Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 339 (2006) 107–114
BBRC
www.elsevier.com/locate/ybbrc

Inhibition of platelet aggregation by anthrax edema toxin ☆

Sheeba Alam, Megha Gupta, Rakesh Bhatnagar *

Centre for Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India

Received 6 October 2005
Available online 9 November 2005

Abstract

Edema toxin is a key virulence determinant in anthrax pathogenesis that causes augmentation of cAMP inside host cells. This exotoxin has been implicated in facilitating bacterial invasion by impairing host defenses. Here, we report for the first time that edema toxin plays an important role in suppression of platelet aggregation; an effect that could be of vital significance in anthrax afflicted subjects. It was found that edema toxin induces a dose dependent and time dependent increase in cAMP inside rabbit platelets. Elevation of cAMP led to suppression of platelet aggregation as demonstrated by in vitro aggregation assays. A 95% suppression of platelet aggregation in response to thrombin and a complete suppression in response to ADP, at toxin concentrations of 7 and 2.2 nM, respectively, were observed. Antibody neutralized wild type edema factor and non-toxic mutants of this binary toxin failed to show any alteration in the normal aggregation pattern. Edema toxin caused the activation of cAMP dependent protein kinase A inside platelets, a phenomenon that could be speculated to initiate the cascade of events responsible for suppressing platelet aggregation. Furthermore, in vivo bleeding time registered a sharp increase in response to edema toxin. These findings can explicate the systemic occurrence of hemorrhage, which is a prominent symptom of anthrax. This study exemplifies how *Bacillus anthracis* has evolved the ability to use host's physiological processes by mimicking the eukaryotic signal transduction machinery, thus inflicting persistent infection.

Keywords: Edema toxin; Platelet aggregation; Hemorrhage; Bleeding time

The pathogenesis of *Bacillus anthracis* is primarily attributed to the exotoxin complex and poly-D-glutamic acid capsule. The tripartite exotoxin comprises of lethal toxin and edema toxin where the common receptor binding moiety, protective antigen (PA) administers the entry of lethal factor (LF) and edema factor (EF) inside cell's cytosol [1]. LF is a zinc dependent protease that inhibits the MAPK signal transduction pathway [2]. Edema factor, a calmodulin dependent adenylate cyclase, causes dramatic elevation in cAMP levels inside target cells [3]. It belongs to a class of adenylate cyclases namely *Bordetella pertussis* adenylate cyclase toxin, Exo Y of *Pseudomonas aeruginosa*, and adenylate cyclase of *Yersinia pestis*, that are produced as secreted virulence factors [4]. The intrinsic adenylate cyclase activity of edema factor leads to disturbance in the deftly regulated intracellular physiological equilibrium. Studies conducted hitherto have shown that EF intoxicates a wide variety of cells. It targets cytokine networking in monocytes, leading to weakening of host response to anthrax infection [5]. It also attenuates antimicrobial activity of neutrophils by inhibiting phagocytosis [6]. Earlier studies conducted in our laboratory have shown that lymphocytes are the most sensitive of all cells to edema toxin-induced cAMP production [7]. Such a massive enhancement of this second messenger inside lymphocytes may, in principle, lead to a suppressed immune response. EF has also been reported to induce hemolysis in the presence of polymorphonuclear cells [8]. The clinical disease of anthrax is currently recognized as cutaneous, inhalational, and

☆ *Abbreviations:* PA, protective antigen; LF, lethal factor; EF, edema factor; EdTx, edema toxin (PA + EF); wt, wild type; cAMP, 3′–5′-cyclic adenosine monophosphate; ADP, adenosine diphosphate; PKA, cAMP dependent protein kinase.

* Corresponding author. Fax: +91 11 26717040.
E-mail address: rakbhat01@yahoo.com (R. Bhatnagar).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.008

gastrointestinal depending upon the route of entry of spores [9]. Distinctive pathologic findings in patients suffering from anthrax point toward the incidence of hemorrhage [10–18]. Histopathological studies carried out on animal models have also indicated a systemic occurrence of hemorrhage [19–21]. The hemorrhagic condition during whooping cough has been linked to suppression of platelet aggregation by *B. pertussis* adenylate cyclase toxin [22]. A recent study has revealed that the antiplatelet activity of lethal toxin during infection can lead to higher mortality rates, thereby implicating platelets as one of the important cellular targets of anthrax toxin [23]. Thus far, there are no reports on effect of EF on platelet function. The best known negative effectors of platelet function are prostaglandins E1, D2, and I2 that activate the platelet adenylate cyclase system leading to increased intracellular cAMP [24]. Elevation of intracellular cAMP has been recognized as a potent endogenous inhibitory pathway that results in the global downregulation of signaling and biochemical events necessary for platelet activation [25]. Our studies show that edema toxin completely suppressed thrombin and ADP-induced platelet aggregation by causing an abrupt increase in intracellular cAMP levels. EF plays a role analogous to LF by causing inhibition of platelet function, albeit through a distinctive pathway and displaying more prominent intoxicating effects. Thus, it follows that anthrax toxin mediated dysfunction of platelets is a crucial phenomenon in manifestation of diseased state.

Experimental procedures

Materials. Thrombin, ADP, fibrinogen, dibutyryl-cAMP, calmidazolium chloride, calcium chloride, forskolin, DEAE Sepharose, SP Sepharose, and phenyl Sepharose were from Sigma (St Louis, MO, USA). Ni–NTA agarose was obtained from Qiagen (Hilden, Germany). cAMP Biotrak Enzymeimmunoassay kit, bovine serum albumin, ampicillin, kanamycin, β-ME, IPTG, lysozyme, EDTA, Luria–Bertani agar and broth, and PMSF were obtained from Amersham Biosciences (Uppsala, Sweden). The PepTag Non-radioactive cAMP dependent protein kinase assay kit was from Promega (Madison, USA). Bradford reagent used for protein estimation was obtained from Bio-Rad (CA, USA.) All other reagents used were of the highest purity commercially available.

