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 Breittmayer and Claude Aussel

*J Immunol* 2005; 175:5637-5648; doi: 10.4049/jimmunol.175.9.5637

http://www.jimmunol.org/content/175/9/5637

**References**

This article *cites 55 articles*, 33 of which you can access for free at:

http://www.jimmunol.org/content/175/9/5637.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
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 Breittmayer,* and Claude Aussel†*‡*

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 rCTB-induced inhibition of CD4+ T lymphocytes. rCTB specifically binds to GM1, a raft marker, and strongly modifies the lipid composition of rafts. First, rCTB 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 Ca phosphorylation and its translocation into the modified lipid rafts. Furthermore, we show that rCTB-induced ceramide production activate NF-κB. Combined all together: raft modification in terms of lipids, ceramide production, protein kinase Ca inhibition, and NF-κB activation lead to CD4+ T cell inhibition. The Journal of Immunology, 2005, 175: 5637–5648.

Cholera toxin B-subunit (CTB), produced by the bacterium Vibrio cholerae, exerts profound immunomodulatory effects on blood cell populations in vitro. CTB triggers the polyclonal activation of B cells. This activation occurs in the absence of significant proliferation and involves the up-regulation of a number of important molecules, namely MHC class II (Ia), B7, CD40, ICAM-1, and IL-2Rα (CD25) (1, 2). CTB induces selective apoptosis of T CD8+ cells. This apoptosis is preceded by enhanced expression of CD25 and is independent of Fas (CD95) or TNF-α. It involves a NF-κB-dependent and caspase-3-dependent pathway (3). A similar effect is exerted by CTB in vivo where oral administration of CTB leads to a demonstrable depletion of CD8+ T cells from both the Peyer’s patch and intraepithelial lymphocyte compartments (4). Heat-labile enterotoxin B-subunit (ETB) from Escherichia coli, which is a close homologue of CTB, induces the release of IL-10, IL-6, and TNF-α by human monocytes (5). CTB modulates Ag processing and presentation by macrophages (6–8). CTB inhibits CD3- and PMA/ionomycin-induced murine T cell proliferation (9, 10). Such proliferation is inhibited even if CTB is added hours after the start of culture (10). CTB also inhibits the proliferation of 2.10 cells (a human IL-2-dependent, CD4+ T cell clone) in response to IL-2 produced endogenously on stimulation with anti-TCR or provided exogenously (11). ETB induces nuclear translocation of NF-κB in Jurkat cells (12). To conclude, CTB displays pleiotropic effects on human PBMCs (hPBMCs), it inhibits CD4+ T cell activation and proliferation, but the exact mechanism remained unclear.

In this study, our results explain the inhibitory effect of rCTB both on the activation and on the proliferation of human CD4+ T lymphocytes. rCTB specifically binds to the monosialoganglioside GM1, a raft marker, and strongly alters the lipid composition of rafts of CD4+ T lymphocytes. First, rCTB inhibits sphingomyelin (SM) synthesis, secondly it enhances phosphatidylcholine (PtdCho) synthesis, and thirdly it activates a raft-resistant neutral sphingomyelinase (SMase) resembling to NSM1, thus generating a transient ceramide production. We demonstrated that these ceramides inhibit protein kinase Ca (PKCa) phosphorylation and its translocation into the modified lipid rafts. We also demonstrated that rCTB as ETB activates the NF-κB transcription factor. Furthermore, we also show that rCTB induces NF-κB activation via the production of ceramides. Combined all together, raft modification in terms of lipids, ceramide production, PKCa inhibition, and NF-κB activation lead to CD4+ T cell inhibition.

Materials and Methods

Cells

Citrate anticoagulated venous blood samples of healthy adult volunteers and buffy coats collected from normal donors by the Etablissement Français du Sang were obtained according to institutional guidelines. hPBMCs were isolated from either blood samples or buffy coats by centrifugation on a Ficoll-Hypaque density-gradient (1.077 g/ml). Interface PBMCs were pelleted, washed, and cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 50 U/ml penicillin G sodium, 50 μg/ml streptomycin sulfate, 2 mM l-glutamine, 1 mM sodium pyruvate, 20 mM HEPES, and...
Abs, reagents, and radioactive products

rCTB was a kind gift of Dr. F. Anjüere and Prof. C. Czernikowsky (U721, Nice, France). rCTB was produced in a mutant strain of Vibriob choleter 01a. of its CT genes and transformed with a multicopy plasmid encoding CTB. The rCTB was used for the purification of the culture medium by a combination of salt precipitation and chromatographic methods, as previously described (13), and has been already used in several studies (14–16).

