

Internalization of a *Bacillus anthracis* Protective Antigen-c-Myc Fusion Protein Mediated by Cell Surface Anti-c-Myc Antibodies

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Abstract

Background: Anthrax toxin, secreted by *Bacillus anthracis*, consists of protective antigen (PA) and either lethal factor (LF) or edema factor (EF). PA, the receptor-binding component of the toxin, translocates LF or EF into the cytosol, where the latter proteins exert their toxic effects. We hypothesized that anthrax toxin fusion proteins could be used to kill virus-infected cells and tumor cells, if PA could be redirected to unique receptors found only on these cells.

Materials And Methods: To test this hypothesis in a model system, amino acids 410–419 of the human p62^{c-myc} epitope were fused to the C-terminus of PA to redirect PA to the c-Myc-specific hybridoma cell line 9E10.

Results: The PA-c-Myc fusion protein killed both mouse macrophages and 9E10 hybridoma cells when adminis-

tered with LF or an LF fusion protein (FP59), respectively. Similar results were obtained with PA, which suggests that PA-c-Myc used the endogenous PA receptor to enter the cells. By blocking the endogenous PA receptors on 9E10 cells with the competitive inhibitor PA SNKEΔFF, the PA-c-Myc was directed to an alternate receptor, i.e., the anti-c-Myc antibodies presented on the cell surface. The c-Myc IgG were proven to act as receptors because the addition of a synthetic peptide containing the c-Myc epitope along with PA SNKEΔFF further reduced the toxicity of PA-c-Myc + FP59.

Conclusion: This study shows that PA can be redirected to alternate receptors by adding novel epitopes to the C-terminus of PA, enabling the creation of cell-directed toxins for therapeutic purposes.

Introduction

Anthrax toxin is a three-part toxin secreted by *Bacillus anthracis* consisting of protective antigen

(PA), lethal factor (LF), and edema factor (EF) (1). PA, the receptor-binding component of anthrax toxin, binds an unidentified receptor and then is cleaved by a cell-surface, furin-like protease (2). The cleaved PA binds LF or EF, undergoes receptor-mediated endocytosis (3,4), and forms a pore-like heptameric structure (5,6). The PA pore transfers LF and EF into the cytosol, where they induce cytotoxic events (6,7). When administered to rats intravenously, lethal toxin (the combination of PA and LF) causes pulmonary edema and death in as little as 38 min (8,9). In cell culture, mouse macrophages lyse completely in 90 min after addition of lethal toxin (3,10). The ability of lethal toxin to rapidly lyse

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cultured cells provides a reliable assay for PA activity.

Several lines of evidence suggest that the C-terminus of PA is the receptor-binding domain. First, the removal of 3, 5, or 7 residues from the carboxyl terminus of PA decreases the toxicity of the PA progressively, creating a final 20-fold decrease in toxicity (11). These truncated proteins exhibit decreased affinity for the receptor in competition assays, indicating that the receptor-binding domain resides in the C-terminus of PA. Second, two monoclonal antibodies against PA, 14B7 and 3B6, fail to interact with a truncated PA (155 amino acids deleted from the C-terminus), but bind to full-length PA (735 amino acids) and inhibit binding of PA to its receptor (12). Third, comparison of the amino acid sequence of PA with that of other binary toxins, such as iota-*b* toxin from *Clostridium perfringens* (13), shows high similarity between the toxins, except in the C-terminal domain (14). This suggests that the C-terminal domain confers receptor specificity to PA.

If PA can be redirected to an alternate receptor, anthrax toxin could be used in vivo to kill tumor cells. Before creating a nonreceptor-binding mutant of PA, a model system was tested to determine if intact PA could be directed to and use an alternate receptor. To test this idea, the C-terminus of PA (because the carboxyl end of PA possesses the receptor-binding domain) was fused to the human p62^{c-myc} (15) epitope (amino acids 410–419 (16)) so as to target the anti-c-Myc hybridoma cell line 9E10 (17). The *myc* family of proto-oncogenes express DNA binding proteins that affect cell proliferation, apoptosis, and cell-cycle progression (for reviews see refs. 18–20). The c-Myc peptide for our fusion experiment was selected because it contains a well-characterized antigenic epitope of c-Myc that is recognized by the anti-c-Myc 9E10 antibody secreted by the 9E10 hybridoma cell line (16,17). Hybridoma cells both secrete antibody and present antibody on their cell surface. The presented antibody acts as a receptor for its antigen (for reviews on Ig receptors see refs. 21–23). The antibodies to c-Myc presented on the surface of the 9E10 hybridoma cell may also be capable of acting as receptors for PA-c-Myc and internalizing the protein by receptor-mediated endocytosis. This study shows that through the addition of a cell-specific epitope to the C-terminus of PA, PA can be targeted to alternate receptors and thereby to specific cell lines.

