A Mechanistically Relevant Cytotoxicity Assay Based on the Detection of Cellular Green Fluorescent Protein

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ABSTRACT

Cell-based assays for measuring ribosome inhibition by proteins such as the plant toxin ricin are important for characterizing decontamination strategies and developing detection technologies for field use. We report here an assay for ricin that provides a response that is relevant to the mechanism of ricin activity and permits a much faster readout than commonly used assays for cytotoxicity. The assay relies on the response of an engineered reporter cell line that was produced by stably transfecting Vero cells to express green fluorescent protein (GFP) under the control of a cytomegalovirus promoter. The results of the GFP-based assay were compared with assay results from three commercially available cytotoxicity assays. The GFP assay reports a sensitive response to ricin after 6 h of treatment, whereas the other assays require a 24-h incubation. Unlike the other assays, monitoring cellular GFP on a per-cell basis allows detection of reduced ribosome activity before significant cell death occurs, and the results are not convoluted by the numbers of cells being assayed.

INTRODUCTION

Ricin is a type 2 ribosome-inactivating protein belonging to the A-B family of toxins[1,2] and is of particular interest because of its potential use as a biological weapon and the dearth of rapid and sensitive assays that could assist detection and decontamination.[3] Ricin is a toxin derived from the plant *Ricinus communis*, which can be found all over the world and mass-produced with little effort. The ricin toxin inhibits protein synthesis by first binding to cell surface galactose residues and then entering the cell via endocytosis, inactivating ribosomes catalytically and irreversibly.[4] Biochemical assays[5] for ricin activity do not sufficiently measure the multiple processes, namely, cell entry, cytoplasmic transport, and ribosomal inactivation, involved in ricin intoxication. A convenient, cell-based assay that was specific to the action of such a toxin would allow the biological activity of the protein to be assessed and would report the sensitivity of the cell to the receptor-mediated endocytosis and cytoplasmic transport of the toxin. In this study, we report the use of a stably transfected line of Vero cells that expressed green fluorescent protein (GFP) and measured the loss of cellular GFP as an indicator for protein synthesis inhibition by the ribosome inhibitor ricin. The GFP construct used here consisted of the sequence for destabilized enhanced GFP ligated to the promoter sequence for cytomegalovirus (CMV). Under control of the CMV promoter, GFP was constitutively expressed by the cells, and because it contained a proteasome-targeting peptide sequence,[6] it was rapidly degraded with a half-life of approximately 2–3 h.[7,8] Changes in the synthesis rate of GFP due to interference with ribosome activity had a rapid effect on decreasing the cellular GFP levels in affected cells.

We compared this assay, based on quantitative imaging to measure GFP levels in cells over time, with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the MultiTox-Fluor™ (MTox) multiplex cytotoxicity assay (Promega, Madison, WI), and the CellTiter-Glo® (CTG) luminescent cell viability assay (Promega), which are based on measurements of an overall response from a well or plate of cells. These whole-well assays are intended to report cellular enzyme activity, but their results are convoluted by the number of cells present.[9–11] A loss of signal from the assay can be caused by either the reduction of activity of the particular enzyme detected

ABBREVIATIONS: CHX, cycloheximide; CMV, cytomegalovirus; CTG, CellTiter-Glo® (Promega, Madison, WI); FBS, fetal bovine serum; GFP, green fluorescent protein; IC_{50}, 50% inhibitory concentration; MEM, Minimum Essential Medium; MTox, MultiTox-Fluor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NA, numerical aperture. The accepted SI unit of concentration, mol/L, has been represented by the symbol *M* in order to conform to the conventions of this journal.

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by the assay or a reduction in the number of cells in the well after cell death occurs. Furthermore, these assays frequently must be performed after the cells die (*i.e*., 24 h after intoxication) because of continued cell activity during the apoptotic process. The GFP assay, on the other hand, is more specific to the mechanism of action of the toxin because the assay reports more directly on ribosome activity.[12] Therefore, the fluorescence readout is sensitive to the translation of GFP, not to proteins that were previously made and remain in the cell. The assay is also a more rapid indicator of toxin activity for the same reason.

MATERIALS AND METHODS

Preparation of GFP-Expressing Vero Cell Line

The CMV/pd2EGFP-1 construct was obtained by ligating the CMV promoter to the gene for a short half-life destabilized GFP. The sequence encoding the immediate early CMV promoter was removed from the pQCXIP vector (Clontech Laboratories, Palo Alto, CA) using restriction enzymes *Age*I and *Bgl*II and subcloned into vector pd2EGFP-1 (Clontech Laboratories) containing a gene encoding a destabilized variant of enhanced GFP, with a half-life of 2 h as reported by the manufacturer. Vero cells (CCL-81, American Type Culture Collection, Manassas, VA) were maintained in Minimum Essential Medium (MEM) Alpha (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin (MEM-FBS medium) and maintained in a humidified 5% CO_2 balanced-air atmosphere at 37°C. Cells were transfected with the construct using an Amaxa (Gaithersburg, MD) electroporator using solution V (proprietary solution, Amaxa) and program V-001 (cell type-specific program, Amaxa) according to the manufacturer's protocol (Amaxa Inc.). In brief, 500,000 cells suspended in solution V were incubated with 2 µg of construct or positive control vector provided by the manufacturer (Amaxa) or no DNA and electroporated with program V-001. Twenty-four hours after transfection, cells were switched to growth medium containing 800–1,200 µg/ml geneticin (Invitrogen) for selection of stable transfectants.[13] Colonies were visualized under fluorescence microscopy and scored based on degree of brightness and fluorescence homogeneity. Individual clones were subjected to two rounds of selection using sterile cloning cylinders and visualized under phase microscopy to ensure the purity of the clone. In brief, cloning cylinders were carefully placed around the colony and harvested by trypsinization. Clones were expanded in individual wells of a 24-well plate containing growth medium with geneticin. The selection agent was eventually removed from the growth medium to yield stable transfectants. The cloning process results in a genetically identical population of cells expressing GFP.

