CLINICAL AND VACCINE IMMUNOLOGY, Sept. 2008, p. 1330–1336 Vol. 15, No. 9
1556-6811/08/$08.00+0 doi:10.1128/CVI.00103-08

Toxicity of Anthrax Toxin Is Influenced by Receptor Expression▽

Sarah C. Taft and Alison A. Weiss*

Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, Ohio 45267-0524

Received 18 March 2008/Returned for modification 29 May 2008/Accepted 13 June 2008

Anthrax toxin protective antigen (PA) binds to its cellular receptor, and seven subunits self-associate to form a heptameric ring that mediates the cytoplasmic entry of lethal factor or edema factor. The influence of receptor type on susceptibility to anthrax toxin components was examined using Chinese hamster ovary (CHO) cells expressing the human form of one of two PA receptors: TEM8 or CMG2. Unexpectedly, PA alone, previously believed to only mediate entry of lethal factor or edema factor, was found to be toxic to CHO-TEM8 cells; cells treated with PA alone displayed reduced cell growth and decreased metabolic activity. PA-treated cells swelled and became permeable to membrane-excluded dye, suggesting that PA formed cell surface pores on CHO-TEM8 cells. While CHO-CMG2 cells were not killed by wild-type PA, they were susceptible to the PA variant, F427A. Receptor expression also conferred differences in susceptibility to edema factor.

The major virulence factor of *Bacillus anthracis* is a three-part toxin consisting of protective antigen (PA), lethal factor (LF), and edema factor (EF). EF is an adenylate cyclase that converts ATP to cyclic AMP (cAMP), while LF is a zinc protease that cleaves mitogen activated protein kinase kinases (8, 16). The interaction of anthrax toxin with target cells has been extensively studied. The process initiates when PA (83 kDa) binds to the host receptor. Bound PA is cleaved to its active 63-kDa form (PA 63) by a furin-like protease, releasing a 20-kDa amino-terminal fragment (14, 24). Receptor-bound PA 63 self-associates to form ring-shaped heptamers that can bind up to a total of three molecules of any combination of LF (90 kDa) and/or EF (89 kDa) (20, 21). The PA complex is endocytosed through a lipid-raft-mediated clathrin-dependent process (1, 3). As the endosome is acidified, the heptameric PA complex forms a membrane pore, and LF and/or EF is translocated into the cytosol (5, 40).

Two human receptors for PA have been characterized: anthrax toxin receptor encoded by the tumor endothelial marker 8 gene (TEM8/ATR/ANTXR1) and capillary morphogenesis protein two (CMG2/ANTXR2) (7, 31). Most human cells appear to express CMG2 (31); however, TEM8 has been reported to be expressed by human epithelial cells of the lungs, skin, and intestines (6) and in endothelial cells (29). Very little is known about receptor expression in other animal species. The two human receptors are ca. 40% identical overall but share 60% identity in the von Willebrand factor A-like region where PA binding occurs (15, 31). In spite of the overall identity, CMG2 has a much higher affinity for PA (K_D = 0.170 nM compared to K_D = 130 nM for TEM8) (32). Membrane insertion of PA is thought to first require dissociation from the receptor, and the higher affinity of PA for CMG2 appears to make dissociation more difficult. For example, dissociation from CMG2 requires a very low pH (5.2), while membrane insertion for the PA-associated TEM8 complex has been reported to occur at a much higher pH (6.0 to 6.5) (27, 41). In addition to differences in the extracellular domains, these receptors also differ in their cytoplasmic domains. The cytoplasmic domain of the CMG2 receptor possesses a region homologous to the WH-1 signaling domain of the Wiskott-Aldrich syndrome proteins (4), while TEM8 appears to lack cytoplasmic signaling domains.

Given these differences, we hypothesized that receptor use may influence the toxic responses to anthrax toxin in disease or perhaps following vaccination. The U.S. Food and Drug Administration-licensed anthrax vaccine, anthrax vaccine adsorbed (AVA; BioThrax), is formulated from formalin-treated filtrate of the culture supernatant from *B. anthracis* and contains predominantly PA, as well as small, but detectable amounts of EF and LF (26, 37). The AVA vaccine is extremely reactogenic, and adverse reactions to the anthrax vaccine have been reported (9, 10, 12, 22, 25, 36, 39). In the present study, we examined the influence of receptor expression on anthrax toxin toxicity utilizing wild-type K1 Chinese hamster ovary cells (CHO-K1), CHO cells lacking functional anthrax toxin receptors (CHO-R1.1), and a well-characterized series of CHO-R1.1 cells expressing one of the two human anthrax toxin receptors, TEM8 or CMG2 (31, 33, 41, 42). Our studies suggest that the CMG2 receptor might signal in response to cAMP and that, unexpectedly, PA in the absence of EF or LF may mediate toxicity in a receptor-dependent manner.

MATERIALS AND METHODS

CHO cell toxicity assays. Chinese hamster ovary cells, CHO-K1, were obtained from American Type Culture Collection (ATCC CCL-61; Manassas, VA). The CHO anthrax toxin receptor-negative cell line, CHO-R1.1, and CHO-R1.1 cells expressing the human forms of TEM8 (CHO-TEM8) and CMG2 (CHO-CMG2) were kindly provided by J. A. T. Young (31). CHO cell lines were cultured in F-12 nutrient mixture (Ham) with L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (10,000 U of penicillin G sodium/ml and 10,000 U of streptomycin sulfate/ml; Invitrogen) at 37°C in 5% CO_2. The anthrax toxin components (List Biologicals Laboratories, Campbell, CA) EF and LF were added at 2.5 μg/ml, PA was added at 5 μg/ml, and PA 63 was added at 10 μg/ml. The PA mutants PA K397D/D425K, PA SSSR, and PA F427A, added at 10 μg/ml, were kindly provided by R. J. Collier (34).

* Corresponding author. Mailing address: Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, 231 Albert Sabin Way, Cincinnati, OH 45267-0524. Phone: (513) 558-2820. Fax: (513) 558-8474. E-mail: Alison.Weiss@uc.edu.

