A Dominant-Negative Approach That Prevents Diphthamide Formation Confers Resistance to Pseudomonas Exotoxin A and Diphtheria Toxin

Vincent Roy, Karim Ghani, Manuel Caruso*

Le Centre de Recherche en Cancérologie de l’Université Laval, L’Hôtel Dieu de Québec, Centre Hospitalier Universitaire de Québec, Québec, Canada

Abstract

Diphtheria toxin (DT), Pseudomonas aeruginosa Exotoxin A (ETA) and cholix toxin from Vibrio cholerae share the same mechanism of toxicity; these enzymes ADP-ribosylate elongation factor-2 (EF-2) on a modified histidine residue called diphthamide, leading to a block in protein synthesis. Mutant Chinese hamster ovary cells that are defective in the formation of diphthamide have no distinct phenotype except their resistance to DT and ETA. These observations led us to predict that a strategy that prevents the formation of diphthamide to confer DT and ETA resistance is likely to be safe. It is well documented that Dph1 and Dph2 are involved in the first biochemical step of diphthamide formation and that these two proteins interact with each other. We hypothesized that we could block diphthamide formation with a dominant negative mutant of either Dph1 or Dph2. We report in this study the first cellular-targeted strategy that protects against DT and ETA toxicity. We have generated Dph2(C-), a dominant-negative mutant of Dph2, that could block very efficiently the formation of diphthamide. Cells expressing Dph2(C-) were 1000-fold more resistant to DT than parental cells, and a similar protection against Pseudomonas exotoxin A was also obtained. The targeting of a cellular component with this approach should have a reduced risk of generating resistance as it is commonly seen with antibiotic treatments.

Introduction

Diphtheria toxin (DT) and Pseudomonas aeruginosa exotoxin A (ETA) are two bacterial A-B toxins that share the same mechanism of toxicity. These toxins are characterized by a B moiety that recognizes the cell surface receptor but that also plays a role in the translocation of the toxin into the cytosol, and an A moiety that contains the catalytic activity of the toxin. After binding to its receptor and endocytosis, the A subunit of DT enters the cytosol from the acidic endosomes and the one from ETA is released from the endoplasmic reticulum. The A subunit can then inactivate the elongation factor-2 (EF-2) by adenosine diphosphate (ADP)-ribosylating a modified histidine residue called diphthamide leading to cell death by blocking protein translation [1,2]. More recently, it was reported that cholix toxin from Vibrio cholerae has also a similar ADP-ribosylating activity on EF-2 [3].

P. aeruginosa is a Gram-negative bacillus ubiquitously present in the environment and, according to the Central for Disease Control, it is the fourth most commonly isolated nosocomial pathogen [4,5]. Nearly all P. aeruginosa clinical cases can be associated with compromised host defence. Systemic infections are common in patients with severe burns, and immunosuppressed AIDS and cancer patients. The infection by P. aeruginosa can also be seen with contact lenses wearers that develop keratitis of the cornea. P. aeruginosa is also responsible for ventilator-acquired pneumonia, and it is the primarily cause of mortality in cystic fibrosis patients due to lung infection [4]. The pathogenicity of P. aeruginosa is associated to several virulence factors but ETA is produced by 95% of P. aeruginosa clinical isolates and it is the most toxic [6]. It has been reported that ETA deficient strains are less pathogenic in mice than wild-type strains [7,8], and that the immunization directed against ETA increased survival in normal and thermally injured mice infected by P. aeruginosa [9–14]. It is also clear that ETA contributes to the organism’s pathogenicity in keratitis [15,16]. Overall, the high incidence of this microorganism, the severity of its infection and the resistance to antimicrobial treatments promote P. aeruginosa as a major human pathogen.

