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Pseudomonas aeruginosa exotoxin A induces pulmonary endothelial cytotoxicity: protection by dibutyryl-cAMP

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Pseudomonas aeruginosa *exotoxin A induces pulmonary endothelial cytotoxicity: protection by dibutyryl-cAMP. W.J. Bourke, C.M. O'Connor, M.X. FitzGerald, T.J. McDonnell. ©ERS Journals Ltd 1994.*

ABSTRACT: In pseudomonal septicaemia, serum levels of antibody to exotoxin A have been demonstrated to be an important independent predictor of survival. Previously, we have demonstrated that exotoxin A directly injures pulmonary endothelial cells, and that dibutyryl-cyclic adenosine monophosphate (Db-cAMP) can attenuate this injury. The object of this study was to examine the mechanisms of this pulmonary endothelial cell injury and the mechanism of Db-cAMP protection.

The effects of differing duration of exposure to exotoxin A and a reduction in temperature on endothelial cell injury were examined. In addition, the effect of post-treatment with Db-cAMP on exotoxin A-induced endothelial cell injury was studied.

A brief, 5 min, exposure to exotoxin resulted in maximum injury comparable to that produced by 18 h exposure. This injury did not occur at low temperatures, which would inhibit receptor-mediated endocytosis. Db-cAMP protected endothelial cells, even when added up to one hour after exotoxin exposure.

These results suggest that, in this model, exotoxin A-induced injury of endothelial cells is receptor-mediated. Furthermore, this injury may be attenuated even after exotoxin A internalization has taken place.

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Pseudomonas aeruginosa is an important Gram-negative pathogen which produces life-threatening pneumonias and sepsis [1, 2]. Although the role of endotoxin as an aetiological agent in the adult respiratory distress syndrome (ARDS) associated with Gram-negative sepsis has been extensively studied [3, 4], the exotoxins elaborated by these same organisms have received less attention. *Pseudomonas aeruginosa* exotoxin A (Exo A) is a proenzyme, which causes cytotoxicity by inhibiting protein synthesis [5, 6], and is 10,000 times more toxic to mice than Pseudomonas endotoxin [7]. POLLACK and YOUNG [8] have demonstrated that a high level of antibody to Exo A is an important independent prognostic indicator of survival in Pseudomonas septicaemia. Previously, we have demonstrated that Exo A is directly toxic to alveolar epithelial and pulmonary endothelial cells [9]. However, the mechanism underlying this injury has not been elucidated.

In the mouse fibroblast, investigators have demonstrated that exotoxin is taken up through receptor-mediated endocytosis [10]. Binding to the cell surface is necessary before exotoxin can produce cytotoxicity in this fibroblast model. This suggests that after internalization of a critical quantity of exotoxin into the cell, subsequent protein synthesis inhibition and cell cytotoxicity will proceed, irrespective of the availability of extracellular exotoxin. We hypothesized that a minimal exposure time is required to achieve this binding to the cell surface and subsequent internalization. Thereafter, cytotoxicity is independent of extracellular exotoxin. If this hypothesis proves to be true, this would have important implications for the clinical use of anti-exotoxin monoclonal antibodies [8, 11], as antibodies to exotoxin will only bind to extracellular exotoxin and will have no influence on exotoxin that has already been internalized.

The purpose of this study was to investigate the duration of exposure and influence of temperature on Exo A injury to pulmonary endothelial cells. In addition, methods of interrupting Exo A-induced injury to pulmonary endothelial cell injury were investigated. Previously, we have shown that dibutyryl-cyclic adenosine monophosphate (Db-cAMP) can prevent Exo A cytotoxicity [9], and to investigate this further, we now examined the effects of adding Db-cAMP post-Exo A to see if post-treatment can attenuate exotoxin-mediated cytotoxicity.

