monocyte functions: O_2^- release, cytolysis of leukaemic cells, and secretion of the cytokines IL-1β and TNF-α.

MATERIALS AND METHODS

Reagents

C_2-ceramide (*N*-acetylsphingosine), C_6-ceramide (*N*-hexanoyl-sphingosine), phorbol myristate acetate (PMA), and cytochrome *c* were purchased from Sigma Chemical (St Louis,

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MO). C_2-dihydroceramide, an inactive analogue that is missing an essential double bond, and C_{16}-ceramide (*N*-palmitoylsphingosine) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Superoxide dismutase was purchased from Diagnostic Data Inc. (Mountain View, CA). Stock solutions of ceramide were made up in dimethyl sulphoxide (DMSO) and stored at −20°; when added to aqueous reaction mixtures, the final concentration of DMSO did not exceed 0·12%. Brief sonication was needed to dissolve C_6-ceramide and C_{16}-ceramide stock solutions in modified Earle's solution (0·9 mM NaH_2PO_4, 14·9 mM $NaHCO_3$, 115 mM NaCl, 0·3 mM KCl, 1·36 mM $CaCl_2$, 0·8 mM $MgSO_4$, 0·1% glucose, pH 7·3). PMA was dissolved in DMSO at 1 mM and stored at −20°. LPS, purified from *Escherichia coli* K235 by phenol extraction and gel filtration chromatography in the presence of deoxycholate, was a gift of F. C. McIntire of the University of Colorado, Denver, CO. LPS was dissolved in sterile pyrogen-free water to make a stock solution of 10 μg/ml and stored at −20°. Recombinant human interferon-γ (IFN-γ, $1·0 \times 10^7$ U/mg) was purchased from Becton Dickinson (Bedford, MA). All reagents that we used, except LPS, were LPS-free, as assessed by the *Limulus* test.

Monocyte isolation

Monocytes were separated from buffy coats (Life Blood Mid-South Regional Center, Memphis, TN) as described previously.[14] Briefly, mononuclear cells were isolated from buffy coats by dextran sedimentation (Calbiochem, La Jolla, CA) and Histopaque gradients (Sigma). The harvested mononuclear cells were then loaded into counterflow centrifugal elutriation. The monocyte rich fraction was obtained at a speed of 2020 r.p.m. (400 *g*) and flow rate of 16–24 ml/min. The purity of monocytes at this step was more than 80%, as assessed by non-specific esterase staining.

Superoxide O_2^- *assay*

Release of O_2^- from monocytes was measured as superoxide dismutase-inhibitable reduction of cytochrome *c* as described previously.[13] Collected monocytes were incubated in LPS-free modified Earle's solution. Monocytes were suspended at 5×10^5 cells/ml and cultured in Teflon bags in a 5% CO_2 incubator for 18 hr with or without LPS (1 ng/ml) or IFN-γ (100 U/ml) in the presence or absence of ceramide. After 18 hr incubation, cytochrome *c* (80 μM) and PMA (1 μM) were added to the monocytes for 40 min, then the cultures were put on ice, centrifuged and the supernatants were transferred to cuvettes for spectrophotometric analysis. The peak of absorbance at 550 nm was compared to isosbestic points at 542 and 556 nm. The differential extinction coefficient $\Delta\varepsilon = 0·021\ \mu M^{-1}$ for reduced versus oxidized cytochrome *c* was used to calculate the amount of O_2^- released. In selected experiments, we confirmed that reduction of cytochrome *c* was inhibited completely by addition of superoxide dismutase (10 μg/ml).

Monocyte culture for cytotoxicity assay

The monocytes were suspended in RPMI-1640 (Life Technologies, Grand Island, NY) containing 5% human heat-inactivated AB serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma) (complete medium) at a concentration of 2×10^6/ml. The monocytes (2×10^5/well) were plated in 96-well microplates (Falcon 3072, Becton Dickinson, Franklin Lakes, NJ). After 2 hr incubation in a 5% CO_2 incubator, the plates were washed to eliminate non-adherent cells. After washing, more than 98% of the adherent cells were monocytes as determined by non-specific esterase stain. The monocytes were treated with IFN-γ (100 U/ml), LPS (5 ng/ml), or both, in the presence or absence of ceramide for 18 hr. After 18 hr incubation, monocyte viability was assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay as described previously.[14] Briefly, 10 μl MTT (5 mg/ml, Sigma) was added to each well for 4 hr. After formation of formazan crystals, the culture supernatants were aspirated from the wells. The formazan crystals were dissolved in 150 μl/well DMSO with the addition of 25 μl/well glycine (0·1 M, Sigma). The absorbance was measured at 550 nm using a microplate spectrophotometer. Only live cells produce formazan.