The implementation of a vaccine program in the 1940s and 1950s based on diphtheria toxoid had nearly eliminated diphtheria in developing countries. However, recent outbreaks of diphtheria have been reported in countries like Russia, in newly independent states of the former Soviet Union, and in poor socio-economically disadvantaged groups living in crowded conditions in Europe and the US [17,18]. One reason for the re-emergence of epidemics in countries where immunization have been performed is that the resistance to antimicrobial treatments promote P. aeruginosa as a major human pathogen. The implementation of a vaccine program in the 1940s and 1950s based on diphtheria toxoid had nearly eliminated diphtheria in developing countries. However, recent outbreaks of diphtheria have been reported in countries like Russia, in newly independent states of the former Soviet Union, and in poor socio-economically disadvantaged groups living in crowded conditions in Europe and the US [17,18]. One reason for the re-emergence of epidemics in countries where immunization have been performed is that the resistance to antimicrobial treatments promote P. aeruginosa as a major human pathogen. The implementation of a vaccine program in the 1940s and 1950s based on diphtheria toxoid had nearly eliminated diphtheria in developing countries. However, recent outbreaks of diphtheria have been reported in countries like Russia, in newly independent states of the former Soviet Union, and in poor socio-economically disadvantaged groups living in crowded conditions in Europe and the US [17,18]. One reason for the re-emergence of epidemics in countries where immunization have been performed is that the resistance to antimicrobial treatments promote P. aeruginosa as a major human pathogen.
to DT in some industrialized countries [17,19,20]. The introduction of a new biotype of toxigenic C. diphtheriae could also be a factor in the re-emergence of diphtheria [17,21]. It is now admitted that a lack of immunity against diphtheria in adults represents a potential threat that could lead to the development of epidemics in industrialized countries [17,21].

The molecular target of DT, ETA and cholix toxin is the diphthamide residue whose biosynthesis consists of stepwise modifications of a histidine residue present at position 715 (699 in yeast) [22–27]. Five proteins named Dph1 to Dph5 are involved in this process in yeast and eukaryotic cells [28–31]. The chemical transformation into diphthamide requires three successive biochemical reactions starting with the transfer of a 3-amino-3-carboxypropyl from AdoMet to the imidazole C-2 of the histidine residue [25]. Studies in yeast and Chinese hamster ovary (CHO) cells indicated that Dph1 to Dph4 are implicated in the first biochemical transformation [29–31]. More recently, WDR85 was identified as the fifth protein involved in the first step of diphthamide formation [32]. In the second step, Dph5 is involved in the trymethylatation of the diphthamide intermediate [29] and, to up to now, no protein involved in the last step of the diphthamide biosynthesis has been identified. Diphthamide can be found in all eukaryotic organisms and in archaeabacteria except eubacteria, suggesting a relevant role in cell physiology [33,34]. The diphthamide residue is located at the tip of a domain loop in EF2 that mimics the anticodon loop of a tRNA. It has been suggested that diphthamide stabilizes the tRNA-anticodon-codon interaction and is necessary to maintain the translation reading frame [35,36]. Nevertheless, except for yeast Dph3 mutants, the other yeast and CHO cell lines deficient in some of the Dph genes do not show distinctive phenotypes except for DT and ETA resistance [27,31,37]. We thus reasoned that the blockade of diphthamide formation could be an efficient therapeutic strategy for treating P. aeruginosa or C. diphtheriae infections.

Dph1 and Dph2 interact in vivo in yeast as well as in eukaryotic cells, and it has also been suggested that these two proteins could be part of a catalytic complex involved in the first step of diphthamide synthesis [31,38]. We then hypothesized that it could be possible to generate a mutant of Dph1 or Dph2 that could inhibit in a dominant manner the formation of diphthamide. We report in this study the identification of a C-terminal deletion mutant of Dph2 that could completely protect cells against the toxicity of DT and ETA.