Purification of toxins. Recombinant PA was prepared and purified as described previously [26]. Briefly, PA was expressed in *E. coli* BL21 (DE3) strain and purified from the periplasmic extract. Purification was carried out on anion exchanger, DEAE Sepharose, followed by hydrophobic interaction chromatography on phenyl Sepharose. Edema factor was expressed as a soluble protein in SG13009 (pREP4) cells. Purification was carried out with Ni–NTA agarose followed by ion exchange chromatography on SP Sepharose [27]. The dominant negative PA mutant, Phe427del [28], and non-toxic EF mutant, Y137A [27] were prepared similarly. Protein content was estimated with Bradford reagent using bovine serum albumin as standard [29]. The proteins were run on a 12% polyacrylamide gel and visualized by staining with Coomassie brilliant blue [30] as shown in Fig. 1. Purified protein fractions were stored in aliquots at −80 °C. Activity of purified proteins was checked by evaluating the elongation response of Chinese hamster ovary (CHO) cells followed by cAMP measurements.

cAMP measurements. Washed rabbit platelets were prepared by the method of Yuan et al. [31]. The final cell pellet was adjusted to a concentration of 1×10^8 cells/ml per reaction. Each assay reaction was incubated with increasing concentrations of EF ranging from 2.2 to 11 nM along with 11 nM PA. Additionally, for each concentration of EF, time dependent incubations were done for 0.5, 1, 1.5, 2, 2.5, and 3 h. The adenylyl cyclase activator, forskolin (12 μM), was incubated with platelets for 5 min. The calmodulin inhibitor, calmidazolium chloride (10 μM), was incubated for 20 min with platelets prior to incubation with edema toxin (11 nM PA and 11 nM EF) for 2 h. EF mutant was incubated at a concentration of 50 nM along with wt PA. All reactions were carried out at 37 °C. The assays were terminated by adding 5 mM EDTA followed by freeze thawing cells three times. The samples were then heated at 80 °C for 20 min and centrifuged at 14,000*g* for 10 min. The supernatant was removed and assayed for cAMP (picomoles of cAMP/10^8 platelets) using the cAMP assay kit, Amersham.

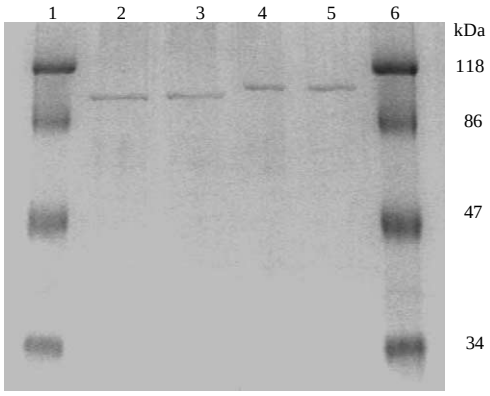

Fig. 1. Coomassie blue-stained SDS–polyacrylamide gel of purified recombinant proteins. Lanes 1 and 6, protein molecular weight marker; lane 2, wild type PA; lane 3, mutant PA; lane 4, wild type EF; lane 5, mutant EF.

Platelet aggregation assay. For the aggregation assay, washed rabbit platelets were prepared by the method of Kitamura et al. [32]. All experiments were carried out within 4 h of isolation of platelets. Platelet aggregation assay was carried out according to the method of Born [33] in a two-channel aggregometer (Chrono-Log). Aggregation was expressed as percent change in transmission of light, with the blank sample (buffer without platelets) defined as 100%. The final cell pellet was resuspended in 450 μl sodium potassium tris buffer (pH 7.4) and 50 μl of 112 mM citrate buffer (pH 6.0) and supplemented with 1 mM calcium chloride. Each aggregation assay reaction (400 μl) was adjusted to contain 2.5×10^8 cells/ml. The cells were preequilibrated to 37 °C and incubated with 11 nM PA and 7 nM EF. Aggregation scans were recorded, with constant stirring at 1000 rpm, after every 30 min. Aggregation was induced with thrombin at a concentration of 0.2 U/ml. For ADP-induced aggregation, platelets were stirred with 1 mg/ml fibrinogen at 37 °C for 5 min followed by addition of 10 μM ADP. The mutant proteins were added at ten times the wt concentration for 2 h before inducing aggregation. The cAMP analog, dibutyryl-cAMP (1 mM), and adenylyl cyclase activator, forskolin (12 μM), were incubated for 5 min each. Platelets were incubated with calmidazolium chloride (10 μM) for 20 min before incubation with edema toxin (11 nM PA and 11 nM EF) for 2 h. Wild type PA or EF only were also incubated for 2 h.

In vitro antibody neutralization assay. Polyclonal antiserum was raised in rabbits against native EF as described previously [34]. The serum titers were determined employing indirect ELISA. The serum was complement deactivated at 56 °C for 30 min followed by incubation with recombinant wt EF for 2 h at room temperature. This reaction mixture was incubated with freshly isolated platelets and supplemented with wt PA for 2 h at 37 °C followed by induction of aggregation with 0.2 U/ml thrombin or 10 μM ADP.

