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Effects of Botulinum Toxin Type D on Secretion of Tumor Necrosis Factor from Human Monocytes

K. IMAMURA, D. SPRIGGS, T. OHNO, AND D. KUFE*

Laboratory of Clinical Pharmacology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

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Botulinum toxins are potent neurotoxins which block the release of neurotransmitters. The effects of these toxins on hematopoietic cells, however, are unknown. Monocytes secrete a variety of polypeptide growth factors, including tumor necrosis factor (TNF). In the study reported here, the effects of botulinum toxin type D on the secretion of TNF from human monocytes were examined. The results demonstrate that botulinum toxin type D inhibits the release of TNF from monocytes activated by lipopolysaccharide (LPS) but not by 12-*O*-tetradecanoylphorbol-13-acetate. Botulinum toxin type D had no detectable effect on intracellular TNF levels in LPS-treated monocytes, indicating that the effects of this toxin involve the secretory process. This inhibitory effect of botulinum toxin type D on TNF secretion from LPS-treated monocytes was partially reversed by treatment with 12-*O*-tetradecanoylphorbol-13-acetate or introduction of guanosine 5′-[γ-thio]triphosphate into these cells. The results demonstrate that TNF secretion is regulated by at least two distinct guanine nucleotide-binding proteins, one responsible for the activation of phospholipase C and another which acts as a substrate for botulinum toxin type D. ADP-ribosylation of monocyte membranes by botulinum toxin type D demonstrated the presence of three substrates with M_rs of 45,000, 21,000, and 17,000. While the role of these substrates in exocytosis is unknown, the results suggest that the M_r 21,000 substrate is involved in a process other than TNF secretion.

Previous studies have demonstrated that botulinum toxin type D (20) specifically ADP-ribosylates an M_r 21,000 protein in bovine adrenal cell membranes (15). Moreover, this toxin inhibits exocytosis in adrenal chromaffin cells (12). These studies have led to the speculation that botulinum toxin type D ADP-ribosylates a specific membrane protein common to secretory cells (15). More recent evidence suggest that exocytotic secretion from different cell types is regulated by a distinct guanine nucleotide-binding protein (3, 4, 8, 14, 19). However, the identity of this putative GTP-binding protein has remained unclear.

Monocytes secrete tumor necrosis factor (TNF) in response to stimulation with lipopolysaccharides (LPS) (13, 16). TNF levels were thus monitored in supernatants of human monocytes cultured in the presence of LPS alone or in the presence of LPS with botulinum toxin type D. Human peripheral blood monocytes were purified by Ficoll-Hypaque separation, adherence for 1 h, and removal of the nonadherent cells (9). The adherent cell population was collected with a plastic policeman, readhered for 1 h, and washed again with three changes of medium. The second adherent cell population was used in these studies and consisted of more than 96% monocytes, as determined by morphologic examination. Monocytes were incubated for 15 h with 2 μg (2×10^8 MLD_{50}/mg of protein [MLD_{50} is the lethal dose for 50% of the mice) of botulinum toxin type D per ml (Wako Chemicals, Osaka, Japan). The cells were then treated with 50 nM LPS or 33 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and monitored for secretion of TNF protein (21).

TNF protein was detectable after 3 h of LPS treatment and continued to increase during 18 h of exposure. In contrast, pretreatment of these monocytes with botulinum toxin type D and subsequent addition of LPS were associated with a marked decrease in TNF secretion in the supernatant (Fig. 1A). TPA also induced TNF secretion, although the pattern of release into the supernatant differed from that obtained with LPS (Fig. 1B). However, botulinum toxin type D had little if any effect on TPA-induced TNF secretion (Fig. 1B). These findings suggested that botulinum toxin type D inhibited an event required for LPS-stimulated TNF production, while TPA induced TNF secretion by a different mechanism. TPA activates protein kinase C in monocytes (9), and activation of this enzyme has been shown to enhance secretion in certain cells by reducing the requirement for calcium (11). Alternatively, TPA-induced TNF secretion could result from subsequent down-regulation of protein kinase C or from other effects of this agent.

