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Cholera toxin differentially regulates nitric oxide synthesis, tumor necrosis factor-α production and respiratory burst in murine macrophages

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Abstract

The aim of this study was to determine if cholera toxin can modulate the expression of several macrophage effector functions. The effect of cholera toxin on the induction of NO synthesis, production of tumor necrosis factor-α and induction of respiratory burst was examined in the J774.A2 macrophage cell line. Pre-incubation of cell cultures with cholera toxin significantly down-regulated lipopolysaccharide-induced NO synthesis and phorbol-12-myristate-13-acetate-induced respiratory burst. Concomitant addition of cholera toxin and lipopolysaccharide to cell cultures enhanced the production of tumor necrosis factor-α. These effects were abrogated when cholera toxin was inactivated by heat or treated with a specific monoclonal antibody.

Keywords: *Vibrio cholerae*; Cholera toxin; Tumor necrosis factor-α; Nitric oxide; Respiratory burst

1. Introduction

Cholera is an acute diarrheal disease consisting in the passage of voluminous stools of rice water character. *Vibrio cholerae* of serogroups O1 and O139, the causative agents of cholera, secrete a potent enterotoxin, cholera toxin (CT), that causes the clinical symptoms of the disease [1,2]. To cause infection, cholera vibrios must overcome the gastric acid barrier, attach to and penetrate the mucus coat and reach the underlying epithelial cells [1]. Vibrios are also taken up by M cells in which they encounter lymphocytes, macrophages and occasionally polymorphonuclear leukocytes (PMNs) [3].

CT catalyzes NAD-dependent ADP-ribosylation of the α subunit of certain G-proteins resulting in activation of adenylate cyclase and subsequent increase in intracellular cAMP concentration [1]. The increased levels of cAMP activate protein kinase A which in turn increases chloride secretion by modifying and up-regulating ion channels [1]. CT is endowed with a second mode of action, which involves activation of phospholipase C, hydrolysis of membrane phospholipids and accumulation of arachidonic acid, the precursor of prostaglandin and leu-

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kotriene secretagogues [4,5]. Elevated levels of jejunal prostaglandin E_2 have been demonstrated during the secretory stage of clinical cholera [6]. Recently, platelet activation factor has been implicated in the pathogenesis of intestinal secretion stimulated by CT [7]. Additionally, CT has a variety of effects on immune cells such as stimulating interleukin-1 proliferation, enhancing antigen presentation by macrophages, promoting B-cell isotype differentiation and inhibiting Th1 cells [8–10].

Although CT is responsible for the clinical symptoms of cholera, volunteers orally immunized with certain recombinant *V. cholerae* strains lacking cholera toxin genes and other toxic factors still produce mild diarrhea and other forms of reactogenicity [11]. The detection of interleukin-8 and lactoferrin in the stools of vaccinees suggests a local inflammation process [12]. Recent studies have demonstrated production of proinflammatory cytokines by intestinal epithelial cells in response to pathogens [13]. However, interaction of cholera vibrios with the immune cells of the lamina propria (monocytes, macrophages, PMNs, etc.) could also contribute to intestinal secretion and other symptoms.

In the present study we examine the effect of *V. cholerae* O1 lipopolysaccharide (LPS) and CT on two macrophage microbicidal effector functions, NO synthesis and respiratory burst, and on the production of proinflammatory cytokine tumor necrosis factor-α (TNF-α). We show that in J774.A2 cells, CT down-regulates LPS-induced NO production, phorbol-12-myristate-13-acetate (PMA)-induced respiratory burst and enhances LPS-induced production of TNF-α.

2. Materials and methods

2.1. Reagents

Tissue culture media, supplements and reagents were purchased from Sigma (St. Louis, MO, USA). CT and cholera toxin B subunit (CTB) purified from *V. cholerae* strain 569B were kindly provided by Dr. R.A. Finkelstein (University of Missouri School of Medicine, Columbia, MO, USA). Monoclonal antibody 4E1G5 reacts with the B subunit of CT [14,15].

2.2. Preparation of lipopolysaccharide

V. cholerae serogroup O1 LPS was purified from strain E7946 (El Tor, Ogawa) as described elsewhere [16]. Briefly, cells were extracted with hot phenol, the aqueous phase dialyzed against phosphate-buffered saline (PBS) and LPS recovered by ethanol precipitation. The preparation was cleared of nucleic acids and proteins by treatment with RNase, DNase and Pronase. The purity of LPS was established by scanning UV spectrophotometry and SDS-polyacrylamide gel electrophoresis with silver staining.