Protein kinase assay. Rabbit platelets were isolated as described for aggregation assay and suspended at a concentration of 1×10^8 cells/ml for each reaction. The experimental aliquots were incubated with 11 nM PA

and 11 nM EF at 37° in a shaker water bath. Appropriate controls were put containing PA or EF only. The cells were also incubated with the PKA inhibitor, H-89. After 2 h, the aliquots were sonicated and centrifuged at 14,000 rpm at 4 °C for 5 min. The supernatant was collected and PKA assay was done as described in the instruction manual (NonRadioactive PKA measurement kit, Promega).

Bleeding time assay. Bleeding time assay was performed on 7- to 8-week-old Swiss albino mice as described earlier [35]. 50 μg wt PA and 22 μg wt EF were injected intravenously at the base of tail [28]. Mutants of PA and EF were injected with the respective wt counterparts. Separate groups of mice were also injected with only wt PA or EF. The mice were anesthetized with intraperitoneal pentobarbital (75 mg/kg) after 2 h. The tail was cut 5–6 mm from the tip and immersed in phosphate-buffered saline (PBS) maintained at 37 °C. Bleeding times were measured from the time of incision of tail until complete cessation of bleeding. The assay was stopped after 20 min. Mice were checked next morning for signs of delayed bleeding.

Results and discussion

Edema toxin elevates cAMP inside platelets

cAMP is a principal secondary messenger whose imbalance may lead to altered cellular responses. The increase in cAMP levels in response to edema toxin has been found to vary in different cell types. Thus, to adjudge the potency of EF activity inside platelets, cAMP levels of edema toxin treated platelets were measured. Platelets were incubated with increasing concentration of EF (2.2–11 nM) along with PA (11 nM) and a time dependent quantification of cAMP was done at each EF concentration. It was observed that EF causes a dose dependent and time dependent

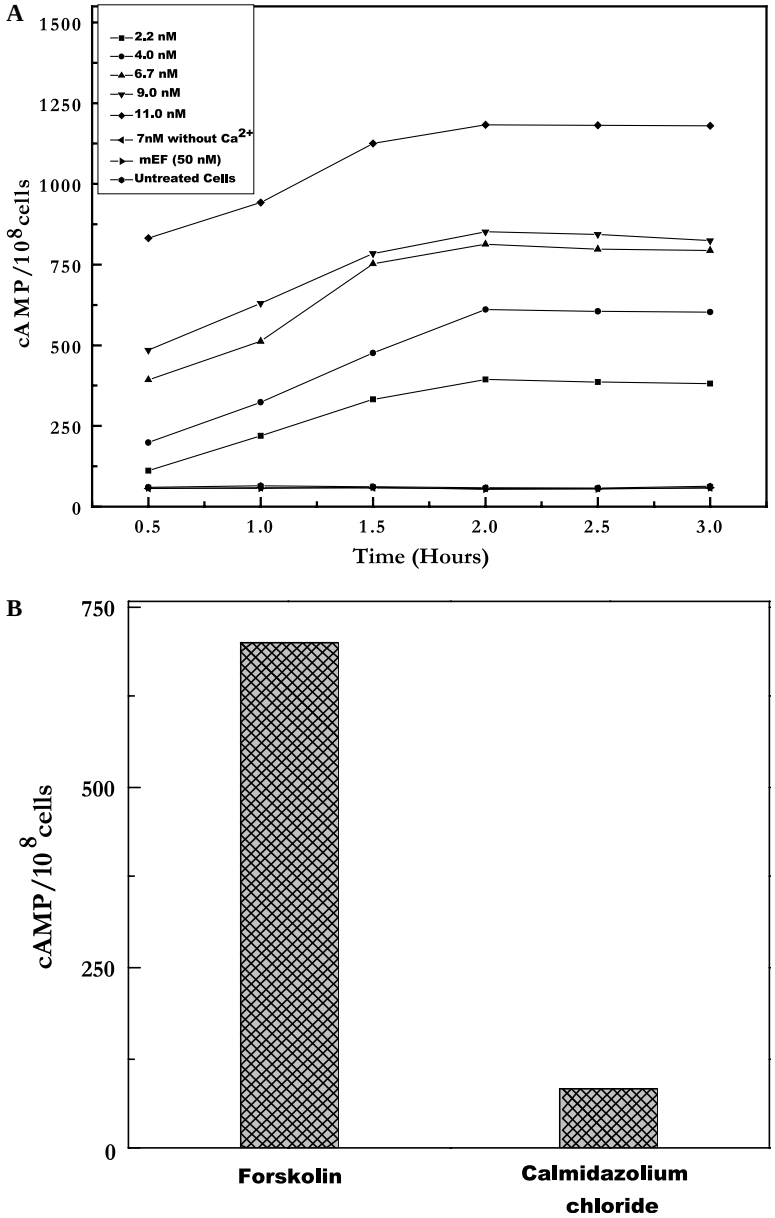

Fig. 2. Measurement of intracellular platelet cAMP. (A) Platelets were incubated with varying EF concentrations along with PA and cAMP levels were measured at different time points. Each value represents average of three experiments carried out in duplicate. The standard error was found to be within 5%. (B) cAMP levels in Forskolin and calmidazolium chloride treated platelets.

increase in cAMP levels inside platelets (Fig. 2A). At each concentration, cAMP gradually increased with time, reaching a peak at 2 h. Thereafter, cAMP levels remained more or less the same up to 3 h, registering only a slight decline. Much lower EF concentrations (6.7 nM) could efficiently raise cAMP levels equivalent to those induced by forskolin (Fig. 2B), a potent host adenylyl cyclase activator. A PA binding defective EF mutant was unable to generate cAMP response inside platelets even at a concentration as high as 50 nM. Furthermore, when platelets were incubated with