**Results**

**A C-terminal Dph2 Deletion Mutant Protects Cells Against DT Toxicity**

Deletion mutants of Dph2 were generated in order to create a dominant negative protein that could block the first step of diphthamide formation. First, the hamster Dph2 cDNA gene was cloned by PCR amplification using degenerated primers based on the mouse and the human sequence. A strong homology was obtained with other human cell lines and CHO cells (data not shown). We are also in the process of defining the shortest deletion mutant that could confer resistance to DT and ETA; preliminary results indicate that the same human Dph2 deletion mutant is functional but not a shorter version with a 133 amino acid deletion (data not shown).

**Cells Expressing Dph2(C-) and Cells Dph2-deficient are Equally Resistant to DT**

In order to investigate to which extent Dph2(C-) could protect cells against DT, CHO cells expressing Dph2(C-) were cultured with increasing concentrations of DT up to $10^{-2}$ μg/μl. There was no significant effect on the proliferation of cells expressing Dph2(C-) even if they were cultured with the highest dose of DT. These cells were as resistant to DT as RPE.33d that is a

---

**Figure 1. A C-terminal Dph2 deletion mutant protects against DT toxicity.** A, Schematic drawings of Dph2 and Dph2 deletion mutants. B, Dph2(C-) confers DT resistance in TE671 cells. TE671 cells untransfected and transfected with plasmids expressing Dph2(C-), Dph2(N-) and Dph2(Z-) were selected with DT for 7 days. Colonies were then fixed with methanol and stained with methylene blue. The colony assay was performed three times; one representative experiment is displayed. doi:10.1371/journal.pone.0015753.g001

---

**Table 1.**
diphthamide-negative CHO subline defective for Dph2 [27]. Wild-type CHO cells were killed with $10^{-2} \, \text{mg/\mu l}$ of DT (Fig. 2). These data demonstrated a strong potency of Dph2(C-) in protecting cells against DT toxicity.

**Dph2(C-) Confers Resistance to ETA**

We next tested the ability of Dph2(C-) to protect against ETA. Proliferation experiments with increasing ETA concentrations were performed on CHO cells that had been transfected with dph2(C-) and selected with DT. These cells were completely resistant to ETA at concentrations as high as $10^{-2} \, \text{mg/\mu l}$, while the viability of parental CHO cells was reduced by 70% with as little as $10^{-3} \, \text{mg/\mu l}$ of the toxin (Fig. 3). This result indicated that DT protection conferred by Dph2(C-) could be extended to ETA.

**Dph2(C-) Prevents the ADP-ribosylation of EF-2 by DT**

The mechanism of toxin protection conferred by Dph2(C-) was investigated in vitro using an EF-2 ADP-ribosylation assay. Protein extracts from different CHO cell lines were incubated with DT and radiolabeled nicotinamide adenine dinucleotide (NAD), and run in an SDS-PAGE gel. As shown by autoradiography, EF-2 was ADP-ribosylated in parental CHO cells and in CHO cells transfected with the inactive N- and Z-deletion mutants. However, no EF-2 ADP-ribosylated form could be detected in CHO cells expressing Dph2(C-), indicating that the absence of diphthamide was responsible for the lack of DT toxicity. As expected, EF-2 from RPE.33d cells could not be ADP-ribosylated but the enzymatic activity of DT could be detected after transfection of dph2 in those cells (Fig. 4A). EF-2 was present in similar amounts in all tested samples as shown by Western blot (Fig. 4B).

**Dph2(C-) Binds Dph1 and Competes with Wild-type Dph2**

One possible explanation for the Dph2(C-) anti-toxin activity was a competition with endogenous Dph2 for the binding to Dph1, leading to the block of the first biochemical step of diphthamide formation. Cotransfection/immunoprecipitation experiments showed that Dph2(C-) fused to a TAP-tag could be immunoprecipitated by an anti-myc antibody recognizing a Dph1-myc fusion protein (Fig. 5; lane 2). Furthermore, cotransfection of Dph2-TAP and Dph2(C-) expression plasmids in a 1:1 ratio (Fig. 5; lane 4) decreased the amount of Dph2-TAP recovered by immunoprecipitation with the anti-myc antibody (Fig. 5; lane 4 versus lane 3). With a 10-fold excess of the Dph2(C-) plasmid over the Dph2 plasmid, the band corresponding to Dph2-TAP on the Western blot was barely detected (Fig. 5; lane 5). These results clearly showed that Dph2(C-) binds to Dph1, and that it competes with wild-type Dph2.
The phenotype of Dph4-null mutants is similar in multiple organ systems. Moreover, embryonic fibroblasts from development and at birth with developmental delay and defects two is displayed.

