Cholera Toxin B Pretreatment of Macrophages and Monocytes Diminishes Their Proinflammatory Responsiveness to Lipopolysaccharide

Volker Burkart, Yoong-Eun Kim, Bettina Hartmann, Iona Ghiea, Ulrike Syldath, Manfred Kauer, Waltraud Fingberg, Pejman Hanifi-Moghaddam, Sylvia Müller and Hubert Kolb

J Immunol 2002; 168:1730-1737; doi: 10.4049/jimmunol.168.4.1730
http://www.jimmunol.org/content/168/4/1730
Cholera Toxin B Pretreatment of Macrophages and Monocytes Diminishes Their Proinflammatory Responsiveness to Lipopolysaccharide

Volker Burkart, Yoong-Eun Kim, Bettina Hartmann, Iona Ghiea, Ulrike Syldath, Manfred Kauer, Waltraud Fingberg, Pejman Hanifi-Moghaddam, Sylvia Müller, and Hubert Kolb

The cholera toxin B chain (CTB) has been reported to suppress T cell-dependent autoimmune diseases and to potentiate tolerance of the adaptive immune system. We have analyzed the effects of CTB on macrophages in vitro and have found that preincubation with CTB (10 µg/ml) suppresses the proinflammatory reaction to LPS challenge, as demonstrated by suppressed production of TNF-α, IL-6, IL-12(p70), and NO (p < 0.01) in cells of macrophage lines. Pre-exposure to CTB also suppresses LPS-induced TNF-α and IL-12(p70) formation in human PBMC. Both native and recombinant CTB exhibited suppressive activity, which was shared by intact cholera toxin. In cells of the human monocyte line Mono Mac 6, exposure to CTB failed to suppress the production of IL-10 in response to LPS. Control experiments excluded a role of possible contamination of CTB by endotoxin or intact cholera toxin. The suppression of TNF-α production occurred at the level of mRNA formation. Tolerance induction by CTB was dose and time dependent. The suppression of TNF-α and IL-6 production could be counteracted by the addition of Abs to IL-10 and TGF-β. IFN-γ also antagonized the actions of CTB on macrophages. In contrast to desensitization by low doses of LPS, tolerance induction by CTB occurred silently, i.e., in the absence of a measurable proinflammatory response. These findings identify immune-deviating properties of CTB at the level of innate immune cells and may be relevant to the use of CTB in modulating immune-mediated diseases. The Journal of Immunology, 2002, 168: 1730–1737.

Materials and Methods

Macrophages

The human monocyte cell line Mono Mac 6 (17) was provided by Dr. H. W. Ziegler-Heitbrock (Institute for Immunology, University of Munich, Munich, Germany). Mono Mac 6 cells were cultured in RPMI 1640 medium containing the oxalacetate-pyruvate-insulin (OPI) supplement (Sigma, Deisenhofen, Germany), 2 mM l-glutamine, antibiotics (120 µg/mL penicillin and 200 µg/mL streptomycin) and 10% FCS (Life Technologies, Munich, Germany). The human monocyte cell line J774A.1 (18) was cultured in RPMI 1640 medium supplemented as

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

described above and with 1 mM sodium pyruvate but without the OPI supplement. Human PBMC were enriched from venous blood freshly drawn from normal healthy donors. Whole blood was centrifuged on a Ficoll–Paque Plus (Amersham Biosciences, Uppsala, Sweden) gradient (800 × g, 15 min), followed by washing of the enriched cells in HBSS (400 × g, 10 min). Cells were resuspended in RPMI 1640 supplemented with the same additions as described for Mono Mac 6 cells, except without OPI. PBMC, isolated according to this method, typically contain ~18% monocytes as judged from FACS analysis.

