Ceramide levels increase in activated polymorphonuclear neutrophils, and here we show that endogenous ceramide induced degranulation and superoxide generation and increased surface $\beta_2$-integrin expression. Ceramide accumulation reveals a bifurcation in integrin function, as it abolished agonist-induced adhesion to planar surfaces, yet had little effect on homotypic aggregation. We increased cellular ceramide content by treating polymorphonuclear neutrophils with sphingomyelinase C and controlled for loss of sphingomyelin by pretreatment with sphingomyelinase D to generate ceramide phosphate, which is not a substrate for sphingomyelinase C. Pretreatment with the latter enzyme blocked all the effects of sphingomyelinase C. Ceramide generation caused a Ca$^{2+}$ flux and complete degranulation of both primary and secondary granules and increased surface $\beta_2$-integrin expression. These integrins were in a nonfunctional state, and subsequent activation with platelet-activating factor or formyl-methionyl-leucyl-phenylalanine induced $\beta_2$-integrin-dependent homotypic aggregation. However, these cells were completely unable to adhere to surfaces via $\beta_2$-integrins. This was not due to a defect in the integrins themselves because the active conformation could be achieved by cation switching. Rather, ceramide affected cytoskeletal organization and inside-out signaling, leading to affinity maturation. Cytochalasin D induced the same disparity between aggregation and surface adhesion. We conclude that ceramide affects F-actin rearrangement, leading to massive degranulation, and reveals differences in $\beta_2$-integrin-mediated adhesive events.

Adhesion of PMNs to vessel walls and their subsequent emigration from the vasculature depend on the heterodimeric adhesion protein $\alpha_{\text{v}}\beta_2$-integrin (MAC-1 or CD11b/CD18), one of four members of the $\beta_2$-integrin (CD18 surface antigen) family. $\beta_2$-Integrins are expressed on resting PMNs, but in a low affinity state (1–3) that does not support adhesion. The $\beta_2$-integrin constitutively expressed on the cell surface can be activated by an incompletely defined process of inside-out signaling (4, 5). The complement of surface $\beta_2$-integrin is also quantitatively augmented by translocation of $\alpha_{\text{v}}\beta_2$ and $\alpha_8\beta_2$ (6) from specialized intracellular granules to the cell surface of activated cells (7, 8), but it is the activation of the constitutively expressed integrin, rather than the newly recruited $\beta_2$-integrin, that is required for PMN aggregation (9). Sequentially increasing agonist concentrations shows that the newly recruited integrin is activated separately from the constitutively expressed integrin and that a subsequent stimulus is needed to promote this $\beta_2$-integrin to an active conformation (10).

$\beta_2$-Integrin function is also regulated by clustering, which strengthens adhesive interactions (5). Microscopy shows the $\beta_2$-integrins to be uniformly distributed over the surface of unactivated PMNs (11, 12), but they appear to be aggregated in clusters in activated cells (3, 13). Clustering is important because it increases the avidity of integrins (14–16). Experiments with cytochalasin D show that the actin cytoskeleton has a key role in developing a fully functional adhesive interaction: this event requires the release of integrins trapped in a low affinity, low avidity state from the cytoskeleton, followed by integrin migration and aggregation and then by reassociation with the cytoskeleton, which locks them in a high affinity state (3, 17).

Ceramide, the product of sphingomyelinase C, has the potential to be an endogenous modulator of leukocyte function because it can inhibit the respiratory burst (18) and phagocytosis (19, 20). Ceramide is rapidly generated in tumor necrosis factor-α-treated PMNs (21) and less rapidly when they are stimulated by fMLP (18) through the stimulation of an endogenous sphingomyelinase activity (22). Here, we examined the effect of cell-derived long chain ceramide on PMN adhesive interactions. Paradoxically, we found that it abolished agonist-stimulated adhesion to surfaces, but had no effect on their adhesion to other leukocytes through the same integrins. Ceramide also induced the complete release of the granular contents of the leukocytes, compared with the few percent release in response to soluble stimuli like fMLP. Ceramide altered the F-actin content of the leukocytes, and we found that cytochalasin D had the same disparate effect on adhesion. We conclude that ceramide alters PMN function by an effect on the cytoskeleton.