Methods

Materials/reagents

Bovine pulmonary artery endothelial cells (BPAEC) were acquired from the American Type Culture Collection

(ATCC), (Rockville, Md, USA), penicillin and streptomycin from Flow, (Irvine, Scotland), trypsin-ethylenediamine tetra-acetic acid (EDTA), ammonium hydroxide (NH_4OH) and Db-cAMP from Sigma, (St Louis, Mo, USA), RPMI 1640, L-glutamine, foetal calf serum, and Hank's balanced salt solution (HBSS) from Gibco, (Paisley, Scotland). Radioactive sodium chromate was purchased from (Dupont, Boston, Mass, USA), and Exo A from List Biological Labs, (Campbell, Ca, USA). Tissue culture flasks were acquired from Becton and Dickinson, (Plymouth, UK). A 1261 MultiGamma counter from Pharmacia, (Turku, Finland) was used to perform radioactivity counts.

Cell Culture

Standard tissue techniques were used to grow BPAECs in RPMI media containing 20% heat inactivated foetal calf serum with 2 mM glutamine, penicillin 100 $U \cdot ml^{-1}$, and streptomycin 200 $U \cdot ml^{-1}$. Experiments were performed on cells between passage numbers 18–25. Trypsin (0.05%) was used to release cells from the flasks. Ethidium bromide staining confirmed that the cells were >98% viable before plating on 24 well plates at a density of 10^5 cells·well^{-1}.

Chromium (Cr-51) assay

When the cells were near confluence (80–90%), Cr-51 was added overnight at a concentration of 2 $\mu Ci \cdot ml^{-1}$. After this labelling process, excess chromium was washed off with HBSS. Subsequent Cr-51 release to the overlying media was then measured to assess cell damage [12, 13]. The total possible Cr-51 counts (total) were determined from the addition of control well cell lysates plus the overlying media counts. The cells were lysed by adding 1 ml of NH_4OH to the well for 10 min. Injury to the cells for each experimental condition was then calculated, using the overlying media in the following manner, as described by KAMP *et al*. [14]: % specific Cr-51 release = (test media counts per minute (cpm)-control media cpm/total cpm) × 100. This calculation corrects for spontaneous Cr-51 release from control wells. The spontaneous %Cr-51 release over 18 h from control wells was 31±2.6% (mean±SEM) of the total releasable Cr-51 (n=6).

Exposure of BPAECs to Exo A

Exo A (10 $\mu g \cdot ml^{-1}$) was added to confluent monolayers of Cr-51-labelled cells in 24 well plates, for times varying from 5 min to 18 h. At the completion of exposure to Exo A, the overlying media (1 ml) was removed, the monolayer rinsed, and fresh media was added to the monolayer. At 18 h from the initial exposure to Exo A, the Cr-51 counts were determined. Each experimental condition was repeated in duplicate. Experiments were also carried out to examine the effects of low temperature (4°C) on Exo A injury over an 18 h time period.

Dosage of Db-cAMP

The optimum dose of db-cAMP was determined by varying the dosage between 0.1, 1, and 10 mM with a standard dose of Exo A (10 $\mu g \cdot ml^{-1}$). The optimal dose of Db-cAMP was then added immediately before Exo A and subsequently at various time-points after Exo A, ranging from 5 min to 4 h, to determine how long after Exo A addition Db-cAMP might confer protection.

Statistics

The experiment results are expressed as the mean±SEM. The Student's t-test was used to compare between two groups. Where there were multiple groups, one way analysis of variance (ANOVA) for time and treatment effects was performed, followed by the Neuman-Keuls range test. A p-value of less than 0.05 was selected as significant.

Results

Effect of Exo A exposure time to endothelial cell injury

Exposure of BPAECs to Exo A for various periods of time indicated that there was no difference in the level of injury whether the endothelial cells were exposed to Exo A for 5 min or 18 h in the media, although there was a trend for chromium release to increase slightly with time. There was 26±3% specific Cr-51 release at 5 min and 33±3% specific Cr-51 release at 18 h (n=6) (fig. 1).

