Quantification of Diphtheria Toxin–Mediated ADP-Ribosylation in a Solid-Phase Assay

Christopher Bachran,1 Mark Sutherland,1† Diana Bachran,1 and Hendrik Fuchs1*

Background: Because of reduced vaccination programs, the number of diphtheria infections has increased in the last decade. Diphtheria toxin (DT) is expressed by Corynebacterium diphtheriae and is responsible for the lethality of diphtheria. DT inhibits cellular protein synthesis by ADP-ribosylation of the eukaryotic elongation factor 2 (eEF2). No in vitro system for the quantification of DT enzymatic activity exists. We developed a solid-phase assay for the specific detection of ADP-ribosylation by DT.

Methods: Solid phase–bound his-tag eEF2 is ADP-ribosylated by toxins using biotinylated NAD as substrate, and the transferred biotinylated ADP-ribose is detected by streptavidin-peroxidase. DT enzymatic activity correlated with absorbance. We measured the amount of ADP-ribosylated eEF2 after precipitation with streptavidin-Sepharose. Quantification was done after Western blotting and detection with anti–his-tag antibody using an LAS-1000 System.

Results: The assay detected enzymatically active DT at 30 ng/L, equivalent to 5 mU/L ADP-ribosylating activity. Pseudomonas exotoxin A (PE) activity was also detected at 100 ng/L. We verified the assay with chimeric toxins composed of the catalytic domain of DT or PE and a tumor-specific ligand. These chimeric toxins revealed increased signals at 1000 ng/L. Heat-inactivated DT and cholera toxin that ADP-ribosylates G-proteins did not show any signal increase.

Conclusions: The assay may be the basis for the development of a routine diagnostic assay for the detection of DT activity and highly specific inhibitors of DT.

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Diphtheria is a highly infective disease caused by Corynebacterium diphtheriae, an aerobic, gram-positive, nonsporulating bacterium. The disease is usually transmitted by droplet contact. The virulence factor of C. diphtheriae is diphtheria toxin (DT), a highly toxic protein expressed after infection by a specific tox© corynebacteriophage (1). Infections by C. diphtheriae occur worldwide. The combination of reduced immunization in Western Europe with increased travel to countries in Eastern Europe accounts for the high risk of new diphtheria infections (2).

The primary infection with corynebacteria takes place in the respiratory tract and affects mainly the pharyngeal tonsil. Death may result from obstruction of the respiratory tract and myocarditis. Because of the danger of diphtheria infections, diagnostic testing must be performed immediately. Although a number of methods are available to detect the gene and the protein, the only method to identify the active conformation of DT is the infection of guinea pigs with phase-infected corynebacteria (3, 4).

DT causes cell death by inhibiting protein biosynthesis. It is composed of a catalytic-, a transmembrane-, and a receptor-binding domain. Binding to eukaryotic cells is mediated by the receptor-binding domain, which interacts with the heparin-binding epidermal growth factor (EGF)-like precursor on the cell surface (5). DT is internalized by receptor-mediated endocytosis to endosomes and subsequently cleaved by a protease of the furin family within a protease-sensitive loop between the catalytic and transmembrane domains (5). The catalytic domain is referred to as the A-chain of DT (DTA); the remainder is referred to as the B-chain. The A- and B-chain remain linked by a disulfide bridge. A cytosolic translocation factor complex of the cell seems to be involved in transfer of the A-chain and subsequent cleav-
age of the intramolecular disulfide bridge, releasing DTA into the cytosol (6).

