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Cholera Toxin B-Subunit Prevents Activation and Proliferation of Human CD4+ T Cells by Activation of a Neutral Sphingomyelinase in Lipid Rafts

Alexandre K. Rouquette-Jazdanian,* Arnaud Foussat,† Laurence Lamy,* Claudette Pelassy*, Patricia Lagadec,† Jean-Philippe Breitmayer,* and Claude Aussel2*

The inhibition of human CD4+ T lymphocyte activation and proliferation by cholera toxin B-subunit (CTB) is a well-established phenomenon; nevertheless, the exact mechanism remained unclear. In the present study, we propose an explanation for the CTB-induced inhibition of CD4+ T lymphocytes. CTB specifically binds to GM1, a raft marker, and strongly modifies the lipid composition of rafts. First, CTB inhibits sphingomyelin synthesis; second, it enhances phosphatidylcholine synthesis; and third, it activates a raft-resident neutral sphingomyelinase resembling to neutral sphingomyelinase type 1, thus generating a transient ceramide production. We demonstrated that these ceramides inhibit protein kinase Cε phosphorylation and its translocation into the modified lipid rafts. Furthermore, we show that CTB-induced ceramide production activate NF-κB. Combined all together: raft modification in terms of lipids, ceramide production, protein kinase Cε inhibition, and NF-κB activation lead to CD4+ T cell inhibition. The Journal of Immunology, 2005, 175: 5637–5648.

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*Abbreviations used in this paper: CTB, cholera toxin B-subunit; ETB, enterotoxin B-subunit; hPBMC, human PBMC; SM, sphingomyelin; PtdCho, phosphatidylcholine; SMase, sphingomyelinase; PKCα, protein kinase Ca; 7-AAD, 7-aminoactinomycin D; m-β-CD, methyl-β-cyclodextrin; NAC, N-acetyl-l-cysteine; GSH, glutathione; PDMP, 1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol; FB1, fumonisin B1; PNS, postnuclear supernatant; PVDF, polyvinylidene difluoride; HBSS, HEPES saline buffer; DAG, diacylglycerol; C-1-P, ceramide-1-phosphate; pCTB, pu- rified CTB; DRM, detergent-resistant membrane; FA, fatty acid; PtdEtn, phosphatidyethanolamine; ASM, acidic SMase; SMS, SM synthase.
rCTB-INDUCED SMase ACTIVATION CAUSES CD4+ T CELL INHIBITION

In vivo T lymphocyte proliferative responses

Positive selection of CD4+ T lymphocytes from freshly isolated hPBMCs was first performed using a fluorescence activated cell sorter (FACStar+; BD Biosciences). Reanalysis of the sorted population showed a purity higher than 98%. Purified CD4+ T lymphocytes were extensively washed then resuspended in prewarmed culture medium at a cellular concentration of 1 x 10^6/ml. Cell suspension was cultured in triplicate sets in flat-bottom 24-well plates containing 200 µl/well. Cells pretreated or not with either rCTB (10 µg/ml) and/or others reagents (as detailed in the figure legends) were stimulated or not by either PMA (10 ng/ml) plus ionomycin (100 nM) or soluble anti-CD3 mAb (5 µg/ml) plus anti-CD28 mAb (5 µg/ml) for 72 h. The cultures were pulsed with 1 µCi/well [3H]thymidine during the last 16 h. Cells were harvested with a semiautomatic cell harvester and counted by liquid scintillation in a Beckman Tricarb scintillation spectrometer. Results are expressed as mean cpm ± SEM of triplicate cultures.

Cell surface receptors staining

hPBMCs or Jurkat cells (1 x 10^6) were washed in cold PBS supplemented with 0.1% BSA (pH 7.4) then incubated for 30 min in the dark at 4°C with the appropriate fluorochrome-conjugated mAb according to the manufacturer’s instructions. For GM1 indirect-staining, Jurkat cells were first incubated with biotin-labeled CTB (10 µg/ml). Then cells were washed and incubated (30 min at 4°C) in 100 µl of 1/25 dilution of RPE-Cy5-conjugated streptavidin. Cells were washed again and fixed with 0.37% formaldehyde. 

Cytometric analysis of T cell activation markers

Freshly isolated hPBMCs were washed then resuspended in prewarmed culture medium at a cellular concentration of 1 x 10^6/ml. Cell suspension was dispensed in triplicate sets into flat-bottom 96-well plates in volume of 200 µl/well. hPBMCs pretreated or not with the indicated drugs (as detailed in the figure legends) were stimulated or not by soluble anti-CD3 mAb (5 µg/ml) plus anti-CD28 mAb (5 µg/ml) for 20 h. Then, cells were costained with a PE-CD25 mAb, a FITC-CD69 mAb, and a PerCP-CD4 mAb. hPBMCs were gated on lymphocytes according to their forward and side angle light scatter. CD25 and CD69 surface expression on CD4+ T lymphocytes was determined flow cytometry after gating lymphocytes on the basis of membrane expression of CD4. CD25 and CD69 up-regulation was also examined on Jurkat cells pretreated or not with the indicated drugs (as detailed in the figure legends) then stimulated or not with PMA (10 ng/ml) plus ionomycin (100 nM) for 20 h. The mean fluorescence intensity of 5000 cells was determined by flow cytometry (FACScan; BD Biosciences).

Viability measurement of treated cells

hPBMCs, treated or not with rCTB (10 µg/ml) for 72 h, were stained with a FITC-CD4 mAb. Then cells were incubated with both annexin-PE and 7-AAD according to the manufacturer’s specifications. 7-AAD can be excited by the 488-nm argon laser line and emits in the far red range of the spectrum; consequently, its spectral emission can be separated from the emissions of FITC and PE. The fluorescence parameters allow characterization of necrotic cells (annexin-PE+7-AAD+), apoptotic cells (annexin-PE-/7-AAD-), and viable cells (annexin-PE-/7-AAD-) in the chosen subset of FITC+ cells. Moreover, viability of FB1- and PDDM-treated Jurkat cells was also compared with control Jurkat using the same technique.