Effect of temperature on Exo A injury to endothelial cells

When experiments were carried out at 4°C, the endothelial cells incubated with Exo A for 18 h demonstrated significantly less Cr-51 release than endothelial cells incubated with Exo A at 37°C (0.5±2.8 4°C *vs* 33.0±2.5 37°C; $p<0.05$)

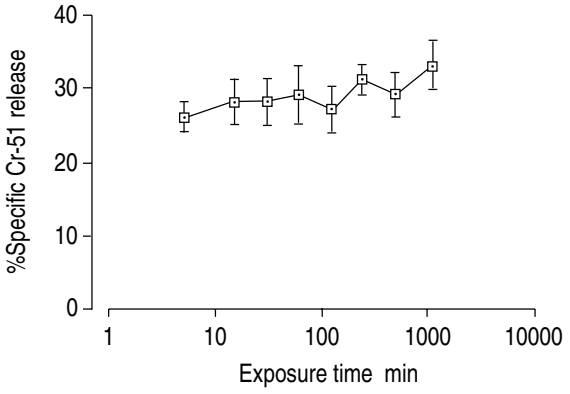

Fig. 1. – The effect of exposure time to exotoxin A (Exo A) on BPAEC injury. BPAECs were exposed to Exo A (10 $\mu g \cdot ml^{-1}$) for time periods from 5 min to 18 h. There were no differences in Cr-51 release between any of the exposure times. Results are expressed as the mean±SEM, n=6. BPAEC: bovine pulmonary artery endothelial cells.

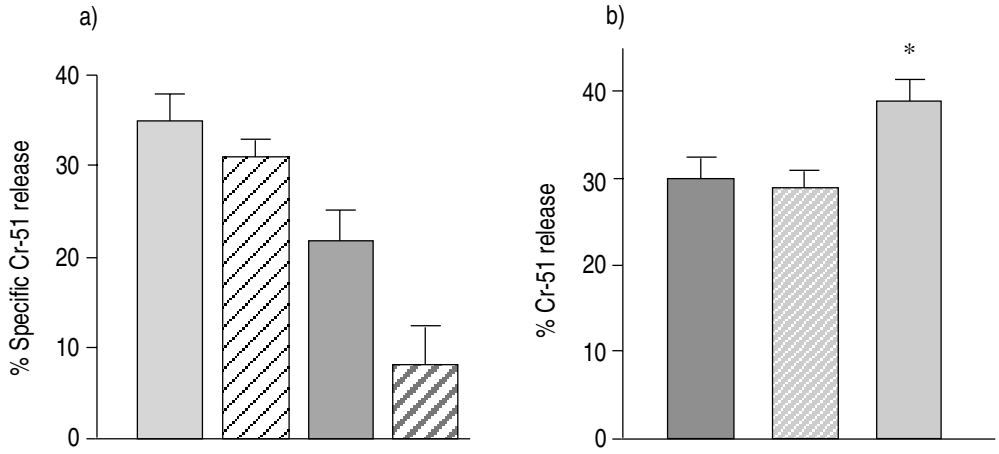

Fig. 2. – a) The optimum dose of Db-cAMP against Exo A injury (10 μg·ml^{-1}) to BPAECs. The optimum dose of Db-cAMP was determined to be 1 mM Db-cAMP. : 10.0 mM Db-cAMP + Exo A; : Exo A alone; : 0.1 mM Db-cAMP + Exo A; : 1 mM Db-cAMP + Exo A. b) 1 mM Db-cAMP did not cause significant Cr-51 release, although 10 mM caused significant Cr-51 release. : control; : control + 1 mm Db-cAMP; : control + 10 mM Db-cAMP. *: $p<0.05$ compared to control wells. Results are expressed as the mean±SEM, n=4. Db-cAMP: dibutyryl cyclic adenosine monophosphate. For further abbreviations see legend to figure 1.