DT is an ADP-ribosylating enzyme. The catalytic domain transfers ADP-ribose from nicotinamide adenine dinucleotide (NAD\(^+\)) to eukaryotic elongation factor 2 (eEF2) and releases nicotinamide (7). eEF2 mediates the translocation step in peptide chain elongation by promoting transfer of peptidyl tRNA from the A- to the P-site of the ribosome (8). In contrast to bacterial EF2, eEF2 and EF2 from archaea contain a unique and strictly conserved posttranslationally modified histidine referred to as diphthamide (9). The ADP-ribosylation takes place on this diphthamide, resulting in inactivated eEF2 and blocked protein biosynthesis (10). eEF2-inactivation by DT is very effective, and 1 molecule modifies a vast number of eEF2 molecules. Therefore, a few toxin molecules are sufficient to inhibit cellular protein synthesis and induce apoptosis (11) with median lethal dose values in humans <100 ng/kg (12). Pseudomonas exotoxin A (PE) is a further bacterial toxin that ADP-ribosylates eEF2 at the diphthamide (13). Like DT, PE consists of separate catalytic and binding domains and has high toxicity, with a median lethal dose of approximately 3 μg/kg in mice (14). The catalytic activity of PE is located at the C-terminus within domain III (15). Both bacterial toxins mediate cell death. When coupled to target-specific ligands, these chimeric toxins can act as specific cytotoxic agents. In modern tumor therapy, these chimeric toxins are a promising tool to overcome the low efficacy and higher incidence of side effects with conventional chemotherapeutics. Chimeric toxins bind specifically to their receptor on the tumor cell, are internalized, and kill the cell by inactivation of protein biosynthesis (16, 17).

We developed an ADP-ribosylation assay for enzymatically active DT. Measuring the toxin activity may improve the current identification of DT by PCR or antibody detection, which fail to discriminate between active and inactive forms of the toxin.

Material and Methods

Purification of Yeast eEF2

Endogenous eEF2 from Saccharomyces cerevisiae strain TKY675 was replaced by a genomically integrated his-tagged analog (kindly provided by Terri Goss Kinzy, Piscataway, NJ) (18). Yeasts were grown at 30 °C in 1 L yeast extract peptone dextrose to an A\(_{600}\) of 2, harvested by centrifugation, resuspended in 15 mL buffer A-10 (50 mmol/L potassium phosphate, pH 7.6, 300 mmol/L potassium chloride, 10 mmol/L imidazole), and lysed by glass beads (0.5 mm diameter). After removal of the glass beads and subsequent centrifugation (30 min, 60 000g, 4 °C) the supernatant was applied to a 2-mL nickel-nitrilotriacetic acid agarose (Qiagen) column, washed with 5 column volumes of A-10 and 4 column volumes of A-20 (A-10 with 20 mmol/L imidazole), and eluted with 3 column volumes of A-250 (A-10 with 250 mmol/L imidazole). The eluate was dialyzed twice against 0.5 L storage buffer (300 mmol/L potassium phosphate, pH 7.0, 50 mmol/L potassium chloride, 50 mM/L glycerol), concentrated with Amicon Ultra-30 columns (Millipore), and stored at −80 °C. The yield was approximately 400 μg eEF2 per liter yeast culture.

Expression and Purification of Chimeric Toxins

The 2 chimeric toxins DTA-EGF (DTA coupled to EGF) (19) and scFv-PE (catalytic domain of PE coupled to an anti–interleukin-2 receptor single chain Fv antibody, kindly provided by Stefan Barth, Aachen, Germany) (20) were transformed into Escherichia coli Rosetta (DE3) pLysS (Merck), expressed in 200 mL Luria broth, and cells were harvested by centrifugation (15 min, 4 °C, 5000 g). We purified DTA-EGF by nickel-nitrilotriacetic acid agarose chromatography (Qiagen) and scFv-PE using Protino nickel Tris(carboxymethyl)ethylene diamine columns (Macherey-Nagel). Both proteins were eluted with buffer containing 250 mmol/L imidazole, renatured by dialysis at 4 °C against 2 mol/L urea/10 mmol/L Tris (pH 7.4)/1 mmol/L reduced glutathione, twice against 20 mmol/L Tris (pH 7.4)/1 mmol/L EDTA, and twice against PBS (150 mM NaCl, 8.3 mM Na\(_2\)HPO\(_4\), 1.7 mM KH\(_2\)PO\(_4\), pH 7.4), concentrated as described above for eEF2 purification, and stored at −20 °C.