MATERIALS AND METHODS

Reagents—Loxosceles deserta venom sphingomyelinase D (Spider Pharm, Yarnell, AZ) was stored in small aliquots at −80 °C, thawed,
and diluted 100-fold into HBSS containing 0.5% human serum albumin (HBSS/HSA; Miles Laboratories, Elkhart, IN) before use. Staphylococcus aureus sphingomyelinase C, FITC-phalloidin, and lysophosphatidylcholine were obtained from Sigma. Blocking monoclonal antibodies 60.3 (which recognizes CD18) and 60.1 (which recognizes CD11b) (23) were a gift from Patrick Beatty (University of Utah). FITC-conjugated anti-CD18 antibody was from Immunotech (Marseille, France). All secondary antibodies were obtained from BIOSOURCE, International (Camarillo, CA).

Cells, Adhesion Assays, and Immunohistochemistry—Neutrophils were isolated from fresh human blood as described (24). For adhesion studies, four-well plates (Nuncclone, Roskilde, Denmark) were coated with 100 μg/ml gelatin, 100 μg/ml fibronectin, or 100 μg/ml laminin in nanopure water at 37 °C for 3 h and blocked with HBSS/HSA for 1 h at 37 °C. The wells were washed twice with HBSS/HSA before addition of 0.25 ml of PMNs (5 × 10^6/ml in HBSS/HSA), followed by an agonist at the indicated concentrations. The plates were incubated at 37 °C for 5 min before non-adherent PMNs were aspirated, and loosely adherent cells were removed by two HBSS/HSA washes. The cells were immediately quantified in the three random microscopic fields using a custom video imaging system. Unless otherwise stated, PMNs at 5 × 10^6/ml were pretreated with 0.5 units/ml sphingomyelinase C for 30 min at 37 °C before addition to adhesion assays. Ion substitution of β2-integrin complexes was performed as described (25).

PMN Aggregation and Ca^2+ Flux Measurement—PMNs (5 × 10^6/ml) were loaded in HBSS with 1 μM Indo-1/AM (Molecular Probes, Inc., Eugene, OR) for 30 min at 37 °C in the dark. Cells were then washed with HBSS/HSA, and Ca^{2+}-dependent fluorescence was measured by emission at 492 nm after excitation at 355 nm. Aggregation was measured by loss of light dispersion during the continuous stirring of 5 × 10^6 PMNs/ml in HBSS/HSA that had been preincubated for 30 min with buffer or sphingomyelinase C before use.

Flow Cytometry, Imaging, and Enzyme-linked Immunosorbent Assay—surface expression of adhesion molecules and F-actin content were quantified by FACS analysis. PMNs (10^6/ml) were treated as described in the figure legends, collected by centrifugation at 500 × g, and resuspended at 4 °C in 0.1% sodium azide and 10% goat serum in PBS for 10 min. Cells were centrifuged and resuspended for 1 h at 4 °C in 10 μg/ml monoclonal antibody 60.3 or 60.1 and washed three times by centrifugation before resuspension in either Alexa 488- or FITC-conjugated goat anti-mouse polyclonal antibody in the same buffer. Cells were finally resuspended in 0.5% formaldehyde at 4 °C before an aliquot was removed for FACS quantification, and a second aliquot was centrifuged onto microscope slides in a Cytospin holder (Shandon Instruments, Astmoor, United Kingdom) for confocal fluorescence microscopy. The cells were not allowed to dry during this procedure. Propidium iodide at 10 μg/ml in PBS was added to the cells on the slide just prior to viewing by confocal microscopy. The F-actin content was determined using FITC-phalloidin. PMNs were incubated with 200 μg/ml lysophosphatidylcholine, and 2 μg/ml FITC-phalloidin in PBS for 4 h on ice; washed with PBS; and resuspended in 1 ml of PBS before FACS analysis. Lactoferrin as a secondary granule marker and elastase as a primary granule marker were detected using sandwich enzyme-linked immunosorbent assay with rabbit anti-human antibodies as described (26). The total content of the marker enzymes was determined by sonication of control PMNs, followed by centrifugation to remove insoluble materials. We confirmed complete release of marker enzymes from sphingomyelinase C-treated PMNs by collecting the treated cells by centrifugation and assaying the sonicated cellular material.

