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# The Effect of Diphtheria Toxin on Nitric Oxide Induction From RAW264.7 Murine Macrophages

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**Abstract:** In this study the effect of diphtheria toxin (DT) on nitric oxide (NO) production from RAW 264.7 murine macrophages was investigated. Griess reagent was used to determine NO production by measuring nitrite levels in the culture supernatants. Corynebacterium diphtheriae G12/6 strain-produced DT (Limes flocculation activity and immunodiffusion assays were positive) demonstrated a dose-dependent effect on RAW 264.7 macrophages to induce NO production. L-NAME, an L-arginine analogue, inhibited NO production from DT-stimulated RAW 264.7 macrophages. At the given concentrations of DT, lipopolysaccharide (LPS or endotoxin)-induced NO production from RAW 264.7 macrophages was also inhibited. RAW 264.7 macrophage cells, incubated with various concentrations of DT for 3 days, were killed by DT in a dose dependent manner. Endotoxin contamination of DT was demonstrated with limulus amoebocyte lysate assay. Polymyxin B, frequently added to neutralize the effects of LPS in vitro, inhibited DT-induced NO production from RAW 264.7 cells. The misleading data concerning NO inducing capacity of DT was seemed to be related to endotoxin contamination. Thus, DT does not seem to be capable of inducing NO production from macrophages.

**Key Words:** Diphtheria toxin, nitric oxide, macrophage

## Introduction

Diphtheria is a bacterial respiratory infection caused by Corynebacterium diphtheriae, a gram positive bacillus. The major manifestations are a membraneous inflammation of the upper respiratory tract, usually of the pharynx but sometimes of the posterior nasal passages, larynx and trachea, plus widespread damage to other organs, including the myocardium, nervous system and kidneys, caused by the organism's exotoxin. A cutaneous form of diphtheria also occurs (1).

Pathogenic Corynebacterium diphtheriae strains secrete a potent protein toxin called diphtheria toxin (DP). The ability to produce DT has been observed only in C. diphtheriae and closely related species (2-4). Diphtheria toxin is a single polypeptide of Mr 58,342 secreted by toxic strains of Corynebacterium diphtheriae, carrying the tox structural gene found in the lysogenic corynebacteriophages $\beta$ tox$^+$, $\gamma$ tox$^+$ and $\omega$ tox$^+$. Avirulent strains also become lysogenic when infected with these phages (5). Diphtheria toxin is composed of two disulfide-linked subunits, both of which are involved in the intoxication process. The A subunit catalyzes ADP-ribosylation of elongation factor-2 (EF-2), thereby stopping protein synthesis and killing the cells. The B subunit has two functions: binding to cell-surface receptors and translocation of the A subunit into the cytosol (6).

It was recently shown that diphtheria toxin can be crosslinked to 10-20-kDa molecules present at the surface of toxin-sensitive cells (7), but evidence that these molecules are the functional receptors is so far lacking. It has been shown earlier by other workers that, when diphtheria toxin or incomplete toxin molecules containing the hydrophobic regions of the B fragment are added to artificial lipid membranes at low pH, ion-conduction channels are formed in the membranes (8, 9). Since the artificial membranes lacked toxin receptors, this would imply that translocation of the A fragment is not dependent on the receptor. In addition, although it may be dependent on the number of the DT receptors on the cell surface, a broad spectrum of various cell lines such as Vero, CHO-K1, Jurkat, K562, CEM, WEHI-7 and mouse L cells binds DT to some extent (10-12). In addition, the binding function of DT can be separated from the translocation function of DT (13).

Little lis known about the effects of diphtheria toxin on cells at the molecular level and there is apparently no specific target organ or cells. Thus, DT seemed to be likely to affect various cellular function via binding to or cross linking specific or non specific receptors on the cell membrane.

In the presence of diphtheria toxin, the adenosine diphosphate ribose moiety of $NAD^+$ is transferred into covalent linkage with transferase II, producing an inactive derivative of the factor. The toxin acts catalytically in this reaction, in which nicotinamide and a proton are released concomitantly (14, 15).

$NAD^+$ + tranferase II $\leftrightarrow$ ADP-ribose-transferase II + nicotinamide + $H^+$

ADP-ribosylation of proteins catalyzed by toxins from bacteria, such as diphtheria, cholera, and pertussis, has been extensively studied (16). Carracedo et al. (17) reported that the G proteins involved in the regulation of activation-induced NK (natural killer) apoptosis are sensitive to pertussis toxin and to exert its apoptosis-inhibitory effect, pertusis toxin must be employed before cells are activated. In addition Zhang and Morrison (18) reported that pertussis toxin, which can ADP-ribosylate the α-subunits of certain types of G-proteins (i.e., Go- and Gi-proteins) (19), have marked effect on NO production from LPS-stimulated mouse peritoneal macrophages.