Optimal dosage and time sequence of Db-cAMP protection

We demonstrated that 1 mM Db-cAMP was the optimum dose conferring protection to endothelial cells against a given dose (10 μg·ml^{-1}) of Exo A (fig. 2a). A 10 mM dose of Db-cAMP did not confer any protection, and 10 mM alone actually caused significant Cr-51 release when compared to control and 1 mM Db-cAMP alone (fig. 2b). Db-cAMP was protective when co-incubated with Exo A. Specific Cr-51 release was determined to be 10±2% in this case.

This protective effect of Db-cAMP against Exo A-induced endothelial injury was also noted with postincubation of Db-cAMP up to 1h after Exo A. If Db-cAMP was added to the wells 1 h after Exo A, the specific Cr-51 release was 13±3% (n=6). The lowest level of specific Cr-51 release was demonstrated when Db-cAMP was added 5 min post-Exo A, where specific Cr-51 release was 4±2% (fig. 3).

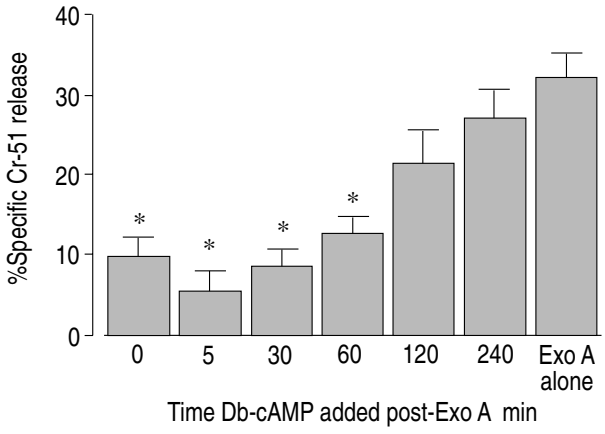

Fig. 3. – Post-treatment with Db-cAMP attenuates Exo A (10 μg·ml^{-1}) injury. Results are expressed as the mean±SEM, n=6. *: $p<0.05$ as compared to Exo A alone. For abbreviations see legend to figure 1.

Discussion

Our results establish that Exo A-induced pulmonary endothelial cell injury is independent of the duration of exposure to Exo A and can be prevented by temperature reduction. In this model, it appears that after one hour of exposure to exotoxin, irreversible injury to endothelial cells has occurred. These observations support the findings of FITZGERALD *et al*. [10], in mouse fibroblasts, that Exo A is taken up by receptor-mediated endocytosis. In our model, binding of Exo A to the cell surface occurs rapidly, as removal of the Exo A following 5 min of exposure did not prevent the occurrence of injury. Our experiments do not clarify whether endocytosis also occurs rapidly, or whether this requires more time. The former observation has important clinical implications, as early interventions, such as monoclonal antibody binding to toxins, may be unhelpful if the toxin has already been bound to the cell surface and possibly been internalized by endocytosis.

We have also demonstrated that the addition of Db-cAMP is protective if added up to one hour after Exo A. In contrast to other studies of lung injury, we were not measuring endothelial cell permeability but severe cell damage as related to Cr-51 release, which is a late event [15]. Consequently, this post-treatment cytoprotection is more significant and is consistent with *in vivo* work of HOFFMANN *et al*. [16], who demonstrated that Db-cAMP and pentoxiphylline attenuated endotoxin-induced lung injury in guinea-pigs *in vivo* when added 30 min post-endotoxin.

Db-cAMP has been demonstrated to attenuate lung injury from a number of injurious stimuli [17–21]. However, the mechanism of protection in these instances is not known, although there is evidence that in some *in vivo* models it may exert a beneficial response by reducing pulmonary vascular pressures [19, 20]. However, there is also evidence that this protection may be related to decreasing endothelial permeability [20, 21]. A further hypothesis to explain the protective influence of

Db-cAMP on pulmonary endothelial cell injury is that Db-cAMP may exert its *in vitro* protective influence by activating protein kinase A and promoting endothelial cell membrane stability [22]. Following the observation of SHASBY *et al*. [23], who demonstrated the importance of actin filaments at the cell membrane in regulating cell permeability, STELZNER *et al*. [24] have shown that increased levels of cAMP increase endothelial apposition and have related this to increased levels of actin at the cell membrane. A further hypothesis has been suggested by KOYAMA *et al*. [25], who postulated that Db-cAMP attenuation of endotoxin lung injury in sheep was related to decreased prostaglandin production.