Figure 5. Dph2(C-) binds to Dph1 and competes with wild-type Dph2. Western blot analysis performed with an anti-TAP antibody of cellular extracts immunoprecipitated with an anti-myc antibody. Lane 1, Dph2. Western blot analysis performed with an anti-TAP antibody of Figure 5. Dph2(C-) binds to Dph1 and competes with wild-type Dph2. Western blot analysis performed with an anti-TAP antibody of cellular extracts immunoprecipitated with an anti-myc antibody. Lane 1, Dph2.

Discussion

In addition of being the target of DT and ETA, it is assumed that the diphthamide residue present on EF-2 plays an important biological role since it is present in all eukaryotic organisms and in archaeabacteria [33,34]. Nevertheless, CHO cells mutated for diphthamide formation are able to synthesize proteins and grow as well as wild-type cells [27,31]. These observations led us to predict that a strategy that prevents the formation of diphthamide to confer DT and ETA resistance is likely to be safe. In the present study, we have designed a dominant-negative strategy that could prevent diphthamide formation and that could render cells resistant to DT and ETA toxicity. We found that Dph2(C-), a C-terminal deletion mutant of Dph2, could completely block DT and ETA induced toxicity by preventing the ADP-ryosylation of EF-2. Dph2(C-) was able to bind Dph1 and to compete with wild-type Dph2, suggesting that the first step of diphthamide formation was impaired.

All Dph proteins involved in the biosynthesis of diphthamide have been well studied. Dph1 is a candidate tumor suppressor gene that has been cloned independently as Ovac1; a loss of Dph1 heterozygosity is frequent in breast and ovarian carcinoma and is associated to a decrease in protein expression [40]. Ovac1 heterozygote mice develop spontaneous cancer, thus, confirming the suppressor function of Dph1. Dph1 is also essential to mouse development as the Ovac1−/- mice die during embryonic development and at birth with developmental delay and defects in multiple organ systems. Moreover, embryonic fibroblasts from these mice revealed a role of Dph1 in the regulation of cell proliferation [40]. The phenotype of Dph1-null mutants is similar to Ovac1−/- mice but heterozygous mice for the Dph1 mutation do not develop tumors, suggesting an additional tumor suppressive function for Dph1 [41]. In addition to DT resistance, yeast Dph3 mutants show defect in growth and drug, and temperature sensitivity suggesting a broader biological role for Dph3 [31,42]. Dph3 knockout mice have also been generated and are associated with lethality during embryonic life [43]. Dph3 has a role outside diphthamide formation that could account for the severe phenotypes observed in knockout mice [44]. It is also most likely that a Dph2 null mutation in mice would be lethal before or at birth. The generation of a transgenic mouse with a conditional expression of Dph2(C-) could circumvent the possible lethality associated to the absence of the wild-type gene and allow the study of the role of the diphthamide residue in adult tissue. This mouse model could be used to assess the efficacy of Dph2(C-) in the treatment of P. aeruginosa infections.

From a therapeutic stand-point, it would be feasible to deliver the Dph2(C-) gene directly at sites of P. aeruginosa infection. For example, viral or non viral delivery systems could be used to introduce Dph2(C-) in corneal cells as a local treatment for refractory P. aeruginosa-induced keratitis [45]. A similar gene therapy strategy could also be offered via aerosol to cystic fibrosis patients that develop P. aeruginosa infections in the lungs [46] or for topical treatments of wounds in burn patients [47]. The crystal structure of a Dph1/Dph2(C-) complex may help in the development of small molecules with similar therapeutic activity as Dph2(C-). High-throughput screening technologies with chemical libraries could also lead to drug candidates for the treatment of P. aeruginosa and C. diphtheriae infection.