Lipid raft isolation

Rabbit polyclonal Ab anti-phospho-PKCα (clone M4, IgG1), rabbit polyclonal Ab anti-p56Lck (IgG), and rabbit polyclonal Ab anti-LAT (IgG) were obtained from Upstate Biotechnology. Rabbit polyclonal Ab anti-PLCγ1 were purchased from Santa Cruz Biotechnology. Anti-CD3 mAb (clone X3, IgG2a) and anti-CD28 mAb (clone 28.2, IgG1) were produced in our laboratory. CD4-PE, PerCP (clone SK3, IgG1), CD69-FITC (clone FN50, IgG1), CD25-PE (clone M-A251, IgG1), and apoptosis detection kit (annexin V-PE, 7-aminoactinomycin D (7-AADD)) were purchased by BD Pharmingen. Peroxidase-labeled anti-rabbit IgG and rabbit anti-mouse IgG conjugated to peroxidase were obtained from Rockland, and R-PE-Cy5-conjugated streptavidin was obtained from DakoCytomation. Methyl-β-cyclodextrin (m-β-CD), CTB, biotin-labeled CTB, monosialoganglioside-GM1, N-acetyl-p-cysteine (NAC), glutathione (GSH), cGMP, PMA, ionomycin, pepstatin, leupeptin, chymostatin, and anti-PMSF were purchased from Sigma-Aldrich. α1,4-macroglobulin was purchased from Roche. 1,2-octanediol-O-[1-14C]Hcholesterol (1.3–1.85 TBq/mmol), methyl-[14C]cholesterol (2.22–3.14 TBq/mmol, 9,10(11)-[3H]palmitic acid (37 MBq/mmol), [methyl-3H]thymidine (740 GBq/mmol), γ-[32P]ATP, and [N-methyl-14C]Choline chloride (2.04 TBq/mmol, 55 mCi/mmol) were purchased from Amersham Biosciences.

Immunoblot analysis

Aliquots (50 µl) of each lane were boiled, and the proteins were separated on an SDS-PAGE gel (15% resolving gel, 4% stacking gel). The proteins were electroblotted onto a PVDF membrane. Membranes were then incubated with a rabbit polyclonal Ab anti-p56Lck (Ser296) for 2 h at room temperature. The rabbit polyclonal Ab anti-phospho-PKCα was purchased from Santa Cruz Biotechnology. Membranes were blocked for 2 h at room temperature in a blocking buffer containing 5% dry milk and incubated in the dark at 4°C. Membranes were incubated for 2 h with rCTB (10 µg/ml) and then incubated for 1 h with the appropriate Ab diluted 1000-fold in the same buffer. Membranes were washed extensively in TBS containing 0.1%Tween 20. Detection was performed with HRP-conjugated anti-rabbit or anti-mouse and ECL reagents (Amer- sham Biosciences) according to manufacturer’s instructions. For phospho-PKCα immunoblotting, aliquots of fraction B (fraction 2 + 3) solubilized in the same volume of 2x Hoeschel buffer were loaded on 10% SDS-PAGE and then transferred to PVDF membranes as described above. Membranes were blocked for 2 h at room temperature in a blocking buffer containing 5% (w/v) BSA in TBS and subsequently incubated with rabbit polyclonal Ab anti-phospho-PKCα (Ser296) for 2 h at room temperature. Phospho-PKCα signals were detected with HRP-conjugated goat anti-rabbit, followed by ECL. Membranes were then stripped in a buffer (pH 6.7) containing 62.5 mM Tris-HCl, 2% SDS, and 0.7% 2-ME and reblotted with an Ab against PKCα.

Sphingolipid content manipulation

To reduce cellular sphingolipid content, Jurkat cells were cultured as control cells in a medium supplemented with either FB1 (10 µM final concentration) or PDDM (10 µM final concentration) for 4 days; FB1 and
PDMP were included in each medium change. Inhibition of sphingolipid synthesis was monitored by analyzing surface expression of GM1 by flow cytometry. GM1 replenishment of sphingolipid-depleted Jurkat cells was conducted by incubating cells in serum-free RPMI 1640 containing GM1 (0.5 mg/ml for 30 min) at 37°C.

Glycerophospholipids and SM content analysis

Jurkat cells were washed then incubated for 16–18 h in a HEPES saline buffer (HSB) (pH 7.4), containing 137 mM NaCl, 2.7 mM KCl, 1 mM Na2HPO4, 12 H2O, 2.5 mM glucose, 20 mM HEPES, 5 mM MgCl2, 1 mM CaCl2, and 0.1% BSA at 37°C in the presence of 4 μCi of either [3H]palmitic acid or [3H]choline chloride. Lipids were extracted and analyzed from either whole cells (a) or fractions obtained after ultracentrifugation (b).

(a) rCTB-treated cells or control cells were rapidly sedimented, supernatants were discarded, and cell lipids were extracted with chloroform/ methanol according to Bligh and Dyer (19) then separated by monodimensional thin-layer chromatography on plates LK60 Silica Gel 60 A (Whatman) in a solvent system composed of chloroform/methanol/acetic acid/water (75/45/12/3). Authentic phospholipid standards (Sigma–Aldrich) were run in parallel and detected with iodide vapors. Radioactivity in lipid spots was determined by using an automatic linear radiochromatography analyzer, Tracermaster 20 (Berthold), equipped with an 8-mm window and the integration software supplied by the manufacturer.

(b) An aliquot (50 μl) of each different fraction obtained after ultracentrifugation on sucrose density-gradient was extracted and analyzed as described above.

SM synthesis measurement

Jurkat cells (2 × 106) were maintained in 500 μl of HSB. At time 0, 4 μCi of either [3H]palmitic acid or [3H]choline chloride were added, with or without rCTB, at the end of the treatment, and lipids were extracted and analyzed as described above.

Cholesterol analysis

[3H]Cholesterol in toluene solution was first evaporated under N2, and dissolved in ethanol just prior its use. Jurkat cells were washed then incubated for 16–18 h in HBS containing 4 μCi of [3H]cholesterol. Raft purification was performed as detailed above. To determine the distribution of [3H]cholesterol, an aliquot (50 μl) of each different fraction obtained after ultracentrifugation on sucrose density-gradient was mixed with Picofluor and counted by liquid scintillation in a Beckman Tricarb scintillation spectrometer.

