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Sulforhodamine B Assay to Determine Cytotoxicity of *Vibrio vulnificus* Against Human Intestinal Cells

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Abstract Sulforhodamine B (SRB) assay is a rapid, sensitive, and inexpensive method for measuring cell proliferation and chemosensitivity. However, the lactate dehydrogenase (LDH) release assay is generally used to measure cytototoxicity of infectious microorganisms against host cells. In this study, we investigated the possibility of applying the SRB assay to determine cytotoxicity for infectious microorganisms, and compared the results with those obtained by the LDH release assay. We used *Vibrio vulnificus* as a model of infectious microorganisms. The SRB assay showed that *V. vulnificus* strongly induced cytotoxic activity against human intestinal cells, Caco-2 and INT-407 cells. The degree of cytotoxicity closely correlated with infection time and number ratios of *V. vulnificus* to intestinal cells (MOI, multiplicity of infection). Furthermore, cytotoxicity values obtained by SRB assay correlated well with results obtained by the LDH release assay, and both assays gave a linear response with respect to MOI. Heat-inactivation of *V. vulnificus* for 35 min at 60°C did not induce cytotoxic activity, indicating that viability of *V. vulnificus* is crucial for cytotoxic activity against intestinal cells. Although both assays are suitable as cytotoxicity endpoints, the SRB assay is recommended for measuring cytotoxicity of infectious microorganisms against host cells because of its significantly lower cost and more stable endpoint than the LDH release assay.

Key words: Cytotoxicity, *Vibrio vulnificus*, intestinal cells, SRB assay

Infection of virulent microorganisms causes cytolysis of host cells *via* several distinct mechanisms. For examples, *Vibrio vulnificus*, a causative agent of food-borne diseases including life-threatening septicemia and possibly gastroenteritis, and seriously induced cytolysis by several virulent factors such as hemolysin, elastase, and other exotoxins [1, 5, 18]. Therefore, it is important to accurately determine the cytotoxicity of infectious microorganisms against host cells, including intestinal cells and immune cells, and also to identify novel virulent factors with cytotoxic potential from infectious microorganisms.

Cell cytotoxicity is classically evaluated by quantification of plasma membrane damage. The need for sensitive, quantitative, reliable, and automated methods to precisely determine cell death led to the development of several standard assays for quantification of cellular viability. In this study, we used two different cytotoxicity assays: the lactate dehydrogenase (LDH) release assay and sulforhodamine B assay. The LDH assay is based on the measurement of cytoplasmic LDH activity released by damaged cells [14, 16]. LDH is a stable cytoplasmic enzyme present in all cells, and is rapidly released into the cell culture supernatant upon damage of the plasma membrane. The LDH activity is enzymatically determined: In the first step, NAD^+ is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. In the second step, the catalyst transfers H/H^+ from $NADH/H^+$ to tetrazolium salt which is reduced to formazan, measurable at 490 nm by a microtiter plate spectrophotometer.

The sulforhodamine B (SRB) protein staining assay was originally established for the *in vitro* measurement of cellular protein content of adherent and suspension cultures by Skehan *et al.* [17]. Subsequently, it was adopted for routine use as an *in vitro* antitumor screen by the National Cancer Institute. The dye binds to basic amino acids of cellular proteins, and colorimetric evaluation provides an estimate of total protein mass which is related to cell number. The method has replaced the tetrazolium-based assays, because of a number of advantages including better linearity, higher sensitivity, a stable endpoint that does not require time-sensitive measurement, and lower cost [12, 13].

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In this study, we have studied the possibility of applying the SRB assay to determine cytotoxicity caused by infectious microorganisms and compared the results with those of the LDH release assay. *V. vulnificus* was used as a model microorganism to induce cell cytotoxicity, and human intestinal Caco-2 and INT-407 cells were used as target cell-lines of *V. vulnificus* infection. Both cell-lines are generally used in the studies of cytotoxicity and pathogenesis of infectious microorganisms, especially enteropathogenic microorganisms such as *Vibrio*, *Shigella*, and *Escherichia* [2, 19, 20].

MATERIALS AND METHODS

Cell lines and Reagents

Human intestinal epithelial cell-lines, Caco-2 cell and INT-407 cell, were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, U.S.A.) and antibiotics (1% penicillin/streptomycin). All other reagents and solutions used in this study were from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.).