ADP-Ribosylation of eEF2 in a Western Blot

We performed the ADP-ribosylation assay with 600 ng of yeast eEF2, 0.5 μL biotinylated NAD\(^+\) (250 μmol/L; BD Biosciences), and 1 μL DT (final concentration 420 μg/L; Sigma) in a total volume of 24 μL reaction buffer (50 mmol/L Tris, pH 7.6, 1 mmol/L EDTA, 1 mmol/L dithiothreitol) for 1 h at 37 °C. We separated the samples by electrophoresis (19) and detected biotinylated eEF2 by Western blotting with goat anti-biotin antibody (Sigma) as described (21). For negative controls, DT was heat-inactivated at 95 °C for 30 min.

ADP-Ribosylation of eEF2 in a Colorimetric Solid-Phase Assay

We precoated U16 MaxiSorp Immuno Modules (Nunc) with rabbit antismouse antibody (RAM) (Dako) (1:100 in 100 μL PBS for 2 h) and incubated them overnight with mouse anti–his-tag antibody (Qiagen) (5 mg/L in 100 μL PBS). We incubated his-tagged yeast eEF2 (1.2 μg) with 2 μL biotinylated NAD\(^+\), 10 μL BSA (2 g/L), 10 μL l-arginine (100 mmol/L), 1 μL ADP-ribosylating compound [DT, PE, and cholera toxin (CT); all obtained from Sigma], chimeric toxins (DTA-EGF or scFv-PE; 10 to 1000 ng/L), and reaction buffer in a total volume of 100 μL at 37 °C for 2 h, transferred it into the precoated wells, and incubated it for an additional 2 h at room temperature. Imprecision of the method was analyzed by using different batches of eEF2. The wells were washed 3 times with PBS containing 0.05 g/L Tween 20 (PBS\(_{10,000}\)), incubated for 5 min, and washed again. Wells were blocked with 200 μL blocking solution I (50 g/L dry milk powder...
in PBS) for 30 min at room temperature followed by 30 min at 37 °C and washed again as above. After incubation with horseradish peroxidase–labeled streptavidin (1 mg/L; Jackson ImmunoResearch Laboratories) in 100 µL blocking solution II (100 mL/L fetal calf serum, 30 g/L BSA in PBS) for 30 min, wells were washed with PBS for 30 min and finally incubated with 3,3′,5,5′-tetramethylbenzidine (Merck) (0.2 g/L in 100 mL 40 mmol/L citrate, pH 3.95) for color development. We stopped the reaction after 30 min by adding 50 µL 2 mol/L H2SO4 and measured the signal as difference of the absorbance at 450 nm and 490 nm by use of a microplate reader (Spectra Max 340PC).

To compare ADP-ribosylation of soluble eEF2, as described above, with immobilized eEF2, 1.2 g eEF2 in PBS was incubated for 2 h directly after precoating with anti-his antibody and then washed to remove unbound eEF2. Thereafter, ADP-ribosylation and color development was performed in the same wells (without transfer) according to the protocol above.

To test the binding of soluble ADP-ribosylated eEF2 to different precoated antibodies, the wells were incubated with RAM followed by anti–his-tag antibody, as described above, or only directly with the anti–his-tag antibody, a commercially available polyclonal antibody (H118) against eEF2 (Santa Cruz Biotechnology), or a rabbit polyclonal peptide antibody against the yeast eEF2 peptide AVGGIYSVLNKVRGC (immunization by Bio Genes).

To find the association of DT enzymatic activity with absorbance, eEF2 was ADP-ribosylated with 1000 ng/L DT for 2 h as described above, bound to streptavidin-Sepharose within 2 h, and separated from unbound eEF2 by centrifugation (5 min, 4 °C, 500 g). Bound and unbound eEF2 were quantified by use of the LAS-1000 System (Fujifilm) after Western blotting and detection with anti–his-tag antibody (Qiagen) as described above.

**DATA ANALYSIS**

We performed statistical analysis by use of SPSS version 11. We calculated the significance of increased signals by the paired t-test and estimated the significance of curve fits for linear regression curves. Statistical significance was assumed at P values <0.05.