In our model of Exo A-induced endothelial cell injury, the protective effects of Db-cAMP are not related to pressor effects. The mechanism of Db-cAMP protection in this model is dependent on intracellular mechanisms, as incubation of Db-cAMP with endothelial cells post-Exo A protects against exotoxin injury, and protection, therefore, is not dependent on binding extracellular exotoxin. The specific intracellular mechanism of protection in our model is not clear. Repletion of intracellular adenosine triphosphate (ATP) levels using cAMP analogues as a substrate for ATP formation, as suggested by ANDREOLI *et al*. [26], is a possibility. They have demonstrated that oxidant injury to endothelial cells can be reversed by augmenting intracellular cAMP levels [26]. In these studies, it appears that oxidant stress can be tolerated for a period of up to 90 min, after which cell "rescue" is impossible. It is interesting to note that high concentrations of Db-cAMP were toxic to the endothelial cells even in the absence of Exo A. This may be related to high levels of adenosine as a breakdown product of Db-cAMP, which have been demonstrated to cause cytotoxicity to endothelial cells in culture [27]. The possibility that after internalization Db-cAMP works as an intracellular secondary messenger in the form of cAMP, which then activates protein kinase A, has not been ruled out [22].

In summary, exposure of endothelial cells to Exo A need only be for a brief period before binding occurs, and the injury that follows is independent of extracellular Exo A. Furthermore, Db-cAMP can interrupt injury after binding of Exo A. This implies that the sequence of events involved in cell injury in this model is interruptable. However, our studies also show that Exo A-induced endothelial cell injury begins early, and, consequently, treatment of exotoxin-induced lung injury early enough to interrupt intracellular pathways will provide formidable problems in the clinical setting.