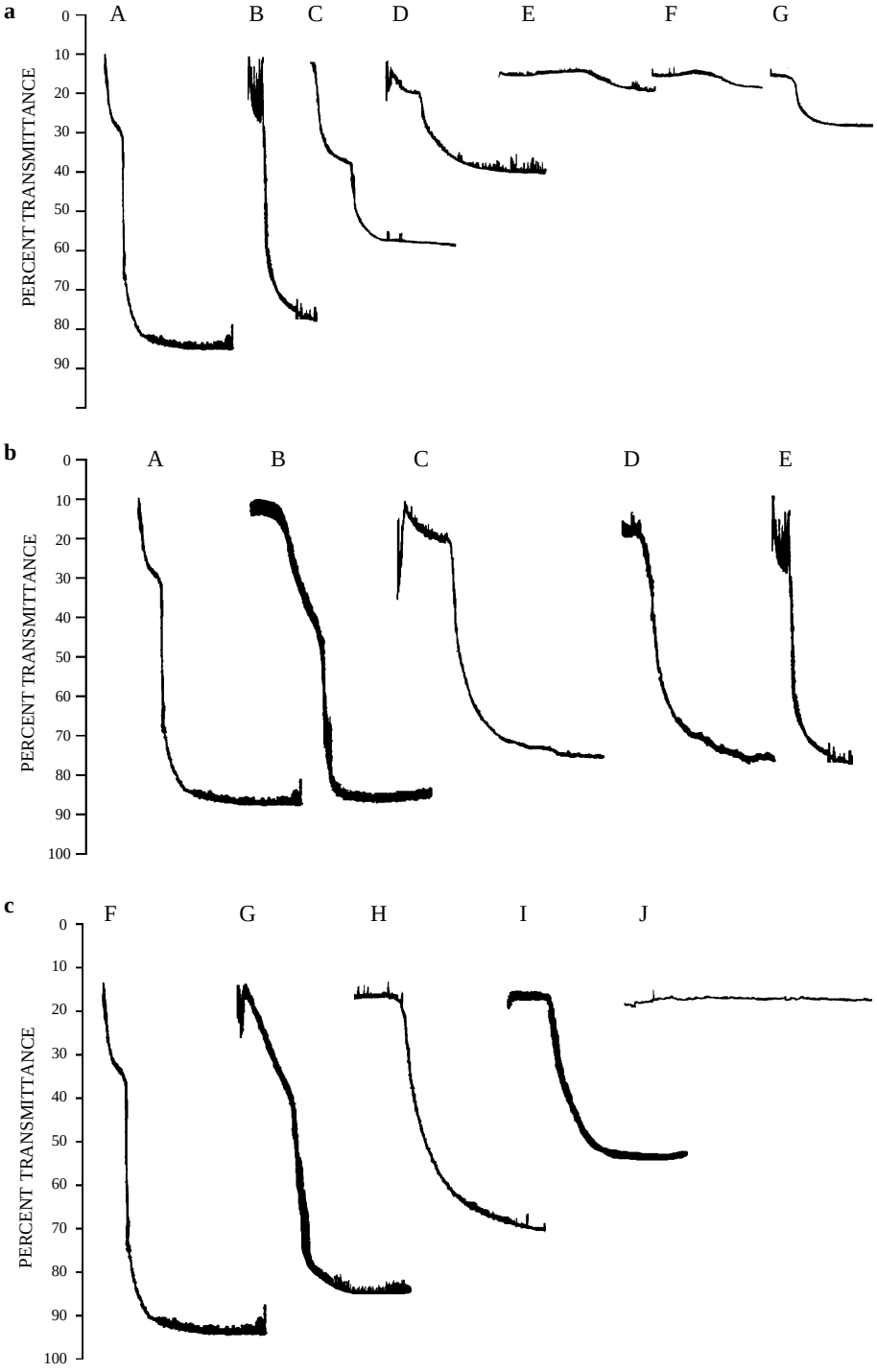

Fig. 3. Suppression of thrombin-induced platelet aggregation. Aggregation assays were carried out as described in Experimental procedures. Each scan was recorded for 10 min. Each graph is representative of three independent scans. (a) Time dependent measurements with 11 nM PA and 7 nM EF. Scan A, untreated cells; B–G are aggregation scans after 0.5, 1, 1.5, 2, 2.5, and 3 h of EdTx treatment, respectively. (b) Aggregation scans of untreated (A); wt PA (B); wt EF (C); mutant PA (D); mutant EF (E) treated platelets. (c) Aggregation curves of untreated cells (F); platelets incubated with EdTx without calcium (G); calmidazolium chloride treated platelets incubated with EdTx (H); dibutyryl-cAMP (I); and forskolin (J) treated platelets.

the translocation defective PA mutant and wt EF, no change in the basal cAMP was observed in view of the fact that the PA mutant would not be able to deliver EF in the cytosol of the cell. Platelets, when pretreated with calmidazolium chloride, a calmodulin inhibitor, did not respond to edema toxin. Additionally, platelets incubated with the toxin in calcium free medium failed to show any increase in cAMP, in agreement with the earlier report, which showed that edema toxin requires calcium for its activity [7]. Thus, we show that EF generates extremely high levels of cAMP inside platelets. The adenylate cyclase toxins represent an important adaptive strategy for pathogens for the reason that augmented cAMP has the capability to disrupt normal functioning of eukaryotic cells.