Leukaemic cells

HL-60 and K562 cell lines were provided by the American Type Culture Collection, Rockville, MD. These leukaemic cells were maintained in complete medium and passaged three times weekly. Mycoplasma contamination was checked and no contamination was found.

Cytotoxicity assay

After treatment of monocytes with IFN-γ, LPS, or both, in the presence or absence of ceramide for 18 hr, the monocyte-mediated cytotoxicity assay was performed as previously described.[14] Briefly, 3×10^6 target leukaemic cells were labelled with ^{111}In-labelled oxine (Amersham Healthcare Medi-physics Inc., Memphis, TN) (40 μCi) for 15 min at room temperature. Labelled target cells (5×10^3) were added to the monocytes in the microplates after monocyte medium was replaced with fresh complete medium. The total volume was 200 μl. The effector : target ratio was 40 : 1. After 48 hr incubation in a 5% CO_2 incubator, the microplates were centrifuged at 250 *g* for 5 min. Supernatants (50 μl) were collected to measure their radioactivity. The specific cytotoxic activity was calculated as follows:

$$\frac{\text{experimental c.p.m.} - \text{spontaneous c.p.m.}}{\text{maximum c.p.m.} - \text{spontaneous c.p.m.}} \times 100\%.$$

The maximum release was that obtained from target cells exposed to 1% sodium dodecyl sulphate (SDS; Sigma). The spontaneous release was that obtained from target cells cultured in monocyte-free medium. The spontaneous release was less than 15% of the maximum release. Each cytotoxicity assay was performed with triplicate cultures.

TNF-α and IL-1β determination

TNF-α or IL-1β content of the culture supernatants was assessed by ELISA as described previously.[14] Briefly, culture supernatants were harvested from the monocytes cultured in the same microplates used for the cytotoxicity assay. The supernatants were assayed for TNF-α and IL-1β activity, using a TNF-α-specific ELISA from BioSource (Camarillo, CA), and IL-1β-specific ELISA from Cistron (Pine Brook, NJ). The supernatants were assayed in duplicate.

RESULTS

C_2-ceramide inhibited priming of monocytes by LPS or IFN-γ for enhanced O_2^- release

Unprimed monocytes cultured in Teflon bags for 18 hr released 15 nmol of O_2^- from 10^6 monocytes when triggered with PMA (1 μM) for 40 min (Fig. 1). When the monocytes were primed by addition of LPS or IFN-γ on the day of cell isolation, monocytes produced a high amount of O_2^- (55–65 nmol/10^6 monocytes). We examined whether C_2-ceramide affected production of O_2^- by monocytes. C_2-ceramide, C_2-dihydroceramide (an inactive analogue),[20] or DMSO (vehicle) was added to primed or unprimed monocytes at the beginning of cultures. LPS and IFN-γ significantly increased PMA-triggered O_2^- release. C_2-ceramide, but not C_2-dihydroceramide or DMSO, almost completely inhibited the effect of LPS or IFN-γ on O_2^- release by monocytes. C_2-ceramide reduced the response of monocytes primed with LPS or IFN-γ to the level of O_2^- release shown by unprimed monocytes (Fig. 1). There were no significant differences by ANOVA between unprimed monocytes with no primer and primed monocytes that had been exposed to C_2-ceramide. The inactive analogue had no significant effect on LPS priming, and only a slight effect on IFN-γ priming. Also noteworthy was the result that C_2-ceramide had no effect on unprimed cells.

The concentration of C_2-ceramide needed for inhibition of O_2^- production by primed monocytes was in the range of 2–6 μM. C_2-ceramide at a concentration of 6 μM almost completely inhibited the increase in O_2^- release by monocytes primed with LPS (Fig. 2 upper).