Sphingomyelin and Ceramide Quantification—Lipids isolated from 10^6 PMNs were extracted (27), and half of the lipids were subjected to diacylglycerol kinase assay using [32P]ATP (Amersham Biosciences, Inc.) of known specific radioactivity as described by the manufacturer with several modifications: only glass tubes were used; purified C14-ceramide was included as a standard in addition to diacylglycerol; and the reaction was extended to 3 h. The completed reaction mixture was separated by high-performance thin-layer chromatography, and low scintillation counting of [32P]phosphorceramide and [32P]phosphatidic acid was quantified to calculate ceramide and diacylglycerol, respectively. PhosphorImager analysis was used to confirm the location and intensity of ceramide phosphate and phosphatidic acid spots. Phosphatidylcholine was quantitated by HPLC using a 4.6 × 300-mm silica gel column developed with a gradient of solvent A (97.5:2.5 acetonitrile/water) and solvent B (85:15 acetonitrile/water; 100% solvent A for 3 min, followed by a gradient to 100% solvent B over 12 min and holding for 10 min. Peaks were detected at 203 nm, collected, and dried, and the

![Diagram](http://www.jbc.org/)

**Fig. 1.** Sphingomyelinase C increases PMN ceramide, and sphingomyelinase D pretreatment blocks this. a. loss of sphingomyelin after treatment with 0.5 units/ml sphingomyelinase C (SMD) or 0.1 μl of sphingomyelinase D (SMD) after sphingomyelinase D pretreatment followed by sphingomyelinase C exposure (SMD→SMC). The phospholipids were separated by HPLC and quantified by inorganic phosphate analysis as described under “Materials and Methods.” b, analysis of ceramide content performed using a diacylglycerol kinase assay system and [32P]ATP of known specific activity as described under “Materials and Methods.” Results are from a single experiment that is representative of two others.

**RESULTS**

### Sphingomyelinase C (but Not Sphingomyelinase D) Increases Ceramide Levels in PMNs—Freshly isolated human PMNs that were exposed to exogenous bacterial sphingomyelinase C lost two-thirds of their cellular sphingomyelin within 30 min, with no corresponding loss of phosphatidylcholine (Fig. 1a). The latter point is important because it clearly demonstrates the specificity of this enzyme for sphingomyelin even though both of these complex lipids possess the same polar head group. Similarly, the sphingomyelinase D activity in Loxosceles reclusa venom (which yields ceramide 1-phosphate and choline (29)) hydrolyzed two-thirds of the sphingomyelin, again without affecting phosphatidylcholine levels. We found that sequential treatment with sphingomyelinase D followed by sphingomyelinase C failed to further deplete sphingomyelin content. We infer from this that the residual sphingomyelin was not accessible to extracellular sphingomyelinase activity and might therefore represent pre-existing intracellular pools or sequestration of sphingomyelin by endovesiculation during sphingomyelinase treatment (30). This loss of membrane lipid did not harm the permeability barrier provided by the plasma membrane, as neither of the sphingomyelinases affected trypan blue exclusion (data not shown).

We determined whether changes in ceramide content inversely correlate with changes in sphingomyelin content. We found that exogenous sphingomyelinase C caused a dramatic increase in ceramide levels within 10 min of exposure to the enzyme (Fig. 1b). This level was maintained for at least 30 min and represents a nearly quantitative conversion of cellular
sphingomyelin to ceramide. We also confirmed that fMLP did not stimulate an increase in cellular ceramide over the first 30 min of exposure to this agonist (Fig. 1b) (18). In contrast, sphingomyelinase D treatment failed to increase cellular ceramide levels, showing that the amount of ceramide phosphate generated by this treatment cannot readily be metabolized to ceramide. We took advantage of this inability to rapidly metabolize ceramide phosphate to ceramide by using a pretreatment with sphingomyelinase D to convert sphingomyelin to ceramide phosphate and thereby deplete the substrate for sphingomyelinase C (31, 32). This maneuver effectively prevented ceramide accumulation in response to sphingomyelinase C (Fig. 1b), demonstrating that sphingomyelinases C and D have access to the same pool of sphingomyelin. This means that we can specifically block ceramide accumulation in sphingomyelinase C-treated cells, and so we can control for the effect of sphingomyelin loss.