The finding that botulinum toxin type D inhibits TNF secretion by LPS-treated monocytes raised the possibility that this effect might be related to decreased TNF production. Thus TNF gene expression was monitored at both the mRNA and protein levels. TNF mRNA levels were low to undetectable in uninduced monocytes, while the human TNF cDNA probe (21) hybridized to a 1.6-kilobase transcript that was induced in LPS-treated cells (Fig. 2A). Pretreatment of these cells with botulinum toxin type D had little if any effect on induction of TNF mRNA levels following exposure to LPS (Fig. 2A). Moreover, treatment with these agents had no detectable effect on constitutive actin gene expression (Fig. 2A).

Intracellular TNF protein was also monitored at various intervals after adding LPS (Fig. 2B). The monocytes were lysed in 50 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–20 mM EDTA–1% Triton X-100–1% sodium deoxycholate–0.1% sodium dodecyl sulfate–1 mM phenylmethylsulfonyl fluoride–10 μg of leupeptin per ml–100 μM sodium orthovanadate for determination of intracellular TNF protein levels (21). While not found in uninduced cells, TNF was detectable by 1 h and remained at relatively constant levels

* Corresponding author.

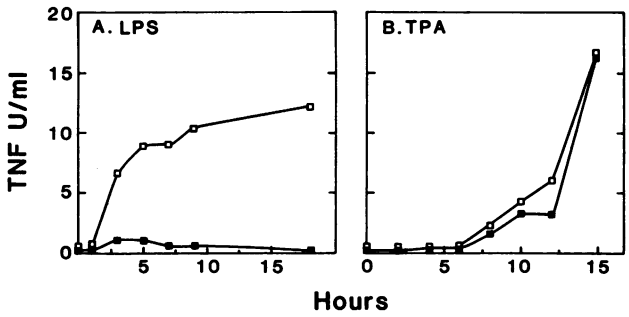

FIG. 1. Effects of botulinum toxin type D on secretion of TNF from LPS- and TPA-stimulated human monocytes. Monocytes were preincubated for 15 h with 2 μg of botulinum toxin type D per ml. The cells (2×10^6/0.5 ml) were then treated with 50 nM LPS (A) or 33 nM TPA (B) for the indicated times (■). Preparations of the same monocytes incubated for 15 h in the absence of toxin were also treated with LPS or TPA (□). TNF protein levels were determined by an enzyme-linked immunosorbent assay (ELISA) (21).

through 9 h of LPS exposure. This pattern of intracellular TNF production was similar in monocytes which had been treated with botulinum toxin type D and then exposed to LPS (Fig. 2B). However, TNF levels were relatively higher in LPS-induced monocytes that had been pretreated with toxin (Fig. 2B). These findings indicated that TNF secretion, rather than production, was inhibited by botulinum toxin type D.

The involvement of GTP-binding proteins in TNF secretion was studied in monocytes treated with botulinum toxin type D and then stimulated with LPS for 12 h. The stimulated cells were permeabilized as previously described (7) with 5 μM ATP in a divalent cation-free salt solution (137 mM NaCl, 2.7 mM KCl, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid] [pH 7.7], 5.6 mM glucose, 1 mg of bovine serum albumin per ml) for 10 min at 37°C. Various concentrations of guanosine 5'-[γ-thio]triphosphate were included as indicated during the permeabilization. $MgCl_2$ (5 mM) was then added for an additional 10 min at 37°C. The monocytes were then pelleted, and the supernatant was analyzed for TNF secretion.

Permeabilization in the absence of guanosine 5'-[γ-thio] triphosphate had no detectable effect on TNF release (Fig. 3A). In contrast, the nucleotide stimulated TNF secretion in a concentration-dependent manner (Fig. 3A). The guanosine 5'-[γ-thio]triphosphate-induced stimulation of TNF secretion from monocytes treated with botulinum toxin type D and LPS was less than that obtained with cells treated with LPS alone (Fig. 3A), while unstimulated monocytes treated with guanosine 5'-[γ-thio]triphosphate failed to secrete detectable levels of TNF (data not shown).

