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Caspases are the main executioners of Fas-mediated apoptosis, irrespective of the ceramide signalling pathway

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Abstract

Tumor necrosis factor α (TNF) or cytotoxic anti-Fas antibodies lead to the activation of apoptotic proteases (caspases) and to sphingomyelinase-mediated ceramide generation. Caspases and ceramide are both known to induce apoptosis on its own, but their relative contribution to Fas- and TNF-induced cell death is not well established. We report here that rapid apoptosis induced by TNF in U937 cells or anti-Fas in Jurkat cells, in the presence of cycloheximide, induced only a very low increase ($<$20%) in the cell ceramide content. Neither treatment with inhibitors of sphingomyelinases nor incubation of cells with fumonisin B_1, which inhibits *de novo* ceramide synthesis, prevented TNF and Fas-mediated apoptosis. Increasing or depleting the cell ceramide content by prolonged culture in the presence of monensin or fumonisin B1, respectively, did not prevent TNF and Fas-mediated apoptosis. Treatment of cells with sphingomyelinase inhibitors did not affect to the activation of CPP32 (caspase-3) induced by TNF or anti-Fas antibodies. Chromatin condensation and fragmentation in cells treated with anti-Fas or TNF was abrogated by peptide inhibitors of caspases, which also inhibited Fas-, but not TNF-induced cell death. These results indicate that while ceramide does not seem to act as a critical mediator of TNF and Fas-induced apoptosis, it is generated as a consequence of CPP32 activation and could contribute to the spread of the intracellular death signal.

Keywords: Fas; TNF; ceramide; sphingomyelinase; CPP32; apoptosis

Abbreviations: TNF, Tumour necrosis factor-α; TNFR, TNF-α receptor; FasL, Fas ligand; FB_1, Fumonisin B_1; MTT, 3-[4,5-dimethylthiazol]-2,5-diphenyl-tetrazolium bromide; Ac-DEVD-CHO, N-acetyl-Asp-Glu-Val-Asp aldehyde; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Boc-D-fmk, t-butyloxy-carbonyl-Asp-fluoromethylketone

Introduction

The Fas molecule and the receptor for tumour necrosis factor α (TNF) belong to a family of surface molecules which possess the so-called death domain, which upon occupancy by their corresponding physiological ligands (FasL and TNF, respectively) trigger apoptosis (Henkart, 1996). The transduction of the apoptotic signal generated by these occupied receptors is not fully known but involves the recruitment and sequential activation of cytosolic cysteine proteases with Asp specificity (ICE/CED3-like proteases or caspases) (Chinnayian *et al*, 1996) and the activation of sphingomyelinases (Kolesnick and Golde, 1994; Tepper *et al*, 1995; Gamen *et al*, 1996). The active caspases and the product of sphingomyelinase activity, ceramide, can both induce apoptosis in sensitive cells when transfected or exogenously added, respectively (Tewari *et al*, 1995; Gamen *et al*, 1996; Henkart, 1996). Active proteases of the CPP32 subfamily of caspases cause the hydrolysis of a number of cell proteins such as ply(ADP-ribose) polymerase (PARP), the 70 kDa protein component of the U1-ribonucleoprotein (U1-70 kDa), DNA-dependent protein-kinase (DNA-PK_{cs}) and lamin A (Casciola-Rosen *et al*, 1996; Greidinger *et al*, 1996). On the other hand, it has been described that ceramide activates a protein kinase, recently identified as c-Raf (Huwiler *et al*, 1996), and a protein phosphatase (Dobrowsky *et al*, 1993). Ceramide has been also proven to be a key mediator of irradiation-induced apoptosis (Santana *et al*, 1996). The cleavage of the above mentioned proteins, and probably others, critical for cell homeostasis, as well as the activation of ceramide-dependent kinases and phosphatases acting on nuclear targets (Huwiler *et al*, 1996) may thus constitute the executive phase of apoptotic cell death.

However, the relative contribution of ceramide to the apoptotic processes triggered by TNF and FasL (or cytotoxic anti-Fas antibodies) is presently ill-defined. We have recently demonstrated that Fas-induced activation of CPP32-like proteases is a pre-requisite for ceramide generation and apoptosis in human cells (Gamen *et al*, 1996). In addition, in the short-time (4 h) Fas-mediated lysis caused by FasL-expressing effector cells, no rise in the levels of cellular ceramide could be noticed (Anel *et al*, 1997). This prompted us to determine if ceramide generation after Fas or TNF-receptor (TNFR) ligation is an essential event in the intracellular pathway of cell death or a secondary mechanism for amplification or diversification of the death signal. Our present results support the latter possibility.

Results

Effect of sphingomyelinase inhibitors on TNF and Fas cytotoxicity

U937 and Jurkat cells possess both neutral and acidic sphingomyelinases that become activated upon TNF and

Fas stimulation (Cifone *et al*, 1993; Wiegmann *et al*, 1994; Boucher *et al*, 1995; Gamen *et al*, 1996). To evaluate the contribution to apoptosis of sphingomyelinase-generated ceramide, as a first approach, U937 and Jurkat cells were treated with cytotoxic anti-Fas antibodies or TNF in the presence or absence of inhibitors of neutral (EDTA) (Wiegmann *et al*, 1994; Tepper *et al*, 1995) and acidic (ammonium chloride, chloroquine and monensin) (Wiegmann *et al*, 1994; Andrieu *et al*, 1996) sphingomyelinases. Since incubations with these compounds (at the concentrations reported to be inhibitory) for the times required for physiological cell lysis (8–12 h for anti-Fas, 24–48 h for TNF), produced a high toxicity, tests were performed in the presence of cycloheximide to accelerate the onset of cell death (Schulze-Osthoff *et al*, 1994) (Figure 1). Under these conditions, the inhibitors did not induce any toxicity *per se*. Only monensin treatment had a slight protective effect on Fas-induced apoptosis in U937 cells at 3 h, but this effect was not observed at longer incubation times (Figure 1b). The other sphingomyelinase inhibitors tested offered very low or no protection to Jurkat and U937 cells treated with anti-Fas or TNF at any time point tested (Figure 1).