**Macrophage activation**

Mono Mac 6 cells or PBMC were cultured at a density of 1 × 10^6 cells/ml in 24-well flat-bottom tissue culture plates at a volume of 2 ml or in 96-well flat-bottom plates (Falcon; BD Biosciences, Heidelberg, Germany) and challenged with LPS from *E. coli* serotype 026 B6 (Sigma) at 1 μg/ml. At the end of the experiments, culture supernatants were collected and stored at −20°C until further analyses. Pretreatment of cells with purified CT or CTB (Sigma), with recombinant CTB (gift from G. C. Cerkinsky), or with 10 ng/ml LPS was performed in 24-well plates, followed by two washes at 800 × g and 4°C for 5 min and resuspension at 1 × 10^6 cells/ml. To neutralize for endotoxin, 0.1 μg/ml polymyxin B sulfate (Sigma) was added to cell cultures. Heat treatment was performed by keeping samples for 10 min at 100°C, followed by rapid cooling in iced water.

J774A.1 macrophages exhibit adherent growth and were seeded at 1 × 10^6 cells/ml in tissue culture dishes (Falcon; BD Biosciences) in a total volume of 2 ml. Pretreatment with medium or CTB, cells were detached by incubation with ice-cold Ca^2+ -Mg^2+-free HBSS (Life Technologies) for 7 min. Cells were washed once at 300 × g and 4°C for 5 min and resuspended at 1 × 10^6 cells/ml. Challenge with LPS was performed in 96-well plates (2 × 10^5 cells in a total volume of 200 μl/well as described for Mono Mac 6 cells.

To exclude possible effect of contaminations with cholera toxin A (CTA subunit) or the holotoxin that causes a rise in intracellular cAMP concentrations, 2',3'-dideoxyadenosine (Sigma), an adenylyl cyclase inhibitor, was together added with 10 μg/ml CTB to the cell culture. After 5 h, the Mono Mac 6 cells were pooled, centrifuged, washed with culture medium, and seeded at a cell density of 1 × 10^6 cells/ml in a 24-well plate. Then the cells were stimulated with 1 μg/ml LPS for the next 5 h, and supernatants were collected for analysis.

In an attempt to prevent macrophage tolerance induction, human Mono Mac 6 cells were preincubated for 24 h without or with 10 ng/ml LPS or 10 μg/ml CTB in the presence of 1200 U/ml human IFN-γ (Biozol, Eching, Germany) or 50 ng/ml GM-CSF (Pharma Biotechnologie, Hannover, Germany). In a second approach to preventing macrophage tolerance, human Mono Mac 6 cells were pretreated for 24 h with 10 μg/ml CTB in the presence of 1 μg/ml anti-IL-10 or 10 μg/ml anti-TGFβ1,2,3 (BD Pharmingen, San Diego, CA) mAbs. To reverse CTB-induced macrophage tolerance, human Mono Mac 6 cells were pretreated for 5 h with 10 μg/ml CTB. After washing with culture medium the cells were cultured without or with 1200 U/ml IFN-γ or 50 ng/ml GM-CSF. After 24 h the cells were washed and stimulated with 1 μg/ml LPS for the next 5 h.

**Determination of TNF-α, IL-6, IL-10, and IL-12(p70)**

The amounts of the cytokines TNF-α, IL-6, IL-10, and IL-12(p70) in the culture supernatants were determined by ELISA (19). For TNF-α determination, microtiter plates (Nunc, Wiesbaden, Germany) were coated with rat anti-mouse or mouse anti-human mAb (BD Pharmingen). TNF-α bound to plates was determined by complexity with biotinylated TNF-α Ab of matching specificity, subsequent binding of avidin-HRP (Dianova, Hamburg, Germany), and addition of 2,3'-azino-bis(3-ethylbenzo-thiazolinedione-6-sulfonic acid) and 3,5,3'-tetramethylbenzidine (TMB).

Concentrations of human IL-6 were determined using the PeliKine Compact kit (CLB, Amsterdam, The Netherlands). Microtiter plates were coated with an anti-human IL-6 mAb before the samples were added. Bound IL-6 was determined by a biotinylated sheep Ab to human IL-6, followed by the addition of HRP-conjugated streptavidin and the substrate 3,5,3'-tetramethylbenzidine (TMB).