Although guanosine 5'-[γ-thio]triphosphate partially reversed the inhibitory effects of botulinum toxin type D on LPS-induced TNF secretion, these findings may have been related to stimulation of phospholipase C by guanosine 5'-[γ-thio]triphosphate and the resultant activation of protein kinase C. Monocytes were therefore permeabilized for introduction of 50 μM guanosine 5'-[γ-thio]triphosphate as well as 100 μM neomycin to inhibit phospholipase C-induced (i) activation of protein kinase C by diacylglycerol and (ii) Ca^{2+} mobilization by inositol 1,4,5-triphosphate formation (5). TNF secretion was stimulated by 50 μM guanosine 5'-[γ-thio]triphosphate in cells treated with LPS alone or with botulinum toxin type D and LPS (Fig. 3B). In contrast, neomycin partially decreased this stimulation by guanosine

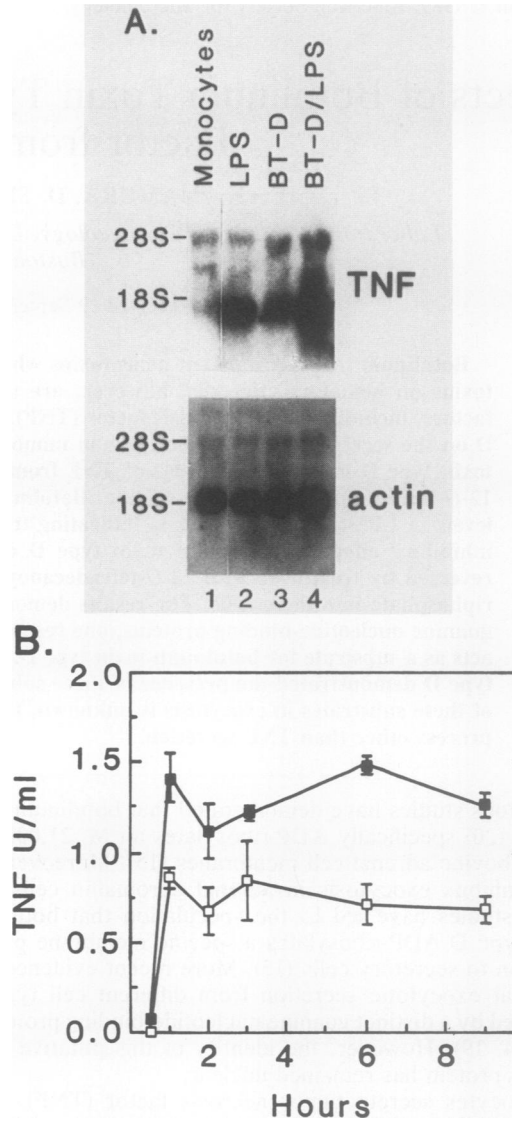

FIG. 2. Effect of botulinum toxin type D on TNF expression at the mRNA and protein levels. (A) Lane 1, Untreated monocytes; lane 2, monocytes incubated in the absence of toxin for 15 h and then treated with LPS for 3 h; lane 3, monocytes treated for 18 h with botulinum toxin type D (BT-D); lane 4, monocytes treated for 15 h with botulinum toxin type D and then with added LPS (BT-D/LPS) for 3 h. Total cellular RNA (20 μg) was isolated, analyzed, and hybridized to ^{32}P-labeled human TNF or chicken beta-actin cDNA probes as described previously (21). (B) Monocytes (10^6) were treated with LPS alone (□) or botulinum toxin type D for 15 h and then with added LPS (■) for the indicated periods. Cell lysates were then monitored for TNF protein levels by ELISA (21).