Cell Culture, Toxin Preparation, and Plate Preparation

The stably transfected, monoclonal Vero cells were then maintained in MEM-FBS medium and maintained in a humidified 5% CO_2 balanced-air atmosphere at 37°C. For the GFP expression stability experiments, three separate T-25 flasks were maintained. Before measurement of the GFP intensity by flow cytometry, cells were removed from tissue culture polystyrene flasks with 500 µl of a 0.25% trypsin/0.53 m*M* EDTA solution (American Type Culture Collection). Trypsin activity was quenched by the addition of an equivalent volume of growth medium (Dulbecco's Modified Eagle's Medium plus 10% FBS).

Flow cytometric analysis of the cellular populations were performed with a Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA), equipped with a 15 mW output laser, tuned to 488 nm. The green fluorescence was collected after a 525/40 nm band pass filter. Samples were gated on forward scatter versus side scatter to exclude debris and clumps and to select cell populations of interest. The GFP-expressing Vero cells and the non-transfected control Vero cells were analyzed separately, and 10,000 events were collected for each sample.

Before each GFP-based imaging assay for ribosome inhibition, the GFP-expressing Vero cells were seeded at 1×10^4 cells per well in 80 µl of the growth medium into 96-well imaging plates (catalog number 353219, BD Falcon, Franklin Lakes, NJ). Ricin (catalog number L-1090, 5 mg/ml, Vector Laboratories, Burlingame, CA) was serially diluted in the MEM-FBS medium to achieve the desired concentrations. Cycloheximide (CHX) (Sigma, St. Louis, MO) was diluted in the MEM-FBS medium from a 100 mg/ml stock solution in dimethyl sulfoxide. The next day, 20 µl of CHX or ricin (Vector Laboratories) solution was added to each of the wells such that the desired final concentrations were reached. The plates were then maintained in a humidified 5% CO_2 balanced-air atmosphere at 37°C. Ten minutes prior to imaging, 100 µl of a 4 µg/ml Hoechst 33342 solution was added to each well, resulting in a final concentration of 2 µg/ml. Immediately before imaging, the wells were aspirated, and the medium was replaced with Dulbecco's phosphate-buffered saline containing glucose. For the kinetic assays, a single plate was seeded for the CHX time points, and a single plate was seeded for the ricin time points. Either CHX or ricin was added to three wells of each plate at 10 h, 8 h, 6 h, 4 h, 2 h, and immediately prior to imaging. The final concentrations of CHX and ricin were 100 µg/ml (360 µ*M*) and 5 ng/ml (76 p*M*), respectively.

Ricin solutions were prepared in a Biosafety Level 2 laboratory. Solutions containing ricin were inactivated at the end of the experiments by contacting with 10% sodium hypochlorite (bleach) for at least 30 min.

Imaging Cytometry

Image collection was performed on a Zeiss 200M inverted microscope (Carl Zeiss, Thornwood, NY) equipped with an automated

stage (Ludl, Hawthorne, NY), a mercury metal-halide fluorescence excitation source (EXFO, Mississauga, ON, Canada), and a CoolSNAP HQ CCD camera (Roper Scientific, Tucson, AZ). To qualitatively compare the cell morphology and GFP intensity of the GFP-expressing Vero cells and the non-transfected controls, phase-contrast and GFP fluorescence images were collected from cells cultured in T-25 flasks. The phase-contrast and GFP fluorescence images were acquired with a 10× objective, numerical aperture (NA) 0.3 using a 0.05 s or 0.4 s exposure time, respectively.

To quantify the GFP response to ricin or CHX, four fields were imaged in each well of a 96-well plate (approximately 7% of the well) with 12-bit depth and 2 × 2 pixel binning using a 10× objective, NA 0.45. At each field, nuclear and GFP fluorescence images were collected using a standard Hoechst filter set (excitation 360/40, part number 38086; dichroic beamsplitter set 84000; emission 460/50, part number 42919 [Chroma, Rockingham, VT]) and a standard GFP filter set (excitation 470/40, part 51359; dichroic beamsplitter set 84000; emission 525/50, part 42345 [Chroma]). The microscopy hardware and image acquisition were controlled by modular routines within ISee image acquisition software (ISee Imaging Systems, Raleigh, NC). The following operations were performed sequentially at each field: (1) an autofocus routine was performed with the Hoechst filter set in place, (2) a nuclear image was collected with a 0.25 s acquisition time, (3) a GFP fluorescence image was collected with a 0.4 s acquisition time, and (4) the stage position was moved to the next field.