Ceramide Is a Leukocyte Agonist—We determined whether ceramide alters intracellular calcium levels in leukocytes using the fluorescent indicator Indo-1. We found that sphingomyelinase C induced an increase in intracellular calcium, although the rate of increase was slower than that induced by fMLP (Fig. 2). The increase in intracellular Ca\(^{2+}\) did not result from lytic cellular injury, as these PMNs still responded to a subsequent stimulation with fMLP in a fashion indistinguishable from that of control PMNs. Sphingomyelinase D treatment of leukocytes had no effect on intracellular calcium levels (Fig. 2), so neither the loss of plasma membrane sphingomyelin nor its conversion to ceramide phosphate directly altered calcium metabolism. We used the ability to deplete cellular sphingomyelin content without activating the cells to determine whether ceramide accumulation accounted for the effect of sphingomyelinase C on intracellular Ca\(^{2+}\) levels. We pretreated the PMNs with sphingomyelinase D and found that, in the absence of its substrate, sphingomyelinase C did not induce an intracellular Ca\(^{2+}\) transient. Sphingomyelinase D pretreatment of PMNs did not alter the Ca\(^{2+}\) transient induced by a subsequent exposure to fMLP (Fig. 2).

Ceramide Accumulation Causes Dramatic Degranulation of PMNs—We explored whether ceramide accumulation has an effect on the release of hydrolytic enzymes from primary and secondary granules. We found that almost 90% of cellular elastase was released from the primary granules of sphingomyelinase C-treated cells (Fig. 3), a level of degranulation that greatly exceeded the release of this marker in response to fMLP. An identical response to sphingomyelinase C was observed when lactoferrin release from secondary granules was determined (Fig. 3). That this was indeed due to ceramide accumulation was demonstrated using sphingomyelinase D pretreatment. We found that sphingomyelinase D treatment on its own did not induce enzyme release from either primary or secondary granules, but that pretreatment with this enzyme abolished the release of both enzymes in response to sphingo-
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Ceramide Stimulates $O_2^−$ Production—The cytochrome $b_{558}$ component of the respiratory burst oxidase complex is a component of specific granules (33), so we determined whether ceramide promotes oxidase complex assembly and activation. We found that sphingomyelinase C treatment of PMNs stimulated $O_2^−$ generation and that, although there was a distinct delay in the onset of $O_2^−$ production, the amount of superoxide generated was comparable to that induced by fMLP (Fig. 4a). We also found that pretreatment with sphingomyelinase C did not enhance the fMLP-induced accumulation of superoxide (Fig. 4b), suggesting that ceramide is as complete an agonist for this response as this bacterial peptide. Sphingomyelinase D pretreatment of leukocytes abolished sphingomyelinase C (but not fMLP) induction of $O_2^−$ accumulation. So ceramide itself is an agonist, whereas fMLP stimulation of the oxidative burst is largely independent of ceramide signaling from the plasma membrane pool of sphingomyelin.

Ceramide Increases Surface $\beta_2$-Integrin Expression, but Inhibits CD18-dependent Adhesion to Protein-coated Surfaces—The complete degranulation induced by ceramide suggested that $\beta_2$-integrins sequestered in the secondary granules should be expressed on the surface of sphingomyelinase C-treated PMNs. We analyzed surface $\beta_2$-integrin expression by flow cytometric analysis and found that quiescent neutrophils constitutively expressed $\beta_2$-integrin and that fMLP stimulation enhanced this expression as expected (Fig. 5a). Sphingomyelinase C treatment also increased surface $\beta_2$-integrin expression, and this expression greatly exceeded that induced by fMLP.

We determined whether the large complement of $\beta_2$-integrin on the surface of sphingomyelinase C-treated cells would support adhesion to gelatinized surfaces, a measure of $\beta_2$-integrin function (34–36). However, sphingomyelinase-treated cells were non-adhesive (Fig. 5b), so their abundant surface integrins were in a low avidity state. When we treated these cells with a second agonist (fMLP) to functionally up-regulate these surface integrins, we discovered that this agonist could not induce integrin-dependent adhesion in ceramide-enriched cells. As in previous experiments (35), the fMLP-induced adhesion was completely inhibited by a blocking monoclonal antibody directed against CD18 (data not shown), so the process affected by ceramide was loss of $\beta_2$-integrin function. We also found that adhesion in response to platelet-activating factor was inhibited by sphingomyelinase C pretreatment, showing that the block created by sphingomyelinase C pretreatment was not specific to fMLP-generated signals. We found that the inhibition of $\beta_2$-integrin function was the result of ceramide accumulation, as sphingomyelinase D pretreatment, which caused no changes on its own, blocked the effect of sphingomyelinase C on agonist-induced adhesion. This effect of ceramide does not arise from interference with the signaling that initiates adhesion because we found that sphingomyelinase C