Concentrations of human IL-10 were determined by coating microtiter plates with rat anti-human IL-10 mAb (BD Pharmingen). Samples from culture supernatants were added, and bound IL-10 was determined by a biotinylated rat mAb directed against human IL-10 (BD Pharmingen). Detection was performed by HRP-conjugated streptavidin and TMB.

Concentrations of human IL-12(p70) were determined by coating microtiter plates with rat anti-human IL-12 mAb (BD Pharmingen). Samples from culture supernatants were added, and bound IL-12 was detected by a biotinylated mouse anti-human IL-12 mAb, followed by the addition of HRP-streptavidin conjugate and TMB.

In all cytokine ELISAs, the ODs of the samples were determined by spectrometry on a microplate reader at 405 nm (TNF-α) and 450 nm (IL-6, IL-10, IL-12(p70)), and the cytokine concentrations were quantified from standard curves, which were obtained from sequential dilutions of the corresponding recombinant cytokines.

**Measurement of nitrite production**

The amount of nitrite (NO_2^-) released by macrophages was detected in cell-free supernatants by the colorimetric Griess reaction as described previously (20). Briefly, 50 μl of supernatant and serial dilutions of NaNO_2 standard solution were placed in 96-well plastic plates and then mixed with an equal volume of Griess reagent containing 1% sulfanilamide, 0.1% naphthylethylene-diame-dihydrorhodioline, and 2.5% H_3PO_4. After incubation for 10 min at room temperature, the OD of reaction products reflecting the concentration of NO_2^- was assessed at 550 nm on a microplate reader. The results were expressed as micromoles of NO_2^- per milliliter.

**TNF-α mRNA analysis**

Cell cultures for RNA studies were performed at cell densities as described above, but in 25-cm² culture flasks (Falcon; BD Biosciences) and a volume of 5 ml of RNA was isolated by Tri-Resagent (Sigma) essentially as suggested by the manufacturer. After spectrophotometric determination of the concentration, an aliquot of the RNA was electrophoresed for quality control on an ethidium bromide-stained formaldehyde agarose gel to check for RNA degradation (data not shown). Before PCR, 10 μg of the total RNA was reverse transcribed with Superscript II (Life Technologies) in a total volume of 40 μl, and 2 μl of each sample was used as a template for the quantitative determination of mRNA molecules by TaqMan PCR (Applied Biosystems PRISM 7700 Sequence Detector System; PE Applied Biosystems, Foster City, CA). This analysis was performed as previously described (21) with minor modifications. Briefly, the standard curve for TaqMan PCR used 1 × 10^1–1 × 10^6 molecules of a cloned cDNA standard for each parameter to be determined. All samples were measured in triplicate and added in a volume of 10–40 μl Master Mix. As an internal reference dye, 6-carboxy-tetramethyl-rhodamine was used instead of 6-carboxy-X-rhodamine, and uracil-n-glycosylase Amperase was omitted. The reaction conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles with 15 s at 95°C and 1 min at 60°C, and a final hold at 25°C. The efficiency of the PCR was monitored by the slope of the standard curve and was near 100%. For analysis of the mRNA levels, the ratio of the molecule numbers of TNF-α/β-actin was determined and plotted as the means of triplicate determinations in four independent experiments.

**Statistical analysis**

Data were expressed as the mean ± SD. Statistical analysis was performed using Student’s two-sided t test. Differences were considered statistically significant at p < 0.05.
Results

CTB suppresses the proinflammatory response of macrophages

Incubation of mouse macrophages with CTB rendered the cells refractory to subsequent activation by treatment with LPS. As shown in Fig. 1A, pretreatment with CTB suppressed the TNF-α response to LPS by >80%. CTB itself did not elicit TNF-α production. The same result was obtained with a human monocyte line. Again, CTB pretreatment suppressed the TNF-α response to LPS (Fig. 1B).

The impact of CTB on inducible NO production could only be studied in mouse macrophages because human monocytes are poor producers of NO. Nitrite as a stable end product of NO formation was seen in the supernatant of LPS-activated macrophages. Pretreatment with CTB significantly decreased LPS-induced nitrite production (p < 0.001; Fig. 2). CTB alone did not induce nitrite formation.