**Results**

**Detection of ADP-ribosylation after Western blotting**

His-tagged yeast eEF2 was successfully purified (>95% pure according to Coomassie staining) (Fig. 1A). Immunologic detection of the toxin-mediated transfer of ADP-ribose from NAD+ to eEF2 is shown in Fig. 1B. Assay conditions gave a final DT concentration of 420 µg/L; however, incubation of eEF2 without DT resulted in a weak but clear signal. Unspecific adsorption of the biotinylated NAD+ was assumed to cause the signal. To minimize this nonspecific signal, the reaction was repeated in the presence of BSA, resulting in a modest reduction at 0.3 g BSA and a complete inhibition of the nonspecific signal after addition of 3 g BSA, without affecting DT activity. This optimized protocol was executed for DT and heat-inactivated DT, both at a concentration of 420 µg/L (Fig. 1C). Whereas DT was able to ADP-ribosylate eEF2, heat-inactivated DT was apparently

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**Fig. 1. Purified his-tagged eEF2, ADP-ribosylation, and reduction of toxin-independent signals by BSA.**

(A), Coomassie-stained SDS-gel with 1 µg BSA and 1 µg eEF2. (B), ADP-ribosylation without DT and with 420 µg/L DT in the absence or presence of BSA (+ with 0.3 µg; ++ with 3 µg BSA). eEF2-bound ADP-ribose was detected with an anti-biotin antibody after Western blotting. (C), heat-inactivation of DT. ADP-ribosylation was performed without DT and with 420 µg/L active and heat-inactivated DT.
denatured after 30-min incubation at 95 °C. The purified chimeric toxins scFv-PE (containing the catalytically active domain of PE) and DTA-EGF (containing the catalytically active A-chain of DT) were obtained at a purity >90% according to Coomassie staining (Fig. 2A). The enzymatic activity of both chimeric toxins was demonstrated by ADP-ribosylation with 420 µg/L protein and detection by Western blotting (Fig. 2B).

**ADP-ribosylation in a colorimetric solid-phase assay**

ADP-ribosylation by both DT and PE was detected by color development in the solid-phase assay, whereas heat-inactivated DT and CT, which ADP-ribosylates G-proteins, did not ADP-ribosylate eEF2, and no increase in the signals was observed (Fig. 3A). The increase in signal intensity for DT is concentration dependent and linear ($R^2 = 0.997; P < 0.001$) (see Fig. 3B for a double logarithmic plot). Fig. 3, A and B, present the results of ADP-ribosylation combining several different eEF2 batches. The lowest detectable DT concentration for specific eEF2 batches is 30 ng/L DT, measured either by analysis of the 95% CIs or the comparison of the signal with the background mean plus the 3-fold SD (Table 1). The imprecision of the method is indicated by varying detection limits (background mean plus 3-fold SD) of different eEF2 batches (batch 1 = 0.248, batch 2 = 0.177). Collier and Kandel (22) defined the enzymatic activity of DT (1 unit DT transfers 1 pmol ADP-ribose in 15 min), and we used this definition to calibrate the solid-phase assay. We measured the amount of ADP-ribosylated eEF2 as 1.3 pmol (10.5% of the total amount of eEF2) after incubation with 1000 ng/L DT for 2 h, which is thus equivalent to 0.163 U/L (or 1 mU/L corresponds to 6 ng/L DT). The lowest detectable concentration of DT (30 ng/L, see above) thus corresponds to 5 mU/L (Table 1).

The linear regression curves for CT and inactivated DT resulted in $R^2$ values of 0.00 and 0.04, with $P$ values of 0.97 and 0.8, respectively, showing no correlation. CT was shown to be enzymatically active in a proliferation assay (data not shown). PE revealed a significant correlation between signal intensity and toxin concentration ($R^2 = 0.96; P = 0.019$) (Fig. 3B); however, 1000 ng/L PE resulted in only a 3.7-fold signal increase. Although 10 and 30 ng/L PE did not result in a measurable increase of the signal in comparison to the toxin-free background, all analyzed concentrations >30 ng/L PE did. A linear signal increase was also observed for both chimeric toxins. At 1000 ng/L, DTA-EGF enhanced the toxin-free signal 7.0-fold and scFv-PE 5.2-fold. The $R^2$ value for the linear regression curves of DTA-EGF and scFv-PE was 0.97 for both chimeric toxins, with $P$ values of 0.016 and 0.014, respectively.