Assays for neutral- and acidic-SMase

The activity of neutral- and acidic-SMase was calculated by using a combination of published protocols (20–22). To prepare a stock solution of 50 μM radioactive SM substrate, 55 μl (1357 μCi, 25 nmol) of [N-methyl-14]C]SM (55 mCi/mmol, 10 μCi/400 μl in toluene/ethanol, 1:1, v/v) were placed in a glass tube, and the organic solvent was removed under N2. The dried [14]C]SM was solubilized in 500 μl of 1% (w/v) β-octylglucoside by brief sonication with a bath-type sonicator. Fifty-microliter aliquots of selected fractions (2 + 3 and 8 + 9) were assayed for the presence of different SMase activities. Reactions were started by adding 50 μl of substrate solution. For the measurement of the neutral-SMase activity, this solution consisted in 10 μl of the stock solution of [14]C]SM (0.5 nmol), 10 μl of a buffer consisting in 250 mM HEPES (pH 7.5), 50 mM MgCl2, and 0.5% (v/v) Triton X-100 and 30 μl of deionized water. After incubation at 37°C for 3 h, the reaction was stopped by adding 800 μl of chloroform/methanol (2/1, v/v) and 200 μl of deionized water. A 100-μl aliquot of the aqueous upper phase containing [14]C]phosphorylcholine released from [N-methyl-14]C]SM was collected and counted by liquid scintillation. The reaction was linear within this frame, and the amount of [N-methyl-14]C]SM hydrolyzed during an assay did not exceed 10% of the total amount of radioactive SM added. For calculation of the specific activities, values were corrected for volume of the aqueous phase, volume of the sample, protein content, reaction time, and specific activity of the substrate. For the assay of acidic-SMase activity, the substrate solution consisted in 10 μl of the stock solution of [14]C]SM (0.5 nmol) and 10 μl of 0.5 M sodium acetate buffer (pH 4.8), consisting of 10 mM EDTA, 0.5% (v/v) Triton X-100, and 30 μl of deionized water. The initiation and termination of the reaction and the determination of the water-soluble radioactivity released from [N-methyl-14]C]SM was proceed as described above.

Diacylglycerol (DAG) kinase assays

Total cellular ceramide levels were quantified by the DAG kinase assay as 32P incorporated upon phosphorylation of ceramide to ceramide-1-phosphate (C1-P) by DAG kinase from Escherichia coli (23). After iCTB treatment (10 μg/ml) for different period of time, Jurkat cells (5 × 106) were washed twice with ice-cold PBS. After centrifugation (1000 × g, 5 min, 4°C), lipids were extracted with 1 ml of chloroform/methanol/hydrochloric acid (1 N) (100/100/1, v/v/v), 170 μl of buffered saline solution (135 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 5.5 mM glucose, and 10 mM HEPES (pH 7.2)), and 30 μl of 100 mM EDTA. The lipids of the organic phase were transferred to a new glass vial and dried under a stream of N2. Lipid extracts were then subjected to mild alkaline hydrolysis (1.0 M KOH in methanol for 1 h at 37°C) to remove glycerophospholipids. Five hundred microliters of chloroform, 270 μl of buffered saline solution, and 30 μl of 100 mM EDTA were added. After drying the organic phase with N2, in vitro phosphorylation of extracted ceramides was performed as described by the manufacturer (RPN 200 kit; Amersham Biosciences). A total of 1 μCi of [γ-32P]ATP (4000 Ci/mmol) was used to start the reaction. After 30 min at room temperature, the reaction was stopped by extraction of lipids with 1 ml of chloroform/methanol/hydrochloric acid (1 N) (100/100/1, v/v/v), 170 μl of buffered saline solution, and 30 μl of 100 mM EDTA. The lower organic phase was dried under N2. The samples were resuspended in 30 μl of chloroform/methanol (95/5, v/v) and spotted on plates LK60 Silica Gel 60 A. C-1-P was resolved by TLC with chloroform/methanol/acetic acid (75/25/5, v/v/v) as solvent and migrated as a single spot at RF 0.25. Linearity of the assay was established using purified C1-ceramide (Sigma–Aldrich). Radioactivity in lipid spots was determined by using an automatic linear radiochromatography analyzer, Tracermaster 20 (Berthold), equipped with an 8-mm window and the integration software supplied by the manufacturer.

Semiquantitative RT-PCR

After cell treatment, total RNA was isolated from Jurkat cells using TRIzol Reagent (Invitrogen Life Technologies) based on method derived by Chomczynski and Sacchi (24). RNA (150 ng) was then reverse transcribed using the SuperScript II RNAase H– reverse transcriptase (Invitrogen Life Technologies) following the manufacturer’s instructions and resuspended in 150 μl of distilled water. cDNAs (5 μl) or water as control were amplified by PCR in a final volume of 25 μl using the Platinum TaqDNA Polymerase (Invitrogen Life Technologies) and 300 nM of forward and reverse primers. RT-PCR was typically performed for 35 cycles (denaturation at 95°C for 20 s, annealing at 68°C for 1 min, extension at 72°C for 1 min). Primers were designed using the PRIMER Express Software 1.5 (Applied Biosystems). The following 5′ and 3′ primers were as follows: human CD69, 5′ primer (5′-GCTGACGAGGAAACGCTTGTGC-3′) and 3′ primer (5′-ACAGGAGCAAGACTTTGAGAAGA-3′), and human CD25, 5′ primer (5′-GAGGGGACTGTCACCGTCAACTCA-3′) and 3′ primer (5′-TTTACACTAGTTTCTCCTTGTAG-3′). β-actin was used as loading control.

EMSA

Total cellular extracts were prepared in Topex lysis buffer (20 mM HEPES (pH 7.9), 350 mM NaCl, 20% glycerol, 1% Nonidet P-40, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, and 10 μg/ml aprotinin). Supernatants from a 15,000 × g for 25 min at room temperature. Complexes were then separated by electrophoresis on a 5% nondenaturing polyacrylamide gel in 0.5 × Tris-borate EDTA. Dried gels were subjected to autoradiography.