Effect of sphingomyelinase inhibitors on ceramide generation

Cells treated for 3.5 h with anti-Fas antibodies or for 6 h with TNF, in the presence of cycloheximide, showed a slight increase (around 20% in both cases) in the ceramide to sphingomyelin ratio (Figure 2). Treatment of Jurkat and U937 cells with a combination of monensin and EDTA, in the absence of TNF and anti-Fas, caused a greater increase in the ceramide to sphingomyelin ratio (47 and 66%, respectively). This fact was probably due to the intracellular accumulation of ceramide by monensin blockade of its transport to the plasma membrane (Kallen *et al*, 1993; Andrieu *et al*, 1996). This increase in the cell ceramide content was not associated, however, with any observable cytotoxicity during the experimental period, in spite of being greater than that induced by anti-Fas or TNF (Figure 2). Anti-Fas or TNF treatment caused a considerable cytotoxicity, comparable to that shown in Figure 1, irrespective of the presence or absence of sphingomyelinase inhibitors. Treatment with anti-Fas or TNF, in the presence of monensin and EDTA produced only a modest increase (28 and 18%, respectively) in the ceramide to sphingomyelin ratio. This increase was probably due to a residual activity of neutral sphingomyelinase, since monensin, at the concentrations used, efficiently inhibits acid sphingomyelinase activity in intact cells (Wiegmann *et al*, 1994; Boucher *et al*, 1995). EDTA was used to chelate extracellular Mg^{2+}, in an attempt to block the activity of neutral sphingomyelinase, which is localised at the plasma membrane and externally oriented (Das *et al*, 1984). However, intracellular Mg^{2+}, unlike calcium, does not exchange with extracellular Mg^{2+} (Murphy *et al*, 1989) and simultaneous incubation of cells with EDTA and an intracellular Mg^{2+}-chelator elicited a great cytotoxicity (data not shown). As an alternative to estimate the possible contribution of the neutral sphingomyelinase activity to

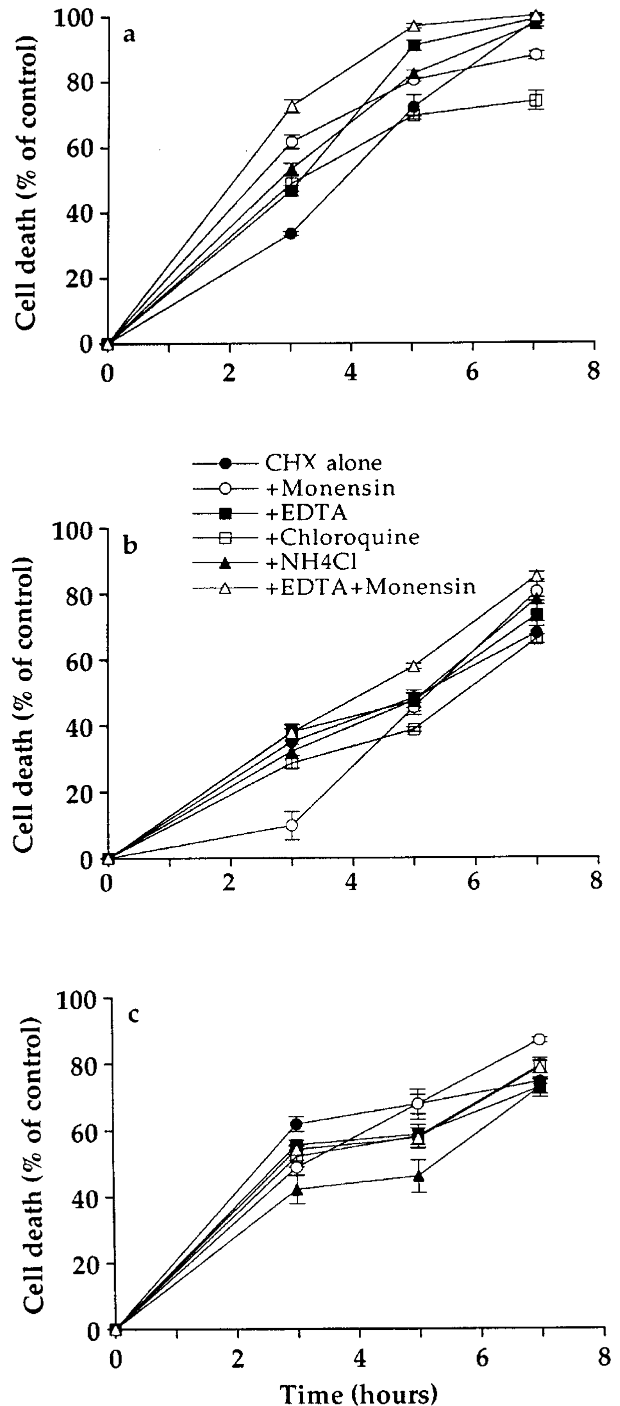

Figure 1 Sphingomyelinase inhibitors did not prevent Fas or TNF-induced apoptosis. Jurkat (**a**) and U937 (**b, c**) cells were pretreated for 30 min in complete medium containing 10 μg/ml cycloheximide and either 10 μg/ml monensin, 10 μM chloroquine, 8 mM ammonium chloride, 1 mM EDTA or a combination of monensin and EDTA, as indicated. Then, anti-Fas (100 ng/ml) (**a, b**) or TNF (300 U/ml) (**c**) were added and cells incubated for the indicated times. Cultures containing the same amounts of inhibitors, without TNF or anti-Fas, were used as controls. Cell death was determined by the MTT-assay. Results are the mean values of determinations on four to five individual cultures. Vertical bars indicate S.D.

apoptosis, cells were treated with bacterial sphingomyelinase (0.4 U/ml) for 16 h, which virtually eliminates all the plasma membrane sphingomyelin (Hidari *et al*, 1996). This treatment did not induce apoptosis *per se* nor affected to anti-Fas and TNF-induced apoptosis (data not shown).

The possible contribution of the *de novo* synthesised ceramide to TNF and anti-Fas induced apoptosis in the presence of cycloheximide was tested by addition to culture media of FB_1, a strong inhibitor of ceramide synthase (Hidari *et al*, 1996; Jaffrézou *et al*, 1996). FB_1 did not reduce TNF and Fas cytotoxicity in any case (Figure 3).