Materials and Methods

Reagents and Supplies

Enzymes for DNA manipulation and modification were purchased from New England Biolabs, Inc. (Beverly, MA), Boehringer Mannheim (Indianapolis, IN), and Amersham Life Sciences (Arlington Heights, IL). Chemicals for protein work were purchased from Sigma (St. Louis, MO). Tissue culture products were obtained from Biofluids (Rockville, MD) or Life Technologies (Grand Island, NY) and the bacterial culture media from Difco Laboratories (Detroit, MI).

Plasmid Construction

Using the previously described plasmid, pYS5 (24), the human p62^{c-myc} (15) epitope corresponding to amino acid residues 410–419 (EQKLISEEDL) (16) was attached to the 3'-end of *pag*, the gene for PA (25). The pYS5 plasmid, encoding *pag* is an *Escherichia coli*–*Bacillus* shuttle vector. A primer spanning the unique *Pst*I site at nucleotide 966 of the mature *pag* sequence and a 98 nt mutagenic primer overlapping the C-terminal *Bam*HI site of *pag* were used for polymerase chain reaction (PCR) amplification of pYS5. The mutagenic 3' *Bam*HI primer encoded the 3'-end of *pag* (nt 2185 to 2205), a 6-amino acid (GGSGGS) linker, the 10-amino acid c-Myc epitope, two stop codons, the *Bam*HI site, and a short extension of DNA, in this order. After digestion with *Pst*I and *Bam*HI the PCR product from the amplification was ligated into the *Pst*I and *Bam*HI digested pYS5 plasmid and transformed into the *E. coli* *dcm dam* strain, GM2163. After plasmids had been sequenced to verify the presence of the c-Myc sequence at the 3'-end of *pag*, the unmethylated GM2163 DNA was transformed into the *Bacillus anthracis* strain, UM23C1-1 (26).

Expression and Purification of Protein

To purify the secreted PA-c-Myc from the medium of transformed *B. anthracis* cultures, the *B. anthracis* was grown in modified FA medium (24) in the presence of kanamycin (5 µg/ml) for 14 hr, at 200 revolutions/min and 37°C. The modified FA media contained a reduced quantity of yeast extract (5 g of yeast extract/liter) as compared with the unmodified FA medium. The PA was precipitated from the filtered culture supernatant by the addition of ammonium sulfate (61% saturation). The precipitate was concentrated by centrifugation at 4,000 × *g* for 30 min.

The precipitated protein was redissolved in 1 mM EDTA, 10 mM Tris, 0.2 M NaCl, pH 8.0, centrifuged at $5000 \times g$ for 5 min, and the supernatant from the centrifugation fractionated by Sephadex G-75 (Pharmacia Biotech, Piscataway, NJ). The fusion protein fractions from the Sephadex column were pooled and separated on a 1-ml MonoQ column (Pharmacia Biotech) using a 50- to 200-mM NaCl gradient in 10 mM CHES (2-[N-cyclohexylamino]ethane-sulfonic acid), 0.06% (v/v) ethanolamine, pH 9.1 (2). The pooled MonoQ fractions were dialyzed against 10 mM HEPES, 0.1 M NaCl, pH 8.0, concentrated with a Centricon 50 (Amicon, Beverly, MA), and analyzed by native 8–25% Phast gel electrophoresis (Pharmacia Biotech).

Cell Culture

RAW264.7 cells (mouse macrophages) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4,500 mg/l D-glucose, 10% (v/v) fetal bovine serum (FBS), 50 $\mu\text{g}/\text{ml}$ gentamicin, 10 mM HEPES, and 2 mM glutamine. The 9E10.2 hybridoma cells, ATCC 1729-CRL (17), and the SP2/0 Ag 14 myeloma cells, ATCC 1581-CRL (27), were maintained in DMEM with 4,500 mg/l D-glucose, 10% (v/v) FetalClone I (HyClone Laboratories, Inc. Logan, UT), 50 $\mu\text{g}/\text{ml}$ gentamicin, 10 mM HEPES, 2 mM glutamine, 1:100 dilution of GMS-G (Insulin-Transferrin-Selenium supplement) (Life Technologies), 150 $\mu\text{g}/\text{mL}$ oxaloacetate, and 10 $\mu\text{g}/\text{ml}$ bovine insulin (Life Technologies).

Antibody Isolation

The monoclonal antibody from cell culture medium of 9E10.2 hybridoma cells was isolated using the MabTrapG Protein G kit from Pharmacia. Purified antibody was concentrated in a Centricon 50 (Amicon) and dialyzed against 10 mM HEPES, 0.1 M NaCl, pH 8.0. Antibody concentration was determined by spectrophotometry at 280 nm.