Bacterial Strain and Growth Conditions

V. vulnificus strain ATCC 29307 used in this study was purchased from ATCC and cultured as previously described [9]. For infection experiments, bacteria were grown overnight at 37°C in Luria-Bertani medium supplemented with 2.0% NaCl (LBS), and diluted to 0.8 OD_{600} (approximately 6×10^8 cfu/ml) in LBS, and then centrifuged and resuspended in complete MEM without antibiotics prior to addition to epithelial cells. In some experiments, bacterial suspension was subjected to heat treatment at 60°C for 35 min. Bacterial concentrations were confirmed by viable-cell count on LBS agar plates after serial dilution in phosphate-buffered saline and overnight culture at 37°C.

Morphologic Studies

Microscopic observation of *V. vulnificus*-infected or uninfected intestinal cells was carried out as previously described [2, 7]. In brief, human intestinal epithelial cells were cultured onto 18-mm-diameter coverglasses in the 3.0 cm diameter tissue culture dish for 24 h. Afterward, the cells were infected with *V. vulnificus* at a multiplicity of infection (MOI) of 10 for 3 h or not infected. The slides were fixed with methanol and dried. The slides were stained with Giemsa staining solution for 20 min and rinsed in deionized water, air-dried, and observed under a microscope with a camera. The stained cells were assessed for size, regularity of the cell margin, and morphological characteristics of the nuclei.

Infection Protocol

Human intestinal cells were infected with *V. vulnificus* as previously described [15]. In brief, intestinal cells were grown in 75-ml culture flasks at 37°C in 5% CO_2 incubator. Cells (2×10^4 cells/well) were seeded onto 96-well plates for 24 h in media without antibiotics. Prior to infection, bacteria were centrifuged at 5,000 rpm for 3 min, resuspended, and adjusted to approximately 6×10^8 cfu/ml in MEM without antibiotics. The suspensions were added to epithelial cells at a multiplicity of infection from 0.1 to 200, and then the infected cells were incubated in a 5% CO_2 incubator for 1 - 4 h at 37°C in MEM without antibiotics.

Cytotoxicity Assays

Cytotoxic activity was determined by two methods, SRB and LDH release assays, as previously described [6, 10]. For the SRB assay, Caco-2 and INT-407 cells were seeded in 96-well culture plates at a concentration of 2×10^4 cells per well and cultured at 37°C in 5% CO_2. After 24 h, the cells were washed twice with prewarmed phenol red-free MEM medium. Human epithelial cells were infected at MOI from 0.1 to 200 for 1 - 4 h. After infection, the supernatants were harvested and stored at - 80°C. Cell layers were fixed to the well bottoms by adding 50 µl of 10% trichloroacetic acid (TCA) in each well, and the plates were incubated at room temperature for 1 h. The wells were then drained, rinsed twice with distilled water, and air dried. SRB (0.4% w/v in 1% glacial acetic acid) was then added (100 µl/well), and the plates were incubated for 30 min. Unbound dye was drained and removed by washing 3 times with 1% glacial acetic acid. After air-drying the plate, the dye was solubilized by adding 100 µl/well of 10 mM Tris base and swirling for 10 min. Absorbance at 520 nm was measured on a microtiter plate reader.

For the LDH release assay, the CytoTox® nonradioactive cytotoxicity assay kit (Promega) was used to quantify cytosolic LDH release as an indicator of cytotoxicity. At indicated times, a sample of supernatant from experimental wells (either infected or uninfected) was centrifuged at 10,000 rpm and 4°C for 5 min to remove cell debris. To measure total LDH activity in either infected or uninfected wells, Triton X-100 was added to the wells, which contained host cells and remaining supernatant, at a final concentration of 9% to lyse host cells. A 50 µl sample of the supernatant was transferred to a new 96-well plate and mixed with the same volume of reconstituted LDH substrate mix. After 30 min of incubation at room temperature in the dark, 50 µl of the stop solution was added to each well, and absorbance at 490 nm was measured. Triplicate wells of each sample were run, and the experiment was performed at least twice.