5'-[γ-thio]triphosphate (Fig. 3B). These findings suggested that activation of phospholipase C and protein kinase C was in part responsible for TNF secretion. Indeed, treatment with TPA reversed the inhibitory effects of neomycin (Fig. 3B). However, as in guanosine 5'-[γ-thio]triphosphate-stimulated monocytes, TPA failed to completely reverse the inhibitory effects of botulinum toxin type D on TNF release (Fig. 3B). These results suggest that TNF secretion is regulated by at least two distinct GTP-binding proteins, one

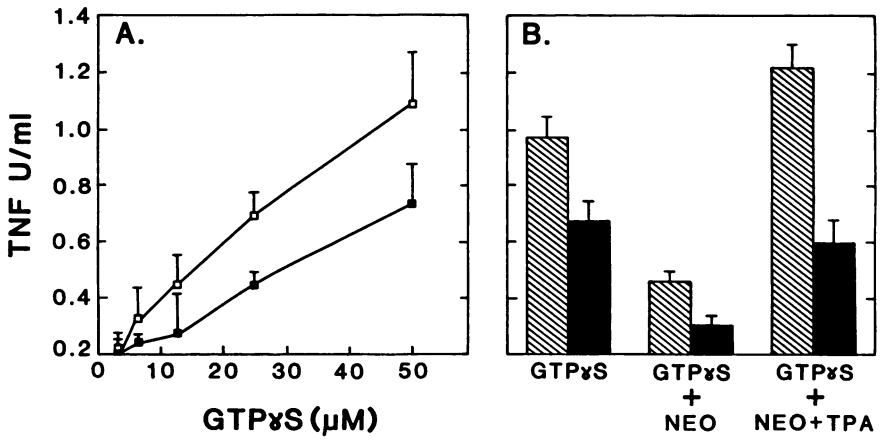

FIG. 3. Effects of guanosine 5'-[γ-thio]triphosphate on secretion of TNF from stimulated monocytes. (A) Monocytes (2×10^5/ml) were treated with botulinum toxin type D for 15 h and then with added LPS for an additional 12 h (■) or with LPS alone for 12 h (□). The cells were then permeabilized in the presence of various concentrations of guanosine 5'-[γ-thio]triphosphate (GTPγS). To demonstrate that the monocytes were permeabilized by this method, [γ-^{35}S]GTP (0.57 μCi/5 ml, 1,627 Ci/mmol; New England Nuclear) was added instead of unlabeled compound. Control cells incubated with [γ-^{35}S]GTP in RPMI 1640 medium retained 258 ± 16 cpm (mean ± standard deviation; n = 3), while permeabilized cells retained 4,624 ± 213 cpm. The results, expressed as units of TNF released per milliliter of supernatant (determined by ELISA), represent the means ± standard errors for three experiments performed in triplicate. (B) Monocytes (2×10^5/ml) were incubated for 15 h in the absence (▨) or presence (■) of botulinum toxin type D. The cells were then permeabilized and incubated with 50 μM guanosine 5'-[γ-thio]triphosphate (GTPγS), 50 μM guanosine 5'-[γ-thio]triphosphate plus 100 μM neomycin (NEO), or both compounds plus 33 nM TPA for 10 min. The results of this representative experiment are expressed as the means ± standard deviations for three determinations. Similar results were obtained in three separate experiments.

responsible for activation of phospholipase C (Gp) and another which acts as a substrate for botulinum toxin type D.

Membranes from human monocytes were also incubated with botulinum toxin type D in the presence of [^{32}P]NAD. Monocyte membranes were prepared by a previously described procedure (22). The membranes (25 μg) were incubated in a reaction mixture (100 μl) containing 67.9 μM [^{32}P]NAD (2,930 Ci/mmol; New England Nuclear Corp., Boston, Mass.), 100 mM Tris hydrochloride (pH 7.5), 10 mM thymidine, 1 mM ATP, 100 μM GTP, 5 mM $MgCl_2$, and 10 mM dithiothreitol for 30 min at 37°C. Proteins in the acid-insoluble fraction were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and analyzed by autoradiography. There was no detectable ADP-ribosylation in the absence of toxin (Fig. 4A, lane 1). In contrast, the addition of botulinum toxin type D resulted in the appearance of distinct radioactive bands with M_rs of 45,000, 21,000, and 17,000 (Fig. 4A, lane 2). Other studies have demonstrated that *Clostridium botulinum* type D strains produce the neurotoxin as well as C2 toxin and the exoenzyme C3 (18). Botulinum C2 toxin ADP-ribosylates actin at M_r 45,000 (1, 17, 18), while the substrate of C3 is an M_r 21,000 protein (2, 18). Thus, the M_r 17,000 protein ADP-ribosylated by the botulinum toxin type D preparation may represent a novel substrate.