ELISA

For the ELISA, purified PA, PA-c-Myc, or c-Myc peptide (residues 408–439, Calbiochem) was diluted to 10 $\mu\text{g}/\text{ml}$ in 10 mM phosphate buffer, pH 9.2, and 75 μL of protein or peptide applied per well of a 96-well Immulon II microtiter plate (Dynex Technologies, Chantilly, VA). After washing the microtiter plate with phosphate

buffer plus 0.1% (w/v) gelatin, 50 μl of 1:100 diluted monoclonal PA antibody 3B6 (12) or monoclonal c-Myc antibody 9E10 was added to the top set of wells and the antibody was serially diluted 1:3 down the column of wells. The wells were washed and secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Cappel, Organon Teknika Corporation, Durham, NC), was then added to the samples. The HRP-bound antibody was visualized with 1 mg/ml of ABTS (2,2'-azinobis[3-ethylbenzthiazoline-sulfonic acid]) in 0.1 M citrate buffer, pH 5.0, and 0.01% (w/v) H_2O_2 . The absorption at 405 nm was read using a UV_{max} Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA).

Immunoassays

To quantitate the relative amounts of mouse IgG presented on the surface of the cells, the 9E10 and the parent SP2/0 cell lines were labeled with anti-mouse IgG and the IgG bound to the cells quantitated by spectrophotometry or analyzed by a fluorescent activated cell sorter (FACS). The cells were washed twice in Hanks buffered saline solution (HBSS), resuspended in phosphate-buffered saline (PBS) plus 0.05% (w/v) gelatin, counted in a hemacytometer (Fisher Scientific, Pittsburgh, PA), and then 1 ml of 1.2×10^5 cells/ml used for the spectrophotometric immunoassay. The 1 ml of cells was incubated with 5 μl of HRP-conjugated goat anti-mouse IgG (Cappel), washed three times, and resuspended in ABTS and citrate buffer or the resuspended sample diluted 1:10 into ABTS and citrate buffer as per the ELISA protocol above. The amount of cell-bound HRP-conjugated antibody was determined at 405 nm in the Molecular Devices UV_{max} Kinetic Microplate Reader. To prepare cells for the FACS, the cells were pelleted, washed two times in PBS, and counted in a hemacytometer. One milliliter of 1×10^5 cells/ml was then placed into 5 ml of PBS plus 0.05% (w/v) gelatin and 1 μl of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Boehringer-Manheim) incubated with the cells. The cells were washed again, resuspended in 400 μl of PBS plus gelatin, and the relative amount of cell-bound FITC-conjugated antibody determined by a FACScan (Becton-Dickinson, Sparks, MD).



Fig. 1. Reverse-complement of the 3' primer used to create PA-c-Myc by PCR amplification of the PA expression vector, pYS5. The top sequence is the 3' wild-type PA sequence (25). The bottom sequence is the reverse complement of the 3' PCR primer encoding the 3' PA sequence, the linker (dashed underlined amino acids), the c-Myc

epitope that corresponds to amino acids 410–419 of c-Myc (underlined amino acids) (17), and the unique *Bam*HI restriction site (underlined nucleotides). The capital letters denote the gene sequence and the lowercase letters denote the noncoding vector sequence.

Cytotoxicity Assays with MTT

The macrophage lysis assay (28) was used to measure the toxicity of PA-c-Myc fusion protein on RAW264.7 macrophages. PA, as a control, or PA-c-Myc was added to one-third confluent, adherent RAW264.7 cells along with 100 ng/ml of LF in a 96-well microtiter dish to give a total volume of 200 μ l/well. PA SNKE Δ FF (2), a non-toxic receptor-binding mutant of PA, was used as a competitor in some of the wells. The PA or PA-c-Myc was serially diluted in the wells in the presence of a constant amount of LF and/or competitor. The cells were incubated for 3 hr at 37°C in the presence of toxin, after which cell viability was assayed by adding 50 μ l of 0.5 mg/ml of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). The cells were incubated with the MTT for 45 min at 37°C, the media removed, and the blue precipitate solubilized with 100 μ l/well of 0.5% (w/v) SDS (sodium dodecyl sulfate), 25 mM HCl, in 90% (v/v) isopropanol. The plates were vortexed and the intensity of the oxidized MTT read at 540 nm using the microplate reader.

Cytotoxicity Assays with XTT

Hybridoma cytotoxicity was assayed using XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide, inner salt, sodium salt) (29). One-third confluent cells was added to PA or PA-c-Myc serially diluted in the presence of a constant amount of 100 ng/ml of FP59, a fusion protein of the amino-terminal domain of LF(1–254) and the ADP-ribosylating domain of *Pseudomonas* exotoxin A (PE) that is similar to the fusion proteins described previously (30,31). A constant amount of PA SNKE Δ FF and/or c-Myc peptide was used as competitors in the assay to give a total volume of 250 μ l/well. The XTT was added

to the samples after the cells had been incubated with toxin for 50–60 hr. The XTT solution (1 mg/ml of XTT in serum-free media and 25 μ l of 1.53 mg/ml of PMS [phenazine methosulfate] in acetone for every 5 ml of XTT solution) was added to the wells at 50 μ l/well and incubated at 37°C for 3–4 hr to permit development of color. The optical density of the samples was determined at 405 nm in the Molecular Devices UV-max Kinetic Microplate Reader. The absorbance reading from the wells containing entirely dead cells was subtracted from the rest of the absorbance readings before calculating percent viability for the XTT assays.