Dph2(C-) might also be useful for the treatment of other pathogens. Indeed, cholix toxin from Vibrio cholerae has the same mechanism of toxicity of DT and ETA [3], and putative ADP-ryosyltransferases with a diphthamide residue as target have also been identified in Neisseria gonorrhoeae and Staphilococcus aureus [48]. Finally, efforts are being made to develop antitoxides to counter the health consequences of bioterrorism; dominant-negative mutants of anthrax toxin have already been identified [49], and Dph2(C-) could also be a valuable asset against potential ETA- or DT-based bioweapons.

In conclusion, our results indicate that it is possible to completely abolish DT and ETA toxicity by blocking the formation of diphthamide with a dominant-negative strategy. This is the first demonstration that the targeting of a cellular component can protect against DT and ETA toxicity. Dph2(C-) could have a broad range of clinical applications, including its use alone or in combination with standard antibiotics for the treatment of P. aeruginosa and C. diphtheriae infection. This approach is likely to have a reduced risk of generating resistance, a common outcome with antibiotic therapy.

Materials and Methods

DNA Constructions

The hamster Dph2 gene was amplified by RT-PCR from CHO-K1 cells using the mouse 5' primer Dph2E5' 5' AGTA-GATT-CAT-GGAGTCTACGTTCAGC-3' containing a EcoRI site (underlined) and a 3' degenerated primer designed according to the mouse and human Dph2 sequence 5' TGAGCNCTGTCGACAGGGTACCATGGAGTCTACGTTCAG-3'. The pBS-Dph2 plasmid was then obtained by cloning the PCR product in pBluescript SK+ (Stratagene, LaJolla, CA) opened in EcoRI/EcoRV. The hamster Dph2 gene was sequenced and deposited to GenBank (accession no. DQ981502). Three deletion mutants of Dph2 were constructed by PCR using pBS-Dph2 as template, and they were cloned in the eukaryotic vectors pMD2iPuro', pcDNA3-TAP and pNC described elsewhere [50-52].

For Dph2, a PCR was performed with the following primers: Dph2K5' 5' CGGCGTACCAATGGAGTCTACGTTCAGC-3' containing a KpnI site (underlined) and a Dph2B3' 3' primer 5' CGCGATCCCGGCGGTGCCCCGATCC-3' containing a BamHI site (underlined). The KpnI/BamHI digested PCR product was then cloned in pcDNA3-TAP opened in KpnI/BamHI to create the pcDNA-Dph2(TAP)-plasmid.

The G-terminal mutant was constructed by PCR with the 5' primer Dph2K5' and the 3' primer 5' AGTA-GATT-CAT-GGAGTCTACGTTCAGC-3'
GAACATA-3'. The pcDNA-Dph2(C-) TAP plasmid was obtained by cloning the KpnI digested PCR fragment in pcDNA3-TAP opened in BamHI blunted by klenow and KpnI. pNC-Dph2-2-TAP and pNC-Dph2(C-) TAP were generated by cloning in pNC opened in BamHI Dph2-TAP and Dph2(C-) TAP linked to a BamHI adaptor.

The pMD2-Dph2iPuro vector was constructed by cloning in pMD2iPuro opened in EcoRI/XhoI Dph2 from pBS-Dph2 digested by EcoRI/XhoI.

The pMD2-Dph2(C-)iPuro plasmid was constructed by inserting a Dph2(C-) PCR product with the 5' primer Dph2E5' and the 3' primer 5'-TTAATTCGGGAGTGGAACAT3'- digested by EcoRI and inserted in pMD2iPuro opened by EcoRI/EcoRV.