Previous work suggested that the M_r 21,000 substrate of botulinum toxin type D preparations is required for exocytosis (12, 15, 20). However, these preparations also contain the exoenzyme C3, which ADP-ribosylates an M_r 21,000 protein (18). Thus studies were performed with a rabbit antiserum against exoenzyme C3 (18) to determine whether the M_r 21,000 substrate is involved in TNF secretion by monocytes. Incubation of botulinum toxin type D with normal rabbit serum had no detectable effect on ADP-ribosylation of the M_r 21,000 substrate (Fig. 4A, lane 3). In contrast, treatment of the botulinum toxin type D preparation with the rabbit antiserum to exoenzyme C3 inhibited ADP-ribosylation of the M_r 21,000 substrate but had little if any effect on labeling of actin (Fig. 4A, lane 4). Moreover, treatment of the type D toxin preparation with the rabbit antiserum to exoenzyme C3 had no detectable effect on the inhibition of TNF secretion by this toxin (Fig. 4B). These findings thus suggest that the M_r 21,000 substrate of exoenzyme C3 found in botulinum toxin type D preparations is probably not involved in TNF secretion by these cells.

The present studies with guanosine 5'-[γ-thio]triphosphate suggest that the substrate of the botulinum type D neurotoxin is a G protein. Nonhydrolyzable guanine nucleotides have been shown to modify the ability of G proteins to serve as substrates for ADP-ribosylating toxin (10). We therefore studied the effects of guanosine 5'-[γ-thio]triphosphate on ADP-ribosylation of the various substrates found for the botulinum toxin type D preparation. Guanosine 5'-[γ-thio]-triphosphate (5 μM) inhibited labeling of the M_r 21,000 substrate by 43% (as determined by densitometric scanning) in the presence of the toxin and [^{32}P]NAD. This finding is in concert with the demonstration that the substrate of exoenzyme C3 is a GTP-binding protein (2, 18). Guanosine 5'-[γ-thio]triphosphate also inhibited ADP-ribosylation of the M_r 45,000 actin and the M_r 17,000 substrates by 40 and 32%, respectively (Fig. 4C, lane 2). These results suggest that ADP-ribosylation of these substrates is also modified by nonhydrolyzable guanine nucleotides.

Adenylate cyclase activity is regulated by stimulatory and inhibitory G proteins, designated G_s and G_i, respectively (6). The alpha subunits of these heterotrimeric G proteins bind guanine nucleotides and act as substrates for bacterial toxins. In this context, cholera toxin ADP-ribosylates G_s-alpha, while pertussis toxin ADP-ribosylates G_i-alpha (6). The present findings confirm that the M_r 21,000 substrate for exoenzyme C3 is also a member of the G protein family and that C3 is detectable in botulinum toxin type D preparations (18). The results further indicate that this M_r 21,000 protein is not involved in the secretion of TNF by monocytes. Thus, the M_r 21,000 substrate first identified by using a botulinum toxin type D preparation is probably not the putative Ge