Statistics

The Student's *t*-test and one-way analysis of variance followed by the Bonferroni method were used to determine

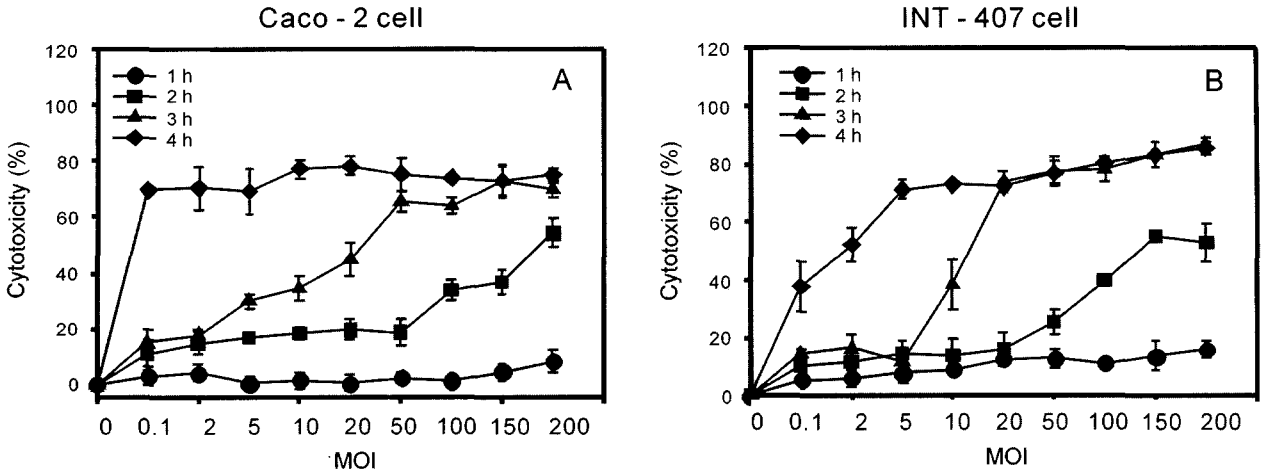

Fig. 1. Cytotoxic effects of *V. vulnificus* infection on human intestinal cells, Caco-2 and INT-407, determined by SRB assay.
Caco-2 and INT-407 cells were infected with *V. vulnificus* at different MOIs for various incubation periods, and cell cytotoxicity was determined by SRB assay using a computer-interfaced, 96-well plate reader at 520 nm. The data represent mean±standard deviations of triplicate determinations in four independent experiments.

statistical differences between values of the various experimental and control groups. The *P*-values <0.05 were considered statistically significant.

Results and Discussion

Determination of Cytotoxicity Against Human Intestinal Cells, Using *V. vulnificus*

To determine whether SRB assay can be applied to determine cytotoxicity induced by infectious microorganisms, *V. vulnificus* as a model of infectious microorganisms was infected at different MOI (multiplicity of infection; ratio of *V. vulnificus* to intestinal cells) into human intestinal cells, Caco-2 and INT-407, for various periods, and the cytotoxic activity was then determined by SRB assay.

As shown in Fig. 1, infection with *V. vulnificus* significantly induced cytotoxicity in Caco-2 and INT-407 cells in both incubation time- and MOI-dependent manners. At an MOI of 200, *V. vulnificus* induced 54%, 69%, and 75% of cytotoxicity, when incubated with Caco-2 cells for 2, 3, and 4 h, respectively. Similar levels of cytotoxic activity