▽ Published ahead of print on 2 July 2008.

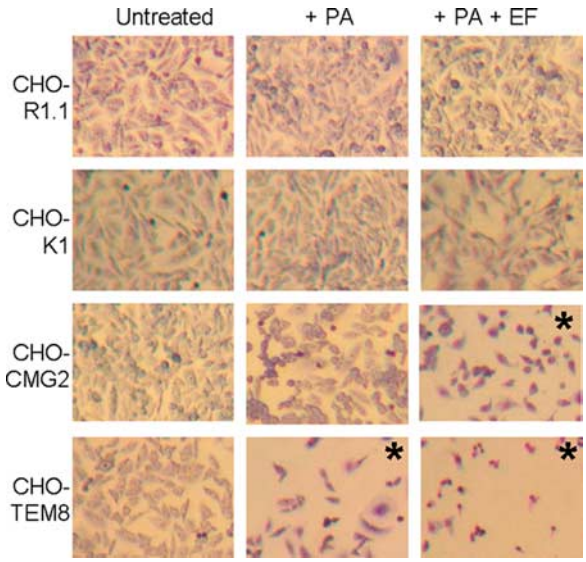

FIG. 1. PA-mediated toxicity to CHO cells is receptor dependent. Mutant CHO cells lacking anthrax toxin receptor (CHO-R1.1), wild-type CHO cells (CHO-K1), and CHO-R1.1 cells expressing the human CMG2 receptor (CHO-CMG2) or TEM8 receptor (CHO-TEM8) were incubated overnight with PA or PA+EF. Cells were fixed, stained with Giemsa, and viewed at ×63 magnification. The asterisk (*) denotes conditions that resulted in a statistically significant loss of cellular metabolic activity (Fig. 2).

To assess anthrax toxicity, CHO cell lines were plated at 10^5 in 96-well flat-bottomed plates and left untreated or incubated with anthrax toxin components. After 18 h, the cells were fixed with 100% methanol for 5 min, stained with 2% Giemsa in distilled water (Sigma-Aldrich, St. Louis, MO) for 20 min, and washed with water. Individual wells were photographed at ×63 magnification with an Olympus C-5060 microscope camera (Center Valley, PA).

The influence of anthrax toxin on metabolic activity was assessed using alamarBlue (Trek Diagnostic Systems, Inc., Westlake, OH). Cells were grown for 18 h in the presence of anthrax toxin components. An 80% solution of alamarBlue in Hanks balanced salt solution (HBSS; Invitrogen) was added at 10% of the well volume, and the cells were incubated for an additional 20 h at 37°C in 5% CO_2. Conversion of oxidized alamarBlue to its reduced form was determined according to the manufacturer's protocol. To assess the intracellular cAMP, cells were incubated with anthrax toxin components for 2 h. Intracellular cAMP was measured utilizing the BioTrak ELISA kit according to the manufacturer's protocol (Amersham Biosciences/GE Healthcare, United Kingdom). Cell lysis was assessed by monitoring lactate dehydrogenase (LDH) release. Cells were incubated with anthrax toxin components for 18 h, and LDH release was measured using a CytoTox-ONE homogeneous membrane integrity assay (Promega, Madison, WI) according to the manufacturer's protocol.

Flow cytometry. CHO cell variants were plated 10^6 in 24-well flat-bottomed tissue culture plates. Cells were incubated with or without anthrax toxin components for various times up to 24 h at 37°C in 5% CO_2. In the low-pH assays, cells were incubated with toxin for 5 min, and MES [2-(*N*-morpholino)ethanesulfonic acid] buffer (pH 5.5; Sigma-Aldrich) was added to decrease the pH to 6.0. After 5 min, the medium was removed and reserved to include nonadherent cells in the assay. The wells were washed with HBSS, treated with trypsin (Invitrogen) to remove adherent cells, and suspended in the medium. For permeability studies, cells were stained with 7-amino-actinomycin D (7-AAD; BD Biosciences, San Jose, CA), and fluorescence (FL3) and cell size (forward scatter) were analyzed by using a BD Biosciences FACSCalibur system.

For PA-receptor binding studies, trypsin-treated cells were incubated with PA for 5 min at 37°C in 5% CO_2, washed with HBSS, incubated with human serum (1/100) from an anthrax vaccinated donor for 5 min (13), washed, incubated with anti-human immunoglobulin G (IgG) Alexa Fluor 647 antibody (1/500) (Invitrogen), washed, and analyzed for fluorescence (FL4). Experimental values were determined as the percentage of total cells exceeding two standard deviations of the mean of control cells. All of these experiments were performed with at least three independent trials, and data were analyzed by using a Student paired *t* test.

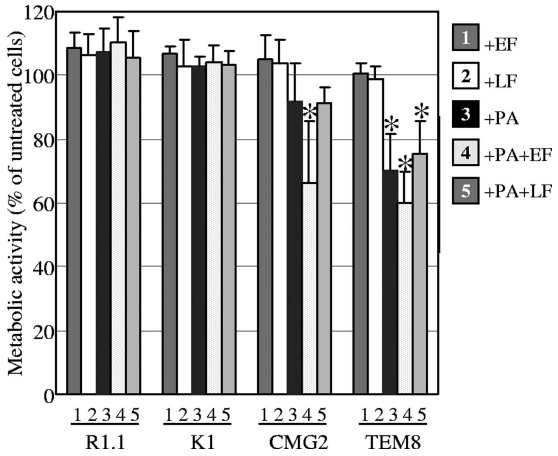

FIG. 2. PA alone reduces metabolic activity in CHO-TEM8 cells. CHO-R1.1, CHO-K1, CHO-CMG2, and CHO-TEM8 cells were incubated overnight with: 1, EF; 2, LF; 3, PA; 4, PA+EF; or 5, PA+LF. The mitochondrial conversion of alamarBlue was measured to determine metabolic activity. Values are shown as percentages of untreated control cells. The data are represented as means ± the standard error of the mean (SEM). An asterisk (*) signifies statistical significance compared to untreated cells ($P < 0.01$).

