1   Parenteral Administration of Capsule Depolymerase EnvD Prevents Lethal Inhalation Anthrax

2   Infection

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22 **ABSTRACT** Left untreated, inhalation anthrax is usually fatal. Vegetative forms of *Bacillus anthracis*

23 survive in blood and tissues during infection due to elaboration of a protective poly-γ-D-glutamic

24 acid (PDGA) capsule that permits uncontrolled bacterial growth *in vivo*, eventually leading to

25 overwhelming bacillosis and death. As a measure to counter threats from multi-drug-resistant

26 strains, we are evaluating the prophylactic and therapeutic potential of the PDGA depolymerase

27 EnvD, a stable and potent enzyme which rapidly and selectively removes the capsule from the

28 surface of vegetative cells. Repeated intravenous administration of 10 mg/kg recombinant EnvD to

29 mice infected with lethal doses of *B. anthracis* Ames spores by inhalation prevented the emergence

30 of symptoms of anthrax and death; all animals survived the five day treatment period and 70%

31 survived to the end of the 14 day observation period. In contrast to sham-treated animals, the lungs

32 and spleen of rEnvD-dosed animals were free of gross pathological changes. We conclude that rEnvD

33 has potential as an agent to prevent the emergence of inhalation anthrax in infected animals and is

34 likely to be effective against drug resistant forms of the pathogen.

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INTRODUCTION

Bacillus anthracis featured in offensive weapons programs in the USA and former Soviet Union during the last century (1) and has been identified by the World Health Organization, the United Nations and the Working Group on Civilian Defense (WGCB) as a pathogen of great concern. The WGCB has highlighted a limited number of microorganisms that could cause infections in sufficient numbers to cripple a city or region and *B. anthracis* is one of the most serious of such threat agents (2). Their spores are able to survive in hostile environments for many decades and, in aerosolized form, can travel significant distances on prevailing winds, disseminating over a wide area. Accidental release of anthrax spores as an aerosol from a military facility in Sverdlovsk in 1979 resulted in at least 79 cases of anthrax and 68 deaths, demonstrating its lethal potential (3). These traits define *B. anthracis* as a potential threat agent, attractive to both rogue states and terrorist groups, and a cause of human and animal disease globally. The vegetative bacilli release toxin complexes that cause hemorrhage, edema and necrosis and are protected from host innate defenses by a capsule comprised of poly-γ-D-glutamic acid (PDGA) (4). In inhalation anthrax, endospores gain access to the alveolar spaces and are ingested by macrophages; they are then transported to regional lymph nodes where spore germination occurs after a variable period of dormancy (4, 5). Toxin-mediated clinical symptoms typically arise soon after the onset of rapid bacillary growth (2).

Effective treatment requires prompt and aggressive antibiotic therapy; a fluoroquinolone and an agent that inhibits protein synthesis such as linezolid are currently recommended by the Centers for Disease Control and Prevention (6). The consensus approach to prophylaxis and treatment of inhalation anthrax could be compromised by the release of *B. anthracis* carrying engineered antibiotic resistance genes and occasional reports have emerged of naturally occurring strains resistant to currently useful antibiotics (7, 8). Clearly, new agents or novel therapeutic and prophylactic modalities should be developed as a part of a comprehensive preparedness strategy. We previously demonstrated that parenteral administration of a capsule depolymerase with the capacity to rapidly and selectively remove the protective capsule from the bacterial surface can

74 resolve potentially lethal *Escherichia coli* infection in the neonatal rat (9, 10). Systemic anthrax is an

75 attractive candidate for this approach as infections are attributable to a single, phylogenetically

76 homogeneous bacterial species, all strains elaborate the unique PDGA capsule essential for

77 pathogenesis (11) and hydrolysis of the outermost layer of the bacilli would confound attempts to

78 circumvent antibiotic chemotherapy by the introduction of antibiotic resistance genes into *B.*

79 *anthracis*. Here we report that early intravenous administration of rEnvD, a recombinant PDGA

80 hydrolase elaborated by a consortium culture of soil bacteria, is able to prevent anthrax in mice

81 infected by the inhalation route.