Luciferase assays

Jurkat cells were transiently transfected by electroporation (320 V, 960 μF) with 10 μg of a luciferase reporter gene controlled by a minimal thymidine kinase promoter and six reiterated xB sites (xB6 thymidine kinase luc). At 36 h after transfection, cells were stimulated as indicated. Cells were washed twice in PBS (pH 7.2) and lysed in 100 μl of reporter lysis buffer (Promega). Luciferase activity was assayed by luminometry (Lumat; EG&G Berthold) using the Promega luciferase assay system. Normalization of luciferase transfection efficiency was done using a cotransfected β-galactosidase expression vector. Luciferase activity was determined in triplicate and expressed as fold increase relative to basal activity seen in untreated unstimulated mock-transfected cells.
Results
GM1-rCTB interaction but not GM1-anti-GM1 Abs interaction inhibits PMA/ionomycin-induced CD4\(^+\) T proliferation

Previous works have demonstrated that CTB is able to inhibit T cell activation and proliferation induced by either polyclonal mitogens or by specific Ags (9–11). To see whether GM1 binding alone is sufficient or not to inhibit PMA/ionomycin-induced CD4\(^+\) T proliferation, CD4\(^+\) T lymphocytes were pretreated with various concentrations of either purified CTB (pCTB), rCTB, or rabbit polyclonal anti-GM1 Abs, then cells were stimulated with PMA/ionomycin, and proliferation was measured. Fig. 1A unambiguously demonstrates that PMA/ionomycin-induced CD4\(^+\) T cell proliferation is inhibited by both pCTB and rCTB in a dose-dependent manner. Our result unambiguously demonstrates that the inhibition of the proliferation of CD4\(^+\) T lymphocytes is not due to cAMP produced by contaminant cholera toxin A-subunit because rCTB gives the same results as pCTB. Furthermore, we investigated whether cGMP inhibit the effects of pCTB. Indeed, if pCTB is contaminated by cholera toxin A-subunit, it will produce cGMP, and it is well known that cGMP and cAMP have antagonistic action on proliferation of T lymphocytes (25). As shown in Fig. 1A, cGMP does not prevent the pCTB-induced inhibition of the PMA/ionomycin-induced proliferation of CD4\(^+\) T lymphocytes, thus indicating that pCTB does not exert its effect via AMPc. In contrast with rCTB, anti-GM1 Abs have no effects on PMA/ionomycin-induced CD4\(^+\) T cell proliferation. In conclusion, GM1 binding by specific Abs is not able to inhibit CD4\(^+\) T cells, whereas GM1-rCTB interaction is necessary to exert inhibitory effect. Furthermore, we questioned whether Ac anti-GM1 would potentiate the effects of rCTB or would block its activity. CD4\(^+\) T lymphocytes incubated with a combination of rCTB plus Ac anti-GM1 were stimulated by PMA/ionomycin and \([^{3}H]\)thymidine incorporation was measured. As shown in Fig. 1A, Ac anti-GM1 neither potentiates nor blocks the effect of rCTB on PMA/ionomycin-induced proliferation. Fig. 1B clearly shows that the epitopes recognized by either rCTB or Ac anti-GM1 are different. There is no competition between rCTB and Ac anti-GM1 for the binding of GM1. This result may explain why Ac anti-GM1 does not block the action of rCTB. The epitope recognized by Ac anti-GM1 may be not involved in the inhibition of CD4\(^+\) T lymphocytes because they do not potentiate the effects of rCTB.

The integrity of cholesterol-rich raft is not required for rCTB-induced inhibition of CD4\(^+\) T lymphocytes

The monosialoganglioside GM1 is certainly the most commonly used lipid raft marker. Cholesterol extraction by m-β-CD disrupts cholesterol-rich rafts and raft-resident molecules leave rafts. However, a recent study (26) showed that depletion of 73% of cell cholesterol with m-β-CD significantly affects the recovery in detergent-resistant membranes (DRMs) of GM1 acetylated or acylated with C\(_{18}\), C\(_{22}\), or C\(_{24}\) FAs. To see whether cholesterol-rich raft integrity is required for rCTB-induced CD4\(^+\) T cell inhibition, we first treated hPBMCs with m-β-CD to disrupt cholesterol-rich rafts. Then cholesterol-depleted hPBMCs were pretreated with rCTB or pCTB and cells were stimulated with PMA/ionomycin. As shown in Fig. 2A, cholesterol depletion of CD4\(^+\) T lymphocytes with m-β-CD does not prevent rCTB and pCTB to inhibit PMA/ionomycin-induced CD69 and CD25 up-regulation. The same results were obtained in Jurkat cells (Fig. 2B).

SM is necessary for rCTB-induced CD4\(^+\) T cell inhibition

Because cholesterol is not required for rCTB-induced inhibition of CD4\(^+\) T lymphocytes, we investigated a role for SM in rCTB-inhibited CD4\(^+\) T Inhibition. To this end, we used Jurkat cells instead of hPBMCs because these cells are more suitable for studying lipid metabolism because their global metabolism is greatly

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

**FIGURE 1.** Effect of rCTB and rabbit polyclonal Abs anti-GM1 on PMA plus ionomycin-induced CD4\(^+\) T lymphocyte proliferation. A, FACS-sorted CD4\(^+\) T lymphocytes were pretreated or not in 96-well flat-bottom plates with either pCTB, rCTB, rabbit polyclonal anti-GM1, pCTB + cGMP, or rCTB + Abs anti-GM1 for 30 min at the indicated concentrations, then CD4\(^+\) T lymphocytes were stimulated or not with 10 ng/ml PMA plus 100 nM ionomycin. Proliferation was monitored by \([^{3}H]\)thymidine incorporation during the last 16 h of culture. CD4\(^+\) T lymphocytes were harvested for beta scintillation counting. Data represent one of three similar experiments. The values represent means ± SEM. B, Jurkat cells (1 \(\times\) 10\(^6\)) were first incubated with Abs anti-GM1 (30 min at 4°C), then GM1 receptors were stained with pCTB-biotin + streptavidin-RPE-Cy5 (30 min at 4°C) as indicated in Materials and Methods. GM1 staining was measured by cytometric analysis.
more rapid than those of hPBMCs. Indeed, Jurkat cells do spontaneously proliferate whereas hPBMCs do not. Incorporation of $[^3H]$palmitic acid or $[^3H]$choline is quite equal in Jurkat cells and in hPBMCs. But the kinetic of SM synthesis is completely different. After a 4 h-incubation with triitated precursors, it is already possible to easily detect SM synthesis in Jurkat cells, whereas it is impossible in hPBMCs. We used two drugs to modulate the level of SM and/or gangliosides: FB1 and PDMP. FB1 prevents the production of both SM and gangliosides (27). PDMP specifically diminishes levels of endogenous gangliosides (28, 29). Note that PDMP treatment also results in a significant accumulation of SM (30).