Statistics

All samples were assayed in duplicate and the entire assay repeated three times. Standard errors were determined for the duplicate samples and presented as error bars on the graphs.

Results

Construction and Purification of PA-c-Myc

The c-Myc epitope was genetically fused to the C-terminus of PA through a linker to ensure that the c-Myc epitope had enough flexibility to bind the antibody receptor (Fig. 1). Sequencing of the pYS5-c-myc plasmid indicated that the linker and c-myc epitope had inserted properly at the 3'-end of *pag* (PA gene). The fusion protein was secreted at a concentration of approximately 10 mg/l into the medium and isolated by column chromatography to 99% purity, as determined by SDS-PAGE. The PA-c-Myc eluted at 140 mM NaCl from the MonoQ column, as compared with the elution point of PA from the MonoQ column at 100 mM NaCl. The purified PA-c-Myc also migrated faster than PA on a native gel, which is

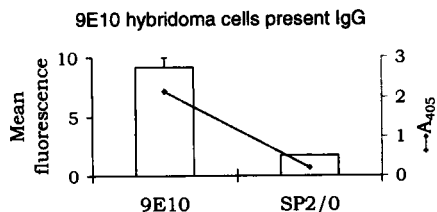


Fig. 2. The 9E10 mouse hybridoma cell line presents IgG on the cell surface. The relative amount of IgG on the cell surface was determined both by FACS and by immunoassay. FITC-conjugated goat anti-mouse IgG bound cells were analyzed by FACS and the results given as the mean fluorescence of the sorted cells (bar graph). For the immunoassay, the HRP-conjugated goat anti-mouse IgG bound cells were resuspended in ABTS and citrate buffer and the absorbance measured at 405 nm (line graph).

consistent with the incorporation of three negative charges from the added c-Myc epitope. In addition, the two proteins migrated to the same position on a denaturing SDS-Phast gel, corresponding to approximately 85 kDa, the M_r of PA (32,33). The fusion protein showed no evidence of degradation on SDS-Phast gels after 6 months of storage at -70°C .

Immunoassays against PA-c-Myc To Verify Presence of c-Myc Epitope

Antibody against c-Myc (9E10) and antibody against PA (3B6) were used in a serial dilution ELISA against PA, PA-c-Myc, and c-Myc peptide. The 9E10 antibody reacted with PA-c-Myc and c-Myc peptide and failed to detect PA, as expected (data not shown). The 9E10 antibody reaction with the c-Myc peptide produced an optical density twice that of the PA-c-Myc. The PA antibody 3B6 reacted with both PA-c-Myc and PA.

Immunoassays of Anti-c-Myc 9E10 Mouse Hybridoma Cells To Confirm Presence of Antibody on the Cell Surface that Could Act as Receptors for PA-c-Myc

The 9E10 cell line was treated with HRP or FITC-conjugated anti-mouse IgG (Fig. 2). The control for the experiments was the parent cell line of the hybridoma cell line, SP2/0, which is known to be Ig-negative (27). When incubated with the fluorescent antibody, the 9E10 cells had a peak mean of 9.18; the SP2/0 cells had a peak mean of 1.72. Similar results were obtained with the

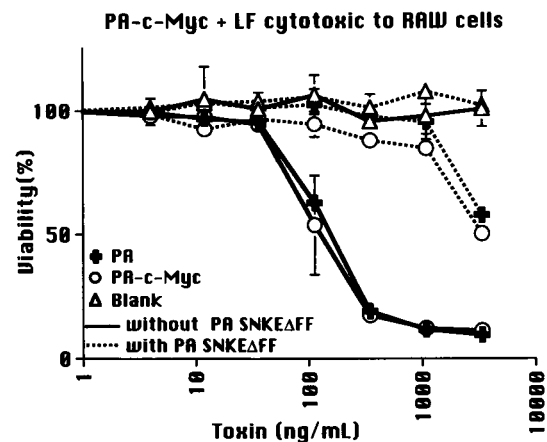


Fig. 3. PA and PA-c-Myc exhibit similar cytotoxic effects against RAW cells. PA and PA-c-Myc were serially diluted into the 96-well plates containing RAW cells, in the presence of 100 ng/ml of LF and with or without 5 $\mu\text{g}/\text{ml}$ of the nontoxic competitive mutant of PA, PA SNKE Δ FF. The control samples without toxin are indicated as triangles. After 3 hr of toxin challenge, MTT was added to determine cell viability.

HRP-conjugated anti-mouse IgG. The 9E10 cell line had 20 times as much absorbance (2.1 A_{450} after dilution factor correction) as the SP2/0 cell lines background absorbance (0.133 A_{450}).