Another cell-permeable ceramide, C_6-ceramide, also inhibited priming for enhanced O_2^- release in monocytes primed with LPS. C_6-ceramide was active in the range of 30–60 μM (Fig. 2, lower). In these experiments, DMSO, which

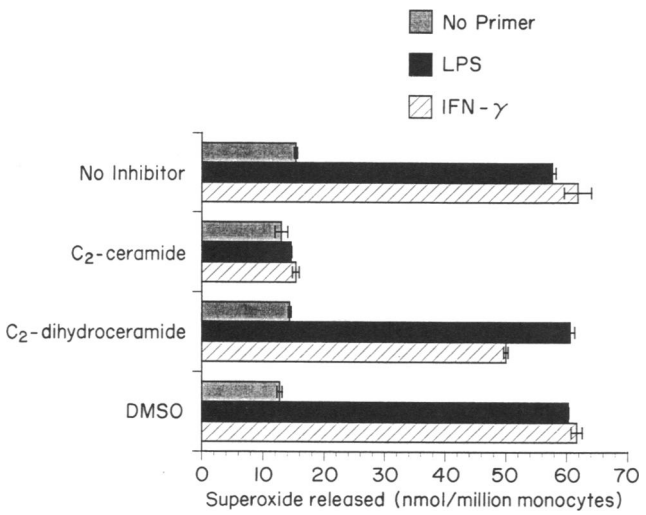

Figure 1. Effect of C_2-ceramide on priming for enhanced O_2^- release from monocytes. Monocytes were unprimed or primed with LPS (1 ng/ml) or IFN-γ (100 U/ml) for 18 hr. Where indicated, C_2-ceramide (6 μM), C_2-dihydroceramide (6 μM), or DMSO (0·02%) was added to some cultures at the same time. After 18 hr incubation, PMA-triggered O_2^- release was determined. Results are means ± SEM for duplicate cultures. This experiment is representative of three performed.

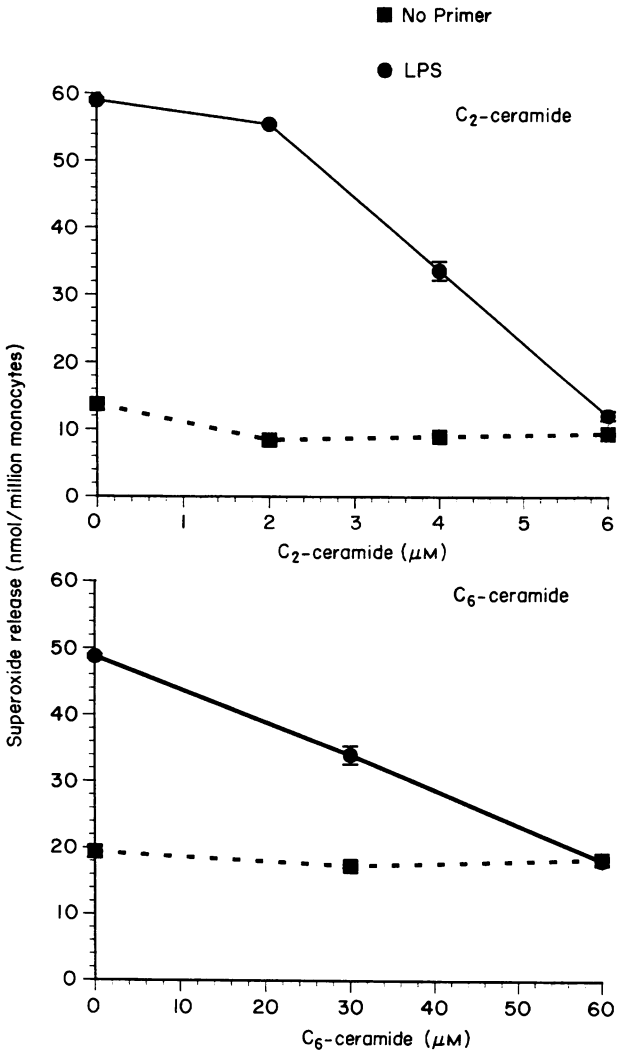

Figure 2. Effect of various concentrations of C_2-ceramide (upper) or C_6-ceramide (lower) on priming for enhanced O_2^- release from monocytes. Monocytes were unprimed or primed with LPS (1 ng/ml) for 18 hr. C_2-ceramide or C_6-ceramide was added to monocytes at the beginning of culture. After 18 hr culture, PMA-triggered O_2^- release was determined. Results are means ± SEM for duplicate cultures. This experiment is representative of two performed.

was used to make the stock solutions of the ceramides, had no effect.