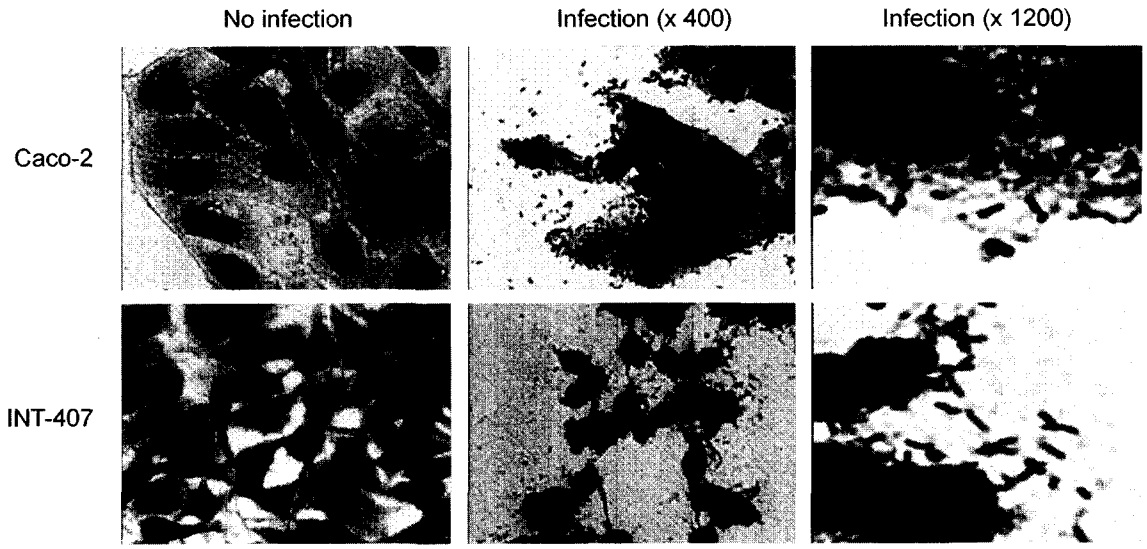

Fig. 2. Microscopic observation of human intestinal cells infected with *V. vulnificus*.
Caco-2 and INT-407 cells were cultured onto 18-mm-diameter coverglasses in culture dish for 24 h. Afterward, the cells were infected with *V. vulnificus* at an MOI of 10 for 3 h or not infected. The cells were observed under a light microscope after Giemsa staining. Left (uninfected control, ×400), middle (infected, ×400), right (infected, ×1,200).

were also observed in INT-407 cells infected with *V. vulnificus*. However, incubation with *V. vulnificus* for 1 h induced little cytotoxicity against both Caco-2 and INT-407 cells at any MOIs used in this study, indicating that the cytotoxic study of *V. vulnificus* against human intestinal cells requires at least 1-h incubation with mammalian cells at an MOI of less than 200.

Morphologic studies were also carried out to confirm the cytotoxicity of the infected cells. As shown in Fig. 2, many Giemsa-stained adherent Caco-2 and INT-407 cells revealed marked cellular damage after infection with *V. vulnificus*: Cytoplasmic loss and nuclear material condensation, a typical phenotype of cell death, were observed in both intestinal cells infected with *V. vulnificus*, and many *V. vulnificus* microorganisms were seen in the disrupted cytoplasmic region of the infected cells. In contrast, dead cells were not observed after incubation with medium alone: The uninfected cells showed a clear shape of cell surface and no cytoplasmic loss.

Comparison of SRB Assay and LDH Release Assay for Cytotoxic Activity of *V. vulnificus* Infection

Since the LDH release assay has been generally used for determination of cytotoxic activity induced by infectious microorganisms, the values obtained by the SRB assay were compared with those determined by the LDH release assay. Thus, both Caco-2 and INT-407 cells were incubated with *V. vulnificus* at various MOIs, and the cytotoxic activity was determined by both the SRB assay and LDH release assay. As shown in Fig. 3, the cytotoxic levels obtained by the SRB assay were approximately the same as those obtained by the LDL release assay. For example, cytotoxicity determined by the SRB assay showed 72.8±6.2 and 83.3±4.5 at an MOI of 100, while the LDH release assay showed 81.1±6.8 and 78.2±6.2, when *V. vulnificus* was infected into Caco-2 and INT-407 cells, respectively.

Effect of Heat Inactivation on Cytotoxicity Induced by *V. vulnificus* Infection

Viability of infectious microorganisms is important in inducing cytotoxicity against mammalian cells, including immune cells [4, 8, 11]. To ascertain whether the cytotoxic activity of *V. vulnificus* infection was affected by heat inactivation and whether this effect could be determined by the SRB assay, *V. vulnificus* was heat-inactivated at 60°C for 35 min and then incubated with the intestinal cells at various MOIs, and the cytotoxic activity was determined by both the SRB assay and LDH release assay. As shown in Fig. 4, infection with live *V. vulnificus* significantly induced cytotoxicity against both intestinal cells in a MOI-dependent manner, as determined by SRB and LDL release assays. Heat inactivation of live *V. vulnificus* at 60°C for 35 min resulted in complete disappearance of the cytotoxicity induced by live *V. vulnificus* infection. For example, heat-inactivated *V. vulnificus* induced little cytotoxicity (1-3%) at MOI of 200, while live *V. vulnificus* induced strong cytotoxicity against both intestinal cells (80-87%). As expected, there was no viable colonies formation on LBS agar, when *V. vulnificus* was preincubated at 60°C for 35 min (data not shown), indicating that the cell viability is critical for *V. vulnificus* infection against human intestinal cells.