**Optimization of the ADP-ribosylation assay**

When testing 2 different blocking solutions, Rotiblock resulted in increased background, larger variations in the results, and an increased lowest detectable amount, with only 1000 ng/L DT showing activity (Fig. 4A). Blocking with 50 g/L dry milk yielded more reproducible results (small error bars) and a concentration-dependent increase in the signal. Performing the ADP-ribosylation either in a separate vial or directly in the wells on immobilized eEF2 resulted in clear detection of DT activity (Fig. 4B). The weaker signal with the immobilized eEF2 may result from decreased accessibility of DT to diphthamide. A comparison of 4 different antibodies for the binding of ADP-ribosylated eEF2 to the solid phase revealed that best results were obtained with a combination of RAM and subsequent coating with anti–his-tag antibody (Fig. 4C). Neither the his-tag antibody alone nor the polyclonal antibodies against eEF2 were effective in binding eEF2 on...
the solid phase, probably owing to superior exposition and accessibility of the his-tag antibody’s paratope after binding to RAM. The prolonged incubation of the ADP-ribosylation reaction provided only a minimal increase in the signal for 30 ng/L DT (Fig. 4D). This result may be linked to the degradation of 6-biotin-17-NAD\(^+\), which is unstable in aqueous solutions.

**Discussion**

Collier and Kandel (22) developed a system for the detection of the toxin-mediated ADP-ribosylation of eEF2. Enriched eEF2 was incubated with the toxin and radioactive-labeled NAD\(^+\), and ADP-ribosylation was detected autoradiographically. Zhang (23) showed that DT accepts 6-biotin-17-NAD\(^+\) as substrate for ADP-ribosylation.

**Table 1.** Response signals of ADP-ribosylation by DT in the solid-phase assay at different toxin concentrations for 2 separate eEF2 batches.\(^a\)

<table>
<thead>
<tr>
<th>DT concentration, ng/L</th>
<th>DT activity, U/L</th>
<th>Mean response, absorbance</th>
<th>95% CI</th>
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<td></td>
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<td>upper bound</td>
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<tr>
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<tr>
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<td>1.44</td>
<td>0.849</td>
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</table>

\(^a\) Means for each batch and DT concentration were calculated from 4 to 10 different samples. The detection limit was determined by addition of the 3-fold SD to the mean of the background and is 0.248 for eEF2 batch 1 and 0.177 for eEF2 batch 2.

Based on these studies, we established a system for the measurement of enzymatically active DT (24). Western blot analyses were only semiquantitative, and detection was limited to approximately 40 \(\mu\)g/L DT. To circumvent these drawbacks, we developed the quantitative colorimetric solid-phase assay. We used yeast his-tagged eEF2, and the source for ADP-ribose was biotinylated instead of radioactive NAD\(^+\). Differences in the lethal doses of DT for several species may be attributed to structural differences of eEF2 or different binding properties of DT to its extracellular receptor. Because DT showed high activity on yeast eEF2, we chose it as substrate for this assay. A certain background signal was observed in Western blotting, even in samples without DT, likely attributable to endogenous ADP-ribosylation activity (25) or unspecific adsorption of the biotinylated NAD\(^+\) or ADP-ribose moiety to eEF2. The addition of a 10- to 100-fold excess of BSA to the reaction successfully blocked this undesired side reaction. Specificity of the solid-phase assay was demonstrated with DT, PE, heat-inactivated DT, and CT (Fig. 3A). Heat-inactivation of DT was furthermore depicted in Western blotting, and the enzymatic activity of CT was measured in a cell culture experiment (data not shown). Because CT ADP-ribosylates G-proteins but not eEF2, it highlights the specificity of the solid-phase assay for ADP-ribosylation of eEF2. The assay is also suitable for PE, because PE shares substrate specificity with DT.