C_{16}-ceramide (20–40 μM), the natural form of ceramide in cell membranes, did not inhibit O_2^- production by monocytes primed with LPS (1 ng/ml) (data not shown). We did not test concentrations higher than 40 μM, because C_{16}-ceramide at higher concentrations did not dissolve in modified Earle's solution.

C_2-ceramide at 6 μM or C_6-ceramide at 60 μM did not interfere with the viability of the monocytes treated with or without LPS, as assessed by trypan blue exclusion assay. All cultures showed >90% viable cells. In addition, as shown below, C_2-ceramide did not interfere with synthesis and secretion of cytokines by these cells, indicating that the cells were not damaged.

C_2-ceramide inhibited priming, but not triggering, of O_2^- production by monocytes. We considered the possibility that

C_2-ceramide inhibited O_2^- production by inhibiting triggering by PMA. However, C_2-ceramide had no effect on unprimed cells, which showed normal triggering. We tried adding C_2-ceramide to monocyte cultures shortly before the addition of PMA, after priming had already occurred. C_2-ceramide had no effect on O_2^- production by primed monocytes when it was added approximately 5 min before PMA addition.

Lack of effect of C_2-ceramide on the killing of leukaemic cells by monocytes

A recent study showed that C_2-ceramide induced enhanced expression of LPS-inducible genes in murine macrophages.[17] So we examined the effect of C_2-ceramide on the killing of leukaemic cells (K562 and HL-60 cells) by human monocytes. Unexpectedly, C_2-ceramide (20 μM) did not activate human monocytes for enhanced cytolytic activity; nor did C_2-ceramide increase the cytolytic activity of monocytes primed with LPS (5 ng/ml), IFN-γ (100 U/ml), or both (Fig. 3). C_2-dihydroceramide (the inactive analogue) or DMSO also had no effect on the killing of leukaemic cells by monocytes (Fig. 3). No significant differences in killing were found by ANOVA, in the presence versus the absence of inhibitors, with either target leukaemic cell line.

We confirmed the viability of C_2-treated monocytes under these conditions of culture by performing the MTT assay, which measures the ability of live cells to produce formazan, a coloured product. Both the primers and C_2-ceramide significantly reduced the amount of formazan produced (absorbance at 550 nm of untreated cells, $0{\cdot}97 \pm 0{\cdot}01$; cells primed with LPS + IFN-γ, $0{\cdot}70 \pm 0{\cdot}04$; unprimed cells treated with 12·5 μM C_2-ceramide, $0{\cdot}85 \pm 0{\cdot}05$; primed cells treated with ceramide, $0{\cdot}70 \pm 0$; unprimed cells treated with inactive analogue, $0{\cdot}90 \pm 0{\cdot}06$; primed cells treated with analogue $0{\cdot}67 \pm 0{\cdot}01$; means $\pm$ SEM, $n = 3$). However, there were no significant differences between the effects of primers and C_2-ceramide, and no significant difference between C_2-ceramide and the inactive analogue C_2-dihydroceramide, by analysis of variance.

Effect of C_2-ceramide on cytokine production by monocytes

We measured TNF-α and IL-1β production from monocytes treated with or without LPS (5 ng/ml), IFN-γ (100 U/ml), or both, in the absence or presence of C_2-ceramide (20 μM) for 18 hr. C_2-ceramide alone induced TNF-α (295 ± 73 pg/ml) and IL-1β (945 ± 165 pg/ml) from monocytes (Fig. 4). C_2-dihydroceramide or DMSO had no effect (not shown). However, C_2-ceramide did not enhance or inhibit production of TNF-α or IL-1β by monocytes treated with LPS, IFN-γ, or both (Fig. 4). No significant differences in cytokine release in primed monocytes were found by analysis of variance, comparing monocytes in the presence and absence of C_2-ceramide.

DISCUSSION

We demonstrated that the cell-permeable ceramides, C_2-ceramide and C_6-ceramide, inhibited priming for enhanced O_2^- release by monocytes (Fig. 1 and 2), whereas an inactive analogue, C_2-dihydroceramide, did not. Therefore, inhibition of priming in monocytes is specific for ceramides which have a double bond in their structures.