Cytotoxicity Assay of PA-c-Myc + LF against RAW Cells

Because fusion of the c-Myc epitope to PA could have created a nonfunctional fusion protein, the fusion protein's cytotoxicity was first tested on RAW cells, which are known to be lysed by the PA + LF toxin (Fig. 3). Both PA and PA-c-Myc killed RAW cells in an MTT cytotoxicity assay in the presence of LF, with a 50% effective concentration (EC_{50}) of approximately 100 ng/mL. The nontoxic receptor-binding competitive inhibitor of PA, PA SNKE Δ FF (5 $\mu\text{g}/\text{ml}$), protected the cells equally well from both PA and PA-c-Myc, shifting the EC_{50} to greater than 3,000 ng/ml.

Cytotoxicity Assay of PA and PA-c-Myc with FP59 against 9E10 Hybridoma Cells

If the PA-c-Myc protein uses the 9E10 antibody to enter and kill hybridoma cells, the addition of the c-Myc peptide should compete with PA-c-Myc binding and thus protect cells from toxin challenge. Alternatively, the PA-c-Myc might enter through the PA receptor. To test for PA

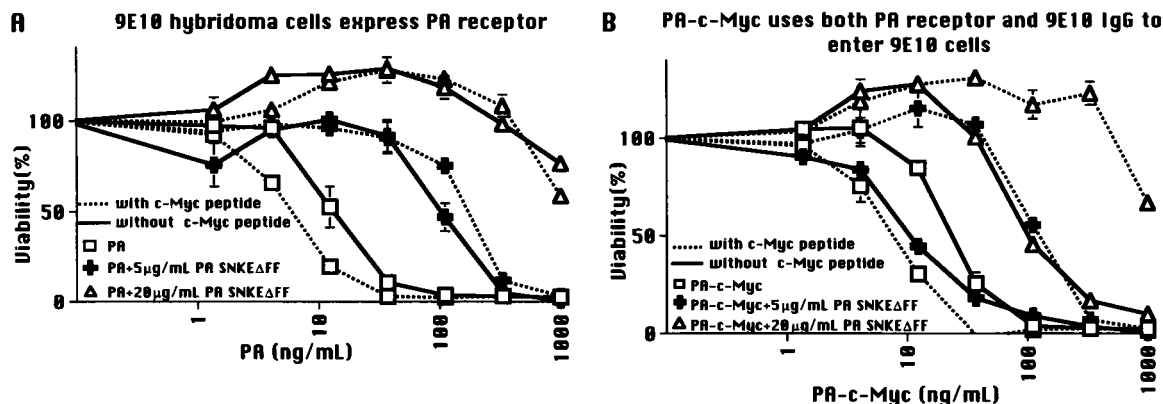


Fig. 4. PA-c-Myc uses c-Myc antibody to enter 9E10 hybridoma cells. PA (A) and PA-c-Myc (B) were serially diluted into 96-well plates in the presence of 100 ng/ml of the LF fusion, FP59, with or without the c-Myc peptide (5.5 μg/ml) as a competi-

tor. Half of the samples also contained the additional competitor PA SNKEΔFF at 0, 5, or 20 μg/ml. After 50–60 hr of toxin challenge, XTT was added to determine cell viability.

receptor, PA was tested on the hybridoma cells using an XTT cytotoxicity assay (Fig. 4A). The cells expressed PA receptor because the PA killed the hybridoma cells with an EC₅₀ of 10–30 ng/ml. In further experiments, the contribution of the PA receptor to the cytotoxicity of PA-c-Myc + FP59 was blocked by the inclusion of the receptor-binding competitive inhibitor of PA, PA SNKEΔFF. The cytotoxicity of PA + FP59 on 9E10 cells could be reduced with 5 and 20 μg/ml of PA SNKEΔFF, shifting the EC₅₀ to greater than 100 and 1000 ng/ml, respectively (Fig. 4 and Table 1). The addition of c-Myc peptide as a competitor to the cytotoxicity assay caused a small increase in potency from 30 to 10 μg/ml. However, this effect was not reproducible and

was attributed to errors introduced during the performance of suspension cell assays.

When PA-c-Myc was tested on the 9E10 cells, the fusion protein killed the hybridoma cells with an EC₅₀ of 50 ng/ml (Fig. 4B). To determine what portion of cell death was caused by entry of the toxin from the c-Myc receptor and what part was caused by entry from the PA receptor, c-Myc peptide and PA SNKEΔFF were added as inhibitors. The PA SNKEΔFF at 20 μg/ml inhibited cytotoxicity of the PA-c-Myc + FP59 to an EC₅₀ of only 200 ng/ml. A concentration of 5 μg/ml of PA SNKEΔFF did not seem to have any effect. The combination of the c-Myc peptide and the PA SNKEΔFF increased the EC₅₀ to greater than 100 and 1000 ng/ml, for the 5

Table 1. Internalization of PA-c-Myc fusion protein mediated by cell surface anti-c-Myc antibodies on 9E10 hybridoma cells

Toxin	EC ₅₀ in ng/mL ^a			
	With Competitor	With c-Myc Peptide ^b	With PA SNKEΔFF ^c	With c-Myc Peptide ^b and PA SNKEΔFF ^c
PA-c-Myc	50	10	100	>1000
PA	30	10	>1000	>1000

^aEC₅₀ is the concentration of toxin required to kill half of the cells compared with untreated controls. EC₅₀ values are interpolated from Fig. 4A and B.

