A Soluble Receptor Decoy Protects Rats against Anthrax Lethal Toxin Challenge

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Successful postexposure treatment for inhalation anthrax is thought to include neutralization of anthrax toxin. The soluble anthrax toxin receptor/tumor endothelial marker 8 and capillary morphogenesis protein 2 (sATR/TEM8 and sCMG2, respectively) receptor decoys bind to anthrax toxin protective antigen (PA) and compete with cellular receptors for binding. Here, we show that, in a tissue-culture model of intoxication, sCMG2 is a 11.4-fold more potent antitoxin than sATR/TEM8 and that this increased activity corresponds to an ~1000-fold higher PA-binding affinity. Stoichiometric concentrations of sCMG2 protect rats against lethal toxin challenge, making sCMG2 one of the most effective anthrax antitoxins described to date.

Anthrax toxin is secreted by *Bacillus anthracis* during infection and is thought to cause many of the disease symptoms. Once symptoms develop, anthrax can progress to death even when antibiotics have been administered, presumably because of the buildup of fatal levels of toxin in the body [1]. Thus, there is an urgent need to develop effective antitoxins that can be used to block the action of toxin in vivo.

Anthrax toxin is composed of 3 components: protective antigen (PA), the receptor-binding moiety; lethal factor (LF), a zinc metalloproteinase; and edema factor (EF), a Ca^{2+}- and calmodulin-dependent adenylate cyclase (reviewed in [2]). PA binds to the integrin-like I domains of either the anthrax toxin receptor/tumor endothelial marker 8 (ATR/TEM8) or the capillary morphogenesis protein 2 (CMG2) receptor [3, 4], undergoes cleavage by furin, and then multimerizes into a heptamer on the cell surface. The PA heptamer binds to EF and LF and delivers them into the cytoplasm after uptake and trafficking of toxin-receptor complexes into low-pH endosomal compartments [5] (figure 1*A*).

Several antitoxins have been developed that interfere with the action of toxin (reviewed in [6]). These include small molecules, peptides, and compounds that neutralize the catalytic activities of LF, EF, or furin. Antibodies that bind to specific toxin components, soluble receptor decoys, and a polyvalent peptide inhibitor block the assembly of functional toxin-receptor complexes. Moreover, dominant negative inhibitory (DNI) forms of PA assemble into heptamers containing wild-type PA subunits and prevent the delivery of EF and LF.

To date, DNI forms of PA and anti-PA antibodies have shown the most promising antitoxin activities in vivo [7–9]. However, anti-PA antibodies may not protect against infection by *B. anthracis* engineered to express functional but antigenically altered forms of PA. Therefore, soluble receptor decoys consisting of either the ATR/TEM8 or the CMG2 I domain (sATR/TEM8 and sCMG2, respectively) may be an important adjunct to antibody-based therapies, because it would be difficult to engineer decoy-resistant forms of the toxin while preserving cellular-receptor use.

It has been shown that mammalian cell–produced sCMG2 protein is more effective than bacteria-produced sATR/TEM8 in protecting cultured CHO-K1 cells against intoxication [3, 4]. Specifically, sCMG2 reduced intoxication by 50% when it was added to PA at a 3:1 ratio [4], whereas, when sATR/TEM8 was added to PA, an 800:1 ratio was required to reduce intoxication to the same extent [3]. Here, we compared the activities of sATR/TEM8 and sCMG2 produced from mammalian cells by use of in vitro intoxication and binding assays as well as in vivo lethal toxin (LeTx) challenge experiments.

Materials and methods. The sCMG2 protein consists of aa residues 1–232 of $CMG2^{489}$ fused to a mycHis tag [4]. The sATR/TEM8 protein consists of aa residues 1–234 of ATR/