Several control experiments were performed to exclude an involvement of possible contaminants in the CTB preparation. Polymyxin B was added to the pretreatment culture to neutralize possible endotoxin contamination. Polymyxin B suppressed LPS-induced macrophage tolerance, but did not interfere with CTB action (Fig. 3A). A contribution of CTA chain was tried by adding increasing amounts of 2',3'-dideoxycytidine. Such treatment did not prevent the suppressive action of CTB (Fig. 3B).

Additional experiments were performed to exclude a possible contamination of the CTB preparation with CT, which has recently been shown to modulate the formation of several costimulatory mediators, such as IL-10, IL-6, IL-1β, IL-12, TNF-α, and NO, from bone marrow-derived macrophages (22). We therefore repeated the experiment with recombinant CTB. As shown in Fig. 4, identical results were obtained when recombinant CTB was used instead of purified CTB. For comparison, we also pretreated macrophages with the holotoxin CT, which is able to exert potent proinflammatory activities, and observed suppression of LPS-induced TNF-α production to a similar extent as with CTB (Fig. 4).

A final control was to test for eventual endotoxin contamination of the recombinant CTB preparation. Following a detailed analysis, boiling of recombinant proteins is the only safe way to check for such contamination (23). Boiling for 10 min completely abolished the suppressive action of recombinant CTB as well as that of CT, whereas LPS retained its biological activity (Fig. 4).

Dose dependence and kinetics of CTB action

Mono Mac 6 cells were pretreated with varying concentrations of CTB. As shown in Fig. 5A, the suppressive action of CTB was dose dependent and no more recognizable at 1 μg/ml or less. Next we analyzed whether CTB-induced desensitization is a permanent or transient state of monocyte function. We therefore exposed Mono Mac 6 cells to CTB for 5 h and challenged the cells with LPS after different periods of time. Our data show that after only 5 h of pretreatment with CTB maximal suppression of TNF-α production was reached (Fig. 5B). Monocyte resistance toward LPS persisted at the same level for 24 h and started to fade by 48 h of culture.

CTB desensitizes monocytes for IL-6 and IL-12, but primes for IL-10 production

Next we examined whether CTB desensitizes not only for the LPS-induced synthesis of the proinflammatory mediators TNF-α and NO, but also for other cytokines involved in the regulation of inflammatory reactions. Fig. 6A shows that CTB pretreatment of

FIGURE 2. Suppression of inducible nitrite formation in macrophages pretreated with CTB. Mouse J774 macrophages were pretreated for 24 h with CTB (10 μg/ml) or medium and challenged with LPS (1 μg/ml) for 24 h. Shown are mean nitrite levels in culture supernatants from nine experiments ± SD. ***, p < 0.001.

FIGURE 3. There was no suppression of CTB effects by polymyxin B (PmB) or 2',3'-dideoxycytidine (DDA). Human Mono Mac 6 monocytes were pretreated with CTB (10 μg/ml) or LPS (10 μg/ml) for 5 h in the presence or absence of PmB (0.1 μg/ml; A) or in the presence of increasing concentrations of DDA (B). The subsequent challenge was with 1 μg LPS/ml for 5 h. Shown are mean TNF-α concentrations in the culture supernatants from six experiments ± SD. **, p < 0.01, *** p < 0.001.
monocytes is able to suppress the production of IL-12(p70), another proinflammatory cytokine. As shown in Fig. 6B the formation of IL-6 was suppressed by about 60% (p < 0.05). By contrast, pretreatment of Mono Mac 6 cells with CTB did not impair the IL-10 response to LPS; rather, cells were primed for significantly enhanced, but not lasting, production of IL-10 (Fig. 6C). CTB alone did not induce measurable IL-12(p70), IL-6, and IL-10 secretion. CTB also did not induce cytokine secretion if cells were washed after 5 h, and new medium was added for another 24 h to mimic the pretreatment protocol (data not shown).