^bc-Myc peptide was used at 5.5 μg/ml.

^cPA SNKEΔFF was used at 20 μg/ml.

and 20 $\mu\text{g/ml}$ of PA SNKE Δ FF, respectively, which is similar to the effect of the PA SNKE Δ FF alone on the PA (see Table 1). When the c-Myc peptide was used alone at 5.5 $\mu\text{g/ml}$, it failed to show a consistent effect on the EC_{50} of PA-c-Myc. As the peptide failed to effect the cytotoxicity of the PA + FP59 toxin, the peptide was determined not to have any nonspecific effects on PA or PA-c-Myc receptor binding. In conclusion, the c-Myc epitope in combination with the PA SNKE Δ FF caused a greater than 10-fold shift in the EC_{50} of PA-c-Myc when tested on 9E10 hybridoma cells, indicating that the PA-c-Myc was using the 9E10 antibody as a receptor to enter the cell.

Discussion

The PA-c-Myc fusion protein was created as a model system to test whether anthrax toxin could be targeted to an alternate receptor. Anti-c-Myc antibodies presented on the cell surface of the hybridoma cell line 9E10 served as the alternate receptor. The addition of the c-Myc epitope to the C-terminus of PA did not interfere with the ability of the fusion protein to bind to the PA receptor. The fusion protein was able to kill RAW cells, which do not express c-Myc receptor, with an EC_{50} similar to that of wild-type PA (Fig. 3). After the endogenous PA receptors on 9E10 cells were specifically blocked by adding the competitive inhibitor PA SNKE Δ FF, the PA-c-Myc still killed the 9E10 cells, indicating that the PA-c-Myc used an alternate receptor, i.e., the 9E10 antibodies, to enter the cell (Fig. 4B). The results show that PA can be redirected to an alternate receptor.

Before testing the fusion protein on the 9E10 cells, immunoassays were used to verify that the fusion protein contained the c-Myc epitope and that the 9E10 hybridoma cell line was presenting antibodies to c-Myc on its cell surface. Analysis of the fusion protein by ELISA showed that the 9E10 anti-c-Myc antibody bound the c-Myc peptide and produced an optical density twice that of the PA-c-Myc. This may be because there was much more c-Myc peptide bound to the plate than PA-c-Myc on a molar basis. The overall results support the conclusion that the c-Myc epitope was attached to the PA-c-Myc fusion protein. Results from the immunoassay with the hybridoma cells verified that the 9E10 hybridoma cells present antibody on the cell surface (Fig. 2).

When PA SNKE Δ FF and the c-Myc peptide were added together to the hybridoma cells, the combination of the two competitors was able to protect the cells from PA-c-Myc toxin challenge, whereas the individual competitors by themselves were only partially effective or ineffective at preventing cell death (Fig. 4B). The PA SNKE Δ FF was less effective as a competitor for PA-c-Myc, compared with the effect of PA SNKE Δ FF on PA, because the PA-c-Myc appeared to use the 9E10 antibody as a receptor to enter the cell as well as the PA receptor. When tested against PA, the PA SNKE Δ FF increased the EC_{50} of PA 1,000-fold, because the PA had no alternative receptor, whereas the PA-c-Myc could use the 9E10 antibody. The experiments clearly indicated that the c-Myc peptide in combination with the PA SNKE Δ FF interfered with the ability of PA-c-Myc to bind to 9E10 cells. No attempt was made to quantitate the effect of the c-Myc peptide alone. As expected, the c-Myc peptide had no effect on PA + FP59 cytotoxicity.

In the present study, c-Myc was fused to PA and targeted to an alternate receptor. In many related studies, bacterial protein toxins have been targeted to alternate receptors using different methodologies. For instance, fusion of diphtheria toxin from *Corynebacterium diphtheriae* to protein A from *Staphylococcus aureus* created a toxin targeted to antibody-coated cells (33). This experiment demonstrated that diphtheria toxin could enter cells by attaching to antibodies instead of the toxin's native receptor. Also, the plant seed toxin, ricin, when fused to monoclonal antibodies against T cells, selectively inhibits the proliferation of T cells when added to a population of peripheral blood mononuclear cells (34). *Pseudomonas* exotoxin fused to single-chain antibodies against interleukin-2 (IL-2) targets IL-2 receptors on human cell lines (35). Alternatively, instead of using antibodies to target a specific cell type, the ligand for the receptor could be used to target the cell. The diphtheria toxin receptor-binding domain was replaced with the 11-amino acid epitope for substance P, thereby targeting substance P receptors on nerve cells (36). A similar strategy was used for *Pseudomonas* exotoxin where several ligands, such as IL-2, IL-4, and insulin growth factor-1, have been fused to the toxin and used to target various cell lines (for reviews on *Pseudomonas* exotoxin, see ref. 37).