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83 **MATERIALS AND METHODS**

84 **Bacteria.** *B. anthracis* Ames (NR-2324; pXO1+, pXO2+) was obtained from the Biodefense and

85 Emerging Infections Research Resources Repository (Manassas, VA). Spores were prepared by fed

86 batch culture in a 2 l bioreactor for 26 h at 37°C with stirring at 400 rpm, collected by centrifugation

87 and washed in sterile distilled water. For spore challenge tests, suspensions (8×10^9 CFU/ml) were

88 prepared in sterile water. *Bacillus licheniformis* ATCC 9945a was purchased from the American Type

89 Culture Collection and grown in Medium E containing 615 μM $MnSO_4$ in an orbital incubator (200

90 orbits/min) at 37°C (12).

91 **Recombinant EnvD.** The enzyme was expressed, refolded and purified as described previously (12).

92 Endotoxin was removed using Proteus Endotoxin Removal Columns (Abd Serotec, Oxford, UK) and

93 removal confirmed with the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher,

94 Rockford, USA). Purified rEnvD was stored in 20 mM Tris (pH 8.5) at -20°C until required.

95 **Impact of rEnvD on bacterial viability.** A culture (50 ml) from a single, heavily mucoid colony of *B.*

96 *licheniformis* 9945a was grown to OD_{600} 0.6 and examined by light microscopy to ensure only

97 vegetative bacilli were present. Two aliquots of 1 ml were removed and rEnvD added to one aliquot

98 to give a final protein concentration of 1μg/ml. An equal volume of phosphate buffered saline (PBS)

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99   was added to the second. Both samples were incubated at 37°C for 15 min, serially diluted in PBS

100   and plated onto Luria-Bertani agar. Plates were incubated at 37°C for 16 h and bacteria enumerated.

101   **Stability of rEnvD in serum.** Aliquots of rEnvD (final concentration 100 nM; in 1.6 ml Eppendorf

102   tubes) were incubated at 37°C in serum from BALB/c mice (Sigma; total volume 200 μl) for up to 24 h

103   and EnvD activity determined at regular intervals by Förster resonance energy transfer (FRET)

104   utilizing the fluorescently labelled synthetic peptide substrate 5-FAM-(D-Glu-γ-)$_5$-K(QXL^{TM}520)-NH$_2$ as

105   previously described (12). Two tubes were used for each time point, to provide duplicate readings. In

106   some experiments heat-inactivated (56°C, 30 min) serum was used and some assays were conducted

107   in the presence of Roche complete protease inhibitor cocktail (Roche, Basel, Switzerland) at

108   concentrations specified by manufacturer's guidelines.

109   **Serum half-life.** Pairs of female adult BALB/c mice were dosed with 10 mg/kg rEnvD in 180 μl 20 mM

110   Tris pH 8.5 by tail vein injection. Paired animals were sacrificed over a 24 h period, blood withdrawn

111   by cardiac puncture and serum obtained. Serum was diluted twofold with 0.1 M Tricine, 0.1%

112   CHAPS, pH 8.5 to a final volume of 200 μl and 100 μl transferred to each well of a black 96 well

113   microtiter plate. All assays were run in duplicate. rEnvD activity was measured using the FRET assay

114   described above with the exception that fluorescence was measured over a 4 min period. The

115   concentration of enzyme in each sample was determined using a standard curve prepared in mouse

116   serum. Area under the curve (AUC) was calculated by GraphPad Prism (GraphPad Software Inc., La

117   Jolla, CA) using the trapezoid rule.

118   **Infection of mice with *B. anthracis*.** All animal studies were carried out in accordance with the UK

119   Animals (Scientific Procedures) Act, 1986 and the Codes of Practice for the Housing and Care of

120   Animals used in Scientific Procedures, 1989 following approval by the local ethical committee and

121   the UK Home Office. Female BALB/c mice (minimum age 10 weeks; approximate body weight 20 g;

122   food and water available *ad libitum*) were obtained from Charles River (Canterbury, UK) and infected

123   by aerosol (13). Groups of ten mice were challenged with 10-50 minimum lethal doses (LD$_{50}$ 6 × 10^4