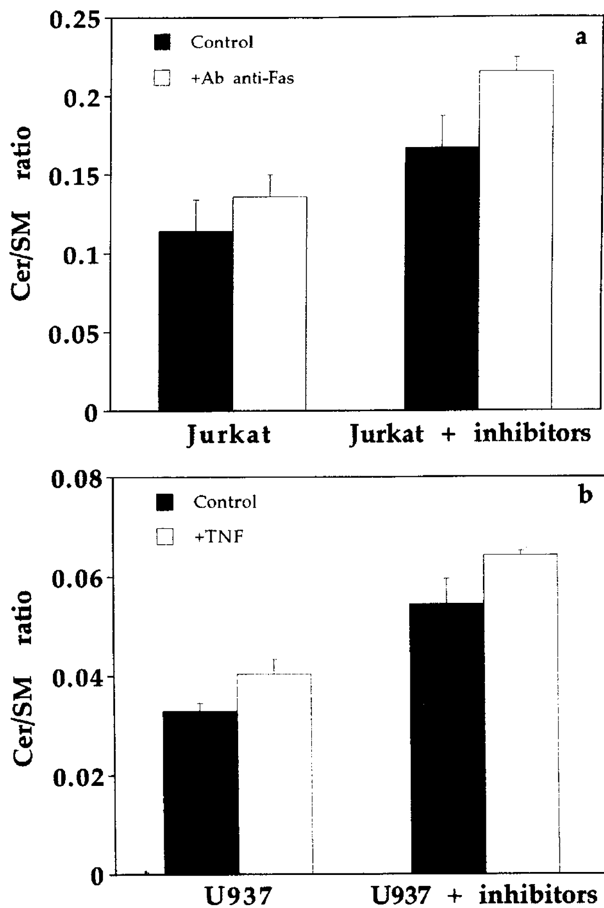

Figure 2 Effect of treatment with sphingomyelinase inhibitors and anti-Fas or TNF on the cell ceramide levels. Jurkat (5×10^5 cells/ml) and U937 (3×10^5 cells/ml) cells, prelabelled for 48 h with 0.5 μCiml of [1-^{14}C]palmitic acid, were incubated for 30 min in complete medium containing 10 μg/ml cycloheximide and a combination of sphingomyelinase inhibitors (10 μg/ml monensin and 1 mM EDTA). Then 100 ng/ml anti-Fas (**a**) or 300 U/ml TNF (**b**) were added and the cells incubated for 3.5 (Jurkat) or 6 h (U937). Cell death was determined in an aliquot of cell suspensions and cell lipids analyzed by TLC as indicated in Materials and Methods. Cultures containing the same amounts of inhibitors alone were used as controls. Results are expressed as the ratio of radioactivity in ceramide (Cer) to sphingomyelin (SM) fractions and correspond to one representative experiment. Data are the mean of three individual determinations. Vertical bars indicate S.D. Radioactivity recovered in ceramide and sphingomyelin fractions from control cells was around 1000 and 12 000 c.p.m., respectively for Jurkat and 12 000 and 42 000 c.p.m., respectively for U937 cells

Effect of chronic treatment with FB_1 or monensin on ceramide content and TNF and Fas cytotoxicity

As an approach to study the contribution of ceramide to TNF and Fas-mediated apoptosis in the absence of cycloheximide, cells were depleted of ceramide and sphingomyelin by prolonged culture in the presence of the ceramide synthase inhibitor FB_1. Treatment with FB_1 reduced the cell ceramide content to about a half and spingomyelin content to the 15 and 2% of that of untreated Jurkat and U937 cells, respectively (Figure 4). Anti-Fas induced approximately a twofold increase in ceramide content in control cells while this increase was marginal in FB_1-treated cells. In spite of this, cell death induced by anti-Fas was similar in FB_1-treated or untreated cells (Table 1). Unexpectedly, treatment of U937 with FB_1 for 72 h significantly diminished the sensitivity to TNF (25 and 10% of cell death at 25 μM and 50 μM FB1, respectively, *versus* 50% in controls). Flow cytometric analysis with specific anti 55 kDa TNFR and anti Fas antibodies revealed a reduction in the number of TNF receptors to about 60% of the value in control cells while no changes in Fas expression were observed (data not shown). Accordingly, the changes in ceramide and sphingomyelin levels in response to TNF triggering were not further evaluated.

Culture of U937 cells with monensin for 36 h caused a near tenfold increase in the cell ceramide content, irrespective of Fas or TNFR ligation (Figure 5). Incubation of U937 cells for 16 h with TNF, in monensin-treated or untreated cells, induced only minor changes in the cell ceramide content (Figure 5), while causing around a 30% of cell death in both experimental conditions (Table 1). Anti-Fas induced a twofold increase in ceramide levels in untreated cells, while the increase was only of 12% in

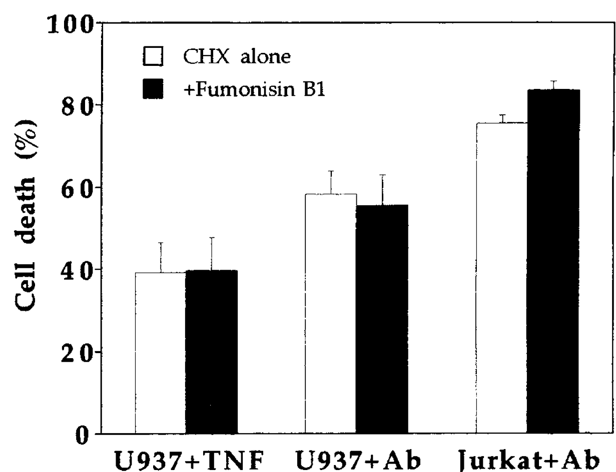

Figure 3 Inhibition of the *de novo* ceramide synthesis has no effect on Fas and TNF-mediated apoptosis. Jurkat (5×10^5 cells/ml) and U937 cells (3×10^5 cells/ml) were preincubated for 30 min in complete medium containing 10 μg/ml cycloheximide and 25 μM fumonisin B_1 an inhibitor of ceramide synthase. Then cells were incubated, in the same media, with 300 U/ml TNF or 100 ng/ml anti-Fas for 6 h and cell death determined by the MTT-assay. Results are the mean values of determinations from four individual cultures. Vertical bars indicate S.D.