RESULTS

Receptor expression mediates differential susceptibility to anthrax toxin. Wild-type CHO-K1 cells, CHO-R1.1 cells lacking functional anthrax toxin receptor, and CHO-R1.1 cells expressing the human forms of TEM8 (CHO-TEM8) or CMG2 (CHO-CMG2) were left untreated or incubated overnight with PA or PA+EF. Cells were fixed and stained with Giemsa (Fig. 1). CHO-K1 cells displayed an elongated morphological form when treated with PA+EF as previously reported (16), while receptor-negative CHO-R1.1 cells had no morphological change. Interestingly, CHO cells expressing the human anthrax toxin receptors displayed differential susceptibility to PA and PA+EF. A visible reduction in cell number was seen for CHO cells expressing the CMG2 receptor after treatment with PA+EF and, surprisingly, cells expressing the TEM8 receptor displayed a visible reduction in cell number when treated with PA alone or PA+EF, suggesting PA alone could mediate toxicity.

To quantify toxicity, we assessed the influence of the anthrax toxin components on cellular metabolism (Fig. 2) using alamarBlue. Mitochondrial activity of living cells can convert oxidized alamarBlue to its reduced form, and the production of reduced alamarBlue can be monitored spectrophotometrically (13, 18). The metabolic activity of CHO-R1.1 and CHO-K1 cells was unaffected by treatment with anthrax toxin. In contrast, PA+EF caused a statistically significant reduction in metabolic activity in CHO cells expressing the CMG2 receptor. In addition, CHO-TEM8 cells displayed a statistically significant decrease when treated with PA+EF, PA+LF, and PA alone. These results confirm the visual observations that PA alone mediates toxicity to CHO cells expressing the TEM8 anthrax receptor, regardless of whether EF or LF is present.

Previous studies suggested that PA can induce apoptosis in mouse macrophages expressing TEM8 (30). Annexin V stain binds to the membrane phospholipid phosphatidylserine (28),

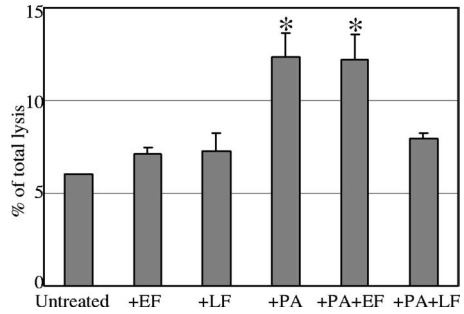

FIG. 3. PA promotes cellular lysis of CHO-TEM8 cells. CHO-TEM8 cells were incubated overnight with EF, LF, PA, PA+EF, or PA+LF. Cellular LDH release was measured and is presented as the percentage of total lysis. The data are represented as mean ± the SEM. An asterisk (*) signifies statistical significance from untreated cells ($P < 0.05$).

which is externalized during the early stages of apoptosis, while the membrane itself remains intact and impermeable to 7-AAD (17). To assess apoptosis, CHO-TEM8 cells were stained with both annexin V and 7-AAD. CHO-TEM8 cells stained positive for both annexin V and 7-AAD at all time points tested, 10 min to 18 h after PA addition (data not shown), suggesting that annexin V staining was due to compromised membrane integrity, which allowed for the staining of cytoplasmic phospholipid phosphatidylserine and not externalized phospholipid phosphatidylserine resulting from an apoptotic process.

PA promotes cellular lysis in CHO-TEM8 cells. Since PA is able to promote pore formation and our studies suggested that incubation with PA resulted in compromised membrane integrity, the ability of PA to mediate cellular lysis was examined by monitoring the release of LDH, a 40-kDa protein, from cells (Fig. 3). A statistically significant increase in the release of LDH was observed for CHO-TEM8 cells incubated overnight with PA or PA+EF. Surprisingly, even though PA+LF reduced metabolic activity (Fig. 2) and increased the 7-AAD permeability of CHO-TEM8 cells (see Fig. 4 and Table 1), cells treated with PA+LF did not show an increase in LDH release, suggesting that LF inhibited the release of cellular LDH. In line with this observation, previous studies have shown that LF can block PA-formed pores (23, 43).

The ability of PA to mediate cellular lysis was also examined by flow cytometry. The dye 7-AAD is excluded from intact, viable cells but stains the DNA of cells with compromised membrane integrity. Cells were treated overnight with EF, LF, or PA alone or in combination. Increased staining with 7-AAD was not observed for CHO-R1.1, CHO-K1, or CHO-CMG2 cells compared to untreated controls (Table 1). In contrast, 15 to 24% of CHO-TEM8 cells were permeable to 7-AAD after overnight incubation with PA in the presence or absence of EF or LF. This increase in the permeability of CHO-TEM8 cells treated with PA closely paralleled the reduced metabolic activity observed in Fig. 2.

TABLE 1. PA-mediated toxicity is receptor dependent

Cell type	Mean 7-AAD-positive cells ± SEM[a]			
	Untreated	With PA	With PA+EF	With PA+LF
CHO-R1.1	2 ± 1	1 ± 1	2 ± 1	2 ± 1
CHO-K1	2 ± 3	2 ± 2	2 ± 2	2 ± 2
CHO-CMG2	2 ± 1	3 ± 1	7 ± 3	3 ± 2
CHO-TEM8	3 ± 2	24 ± 7*	24 ± 12*	15 ± 4*

[a] Cells were left untreated or incubated overnight with anthrax toxin components. Viability was analyzed by flow cytometry measuring 7-AAD permeability. Values represent the percentage of total cells exceeding two standard deviations of the mean of control cells. *, Statistically significant compared to untreated control cells ($P < 0.01$).