Rabbit polyclonal Ab anti-PLC-γ (clone M4, IgG1), rabbit polyclonal Ab anti-phospho-PKCα (Ser657) (IgG), rabbit polyclonal Ab anti-pi56Lecl (IgG), and rabbit polyclonal Ab anti-LAT (IgG) were obtained from Upstate Biotechnology. Rabbit polyclonal Ab anti-PLCγ1 (sc-81) was 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-deleted of its CT genes and transformed with a multicopy plasmid encoding CTB. CD4-PerCP (clone SK3, IgG1), CD69-FITC (clone FN50, IgG1), CD25-PE (clone M-A251, IgG1), and apoptosis detection kit (annexin V-PE, 7-amino-actinomycin D (7-AAD)) were purchased from BD Pharmingen. Peroxidase-labeled anti-rabbit IgG and rabbit anti-mouse IgG coupled to peroxidase were obtained from Rockland, and R-PE-Phyco-cyanine-conjugated streptavidin was obtained from DakoCytomation. Methyl-β-cyclodextrin (m-β-CD), CTB, biotin-labeled CTB, monosialoganglioside-GM1, N-acetyl-t-cysteine (NAC), glutathione (GSH), cGMP, PMA, ionomycin, pepstatin, leupeptin, chymostatin, and anti-PSMS were purchased from Sigma-Aldrich. α-2-macroglobulin was purchased from Roche. 1,2-oxan-2[H]cholesterol (1.3–1.85 TBBq/mmol), methyl-2[H]hemin chloride (2.22–3.14 TBBq/mmol), 9,10(Ω)-[3H]palmitic acid (37 MBq/mmol), [methyl-2H]thymidine (740 GBq/mmol), [3H]-ATP, and [N-methyl-3H]cysteine (2.04 Ci/mmol, 55 nCi/mmol) were purchased from American Biosciences.

In vitro 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 × 10⁶/ml. Cell suspension was cultured in triplicate sets in flat-bottom 96-well plates in a volume of 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 anti-CD28 mAb (5 μg/ml) or soluble anti-CD3 mAb (5 μg/ml) plus anti-CD28 mAb (5 μg/ml) for 20 h. Then, cells were contained 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 PDMT-treated Jurkat cells was also compared with control Jurkat using the same technique.

Lipid rafts isolation

Raft isolation was accomplished using a combination of published protocols (17, 18). After radioactive labeling and/or pretreatment with rCTB (10 μg/ml), Jurkat cells (80–100 × 10⁶) were sonicated gently with a Vibracell sonicator (five bursts of 5 × 5 s; Bioblock Scientific) in 1 ml of ice-cold buffer 0.1% BSA and 150 mM NaCl, 5 mM EDTA, 10 mM sodium pyrophosphate, 5 mM Na3VO4, and 10 mM NaF supplemented with a mixture of protease inhibitors (1 mM a-phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM pepstatin, 2 mg/ml chymotain, and 5 mg/ml α-2-macroglobulin) and centrifuged at 800 g for 10 min to remove nuclei and large debris. The resulting supernatant called postnuclear supernatant was incubated in the presence of 2.5% Triton X-100 (PEG9(10-ocetyl)phor) for 30 min at 2°C. The lysate was then added to 1.35 mM sucrose by the addition of 2 ml of 2 M sucrose and placed at the bottom of an ultracentrifuge tube (Ultra-Clear; Beckman Instruments). A step sucrose gradient (0.2–0.9 M with 0.1 M steps, 1 ml each) was placed on top. The weight percentage of sucrose was checked at room temperature using an Abbe-3L refractometer (Bioblock Scientific). The tubes were centrifuged at 38,000 rpm (~250,000 × g) using the radial distance maximal (rmax: 158.8 mm) for conversion with normogram) for 16–18 h (L8-70M Ultracentrifuge: Beckman Instruments) in a SW41Ti rotor (Beckman Instruments) at 2°C. One-millilitre fractions were harvested from the top. Rafts were recovered from the low-density fractions 2 and 3 while the heavy/H fractions (soluble material) were recovered from the high-density fractions 8 and 9 at the bottom of the ultracentrifuge tube.

Immunoblot analysis

Aliquots (50 μl) of each sucrose density-gradient fraction were solubilized in 50 μl of 2×DCT buffer (150 mM Tris-HCl (pH 8.5), 20% glycerol, 5 mM EDTA, 5% SDS, and 10% 2-ME) and then resolved by 10% SDS-PAGE under reducing conditions, and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). Membranes were blocked for 2 h at room temperature in a blocking buffer containing 5% (w/v) nonfat dry milk in TBS (10 mM Tris-HCl and 140 mM NaCl (pH 7.4)) and then incubated for 1 h with the appropriate Ab diluted 1000-fold in the same buffer. The 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-PKCa immunoiblotting, aliquots of fraction B (fraction 2 + 3) solubilized in the same volume of 2×DCT 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-PKCa (Ser657) for 2 h at room temperature. Phospho-PKCa 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 Ab against PKCa.