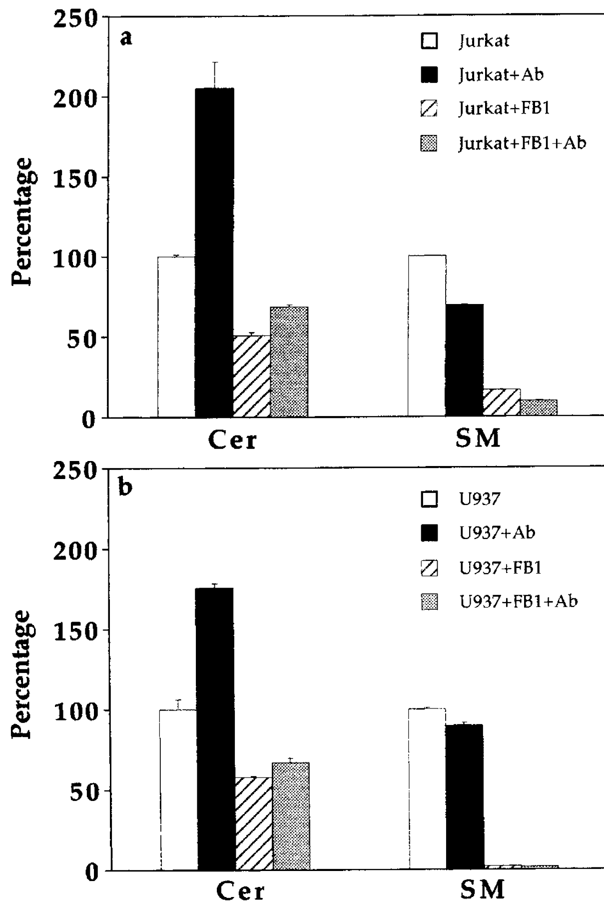

Figure 4 Chronic treatment with fumonisin B_1 greatly reduces the cell ceramide and sphingomyelin content. Jurkat (**a**) and U937 (**b**) cells (3×10^5/ml) were pretreated with 50 μM fumonisin B_1 (FB_1) for 24 h. Then, 0.5 μCi/ml of [1-^{14}C]palmitic acid were added to FB_1-containing medium and cells cultured for a further 48 h. Cells were resuspended at 5×10^6 (Jurkat) or 3×10^6 (U937) cells/ml, in fresh medium containing 50 μM FB_1 and 50 ng/ml anti-Fas. After 15 h of incubation, cell death was estimated in an aliquot of the cell suspensions and the cellular lipids analyzed by TLC. Radioactivity in the ceramide (Cer) and sphingomyelin (SM) fractions was determined by liquid scintillation counting (around 500–1000 for Cer and 12 000–23 000 c.p.m. for SM, respectively in control cells). Results are expressed as percentage of radioactivity in untreated controls and are the mean of individual determinations on three separate cultures. Vertical bars indicate S.D.

Table 1 Anti-Fas and TNF-induced apoptosis in cells cultured with monensin or fumonisin B_1

	Cell death (%)	
Treatment	**U937**	**Jurkat**
+anti-Fas	58.3	72.8
+anti-Fas+monensin	58.6	ND
+anti-Fas+FB_1	51.1	84.0
+TNF	28.1	ND
+TNF+monensin	39.3	ND

U937 cells were cultured for 36 h in complete medium containing 1 μg/ml monensin and then treated with either 50 ng/ml anti-Fas or 300 U/ml TNF for 15 and 22 h, respectively. In other experiments, U937 and Jurkat cells were cultured in medium containing 50 μM fumonisin B_1 (FB_1) for 72 h and then treated with 50 ng/ml anti-Fas for 15 h. Cells treated with anti-Fas or TNF in the same way but without the inhibitors were used as controls. Cell death was determined by the MTT-assay, as described in Materials and Methods and are the mean of quintuplicate individual determinations. ND, not done

monensin-treated cells (Figure 5). However, cell death was similar in both cases (around 58%, Table 1).

Effect of inhibitors of sphingomyelinases on Fas-induced CPP32 activation

Western blot analysis of U937 and Jurkat cells treated with cytotoxic anti-Fas antibodies showed the rapid cleavage of the apoptotic protease CPP32, which is known to be associated with its activation (Nicholson *et al*, 1995; Fernandes-Alnemri *et al*, 1996). Upon stimulation, the 32 kDa-proenzyme was completely cleaved to the active form (Figure 6) producing the characteristic p20, p19 and p17 fragments, corresponding to the different processing of the large N-terminal subunit, and the p12 fragment, correspondent to the small C-terminal subunit (Fernandes-Alnemri *et al*, 1996). Treatment of U937 cells with TNF caused only a partial cleavage of CPP32, as was also previously observed (Erhardt and Cooper, 1996). Metabolic inhibitors alone did not activate CPP32 (Figure 6), and have no effect on anti-Fas or TNF-induced CPP32 cleavage.

Effect of caspase inhibitors on Fas and TNF-mediated apoptosis

Contrary to that observed with sphingomyelinase inhibitors, Fas-induced apoptosis in Jurkat cells was prevented by peptide inhibitors of caspases (Table 2). Both Ac-DEVD-CHO (an inhibitor of CPP32-like proteases) and Z-VAD-fmk (which prevents CPP32 activation by inhibition of FLICE/Mch5/MACH (Fernandes-Alnemri *et al*, 1996; Muzio *et al*, 1996)) blocked Fas-mediated apoptosis. Anti-Fas induced apoptosis in U937 cells was also completely blocked by a combination of Ac-DEVD-CHO and YVAD-cmk (Gamen *et al*, 1996) or Boc-D-fmk, a general inhibitor of caspases (Sarin *et al*, 1996) (data not shown). Ac-DEVD-CHO, Z-VAD-fmk and Boc-D-fmk did not prevent TNF-induced cell death, as determined by annexin V-labeling, MTT-reduction assay (Table 2) or Trypan blue staining (around 60–70% of positive cells, irrespective of treatment with peptides). However, these peptide inhibitors completely prevented the development of apoptotic nuclear morphology, i.e., chromatin condensation and fragmentation in U937 cells, as determined by staining with *p*-phenylenediamine and microscopical inspection (Table 2). Other protease inhibitors tested, including calpeptin, a specific calpain inhibitor (Vanags *et al*, 1996), Z-Asp-2,6-dichlorobenzoyloxymethylketone, 3,4-dichloroisocoumarin and IGA (Anel *et al*, 1997), were also ineffective in blocking TNF toxicity (not shown). Treatment with a combination of Boc-D-fmk and calpeptin caused a slight reduction ($P<0.05$, two tailed Student's *t*-test) in the proportion of dead cells (Table 2).