Edema toxin suppresses thrombin and ADP-induced platelet aggregation

We found that supraphysiological levels of cAMP produced by edema toxin inside platelets led to alteration in agonist-induced aggregation pattern. There was a dose dependent suppression of aggregation by EF in response to 0.2 U/ml thrombin. The minimum concentration of EF that completely suppressed aggregation was found to be 7 nM. Time dependent studies at this concentration exhibited maximum suppression after 2 h of toxin treatment correlating with the highest cAMP levels achieved at that time (Fig. 3a). The fact that EF could inhibit thrombin action, which is the most potent agonist of aggregation, confirms the pronounced effect of EF on platelets. Platelets incubated even with very high concentrations (50 nM) of mutants of PA or EF failed to elicit any suppression. Wild type PA or EF, separately were also not able to induce any suppression (Fig. 3b). Compounds such as forskolin and dibutyryl-cAMP closely paralleled the effects induced by wt EF (Fig. 3c). Aggregation proceeded uninhibited in the presence of calmidazolium chloride, which impresses upon the fact that the suppressed aggregation response is dependent on EF activity that requires calmodulin as a cofactor. In addition, when platelets were incubated with edema toxin sans calcium, a normal aggregation curve was obtained (Fig. 3C). ADP being a mild agonist required low concentrations of toxin (2.2 nM) for inhibition of its activity and complete suppression was achieved within 2 h (Fig. 4). To further appreciate that the effects being produced by edema toxin were indeed due to edema factor activity attained after internalization, in vitro antibody neutralized EF was incubated with platelets along with PA, which failed to induce any suppression (Fig. 5). Therefore, it follows that EF could mediate its effects on platelets strictly under conditions that are conducive for its entry into cells and favorable for attaining optimal activity within. In vitro aggregation assays clarified that cAMP is the effector molecule that is causing suppression of platelet aggregation as the level of suppression was directly linked to the amount of cAMP raised inside platelets. Since cAMP/PKA pathway has been implicated in several processes leading to attenuation of platelet function, we investigated the effect of edema toxin on PKA activity. Treatment with edema toxin led to PKA activation inside platelets whereas PA or EF alone did not have any effect on PKA activity. When cells were treated with the PKA inhibitor (H-89) followed by incubation with edema toxin, PKA activity was found to be inhibited (Fig. 6a). Significantly, normal aggregation curves were obtained when H-89 treated cells were induced with thrombin and ADP (Fig. 6b). These results indicate that edema toxin exerts its effects on platelets through cAMP dependent protein kinase pathway. Recently, Kau et al. discovered that the antiplatelet lethal toxin action is correlated with the downregulation of p42/44 or p38 MAPK activity. However, lethal toxin did not show any noteworthy effect on thrombin-induced platelet aggregation. Our study, on the other hand, demonstrates that one of the principal effects of edema toxin is the inhibition of thrombin and ADP-induced platelet aggregation and that the suppression of signaling apparatus, in all likelihood, is occurring through the cAMP/PKA pathway since a normal aggregation response was obtained when cells were

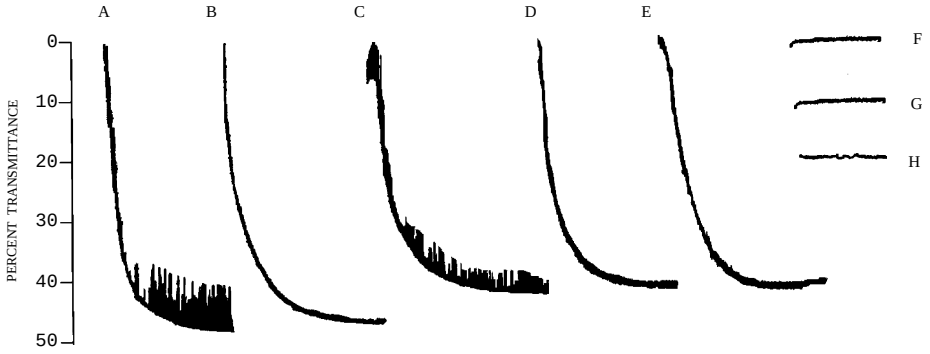

Fig. 4. Suppression of ADP-induced platelet aggregation. Aggregation assays were carried out as described in methodology. Each scan was recorded for 5 min and each curve is a representative of three independent scans. A, untreated cells; B, PA mutant; C, EF mutant; D, EdTx treatment without calcium; E, treatment with calmidazolium chloride; F, cells incubated with EdTx for 2 h; G, forskolin treated platelets; H, dibutyryl-cAMP treated platelets. Scans of wt PA or EF alone are not shown.

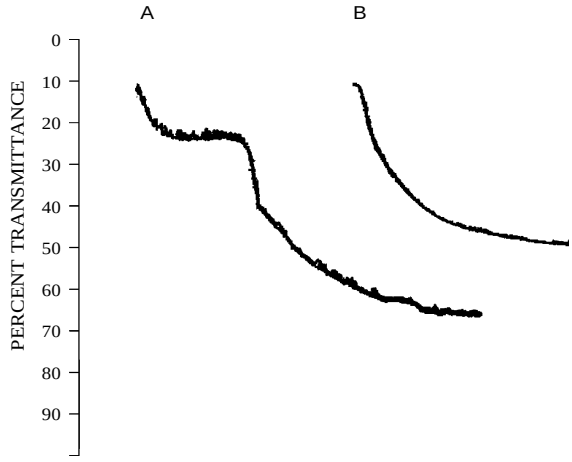

Fig. 5. Aggregation assays carried out after antibody neutralization. Thrombin-induced (A) and ADP-induced (B) scans obtained after incubating the antibody neutralized EF with platelets along with PA for 2 h.