The SRB assay used in this study provides a sensitive measure of drug-induced cytotoxicity and is suitable for very large-scale applications such as *in vitro* anticancer drug-discovery screen. SRB is a bright pink aminoxanthine dye with two sulfonic groups. It binds to basic amino acid

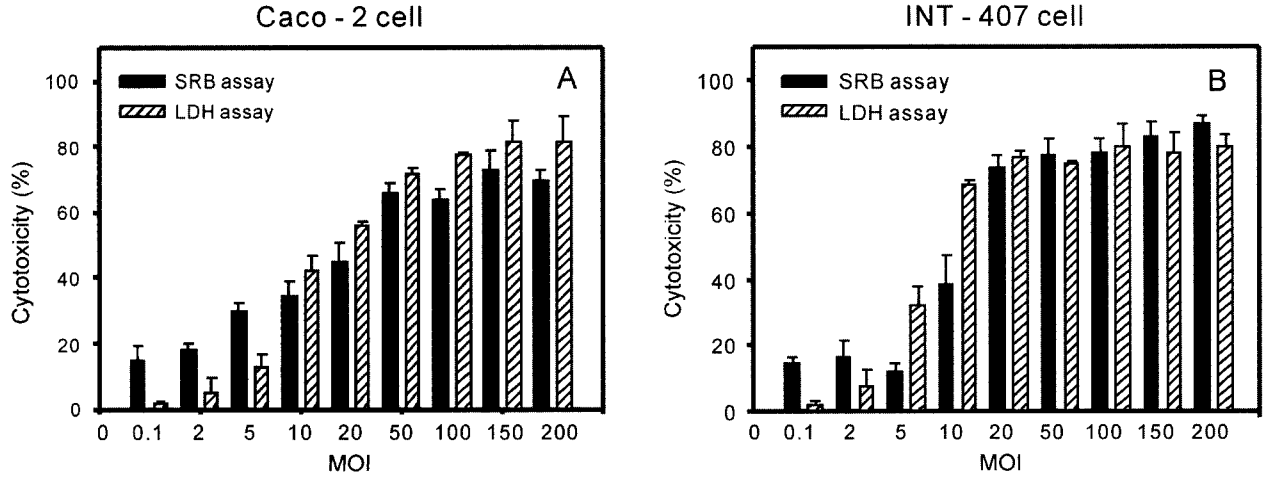

Fig. 3. Comparison of the SRB assay and LDH release assay for determining the cytotoxicity of *V. vulnificus* infection.
Caco-2 and INT-407 cells were infected with *V. vulnificus* at various MOIs for 3 h. Afterward, cytotoxic activity was determined by two different assay systems, the SRB assay and LDH release assay. The data represent mean±standard deviations of triplicate determinations.