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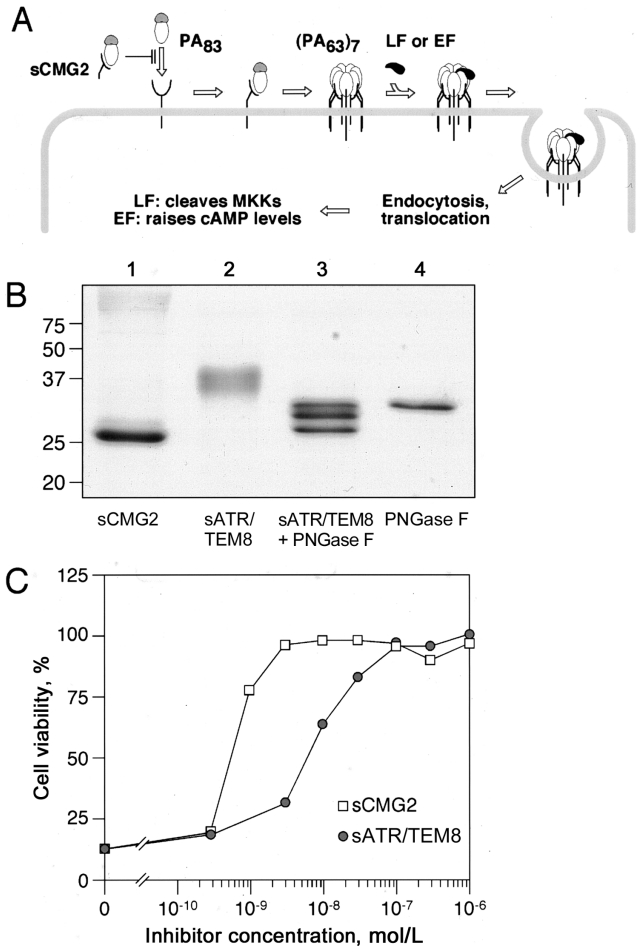

Figure 1. Higher in vitro antitoxin activity of soluble capillary morphogenesis protein 2 (sCMG2) than of soluble anthrax toxin receptor/tumor endothelial marker 8 (sATR/TEM8). *A,* Schematic of anthrax intoxication and inhibition with sCMG2. Full-length protective antigen, PA_{83}, binds to cell surface receptors, a step that is thought to be competitively inhibited by sCMG2. PA_{83} is proteolytically cleaved into a 63-kDa form, PA_{63}, that multimerizes into a heptameric ring structure, $(PA_{63})_7$, that is competent for binding lethal factor (LF) or edema factor (EF). The receptor-toxin complex is endocytosed, and the catalytic subunits are translocated into the cytoplasm, where they exert their toxic effects. *B,* Analysis of sCMG2 and sATR proteins by SDS-PAGE. The proteins (2 μg/lane) were subjected to electrophoresis on a 20% polyacrylamide gel containing SDS and then were stained with Coomassie blue. sATR/TEM8 after deglycosylation with PNGase F (New England Biolabs) is shown in lane 3, and PNGase F alone (500 U) is shown in lane 4. The presence of glycans on sATR/TEM8 is indicated by a change in mobility shift of the sATR/TEM8 species after PNGase F treatment. *C,* Intoxication inhibition. The receptor decoys were tested for their ability to inhibit intoxication in vitro by incubating CHO-K1 cells with 10^{-9} mol/L PA and 10^{-10} mol/L LF_N-DTA (a recombinant toxin composed of the N-terminal region of LF fused to the catalytic portion of diphtheria toxin A chain) and varying amounts of sCMG2 or sATR/TEM8. Cell viability was then assessed 45 h later by use of a commercially available assay (Celltiter-glo; Promega). Cell viability is expressed as a percentage of that seen with control cell populations that were incubated with LF_N-DTA alone (100%). Data points represent the mean $\pm$ SD values for triplicate samples. MKKs, mitogen-activated protein kinase kinases.

TEM8 sv2 [3] fused to a mycHis tag. Extracellular supernatants were obtained from human FreeStyle 293 cell populations (Invitrogen) that were stably transfected with plasmid DNA constructs encoding either sCMG2 or sATR/TEM8. Supernatants were harvested every 24 h for 4 days, beginning when a concentration of 2×10^6 cells/mL was reached. Collected media were pooled, concentrated 30-fold with Amicon concentrators and YM10 membranes (Millipore), and then diluted 5-fold in PBS and reconcentrated. A 40-mL aliquot of the concentrated protein sample was then passed over a column consisting of 1 mL of nickel–nitrilotriacetic acid beads (Qiagen). The soluble receptor proteins were eluted in a range of 50–400 mmol/L imidazole. These fractions were then dialyzed against TBS (20 mmol/L Tris and 150 mmol/L NaCl) and were concentrated in Vivaspin 15R (10-kDa molecular weight cutoff) concentrators (Vivascience). The sATR/TEM8 protein was further purified by fast-protein liquid chromatography (FPLC) with Superose 12 (Amersham). For protein deglycosylation experiments, a 10-μg aliquot of sATR/TEM8 protein was incubated with 1750 U of PNGase F (New England Biolabs), in accordance with the manufacturer's instructions.