All image analysis was performed using combinations of standard algorithms in NIH ImageJ (http://rsb.info.nih.gov/ij/). First, the proprietary ISee image files were converted to TIFF format using homebuilt ImageJ routines. Prior to processing, a background image was subtracted from each nuclear image. The background image was generated by taking the median pixel value from 12 total images, corresponding to four fields acquired from three different blank wells. After preprocessing, an empirically set threshold was used to initially segment nuclei from the background. The same threshold value was used for all images. Then the following ImageJ algorithms were performed on each thresheld, binary image: (1) "Fill Holes," to remove interior holes for nuclear objects, (2) "Watershed," to separate overlapping nuclei, and (3) "Particle Analyzer," for object counting with a minimum size threshold of 50 pixels. The GFP intensities for each field were computed by calculating the total integrated intensity over the entire field. For all of the tabulated and plotted data, a background intensity was subtracted from each of the GFP intensities. The background intensity was computed by taking the average total integrated intensity of 12 images, corresponding to four fields acquired from three different blank wells.

Cytotoxicity Assays

Three commercially available cytotoxicity assays were used to measure the cytotoxic response to ricin: the MTT assay based on the detection of mitochondrial reductase,[14] the MTox assay (catalog number G9200) based on the detection of cellular protease activity (see Promega's description), and the CTG assay (catalog number G7571) based on the detection of ATP (see Promega's description). Vero cells were seeded in opaque-walled clear-bottom 96-well tissue culture plates at 6 × 10^3 cells per well (100 µl per well) and placed in the incubator overnight at 37°C with 5% CO_2. Ricin was added (20 µl) to triplicate wells and placed in the incubator for 22–24 h. MTT reagents (catalog number 30-1010k, American Type Culture Collection) were added according to the manufacturer's protocol. In brief, 12 µl of MTT reagent was added to each well and incubated for 2 h in the dark (or until intracellular crystals were visible), followed by the addition of 100 µl of detergent per well. The plate was incubated overnight at 37°C in the dark, and absorbance was read at 570 nm using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The MTox assay was performed according to the manufacturer's protocol. Fluorescence was measured using the plate reader at 400 nm excitation/505 nm emission for viability and 485 nm excitation/520 nm emission for cytotoxicity after incubation with reagents for 90 min. The ratio of the viability reading to the cytoxicity reading was used for each well. The CTG assay was performed according to the manufacturer's protocol. Luminescence was measured after a 10-min incubation using the SpectraMax M2 plate reader.

Data Analysis

The 50% inhibitory concentration (IC_{50}) values for all assays were determined by fitting the measured parameter versus ricin concentration to the Hill equation[15,16] using four free parameters with Igor Pro (WaveMetrics, Inc., Lake Oswego, OR). The reported errors for the IC_{50} values are the estimated standard deviations from the fitting routine. In the fitting procedure, the standard deviations calculated for each of the triplicate wells were used as weights for each data point and in calculating the fitting error. Statistical significance was ascertained using Student's *t* test.

RESULTS

GFP Expression by Stably Transfected Vero Cells

The strong expression of GFP in transfected cells is the basis of this cell assay for detecting ribosome inhibition. The relative fluorescence intensities of the transfected Vero cells used in this assay are illustrated by the representative fluorescence image in *Fig. 1B* compared to a corresponding image acquired under identical microscope settings from the non-transfected Vero cells shown in *Fig. 1D* and by flow cytometry (*Fig. 1E*), which indicates that the transfected cells were approximately 65-fold brighter than the control cells. The phase-contrast images in *Fig. 1A* and *C* suggest that, qualitatively,

the transfected cells appear to be morphologically similar to the non-transfected cells, and measurement of doubling times for those cells indicated no significant difference between the two cell lines. The transfected GFP expressing cell line had a doubling time of 19.8 ± 1.1 h, which was comparable to the doubling time of 19.3 ± 1.9 h for the non-transfected control Vero cell line. This suggests that the GFP transfection has not substantially altered the proliferative capacity of the original cells, and the culture can be easily expanded to large numbers suitable for screening in assays. The stability of GFP expression in the transfected cell line was quantified by flow cytometry (*Fig. 1F*). These data indicated that the mean GFP intensity of the population was stable over at least eight passages (passages 19–27).

The expression of GFP by the Vero cells apparently did not alter the cytotoxic response of the cells to ricin. The cytotoxic response of the non-transfected Vero cell line is compared with that of the GFP-expressing cell line in *Fig. 2A*. The data were acquired after 24 h in contact with ricin using the MTox assay and indicate that the cytotoxic responses over concentrations of ricin ranging from 0 to 20 ng/ml were not significantly different for the two cell lines.