Discussion

Stimulation of sensitive cells with TNF or anti-Fas antibodies induces the activation of acidic and/or neutral sphingomyelinases and the generation of ceramide, which has been associated in many, but not all, cases with apoptosis (Hannum 1994; Wiegmann *et al*, 1994; Boucher *et al*, 1995;

Cifone *et al*, 1995). Recent and elegant work from the group of M Kronke has led to the identification of two distinct intracellular domains in the 55 kDa TNFR, and also probably in Fas, which are differentially involved in the activation of neutral or acidic sphingomyelinase (Adam *et al*, 1996; Adam-Klages *et al*, 1996). Upon TNF ligation, the activated TNFR recruits a 104 kDa protein, named FAN, through the interaction with a nine aminoacids domain, located upstream of the death domain. The FAN protein in turn mediates the activation of neutral sphingomyelinase and the generation of ceramide at the plasma membrane (Adam-Klages *et al*, 1996). This ceramide specificially activates a Ser/Thr protein kinase (Yao *et al*, 1995), recently identified as c-Raf (Huwiler *et al*, 1996), and initiates the MAPK cascade leading ultimately to cell proliferation/differentiation or generation of inflammatory metabolites. Another member of this pathway may be the ceramide-dependent protein phosphatase 2A (Dobrowsky *et al*, 1993) which control down-regulation of c-*myc* expression (Wolff *et al*, 1994) and c-Jun function (González-Reyes *et al*, 1996). On the other hand, the death domain of TNF receptor, which is responsible for the activation of apoptotic proteases through binding to adaptor proteins such as TRADD (Hsu *et al*, 1995), also mediates the activation of acid sphingomyelinase (Wiegmann *et al*, 1994). The ceramide produced by this endosomal/lysosomal enzyme, not by neutral sphingomyelinase, has been clearly implicated in growth arrest and apoptosis induced by irradiation (Santana *et al*, 1996). Ceramide-mediated apoptosis is thought to be dependent on a distinct subset of kinases, known as stress-activated or c-Jun N-terminal kinases (Xia *et al*, 1995; Coroneos *et al*, 1996).

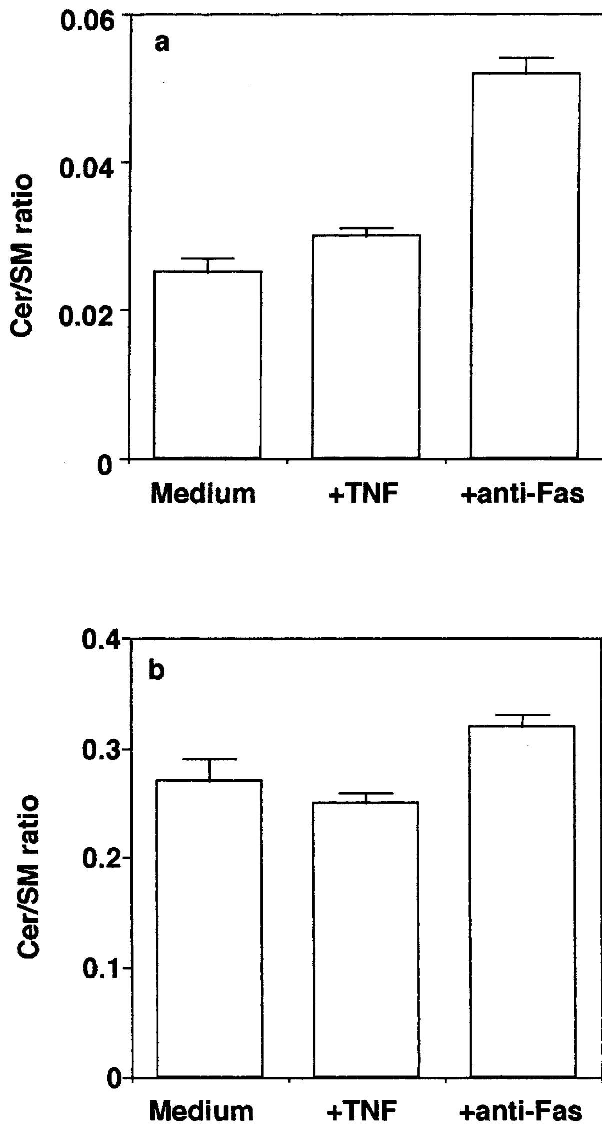

Figure 5 Monensin induces the cellular accumulation of ceramide. U937 cells were labelled for 36 h in complete medium containing 0.5 μCi/ml of [1-^{14}C]palmitic acid in the absence (**a**) or presence (**b**) of 1 μg/ml monensin. Then, cells were resuspended in fresh medium containing 1 μg/ml monensin and 100 ng/ml anti-Fas or 300 U/ml TNF. After 15 or 22 h of incubation, respectively, cell viability was estimated in an aliquot of the cell suspensions and cellular lipids analyzed by TLC. Radioactivity in the ceramide (Cer) and sphingomyelin (SM) fractions was determined by liquid scintillation counting (around 800 and 27 000 c.p.m. in controls, respectively). Results are expressed as the ratio of radioactivity in the ceramide to sphingomyelin fractions and are the mean of individual determinations on three separate cultures. Vertical bars indicate S.D. Note the tenfold difference in the Y-axis scale between parts (**a**) and (**b**)

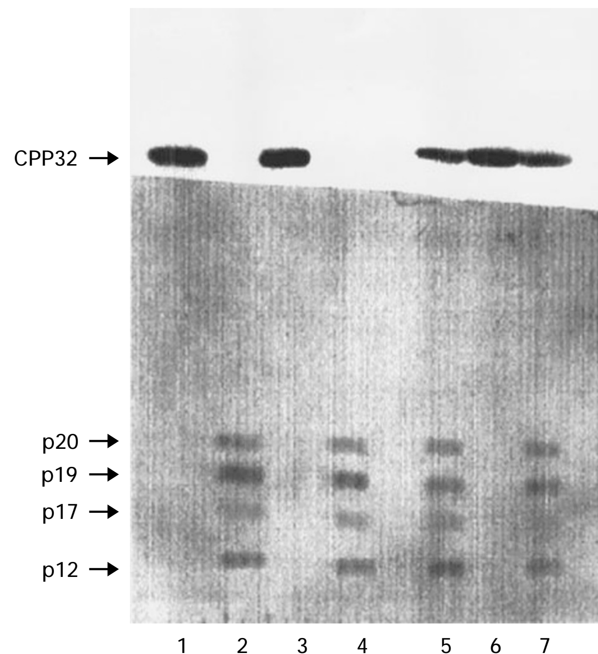

Figure 6 Sphingomyelinase inhibitors do not affect to anti-Fas and TNF-induced CPP32 activation. Jurkat (lanes 1–4) and U937 (lanes 5–7) cells were preincubated for 30 min in complete medium containing 10 μg/ml cycloheximide, 10 μg/ml monensin and 1 mM EDTA (lanes 3, 4, 6 and 7) or cycloheximide alone (lanes 1, 2 and 5). Then, cells were treated with 100 ng/ml of anti-Fas antibody (lanes 2 and 4) or 300 U/ml TNF (lanes 5 and 7) and the incubations prolonged for another 3.5 h (anti-Fas) or 5 h (TNF). Proteins from cell lysates were separated by 12% SDS–PAGE, transferred to nitrocellulose membranes and revealed with a specific anti-CPP32 antibody, as described in Materials and Methods. The upper part of the blot was developed for 5 min, and the lower part was further developed for 6 h to allow detection of the active protease fragments