For the construction of the N-terminal mutant, the PCR amplification was performed using the 5' primer 5'-CGCGGTACC-ATGGAGCCAGCTTGTC-3' containing a KpnI site (underlined) and the 3' primer Dph2B3'. The PCR product was digested by KpnI and BamHI and ligated in the pcDNA3-TAP plasmid to give the pcDNA-Dph2(N-) TAP plasmid.

The leucine zipper mutant (Z-) was created using two different PCR fragments ligated together in pcDNA3-TAP. The first segment was amplified with the 5' primer Dph2K5' and the 3' primer 5'-GGCATGCGGCAACGCTG-3', and the second fragment was amplified with the 5' primer 5'-ATCTCCAGGCCACGCTTT-3' and the 3' primer Dph2B3'. The pcDNA-Dph2(Z-) TAP plasmid was constructed by cloning the first amplification product digested by KpnI and the second one digested by BamHI in pcDNA3-TAP.

The pMD2-Dph2(2N/-iPuro) and pMD2-Dph2(Z/-iPuro) plasmids were constructed similarly. The KpnI/BamHI fragment from pcDNA-Dph2(2N/-TAP) or pcDNA-Dph2(Z/-TAP) was blunt klenow and ligated in pMD2iPuro vector digested by EcoRV and XhoI bluntly digested by klenow. A stop codon was created at the C-terminal end due to the cloning procedure.

The mouse Dph1 gene was amplified by RT-PCR using cDNAs prepared from mouse PG13 cells with the following primers: the 5' primer 5'-CGCGGTACC-ATGGAGCCAGCTTGTC-3' containing a KpnI site (underlined) and the 3' primer Dph2B3'. The PCR product was digested by KpnI and BamHI and ligated in the pcDNA3-TAP plasmid to give the pcDNA-Dph1-myc vector.

Cell Proliferation Assay [54]

The cells were plated at a concentration of 3 x 10^3 cells/well in 96-well plates in six replicates for each toxin concentration. The next day, increasing concentrations of DT or ETA (List Biological laboratories) were added to the wells for 3 days. Cell proliferation was then measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay, which consists of adding 37.5 µl of MTT (1 mg/ml) to the 150 µl of medium in each well for 4 hours at 37°C. After gently removal of the medium, 150 µl of dimethyl sulfoxide (DMSO) was added and plates were gently shaken for 10 minutes to dissolve the formazan blue crystals. The absorbance was then measured at 595 nm with a microplate reader (Tecan, Research Triangle Park, NC).

ADP-ribosylation Assay

Confluent cells grown in 60-mm plates were lysed in 400 µl of modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxychylate, 150 mM NaCl, 1 mM EDTA and protease inhibitors) at 4°C for 45 minutes. Cell extracts were then centrifuged at 13,000 x g for 30 minutes at 4°C, and the supernatant fraction was determined by the Bradford protein assay (Biorad, Hercules, CA). DT was nicked by mixing together 27 µl of DT (1 mg/ml) with 3 µl trypsin (10 mg/ml) for 15 minutes. The reaction was stopped by adding 1 µl of protein inhibitors (Sigma, P8340). For the ADP-ribosylation assay, 100 µg of protein extract were mixed with 500 ng nicked DT, 2 µl [3H]NAD, 285 mM Cl/mmol (GE Healthcare, Baie d’Urfe, Canada), 20 mM Tris-HCl, pH 7.5, 50 mM dithiothreitol (DTT), 1 mM EDTA at 30°C for 30 minutes in a final volume of 120 µl. From the 120 µl reaction, 50 µl were mixed with 17 µl of 4X sample buffer (200 mM Tris-HCl, 5% SDS, 0.4% bromophenol blue, 40% glycerol, 400 mM DTT) and analysed by SDS-PAGE on a 10% acrylamide gel followed by autoradiography.