For in vitro intoxication experiments, 5000 CHO-K1 cells were plated in individual wells of a 96-well tissue culture plate (Costar). Aliquots of 100 μL of medium containing 10^{-9} mol/L PA, 10^{-10} mol/L LF_N-DTA (a recombinant toxin composed of the N-terminal region of LF fused to the catalytic portion of diphtheria toxin A chain [DTA]), and varying amounts of either sATR/TEM8 or sCMG2 were added to the cells. Approximately 45 h later, the medium was removed, 50 μL of Celltiter-glo reagent (Promega) diluted 1:1 with PBS was added for 10 min, and the samples were analyzed for luciferase activity by use of a luminometer (Topcount NXT; Packard Instruments). Calculation of IC_{50}s was done by nonlinear regression analysis with a 1-site binding model (Prism, version 4.0a; GraphPad Software).

All binding-kinetics experiments were performed in accordance with the method of Wigelsworth et al. [10], using the Biacore 2000 system and CM5 sensor chips. Protein concentrations of sATR/TEM8 ranged from 24 nmol/L to 4.8 μmol/L in HBS buffer (10 mmol/L HEPES [pH 7.6] and 150 mmol/L NaCl) with either 1 mmol/L $CaCl_2$ or 1 mmol/L $MgCl_2$. Serial injections were made at 10 μL/min, followed by a 40-μL buffer injection to allow for off-rate measurements. All kinetic data were analyzed by use of Origin software (OriginLab). Equilibrium dissociation constants were calculated on the basis of the kinetic measurements of the association and dissociation rate constants according to the formula $K_D = k_d/k_a$, and errors were propagated. Results are the average of 2 independent trials.

Rat LeTx challenge experiments were performed in accordance with protocols approved by the Scripps Institutional Animal Care and Use Committee. Male Fisher 344 rats (180–200

Table 1. Higher binding affinity for anthrax toxin protective antigen (PA) of soluble capillary morphogenesis protein 2 (sCMG2) than of soluble anthrax toxin receptor/tumor endothelial marker 8 (sATR/TEM8).

I domain, metal	k_a	k_d	K_D, nmol/L
ATR			
Ca^{2+}	1.3 ± 0.046	140 ± 0.56	1100 ± 41
Mg^{2+}	0.22 ± 0.018	2.9 ± 1	130 ± 46
CMG2			
Ca^{2+}	11 ± 0.5	0.84 ± 0.005	0.78 ± 0.03[a]
Mg^{2+}/EGTA	5.3 ± 0.09	0.092 ± 0.001	0.17 ± 0.0009[a]

NOTE. Data are mean ± SE. The binding affinity of the PA–sATR/TEM8 interaction was determined by surface plasmon resonance (SPR) analysis. k_d ($[mol/L]^{-1}s^{-1} \times 10^4$) and k_a ($s^{-1} \times 10^4$), where *s* is seconds, are dissociation and association rate constants, respectively, and K_D is the equilibrium dissociation constant. Results from control SPR experiments of PA binding with sCMG2 I domains were similar to those published by Wigelsworth et al. [10], but the off-rate was too slow to calculate binding affinity without high error.

[a] Data are from Wigelsworth et al. [10] and are provided for comparison purposes. These results were obtained in kinetic fluorescence energy resonance transfer experiments with AlexaFluor 488–labeled PA-K563C proteins and AlexaFluor 546–labeled $CMG2^{40-217}$-R40C/C175A proteins in the presence of 1 mmol/L Ca^{2+} or of 1 mmol/L Mg^{2+} and 2 mmol/L EGTA.

g; Harlan) were anesthetized with isofluoranes and were inoculated with LeTx mixture through a jugular-vein cannula. LeTx was prepared with 40 μg of PA and 8 μg of LF per rat (List Biological Laboratories). For rats that received sCMG2 and LeTx, sCMG2 was added to the LeTx mixture and was coinjected in a 500-μL volume. Rats recovered from anesthesia within 5 min and were monitored for symptoms of intoxication and death. Statistical analysis was conducted on the basis of Student's unpaired *t* test (Prism, version 4; GraphPad). For rats that died overnight, statistical analysis was based on the last observed postinoculation time point.