Recently Brune and Lapetina (20) reported that nitric oxide (NO) stimulates ADP-ribosylation of a protein in platelets. Because of the apparent importance of NO as a biological messenger in mediating the tumoricidal and bactericidal actions of macrophages (21), dilating blood vessels (22), and serving as a neurotransmitter in the central and peripheral nervous system (23), characterization of potential effect of DT on NO induction from macrophages may be of importance.

When taken into account all the above-mentioned data together, DT seemed to have a potential effect on NO production from RAW 264.7 macrophage cell line which is capable of generating nitric oxide. Thus, the primary aim of this study was to investigate whether DT has the potential effect on nitric oxide induction from macrophages.

## Materials and Methods

**Reagents:** RPMI-1640 (supplemented with 0.3 g/l L-glutamine and NaHCO3), Limulus Amoebocyte Lysate (LAL) kit (E-Toxate), Salmonella minnesota LPS, L-NAME (Nω-Nitro-L-Arginine Methyl Ester), NED (N-[1-naphthyl] ethylenediamine dihydrochloride), Sulfanilamide, polymyxin B sulfate, and MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Co. St. Louis MO, USA. Fetal Calf Serum (FCS) was purchased from Seromed, Biochrom KG, D-12247 Berlin, Germany. FCS was heat-inactivated by keeping at 56°C for 30 min before using in experiments.

**Diphtheria toxin:** The toxinogenic strain of Corynebacterium diphtheriae used in this study was G12/6 (originally obtained from The Lister Institute, Elstree, U.K.). Procedure for production of diphtherial toxin was performed according to the WHO Expert Committe on Biological Standardization (24).

**Purification of DT:** G12/6 strain-produced crude DT dialysate was fractionated on a size-exclusion chromatography (a paper concerning DT purification method by size exclusion chromatography is being currently under preparation by the DT supplying investigators). The protein content (0.96 mg/ml) and the potency (290 Lf/ml) of the pooled fractions were measured by spectrophotometrically ([protein] mg/ml=1.55 A280 (1 cm) - 0.76 A260 (1 cm) and by Limes flocculation (Lf) respectively as described elsewhere (25, 26). Active fractions were pooled and lyofilized (with the Virtis Benchtop 3L, New York). All of the experiments in this paper were performed with a single preparation of diphtheria toxin produced by growth of Corynebacterium diphtheria G12/6.

**Standard Diphtheria Antitoxin:** National Standard Diphtheria Antitoxin (ST-AT; Lot:218/7752) (Equine) has the characteristics as follows; Contents=3000 IU/Amp (1000 IU per ml). ST-AT; Lot: 218/7752 was obtained from the R.S. Central Institute of Hygiene, Vaccine-Sera Production and Research Department.

**Immunodiffusion assay:** Active fraction and standard antitoxin were added into two seperate holes, which were prepared 1 cm apart from each other on a 2% noble agar supplemented with 2% PEG, and incubated for 24 h at 37°C in a humidified atmosphere.