124   CFU; presented dose ~1.45×10^6 CFU) of *B. anthracis* spores with the AeroMP-Henderson apparatus.

125   The challenge aerosol was generated using a six-jet Collison nebuliser (BGI Inc., Waltham, MA), the

126   aerosol mixed with conditioned air in the spray tube and delivered to the nose of each animal

127   through an exposure tube in which non-anesthetized mice were held in restraint tubes. Samples of

128   the aerosol were obtained with an AGI30 glass impinger (Ace Glass Inc., Vineland, NJ) and the mean

129   particle size determined with an aerodynamic particle sizer (TSI Instruments Ltd., High Wycombe,

130   UK); these processes were controlled and monitored from an AeroMP management platform (Biaera

131   Technologies, Hagerstown, MD) . All-glass impinger samples were titrated by serial dilution and

132   plated on trypticase soy agar prior to incubation at 37°C for 16-24 h.

133   Intravenous (i.v.) administration of rEnvD was initiated 12 h after spore challenge. The

134   dosing regimen was guided by the stability of the enzyme in commercial mouse serum and by the

135   rate of clearance of rEnvD from the circulation of adult female BALB/c mice. Each mouse received

136   rEnvD (0.5-10 mg/kg) by injection at regular intervals up to 120 h after spore challenge; groups were

137   comprised of ten individual animals. Control mice received i.v. injections of PBS (180 µl) at these

138   time points. Additional groups of ten mice received oral doses of ciprofloxacin (118 mg/kg every 12 h

139   for 14 days). Animals were monitored and assigned a clinical score at least twice daily up to 14 days

140   after spore challenge and at least four times daily during critical periods (13). Clinical scores were

141   based on severity of symptoms (ruffled fur, closed eyes, arched back, immobility, weight loss).

142   Animals surpassing a threshold score were euthanized humanely by pentobarbital overdose.

143   Surviving mice from each group were euthanized at day 14 after challenge. *Post mortem*, blood, lung

144   and spleen samples were taken for enumeration of bacterial load: tissues were weighed and

145   homogenized in sterile water using a Precellys24 tissue homogenizer (Bertin Technologies,

146   Villeurbanne, France), the homogenates serially diluted in sterile water, plated onto trypticase soy

147   agar and the plates incubated at 37°C for 16-24 h before enumeration. Further lung and spleen

148   samples were placed in 10% neutral-buffered formalin for pathological evaluation. An additional

149   group of ten mice was employed to evaluate pathological changes six days after spore challenge;

150 animals were culled 24 h after receiving their final dose of rEnvD on day five and blood and tissue

151 samples removed. Kaplan-Meier log rank test was used to determine the significance of differences

152 in survival between groups of animals and GraphPad Prism software (GraphPad, La Jolla, USA) was

153 employed. For histological evaluation, formalin-fixed tissue samples were processed to paraffin wax

154 and 3-5 μm sections cut and stained with hematoxylin and eosin. Sections were examined by light

155 microscopy and evaluated subjectively. Slides were randomized by a third party before microscopic

156 examination to avoid prior knowledge of group or treatment.

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158 **RESULTS**

159 **rEnvD is a promising candidate for *in vivo* attenuation of *B. anthracis* capsule expression.**

160 Unusually, *envD* resides on the genome of a strain of *Pusillimonas nortemannii* but the enzyme is

161 only produced when the bacteria are co-cultured with a strain of *Pseudomonas fluorescens* (12, 14).