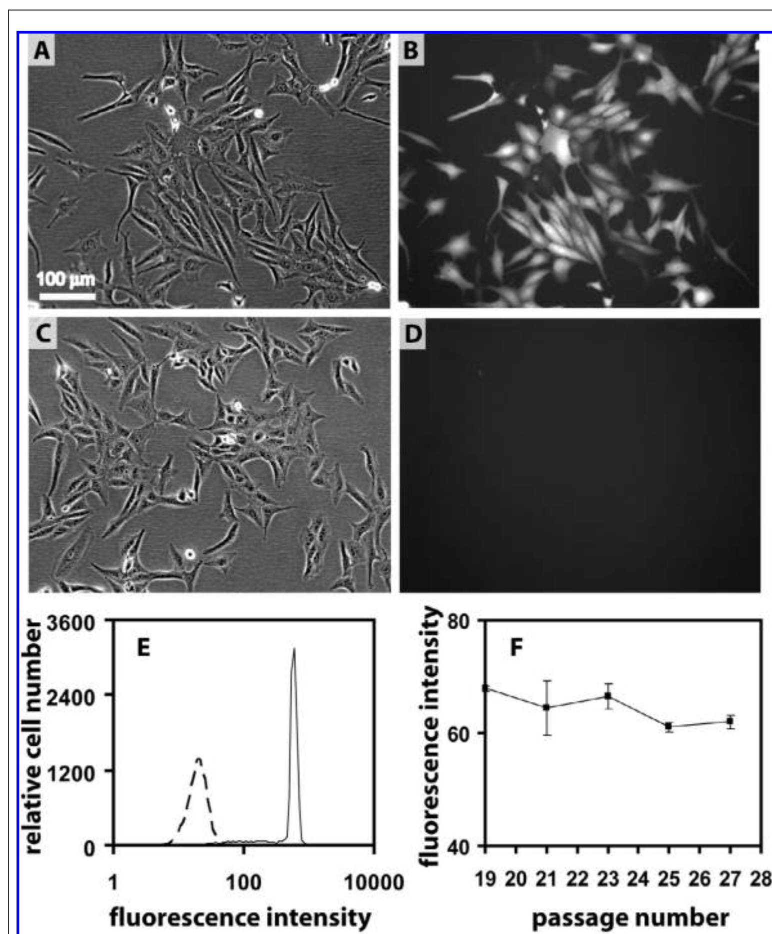

Fig. 1. Characterization of the Vero cell line expressing destabilized GFP driven by the CMV promoter. (**A**) Phase-contrast and (**B**) GFP fluorescence microscopy images of the stably transfected Vero cell line. (**C**) Phase-contrast and (**D**) GFP fluorescence microscopy images of the non-transfected Vero cell line. (**E**) Flow cytometry histograms comparing the intensity of the non-transfected Vero cell line (dashed line) with the GFP-expressing cell line (solid line). (**F**) Flow cytometry data showing the mean fluorescence intensity of the GFP-expressing cell line plotted against the passage number. Fluorescence intensities are reported in arbitrary fluorescence units. Each data point is the average intensity ± standard deviation ($n = 3$, three different GFP-expressing Vero cell cultures maintained in separate flasks).

The responses of the non-transfected cells to ricin as determined by the three cytoxicity assays—the MTT assay, the MTox assay, and the CTG assay—are shown in *Fig. 2B*. All three assays were conducted using 6×10^3 cells per well and 24-h contact with ricin. Under these conditions, all of the assays were sensitive to ricin. The different assays provided somewhat different IC_{50} values: 0.28 ± 0.03 ng/ml, 0.14 ± 0.01 ng/ml, and 0.40 ± 0.03 ng/ml for the MTT assay, the MTox assay, and the CTG assay, respectively. After 24 h of exposure to ricin, inspection of the plate by microscopy indicated that cells clearly decline in number for concentrations of ricin >1 ng/ml.

GFP Intensity as an Indicator of Protein Synthesis Inhibition

In *Fig. 3*, data collected from the GFP-expressing cells in response to inhibition of protein synthesis are shown. The kinetics of the response to treatment with ricin were compared with the response to CHX, a small molecule that inhibits protein synthesis by blocking translational elongation and is known to work rapidly and reversibly.[17,18] The mechanism of protein synthesis inhibition by ricin is different. Ricin enters the cell by endocytosis, which is expected to be slow compared to CHX, and then inactivates ribosomes catalytically and irreversibly.[4] To compare the response to these agents, cells were treated with 100 µg/ml (360 µ*M*) CHX or 5 ng/ml (76 p*M*) ricin for the indicated time prior to imaging. Representative images showing GFP fluorescence and nuclear staining after 0 h and 6 h of contact with CHX are shown in *Fig. 3A–D*. Similar images were collected for the ricin treated cells (data not shown).

Figure 3E shows the response of cellular GFP intensity in live cells over time in response to CHX and ricin. The data shown were the result of integrating the GFP intensity over the entire imaged field. The GFP intensity decreases to 62% ($P < 0.002$) of its initial intensity 2 h after the addition of

CHX, indicating the sensitivity of GFP intensity to ribosome inhibition. In contrast, the GFP intensity in the cells exposed to ricin did not change significantly until the cells have been in contact with ricin for 6 h or longer. This observation is consistent with the lag time involving ricin binding to receptors, endocytosis, and transport through the cytoplasm before reaching the ribosome.[19]

The images of the nuclear staining provided a method to determine the number of cells in the field and to normalize the GFP intensity data to the number of cells on the plate. *Figure 3F* shows the relative change in the number of nuclei determined from the nuclear images at each time point plotted against the time the cells were in contact with CHX or ricin. The data in *Fig. 3F* indicate a trend of decreasing cell density with increasing contact time for both CHX and ricin. The decreasing number of cells on the plate with increasing contact with these toxins shows that even at relatively short times, either cellular proliferation was affected, or some cells died and detached from the plate. *Figure 3G* shows the GFP intensity data normalized by the nuclear count, plotted against contact time with CHX or ricin. Because the change in numbers of cells over time was small, the normalized trends for both CHX and ricin are similar to the non-normalized data shown in *Fig. 3E*. These data show that for short assay times, the change in GFP intensity in cells was greater than the change in the number of cells adhered to the plate.