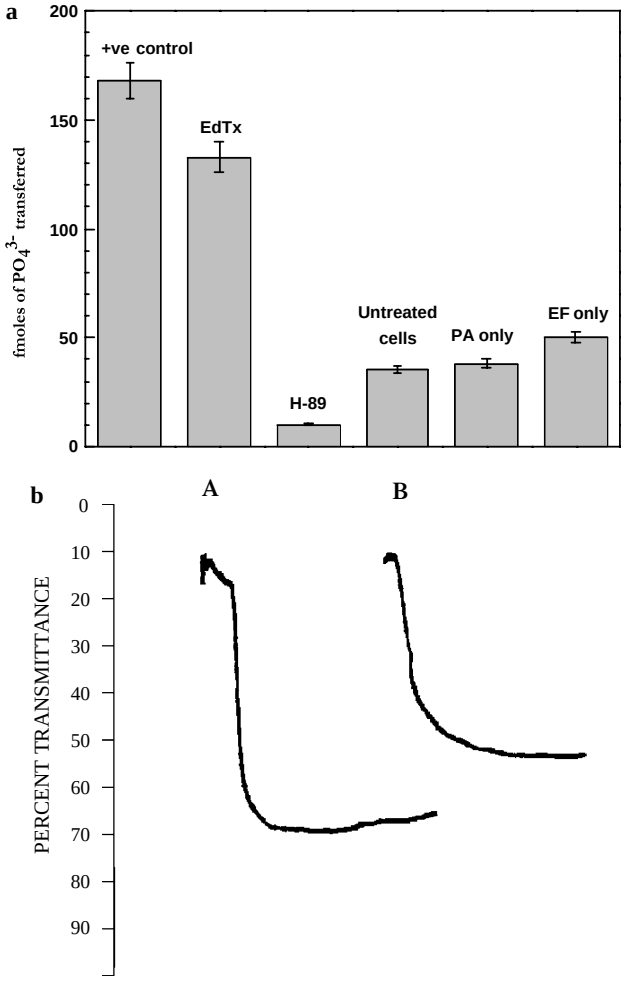

Fig. 6. (a) Protein kinase A activation by EdTx. Protein kinase assay was carried out as described in text. Histogram represents the fmoles of phosphate transferred by PKA to the peptide substrate. (b) Aggregation curves of H-89 treated platelets induced with thrombin (A) and ADP (B).

treated with PKA inhibitor. This finding is in support of the fact that cAMP/PKA pathway is one of the most important negative modulators of platelet function and it exerts its effects through multifarious substrates. Previously, it has also been shown that cAMP-induced activation of PKA causes inhibition of agonist-induced phosphorylation/activation of both p42 and p38 MAPKs in human platelets [36]. Also, inhibition of P-selectin expression by PKA is an important event that mediates the inhibitory effects on cohesive properties of platelets. Thus, it is quite possible that EF is targeting a plethora of substrates in platelets to entirely subdue the platelet system and LF might potentiate EF activity by exerting similar effects inside platelets.

Edema toxin prolongs bleeding time in vivo

To evaluate the effect of edema toxin on platelets in vivo, we injected Swiss albino mice with wt toxin intravenously and bleeding time measurements were done. Interaction of platelets with blood vessel walls to form blood clots is an important function to attain normal bleeding time. It was found that bleeding time in mice was heightened in response to wt EdTx and bleeding continued even after 20 min of observation time (Table 1). Administration of wt PA or EF alone did not have any effect on bleeding time. Also, EF and PA mutants did not alter the normal bleeding time. These observations corroborated the results obtained by in vitro aggregation assays of EdTx treated platelets. In fact, earlier studies have shown that, in vitro, cAMP exercises control over initial attachment of platelets to thrombi [37,38] and in vivo, initial platelet accrual at the site of vascular injury is inhibited by elevation of platelet cAMP [39]. Thus, suppression of platelet aggregation, in vitro and drastic increase in bleeding time, in vivo, point towards the disruption of customary functions of thrombosis and hemostasis of platelets by edema toxin.

Concluding remarks

Edema factor is an important pathogenic element of anthrax exotoxin complex. This is reiterated by 10-fold reduction in pathogenicity in *cya* gene deficient strain of

Table 1
Bleeding time in mice

Type	Mean bleeding time (s)
Non-injected	98
Edema toxin	>1200
wt PA	93
wt EF	115
Mutant EF (Y137A) + wt PA	135
Mutant PA (Phe427del) + wt EF	116
Buffer (5 mM Hepes)	103

Female Swiss albino mice were injected with 50 μg PA and 22 μg EF in a final dose of 100 μl. Values mentioned are the average of two experiments, each done with a batch of 6 mice. The error did not exceed 10%.

B. anthracis [40]. Our study illustrates the role played by edema toxin on modulation of platelet functions. The rise in cAMP brought about by edema toxin and the subsequent activation of cAMP dependent protein kinase A leading to suppression of platelet aggregation emphasizes that edema toxin is an invasive adenylyl cyclase toxin that imposes systemic infectivity on the host. Henceforth, our study identifies platelets as another vital target of edema toxin.

Acknowledgments

We thank Dr. Renu Saxena (All India Institute of Medical Sciences, New Delhi) for providing aggregometer facility and Arijit Biswas for helping with aggregometer experiments. Sheeba Alam and Megha Gupta are recipients of Senior Research Fellowship from CSIR, Government of India.