162 rEnvD showed strong sequence homology to bacterial dienelactone hydrolases and its enzymatic

163 activity is restricted to the hydrolysis of γ-linkages in D- and L-glutamic acid-containing polymers (k_{cat}

164 [h^{-1}] 72.6; k_m [μM] 0.65; k_{cat}/k_m [$M^{-1}s^{-1}$ x 10^4] 3.08 at 37°C). The enzyme retained enzymatic activity

165 after accelerated storage at 37°C for 30 days and completely removed the capsule from *B. anthracis*

166 Pasteur strain within 5 min at 37°C (12). Exposure of *Bacillus licheniformis* ATCC 9945a (induced to

167 elaborate a PDGA polymer) to rEnvD resulted in rapid stripping of the capsule (12) but the viability of

168 this surrogate strain was not significantly altered (2.5×10^8 to 2.9×10^8 CFU/ml over 15 min; *n*=6,

169 Student's *t* >0.05).

170 There is limited capacity for repeated parenteral injections in small animals. To guide the

171 design of dosing regimens for the administration of rEnvD to infected BALB/c mice, we determined

172 the retention of depolymerase activity in mouse serum and the serum half-life ($t_{1/2}$) following

173 intravenous (i.v.) administration. The reduction in rEnvD activity following incubation at 37°C in

174 murine serum as determined by FRET assay (12) followed first-order kinetics with $t_{1/2}$ of 177 min (Fig.

175 1A); approximately 20% of activity remained after 8 h incubation. Neither heat inactivation of serum

176   nor the presence of protease inhibitors had any impact on the rate of reduction of activity.

177   Elimination of rEnvD from the blood circulation of BALB/c mice was biphasic, with an initial rapid

178   decrease in serum concentration (0.5 h to 1 h) followed by a slower elimination phase (2 h to 24 h)

179   characteristic of first order kinetics (Fig. 1B). This elimination profile is typical of agents administered

180   by the intravenous route (15); a rapid decrease in serum concentration due to distribution from the

181   central circulation into the peripheral body tissues (alpha phase) followed by a gradual decrease in

182   plasma concentration attributable to metabolism and excretion of the drug (beta phase). AUC was

183   determined as 118 nM.h/l. Based on this data, we established a dosing regimen in which mice

184   received rEnvD (0.5-10 mg/kg body weight) by i.v. injection 12 h, 24 h, 48 h, 72 h, 96 h and 120 h

185   after spore challenge.

186   **rEnvD administration prevents inhalation anthrax in aerosol-challenged mice.** Typically, all animals

187   in sham-treated (PBS) control groups met humane endpoints within 72 h (median time to death 48

188   h) whereas all mice treated with 10 mg/kg of rEnvD survived the treatment period ($P<0.0001$; log

189   rank) (Fig. 2A). Nine days after termination of treatment, 70% of rEnvD-treated mice had survived

190   ($P<0.0001$, compared to control animals). The protective effect of rEnvD was reflected in the

191   comparative health status of the mice: abnormal clinical signs were absent from rEnvD-treated

192   animals during the five-day period of enzyme administration (Fig. 2B). At six and fourteen days post-

193   challenge and in contrast to controls, bacteria were not cultured from the blood or spleen of

194   surviving rEnvD-treated mice from the tissue group; sham-treated mice were found to carry a high *B.*

195   *anthracis* bioburden in the blood (mean 2.4×10^4 CFU/ml) and spleen (mean 5.84×10^4 CFU/mg) at

196   time of *post mortem* examination (based on severity threshold score). High numbers of viable

197   bacteria were also present in the lung of sham-treated animals at the same time point (mean 1.37 ×

198   10^5 CFU/mg). In comparison to controls, a significant ($P=0.006311$) reduction in the lung bioburden

199   was noted in EnvD-treated animals six days after spore challenge (mean 1.55×10^2 CFU/mg)

200   compared to controls and a lower number (mean 2.07×10^1 CFU/mg) were present in the lung of

201   surviving animals 14 days after challenge (Fig. 3).

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202   Microscopic changes referable to infection with *B.anthracis* were observed in the lung and

203   spleen of all control animals. In the lung, there was prominent pulmonary congestion and patchy

204   haemorrhage, expanding septal cavities and numerous bacilli located in alveolar spaces, walls and

205   within vessel lumena (bacteremia) (Fig. 4A). In the spleen, numerous bacilli within the red pulp

206   sinusoids and vascular lumena were present in these control animals (data not shown). Further,

207   splenic white pulp contained prominent degeneration and loss of lymphocytes, characterized by

208   nuclear fragmentation and cellular paucity. In contrast, animals receiving rEnvD and surviving until

209   study endpoints were found to be clear of gross pathological changes and bacilli were not visible

210   within lung (Fig. 4B) or spleen tissue.