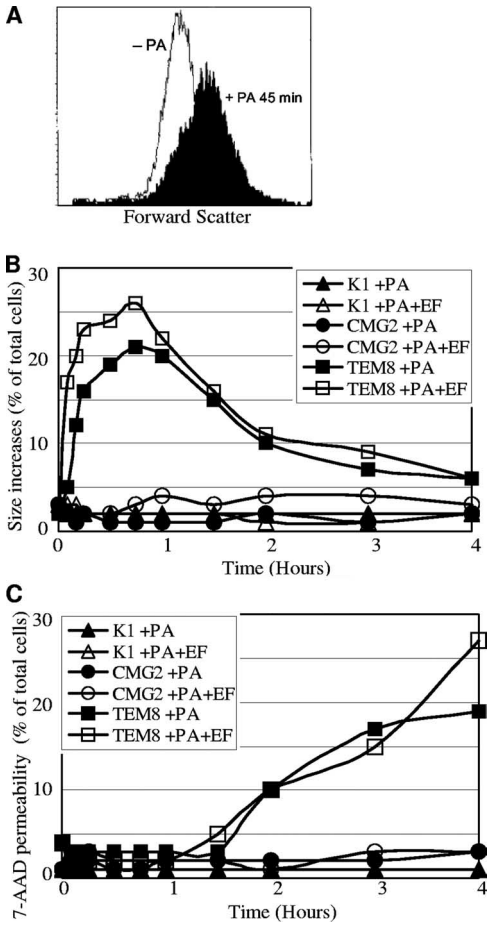

FIG. 4. PA induces size changes and 7-AAD permeability in CHO-TEM8 cells. CHO-K1, CHO-CMG2, or CHO-TEM8 cells were incubated with PA or PA+EF. (A) Forward scatter (FSC-H) as a measure of cell size for CHO-TEM8 cells untreated (open) or incubated with PA for 45 min (filled). Cell size (B) and permeability to 7-AAD (C) were analyzed by flow cytometry. Values are shown as the percentage of total cells exceeding two standard deviations of the mean of control cells.

It is of interest that while a 34% decrease in metabolic activity was observed for PA+EF-treated CHO-CMG2 cells (Fig. 2), treatment of CHO-CMG2 cells with PA+EF did not result in significantly increased staining with 7-AAD compared to untreated cells (Table 1). These results suggested that the inhibition of CHO-CMG2 cell proliferation by PA+EF was not due to compromised membrane integrity. We examined intracellular cAMP levels in cells treated with PA+EF. After 2 h, cAMP levels for the wild-type CHO-K1 cells (1,839 ± 428 fmol/well) were not significantly different from those seen in the CHO-CMG2 cells

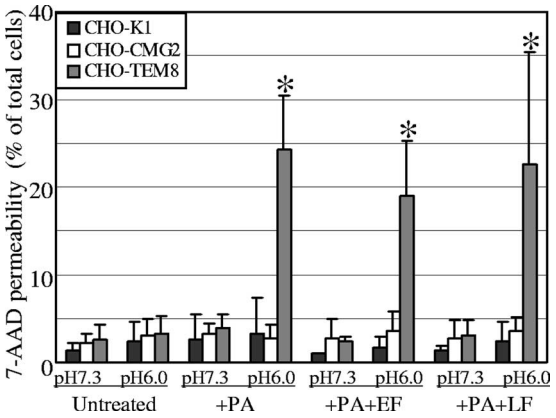

FIG. 5. Influence of low pH on PA-mediated permeability. CHO-K1, CHO-CMG2, and CHO-TEM8 cells were incubated with PA for 5 min, and the pH was adjusted to 6.0. The cells were stained and analyzed by flow cytometry. Values are shown as the percentage of total cells exceeding two standard deviations of the mean of control cells. The data are represented as means ± the SEM. An asterisk (*) signifies statistical significance from untreated cells ($P < 0.05$).

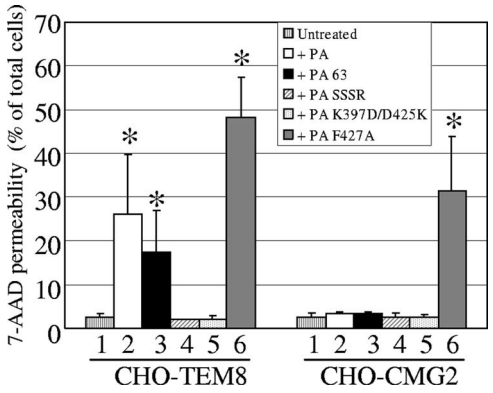

FIG. 6. Analysis of PA mutants. CHO-CMG2 and CHO-TEM8 cells were: 1, untreated; 2, incubated overnight with wild-type PA; 3, incubated overnight with proteolytically activated PA 63; 4, incubated overnight with furin cleavage resistant mutant (PA SSSR); 5, incubated overnight with pore formation resistant mutant (PA K397D/D425K); or 6, incubated overnight with domain 2 mutant (PA F427A). Increases in 7-AAD permeability were analyzed by flow cytometry. Values are shown as the percentage of total cells exceeding two standard deviations of the mean of control cells. The data are represented as means ± the SEM. An asterisk (*) signifies statistical significance from untreated cells ($P < 0.02$).

(3,065 ± 1,149 fmol/well). These results suggest cAMP may inhibit cellular proliferation in a receptor-dependent way.

While examining permeability to 7-AAD, we observed that the CHO-TEM8 cells increased in size after treatment with PA (Fig. 4A), prompting us to examine the time course of cellular responses to PA and PA+EF. CHO-R1.1 (data not shown), CHO-K1, and CHO-CMG2 cells did not show any increase in cell size (Fig. 4B) or increase in 7-AAD permeability (Fig. 4C) at any time after treatment with anthrax toxin. In contrast, CHO-TEM8 cells showed an increase in size within 5 min after treatment with PA alone or in combination with EF (Fig. 4B). However, PA-mediated permeability to 7-AAD was delayed. Increased staining with 7-AAD was first observed about 1.5 h after toxin treatment and continued to increase up to 4 h posttreatment (Fig. 4C). These experiments were repeated with two different preparations of PA, as well as proteolytically activated PA, PA 63, and similar results were seen. These results suggest that PA induces rapid cellular swelling in TEM8-expressing cells that eventually leads to cellular lysis. PA has been reported to form univalent cation specific channels (5). Unregulated entry of sodium ions can lead to cell lysis by breaking down the sodium gradient, allowing the cell to accumulate water.