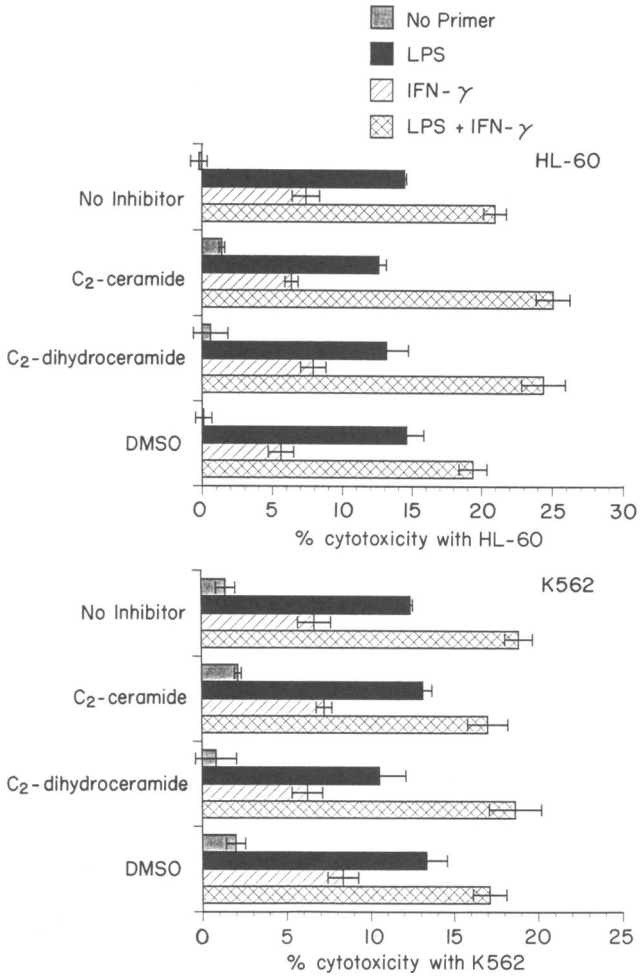

Figure 3. Effect of C_2-ceramide on lysis of leukaemic cells by monocytes. Monocytes were unprimed or primed with IFN-γ (100 U/ml) and/or LPS (5 ng/ml) for 18 hr. C_2-ceramide (20 μM), C_2-dihydroceramide (20 μM), or DMSO (0·07%) was added to the indicated monocyte cultures at the beginning of culture. After 18 hr, ^{111}In-labelled leukaemic cells (HL-60, upper; K562, lower) were added to the monocytes for a further 48 hr, and then release of radiolabel was assessed. Results are means $\pm$ SEM for triplicate cultures. This experiment is representative of three performed.

C_2-ceramide had no effect on unprimed monocytes (Fig. 1), and C_2-ceramide added just before PMA addition failed to inhibit O_2^- release by monocytes. These results indicated that ceramide inhibited priming for enhanced O_2^- release, but did not interfere with triggering by PMA. The mechanism by which C_2- and C_6-ceramide inhibited priming for PMA-triggered O_2^- release in monocytes remains to be established. However, this result is compatible with the evidence that O_2^- and H_2O_2 production in neutrophils is also inhibited by ceramide.[18,19] Without priming, neutrophils produce almost no O_2^-, so the neutrophils in these earlier experiments were probably primed by adherence or by endotoxin contamination, and it may have been this priming that was inhibited by ceramide.

Some ceramides have been shown to inhibit protein kinase C (PKC).[21] However, C_2-ceramide is thought not to affect PKC activity.[22] Wong *et al.* confirmed that C_2-ceramide did