References

[1] R. Bhatnagar, S. Batra, Anthrax toxin, Crit. Rev. Microbiol. 27 (2001) 167–200.
[2] N.S. Duesbery, C.P. Webb, S.H. Leppla, V.M. Gordon, K.R. Klimpel, T.D. Copeland, N.G. Ahn, M.K. Oskarsson, K. Fukasawa, K.D. Paull, G.F. Vande Woude, Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor, Science 280 (1998) 734–737.
[3] S.H. Leppla, Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells, Proc. Natl. Acad. Sci. USA 79 (1982) 3162–3166.
[4] N. Ahuja, P. Kumar, R. Bhatnagar, The adenylate cyclase toxins, Crit. Rev. Microbiol. 30 (2004) 187–196.
[5] D.L. Hoover, A.M. Friedlander, L.C. Rogers, I.K. Yoon, R.L. Warren, A.S. Cross, Anthrax edema toxin differentially regulates lipopolysaccharide-induced monocyte production of tumor necrosis factor alpha and interleukin-6 by increasing intracellular cyclic AMP, Infect. Immun. 62 (1994) 4432–4439.
[6] J. O'Brien, A. Friedlander, T. Dreier, J. Ezzell, S. Leppla, Effects of anthrax toxin components on human neutrophils, Infect. Immun. 47 (1985) 306–310.
[7] P. Kumar, N. Ahuja, R. Bhatnagar, Anthrax edema toxin requires influx of calcium for inducing cyclic AMP toxicity in target cells, Infect. Immun. 70 (2002) 4997–5007.
[8] A.G. Wu, D. Alibek, Y.L. Li, C. Bradburne, C.L. Bailey, K. Alibek, Anthrax toxin induces hemolysis: an indirect effect through polymorphonuclear cells, J. Infect. Dis. 188 (2003) 1138–1141.
[9] T.C. Dixon, M. Meselson, J. Guillemin, P.C. Hanna, Anthrax, N. Engl. J. Med. 341 (1999) 815–826.
[10] J.A. Jernigan, D.S. Stephens, D.A. Ashford, C. Omenaca, M.S. Topiel, M. Galbraith, M. Tapper, T.L. Fisk, S. Zaki, T. Popovic, R.F. Meyer, C.P. Quinn, S.A. Harper, S.K. Fridkin, J.J. Sejvar, C.W. Shepard, M. McConnell, J. Guarner, W.J. Shieh, J.M. Malecki, J.L. Gerberding, J.M. Hughes, B.A. Perkins, Anthrax bioterrorism investigation team, bioterrorism-related inhalational anthrax: the first 10 cases reported in the United States, Emerg. Infect. Dis. 7 (2001) 933–944.
[11] D.B. Jernigan, P.L. Raghunathan, B.P. Bell, R. Brechner, E.A. Bresnitz, J.C. Butler, M. Cetron, M. Cohen, T. Doyle, M. Fischer, C. Greene, K.S. Griffith, J. Guarner, J.L. Hadler, J.A. Hayslett, R. Meyer, L.R. Petersen, M. Phillips, R. Pinner, T. Popovic, C.P. Quinn, J. Reefhuis, D. Reissman, N. Rosenstein, A. Schuchat, W.J. Shieh, L. Siegal, D.L. Swerdlow, F.C Tenover, M. Traeger, J.W. Ward, I. Weisfuse, et al., National anthrax epidemiologic investigation team, investigation of bioterrorism-related anthrax, United States, 2001: epidemiologic findings, Emerg. Infect. Dis. 8 (2002) 1019–1028.
[12] F.A. Abramova, L.M. Grinberg, O.V. Yampolskaya, D.H. Walker, Pathology of inhalational anthrax in 42 cases from the Sverdlovsk outbreak of 1979, Proc. Natl. Acad. Sci. USA 90 (1993) 2291–2294.
[13] L.M. Grinberg, F.A. Abramova, O.V. Yampolskaya, D.H. Walker, J.H. Smith, Quantitative pathology of inhalational anthrax I: quantitative microscopic findings, Mod. Pathol. 14 (2001) 482–495.
[14] L. Borio, D. Frank, V. Mani, C. Chiriboga, M. Pollanen, M. Ripple, S. Ali, C. DiAngelo, J. Lee, J. Arden, J. Titus, D. Fowler, T. O'Toole, H. Masur, J. Bartlett, T. Inglesby, Death due to bioterrorism-related inhalational anthrax: report of 2 patients, JAMA 286 (2001) 2554–2559.
[15] T.A. Mayer, S. Bersoff-Matcha, C. Murphy, J. Earls, S. Harper, D. Pauze, M. Nguyen, J. Rosenthal, D. Cerva Jr, G. Druckenbrod, D. Hanfling, N. Fatteh, A. Napoli, A. Nayyar, E.L. Berman, Clinical presentation of inhalational anthrax following bioterrorism exposure: report of 2 surviving patients, JAMA 286 (2001) 2549–2553.
[16] S.W. Chensue, Exposing a killer: pathologists angle for anthrax, Am. J. Pathol. 163 (2003) 1699–1702.
[17] W.J. Shieh, J. Guarner, C. Paddock, P. Greer, K. Tatti, M. Fischer, M. Layton, M. Philips, E. Bresnitz, C.P. Quinn, T. Popovic, B.A. Perkins, S.R. Zaki, Anthrax bioterrorism investigation team. The critical role of pathology in the investigation of bioterrorism-related cutaneous anthrax, Am. J. Pathol. 163 (2003) 1901–1910.
[18] J. Guarner, J.A. Jernigan, W.J. Shieh, K. Tatti, L.M. Flannagan, D.S. Stephens, T. Popovic, D.A. Ashford, B.A. Perkins, S.R. Zaki, Inhalational anthrax pathology working group. Pathology and pathogenesis of bioterrorism-related inhalational anthrax, Am. J. Pathol. 163 (2003) 701–709.
[19] N.C. Culley, D.M. Pinson, A. Chakrabarty, M.S. Mayo, S.M. Levine, Pathophysiological manifestations in mice exposed to anthrax lethal toxin, Infect. Immun. 73 (2005) 7006–7010.
[20] C.R. Lyons, J. Lovchik, J. Hutt, M.F. Lipscomb, E. Wang, S. Heninger, L. Berliba, K. Garrison, Murine model of pulmonary anthrax: kinetics of dissemination, histopathology, and mouse strain susceptibility, Infect. Immun. 72 (2004) 4801–4809.
[21] M. Moayeri, D. Haines, H.A. Young, S.H. Leppla, *Bacillus anthracis* lethal toxin induces TNF-alpha-independent hypoxia-mediated toxicity in mice, J. Clin. Invest. 112 (2003) 670–682.
[22] M. Iwaki, K. Kamachi, N. Heveker, T. Konda, Suppression of platelet aggregation by *Bordetella pertussis* adenylate cyclase toxin, Infect. Immun. 67 (1999) 2763–2768.
[23] J.H. Kau, D.S. Sun, W.J. Tsai, H.F. Shyu, H.H. Huang, H.C. Lin, H.H. Chang, Antiplatelet activities of anthrax lethal toxin are associated with suppressed p42/44 and p38 mitogen-activated protein kinase pathways in the platelets, J. Infect. Dis. 192 (2005) 1465–1474.
[24] R.H. Harris, P.W. Ramwell, P.J. Gilmer, Cellular mechanisms of prostaglandin action, Annu. Rev. Physiol. 41 (1979) 653–668.
[25] U.R. Schwarz, U. Walter, M. Eigenthaler, Taming platelets with cyclic nucleotides, Biochem. Pharmacol. 62 (2001) 1153–1161.
[26] N. Ahuja, P. Kumar, R. Bhatnagar, Rapid purification of recombinant anthrax protective antigen under nondenaturing conditions, Biochem. Biophys. Res. Commun. 286 (2001) 6–11.
[27] P. Kumar, N. Ahuja, R. Bhatnagar, Purification of anthrax edema factor from *Escherichia coli* and identification of residues required for binding to anthrax protective antigen, Infect. Immun. 69 (2001) 6532–6536.
[28] N. Ahuja, P. Kumar, S. Alam, M. Gupta, R. Bhatnagar, Deletion mutants of protective antigen that inhibit anthrax toxin both in vitro and in vivo, Biochem. Biophys. Res. Commun. 307 (2003) 446–450.
[29] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the Principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[30] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[31] Y. Yuan, S.M. Schoenwaelder, H.H. Salem, S.P. Jackson, The bioactive phospholipid, lysophosphatidylcholine, induces cellular