Results. To determine whether the different activities of the sATR/TEM8 and sCMG2 decoys were due to their inherent PA-binding properties or, instead, to their method of production, both sCMG2 and sATR/TEM8 proteins were produced from the extracellular supernatants of human FreeStyle 293 cells (figure 1*B*). The sCMG2 protein, which contains no N-linked glycosylation sites, was produced as a single protein species, ~25 kDa in size (figure 1*B*, lane 1). By contrast, the sATR/TEM8 protein contains 3 N-linked glycosylation sites [3] and was produced as several heterogeneous species ~37 kDa in size (figure 1*B*, lane 2), and PNGase F treatment of this protein yielded 2 smaller protein species (figure 1*B*, lane 3). It is not known why there are 2 distinct deglycosylated sATR/TEM8 species. As predicted, PNGase F treatment had no effect of the mobility of the sCMG2 protein in SDS-PAGE (data not shown).

The sATR/TEM8 and sCMG2 proteins were tested over a range of concentrations for their capacities to inhibit intoxication of CHO-K1 cells by PA and LF_N-DTA. A concentration of 3 nmol/L sCMG2 was sufficient to confer complete protection, whereas 100 nmol/L sATR/TEM8 was required to produce the same effect (figure 1*C*). The IC_{50} was 0.5 nmol/L for sCMG2 (PA:sCMG2 molar ratio, 1:0.5) and 5.7 nmol/L for sATR/TEM8 (PA:sATR/TEM8 molar ratio, 1:5.7). Thus, when both of the receptor decoys were produced by use of the mammalian cell expression system, sCMG2 was 11.4-fold more potent than sATR/TEM8 with respect to protecting CHO-K1 cells in vitro.

The greater potency of sCMG2 indicated that PA might have a higher binding affinity for the I domain of this receptor than for that of sATR/TEM8. Indeed, it is known that PA has an extremely high binding affinity for sCMG2: in 1 mmol/L Mg^{2+} and 2 mmol/L EGTA, the K_D is 0.17 nmol/L, and in 1 mmol/L Ca^{2+}, the K_D is 0.78 nmol/L [10]. To determine the binding kinetics of the PA–sATR/TEM8 interaction, surface plasmon resonance analysis was performed. The observed K_D values were 130 nmol/L in 1 mmol/L Mg^{2+} and 1.1 μmol/L in 1 mmol/L Ca^{2+}—or 760-fold and 1400-fold higher, respectively, compared with those for sCMG2 (table 1). Therefore, the higher binding affinity of sCMG2 correlates with the increase in potency of sCMG2 over sATR/TEM8.

Because the in vitro potency of sCMG2 compared favorably with that of other inhibitors of PA-receptor interactions, the efficacy of sCMG2 was tested in vivo. Male Fischer 344 rats were inoculated intravenously with LeTx (480 pmol of PA and 89 pmol of LF), either alone or in combination with sCMG2 in amounts ranging from 48 to 480 pmol. The amount of anthrax toxin in vivo during *B. anthracis* infection is unknown, and coinjection experiments with LeTx and antitoxins in this partic-

Table 2. Protection against intoxication in vivo provided by soluble capillary morphogenesis protein 2 (sCMG2).

Experiment, treatment (molar ratio[a])	No. of survivors/total	Time to death, mean ± SD, min	P[b]
Experiment 1			
PBS[c]	3/3	NA	…
LeTx only[d]	0/3	64 ± 1.5	…
LeTx/sCMG2 (1:1)	3/3	NA	…
LeTx/sCMG2 (1:0.9)	0/3	Overnight[e]	<.0001
LeTx/sCMG2 (1:0.6)	0/3	84 ± 7.8	.0115
LeTx/sCMG2 (1:0.3)	0/3	70 ± 1.7	.0090
LeTx/sCMG2 (1:0.1)	0/3	59 ± 3.5	.0996
Experiment 2			
PBS	6/6	NA	…
LeTx only	0/6	60 ± 3.1	…
LeTx/sCMG2 (1:2)	6/6	NA	…
LeTx/sCMG2 (1:1)	1/6	Overnight	<.0001
LeTx/sCMG2 (1:0.6)	0/5	70 ± 4.5	.0015

NOTE. NA, not applicable.

[a] Anthrax toxin protective antigen (PA):sCMG2 ratio when sCMG2 is mixed with lethal toxin (LeTx).

[b] For comparisons to the LeTx-only control group, by Student's unpaired *t* test.

[c] Negative control group receiving PBS and no LeTx.