Table 2 Effect of protease inhibitors on Fas or TNF-induced cell death

	PS exposure (% of cells)	Apoptotic nuclei (% of cells)	Cell death (%)
Jurkat			
Control	1.8±0.1	1.9	0
+anti-Fas	62.6±2.0	71.2	66.1±3.9
+anti-Fas+Ac-DEVD-CHO	3.0±0.2	2.6	2.6±0.5
+anti-Fas+Z-VAD-fmk	1.6±0.2	1.0	0.1±4.0
U937			
Control	1.1±0.5	3.0	0
+TNF	42.9±2.5	64.0	51.6±3.0
+TNF+Ac-DEVD-CHO	41.0±3.0	ND	48.8±5.5
+TNF+Z-VAD-fmk	43.5±3.5	2.1	63.9±2.7
+TNF+Boc-D-fmk	54.3±4.3	4.1	52.7±2.0
+TNF+calpeptin	ND	ND	50.5±3.9
+TNF+Boc-D-fmk +calpeptin	32.5±1.7	3.9	41.0±1.5

Jurkat and U937 cells were preincubated or not (controls) for 3 h with Ac-DEVD-CHO (600 μM) or 1 h with Z-VAD-fmk (100 μM), Boc-D-fmk (50 μM) or calpeptin (10 μM), alone or in combination. Apoptosis was then induced by addition of either 50 ng/ml anti-Fas or 300 U/ml TNF and incubation for 15 h or 20 h, respectively. Cell death was determined by the MTT-assay, cells showing apoptotic nuclei by staining with p-phenylenediamine and phosphatidylserine (PS) exposure by annexin V-FITC binding, as described in Materials and Methods. Results are the mean±S.D. of at least four independent determinations. None of the peptide inhibitors at the concentrations used was cytotoxid for the cells *per se*. ND, not done

Fas is a membrane protein that also possesses a death domain, homologous to that of 55 kDa TNFR (Tartaglia *et al*, 1993). When cross-linked with antibodies or by FasL, the Fas death domain binds to several adaptor proteins which in turn bind and activate apoptotic proteases (caspases) (Boldin *et al*, 1996; Fernandes-Alnemri *et al*, 1996; Muzio *et al*, 1996). It has also been described that acidic and/or neutral sphingomyelinases became activated during Fas-mediated apoptosis and the available evidence suggests that, as in the case of TNF receptor, different regions of Fas may mediate the activation of each type of sphingomyelinases (Cifone *et al*, 1993; Adam-Klages *et al*, 1996). It remains unclear, however, which of these enzymes is relevant for Fas-mediated apoptosis (Cifone *et al*, 1993; Tepper *et al*, 1995; Gamen *et al*, 1996).

To address the question of the relative contribution of ceramide as a mediator of TNF and Fas-mediated apoptosis, we induced these processes in sensitive cells treated with inhibitors of acidic and/or neutral sphingomyelinases. We also evaluated the induction of apoptosis in cells treated with drugs that greatly increase or decrease the intracellular amount of ceramide. The results obtained indicate a lack of correlation between cellular ceramide generation and the extent of Fas-or TNF-induced apoptosis. Rapid apoptosis induced by TNF and anti-Fas in the presence of cycloheximide was associated only with a slight increase in cellular ceramide content (Figure 3). Cell death was not prevented by inhibitors of neutral and acidic sphingomyelinases (Figure 1) or of ceramide synthase (Figure 2). A similar lack of augmentation of the cell ceramide content in rapid Fas-mediated apoptosis induced by T-cell effectors expressing FasL (Anel *et al*, 1997), in apoptosis induced with anti-Fas antibodies in murine thymocytes (Redondo *et al*, 1996) and in murine hepatocytes (Rouquet *et al*, 1996) has also been observed. In addition, a substantial reduction in the cell ceramide and sphingomyelinase content by chronic treatment with FB_1 did not affect to TNF- or anti-Fas cytotoxicity (Figure 4).

There are several differences, that still remain unexplained, concerning the mechanisms of apoptosis induced by Fas, TNF or ceramide. Fas or TNF-induced apoptosis is blocked by the viral serpin *CrmA* (Tewari and Dixit, 1995) but it is not prevented by inhibitors of protein kinases and phosphatases (Wright *et al*, 1992; Anel *et al*, 1994; Schulze-Osthoff *et al*, 1994; Ji *et al*, 1995). On the contrary, ceramide-dependent activities are sensitive to kinase and phosphatase inhibitors (Wright *et al*, 1992; Yao *et al*, 1995) and ceramide-mediated apoptosis is not blocked by Ac-DEVD-CHO (Gamen *et al*, 1996) or *CrmA* (Geley *et al*, 1997). Moreover, TNF causes no toxicity in Jurkat and endothelial cells (Gamen *et al*, 1996; Slowik *et al*, 1996) (where it induces activation of NF-κB (Slowik *et al*, 1996; Van Antwerp *et al*, 1996)), differentiation in HL-60 cells (Greenblatt and Elias, 1992) and apoptosis in U937 cells (Gamen *et al*, 1996), in spite of causing comparable increases in the cell ceramide content in all these cell types (Kolesnick and Golde, 1994). However, addition of ceramide to culture media promotes apoptotic cell death in all Jurkat, HL-60 and U937 cell lines (Jarvis *et al*, 1994; Cuvillier *et al*, 1996; Gamen *et al*, 1996). In addition, induction of sphingomyelinase hydrolysis may be non-specifically triggered by the Fc region of any IgG, without causing any concomitant toxicity (Glick and Barenholz, 1996). The notion that the apoptotic pathways triggered by Fas and ceramide are different is also underscored by the fact that ceramide causes apoptosis in Jurkat-ρ° cells (Gamen *et al*, 1996) which lack the expression of the apoptotic protease CPP32 (Gamen S., Anel A. and Naval J., unpublished work), the main mediator of Fas-based apoptosis in Jurkat cells (Gamen *et al*, 1996; Schlegel *et al*, 1996).