Influence of pH on cellular permeability in CHO-TEM8 cells. PA heptamers have been shown to form pores on the cytoplasmic membrane when the pH is reduced (19), and a recent study by Rainey et al. (27) found that PA-TEM8 complexes form pores at a higher pH (6.0 to 6.5) than PA-CMG2 (pH 5.2). We examined the influence of low pH on cellular permeability. CHO cells were incubated with or without PA for 5 min, and the pH was decreased to 6.0 by the addition of buffer. After an additional 5 min, the cells were stained with 7-AAD, and the permeability was analyzed by flow cytometry. Incubation at pH 6 did not affect wild-type CHO-K1 or CHO-CMG2 cells under any circumstances (Fig. 5). However, decreasing the pH of PA-treated CHO-TEM8 cells caused a statistically significant increase in cellular permeability, independent of the presence of EF or LF. The pH-mediated increase in permeability observed in Fig. 5 was equivalent to the cellular permeability observed 4 h after PA treatment (Fig. 4), under conditions where the pH remained unchanged (pH 7.3). These results suggest that PA forms pre-pore complexes on the plasma membranes of cells expressing the TEM8 receptor. These pores can open spontaneously in the absence of a pH change (Fig. 4), but lowering the pH accelerates this process (Fig. 5).

Analysis of PA mutants. PA internalization and pore formation is a complex, multistep process. We examined the ability of a panel of well-characterized mutants of PA, which are defective in various stages of the intoxication process, to mediate cellular lysis. The PA mutant SSSR has a mutation in the PA domain 1 furin cleavage site in which RKKR has been changed to SSSR. PA SSSR binds to cellular receptors but is not cleaved by furin and is therefore unable to oligomerize, bind EF or LF, or form pores (2, 3, 11). The PA mutant K397D/D425K has two point mutations in PA domain 2, which is mainly responsible for pore formation and EF/LF translocation. PA K397D/D425K binds to cellular receptors, oligomerizes, and binds EF and LF but is unable to form pores (35). The PA mutant F427A also possesses a point mutation in PA domain 2. PA F427A binds to the receptor, oligomerizes, binds EF and LF, and forms sodium dodecyl sulfate-resistant heptamers indicative of pore formation but does not promote translocation of EF or LF into the cytoplasm (35).

To assess toxicity of the various forms of PA, CHO-CMG2 and CHO-TEM8 cells were left untreated or treated overnight with wild-type PA, proteolytically activated PA 63, or mutant PA (Fig. 6). Statistically significantly increased 7-AAD permeability was observed for CHO-TEM8 cells treated with wild-type PA and the cleaved form, PA 63. Increased cellular permeability was not seen for CHO-TEM8 cells treated with PA SSSR or PA K397D/D425K. These results suggested that PA