Immunoprecipitation

293T cells plated in 60-mm plates were transfected with 1 µg of the pcDNA-Dph1-myc plasmid with 1 µg of pNC-Dph2-TAP, and with 0, 1 or 10 µg of pMD2-Dph2(C-) plasmids. One µg of the pcDNA-Dph1-myc plasmid with 1 µg of pNC-Dph2(C-) TAP were also transfected to assess the binding of Dph2(C-) with Dph1. Two days post-transfection, cells were lysed in 500 µl of E1A buffer (20 mM HEPES pH 7.9, 250 mM NaCl, 0.1% IGEPAI, 10% glycerol, 1 mM β-mercaptoethanol and protease inhibitors) at 4°C for 30 minutes. The lysis product was then incubated with 1 µg of the α-myc antibody (clone 9E10) (Sigma) at 4°C for 4 hours. Following this incubation, the extracts were treated with protein A-Sepharose (GE Healthcare) at 4°C for 1 hour. The beads were then washed three times with E1A buffer and resuspended in 40 µl loading buffer (20 mM Tris-Cl pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM DTT). The samples were then analyzed by Western blot.

Western Blot Analysis

Cell extracts prepared for the ADP-ribosylation assays were also analysed by Western blot. Thirty µg of total protein extract were mixed with sample buffer (as described above) and loaded on a 10% SDS-polyacrylamide gel and separated by electrophoresis. The proteins were transferred on nitrocellulose membranes (GE Healthcare) followed by Western blotting using a goat antibody directed against a linear peptide of the carbonyl terminus of EF-2 (Santa Cruz Biotechnology, Santa Cruz, CA). For the immunoprecipitation experiment, the samples were analysed with an anti-TAP antibody (Open Biosystems, Huntsville, AL). The reactive
bands were detected using the Western Lightning Chemiluminescence Reagent Plus kit (Perkin Elmer Life Sciences, Boston, MA).

Statistical Analysis

One-way ANOVA followed by Dunnett’s multiple comparison posttest was performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA) on data presented in figures 2 and 3.