2.3. Cell culture

Murine monocytic leukemia J774.A2 cells were grown in RPMI-1640 medium supplemented with fetal calf serum (10%), glutamine (20 mM), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}). Cells were seeded in 96-well trays at a density of 10^6 cells ml^{-1} and stimulated for NO and TNF-α synthesis with *V. cholerae* O1 LPS (1 μg ml^{-1}). CT or CTB was added to cell cultures prior to or concomitantly with LPS. Culture supernatants were analyzed for TNF-α and NO production at 18 and 48 h respectively after stimulation.

2.4. NO determination

Nitrite levels were measured as an indication of NO production by diazotation with Griess reagent as described previously [17]. Briefly, aliquots of cell-free culture supernatants were mixed with one volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl-ethylenediamine in 2% phosphoric acid). After 10 min at room temperature, absorbance was read at 540 nm. Nitrite concentration was calculated from a standard curve using sodium nitrite.

2.5. TNF-α determination

TNF-α levels were determined by the cytotoxic activity assay on mouse L929 cells [18]. The amount of TNF-α in cell-free culture supernatants was calculated using a standard curve of human recombinant TNF-α (10^6 U mg^{-1}, BASF-Knoll, Ludwigshafen, Germany).

2.6. Respiratory burst chemiluminescence assay

J774.A2 cells were adjusted to a density of 10^6 cells ml^{-1} in PBS. Aliquots of 0.4 ml were dispensed into polystyrene tubes and placed at 37°C. After addition of luminol (40 μg ml^{-1}), cells were stimulated with PMA (3 μg ml^{-1}). The tubes were shaken gently and placed in a luminometer (LKB, model 1250). Chemiluminescence signals were read at 1-min intervals over a 5-min period and the results are expressed as the integrated area under the response curve. In some experiments, the cell suspension was pre-incubated 30 min at 37°C with 0.1 μg ml^{-1} CT or CTB.

3. Results

3.1. NO production

V. cholerae O1 LPS induced a potent NO response in the murine macrophage cell line (Fig. 1). Nitrite production was markedly reduced by 500 μM N^G-monomethyl-L-arginine (data not shown). Neither CT nor CTB induced NO production. No differences were observed in NO production when LPS was added together with 0.1 μg ml^{-1} CT or CTB (Fig. 1). However, a marked reduction in nitrite production was observed when cells were pre-incubated 30 min to 1 h with CT prior to the addition of LPS (Fig. 2A). The induction of NO by LPS was practically abrogated by pre-incubation with increasing amounts of CT (Fig. 2B). Heat-inactivated CT did not affect LPS-induced NO production while addition of mAb 4E1G5 prevented down-regulation of NO synthesis.

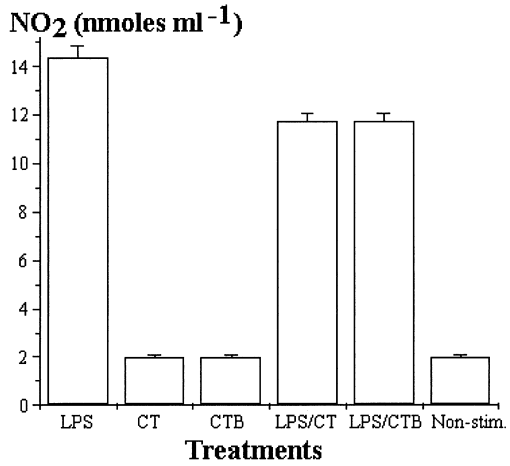

Fig. 1. Effect of CT and CTB on LPS-induced NO production. CT and CTB (0.1 μg ml^{-1}) were added concomitantly with LPS (LPS/CT, LPS/CTB) and cell cultures incubated 48 h for nitrite determination. Basal nitrite levels correspond to non-stimulated cell cultures (Non-Stim.). Results are expressed as means ± S.E.M. of three independent experiments.