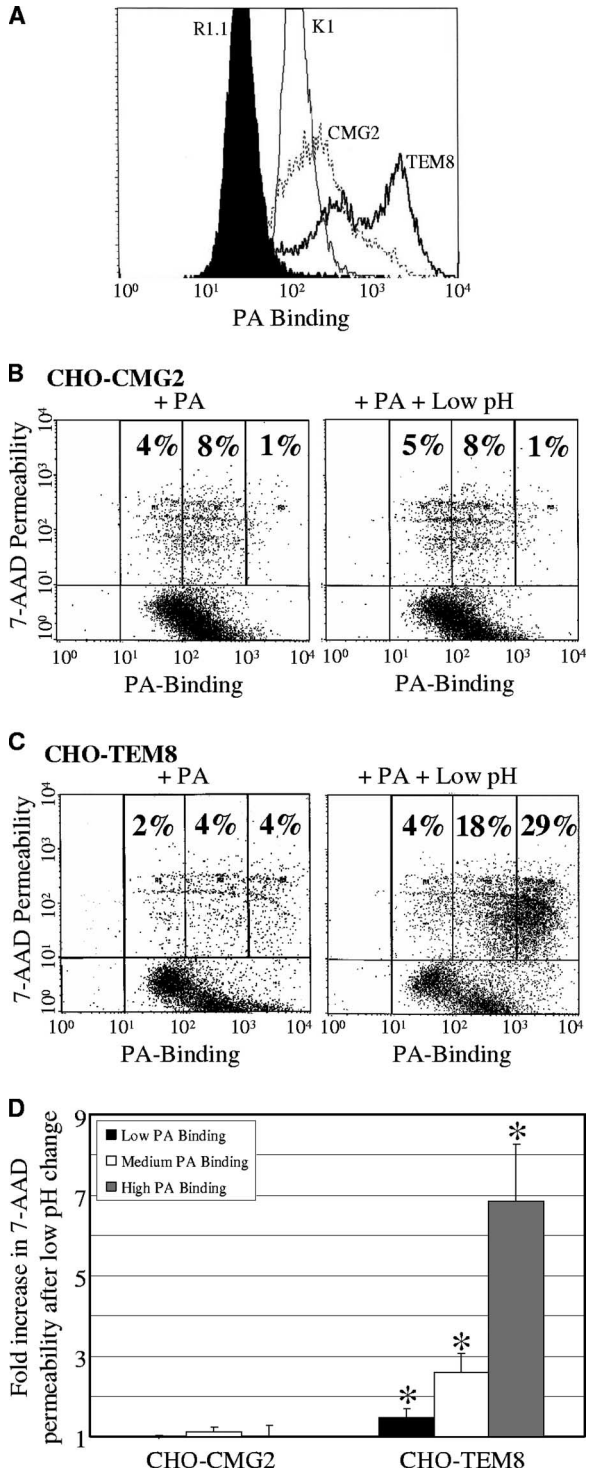

FIG. 7. Susceptibility to PA-mediated toxicity is influenced by receptor expression. (A) PA-receptor binding on CHO cells; CHO-R1.1 (filled histogram), CHO-K1 (thin line), CHO-CMG2 (dotted line), and CHO-TEM8 (heavy line) cells were incubated with PA for 5 min, washed, incubated with anti-PA serum for 10 min, washed, incubated with anti-IgG Alexa Fluor 647 antibody for 20 min, and analyzed by flow cytometry. CHO-CMG2 cells (B) and CHO-TEM8 cells (C) were incubated with PA for 5 min and left untreated or treated with buffer to decrease the pH for 5 min. The cells were double labeled for PA-receptor binding and cell permeability with 7-AAD and then analyzed by flow cytometry. (D) Fold increase in 7-AAD permeability after low pH change of PA treated CHO-CMG2 and CHO-TEM8 cells as a function of PA binding (high, >1,000; medium, 100 to 1,000; low, <100). The data are represented as means ± the SEM. An asterisk (*) signifies statistical significance from cells only treated with PA ($P < 0.05$).

furin cleavage, oligomerization, and pore formation are required for PA-mediated toxicity. Interestingly, increased 7-AAD permeability was seen when CHO-TEM8 cells were treated with mutant PA F427A, the mutant that fails to induce EF and LF translocation; furthermore, this mutant also induced permeability in CHO-CMG2 cells.

Level of receptor expression influences toxicity. The influence of receptor density on PA sensitivity was also examined. The amount of PA binding to the different CHO cell lines was determined by flow cytometry using antibody to PA (Fig. 7A). All of the receptor-expressing cells bound more PA than the CHO-R1.1 receptor-negative cell line. Although the expression of the endogenous CHO cell anthrax receptor on the CHO-K1 cells was very uniform, the CHO-R1.1 cells expressing the human forms of the anthrax toxin receptors, CMG2 and TEM8, exhibited a broad range of PA binding.

The susceptibility of CHO-CMG2 and CHO-TEM8 cells to PA was examined as a function of PA binding. Cells were treated with PA or treated with PA followed by treatment with low-pH buffer to promote PA-mediated lysis. The cells were stained with antibody to PA to assess PA binding levels and with 7-AAD to assess viability. As observed in Fig. 5, brief PA and low-pH treatment does not result in increased permeability to 7-AAD for CHO-CMG2 cells (Fig. 7B). In contrast, treatment of CHO-TEM8 cells with PA and low pH resulted in significantly increased permeability to 7-AAD compared to that for cells only exposed to PA (Fig. 7C). Susceptibility to PA-mediated permeability was assessed as a function of high (>1,000), medium (1,000 to 100), and low (<100) fluorescence (FL4) of bound PA (Fig. 7D). Receptor expression levels influenced susceptibility to lysis. Cells with the highest level of PA binding exhibited an ~7-fold increase in 7-AAD permeability after treatment with PA and low pH. However, even CHO-TEM8 cells that bound the intermediate and low levels of PA displayed statistically significant 4.5- and 2-fold increases, respectively, in 7-AAD permeability (Fig. 7D).

DISCUSSION

Our studies have shown that receptor type can influence the cellular responses to anthrax toxin in two ways. First, CHO cells expressing different anthrax toxin receptors displayed different responses to anthrax edema toxin (PA+EF) even though the cAMP levels were similar. Wild-type CHO-K1 cells displayed a morphological response to elevated cAMP levels (Fig. 1) and assumed the elongated form described in previous studies (16), but toxin treatment did not alter their metabolic activity compared to that of untreated control cells (Fig. 2). In contrast, CHO-CMG2 cells treated with PA+EF displayed reduced cell numbers (Fig. 1) and decreased metabolic activity (Fig. 2), which appeared to be due to an arrest in cellular division and not cellular death. The cytoplasmic domain of the

CMG2 receptor is homologous to the WH-1 signaling domain of the Wiskott-Aldrich syndrome proteins (4), and it is possible that in addition to delivering anthrax toxin to the cytoplasm, the CMG2 receptor may signal in response to elevated cAMP. The endogenous receptor on CHO-K1 cells is uncharacterized, and therefore its signaling capabilities are unknown. The TEM8 receptor appears to lack an intracellular signaling domain and would have been an interesting control for the responses of CMG2 to PA+EF, except that we made the unexpected discovery that PA alone was toxic for cells expressing the TEM8 receptor.

The PA-mediated toxicity to CHO-TEM8 cells appears to be due to pore formation since PA mutants deficient in pore formation did not display any toxicity (Fig. 6). Salles et al. (30) reported PA-mediated apoptosis of RAW 264.7 mouse macrophages overexpressing TEM8 and hypothesized that PA-mediated pore formation in the endosomal vesicles released proteases that triggered the activation of apoptosis (30). However, we were unable to detect any signs of apoptosis in CHO-TEM8 cells, and our studies suggest that toxicity is due to pore formation at the cell surface. Biochemical differences in PA binding to CMG2 and TEM8 give some insight into why TEM8 and not CMG2 supports cell surface pore formation. PA has been reported to have nearly 1,000-fold greater affinity for CMG2 than for TEM8 (32). PA insertion is thought to first require dissociation of PA from the receptor (5, 27), and insertion for PA associated with the CMG2 complex requires more extreme conditions (pH = 5.2) than PA associated-TEM8 complex (pH = 6.0 to 6.5) (27, 41). We observed spontaneous pore formation for the CHO-TEM8 cells grown in culture medium at pH 7.3 (Fig. 4), and this process could be accelerated by decreasing the pH (Fig. 5). Interestingly, the PA variant, F427A, with a mutation in the receptor-interaction domain (domain 2) was able to promote pore formation on CMG2-expressing cells (Fig. 6). It is possible that this mutation reduces the affinity of PA for the CMG2 receptor, allowing pore formation to occur prematurely on the cell surface. Although we have shown that susceptibility to PA is influenced by the level of TEM8 receptor expression (Fig. 7), it is important to note that even cells expressing very low levels of the TEM8 receptor displayed a 4% loss in viability after treatment with PA, a result that was statistically significant.