![Image](http://www.jbc.org/)

**Fig. 4.** Ceramide induces superoxide generation. $a$, rate of $O_2^−$ production. Production of $O_2^−$ was assayed by superoxide dismutase-inhibitable oxidation of ferricytochrome $c$ as described under "Materials and Methods." $b$, amount of superoxide generation in 1 h. Each pair of bars presents superoxide generated by the specified treatments with additional exposure to fMLP (shaded bars) or by cells incubated in buffer (hatched bars). $a$ is representative of three individual experiments. $b$ is the normalized values from two different donor PMNs, with the assay done in triplicate. SMC, sphingomyelinase C; SMD, sphingomyelinase D.

**Fig. 5.** Ceramide increases surface $\beta_2$-integrin expression, but abolishes agonist-induced integrin-dependent adhesion. $a$, shown are the results from flow cytometric analysis of CD18 surface expression. PMNs were treated with buffer, fMLP, or sphingomyelinase C (SMC) and then stained with anti-CD18 monoclonal antibody and FITC-conjugated goat anti-mouse IgG for flow analysis. This is a normalized representation of three data sets. $b$, PMNs were incubated with buffer or the indicated agonist for 25 min and then exposed to buffer (shaded bars), $10^{-7}$ M fMLP (hatched bars), or $10^{-7}$ M platelet-activating factor (PAF; open bars) during a subsequent 5-min adhesion assay. Quantification of PMN adhesion to a gelatinized surface, a $\beta_2$-integrin-dependent function (data not shown), was performed as described under "Materials and Methods." Shown is an individual experiment representative of two others. SMD, sphingomyelinase D.
would reverse established adhesion well after such signaling had successfully initiated adhesion (data not shown).

**Ceramide Does Not Induce the High Affinity State of β₂-Integrins—**β₂-Integrins can be activated to a high affinity state in the absence of inside-out signaling by cation switching (25, 37–39) where Mn²⁺ is substituted for constitutively bound inhibitory ions. To determine whether integrins displayed by ceramide-enriched cells could be promoted to a high affinity state, we treated PMNs with buffer or sphingomyelinase C, removed surface cations with EDTA, and reconstituted the integrins with either Mn²⁺ or Mg²⁺ and Ca²⁺ as a control. We then examined their ability to adhere to a laminin-coated surface (a surface previously used in cation replacement experiments (34)). We found that substitution of Mn²⁺ for endogenous divalent cations allowed unactivated PMNs to adhere to this surface just as if they had been activated by an agonist (Fig. 6). This adhesion was suppressed by an antibody against CD18, as previously reported (25). When sphingomyelinase C-treated neutrophils were subjected to the cation-switching procedure, Mn²⁺ substitution induced β₂-integrin-dependent adhesion to the same extent as in cells that had not been exposed to sphingomyelinase C. The cation replacement procedure per se did not stimulate β₂-integrin function of quiescent PMNs, treated or not with sphingomyelinase C, as stripping and replacement with Mg²⁺ and Ca²⁺ did not induce adhesion. Moreover, sphingomyelinase C-treated PMNs whose integrins were reconstituted with Mg²⁺ and Ca²⁺ displayed the same adhesion defect as control cells (as shown by the last two bars in Fig. 6). These data also show that the effect of ceramide on leukocyte adhesion is independent of the nature of the β₂-integrin-binding partner.