We studied the surface expression of GM1 both in FB1- and PDMP-treated Jurkat cells. As shown in Fig. 38, FB1 and PDMP considerably diminish the level of the ganglioside GM1 after a 4-day treatment. Reduction of GM1 is time dependent, but treatments cannot be longer extended because beyond a 4-day incubation, cell viability starts to decline. After a 4-day treatment, FB1-treated cells exhibits only 34% of control GM1 and cell viability is greater than 93%. PDMP-treated Jurkat only possess 29% of GM1, and cell viability is not affected at all. Incubation of GM1-depleted Jurkat with exogenous GM1 (0.5 mg/ml for 30 min) approximately multiplies by 3.5 the basal content.

PDMP inhibits GM1 synthesis whereas FB1 inhibits both SM and GM1 synthesis. As shown in Fig. 3C, rCTB largely inhibits PMA/ionomycin-induced CD69 and CD25 up-regulation (bar 4 vs bar 2). As expected, rCTB has little effect on CD69 and CD25 expression on PDMP-treated Jurkat cells (bar 7 vs bar 6), but rCTB re-exerts its important inhibitory effect on GM1-restored Jurkat cells (bar 8 vs bars 7 and 4). It indicates that rCTB can inhibit PMA/ionomycin-induced CD69 and CD25 up-regulation either via endogenous or exogenous GM1. It means that exogenous GM1 is as active as endogenous one. Furthermore, as expected, rCTB has little effect on CD69 and CD25 expression on FB1-treated Jurkat cells (bar 11 vs bar 10). In sharp contrast with PDMP treatment, GM1 restoration of FB1-treated Jurkat cells does not allow rCTB to exert its inhibitory effect on PMA/ionomycin-induced CD69 and CD25 up-regulation (bar 12 vs bar 4). This result clearly indicates that SM is necessary for rCTB to exert its inhibitory effect on PMA/ionomycin-induced CD69 and CD25 up-regulation.

To determine whether mRNA correlates with the cell surface expression of CD69 and CD25, RT-PCRs were performed (Fig. 3D). In accordance with the flow cytometry, CTB inhibits mRNA synthesis of CD69 and CD25. FB1 and PDMP treatment prevent the inhibitory effect of CTB on PMA/ionomycin-induced CD69 and CD25 up-regulation. Exogenous GM1-addition in PDMP-treated Jurkat cells allows CTB to inhibit T cell activation. In contrast, exogenous GM1-addition in FB1-treated Jurkat cells does not allow CTB to inhibit T cell activation. It unambiguously demonstrates that if cholesterol is not required, SM is necessary to GM1 signaling via the binding of CTB.

**FIGURE 2.** Cholesterol rich-raft disruption by m-β-CD does not prevent rCTB to inhibit PMA/ionomycin-induced CD69 and CD25 up-regulation. A, Freshly isolated hPBMCs were treated or not with m-β-CD (10 mM, 10 min at 37°C). Then cells pretreated or not with rCTB (10 μg/ml) or pCTB (10 μg/ml) for 30 min were stimulated or not by PMA (10 ng/ml) plus ionomycin (100 nM) for 20 h. Then cells were costained with a PE-CD25 mAb, a FITC-CD69 mAb, and a PerCP-CD4 mAb. hPBMCs were gated on lymphocytes according to their forward and side angle light scatter. CD25 and CD69 surface expression on CD4+ T lymphocytes was determined flow cytometry after gating lymphocytes on the basis of membrane expression of CD4. Data represent one of three similar experiments. B, CD25 and CD69 up-regulation was also examined on Jurkat cells pretreated or not with m-β-CD, pretreated or not with either rCTB or pCTB, and stimulated or not with PMA/ionomycin as in A. Data represent one of three similar experiments. The values represent means ± SEM.

**rCTB inhibits SM synthesis and enhances PtdCho synthesis**

Because we demonstrated that SM is required for GM1 signaling via the binding of rCTB (Fig. 3), we analyzed the synthesis of SM in control and in rCTB-treated Jurkat cells. We also analyzed the synthesis of PtdCho and phosphatidylethanolamine (PtdEtn) (Fig. 4). The uptake of $[^3H]$palmitic acid and $[^3H]$choline chloride is not affected by rCTB treatment (data not shown). We show that $[^3H]$palmitic acid-labeled SM synthesis and $[^3H]$choline-labeled SM synthesis are both inhibited in rCTB-treated cells compared with control cells (−54 and −56%, respectively). In contrast, $[^3H]$palmitic acid-labeled PtdCho synthesis and $[^3H]$choline-labeled PtdCho synthesis are both enhanced in rCTB-treated Jurkat cells (±122 and + 84% respectively). $[^3H]$Palmatic acid-labeled PtdEtn is not affected by rCTB treatment (data not shown).

**rCTB induces SM hydrolysis**

Jurkat cells prelabeled with either $[^3H]$palmitic acid (Fig. 5A, upper graph) or $[^3H]$choline (Fig. 5A, lower graph) were left untreated or incubated with rCTB for different period of time varying from 0 to 30 min. The analysis of lipids extracted from whole cells indicates that rCTB treatment results in a time-dependent decrease of SM (Fig. 5A). The decrease of SM is maximal at time 30 min and reaches 47% for $[^3H]$palmitic acid-labeled SM and 46% for $[^3H]$choline-labeled SM.