**CTB desensitizes human PBMC for LPS-induced TNF-α and IL-12 production**

To investigate whether the desensitizing effect of CTB is limited to macrophage cell lines we pretreated freshly isolated human PBMC with medium or recombinant CTB (10 μg/ml) for 5 h and challenged the cells with LPS (1 μg/ml). In control samples without CTB preincubation, LPS induced TNF-α production of 11.6 ng/ml and IL-12 production of 246 pg/ml (Fig. 7), which is about 40-fold higher than the amount of IL-12 released by LPS-exposed Mono

---

**FIGURE 4.** Suppression of TNF-α response to LPS by recombinant CTB or CT. Human monocytes were pretreated with medium, recombinant CTB (10 μg/ml), or CT (100 ng/ml) for 5 h and challenged with LPS (1 μg/ml) for 6 h. In parallel, treatment was performed with reagents exposed for 10 min to 100°C (boiled). Shown are the mean TNF-α concentrations in culture supernatants from three to six experiments ± SD. ***, p < 0.001.**

**FIGURE 5.** Dose dependence and kinetics of CTB action. Human Mono Mac 6 monocytes were pretreated with CTB for 5 h at varying concentrations (A), or for increasing time periods (B) and subsequently challenged with LPS (1 μg/ml) for 5 h. Shown are mean TNF-α concentrations in the culture supernatants from six experiments ± SD. *, p < 0.05, **, p < 0.01.

**FIGURE 6.** CTB suppresses IL-6 and IL-12 production and primes for IL-10 production. Human Mono Mac 6 monocytes were pretreated with CTB (10 μg/ml) for 5 h and challenged with LPS at time 0 (CTB→LPS). In parallel, samples were pretreated with medium and challenged with LPS or CTB at time zero. The concentrations of IL-12(p70) (A) and IL-6 (B) were determined in the culture supernatants after 24 h. Shown are mean cytokine concentrations from six experiments ± SD. *, p < 0.05; **, p < 0.01. For IL-10 concentrations (C) the kinetics are shown in the culture supernatants (mean of three experiments performed in triplicate ± SD). At 5 h the difference between CTB→LPS and LPS was significant (p < 0.01).
Mac 6 cells (Fig. 6A). Pre-exposure to CTB significantly inhibited LPS-induced formation of TNF-α (Fig. 7A) and IL-12 (Fig. 7B).

**Mechanism of CTB action**

To determine whether the suppressive action of CTB occurred at the level of gene expression, RNA was isolated from monocytes, and the level of TNF-α mRNA was determined by quantitative RT-PCR. Incubation of Mono Mac 6 cells with LPS led to a 19.2-fold increase in TNF-α mRNA levels. Pretreatment with CTB almost abolished TNF-α mRNA levels in response to LPS ($p < 0.01$; Fig. 8A). CTB alone did not increase TNF-α mRNA levels. For comparison, macrophage tolerance was induced by pretreatment with low dose LPS (10 ng/ml), which also significantly suppressed TNF-α mRNA levels after challenge with high dose LPS (Fig. 8A). As a consequence, significantly less TNF-α was released into the supernatant after either CTB or LPS pretreatment and challenge with LPS ($p < 0.01$; Fig. 8B). TNF-α secretion in these experiments occurred in 25-cm² flasks and was ~3.2 times lower than that described above for assays in 96-well tissue culture plates.

Since CTB pretreatment had primed monocytes for enhanced IL-10 production, we tested whether macrophage-deactivating cytokines such as IL-10 or TGF-β were involved in CTB suppression. Mono Mac 6 cells were incubated with a combination of neutralizing Abs to IL-10 and TGF-β during pretreatment with CTB.
CTB. As shown in Fig. 9A such treatment resulted in a partial and weakly significant ($p < 0.05$) restoration of the TNF-α response to subsequent LPS challenge. The presence of the individual anti-IL-10 and anti-TGF-β Abs during CTB pretreatment did not significantly counteract CTB-mediated desensitization (data not shown). Pretreatment with Abs directed against IL-10 and TGF-β successfully counteracted CTB-mediated desensitization of the LPS-induced production of the cytokine IL-6. As shown in Fig. 9B the IL-6 response was restored to about 60–80% of the value achieved without CTB preincubation ($p < 0.01$).