**Ceramide Does Not Block β₂-Integrin-dependent Aggregation—**We examined a second β₂-integrin-dependent function, homotypic aggregation. We found that sphingomyelinase C treatment did not cause PMNs to adhere to one another (data not shown, but see Fig. 7b), again indicating that the very large number of β₂-integrin molecules exposed on the surface are in an inactive state. However, in marked contrast to the effect of ceramide on agonist-stimulated adhesion to immobilized proteins (Figs. 5 and 6), we found that a subsequent exposure of sphingomyelinase C-treated PMNs to fMLP induced homotypic aggregation (Fig. 7a). The initial rate of aggregation of sphingomyelinase C-treated and -untreated cells differed by only 13% in this experiment, but in aggregate showed a modest reduction (20%, n = 5) following sphingomyelinase treatment. Aggregation of control and sphingomyelinase C-treated PMNs in response to fMLP was inhibited by antibody 60.3, demonstrating that the aggregation was β₂-integrin-dependent (66 ± 9% reduction, n = 5). Because of the complete disparity between adhesion to immobilized ligands and aggregation, we considered the possibility that the changes in light transmission only reflected changes in leukocyte optical properties subsequent to their massive release of granular material. However, PMA, which is a more potent degranulating agent than fMLP, produced similar aggregometer recordings (Fig. 8a). Overall, we found that sphingomyelinase pretreatment modestly (16%, n = 6) reduced the rate of aggregation in response to PMA. Moreover, microscopic analysis clearly demonstrated the presence of aggregates following fMLP or PMA stimulation in both control and sphingomyelinase C-treated populations (Fig. 7b). These leukocytes were assayed in parallel for adhesion to immobilized gelatin surfaces (data not shown) and again demonstrated the same defect in binding as shown in earlier experiments (Figs. 5 and 6). Therefore, enhanced ceramide levels block β₂-integrin-dependent surface adhesion, but not β₂-integrin-dependent aggregation.

**Ceramide Alters the F-actin Content of PMNs—**Close inspection of the morphology of PMNs exposed to sphingomyelinase C left the impression that they were slightly larger than control cells. We confirmed this by assessing deformation after hydrostatic filtration through pores of defined size, which reorganizes and stabilizes the cytoskeleton (40). We found that unmanipulated PMNs forced through 8-µm pores, which are just larger than the cells, showed no morphologic effects of their passage through the filter, but cells pre-exposed to sphingomyelinase C displayed a prolate shape, showing they were larger than the pore and that they were unable to rapidly regain their shape after passage (data not shown). These results suggest that the cytoskeleton might have been affected by the sphingomyelinase C treatment and that perhaps this in turn influences surface adhesion and spreading. Accordingly, we examined FITC-phalloidin-stained cells and found that the F-actin content in sphingomyelinase C-treated PMNs was less than in control cells (Fig. 8a). Furthermore, it was apparent that, although fMLP stimulation increased F-actin content in sphingomyelinase C-treated cells, ceramide attenuated this F-actin accumulation such that there was only a small difference in F-actin content compared with quiescent control cells.

**Cytochalasin D Mimics the Effect of Ceramide on PMN Adhesion and Aggregation—**The foregoing suggests that ceramide could modulate surface β₂-integrin function through an effect on the cytoskeleton. To test this, we used cytochalasin D, an agent that alters cytoskeletal reorganization and primes degranulation. We found that, like ceramide, cytochalasin D treatment abolished the ability of PMNs to adhere to a gelatin-coated surface in response to fMLP stimulation (Fig. 8b). Cytochalasin D pretreatment also greatly decreased PMN adhesion to a gelatinized surface in response to the powerful stimulus PMA. We next examined the effect of cytochalasin D on aggregation and found that, although cytochalasin D did not by itself induce aggregation, neither did it block homotypic aggregation in response to a second stimulus (Fig. 8c). Sphingomyelinase C and cytochalasin D pretreatment both reduced the rate of aggregation by about half in this experiment, whereas in two other experiments, the rate of aggregation in response to fMLP was enhanced (overall 204 ± 15%). Therefore, despite a marked effect on surface adhesion (Fig. 8b),
PMNs were clearly able to aggregate after either treatment (Fig. 7b).