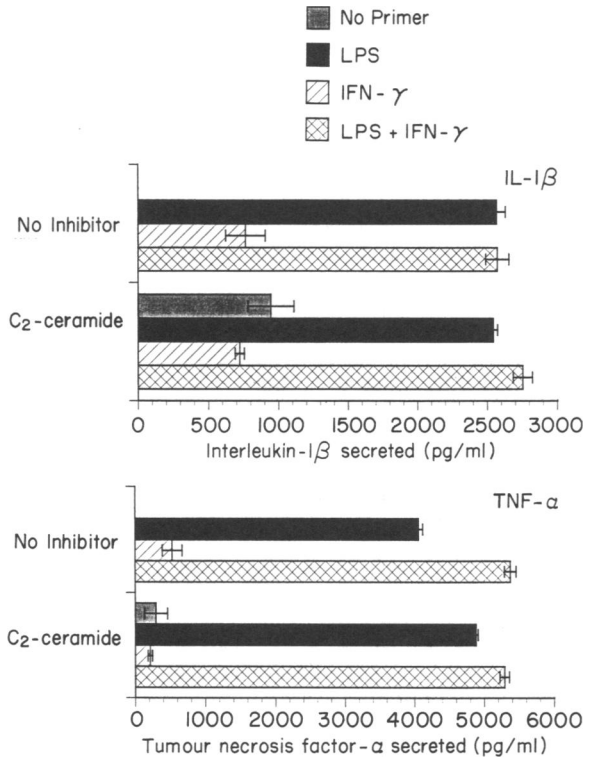

Figure 4. Effect of C_2-ceramide on cytokine production by monocytes. Monocytes were unprimed or primed with IFN-γ (100 U/ml) and/or LPS (5 ng/ml) for 18 hr. C_2-ceramide (20 μM), C_2-dihydroceramide (20 μM), or DMSO (0·07%) was simultaneously added to monocyte cultures. After 18 hr incubation, culture supernatants were assayed for TNF-α or IL-1β by enzyme-linked immunosorbent assay. Results are means $\pm$SEM for duplicate cultures. This experiment is representative of two performed.

not inhibit PKC in neutrophils. However, this group did demonstrate that C_2-ceramide inhibited Ca^{2+} influx in FMLP-activated neutrophils.[19] They concluded that inhibition of Ca^{2+} influx by ceramide caused reduced production of O_2^- in neutrophils. We showed earlier that priming for enhanced O_2^- release in neutrophils is controlled by the levels of cytosolic Ca^{2+}.[23]

Another possible explanation for inhibition of priming for enhanced O_2^- release by ceramide is that C_2-ceramide and C_6-ceramide might induce apoptosis in monocytes. C_2-ceramide is known to induce apoptosis in leukaemic cells,[24] and IL-4 inhibits human monocyte functions at least partly by inducing apoptosis.[25] However, in our study, C_2-ceramide was not cytotoxic for monocytes at the concentrations we used, as assessed by the trypan blue exclusion assay and the MTT assay. Thus, apoptosis seems an unlikely mechanism.

We recently demonstrated that the killing of leukaemic cells by human macrophages was inhibited by anti-TNF-α antibody but not by oxygen radical scavengers.[14] Therefore, TNF-α is involved in the killing of leukaemic cells by human macrophages, although the leukaemic cells that we used are resistant to recombinant TNF-α alone. Thus, additional factors, other than TNF-α, that are presently unknown, are involved in leukaemic cell lysis by human macrophages. The results here, that C_2-ceramide inhibited O_2^- release, but not TNF-α secretion or lysis of leukaemic cells, is consistent with our earlier results, which showed a role for TNF-α, but not O_2^-, in cytolysis. A potential complication in this area is that ceramide itself has a direct effect against tumour cells,[24] although we did not observe that effect here.

LPS has been proposed to act like an analogue of ceramide.[16] However, unlike LPS, C_2-ceramide inhibited priming for enhanced O_2^- release, and C_2-ceramide did not induce the killing of leukaemic cells by monocytes (Fig. 3). C_2-ceramide did induce synthesis and secretion of TNF-α and IL-1β in monocytes (Fig. 4), although the response was weak compared with the response to LPS. C_2-ceramide did not enhance TNF-α or IL-1β production from monocytes treated with IFN-γ and LPS (Fig. 4). Rather than act like an analogue of LPS, the cell-permeable ceramides might act like the inactive biosynthetic precursor of LPS, Lipid IVA. Lipid IVA is an incomplete partial structure of lipid A, which lacks two fatty acids.[26] Lipid IVA inhibits priming for enhanced O_2^- release.[26,27] In a similar way, C_2-ceramide and C_6-ceramide are incomplete partial structures of natural C_{16}-ceramide, because they lack a complete long-chain fatty acid. The cell-permeable ceramides might therefore act as antagonists, rather than agonists, of LPS-activated signal transduction pathways.

Because C_2-ceramide and C_6-ceramide inhibited priming for enhanced O_2^- release, but did not inhibit or enhance lysis of leukaemic cells, the results show that, in monocytes, the signal transduction pathway that regulates production of microbicidal oxygen radicals differs from the pathway that regulates cytolysis. The results also confirm that oxygen radicals are not involved in killing of leukaemic cells by monocytes.

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