Finally, we tested the concept that the suppressive signaling of CTB could be overcome by the presence of a second stimulatory signal. Mono Mac 6 cells were treated with IFN-γ and/or GM-CSF in addition to CTB. As shown in Fig. 10A, IFN-γ completely abolished the suppressive action of CTB. The addition of GM-CSF did not counteract CTB action, nor did GM-CSF antagonize or synergize with IFN-γ. We determined whether IFN-γ would directly interfere with CTB signal transduction or would yield an independent signal antagonizing CTB signals. Monocytes were first treated with CTB for 5 h, washed, and then cultured in the presence of cytokines for 24 h. IFN-γ, when given after CTB, reversed suppression and restored normal reactivity to LPS (Fig. 10B). As before, GM-CSF was neutral and did not modulate the actions of CTB or IFN-γ.

Discussion

Incubation of macrophages with CTB suppressed the ability to mount a proinflammatory response to challenge with LPS, as demonstrated by the significantly decreased production of TNF-α or nitrite. In control experiments possible endotoxin contaminations were neutralized by the addition of polymyxin B, and this did not prevent suppressive effects of CTB. Since the CTB preparation might contain traces of the intact toxin or of the A subunit, an inhibitor of toxin action was added, but this did not interfere with CTB activity over a wide concentration range. More importantly, recombinant CTB, which is devoid of any CT contamination, exerted suppressive activity to a similar degree as the native CTB preparation, although CTB does not activate adenylate cyclase (24). These findings clearly establish that the lectin CTB exhibits immunosuppressive activities and that such properties of CTB are not due to trace contamination with CT as has been suggested previously (25).

The suppressive action of CTB was dose dependent and required $\sim 0.1$ μM of the CTB pentamer for a significant biological effect. CTB induces not an irreversible but, rather, a transient state of unresponsiveness in the monocytes. The desensitizing effect of CTB on the macrophage response to LPS lasted for at least 48 h and was already fully established within 5 h of incubation.

CTB-induced LPS tolerance is reminiscent of desensitization of macrophages to LPS challenge by pretreatment of cells with small endotoxin doses (26–29). In the latter case macrophage function is not completely down-regulated, but cells acquire an alternate state of activation upon LPS rechallenge, characterized by the suppression of proinflammatory mediators such as TNF-α or NO. Our findings show that CTB pretreatment suppresses the formation of the proinflammatory mediators TNF-α, IL-6, NO, and IL-12, a major Th1-associated cytokine. In primary human PBMC pre-exposure to CTB also resulted in an inhibition of LPS-induced TNF-α and IL-12 formation. Although the desensitization of PBMC was not as pronounced as in Mono Mac 6 cells, these results indicate that the sensitivity to CTB-mediated desensitization is not restricted to monocytic cell lines, but represents a general property of monocytes. The desensitizing effect of CTB may also be relevant in murine experimental colitis, which resembles human Crohn’s disease (30). In this animal model of a Th1-driven mucosal inflammation the administration of CTB resulted in an inhibition of the disease associated with a reduction of IFN-γ secretion and an inhibition of IL-12 production. In contrast to the strong suppression of proinflammatory mediators by LPS desensitization the production of antagonistic mediators such as IL-10 is still inducible and may even be potentiated (28, 31). We therefore also analyzed for IL-10 production in CTB-pretreated monocytes and found no suppression of cytokine formation. Rather, elevated peak levels of IL-10 were observed after 5 h of LPS exposure, thereby coinciding with the strong suppression of the proinflammatory mediator TNF-α. Subsequently, the high IL-10 levels declined more rapidly than in control cells without CTB pretreatment, which may be explained by the macrophage-deactivating properties of IL-10. From these observations it may be concluded that CTB induces split tolerance in macrophages by suppressing.