References

1. Collier RJ (2001) Understanding the mode of action of diphtheria toxin: a perspective on progress during the 20th century. Toxicon 39: 1793–1803.
2. Deng Q, Barbieri JT (2008) Molecular mechanisms of the cytotoxicity of ADP-ribosylating toxins. Annu Rev Microbiol 62: 271–286.
3. Jorgensen R, Purdy AE, Fieldhouse RJ, Kimber MS, Bartlett DH, et al. (2008) Cholix toxin, a novel ADP-ribosylating factor from Vibrio cholerae. J Biol Chem 283: 10671–10678.
4. Lyyczak JB, Cannon CL, Pier GB (2000) Establishment of Pseudomonas aeruginosa aerosol inocion: lessons from a versatile opportunist. Microbes Infect 2: 1051–1060.
5. Kipnis E, Sawa T, Wiener-Kronish J (2006) Targeting mechanisms of Pseudomonas aeruginosa pathogenesis. Med Mal Infect 36: 78–91.
6. Bjorn MJ, Vasil ML, Sardot JC, Igleslwi BH (1977) Incidence of exotoxin A production by Pseudomonas species. Infect Immun 16: 362–367.
7. Nicas TI, Igleslwi BH (1985) The contribution of exoproteins to virulence of Pseudomonas aeruginosa. Can J Microbiol 31: 367–392.
8. Miyazaki S, Matsumoto T, Tateda K, Ohno A, Yamaguchi K (1995) Role of exotoxin A in inducing severe Pseudomonas aeruginosa infections in mice. J Med Microbiol 44: 169–175.
9. Pavlovsii OR, Pollack M, Callahan LT, 3rd, Igleslwi BH (1977) Passive protection by antitoxin in experimental Pseudomonas aeruginosa burn infections. Infect Immun 18: 596–602.
10. Matsumoto T, Tateda K, Furuya N, Miyazaki S, Ohno A, et al. (1999) Effect of passive immunization on murine gut-derived sepsis caused by Pseudomonas aeruginosa. J Med Microbiol 48: 765–770.
11. El-Zain HS, Chopra AK, Petersen JW, Vasil ML, Heggars JP (1998) Protection against exotoxin A (ETA) and Pseudomonas aeruginosa aerosol infection in mice with ETA-specific antipeptide antibodies. Infect Immun 66: 5551–5554.
12. Fogle MR, Griswold JA, Oliver JW, Hamwood AN (2002) Anti-ETA IgG neutralizes the effects of Pseudomonas aeruginosa exotoxin A. J Surg Res 106: 98–99.
13. Manafi A, Kohanbaf J, Mehrabani D, Japoni A, Amini M, et al. (2009) Active immunization using exotoxin A confers protection against Pseudomonas aeruginosa infection in a mouse burn model. BMC Microbiol 9: 23.
14. Ohman DE, Burns KP, Igleslwi BH (1980) Corneal infections in mice with toxin A and elastase mutants of Pseudomonas aeruginosa. J Infect Dis 142: 547–555.
15. Pillar CM, Hobden JA (2002) Pseudomonas aeruginosa aerosolxA and keratitis in mice. Invest Ophthalmo1 Vis Sci 43: 1437–1444.
16. Galazka A (2000) The changing epidemiology of diphtheria in the vaccine era. J Infect Dis 181 Suppl 1: S2–S9.
17. Danilova E, Jeram PA, Skogen VG, Pihlakow VJ, Sjurs H (2006) Antidiphtheria antibody responses in patients and carriers of Corynebacterium diphtheriae in the Arkhangelsk region of Russia. Clin Vaccine Immunol 13: 627–632.
18. Furino KM, Strehbl PM, Chen RT, Kinbaler A, Cleary TJ, et al. (1990) Fatal respiratory disease due to Corynebacterium diphtheriae: case report and review of guidelines for management, investigation, and control. Clin Infect Dis 16: 59–68.
19. Speranza FA, Ishii KK, Hiraiz R Jr., Mattos-Guazzal Al, Milagres LG (2010) Diphtheria toxin. IgG levels and in military and civilian blood donors in Rio de Janeiro, Brazil. Braz J Med Biol Res 43: 120–126.
20. Mattos-Guazzal Al, Morriera LO, Damasco PV, Hiraiz Jurer R (2003) Diphtheria remains a threat to health in the developing world—an overview. Mem Inst Oswaldo Cruz 98: 897–903.
21. Robinson EA, Henriksen O, Maxwell ES (1974) Elongation factor 2. Amino acid sequence at the site of adenine diphosphate ribosylation. J Biol Chem 249: 5098–5099.
22. Van Ness BG, Howard JB, Bodley JW (1980) ADP-ribosylation of elongation factor 2 by diphtheria toxin. Isolation and properties of the novel ribo- nucelic acid and its hydrolysis products. J Biol Chem 255: 10717–10720.
23. Van Ness BG, Howard JB, Bodley JW (1980) ADP-ribosylation of elongation factor 2 by diphtheria toxin. NMR spectra and proposed structures of ribosyl-diphthamide and its hydrolysis products. J Biol Chem 255: 10710–10716.
24. Chen JY, Bodley JW (1980) Biosynthesis of diphthamide in Saccharomyces cerevisiae. Partial purification and characterization of a specific S-adenosyl-
50. Doyon Y, Selleck W, Lane WS, Tan S, Cote J (2004) Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans. Mol Cell Biol 24: 1884–1896.

51. Roy V, Qiao J, de Campos-Lima P, Caruso M (2005) Direct evidence for the absence of intercellular trafficking of VP22 fused to GFP or to the herpes simplex virus thymidine kinase. Gene Ther 12: 169–176.

52. Ghani K, Cottin S, Kamen A, Caruso M (2007) Generation of a high-titer packaging cell line for the production of retroviral vectors in suspension and serum-free media. Gene Ther 14: 1705–1711.

53. DuBridge RB, Tang P, Hsia HC, Leong PM, Miller JH, et al. (1987) Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol Cell Biol 7: 379–387.

54. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63.