211   Experiments with 5 mg/kg rEnvD dosed over three days also demonstrated a high degree of

212   protection from anthrax infection (100% survival at 3 days; 60% survival at 14 days) but 0.5 mg/kg

213   rEnvD did not prevent the emergence of clinical symptoms and death; rEnvD administered for five

214   days provided better protection than orally dosed ciprofloxacin administered by the oral route

215   throughout the fourteen day period (Fig. 2C & D).

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217   **DISCUSSION**

218   This study provides clear evidence that prompt serial i.v. administration of small quantities

219   of EnvD prevents the onset and progression of inhalation anthrax in a robust murine model of

220   invasive disease. Even though the strain employed in this study is highly toxigenic, removal of the

221   capsule during the early stages of infection appears sufficient to confound the pathogenic potential

222   of the invading bacteria and further supports the key role of the protective PDGA capsule in anthrax

223   pathogenesis (4, 11), highlighting the requisite nature of the capsule for *in vivo* dissemination of

224   vegetative bacilli. The study also adds to growing evidence that prophylaxis and treatment of severe

225   systemic infections can be realised by agents that do not kill the target bacterial population *per se*

226   but modify the phenotype of the pathogen in a way that is beneficial to the host (16). Further, this

227 approach has the potential to deliver exquisitely selective therapeutics that are unaffected by the

228 presence of antibiotic resistance mechanisms.

229   Treatment of bacterial infections with capsule depolymerases was first explored over 80

230 years ago by Dubos, Avery and colleagues at the Rockefeller Institute for Medical Research. They

231 used an enzyme preparation from cultures of a peat soil bacterium to selectively remove the

232 polysaccharide capsule from the surface of type III pneumococci (17), the pathogen's principle

233 means of defense against immune attack. Intraperitoneal administration of enzyme extracts to mice

234 prior to challenge with type III pneumococci gave rise to type III-specific protection (18), i.v.

235 administration to rabbits with type III dermal infections resulted in early termination of the normally

236 fatal infection (19) and the enzyme prevented dissemination, sterilized the blood and promoted

237 early recovery in non-human primates infected by the intratracheal and intrabronchial routes (20).

238 In addition to our previous work on systemic neonatal *E. coli* infections (9, 10), capsule

239 depolymerases have been shown to resolve potentially lethal experimental *Klebsiella pneumoniae*

240 K1 infections in mice (21).

241   Recently, other attempts have been made to exploit PDGA depolymerases as anti-anthrax

242 therapeutics. CapD is a γ-glutamyltranspeptidase elaborated by *B. anthracis* and catalyzes the

243 attachment of PDGA to peptidoglycan, but also functions as a depolymerase, effecting the release of

244 diffusible PDGA fragments from the surface of producer strains (22). CapD mediates removal of the

245 capsule and induces macrophage uptake and neutrophil killing *in vitro* (23). Intraperitoneal co-

246 injection of CapD and vegetative *B. anthracis* Ames bacteria afforded some protection against

247 infection in mice but no significant protection could be demonstrated when the enzyme was

248 administered after challenge with Ames spores (24), almost certainly due to the labile nature of

249 CapD (12, 20). rEnvD is a far more robust enzyme (12) and a better candidate for therapeutic

250 development.

251   Current evidence suggests that although the toxin complex undoubtedly plays a vital role in

252 anthrax pathogenesis, probably by suppression of the immune response in early stages of the

253 disease, death occurs from overwhelming bacteremia and sepsis due to uncontrolled bacterial

254 proliferation and release of pro-inflammatory mediators (25). Thus, a therapeutic window may be

255 available if treatment is initiated before extensive bacterial division occurs in the blood. Our results

256 support this hypothesis: depolymerase administration initiated 12 h after aerosol challenge provided

257 significant protection against systemic anthrax and prevented bacteremia and dissemination of

258 bacilli to the spleen. This concurs with a previous report that the capsule is essential for

259 hematogenous bacillary spread, as capsule negative mutants did not migrate to the spleen in

260 experimental infections (11). In the current study, deaths generally occurred following cessation of

261 treatment. Viable bacteria were present in the lung of rEnvD-treated mice after the treatment

262 period and animals that subsequently succumbed to infection almost certainly died due to delayed

263 germination of latent spores and after enzyme had been cleared from the blood circulation. *B.*

264 *anthracis* spores are known to persist in the lung for extended periods: for example, latent spores

265 have been isolated from the lung tissue of non-human primates months after initial exposure (26).