GFP Intensity Provides a Rapid Indication of Ribosome Inhibition by Ricin

Figure 4 shows the change in cellular GFP intensity after 6 h as a function of ricin concentration from 0 to 50 ng/ml. After 6 h, concentrations of ricin greater than 1 ng/ml resulted in a significant reduction in the GFP intensity as indicated by the plot in *Fig. 4A*. As shown in *Fig. 4B*, the number of cells on the plates, as determined from nuclear staining, indicates no significant decrease in the number of adherent cells after 6 h for all concentrations of ricin treatment. The well-to-well variability in cell number observed in wells not exposed to ricin (indicated as 0 h) was likely due to pipetting errors and inhomogeneous cell seeding. As a result of the negligible change in cell number in 6 h of exposure to ricin, normalizing the GFP data by numbers of nuclei (as shown in *Fig. 4C*) had little to no effect on the apparent response of cells to ricin. Therefore, at short time points where nuclear number is unaffected, the nuclear normalized GFP fluorescence measurement had no advantage over the GFP fluorescence measurement alone.

Figure 4D is a plot of the results of the MTT assay, the MTox assay, the CTG assay, and the normalized GFP data at 6 h after addition of ricin. In contrast to the GFP-based assay, which indicates that cells were clearly responding to the ricin treatment within this time, no clear trends are apparent for the MTT assay and the CTG assay. The MTox assay response shows a loss of signal with increasing ricin concentration, and the signal measured at the highest ricin concentration, 55.8 ng/ml, was approximately 20% less than the untreated control. Since the MTox assay did not achieve 50% inhibition, an IC_{50} value was not calculated. The IC_{50} value for the GFP-based assay, determined by fitting the data in *Fig. 4D*, was 1.8 ± 0.3 ng/ml. Even though the MTox assay might be used at 6 h because there is a detectable response, the MTox assay requires additional reagents and two measurements (viability and cytotoxicity), whereas the GFP-based assay needs one measurement at 6 h. The MTT assay and the CTG assay did not show any measurable response. Mechanistically, this indicates that the 6-h ricin treatment does not alter the per cell enzyme activities measured by the MTT assay (based on the detection of mitochondrial reductase activity) or the CTG assay (based on the detection of available ATP).

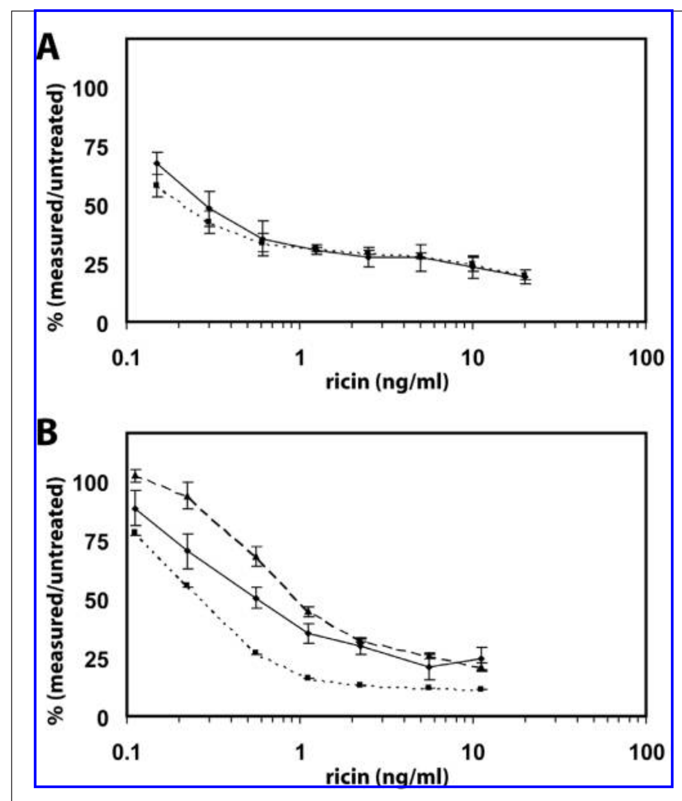

Fig. 2. (**A**) Cytotoxicity data acquired using the MTox assay that compares the response of the non-transfected Vero cell line (◆) with the GFP-expressing cell line (■) after 24 h of contact with ricin. (**B**) Data collected from three different conventional cytotoxicity assays—the MTT assay (◆), the MTox assay (■), and the CTG assay (▲)—after exposure to ricin for 24 h in the parent Vero cell line (non-transfected). Each data point is the average ± standard deviation (n = 3) from triplicate wells.