PA-mediated cell lysis has not been previously reported. Although toxicity due to the holotoxin is observed via both receptors, lysis appears to be produced only by binding of PA to the low-affinity TEM8 receptor, but not by binding to the high-affinity CMG2 receptor. Currently, it is not clear whether other mammalian species express a low-affinity receptor similar to human TEM8, and the failure to observe PA-mediated toxicity in animal models could therefore be due to the lack of TEM8 expression. TEM8 has been reported to be expressed by human epithelial cells of the lungs, skin, and intestines (6), and Rmali et al. (29) reported that interleukin-1β (which is likely to be produced during infection with *B. anthracis*) can upregulate the expression of TEM8 in human endothelial cells. PA has been widely used in the human anthrax vaccine and is a component of new recombinant vaccines under development (38). In addition, dominant-negative mutants of PA have been proposed as therapeutics for anthrax (20). Although the current anthrax vaccine is generally regarded to be safe, it is extremely reactogenic (9, 10, 12, 22, 25, 36, 39). Although susceptibility to PA is influenced by the level of TEM8 expression (Fig. 7), even the low, but statistically significant, levels of PA-mediated swelling and lysis (seen in Fig. 4) could lead to problems, particularly in the vasculature. The CHO-TEM8 cells described here could serve as sensitive indicators for potential PA-mediated toxicity.

ACKNOWLEDGMENTS

This work was sponsored by the NIH/NIAID Regional Center of Excellence for Biodefense and Emerging Infectious Disease Research (RCE) Program. We acknowledge membership within and support from the Region V (Great Lakes) RCE (NIH award 1-U54-AI-057153). S.C.T. was supported by NIH/NIAID training grant T32-AI055406 (Training in Biologic Threat Agents) and a University Research Council Summer Fellowship.

We thank J. A. T. Young for kindly providing the CHO cell lines and R. J. Collier for kindly providing the PA mutants.

REFERENCES

1. **Abrami, L., S. Liu, P. Cosson, S. H. Leppla, and F. G. van der Goot.** 2003. Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. J. Cell Biol. **160:**321–328.
2. **Ballard, J. D., A. M. Doling, K. Beauregard, R. J. Collier, and M. N. Starnbach.** 1998. Anthrax toxin-mediated delivery in vivo and in vitro of a cytotoxic T-lymphocyte epitope from ovalbumin. Infect. Immun. **66:**615–619.
3. **Beauregard, K. E., R. J. Collier, and J. A. Swanson.** 2000. Proteolytic activation of receptor-bound anthrax protective antigen on macrophages promotes its internalization. Cell. Microbiol. **2:**251–258.
4. **Bell, S. E., A. Mavila, R. Salazar, K. J. Bayless, S. Kanagala, S. A. Maxwell, and G. E. Davis.** 2001. Differential gene expression during capillary morphogenesis in 3D collagen matrices: regulated expression of genes involved in basement membrane matrix assembly, cell cycle progression, cellular differentiation and G-protein signaling. J. Cell Sci. **114:**2755–2773.
5. **Blaustein, R. O., T. M. Koehler, R. J. Collier, and A. Finkelstein.** 1989. Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers. Proc. Natl. Acad. Sci. USA **86:**2209–2213.
6. **Bonuccelli, G., F. Sotgia, P. G. Frank, T. M. Williams, C. J. de Almeida, H. B. Tanowitz, P. E. Scherer, K. A. Hotchkiss, B. I. Terman, B. Rollman, A. Alileche, J. Brojatsch, and M. P. Lisanti.** 2005. ATR/TEM8 is highly expressed in epithelial cells lining *Bacillus anthracis*' three sites of entry: implications for the pathogenesis of anthrax infection. Am. J. Physiol. Cell Physiol. **288:**C1402–C1410.
7. **Bradley, K. A., J. Mogridge, M. Mourez, R. J. Collier, and J. A. Young.** 2001. Identification of the cellular receptor for anthrax toxin. Nature **414:**225–229.
8. **Duesbery, N. S., 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, and G. F. Vande Woude.** 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science **280:**734–737.
9. **Geier, D. A., and M. R. Geier.** 2002. Anthrax vaccination and joint related adverse reactions in light of biological warfare scenarios. Clin. Exp. Rheumatol. **20:**217–220.
10. **Geier, M. R., and D. A. Geier.** 2004. Gastrointestinal adverse reactions following anthrax vaccination: an analysis of the Vaccine Adverse Events Reporting System (VAERS) database. Hepatogastroenterology **51:**762–767.
11. **Gordon, V. M., K. R. Klimpel, N. Arora, M. A. Henderson, and S. H. Leppla.** 1995. Proteolytic activation of bacterial toxins by eukaryotic cells is performed by furin and by additional cellular proteases. Infect. Immun. **63:**82–87.
12. **Greidanus, T. G., and B. A. Honl.** 2002. Delayed-type hypersensitivity reaction to anthrax vaccine. Mil. Med. **167:**74–75.
13. **Hanson, J. F., S. C. Taft, and A. A. Weiss.** 2006. Neutralizing antibodies and persistence of immunity following anthrax vaccination. Clin. Vaccine Immunol. **13:**208–213.
14. **Klimpel, K. R., S. S. Molloy, G. Thomas, and S. H. Leppla.** 1992. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. Proc. Natl. Acad. Sci. USA **89:**10277–10281.
15. **Lacy, D. B., D. J. Wigelsworth, R. A. Melnyk, S. C. Harrison, and R. J. Collier.** 2004. Structure of heptameric protective antigen bound to an anthrax toxin receptor: a role for receptor in pH-dependent pore formation. Proc. Natl. Acad. Sci. USA **101:**13147–13151.
16. **Leppla, S. H.** 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. Proc. Natl. Acad. Sci. USA **79:**3162–3166.
17. **Martin, S. J., C. P. Reutelingsperger, A. J. McGahon, J. A. Rader, R. C. van Schie, D. M. LaFace, and D. R. Green.** 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of