**Stimulation of RAW 264.7 macrophages:** RAW 264.7 cell line was used for nitric oxide (NO) generating cells (27). The macrophage cell line RAW 264.7 (certified mycoplasma free at the time of purchase) was obtained from American Type Culture Collection. RAW 264.7 cells were plated at a density of $5 \times 10^5$ cells/250 μl (in RPMI-1640 supplemented with 20% FCS) per well in 24-well tissue culture plates (Falcon, Oxnard, CA 93030 USA). In order to get the desired concentrations of DT, antitoxin, polymyxin B. and L-NAME (each was added into
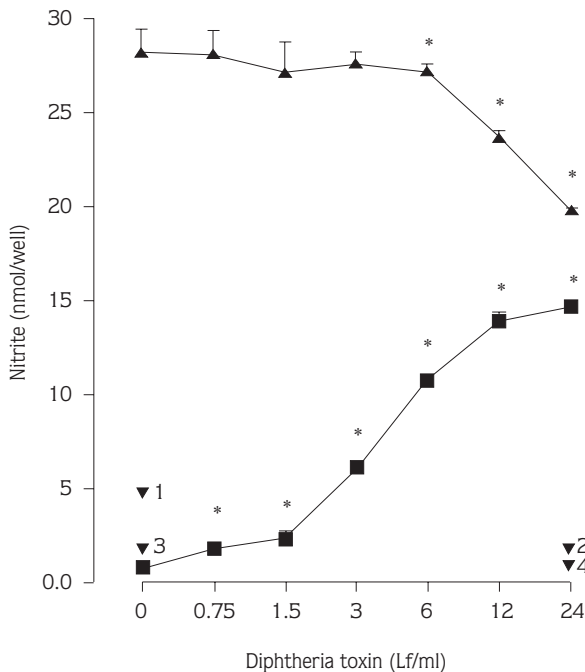
Figure 1.   Effect of diphtheria toxin on $NO_2^-$ production by RAW 264.7. Cells were stimulated with various concentrations of DT as indicated. Then 4 h later following pretreatment with DT, the cells were incubated either without (■) or with (▲) the presence of 1 µg/ml of S. minnesota LPS for an additional 20 h. Controls (no toxin and no LPS) were included in each plate. $NO_2^-$ levels were almost undetectable (<1 nmol/well) in the control supernatants. Active fraction induced $NO_2^-$ production from RAW cells in a dose dependent manner. In contrast, it inhibited LPS-depentent $NO_2^-$ production at>6 Lf/ml. Symbol ▼1 and ▼2 denote the $NO_2^-$ levels of the culture supernatants of LPS- (1 µg/ml) and DT (24 Lf/ml)-stimulated macrophages respectively in the presence of 5 mM L-NAME. Symbol ▼3 and ▼4 denote the $NO_2^-$ levels of culture supernatants of RAW 264.7 cells stimulated either with LPS (1 µg/ml) or DT (24 Lf/ml) respectively in the presence of polymyxin B at 20 µg/ml. Asterisk denotes a significant difference (p<0.05) from control (0). Values are expressed as the mean ± SD of six independent experiments.

appropriate wells in a volume of 250 µl), appropriate amounts were added to the macrophage cultures as indicated. The cells were then incubated at 37°C in a humidified atmosphere of 95% air/5% $CO_2$ incubator for 28 h.

The Salmonella minnesota LPS stock solution prepared in RPMI-1640 at 4 µg/ml was sonicated for 5 min, using a sonicator (Transsonic 660/H, Elma, Germany) before being adding (in a volume of 250 µl) into DT-stimulated RAW 264.7 cells. After a 4 h
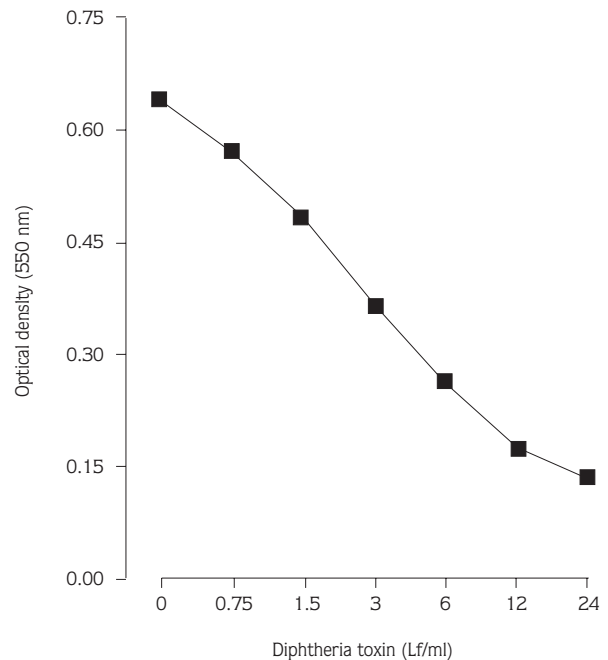


Figure 2.   Effect of DT on the viability of RAW264.7 cells. Macrophages ($5 \times 10^5$ cells/250 µl per well) were seeded at wells and incubated for 3 d with various concentrations of DT as indicated. Optical density was directly related to the number of viable RAW 264.7 cells (data not shown). After an incubation period of 3d, DT demonstrated a dose-dependent (r=-0.8442, p=0.0169) lethal effect upon RAW 264.7 cells. Data are expressed as the mean for two independent experiments.

incubation period, LPS was added into RAW cells either stimulated with DT or unstimulated and incubated for 24h. Details of experimental designs are outlined in the text and figure legends. At the end of cell culture, supernatants from six identically treated wells were assayed for the presence of nitrite.