To study SM hydrolysis in lipid rafts, Jurkat cells, prelabeled with either $[^3H]$palmitic acid (Fig. 5B) or $[^3H]$choline (Fig. 5C), were treated or not with rCTB for 30 min and rafts were purified. The analysis of the fractions was first performed by liquid scintillation counting (data not shown). The distribution of the tritiated FA clearly indicates that this saturated FA is preferentially incorporated into fractions 2 and 3 corresponding to membrane rafts compared with fractions 8 and 9 corresponding to the detergent-soluble material. A further analysis by TLC of the lipid composition of the sucrose density-fractions unambiguously indicates that the raft fraction is highly enriched in $[^3H]$palmitic acid-labeled SM.
Comparison between fractions 2 and 9 shows that [3H]choline-labeled SM is less predominant than [3H]palmitic acid-labeled SM in raft fractions (Fig. 5C). rCTB treatment results in an important loss of SM from raft fractions while soluble fractions remain unchanged (Fig. 5B and C). Fig. 5B and C also show that rCTB enhances PtdCho synthesis. When it is labeled with palmitic acid, PtdCho accumulation only occurs in raft fractions; by contrast, when it is labeled with choline, PtdCho is found in rafts as well as in soluble fractions.

Because saturated FA carbon chains are known to interact with cholesterol, we investigated the effect of SM degradation on cellular [3H]cholesterol content. In control cells, a clear enrichment of [3H]cholesterol is observed in raft fractions compared with detergent-soluble fractions (Fig. 6). rCTB treatment results in a decrease of cholesterol in agreement with previous reports (31, 32), which demonstrated that the hydrolysis of plasma membrane SM alters cellular cholesterol homeostasis. We observed that this cholesterol decrease specifically occurs in rafts.

rCTB activates a NSM1-like enzyme in lipid rafts that produces ceramides

Because we observed an important decrease of raft-SM in rCTB-treated Jurkat, we were interested in characterizing the rCTB-induced SMase activity. For that purpose, Jurkat cells were treated or not with rCTB and raft isolation was performed. Raft fractions, and fractions containing the Triton X-100-soluble material were assayed for either neutral or acidic SMase (ASM) activity. As shown in Fig. 7A, a neutral pH optimum SMase activity was found in raft of rCTB-treated Jurkat cells. This result indicates that a raft-resident neutral SMase is involved in GM1 signaling via the

**FIGURE 3.** SM is necessary for rCTB-induced CD4+ T inhibition. A, FB1 inhibits both SM and gangliosides. PDMP specifically prevents the formation of gangliosides while enhancing SM production. B, Jurkat cells (1 × 10⁶) were cultured with or without FB1 or PDMP (in both case, 10 μM final concentration) for 24, 48, 72, and 96 h. At 96 h, half the culture was supplemented with GM1 (0.5 mg/ml for 30 min). Surface expression of GM1 was measured by flow cytometry. Data represent one of three similar experiments. The values represent means ± SEM. C, Jurkat cells (1 × 10⁶) pretreated or not with either FB1 (10 μM) or PDMP (10 μM) for 4 days were incubated or not with exogenous GM1 (0.5 mg/ml) for 30 min. Then cells pretreated or not with rCTB (10 μg/ml) for 30 min were stimulated or not by PMA (10 ng/ml) plus ionomycin (100 nM) for 20 h. Surface expression of CD69 and CD25 was measured by flow cytometry as in Fig. 2B. Data represent one of three similar experiments. The values represent means ± SEM. D, Jurkat cells were treated as in C, except that they were stimulated by PMA/ionomycin for 6 h. RNA was extracted, and RT-PCR was performed using primers amplifying either CD69, CD25, or β-actin. Data represent one of three similar experiments.

**FIGURE 4.** rCTB inhibits SM synthesis and enhances PtdCho synthesis. Jurkat cells (2 × 10⁶) were maintained in 500 μl of HSB. At time 0, 4 μCi of either [3H]palmitic acid (A) or [3H]choline chloride (B) were added, with (10 μg/ml) or without rCTB ([]), at the end of the treatment (120 min); lipids were extracted and analyzed as described in Materials and Methods. Data represent one of three similar experiments. The values represent means ± SEM.
rCTB. Furthermore, this neutral SMase activity is almost entirely inhibited by GSH. It indicates that the involved enzyme is probably a NSM1-like one. ASM has not been found implicated in that process (data not shown).

SM hydrolysis results in the formation of ceramides and phosphocholine. The topology of ceramide formation determines its function (33). When SM from the outer leaflet is hydrolyzed, ceramides generated in this outer leaflet form ceramides-rich domains, while ceramides generated from the small SM pool in the plasma membrane inner leaflet serve as second messengers in signal transduction. Using the DAG kinase assay, we dosed the ceramide production upon the binding of GM1 by rCTB. As shown in Fig. 7, rCTB induces a rapid and transient production of ceramides. The ceramide production reaches its maximal at 30 min and returns near to the basal level at 45 min. This result suggests that ceramides produced by rCTB are rapidly metabolized and re-enter into the SM cycle.

GSH and NAC pretreatment inhibits the effects of rCTB

On one hand, we demonstrated that rCTB-GM1 association inhibits CD4⁺ T lymphocyte activation (Figs. 2, A and B, 3, D and E) and proliferation (Fig. 1). In the other hand, we demonstrated that 1) rCTB inhibits SM synthesis (Fig. 4) and 2) activates a NSM1-like enzyme in lipid rafts that produces transient ceramides (Fig. 7). To demonstrate that rCTB-induced CD4⁺ T lymphocyte inhibition is due in part to SM level modifications, we pretreated Jurkat cells with either the antioxidant GSH or NAC. NAC acts as a precursor of reduced GSH biosynthesis and consequently it inhibits neutral SMase. As shown in Fig. 8A, pretreatment with GSH or NAC inhibits the inhibitory effect of rCTB on Jurkat cells. rCTB-treated Jurkat do not up-regulate CD69 and CD25 when they are stimulated by PMA/ionomycin. By contrast, rCTB-treated cells that have been pretreated before with NAC or GSH are able to up-regulate the activation markers CD69 and CD25.

To determine whether mRNA correlates with the cell surface expression of CD69 and CD25, RT-PCRs were performed (Fig. 8B). In total accordance with the flow cytometry, the inhibitory effect of CTB on Jurkat cells is abolished when cells are pretreated with NAC.