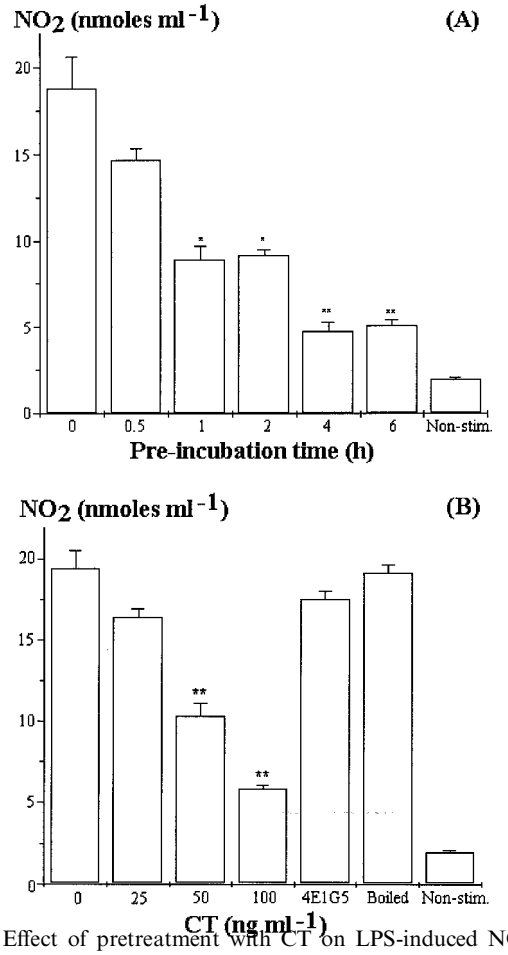

Fig. 2. Effect of pretreatment with CT on LPS-induced NO production. A: CT (0.1 μg ml^{-1}) was added to J774.A2 cultures at different times prior to addition of LPS (1 μg ml^{-1}) and nitrite levels were determined 48 h after LPS stimulation. B: Dose-response relationship between CT pretreatment and LPS-induced NO production. The effect was abrogated when CT (0.1 μg ml^{-1}) was inactivated by heat or treated with mAb 4E1G5. Basal nitrite levels correspond to non-stimulated cultures (Non-Stim.). Results are expressed as means ± S.E.M. of three independent experiments. Significance in *t*-test, $*P < 0.05$, $**P < 0.01$.

3.2. TNF-α production

J774.A2 cells produced significant amounts of

TNF-α when stimulated with *V. cholerae* O1 LPS. TNF-α was not detected in culture supernatants of cells incubated with CT or CTB (0.1 μg ml^{-1}) in the absence of LPS. Treatment with CT and LPS yielded higher TNF-α levels than treatment with LPS alone regardless of CT being added together or 4 h prior to the addition of LPS (Fig. 3A). CTB had no detectable effect on the stimulation of TNF-α production by LPS. As shown in Fig. 3B, the effect of CT on LPS-induced TNF-α production in J774.A2 cells was dose-dependent. As expected, heat-inactivated CT did not stimulate LPS-induced TNF-α production and neutralization of CT (0.1 μg) significantly reduced the induction of TNF-α.

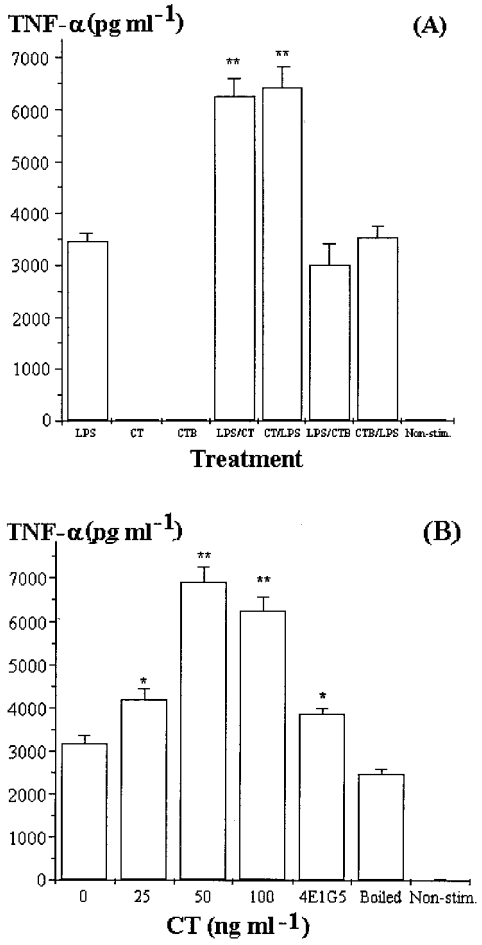

Fig. 3. Effect of CT and CTB on LPS-induced TNF-α production. A: CT or CTB (0.1 μg ml^{-1}) were added 4 h prior to LPS stimulation (CT/LPS, CTB/LPS) or added concomitantly with LPS (LPS/CT, LPS/CTB) and incubated for 18 h. B: Dose-response relationship between CT and TNF-α production. The effect was abrogated when CT (0.1 μg ml^{-1}) was inactivated by heat or treated with mAb 4E1G5. Results are expressed as means ± S.E.M. of three independent experiments. Significance in *t*-test, $*P < 0.05$, $**P < 0.01$.