References

1. Andriole VT. Pseudomonas bacteremia: can antibiotic therapy improve survival? *J Lab Clin Med* 1979; 94: 196–200.
2. Fick RB, Hata JS. Pathogenetic mechanisms in lung disease caused by *Pseudomonas aeruginosa*. *Chest* 1989; 95: 206S–213S.
3. Brigham KL, Meyrick B. Endotoxin and lung injury. *Am Rev Respir Dis* 1986; 133: 913–927.
4. Rinaldo JE, Christman JW. Mechanisms and mediators of the adult respiratory distress syndrome. *Clin Chest Med* 1990; 11: 621–632.
5. Liu PV, Yoshii S, Hsieh H. Exotoxins of *Pseudomonas aeruginosa*: concentration, purification, and characterisation of exotoxin A. *J Infect Dis* 1973; 128: 514–519.
6. Pollack M. *Pseudomonas aeruginosa* exotoxin A. *N Engl J Med* 1980; 302: 1360–1362.
7. Iglewski BH, Kabat D. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. *Proc Natl Acad Sci USA* 1975; 72: 2284–2288.
8. Pollack M, Young S. Protective activity of antibodies to exotoxin A and lipopolysaccharide at the onset of *Pseudomonas aeruginosa* septicaemia in man. *J Clin Invest* 1979; 63: 276–286.
9. Bourke W, Kamp D, Chang S. *Pseudomonas aeruginosa* exotoxin A cytotoxicity: modulation by Db-cAMP. *Am Rev Respir Dis* 1991; 141: A769.
10. Fitzgerald D, Morris R, Saelinger C. Receptor-mediated internalisation of Pseudomonas toxin by mouse fibroblasts. *Cell* 1980; 21: 867–873.
11. Kohzuki T, Kato M, Irie K, Ohtsuka H, Higuchi A, Noguchi H. Protective activity of anti-exotoxin A monoclonal antibody against mice infected with toxin-producing *Pseudomonas aeruginosa*. *J Infect Dis* 1993; 167: 119–125.
12. Baker JR, Bullock GR, Crawford N, Taylor DG. Localisation in platelets of sodium ^{51}Cr ^{125}I antibody to whole membrane and ^{3}H-diisopropylfluorophosphate using EM auto-radiography. *Am J Pathol* 1977; 88: 277–290.
13. Steinzer M, Baldini M. Subcellular localization of ^{51}Cr and characterization of its binding sites in human platelets. *Blood* 1970; 35: 727–739.
14. Kamp DW, Dunne M, Weitzman SA, Dunn MM. The interaction of asbestos and neutrophils injures cultured human pulmonary epithelial cells: role of hydrogen peroxide. *J Lab Clin Med* 1989; 114: 604–612.
15. Andreoli SP, Baehner RL, Bergstein JM. *In vitro* detection of endothelial cell damage using 2-deoxy-D-H-glucose: comparison with chromium-51, H-leucine, H-adenine, and lactate dehydrogenase. *J Lab Clin Med* 1985; 106: 253–261.
16. Hoffmann H, Hatherhill JR, Crowley J, *et al*. Early post-treatment with pentoxifylline or dibutyryl cAMP attenuates *Escherichia coli*-induced acute lung injury in guinea-pigs. *Am Rev Respir Dis* 1991; 143: 289–293.
17. Kobayashi H, Kobayashi T, Fukushima M. Effects of dibutyryl cAMP on pulmonary air embolism-induced lung injury in awake sheep. *J Appl Physiol* 1988; 63(6): 2201–2207.
18. Chang S, Sakai A, Voelkel NF. Dibutyryl-cAMP blocks endotoxin-induced lung injury in rats. *Am Rev Respir Dis* 1989; 140: 1814–1817.
19. Farrukh IS, Gurtner GH, Michael JR. Pharmacological modification of pulmonary vascular injury: possible role of cAMP. *J Appl Physiol* 1987; 62: 47–54.
20. Casnocha S, Eskin S, Hall E, McIntire L. Permeability of human endothelial monolayers: effect of vasoactive agonists and cAMP. *J Appl Physiol* 1989; 67(5): 1997–2005.
21. Kennedy TP, Michael JR, Hoidal JR, *et al*. Dibutyryl cAMP, aminophylline, and beta-adrenergic agonists protect against pulmonary edema caused by phosgene. *J Appl Physiol* 1989; 67(6): 2542–2552.
22. Taylor SS, Buechler JA, Yonemoto W. cAMP dependent protein kinase: framework for a diverse family of

regulatory enzymes. *Annu Rev Biochem* 1990; 59: 971–1005.
23. Shasby DM, Shasby S, Sullivan JM, Peach MJ. Role of endothelial cell cytoskeleton in control of endothelial permeability. *Circ Res* 1982; 51: 657–661.
24. Stelzner TJ, Veil JV, O'Brien RF. Role of cyclic adenosine monophosphate in the induction of endothelial barrier properties. *Cell Physiol* 1989; 39: 159–166.
25. Koyama S, Toyofuku T, Hirai K, Yoshimura K, Kobayashi T. Dibutyryl cyclic AMP attenuates lung responses induced by endotoxin in conscious sheep. *Am Rev Respir Dis* 1992; 146: 32–38.
26. Andreoli SP, Liechty EA, Mallett M. Exogenous adenine nucleotides replete endothelial cell adenosine triphosphate after oxidant injury by adenosine uptake. *J Lab Clin Med* 1990; 115: 304–313.
27. Pearson JD, Gordon GL. Nucleotide metabolism by endothelium. *Annu Rev Physiol* 1985; 47: 617–627.