To link the absence of proliferation of rCTB-treated CD4⁺ T lymphocytes and the neutral SMase activity (i.e., the transient accumulation of ceramides), purified CD4⁺ T lymphocytes were pretreated or not with GSH or NAC before being treated or not with rCTB (10 μg/ml, 30 min). Then cells were stimulated either by
CD3/CD28 or by PMA/ionomycin. As expected, rCTB inhibits less the proliferation of GSH- or NAC-pretreated CD4+ T lymphocytes than the proliferation of control lymphocytes.

Thus, these results demonstrate that the rCTB-induced SMase activation is responsible for the observed inhibition (activation and proliferation) of CD4+ T lymphocytes when they are preincubated with rCTB.

SM hydrolysis is required for rCTB-induced NF-κB activation

Heat-labile ETB from Escherichia coli, a close homologue of CTB, is known to activate nuclear translocation of NF-κB in Jurkat cells (12). Activation of NF-κB by rCTB has never been investigated before. Translocation of NF-κB was visualized after 1 h of rCTB (10 μg/ml) stimulation by its binding to a radioactive probe containing κB sites from the Igx promoter (Fig. 9A, lane 2 compared with unstimulated cells, lane 1). In PDMP-treated Jurkat cells, rCTB fails to translocate NF-κB. In addition, in FB1-treated cells, rCTB also fails to translocate NF-κB (Fig. 9A, lane 11 vs lane 2). Furthermore, rCTB is able to translocate NF-κB in GM1-restored PDMP-treated cells (Fig. 9A, lane 8 vs lane 2); by contrast, rCTB cannot translocate NF-κB in GM1-restored, FB1-treated cells (Fig. 9A, lane 12 vs lane 2), indicating that SM is necessary for NF-κB translocation by rCTB. Preincubation with GSH leads to an inhibition of NF-κB DNA-binding activity (Fig. 9A, lane 4 vs lane 2), indicating that SM hydrolysis is required for rCTB-induced NF-κB translocation.

NF-κB activation was measured in Jurkat cells transfected with a reporter luciferase gene under the control of NF-κB (Fig. 9B). In total accordance with EMSAs, NF-κB activation was inhibited both in PDMP- and in FB1-treated cells and was restored only in GM1-reconstituted, PDMP-treated cells, indicating that SM is required for NF-κB activation by rCTB. rCTB stimulation leads to an 8-fold increase in luciferase activity that is strongly decreased by ~50% by GSH or NAC-pretreatment, indicating that SM hydrolysis is involved in rCTB-induced NF-κB activation. GSH and NAC has no effect on baseline luciferase activity.

rCTB treatment prevents PKCa phosphorylation and translocation into modified lipid rafts

Because rCTB treatment strongly modifies the lipid composition of rafts (1) rCTB diminishes SM synthesis (Fig. 4), 2) hydrolyzes SM, and 3) enhances PtdCho synthesis (Fig. 5), we were interested in studying the distribution of raft-resident proteins highly involved in T cell activation. As shown by Fig. 10A, rCTB treatment modifies neither the distribution of linker for activation of T cells nor Lck. Then, we studied the effect of rCTB on PMA-induced recruitment of PKCa into lipid rafts (Fig. 10B, upper panel). As shown in Fig. 10B, upper panel, PMA induces a partial translocation of PKCa into lipid rafts. rCTB treatment (i.e., raft modifications in terms of lipids) importantly prevents PKCa translocation into lipid rafts. Interestingly, GSH and NAC pretreatment, which prevents rCTB to inhibit Jurkat activation, allows PKCa to redistribute itself after rCTB pretreatment and PMA stimulation.

Then, we investigated the phosphorylation status of PKCa within rafts (Fig. 10B, lower panel). The same amount of PKCa in rafts for control and rCTB-treated cells was subjected to SDS-PAGE, and the membrane was immunoblotted with anti-phosphoPKCa mAb. Fig. 10B, lower panel, clearly shows that rCTB inhibits PKCa phosphorylation on Ser657.

Discussion

In the present article, we propose a mechanism for the inhibitory effect of rCTB both on the activation and on the proliferation of human CD4+ T lymphocytes. rCTB specifically binds to GM1, a raft marker, and strongly modifies the lipid composition of rafts. First, rCTB inhibits SM synthesis; second, it enhances PtdCho synthesis; and third, it activates a raft-resident neutral SMase, thus...
CD59 and GM1 cluster in different membrane subdomains of Jurkat cells. Furthermore, m-CD extracts cholesterol from Triton X-100-resistant membranes without affecting the buoyant properties of Thy-1 and GM1 (37, 38). The occurrence of GM1 in DRMs depends on its ceramide moiety. Depletion of 73% of cellular cholesterol with m-CD does not affect the recovery in DRMs of GM1 acylated with C18-, C22-, or C24-saturated FAs (26). Combined all together, these data suggest that GM1 resides in a subset of lipid raft that is insensitive to cholesterol depletion by m-CD. Our data support and extend this earlier observation because we show that m-CD treatment does not affect the inhibitory effect of rCTB via GM1.

CTB and ETB are known to modulate leukocyte function. This property is attributed to the ability of the B-subunit to bind GM1. Nevertheless, it has been demonstrated that GM1 binding alone, contrary to expectations, is not sufficient to initiate toxin action. Fraser et al. (12) described the properties of ETB (H57S), a mutant B-subunit with a His→Ser substitution, at position 57. The mutant still binds to GM1 but is found to be severely defective in inducing generating a transient ceramide production. These ceramides inhibit PKCa phosphorylation and its translocation into the modified lipid rafts. Furthermore, ceramides activate NF-kB. We hypothesize that combined all together, raft modification in terms of lipids, ceramide production, PKCa inhibition, and NF-kB activation lead to T cell inhibition.