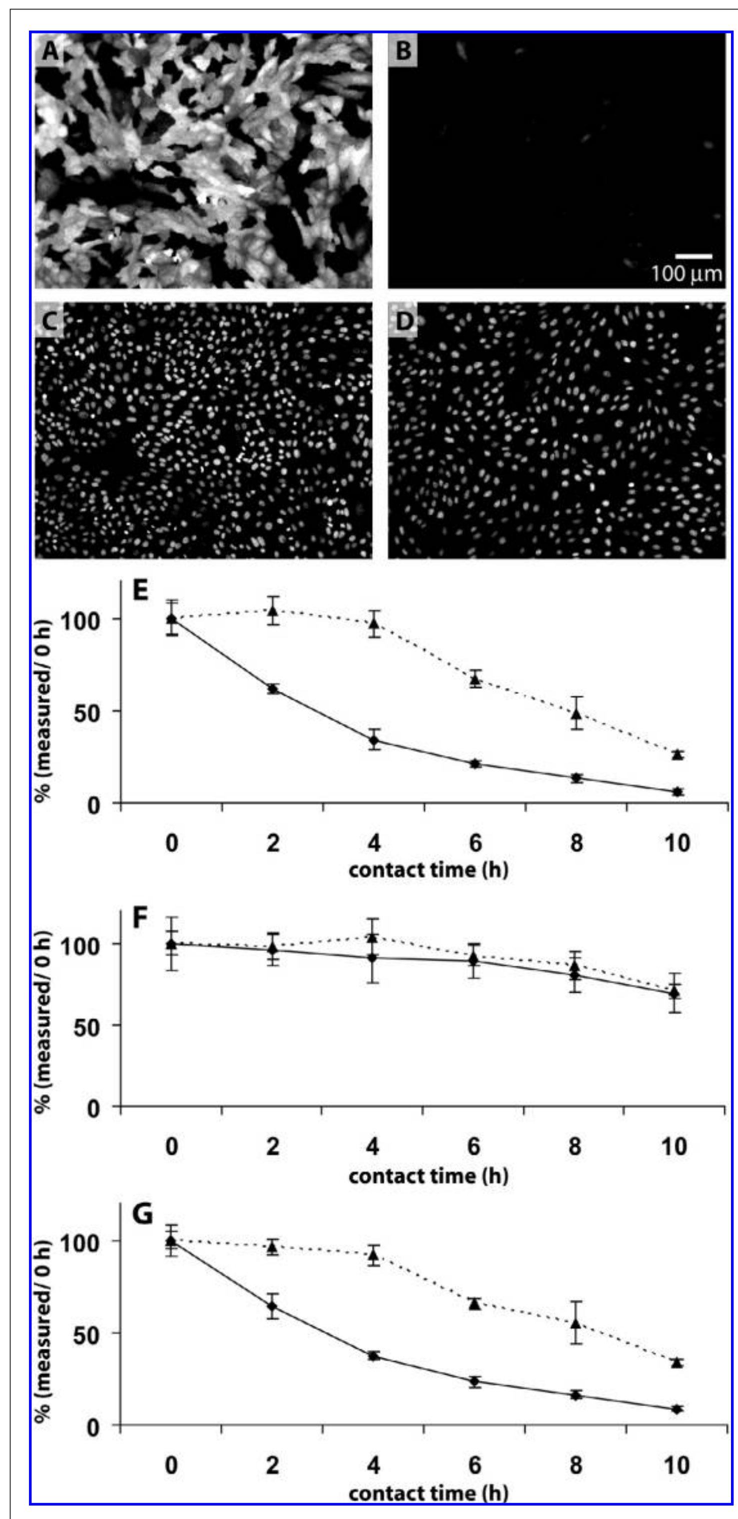

Fig. 3. Representative (**A** and **B**) GFP images and (**C** and **D**) Hoechst 33342-stained nuclear images. (A and C) Corresponding GFP and nuclear images acquired from control cells not incubated with CHX. (B and D) Corresponding GFP and nuclear images acquired from cells incubated with CHX for 6 h. All of the nuclear images and all of the GFP images were collected under identical imaging conditions and are displayed with the same contrast and brightness settings. (**E**) GFP intensity, (**F**) number of nuclei/mm^2, and (**G**) GFP intensity normalized to numbers of nuclei. (E–G) are plotted as relative change to the 0 h control in response to exposure to 100 µg/ml (360 µ*M*) CHX (◆) or 5 ng/ml ricin (76 p*M*) (▲). For the plots in (E–G), each data point is the average ± standard deviation (*n* = 4).

GFP-Based Assay Response at 18 h

Data from the GFP expression assay after 18 h of contact with ricin concentrations are shown in *Fig. 5*. The calculated IC_{50} values determined from the data in *Fig. 5A*, which were not normalized for cell number, and the data in *Fig. 5C*, which were normalized for cell number, were 0.23 ± 0.03 and 0.25 ± 0.03 ng/ml, respectively. These values are summarized in *Table 1*, where all of the IC_{50} values calculated in this study have been tabulated. It is important to note that the GFP intensity was approaching background levels for ricin concentrations >1 ng/ml and that few cells remained attached to the plate for concentrations >10 ng/ml as illustrated by the nuclear staining data in *Fig. 5B*. Normalizing the GFP intensity to cell number for these concentrations is problematic because the GFP intensity is divided by a small number (approaching 0) of cells.

DISCUSSION

There are several advantages in using an engineered and stably transfected reporter cell line and GFP degradation as a readout for protein synthesis inhibition. First, the ribosome inhibition assay based on the detection of GFP can be performed in live cells and monitored over time, which reduces experimental uncertainty. Second, unlike a similar approach using a luciferase expressing cell line,[20] no additional reagents are required, which makes it a convenient assay and also reduces measurement uncertainty associated with pipetting errors. The response can easily be quantified with microscopic imaging, as shown here, or by using flow cytometry. Most importantly, the GFP expression assay responds more rapidly than conventional cytotoxicity assays because the GFP expression reports more directly on the activity of the ribosome. The interpretation of the assay is simplified because the reduction in signal from the loss of ribosome activity is not confounded by a loss in cell number at longer times.