**Analysis of Nitric Oxide (NO):** The presence of NO in macrophage culture supernatants was determined by measuring the amount of nitrite ($NO_2^-$), a metabolic product of NO (28). Briefly, 100 µl of macrophage culture supernatant was directly mixed with 100 µl of Griess reagent (1:1, [vol/vol] of 0.1 % N-[1-naphthyl] ethylenediamine dihydrochloride (NED) in $H_2O$/ 1% sulfanilamide in 5% $H_2PO_4$) in flat-bottomed 96-well microtiter immunoassay plates (Corning), and the absorbance at 550 nm was measured on a microplate reader (LP-400 Diagnostics Pasteur). The nitrite amount was calculated from a $NaNO_2$ standard curve.

**Toxicity Assay:** Toxicity of DT on RAW 264.7 cells was assessed with a colorimetric assay using MTT which is converted to water-insoluble formazan by viable eukaryotic cells (28). Briefly, two-fold dilutions of diphtheria toxin was prepared and added to macrophages ($5x10^5$ cell/ml per well of 24-well plate) in order to get the final concentrations from 24 to 0.75 Lf/ml. Dublicate wells for cell control (no toxin) were included in each plate. After an incubation period for 3 days in a humidified 5% CO2 incubator, 50 µl of MTT solution (5 mg/ml in saline) was added to each well and incubated for 3h more. Plates were centrifuged at 800xg for 15 min. Supernatants were aspirated and the formazan dye was dissolved in 1 ml of 2-propanol (Merck). A volume of 100 µl from each well was transferred to flat-bottomed 96-well microtitre plate and the OD values were quantitated with an ELISA reader at 550 nm wave length.

**Data presentation:** Data reported are means ± standard deviation from a representative experiment. When not shown in the figures, the standard deviation bars were smaller than the symbol indicating the value of mean. The students-t test was used to determine statistical significance between groups. Nitrit contents in culture supernatants were determined by Corelation-Regression Analysis based on a $NaNO_2$ standard curve. $p < 0.05$ was regarded as significant.

## Results

Corynebacterium diphtheriae G12/6 -produced diphtheria toxin was extracted and checked for it's Lf activity. Extracted DT demostrated an Lf activity of 800 Lf/ml. DT dialysate was further purified by high-performance liquid chromatography on a size-exclusion based column. Among the fractions collected, the active fraction demonstrated an immunoprecipitation band with the use of standard antitoxin on a 2% agar matrix (data not depicted). Thus, throughout the study the active fraction (also reffered to as DT from now on) was used in all experiments.

Diphtheria toxin demonstrated a dose dependent effect on NO induction from RAW 264.7 murine macrophages. At the concentrations of equal and higher than 6 Lf/ml, DT demonstrated an inhibitory action on LPS-dependent NO production from RAW 264.7 cells. Polymyxin B, at a concenration of 20 µg/ml abolished the NO inducing capacity of active fraction as well as of LPS. NO inducing capacities of active fraction and LPS were also inhibited by L-NAME (Fig. 1). Since L-NAME is a specific inhibitor of inorganic nitrogen oxide synthesis from L-arginine, nitrite ($NO_2^-$) accumulated in the culture supernatant was originally derived from NO.

Diphtheria toxin demostrated a dose dependent killing effect on RAW 264.7 macrophage cells after a 3 days of incubation period (Fig. 2).

## Discussion

Experiments in rats showed that the urinary $NO_3^-$ levels could be elevated about tenfold when fever was induced by an intraperitoneal injection of E. coli LPS (29). This finding suggested that this elevated synthesis might be related to the immunostimulation known to be brought about by LPS. This LPS-induced synthesis was then reproduced by treatment of murine peritoneal macrophages in culture (30). Further in vivo studies showed that strong immunostimulants such as Bacillus Calmette-Guerin increased urinary $NO_3^-$ excretion in mice up to 50-fold (30), suggesting that endogeneous lymphokines could also stimulate macrophages to carry out this synthesis. Subsequent experiments showed that a major lymphokine involved in this response was interferon-γ (IFN-γ) (31). Further studies with the RAW 264.7 macrophage cell line showed that the chemical precursor to $NO_2^-$ and $NO_3^-$ was exclusively the amino acid L-arginine which was converted to citrulline (27). Investigations revealed that nitric oxide (.N=O) was an intermediate in the production of both $NO_2^-$ and $NO_3^-$ from L-arginine. NO is derived from the oxidation of the terminal guanidino nitrogen atom of L-arginine by the NADPH-dependent enzyme NO synthase, of which there are two types (22). One is constitutive, $Ca^{2+}$/calmodulin dependent and is found mainly in the brain, endothelial cells and platelets. The other is inducible $Ca^{2+}$ independent and is found in a variety of sites, including smooth muscle and macrophages. The activity of both enzymes can be specifically inhibited by some L-arginine analogues.