Gangliosides and glycosylphosphatidylinositol-anchored proteins are frequently used as positive controls for raft purification. GM1 is certainly the most commonly used raft marker. It is to note that gangliosides and glycosylphosphatidylinositol-anchored proteins are almost ever used indiscriminately as if they were equal. Nevertheless, Vyas et al. (34) demonstrated that GM1 does not cocluster with GD3 on intact neurons. Gomez-Mouton et al. (35) show that the acquisition of a motile phenotype in T lymphocytes cocluster with GD3 on intact neurons. Gomez-Mouton et al. (35) showed that the acquisition of a motile phenotype in T lymphocytes results in the asymmetric redistribution of GM3- and GM1-enriched raft domains to the leading edge and to the uropod, respectively. Furthermore, Millán et al. (36) have demonstrated that
leukocyte signaling. For example, it fails to trigger caspase-3-mediated T CD8+ lymphocyte apoptosis. It does not activate NF-κB in Jurkat cells. It fails to induce a potent anti-B-subunit response in mice, and it also fails to serve as mucosal adjuvant. In the same manner, CTB (H57A) binds GM1 but lacks immunomodulatory or toxic activity (39). In the present study, we demonstrate that GM1 ligation alone, via rabbit polyclonal Abs anti-GM1, is not able to inhibit CD4+ T lymphocyte activation and cell proliferation (data not shown). Aman et al. (39) hypothesize that CT may require interaction, not only with GM1, but also with another molecule to exert its biological activity. For Aman et al. (39), it is conceivable that rCTB binding to GM1 in lipid rafts would position it to interact with signaling molecules that participate in toxin-mediated immune cell modulation. In respect with our experimental data, it is quite conceivable that the intermediary molecule involved in the toxin action would be a rafts resident neutral SMase.

The isoform of SMase (acidic or neutral), thus the topology of ceramide formation, dictates its outcome (33). The activation of ASM is believed to theoretically allow the hydrolysis of the main pool of SM from the outer plasma membrane leaflet, thus resulting in the formation of ceramide-rich domains (40), while the activation of neutral SMase would rather allow the hydrolysis of the small pool of SM from the inner leaflet resulting, this time, in the generation of ceramides that will act as second messengers. In this study, we demonstrated that rCTB hydrolyzes SM in a time-dependent manner via the activation of a neutral SMase that is inhibited by GSH. Thus, this enzyme involved in that process resembles to NSM1. However, the quantity of hydrolyzed SM by rCTB reaches 47% for [3H]palmitic acid-labeled SM and 46% for [3H]choline-labeled SM. It probably represents more than the minor pool of SM located in the inner leaflet. Thus, what remains unclear is how neutral SMase gains access to the major pool of SM. It has been hypothesized that neutral SMase would hydrolyze SM after its flip-flop from the outer leaflet to the inner leaflet (33), but we did not observe any flip-flop of phospholipids as studied by the externalization of phosphatidylserine (data not shown).

In this study, we demonstrated that rCTB regulates SM at two levels. First, rCTB inhibits SM synthesis, and second, rCTB activates SM hydrolysis. These two complementary actions both contribute to cell cycle arrest of CD4+ T lymphocytes. Indeed, the inhibition of SM synthesis (1) causes the inhibition of DAG synthesis and (2) increases the level of ceramides; furthermore, SM hydrolysis generates ceramides. Consequently, rCTB enhances ceramides content by two different manners. Ceramides/DAG ratio plays an important role in cell proliferation, and numerous targets of ceramides are known to negatively regulate cell proliferation.

The biochemical synthesis of SM occurs via the action of a PtdCho:ceramide choline phosphotransferase (SM synthase (SMS)), which transfers the phosphorylcholine (phosphocholine) moiety from PtdCho onto the primary hydroxyl of ceramide, thus producing SM and DAG (41, 42). Thus, the inhibition of SMS reduces the intracellular level of DAG. It has been published that DAG generated during SM synthesis is involved in PKC activation and cell proliferation (43). It constitutes the first way by which rCTB inhibits PKCα in CD4+ T lymphocytes. Furthermore, the inhibition of SMS also increases the level of ceramides (44, 45). It may constitute a supplementary way, in addition to SM hydrolysis, to elevate ceramide levels. Flores et al. (46) studied the changes in the balance between DAG and ceramides during cell proliferation, cell arrest, and apoptosis in T lymphocytes. Accordingly, augmentation of ceramides and diminution of DAG favor cell arrest.

Ceramides also target specific proteins inducing cell cycle arrest. Ceramides induce a G_{1}-G_{2} cell cycle arrest, and this was mechanistically shown to be due to the induction of dephosphorylation of the retinoblastoma gene product (Rb) (47). Furthermore, it has been also reported that the treatment of NIH 3T3 cells with a specific inhibitor of glucosylceramide synthase, which induces ceramide accumulation, causes a G_{1}-M cell cycle arrest, possibly mediated by ceramide-induced inhibition of the cyclin-dependent p34^{ck2} and Cdk2 kinases (48). Another study has shown that ceramides specifically inactivate the cyclin-dependent kinase Cdk2 through activation of PPI and PP2A phosphatases (49). Ceramides also inactivate protein kinase B/Akt (50). In addition, it has been
demonstrated that endogenous ceramides, produced either by overexpression of bacterial SMase or by daunorubicin treatment, inhibit mRNA synthesis of telomerase RT and telomerase activity via inactivation of c-Myc transcription factor (51, 52).

In this study, we observed that rCTB inhibits PKCα phosphorylation and prevents its translocation into rfts. PKCα is a pro-growth cellular regulator, and its inactivation can explain cell cycle arrest. PKCα inhibition by rCTB can be easily explained by three ways. First, PKCα is activated by DAG, and because of the inhibition of SM synthesis, DAG level is diminished in rCTB-treated lymphocytes. Consequently, PKCα is less activated in rCTB-treated cells. Second, ceramides are known to inactivate PKCα via a phosphatase (53, 54). Third, rafts are markedly modified in terms of lipids. Indeed, rCTB-treated lymphocytes possess rafts with less SM, less cholesterol, but more PtdCho than control cells. It is quite conceivable that these lipid modifications strongly alter the anchoring of PKCα into these modified rafts because ceramides do not inhibit PMA-induced translocation of PKCα by themselves (53). Collectively, all of these explanations can explain the inhibition of PKCα by rCTB.

Finally, we observed that rCTB induces the activation of NF-κB. rCTB-induced NF-κB activation is inhibited in FB1-treated cells, suggesting that SM is required. Furthermore, NAC and GSH prevent rCTB-induced NF-κB activation, it means that SM hydrolysis i.e., ceramide production is responsible for the rCTB-induced NF-κB activation. Ceramides are well known to activate NF-κB (55), but the genes involved remain to be elucidated.

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Disclosures

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References