Anthrax toxin may have advantages over other single-chain toxins like diphtheria toxin and *Pseudomonas* exotoxin. *Bacillus anthracis* syn-

thesizes as separate proteins the two catalytic components of the toxin and the single receptor-binding component. Thus, while the single-chain toxins mentioned above are intrinsically toxic, the anthrax toxin components are synthesized as independent proteins that are not toxic until combined (3). Because lethal toxin and edema toxin are two-protein toxins, the receptor binding component, PA, can be manipulated independently without affecting the catalytic moieties of the toxins LF and EF. Also, LF can be separately engineered to deliver novel proteins to eukaryotic cells, such as the catalytic domains of tetanus toxin (38), shiga toxin (39), and diphtheria toxin (39, 40). Fusion of the catalytic domains of these toxins to the 254-amino acid N-terminal domain of LF creates proteins that are able to bind PA and use PA to enter cells expressing receptors for PA.

Even though the fusion of c-Myc to PA created a toxin capable of using an alternate receptor, the toxin was still capable of using the native PA receptor. Future studies on PA will focus on the replacement of the receptor-binding domain of PA with cell-specific ligands, such as CD4, IL-2, and single-chain antibodies (as discussed in a recent review, see ref. 41). These fusions can be used to target HIV-infected cells or tumor cells. Alternatively, the receptor-binding domain of PA can be mutated to create a PA that does not bind its own receptor, so that the addition of novel epitopes to the C-terminus of the altered PA will enable the creation of cell-specific anthrax toxins.

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References

- Smith H, Keppie J, Stanley JL (1955) The chemical basis of the virulence of *Bacillus anthracis*. V. The specific toxin produced by *B. anthracis* in vivo. *Br. J. Exp. Pathol.* **36**: 460–472.
- Klimpel KR, Molloy SS, Thomas G, Leppla SH (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. U.S.A.* **89**: 10277–10281.
- Friedlander AM (1986) Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. *J. Biol. Chem.* **261**: 7123–7126.
- Gordon VM, Leppla SH, Hewlett EL (1988) Inhibitors of receptor-mediated endocytosis block the entry of *Bacillus anthracis* adenylate cyclase toxin but not that of *Bordetella pertussis* adenylate cyclase toxin. *Infect. Immun.* **56**: 1066–1069.
- Milne JC, Furlong D, Hanna PC, Wall JS, Collier RJ (1994) Anthrax protective antigen forms oligomers during intoxication of mammalian cells. *J. Biol. Chem.* **269**: 20607–20612.
- Leppla SH (1991) The anthrax toxin complex. In: (ed Alouf JE, Freer JH) *Sourcebook of Bacterial Protein Toxins*. Academic Press, London, pp. 277–302.
- Leppla S (1995) Anthrax toxins. In: (ed Moss J, Iglewski B, Vaughan M, Tu A) *Bacterial Toxins and Virulence Factors in Disease. Handbook of Natural Toxins, Vol. 8* Marcel Dekker, New York, pp. 543–572.
- Beall FA, Taylor MJ, Thorne CB (1962) Rapid lethal effect in rats of a third component found upon fractionating the toxin of *Bacillus anthracis*. *J. Bacteriol.* **83**: 1274–1280.
- Ezzell JW, Ivins BE, Leppla SH (1984) Immunoelectrophoretic analysis, toxicity, and kinetics of *in vitro* production of the protective antigen and lethal factor components of *Bacillus anthracis* toxin. *Infect. Immun.* **45**: 761–767.
- Hanna PC, Kouchi S, Collier RJ (1992) Biochemical and physiological changes induced by anthrax lethal toxin in J774 macrophage-like cells. *Mol. Biol. Cell* **3**: 1269–1277.
- Singh Y, Klimpel KR, Quinn CP, Chaudhary VK, Leppla SH (1991) The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. *J. Biol. Chem.* **266**: 15493–15497.
- Little SF, Leppla SH, Cora E (1988) Production and characterization of monoclonal antibodies to the protective antigen component of *Bacillus anthracis* toxin. *Infect. Immun.* **56**: 1807–1813.
- Perelle S, Gibert M, Boquet P, Popoff MR (1993) Characterization of *Clostridium perfringens* iota-toxin genes and expression in *Escherichia coli*. *Infect. Immun.* **61**: 5147–5156.
- Petosa C, Collier RJ, Klimpel KR, Leppla SH, Lidington RC (1997) Crystal structure of the anthrax toxin protective antigen. *Nature* **385**: 833–838.
- Ramsay G, Evan GI, Bishop JM (1984) The protein encoded by the human proto-oncogene c-myc. *Proc. Natl. Acad. Sci. U.S.A.* **81**: 7742–7746.
- Kolodziej PA, Young RA (1991) Epitope tagging and protein surveillance. *Methods Enzymol.* **194**: 508–519.
- Evan GI, Lewis GK, Ramsay G, Bishop JM (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* **5**: 3610–3616.
- Koskinen PJ, Alitalo K (1993) Role of *myc* amplification and overexpression in cell growth, differentiation and death. *Semin. Cancer Biol.* **4**: 3–12.
- Vastrik I, Makela TP, Koskinen PJ, Klefstrom J,