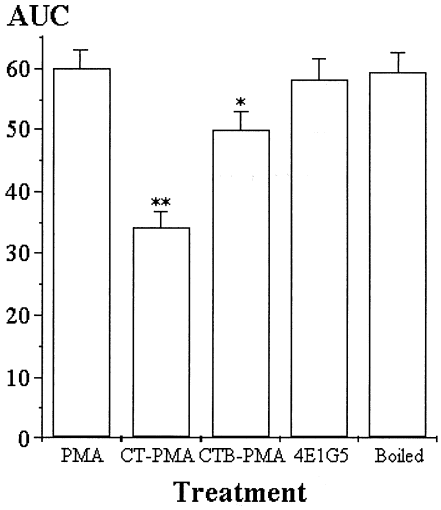

Fig. 4. Effects of CT and CTB pretreatment on PMA-induced respiratory burst. CT or CTB (0.1 μg ml^{-1}) were added 30 min prior to PMA stimulation (CT-PMA, CTB-PMA). The response was abrogated when CT (0.1 μg ml^{-1}) was inactivated by heat or treated with mAb 4E1G5. Results are expressed as means ± S.E.M. of three independent experiments. Significance in Duncan's test for multiple mean comparisons, $*P < 0.05$, $**P < 0.01$. AUC, area under curve.

3.3. Respiratory burst

The pre-incubation of J774.A2 cells with CT for 30 min before stimulation markedly reduced PMA-induced respiratory burst (Fig. 4). CTB was also effective but to a lesser extent. Heat-inactivated CT and CTB or neutralization with mAb eliminated this effect.

4. Discussion

Although the interaction of cholera vibrios or their components with macrophages is crucial to immune response and could trigger the synthesis of mediators causing discomfort upon oral vaccination with recombinant attenuated strains, studies in this area have been neglected.

In the present report we demonstrate that CT down-regulates LPS-induced NO synthesis, diminishes PMA-induced respiratory burst and enhance LPS-induced TNF-α production. Repression of in-

ducible NO synthase by prostaglandin E_2 and cAMP has been reported in several cell types including J774.A2 [19,20]. It is likely that CT exerted its inhibitory action on NO production by increasing cAMP and/or prostaglandin E_2 levels. Volunteers challenged with virulent *V. cholerae* developed increased nitrite and nitrate levels in blood and urine [21] suggesting oxygen-independent killing mechanisms to be an important host defense in cholera infection. The mechanism by which infected volunteers developed increased NO levels is not known. It is noteworthy that the effect of CT observed in monocytic cells was to decrease rather than stimulate NO production. It is conceivable that interactions of bacteria with epithelial cells leading to pathogenesis could differ from those involving other cell types such as the immunocompetent cells of the lamina propria.

Cyclic AMP elevating agents have also been widely associated with a marked reduction of respiratory burst by a mechanism involving activation of cAMP-dependent protein kinases [22]. A role for oxidative stress in host defense is suggested by the observation that some *V. cholerae recA* mutants which display a peroxide-stress hypersensitive phenotype are defective in intestinal colonization [23,24]. The finding that CT could diminish host nonspecific defense mechanisms is significant for ensuring vibrios a higher chance to survive in the intestinal milieu.

The mechanism involved in up-regulation of LPS-induced TNF-α production by CT is not clear. Increases of intracellular cAMP and prostaglandin concentrations are known to be potent down-regulation signals for TNF-α production [25]; however, modulation of TNF-α production by CT in other cell lines has yielded variable results [26,27]. The recent detection of inflammatory markers in the stools of volunteers immunized with live recombinant attenuated *V. cholerae* strains is suggestive of an inflammatory host response to colonizing vibrios [12]. TNF-α is known to exert proinflammatory effects by inducing the expression of adhesion molecules that recruit leukocytes, by priming leukocytes for oxidant production and by inducing the production of prostaglandin and other mediators [28]. The production of proinflammatory cytokines and NO in absorptive epithelial cells has been reported [13,29]. Thus, work is in progress to examine the role of LPS, CT and other bacterial factors in the production of inflammatory mediators in cultured differentiated human intestinal cells to which *V. cholerae* adhere [30].

We conclude from the present data that, in addition to its previously reported physiological effects, CT is a strong modulator of the expression of several macrophage-associated functions.

Acknowledgments

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