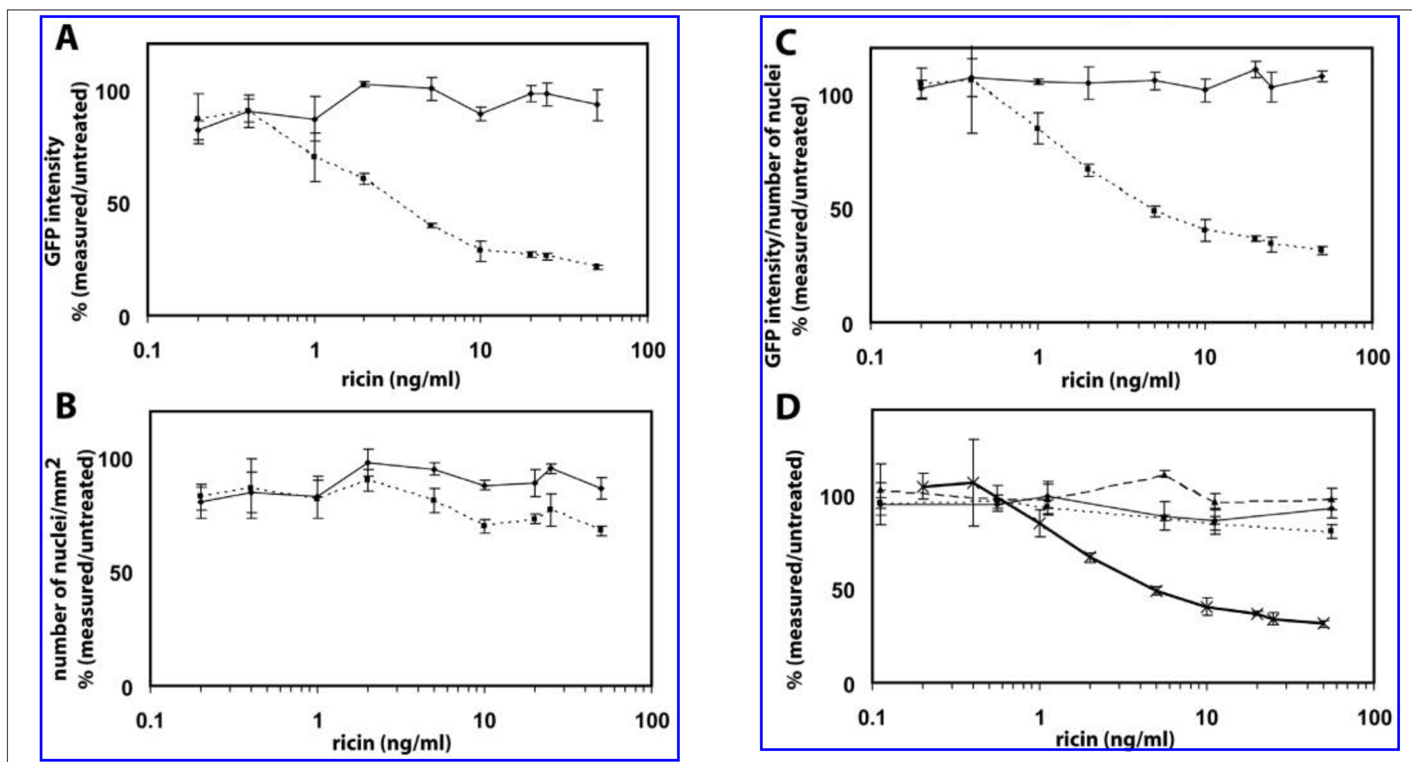

Fig. 4. GFP intensity and nuclear counts in transfected GFP Vero cells at 0 h (■) and 6 h (◆) after exposure to ricin: (**A**) GFP intensity, (**B**) number of nuclei/mm², and (**C**) GFP intensity normalized to numbers of nuclei plotted as relative change to the untreated control. (**D**) Data collected from three different conventional cytotoxicity assays—the MTT assay (◆), the MTox assay (■), and the CTG assay (▲)—after exposure to ricin for 6 h in the parent Vero cell line (non-transfected). The data from (C) are replotted in (D) for comparison (×). Each data point is the average ± standard deviation (*n* = 3).

The sensitivity of this assay was in part due to the high levels of fluorescence of GFP in cells, which provided the dynamic range that enables the sensitive detection of the reduction in GFP fluorescence intensity. The degradation of GFP was a rate-limiting step for generating detectable change in intensity. The destabilized GFP used in this study had a half-life of approximately 2–3 h.[7] If the GFP were more rapidly degraded, it would result in a larger reduction in fluorescence intensity in shorter times; however, it might also require increased levels of synthesis to reach the same initial cellular intensity. The data shown in *Fig. 3E* indicated that CHX activity could be detected at 2 h for a toxin that rapidly enters the cells. These data showed that an additional time of approximately 4 h was required to detect ricin. This 4 h was most likely due to the time required for the uptake and processing of ricin in the cells.[4,19] This assay allows the accurate and rapid determination of the kinetics of the cytotoxicity, which provides additional valuable information about the mechanism of cytotoxicity. Comparing the time course of the GFP response between two different toxins could be used to determine differences in rates of uptake, cytoplasmic transport, and ribosome inhibition. For example, a toxic compound might begin reducing ribosome activity immediately but act on the ribosome with relatively slow kinetics. The reduction in GFP intensity would be immediate but with a slow decay. Alternatively, the compound might require more time for internalization and transport, but act on the ribosome with fast kinetics. The reduction in GFP would begin after an incubation period, and then the decay in intensity would be more rapid.