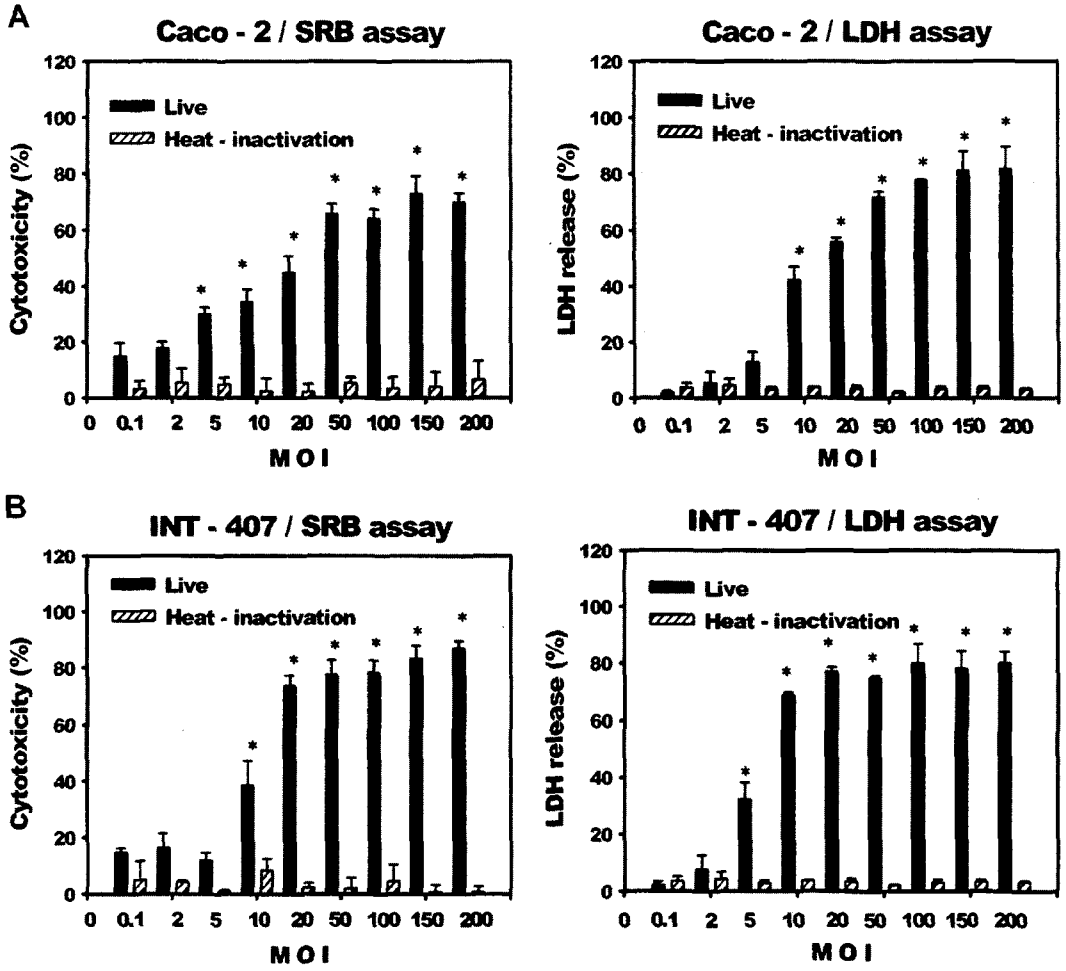

Fig. 4. Effect of heat-inactivation on the cytotoxic effect of *V. vulnificus* infection against human intestinal cells.
Caco-2 and INT-407 cells were infected with live or heat-inactivated *V. vulnificus* at various MOIs for 3 h. Afterward, cell cytotoxicity was determined by both the SRB assay and LDH release assay. The data represent mean±standard deviations of triplicate determinations. *$P<0.01$, relative to groups infected with heat-inactivated *V. vulnificus* at each MOI.

residues of cellular proteins to provide a sensitive index of protein content, which is linear over a cell density range of at least 2 orders of magnitude. Its sensitivity is comparable to the sensitivities of some fluorescent dyes and superior to those of conventional visible dyes. In addition, the 100-fold range of linearity of the SRB assay far exceeds those of the Lowry and Bradford protein assays, eliminating the need for time-consuming and error-prone dilutions of high-protein contents. Color development in the SRB assay is rapid, stable, visible, and can be measured over a broad range of visible wavelength [3]. In the present study, we demonstrated that the SRB assay could also be applied to determine cytotoxicity induced by infection with infectious microorganisms, and that the sensitivity of the SRB assay was favorably comparable with that of the LDH release assay generally used for cytototoxic activity of infectious microorganisms against host cells.

The SRB assay has three additional advantages over the LDH release assay. First, the approximate cost of the SRB assay per sample is more than 500-fold cheaper than that of the LDH release assay and it is, therefore, more suitable for very large-scale application. Second, the LDH release assay is likely to be error-prone to determine cytotoxicity under some experimental conditions. In our study, some of the cytotoxic cells demonstrated by the LDL release assay were found to be viable after re-culture (data not shown): There might be some cells viable but in reversible apoptotic state, although the plasma membrane was ruptured and LDH enzyme was released to culture supernatants. Third, SRB staining reaches a true and stable endpoint that does not have to be measured necessarily within any fixed period of time: When air dried, both TCA-fixed and SRB-stained samples can be stored indefinitely without deterioration. Further study is needed to determine the optimal assay

conditions for cytotoxicity against host cells, using other infectious microorganisms.

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