**FIGURE 10.** IFN-γ reverses CTB-mediated suppression. A, Human Mono Mac 6 monocytes were pretreated with CTB (10 μg/ml) for 24 h in the presence or absence of IFN-γ (1200 U/ml) and/or GM-CSF (50 ng/ml), followed by a challenge with LPS (1 μg/ml) for 5 h. Mean TNF-α concentrations in LPS-treated cultures without CTB preincubation were 9.1 ng/ml. B, Mono Mac 6 cells were treated with CTB for 5 h, washed, and subsequently cultured for 24 h with IFN-γ and/or GM-CSF before the subsequent challenge with 1 μg of LPS/ml for 5 h. In medium-pretreated cultures the LPS-induced TNF-α level was 6.4 ng/ml. Shown are mean values of six experiments with the inhibited TNF-α response to LPS set at 100%. **, $p < 0.01$. 

The Journal of Immunology 1735

Downloaded from http://www.jimmunol.org/ by guest on October 29, 2017
proinflammatory responses while preserving the inducibility of antigenic mediators, i.e., IL-10. Therefore, LPS receptor function is still present in CTB-pretreated macrophages, but the outcome of signaling is different.

Since CTB transiently shifted macrophage reactivity toward IL-10 secretion during tolerance induction, we wondered whether tolerance induction could be overcome by Th1-associated cytokines. Indeed, pretreatment with IFN-γ completely prevented tolerance induction by CTB. Pretreatment with GM-CSF did not prevent macrophage tolerance induction. When the cytokines were added to macrophages after their exposure to CTB, macrophage reactivity to LPS could largely be restored by IFN-γ, but not by GM-CSF. It appears that macrophages can be shifted from the proinflammatory to the antagonistic state and vice versa by the corresponding mediators.

LPS-induced macrophage tolerance is prevented in the presence of Abs to IL-10 and TGF-β, suggesting that these two cytokines are involved in the desensitization process (32). We found that the cytokine Abs also counteract CTB-induced macrophage tolerance. The preventive effect of the Abs was much pronounced for IL-6 than for the proinflammatory mediator TNF-α. This observation indicates that IL-10 and TGF-β only partially account for the suppression of TNF-α production. The preferential activity of CTB to suppress proinflammatory mediators may be important in weakening the defense response of the innate immune system against cholerabacteria. Since IL-10 and TGF-β are key mediators of oral tolerance responses (33, 34), our findings may explain the mechanism by which CTB potentiates oral tolerance responses when physically linked to Ags (1).

An important characteristic of CTB is that it shares with LPS the ability of inducing macrophage tolerance, but, in contrast to LPS, is devoid of any proinflammatory properties. CTB did not elicit a TNF-α or nitrite response while deviating macrophage reactivity. In contrast, tolerizing doses of LPS usually elicit a substantial proinflammatory response before rendering cells refractory to a second challenge with LPS (28). Very low doses of LPS may result in an enhanced secondary response (35, 36). Interestingly, preincubation with CT holotoxin, which is known to be a potent proinflammatory and macrophage-activating agent (37–40), also was found to desensitize Mono Mac 6 cells to a similar extent as after pre-exposure to LPS. The observed macrophage-desensitizing capacity of CT corresponds to previous studies demonstrating tolerizing properties of the holotoxin in models of allograft transplantation (41, 42), delayed-type hypersensitivity (43), and experimental autoimmune neuritis (44). Hence, similar to LPS, CT shares pro- and anti-inflammatory properties.

Because of the lack of a proinflammatory response, macrophage tolerance induction by CTB is a silent process, which could also be relevant when CTB is used for the treatment of autoimmune diseases. In these situations CTB may desensitize APC to release proinflammatory mediators in response to inflammatory signals, thereby down-regulating the progression of the autoaggressive process. Synthetic compounds mimicking the immunomodulatory effects of CTB on macrophages may provide a new approach for deviating the proinflammatory activity of innate immune cells.

Acknowledgments

We are grateful to Drs. C. Cerkinsky and F. Anjuere (Institut National de la Santé et de la Recherche Médicale, Unité de Recherches 364, Nice, France) for the gift of recombinant CTB. We thank Christine Paul and Jennifer Skopnik for excellent technical assistance, and Dr. Stephen Martin for help with the RT-PCR analysis.

References