Another advantage of the GFP expression assay presented here was that a nuclear stain was used to count cells. This allowed the GFP intensity to be normalized to cell number for each field, and differences in cell number caused by changes in proliferation or cell detachment could be identified. Staining and counting nuclei are not possible in ribosome activity assays based on uptake of radiolabeled amino acids, or with the ribosome inhibition assay based on luciferase.[20] Because single cells expressing GFP can be identified, it may be possible to identify cells intoxicated with ricin based on a

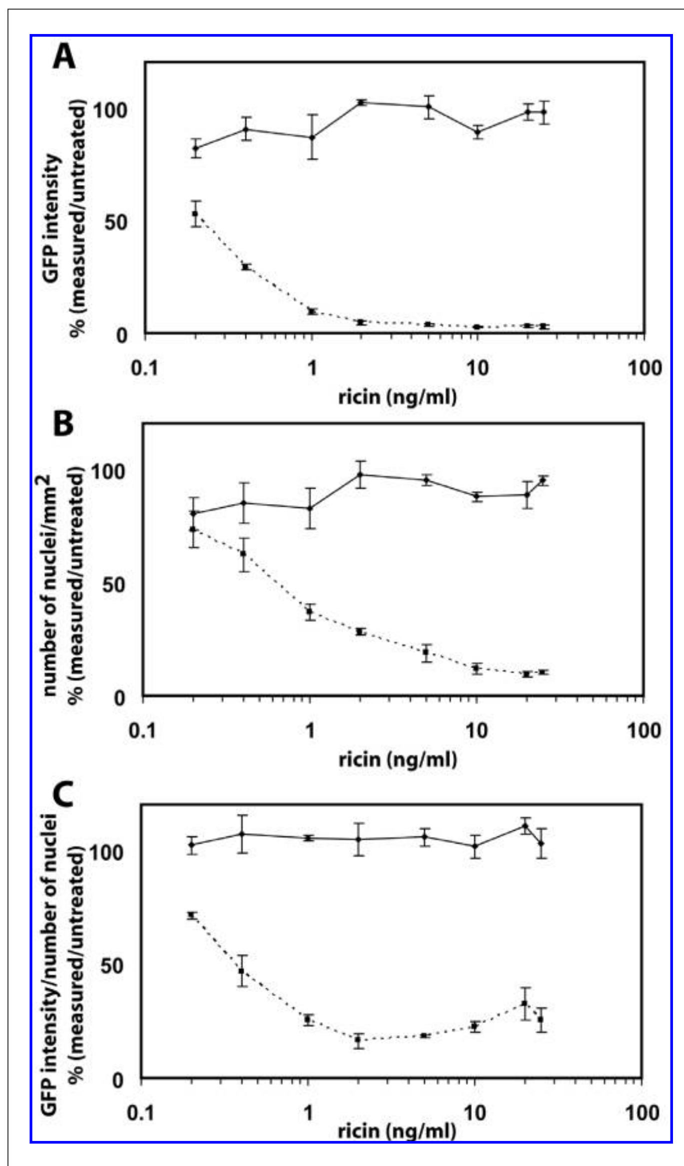

Fig. 5. GFP intensity and nuclear counts in transfected GFP Vero cells at 0 h (◆) and 18 h (■) after exposure to ricin: (**A**) GFP intensity, (**B**) number of nuclei/cm^2, and (**C**) GFP intensity normalized to numbers of nuclei plotted as relative change to the untreated control. Each data point is the average ± standard deviation ($n = 3$).

decrease in cellular GFP. Cells that are not intoxicated would presumably continue to express GFP.

In samples collected for bioterrorism analysis, the GFP-based assay could be used as a neutralization assay in which ricin biological activity is neutralized by an anti-ricin immunoglobulin G, for example. Because other factors in field samples could inhibit translation of GFP, this would be a simple way to identify ricin.

CONCLUSIONS

We have demonstrated a GFP expression assay for monitoring ribosome inhibition by small molecule inhibitors and protein toxins. The GFP-based assay described here is convenient, specific for ribosome inhibition, and more rapid when compared with conventional cytotoxicity assays such as the MTT assay, the MTox assay, and the CTG assay. The assay provided an IC_{50} for ricin of 1.8 ± 0.3 ng/ml at 6 h and 0.25 ± 0.03 ng/ml at 18 h. The effect of ricin on GFP intensity occurred before significant cell death had occurred, so the measurement was not confounded by a significant reduction in cell number. The assay provided a limit of detection for ricin of 1 ng/ml (P = 0.02) after only 6 h of exposure. The assay will be useful for evaluating the effect of decontamination strategies for inactivating biological toxins such as ricin,[21] where activity, not just presence of protein, is the critical measurement.

ACKNOWLEDGMENTS

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Table 1. Summary of Calculated IC_{50} Values

Time (h)	Assay					
	GFP	Nuclear stain	GFP/nuclear	MTT	MTox	CTG
6	1.8 ± 0.3	a	4.0 ± 1.5	a	a	a
18	0.23 ± 0.03	0.5 ± 0.1	0.25 ± 0.03	b	b	b
24	b	b	b	0.3 ± 0.2	0.19 ± 0.03	0.5 ± 0.1

The data are presented as best fit IC_{50} values (in ng/ml) ± the standard deviation calculated as described in Materials and Methods.
[a]Fifty percent inhibition by ricin was not achieved, and a reasonable fit to the data was not obtained.
[b]Data not collected.

DISCLOSURE STATEMENT

Certain commercial products are identified in order to adequately specify the experimental procedure; this does not imply endorsement or recommendation by the National Institute of Standards and Technology.

No competing financial interests exist.

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