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 PDMT (10 μM final concentration) for 4 days; FB1 and PDMT reduced cellular sphingolipid content, Jurkat cells were cultured as control cells in a medium supplemented with either FB1 (10 μM final concentration) or PDMT (10 μM final concentration) for 4 days; FB1 and PDMT reduced cellular sphingolipid content.
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, 12.0 mM glucose, 2 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) cCTB-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 (75/5/45/2). Authentic phospholipid standards (Sigma-Aldrich) were run in parallel and detected with iodine vapors. Radioactivity in lipid spots was determined by using an automatic linear radiochromatography analyzer. Traceremaster 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 cCTB, 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]cholesteral. Raft purification was performed as described above. To determine the distribution of [3H]cholesteral, 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 (1375 μCi, 25 mmol) of [N-methyl-14C]SM (55 mCi/mmol, 10 μCi/004 μl in toluene/ethanol, 1/1, v/v) were placed in a glass tube, and the organic solvent was removed under N2. The dried [14C]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 [14C]SM (0.5 mmol), 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 [14C]phosphorycholine released from [N-methyl-14C]SM was collected and counted by liquid scintillation. The reaction was linear within this frame, and the amount of [N-methyl-14C]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 acid-SMase activity, the substrate solution consisted in 10 μl of the stock solution of [14C]SM (0.5 mmol) 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-14C]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 cCTB 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 tube under a stream of N2. Lipid extracts were then subjected to mild alkaline hydrolysis (0.1 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. Radioactivity in lipid spots was determined by using an automatic linear radiochromatography analyzer. Traceremaster 20 (Berthold), equipped with an 8-mm window and the integration software supplied by the manufacturer.

Semi quantitative 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 RNase A+H− reverse transcriptase (Invitrogen Life Technologies) following the manufacturer’s instructions and resuspended in 150 μl final volume. 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'-CTGAGACGGAGAACACGTCCTTG-C-3’) and 3’ primer (5’-ACAGGACAGAAGTCTGAAGAAGAG-3’), and human CD25, 5’ primer (5’-GGGACTCTGTCACGGTTACATCA-3’) and 3’ primer (5’-TTCACATTGGCTCCTCCTCTTGTAG-3’). β-actin was used as loading control.

EMSAs
Total cellular extracts were prepared in Totex 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 DTT, 1 mM PMSF, and 10 μg/ml aprotinin). Supernatants from a 15,000 g, 10 min centrifugation at 4°C were collected. The NF-κB probe used for mobility shift assay was constructed of a synthetic double-stranded oligonucleotide containing the NF-κB site of the Igk promoter (5’-GATCCAAGGGACTTTCCATG-3’ and 3’-GATCCAAGGGACTTTCCATG-3’). EMSA was incubated with Totex extract (20 μg) 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 (xBX6 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 the 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 cell 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 cAMP, 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 with PMA/ionomycin and [³H]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 C₁₈, C₂₂, or C₂₄ 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-induced 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

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 Abs 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 [³H]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 × 10⁵) 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.

rCTB-INDUCED SMase ACTIVATION CAUSES CD4⁺ T CELL INHIBITION
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 PMA-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 to 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.

**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]Palmmitic 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 prelabeled 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. 5, B 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
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...
Heat-labile ETB from *Escherichia coli*, treated cells (Fig. 9A) induces NF-kB activation. rCTB treatment prevents PKC phosphorylation on Ser657.

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 Igκ 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 PKCo into lipid rafts (Fig. 10B, upper panel). As shown in Fig. 10B, upper panel, PMA induces a partial translocation of PKCo into lipid rafts. rCTB treatment (i.e., raft modifications in terms of lipids) importantly prevents PKCo translocation into lipid rafts. Interestingly, GSH and NAC pretreatment, which prevents rCTB to inhibit Jurkat activation, allows PKCo to redistribute itself after rCTB pretreatment and PMA stimulation.

Then, we investigated the phosphorylation status of PKCo within rafts (Fig. 10B, lower panel). The same amount of PKCo in rafts for control and rCTB-treated cells was subjected to SDS-PAGE, and the membrane was immunoblotted with anti-phospho-PKCo mAb. Fig. 10B, lower panel, clearly shows that rCTB inhibits PKCo 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...
generating a transient ceramide production. These ceramides inhibit PKCα phosphorylation and its translocation into the modified lipid rafts. Furthermore, ceramides activate NF-κB. We hypothesize that combined all together, raft modification in terms of lipids, ceramide production, PKCα phosphorylation and its translocation into the modified lipid rafts 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 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 CD59 and GM1 cluster in different membrane subdomains of Jurkat cells. Furthermore, m-β-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-β-CD. Our data support and extend this earlier observation because we show that m-β-CD-treatment does not affect the inhibitory effect of rCTB via GM1.

CTB and E TB 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
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 (HS7A) 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 proliferation. We demonstrate that GM1 ligation by Abs does not lead to SMase activation and SM hydrolysis (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 isofrom 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 PKCa 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 G1-S 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 G2-M cell cycle arrest, possibly mediated by ceramide-induced inhibition of the cyclin-dependent p34<sup>cdk2</sup> and Cdk2 kinases (48). Another study has shown that ceramides specifically inactivates the cyclin-dependent kinase Cdk2 through activation of PP1 and PP2A phosphatases (49). Ceramides also inactivates 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 rafts. 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 evidences 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.

Acknowledgments
We thank Dr. Laurence Lamy, Dr. Isabelle Foucault, Romain Gallais, and Frank Leporati. We also thank Prof. Cecil Czerniksky and Dr. Fabienne Anjouère for providing us the rCTB. We are also grateful to Alexis de la Duaffa for helpful discussion.

Disclosures
The authors have no financial conflict of interest.

References