[d] LeTx-only control group (40 μg of PA and 8 μg of lethal factor per rat).

[e] Rats were monitored for 180 min and then checked overnight, or 14 h after inoculation. *P* values are calculated for the 180-min time point.

ularly sensitive strain of rat are a standard method for a first in vivo trial [8, 9]. In the first experiment, the rats receiving 480 pmol of sCMG2 (corresponding to a PA:sCMG2 molar ratio of 1:1) survived intoxication (table 2). The rats receiving PA and sCMG2 at molar ratios of 1:0.9, 1:0.6, and 1:0.3—but not 1:0.1—exhibited statistically significant delays in time to death (TTD), compared with the rats receiving LeTx alone (table 2). A second, larger experiment confirmed that the rats receiving PA and sCMG2 at ratios of 1:1 and 1:0.6 experienced statistically significant delays in TTD (table 2). However, in this larger experiment, which used a separate lot of LeTx, several rats receiving PA and sCMG2 at a molar ratio of 1:1 died, and complete protection was instead observed in the rats receiving PA and sCMG2 at a molar ratio of 1:2. These results were confirmed in a repeat experiment. Together, these data indicate that the PA:sCMG2 molar ratio required for protection lies between 1:1 and 1:2. For comparison purposes, the efficacy of sATR/TEM8 was also tested in vivo, with 1 rat each receiving LeTx in combination with sATR/TEM8 in amounts ranging from 0.24–7.3 nmol, corresponding to PA:sATR/TEM8 molar ratios of 1:15, 1:5, 1:1, and 1:0.5. No rats receiving sATR/TEM8 survived, although the rat receiving PA and sATR/TEM8 at a molar ratio of 1:15 exhibited a 2.5-fold delay in TTD (160 min), compared with the rat receiving LeTx alone (64 min) (data not shown).

Discussion. By preventing intoxication in vivo with a PA:inhibitor molar ratio between 1:1 and 1:2, sCMG2 compares favorably to the most effective antitoxins tested to date. Indeed, sCMG2 is more effective against PA than are affinity-matured Fabs, which prevent the death of LeTx-challenged rats at a PA:inhibitor ratio of ~1:4 [9]. The protection afforded by sCMG2 is similar to that seen with dimerized full-length IgG_1 versions of the aforementioned Fabs (PA:inhibitor molar ratio, 1:0.6) [9], antibodies from anthrax vaccine–immunized donors (PA:inhibitor molar ratio, 1:0.5) [7], and DNI forms of PA (PA:inhibitor molar ratio, 1:0.25) [8]. With toxin-blocking antibodies, increases in apparent binding affinity and serum half-life were observed when dimeric versus monomeric antibody reagents were compared [11]. Similarly, multimeric forms of sCMG2 are also likely to show enhanced effectiveness in vivo. It will also be important to evaluate the antitoxin capabilities of sCMG2 in other relevant models of anthrax-toxin action, particularly inhalational challenge experiments with live *B. anthracis* spores.

PA antibodies are the most potent antitoxins to be tested in inhalational spore challenge experiments with a common strain of *B. anthracis* (reviewed in [6]). However, it is not known whether these antibodies are efficacious against weaponized strains of *B. anthracis* that, to overcome the protective effects of existing PA-based vaccines, are engineered to express antigenically altered forms of PA. This is of concern, because an altered form of PA that is functional but that is no longer sensitive to neutralization by a monoclonal antibody has already been described [12]. Indeed, saturation mutagenesis has revealed that most of the surface of PA can be mutated without adversely affecting toxin function [13].

A strategy based on a receptor decoy, such as sCMG2, overcomes the possible limitations of antibody-based therapies, because any altered form of PA that retains function will, presumably, still bind to cellular receptors and, thus, will not affect the therapeutic benefit of sCMG2. By virtue of its high binding affinity for PA, the sCMG2 receptor decoy shows promise as either a stand-alone therapy against anthrax toxin or an important adjunct for antibody-based therapies. There are several strong precedents for the use of soluble forms of cellular receptors as therapeutic agents, such as a multivalent form of the CD4 receptor as a therapy for HIV-1 infection and AIDS [14] and soluble forms of the tumor necrosis factor and interleukin-1 receptors as new therapies for rheumatoid arthritis (reviewed in [15]). In conclusion, the effectiveness of the sCMG2 receptor decoy in protecting rats against LeTx challenge indicates the potential that soluble receptor decoys have as candidate therapeutic agents for anthrax.

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