G12/6 strain-produced DT was purified and fractionated on a size exclusion chromatography. Among the fractions detected, only major fraction (active fraction, DT) demonstrated Lf activity. Major fraction was specifically reacted with standard antitoxin and demonstrated a single immunoprecipitation band on an agar matrix. In addition, active fraction demonstrated a conventional lethal effect upon Vero cells (data not shown) and RAW 264.7 macrophages after a 3 days of incubation period (Fig. 2). Hence, the active fraction was regarded as DT. It seemed to be likely that specific or nonspecific receptors are present on RAW 264.7 macrophages since DT killed macrophages in a dose dependent manner. Although diphtheria toxin is secreted

by Corynebacterium diphtheriae as a single polypeptide chain, it is usually partially digested in the culture medium by proteolytic enzymes released from the bacteria. Preperations of toxin may contain peptides smaller than whole toxin and are present even in highly purified toxin preperations. Gill and Pappenheimer (15) showed that some of these fragments are responsible for the entire enzymatic activity of toxin in vitro. But in the aspect of Lf activity and NO inducing capacity, only with the active fraction these were observed. It was clear that active fraction-induced $NO_2^-$ accumulation in the culture supernatant was originally derived from NO, since L-NAME, a specific inhibitor of inorganic nitrogen oxide synthesis from L-arginine, was able to inhibit the stimulatory effect of active fraction (Fig. 1).

At this stage of all the experiments performed, one may understand that DT has the capacity to induce NO production from macrophages. It is well known that LPS (endotoxin) has the capacity to induce NO from macrophages. In order to exclude any endotoxin contamination we checked the induce NO from macrophages. In order to exclude any endotoxin contamination we checked the active fraction for the presence of LPS. Active fraction demonstrated a positive result (contaminated with endotoxin) with a commercially available endotoxin search kit (LAL). Polymyxin B is the polycationic antibiotic most frequently added to neutralize the effect of LPS in vitro (32). When RAW 264.7 cell were stimulated either with DT or LPS in the presence of polymyxin B, $NO_2$ synthesis was inhibited (Fig. 1). Thus, we concluded that results concerning the NO inducing capacity of DT were misleading and might be directly related to it's endotoxin contents.

Zhang and Morrison (18) demonstrated that pertussis toxin (PT) treatment of mouse peritoneal macrophages enhanced LPS-induced TNF-$\alpha$ production and inhibited LPS-mediated NO production. The effect of PT on these LPS responses has been shown to correlate with PT-mediated ADP-ribosylation of a 41-kDa protein (s). These result suggested that a PT-sensitive G-protein(s) might be involved in these LPS-dependent macrophage activation processes. As that of PT, diphtheriae toxin also demonstrated a similar action on LPS-induced NO production from macrophages (Fig. 1). Thus, apart from its direct effect on NO induction, it is reasonable to speculate that DT may serve a PT-like signal transduction pathways.

In addition to exhibiting antitumor cell activity, reactive nitrogen intermediates exert potent antimicrobial activtiy in vitro against a variety of pathogens including Cryptococcus neoformans (33), Leishmania major (34) Schistosoma mansoni (35), Toxoplasma gondii (36). and Francisella tularensis (37). The mechanism of the NO-mediated nonspecific killing of tumour cells and pathogenes has yet to be clarified. Several workers (38) have postulated that NO reacts with Fe-S groups, resulting in the formation of iron-nitrosyl complexes that cause the inactivation and degradation of Fe-S prosthetic groups of aconitase and complex I and complex II of the mitochondrial electron transport chain.

Despite earlier hopes that the induction of nonspecific immunity might be a valuable tool in the fight against infectious diseases and tumours with the subsequent production of immunostimulants of various kinds, it is now clear that such responses can be extremely dangerous unless carefully controlled. Reactive oxygen intermediates, such as superoxide, cause vascular endothelial damage (39), TNF is largely responsible for he pathology associated with malaria (40) and nitric oxide can profoundly effect blood pressure and flow (41), inhibits platelet adhesion and aggregation (42).

Whether the capacity of DT to modulate NO production from LPS-stimulated macrophages would be detrimental or beneficial for host remains to be further investigated.

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