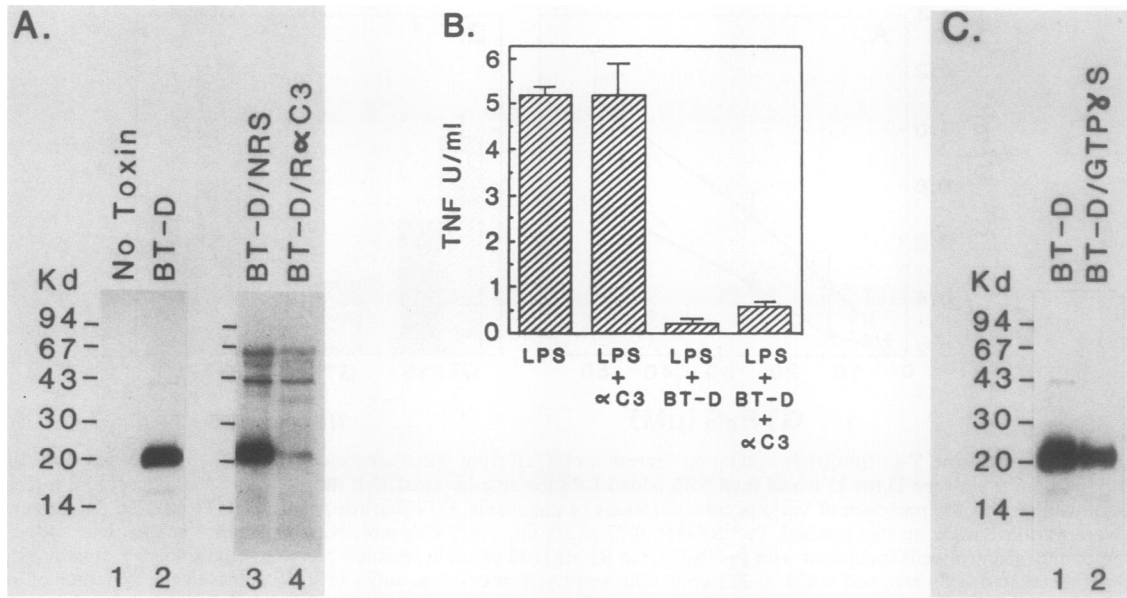

FIG. 4. (A) ADP-ribosylation of human monocyte membranes with botulinum toxin type D and effects of rabbit antiserum to exoenzyme C3. Lane 1, No toxin; lane 2, 20 μg of botulinum toxin type D (BT-D); lane 3, 20 μg of botulinum toxin type D pretreated with 300 μg of nonimmune rabbit serum for 2 h at 4°C (BT-D/NRS); lane 4, 20 μg of botulinum toxin type D pretreated with 300 μg of rabbit antiserum to exoenzyme C3 (BT-D/RαC3) (18) for 2 h at 4°C. (B) Effects of rabbit antiserum to exoenzyme C3 on inhibition of TNF secretion by botulinum toxin type D. Monocytes were treated with LPS for 3 h (LPS), antiserum to exoenzyme C3 for 15 h followed by the addition of LPS for 3 h (LPS + αC3), botulinum toxin type D for 15 h followed by the addition of LPS for 3 h (LPS + BT-D), or botulinum toxin type D and antiserum to exoenzyme C3 for 15 h followed by the addition of LPS for 3 h (LPS + BT-D + αC3). TNF protein levels in the supernatant fluids were determined by ELISA (21). The results represent the means ± standard deviations for three determinations. (C) Effect of guanosine 5′-[γ-thio]triphosphate on ADP-ribosylation of monocyte membranes by botulinum toxin type D. Monocyte membranes (25 μg) were incubated with 20 μg of botulinum toxin type D and [^{32}P]NAD for 30 min at 37°C (lane 1). Membranes (25 μg) were also preincubated with 5 μM guanosine 5′-[γ-thio]triphosphate for 5 min at 37°C and then monitored for botulinum toxin type D-induced ADP-ribosylation (lane 2). Kd, Kilodaltons.

protein. Nonetheless, other studies have suggested that exocytotic secretion from different cell types is regulated by a distinct GTP-binding protein (3, 4, 8, 14, 19).

The present results further indicate that TNF secretion by monocytes is regulated by at least two GTP-binding proteins, one functioning through the activation of phospholipase C (Gp) and another acting as a substrate for the botulinum type D neurotoxin. The identity of the latter G protein, however, remains unclear. While botulinum toxin type D preparations also result in the ADP-ribosylation of actin, this activity has been attributed to the presence of C2 toxin (1, 17, 18). Furthermore, while C2 toxin is cytopathic and lethal, it has not been identified as an agent that inhibits secretion (1, 17). The present studies have also detected a previously undescribed M_r 17,000 substrate of botulinum toxin type D in monocyte membranes. The role of this substrate is unknown; however, it may be a GTP-binding protein on the basis of the ability of guanosine 5′-[γ-thio]-triphosphate to modify it as a substrate for ADP-ribosylation. Thus, further studies are needed to determine whether this M_r 17,000 protein is a toxin substrate in vivo and whether it is involved in exocytosis.

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