While these results indicate that ceramide generation is not essential for Fas- and TNF-induced apoptosis, these processes were associated with the cleavage and activation of the caspase CPP32, especially in the case of Fas-induced apoptosis (Figure 5). Apoptosis via Fas is mediated by CPP32-like proteases, since it is prevented by Ac-DEVD-CHO (Anel *et al*, 1996; 1997; Gamen *et al*, 1996), which inhibits CPP32 activity, or by caspase inhibitors that block CPP32 activation (Table 2). The situation in the case of TNF-induced apoptosis is rather different. TNF also induces the cleavage of CPP32, but to a lesser extent than anti-Fas (Figure 5) and Ac-DEVD-CHO, while blocking CPP32 activity, did not prevent TNF-induced death of U937 cells. However, Z-VAD-fmk or Boc-D-fmk do effectively inhibit the characteristic apoptotic nuclear morphology (chromatin condensation and fragmentation) induced by TNF. A similar result, inhibition of nuclear fragmentation but not U937 cell death, has been recently

reported (Vanags *et al*, 1996). TNF-induced apoptosis has been reported to be prevented by the serpin *CrmA* in other cell lines, suggesting the implication of caspases (Tewari and Dixit, 1995), but the criteria followed for the inhibition of apoptosis was the absence of apoptotic nuclei. The present and previous data (Vanags *et al*, 1996) suggest that the known caspases alone do not seem to be the sole mediators of TNF-induced apoptosis, at least in U937 cells.

One of the consequences of Fas-induced apoptosis is the progressive cellular accumulation of ceramide (Tepper *et al*, 1995; Chinnayian *et al*, 1996; Gamen *et al*, 1996). This increase in ceramide levels is dependent on CPP32-like caspases, since the blockade of CPP32-like activity prevents both cell death and ceramide accumulation (Gamen *et al*, 1996). Cellular ceramide levels are also augmented during TNF-induced apoptosis (Erhardt and Cooper, 1996) and cell transfection with *CrmA* prevents again caspase activation and ceramide generation (Dbaibo *et al*, 1997). Exogenous ceramide is able to induce apoptosis in virtually all kinds of cultured cells and ceramide produced by acid sphingomyelinase is a key mediator of apoptosis induced by irradiation (Santana *et al*, 1996). Therefore, ceramide generation in late steps of TNF and Fas-mediated apoptosis could contribute to the spread of the death signal. In this respect, ceramide has been recently reported to be rather a 'gauge' or a 'sensor' than an 'executor' of apoptosis induced by physiological means (Zhang *et al*, 1996).

In conclusion, while ceramide does not seem to be an essential downstream mediator of TNF and Fas-mediated apoptosis, it is generated after TNF receptor and Fas triggering and can function as a molecular sensor of apoptosis and/or participate in the secondary spread of the death signal.

Materials and Methods

Materials

Fumonisin B_1 (FB_1), from *Fusarium moniliforme*, neutral sphingomyelinase from *Staphylococcus aureus*, chloroquine, monensin, cycloheximide, EDTA and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were products from Sigma (Madrid, Spain). Human recombinant TNF-α was kindly provided by The National Institute for Biological Standards and Control (Hertfordshire, UK). Cytotoxic mouse monoclonal anti human Fas IgM antibody (clone CH-11) was from UBI (Lake Placid, USA) and mouse IgG2a anti human CPP32 (clone 19) from Transduction Laboratories (Affiniti, Mamhead, UK). Acetyl-Asp-Glu-Val-Asp aldehyde (Ac-DEVD-CHO), benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) were from Bachem (Bubendorf, Switzerland). t-butyloxycarbonyl-Asp-fluoromethylketone (Boc-D-fmk) was from Enzyme Systems (Dublin, CA, USA) and benzyloxycarbonyldipeptidyl aldehyde (Calpeptin) from Novabiochem (Läufelfingen, Switzerland).

Cell culture and analysis of cell death

Human promonocytic leukemia U937 and the Jurkat T-cell leukemia (ATCC, clone E6.1) were cultured in RPMI 1640 medium (Biowhittaker, Barcelona, Spain) supplemented with 5% fetal calf serum, L-glutamine and penicillin/streptomycin (hereafter, complete medium), using standard cell culture procedures. For cell death assays, cells were seeded in flat-bottom, 96-well plates at an initial density of $3-5\times10^5$ cells/ml (100 μl/well), and cultured for 3–6 h in complete medium containing 10 μg/ml cycloheximide and 300 U/ml TNF or 100 ng/ml anti-Fas antibodies. The effect of several drugs, which interfere with ceramide metabolism, on TNF and Fas-mediated apoptosis was also evaluated. These were FB_1 (25–50 μM), an inhibitor of ceramide synthase (Jaffrézou *et al*, 1996), the lysosomotropic agents chloroquine (10 μM), ammonium chloride (8 mM) and monensin (1–10 μg/ml), all of which inhibit acid sphingomyelinase activity (Wiegmann *et al*, 1994; Tepper *et al*, 1995; Andrieu *et al*, 1996), and EDTA (1 mM), a Ca^{2+}- and Mg^{2+}-chelator which inhibits the activity of neutral, Mg^{2+}-dependent, sphingomyelinase (Futerman *et al*, 1990). Chloroquine was dissolved in RPMI medium, EDTA and FB_1 in PBS and monensin in ethanol. At the doses used (0.1%, final concentration), ethanol had no effect on cell proliferation. Cells were incubated with inhibitors for 30 min prior to the addition of TNF or anti-Fas antibodies.