\( \beta_2 \)-Integrin Clustering Is Not Detectably Altered by Ceramide or Cytochalasin D — One way for the cytoskeleton to alter integrin function would be through an effect on integrin migration and clustering. We employed confocal microscopy and fluorescent immunochemistry to image surface \( \beta_2 \)-integrin organization to find that resting cells already demonstrated punctate areas of staining (Fig. 9a). This staining derives from surface integrins, and not intracellular storage pools, as shown by a cross-section of these cells (Fig. 9b). There was an increase in the total level of \( \beta_2 \)-integrin on the cell surface after exposure to sphingomyelinase C (see Fig. 5a), but there was no discernible alteration in the organization of this surface \( \beta_2 \)-integrin after this treatment. The larger clusters of \( \beta_2 \)-integrins on sphingomyelinase C-treated cells were brighter and saturated the detector; and initially, this gave the appearance of the clusters being larger and having a different surface distribution between activated and quiescent cells. However, when the detector gain was adjusted to yield equivalent brightness between samples, we found that the surface distribution and staining pattern appeared identical between samples, as shown in Fig. 9. Cytochalasin D, which had the same effect on PMN adhesive functions as sphingomyelinase C treatment, also failed to alter the pattern of surface \( \beta_2 \)-integrin distribution (Fig. 9a). Therefore, ceramide and cytochalasin D do not greatly affect \( \beta_2 \)-integrin clustering, but rather may prevent the transformation of \( \beta_2 \)-integrins to a high affinity state or the solidification of the high affinity state by reassociation with the cytoskeleton.

DISCUSSION
Ceramide functions as an intracellular signaling intermediate (41) through a stimulatable metabolic cycle (42); it participates in apoptosis (41, 43); and it is a component of the signaling pathway used by tumor necrosis factor family members (44, 45). Stimulated ceramide metabolism occurs in PMNs exposed to tumor necrosis factor-\( \alpha \) (21), phagocytizable particles (20, 22), or a soluble agonist like fMLP (18). Ceramide accumulation is associated with termination of the respiratory burst (18, 46) and suppression of cellular spreading (47). It inhibits protein kinase Ca (48), MAPK activity (19), and p21\(^{\text{PAK}} \) activity (49). Conversely, ceramide primes PMNs for the respiratory burst (50) and stimulates MAPK activity in the HL-60 myeloid cell line (51).

Ceramide is distinctly hydrophobic, and water-soluble analogs are often employed to circumvent this property to explore ceramide’s biology. Whether such analogs accurately substitute for ceramide is unknown. Here we defined a novel approach to defining a role for intracellular ceramide by generating ceramide \textit{in situ} with exogenous sphingomyelinase C. We then took advantage of the inability of sphingomyelinase C to readily hydrolyze ceramide 1-phosphate, the product of sphingomyelinase D, and used the latter enzyme to specifically deplete cells of the substrate for sphingomyelinase C. This was possible because sphingomyelinase D did not stimulate PMNs (but this is not true for all cell types (52)) and because both types of sphingomyelinase acted on the same pool of sphingomyelin. As shown in Fig. 1, a combination of the two enzymes did not hydrolyze more sphingomyelin than either individual enzyme. We found that approximately one-third of the cellular sphingomyelin was resistant to exogenous sphingomyelinase hydrolysis, as was found in the promyelocytic cell line HL-60 (53). This pool may be refractory to enzymatic attack by virtue of its localization or subsequent vesiculation induced by sphingomyelin hydrolysis (30). The key observation in subsequent experiments is that each response induced by sphingomyelinase C
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Fig. 8. Ceramide alters F-actin content, and altering cytoskeletal reorganization with cytochalasin D mimics the effect of ceramide on adhesion. a, flow cytometric analysis of FITC-phalloidin binding to cytoskeletal F-actin. PMNs were incubated in buffer alone or with sphingomyelinase C (SMC). b, adhesion to a gelatinized surface. PMNs were pretreated with sphingomyelinase C or 10 μg of cytochalasin D (Cyto D) for 25 min, and then adhesion in response to fMLP, PMA, or buffer was determined as described. c, PMN homotypic aggregation. Half of the PMNs pretreated for the adhesion assay were added to a stirred cuvette, and their ability to aggregate in response to the indicated agonist was determined as described in the legend to Fig. 7. These data are representative of three individual experiments.

Ceramide-induced degranulation likely derives from an effect on the cytoskeleton, but the role of the cytoskeleton in degranulation is ill defined. Disruption of the cytoskeleton by cytochalasin D or direct ribosylation of actin by botulinum C2 toxin (57) enhances agonist-induced degranulation, yet disorganization of the cytoskeleton with botulinum C3 toxin (58) has no effect on release of primary granule contents. That the effect of ceramide on degranulation is only partly mimicked by cytochalasin D suggests that additional, yet to be identified targets of ceramide signaling are involved in this marked secretory response.