266 The size of the mouse restricts the number of i.v. injections that can be given over a relatively short

267 period of time and this issue will be addressed using larger species such as the rabbit. In addition,

268 the mouse is particularly susceptible to death from systemic anthrax due to uncontrolled *in vivo*

269 bacterial growth and a high quantitative level of bacteremia (27), factors which do not favour an

270 anti-capsule therapeutic strategy. The relative susceptibility of humans to toxemia and infection in

271 anthrax is poorly documented but the rabbit is used as an equivalent to human infection (27) and

272 examination of rEnvD in this species will be an important next step.

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FIG. 1 *In vitro* stability in serum and elimination of rEnvD from the circulation of BALB/c mice. A: Stability of rEnvD in BALB/c mouse serum; 100 nM of enzyme was incubated at 37°C in serum and activity determined by FRET assay (12). Enzyme activity in relative fluorescent units was converted to concentration of active enzyme by comparison to a standard curve. Error bars represent the range of three separate determinations; $t_{1/2}$ in serum of rEnvD was 2.95 h (177 min). B: rEnvD in serum obtained from mice intravenously dosed with 10 mg/kg rEnvD. Serum was obtained by terminal bleed and enzyme activity determined by FRET assay; Serum concentration (Cp) of rEnvD was obtained by comparison to a standard curve. Error bars represent the range of three separate determinations performed in duplicate; $t_{1/2}$ was calculated by determination of the elimination rate constant (K_e) and transformation of data to the natural log (ln) to produce a line of best fit for each phase with the slope equal to K_e: $t_{1/2} = \ln(2)/K_e$. The $t_{1/2}$ for the initial alpha phase between 0.5 h and 1 h was 0.22 h (13 min) and for the beta phase between 2 h and 24h was 2.71 h (163 min).

FIG. 2 Impact of intravenous administration of rEnvD on inhalation anthrax in mice. Combined Kaplan-Meier survival plots (A, C) and cumulative mean clinical observation scores (B, D) for rEnvD-dosed, infected BALB/c mice. Mice were infected with *B. anthracis* Ames on day 0 by aerosol followed by tail vein administration of either 10 mg/kg rEnvD or PBS vehicle (A, B) or 0.5 mg/kg rEnvD (C, D) at the times indicated by arrows. Ciprofloxacin (118 mg/kg) was also administered orally for 14 days (C, D). Clinical observations were scored as described (13) and were based on severity of symptoms (ruffled fur, closed eyes, arched back, immobility, weight loss).

FIG. 3 *B. anthracis* (CFU/mg tissue) in the spleen and lung of mice following rEnvD or PBS administration by the intravenous route; *n*=7-10, mean ±1 SD. PBS controls were culled when the clinical score reached threshold levels, as the animals were then close to death (13). Tissues were weighed and homogenized in sterile water, the homogenates serially diluted in sterile water, plated onto trypticase soy agar and the plates incubated at 37°C for 16-24 h.

404   **FIG. 4** Pathology of lung tissue six days after inhalation of spores. Samples were fixed in 10% neutral-

405   buffered formalin, processed to paraffin wax, sectioned to 3-5 µm, stained with HE and examined by

406   light microscopy. Slides were randomized by a third party before microscopic examination to avoid

407   prior knowledge of group or treatment. A: From animals receiving only PBS vehicle; region shows

408   iatrogenic thickening of the alveolar walls due to the collapsed nature of the tissue. Arrows indicate

409   bacilli located in alveolar spaces; Ve, vessel lumena. B: From animals receiving 10 mg/kg rEnvD over

410   5 days; image from region of inflated lung.