In other experiments, Jurkat and U937 cells were also cultured for 3 days in complete medium containing 50 μM FB_1 to deplete cells of sphingomyelin and ceramide (Hidari *et al*, 1996; Jaffrézou *et al*, 1996). Conversely, the intracellular levels of ceramide were raised by culturing U937 cells for 36 h in medium containing 1 μg/ml monensin (Kallen *et al*, 1993). Then 50 ng/ml anti-Fas or 300U/ml TNF were added to cultures and cells incubated for another 15 or 20 h, respectively. Cultures containing the same amounts of drugs, but no TNF or anti-Fas, were used as controls. To assess the effect of caspase inhibitors on Fas and TNF-mediated apoptosis cells were incubated for 1 h with either Z-VAD-fmk (100 μM), Boc-D-fmk (50–100 μM) or calpeptin (10 μM) or for 3 h with Ac-DEVD-CHO (600 μM), prior to the treatment with anti-Fas or TNF, respectively. In all cases, cell viability was determined by the Trypan blue-exclusion test and by a modification of the MTT-reduction method of Mosmann (Alley *et al*, 1988). Percentage of cell death was calculated as follows:

$$\text{Cell death }(\%) = (1-\frac{A_{550}\text{ (samples)} - A_{550}\text{ (blanks)}}{A_{550}\text{ (controls)} - A_{550}\text{ (blanks)}}\times 100$$

Cell death was also quantified by measuring phosphatidylserine exposure by using annexin V-FITC (Bender Medsystems, Germany), according to the instructions of manufacturer. Briefly, cells were incubated in the dark with 0.5 μg/ml annexin V-FITC in binding buffer (10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl, 2.5 mM $CaCl_2$) at 4°C for 30 min, washed and annexin binding analyzed in an Epics XL-MCL (Coulter, Spain) flow cytometer. Nuclear apoptosis (chromatin condensation and fragmentation) in cells treated with anti-Fas or TNF was evaluated by staining cytocentrifuge-prepared cell smears with the nuclear stain *p*-phenylenediamine, as described the previously (Gamen *et al*, 1996).

Analysis of Fas and TNFR expression

The membrane expression of Fas and the 55 kDa TNFR was analyzed by flow cytometry by using the mouse monoclonal antibodies anti human Fas (IgG2b, clone SM1/23) and anti human 55 kDa TNFR (IgG2a, clone H398) both from Bender Medsystems. Cells (5×10^5 in 50 μl) were incubated at 4°C in PBS containing 0.2% BSA and 0.02% sodium azide and 5 μg/ml of the corresponding antibody for 1 h. Then, cells were washed with

PBS, incubated with a 1:250 dilution of a FITC-labelled goat anti mouse IgG (Caltag, Barcelona, Spain) for 30 min, fixed with 1% paraformaldehyde in PBS, pH 7.4, for 15 min, and 5000 cells/sample analyzed by flow cytometry.

Analysis of CPP32 activation

CPP32 activation was evaluated by Western blot analysis of cell homogenates with a specific anti-human CPP32 antibody. Jurkat and U937 cells (5×10^6 in 1 ml of RPMI 1640 medium containing 1 mg/ml BSA and 10 μg/ml cycloheximide) were treated, in the presence or absence of a combination of 1 mM EDTA and 10 μg/ml monensin, at 37°C with 100 ng/ml anti-Fas for 3.5 h or with 300 U/ml TNF for 5 h, respectively. At the end of incubations, cells were recovered by centrifugation at 4°C, washed twice with cold PBS and lysed in 1 ml of lysis buffer (50 mM Tris/HCl, pH 7.6, containing 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 30 mM NaF, 10 μg/ml leupeptin and 1 mM PMSF), as previously described (Alava *et al*, 1992). Cell lysates were centrifuged (4°C, 12 000 *g*, 15 min) and proteins separated by SDS–PAGE in a SDS/12%-polyacrylamide gel under reducing conditions and transferred to nitrocellulose membranes (Hybond-C extra, Amersham, Madrid, Spain). Membranes were then sequentially incubated with 50 ng/ml anti-CPP32 antibody in PBS containing 5% BSA and with 0.2 μg/ml goat anti-mouse IgG coupled to alkaline-phosphatase (Sigma) for 1 h and revealed with BCIP/NBT. After 5 min of colour development, blotting membranes were cut after the position of the 32 kDa bands, corresponding to the inactive CPP32 protease, and the portion of blotting membrane containing the p20 to p12 fragments of the active protease further developed for 6 h to allow their detection.

Analysis of ceramide and sphingomyelin levels

Cells (5×10^6 in 10 ml of complete medium) were labelled for 48 h with 5 μCi of [1-^{14}C]palmitic acid (specific activity 55.0 mCi/mmol, Amersham) bound to fatty acid-free serum albumin (1:1, molar ratio). Cells were harvested, resuspended at 5×10^5 cells/ml in complete medium containing 10 μg/ml cycloheximide or the same medium plus 1 mM EDTA and 10 μg/ml monensin, and preincubated for 30 min. Then, Jurkat cells were treated with 100 ng/ml anti-Fas antibody for 3.5 h, and U937 cells with 300 U/ml TNF for 6 h. In other experiments, Jurkat and U937 cells were cultured in complete medium containing 50 μM FB_1 for 24 h. Then, 0.5 μCi/ml of [1-^{14}C]palmitic acid were added to cultures and incubation prolonged for a further 48 h period (Jaffrézou *et al*, 1996). Jurkat and U937 cells were also labelled with [1-^{14}C]palmitic acid in medium containing 1 μg/ml monensin for 36 h. Then, FB_1 and monensin-treated cells were incubated with 50 ng/ml anti-Fas or 300 U/ml TNF in fresh medium containing the same concentration of either FB_1 or monensin and incubated for 15 h. In all cases, at the end of incubations, cells were harvested, counted and washed with ice-cold PBS. Total cell lipids were extracted with chloroform/methanol (2:1, v/v) (Anel *et al*, 1992), and cholesterol, ceramide and sphingomyelin resolved by TLC in silica gel G plates (20×20 cm), essentially as described previously (Gamen *et al*, 1996). Briefly, the radioactivity recovered in cell lipids was determined in aliquots of chloroform extracts and equal amounts of radioactivity (around 3×10^5 c.p.m.) for each sample were applied to TLC plates. Sequential one-dimensional TLC was performed with chloroform/methanol/water (60:30:5, v/v) up to 12 cm from the bottom of the plate followed by a second development to its full length with *n*-hexane/diethyl ether/acetic acid (80:20:2, v/v). Radiolabelled lipids were located by autoradiography (Hyperfilm β-max, Amersham) at room temperature for 2 days and radioactivity quantitated by liquid scintillation counting. Results were expressed as ceramide to sphingomyelin ratio. Similar results were obtained when ceramide levels were normalised to cholesterol or total phospholipid levels (Gamen *et al*, 1996).

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