The second highly unusual effect of cellular ceramide on PMNs is the appearance of large amounts of β2-integrins on the cell surface, a consequence of the complete degranulation, in a functionally inactive state. PMNs in their quiescent state normally express inactive β2-integrin on their surface. It is these molecules that become competent after stimulation with low levels of agonists, whereas higher concentrations activate the β2-integrins newly recruited to the cell surface (9, 10, 59). However, agents that increase surface β2-integrin expression also activate inside-out signaling, which promotes the transition of β2-integrins to their high affinity state. Ceramide does not do this. Even for homotypic aggregation, where the β2-integrin in sphingomyelinase C-treated PMNs could be transformed to the high affinity state, this transformation required an additional agonist. The integrins on sphingomyelinase C-treated PMNs could be forced into an active conformation with Mn²⁺ (37, 39), suggesting that the integrins themselves are not defective in ceramide-enriched cells.

One way by which β2-integrins become competent and support leukocyte adhesion is through changes in their cytoskeletal association that increase avidity (1, 60, 61). For example, adhesion to an immobilized ligand through αδβ2-integrin is increased by cell activation and inhibited by cytochalasin D, all without changing the affinity state of the receptor (16). Receptor clustering is one well established mechanism that increases the avidity of the receptor-ligand pair. Integrins do cluster, and clustering may be induced from the outside by multivalent ligands (15, 62–64) or from within following outside-in signaling (11, 14, 16, 65). We did not observe changes in the size or numbers of integrin clusters upon stimulation or following sphingomyelinsinase treatment. However, other cytoskeleton-associated events contribute to integrin function. Constitutively expressed β2-integrins appear to be held in a functionally inactive conformation by interaction with cytoskeletal-associatet tadin (17). With appropriate stimulation, this linker is proteolyzed (13), and the transiently free integrin (3, 66) is free to migrate into domains where its mobility is restricted by association with α-actinin (17, 67, 68). We do not know whether cytoskeletal reorganization is a physiologic way to increase integrin mobility, but tumor necrosis factor-α transiently increases ceramide content in PMNs (21, 47), and it reorganizes the cytoskeleton, α-actinin, and β2-integrins to form stable domains (69).

There are dynamic changes in integrin tethering and release during cytoskeletal reorganization as PMNs begin to actively change shape and migrate. This connection is apparent when cytochalasin D disruption of cytoskeletal reorganization blocks αδδβ2-integrin capping and lymphocyte intercellular adhesion (70). Ceramide does not transform these adhesion molecules to their high affinity ligand-binding state, yet is more than a passive bystander after the recruitment of the intracellular integrin stores to the surface because it affects adhesion to planar surfaces. We propose that ceramide might disrupt the tightly regulated interaction of the integrin with the cytoskeletal components that is required for affinity maturation. A key
argument that the changes in F-actin content after ceramide accumulation affect cell function is that cytochalasin D disruption of the cytoskeleton has the same precise effect on adhesion. We cannot ascribe the inability to adhere to surfaces to gross alterations in integrin clustering because we saw no changes in clustering and because the PMNs still could be induced to bind to one another. Instead, we postulate that some step in the release of integrins trapped in a low affinity state from the cytoskeleton, integrin migration, or reassociation with the cytoskeleton that locks them in a high affinity state (3, 17) is affected by cellular ceramide. A new observation here is that a role for cytoskeletal reorganization was only present when the cells were assayed by adhesion to an extracellular matrix; alterations in integrin clustering because we saw no changes in

Fig. 9. Integrin clustering is independent of actin reorganization. a, surface β₂-integrin distribution visualized by a 0.5-μm confocal fluorescence microscopic section representing the surface of the PMNs. Visualization of β₂-integrins with Alexa 488-conjugated goat anti-mouse IgG was performed as described under “Materials and Methods.” b, surface β₂-integrin distribution visualized by a 0.5-μm confocal fluorescence microscopic section representing the center of the PMNs. The red stain in both panels resulted from propidium iodide staining of the PMN nucleus. These data are representative of one of three experiments.

SMC, sphingomyelinase C; CytoD, cytochalasin D; HBSSA, HBSS/HSA.

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