Analysis of ADP-ribosylation by DT and PE has previously used radioactively labeled NAD\(^+\) (26). Armstrong and Merrill (27) used etheno-\(\beta\)-NAD\(^+\) to analyze the enzymatic activity of PE and the kinetic parameters of ADP-ribosylation in a fluorometric assay. This assay resulted in a specific signal at a concentration of 300 \(\mu\)g/L. Other methods with lower detection limits have been based on the indirect measurement of toxin action, as shown by the detection of inhibited luciferase expression in cell culture experiments (28). In our solid-phase assay, the activity of the toxins appears not to be altered by the exchange of the binding domains by an antibody scFv or natural ligands such as EGF. Our results demonstrated
that the catalytic domain of both enzymes is sufficient for detection. The solid-phase assay is thus a reliable method for the in vitro detection and quantification of therapeutic toxins that contain the catalytic domains of DT or PE.

A rapid and unquestionable identification of corynebacteria that are tox-positive and express active DT is necessary for an appropriate diagnosis of a diphtheria infection. All techniques known to date rely on 3 basic principles: (a) the immunologic detection of DT such as the common Elek test \( (29) \) and a number of derivatives such as immunoblots, ELISAs \( (30, 31) \), immunochromatographic strip assays \( (32) \), assays based on fluorescence nanocrystal quantum-dots \( (33) \), hydrogel-based protein microchips \( (34) \), and antibody-based microarrays \( (35) \); (b) the molecular biological detection of the tox-gene by PCR assays \( (36) \); and (c) the detection of the enzymatic activity of DT by infecting guinea pigs \( (4, 31) \), in vitro translation assays, and ADP-ribosylation assays \( (22) \).

These procedures have general disadvantages such as poor reliability, long analysis time, or a requirement for expensive and technically advanced equipment, as well as special reagents and experienced personnel \( (31) \). The methods in categories a and b above do not enable differentiation between harmful strains and a number of less dangerous strains containing the tox-gene but producing enzymatically inactive DT \( (30, 37) \). These bacteria are less toxic and have been found in North America. Nontoxicogenic strains have also been reported with in-

Fig. 4. Variations in ADP-ribosylation of eEF2 in the solid-phase assay.
All errors are SE. (A), effect of different blocking solutions on ADP-ribosylation. (B), comparison of ADP-ribosylation using free and immobilized eEF2. (C), different antibodies for the binding of ADP-ribosylated eEF2 to the solid phase. (D), kinetics of ADP-ribosylation performed with 30 ng/L DT for either 2 or 6 h.
increasing frequency in Russia and the Ukraine (31, 38, 39). Although the 3rd analytical principle described earlier (c) provides the most immediate proof for active DT, the only existing assay for diagnostic purposes is guinea pig infection. In vitro translation assays are too indirect for sufficient reliability, and Western blot–based ADP-ribosylation assays do not allow quantification of DT activity.

Our assay overcomes a number of the problems with other methods. The imprecision of the assay reflects differences between the results obtained for individual eEF2 batches. To avoid batch-specific variations in a diagnostic assay, huge batches of eEF2 with acceptable results in DT detection have to be produced. Because of the low detection limit, this assay could detect enzymatically active DT directly from eluted patients’ throat swabs. However, it is possible that bacterial cells and other cellular impurities may influence the assay. Removal of these cellular contaminants can be achieved by centrifugation, while the secreted DT remains in the supernatant. According to the studies of Engler and Efstratiou (40), different strains of C. diphtheriae produce different quantities of DT, with a mean of approximately 1 µg/L DT produced by approximately 1 × 10^7 colony-forming units of C. diphtheriae. Thus, we believe that 1 × 10^7 DT-producing bacteria should be sufficient for the detection of active toxin in our assay. If, after final diagnostic assay development, the amount of DT in a positive throat swab is not sufficient for the detection of enzyme activity, the development of selective cultures as described by Engler et al. (32) must be performed in all samples. Although it will not be possible to distinguish low DT concentrations from less active DT, by combining the antibody-based assay of Engler and Efstratiou (40) and our activity assay, it should be possible to characterize those C. diphtheriae strains that produce less active DT. Nevertheless, most important for diagnostic purposes is the ability to determine whether DT activity exists at all.

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References