- Alitalo K (1994) Myc protein: Partners and antagonists. *Crit. Rev. Oncog.* **5**: 59–68.
20. Ryan KM, Birnie GD (1996) Myc oncogenes: The enigmatic family. *Biochem. J.* **314**: 713–721.
 21. DeFranco AL (1992) Tyrosine phosphorylation and the mechanism of signal transduction by the B-lymphocyte antigen receptor. *Eur. J. Biochem.* **210**: 381–388.
 22. Reth M (1992) Antigen receptors on B lymphocytes. *Annu. Rev. Immunol.* **10**: 97–121.
 23. Reth M, Hombach J, Wienands J, Campbell KS, Chien N, Justement LB, Cambier JC (1991) The B-cell antigen receptor complex. *Immunol. Today* **12**: 196–201.
 24. Singh Y, Chaudhary VK, Leppla SH (1989) A deleted variant of *Bacillus anthracis* protective antigen is non-toxic and blocks anthrax toxin action *in vivo*. *J. Biol. Chem.* **264**: 19103–19107.
 25. Welkos SL, Lowe JR, Eden-McCutchan F, Vodkin M, Leppla SH, Schmidt JJ (1988) Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis*. *Gene* **69**: 287–300.
 26. Quinn CP, Dancer BN (1990) Transformation of vegetative cells of *Bacillus anthracis* with plasmid DNA. *J. Gen. Microbiol.* **136**: 1211–1215.
 27. Shulman M, Wilde CD, Kohler G (1978) A better cell line for making hybridomas secreting specific antibodies. *Nature* **276**: 269–270.
 28. Quinn CP, Singh Y, Klimpel KR, Leppla SH (1991) Functional mapping of anthrax toxin lethal factor by in-frame insertion mutagenesis. *J. Biol. Chem.* **266**: 20124–20130.
 29. Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR (1988) Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res.* **48**: 4827–4833.
 30. Arora N, Klimpel KR, Singh Y, Leppla SH (1992) Fusions of anthrax toxin lethal factor to the ADP-ribosylation domain of *Pseudomonas* exotoxin A are potent cytotoxins which are translocated to the cytosol of mammalian cells. *J. Biol. Chem.* **267**: 15542–15548.
 31. Arora N, Leppla SH (1993) Residues 1–254 of anthrax toxin lethal factor are sufficient to cause cellular uptake of fused polypeptides. *J. Biol. Chem.* **268**: 3334–3341.
 32. Leppla SH (1988) Production and purification of anthrax toxin. *Methods Enzymol.* **165**: 103–116.
 33. Madshus IH, Stenmark H, Sandvig K, Olsnes S (1991) Entry of diphtheria toxin-protein A chimeras into cells. *J. Biol. Chem.* **266**: 17446–17453.
 34. Vallera DA, Ash RC, Zanjani ED, Kersey JH, LeBien TW, Beverley PC, Neville, Jr, DM, Youle RJ (1983) Anti-T-cell reagents for human bone marrow transplantation: Ricin linked to three monoclonal antibodies. *Science* **222**: 512–515.
 35. Chaudhary VK, Queen C, Junghans RP, Waldmann TA, FitzGerald DJ, Pastan I (1989) A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin. *Nature* **339**: 394–397.
 36. Fisher CE, Sutherland JA, Krause JE, Murphy JR, Leeman SE, vanderSpek JC (1996) Genetic construction and properties of a diphtheria toxin-related substance P fusion protein: In vitro destruction of cells bearing substance P receptors. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 7341–7345.
 37. FitzGerald D, Pastan I (1991) Redirecting *Pseudomonas* exotoxin. *Semin. Cell Biol.* **2**: 31–37.
 38. Arora N, Williamson LC, Leppla SH, Halpern JL (1994) Cytotoxic effects of a chimeric protein consisting of tetanus toxin light chain and anthrax toxin lethal factor in non-neuronal cells. *J. Biol. Chem.* **269**: 26165–26171.
 39. Arora N, Leppla SH (1994) Fusions of anthrax toxin lethal factor with shiga toxin and diphtheria toxin enzymatic domains are toxic to mammalian cells. *Infect. Immun.* **62**: 4955–4961.
 40. Milne JC, Blanke SR, Hanna PC, Collier RJ (1995) Protective antigen-binding domain of anthrax lethal factor mediates translocation of a heterologous protein fused to its amino- or carboxy-terminus. *Mol. Microbiol.* **15**: 661–666.
 41. Leppla SH, Klimpel KR, Arora N (1994) Development of anthrax toxin-based fusion proteins for targeting of HIV-1-infected cells. In: Fehrenbach F (ed). *Bacterial Protein Toxins*. Gustav Fischer Verlag, Stuttgart, pp. 431–442.