effects via G-protein-dependent activation of adenylyl cyclase, J. Biol. Chem. 271 (1996) 27090–27098.
[32] K. Kitamura, K. Kangawa, M. Kawamoto, Y. Ichiki, H. Matsuo, T. Eto, Isolation and characterization of peptides which act on rat platelets, from a pheochromocytoma, Biochem. Biophys. Res. Commun. 185 (1992) 134–141.
[33] G.V.R. Born, Aggregation of blood platelets by adenosine diphosphate and its reversal, Nature 194 (1962) 927–929.
[34] H.C. Flick-Smith, N.J. Walker, P. Gibson, H. Bullifent, S. Hayward, J. Miller, R.W. Titball, E.D. Williamson, A recombinant carboxy-terminal domain of the protective antigen of *Bacillus anthracis* protects mice against anthrax infection, Infect. Immun. 70 (2002) 1653–1656.
[35] A. Moers, B. Nieswandt, S. Massberg, N. Wettschureck, S. Gruner, I. Konrad, V. Schulte, B. Aktas, M.P. Gratacap, M.I. Simon, M. Gawaz, S. Offermanns, G13 is an essential mediator of platelet activation in hemostasis and thrombosis, Nat. Med. 9 (2003) 1418–1422.
[36] U.R. Schwarz, A.L. Kobsar, M. Koksch, U. Walter, M. Eigenthaler, Inhibition of agonist-induced p42 and p38 mitogen-activated protein kinase phosphorylation and CD40 ligand/P-selectin expression by cyclic nucleotide-regulated pathways in human platelets, Biochem. Pharmacol. 60 (2000) 1399–1407.
[37] R.J. Bodnar, X. Xi, Z. Li, M.C. Berndt, X. Du, Regulation of glycoprotein Ib-IX-von Willebrand factor interaction by cAMP-dependent protein kinase-mediated phosphorylation at Ser 166 of glycoprotein Ib(beta), J. Biol. Chem. 277 (2002) 47080–47087.
[38] M. Mazzucato, P. Pradella, M.R. Cozzi, L. De Marco, Z.M. Ruggeri, Sequential cytoplasmic calcium signals in a 2-stage platelet activation process induced by the glycoprotein Ibalpha mechanoreceptor, Blood 100 (2002) 2793–2800.
[39] D.S. Sim, G. Merrill-Skoloff, B.C. Furie, B. Furie, R. Flaumenhaft, Initial accumulation of platelets during arterial thrombus formation in vivo is inhibited by elevation of basal cAMP levels, Blood 103 (2004) 2127–2134.
[40] C. Pezard, P. Berche, M. Mock, Contribution of individual toxin components to virulence of *Bacillus anthracis*, Infect. Immun. 59 (1991) 3472–3477.