the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J. Exp. Med. **182:**1545–1556.
18. **Millen, S. H., D. I. Bernstein, B. Connelly, J. I. Ward, S. J. Chang, and A. A. Weiss.** 2004. Antibody-mediated neutralization of pertussis toxin-induced mitogenicity of human peripheral blood mononuclear cells. Infect. Immun. **72:**615–620.
19. **Milne, J. C., and R. J. Collier.** 1993. pH-dependent permeabilization of the plasma membrane of mammalian cells by anthrax protective antigen. Mol. Microbiol. **10:**647–653.
20. **Milne, J. C., D. Furlong, P. C. Hanna, J. S. Wall, and R. J. Collier.** 1994. Anthrax protective antigen forms oligomers during intoxication of mammalian cells. J. Biol. Chem. **269:**20607–20612.
21. **Mogridge, J., K. Cunningham, and R. J. Collier.** 2002. Stoichiometry of anthrax toxin complexes. Biochemistry **41:**1079–1082.
22. **Muniz, A. E.** 2003. Lymphocytic vasculitis associated with the anthrax vaccine: case report and review of anthrax vaccination. J. Emerg. Med. **25:**271–276.
23. **Neumeyer, T., F. Tonello, F. Dal Molin, B. Schiffler, F. Orlik, and R. Benz.** 2006. Anthrax lethal factor (LF) mediated block of the anthrax protective antigen (PA) ion channel: effect of ionic strength and voltage. Biochemistry **45:**3060–3068.
24. **Petosa, C., R. J. Collier, K. R. Klimpel, S. H. Leppla, and R. C. Liddington.** 1997. Crystal structure of the anthrax toxin protective antigen. Nature **385:** 833–838.
25. **Pittman, P. R., P. H. Gibbs, T. L. Cannon, and A. M. Friedlander.** 2001. Anthrax vaccine: short-term safety experience in humans. Vaccine **20:**972–978.
26. **Puziss, M., L. C. Manning, J. W. Lynch, E. Barclay, I. Abelow, and G. G. Wright.** 1963. Large-scale production of protective antigen of *Bacillus anthracis* in anaerobic cultures. Appl. Microbiol. **11:**330–334.
27. **Rainey, G. J., D. J. Wigelsworth, P. L. Ryan, H. M. Scobie, R. J. Collier, and J. A. Young.** 2005. Receptor-specific requirements for anthrax toxin delivery into cells. Proc. Natl. Acad. Sci. USA **102:**13278–13283.
28. **Raynal, P., and H. B. Pollard.** 1994. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochim. Biophys. Acta **1197:**63–93.
29. **Rmali, K. A., M. A. Al-Rawi, C. Parr, M. C. Puntis, and W. G. Jiang.** 2004. Upregulation of tumour endothelial marker-8 by interleukin-1beta and its impact in IL-1β induced angiogenesis. Int. J. Mol. Med. **14:**75–80.
30. **Salles, I. I., D. E. Voth, S. C. Ward, K. M. Averette, R. K. Tweten, K. A. Bradley, and J. D. Ballard.** 2006. Cytotoxic activity of *Bacillus anthracis* protective antigen observed in a macrophage cell line overexpressing ANTXR1. Cell. Microbiol. **8:**1272–1281.
31. **Scobie, H. M., G. J. Rainey, K. A. Bradley, and J. A. Young.** 2003. Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. Proc. Natl. Acad. Sci. USA **100:**5170–5174.
32. **Scobie, H. M., D. Thomas, J. M. Marlett, G. Destito, D. J. Wigelsworth, R. J. Collier, J. A. Young, and M. Manchester.** 2005. A soluble receptor decoy protects rats against anthrax lethal toxin challenge. J. Infect. Dis. **192:**1047–1051.
33. **Scobie, H. M., and J. A. Young.** 2005. Interactions between anthrax toxin receptors and protective antigen. Curr. Opin. Microbiol. **8:**106–112.
34. **Sellman, B. R., M. Mourez, and R. J. Collier.** 2001. Dominant-negative mutants of a toxin subunit: an approach to therapy of anthrax. Science **292:**695–697.
35. **Sellman, B. R., S. Nassi, and R. J. Collier.** 2001. Point mutations in anthrax protective antigen that block translocation. J. Biol. Chem. **276:**8371–8376.
36. **Swanson-Biearman, B., and E. P. Krenzelok.** 2001. Delayed life-threatening reaction to anthrax vaccine. J. Toxicol. Clin. Toxicol. **39:**81–84.
37. **Taft, S. C., and A. A. Weiss.** 2008. Neutralizing activity of vaccine-induced antibodies to two *Bacillus anthracis* toxin components, lethal factor and edema factor. Clin. Vaccine Immunol. **15:**71–75.
38. **Turnbull, P. C.** 1991. Anthrax vaccines: past, present and future. Vaccine **9:**533–539.
39. **Vasudev, M., and M. C. Zacharisen.** 2006. New-onset rheumatoid arthritis after anthrax vaccination. Ann. Allergy Asthma Immunol. **97:**110–112.
40. **Wesche, J., J. L. Elliott, P. O. Falnes, S. Olsnes, and R. J. Collier.** 1998. Characterization of membrane translocation by anthrax protective antigen. Biochemistry **37:**15737–15746.
41. **Wolfe, J. T., B. A. Krantz, G. J. Rainey, J. A. Young, and R. J. Collier.** 2005. Whole-cell voltage clamp measurements of anthrax toxin pore current. J. Biol. Chem. **280:**39417–39422.
42. **Young, J. A., and R. J. Collier.** 2007. Anthrax toxin: receptor-binding, internalization, pore formation, and translocation. Annu. Rev. Biochem. **76:**243–265.
43. **Zhao, J., J. C. Milne, and R. J. Collier.** 1995. Effect of anthrax toxin's lethal factor on ion channels formed by the protective antigen. J. Biol. Chem. **270:**18626–18630.