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A non-radioactive method for detecting neutralizing antibodies against therapeutic proteins in serum

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Abstract

The presence of neutralizing antibodies against protein therapeutics continues to cause concern in the biomedical field. These antibodies not only reduce the efficacy of the protein therapeutics, but may also block the normal function of their endogenous counterparts, which can result in serious health risks to the patient. To date, a limited number of in vitro cell-based bioassays for detecting neutralizing antibodies against therapeutic proteins have been developed. However, many of the existing assays involve the use of radioactive materials. We have established a novel and non-radioactive bioassay system for detecting neutralizing antibodies in patient serum samples. Our assay measures the cell metabolic activities that are closely associated with cell proliferation and apoptosis. The biologic effect of the therapeutic protein and the capability of the antibodies to neutralize the therapeutics are reflected by changes of the cellular metabolic activities triggered by the administration of the therapeutics or presence of the anti-therapeutic protein antibodies. Compared with existing assays, this new assay is equally or more sensitive, and completely eliminates the use of radioactive materials.
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Keywords: Non-radioactive method; Neutralizing antibodies; Cell-based bioassay; Metabolic activity

1. Introduction

Almost all therapeutic proteins induce some antibody responses when administered to humans [1,2]. These antibodies can be categorized based on their clinical relevance to the drug such as binding, clearing, sustaining, and neutralizing antibodies. Binding antibodies simply bind to an epitope of the therapeutic protein but do not affect the biological function of the protein. Clearing antibodies increase the clearance of the therapeutic protein. In contrast, sustaining antibodies decrease the clearance of the therapeutic protein and therefore cause accumulation of the drug in the system. In both instances the PK/PD profile may be altered, albeit toward opposite directions. Neutralizing antibodies are clinically most significant since they bind to the epitope that is also recognized by the drug target, causing the drug biologically ineffective. In addition, these antibodies may bind to and inactivate the endogenous counterpart of the therapeutic protein and therefore pose a health risk to the patient [3–6]. Therefore, effective detection of neutralizing antibodies against therapeutic proteins is an important safety measurement that helps to reduce the risks to the patient. It also allows pharmaceutical companies to more accurately evaluate their drug candidates and monitor their products for antibody induction. As such, the need to develop assays capable of detecting neutralizing antibodies against therapeutic proteins has increased in the past years.

Bioassays that detect and characterize neutralizing antibodies are commonly converted from potency assays that determine the biological activities of the therapeutic proteins [7,8]. Among various potency assays, the cell-based bioassays are most effective, convenient, and widely employed methods that measure a number of cellular activities, including cell proliferation, survival, apoptosis, and gene expression, etc. [9–12]. Selective use of these measurements in a particular bioassay is solely dependent upon the biological activities of the product to be analyzed. The ability of a bio-sample, such as a serum sample, to inhibit the cellular activities of the therapeutic protein indicates the possible existence of neutralizing antibodies. Most of the bio-samples used in antibody assays are serum samples. Non-specific matrix effects arising

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from bio-samples can affect the assay and therefore deciding on an optimal serum concentration is critical to a successful assay.

Among commonly used cell-based bioassays, tritiated thymidine ([^{3}H]-thymidine) incorporation, which detects changes in DNA synthesis, is widely used [13–15]. This method is very sensitive in measuring cell proliferation activities. However, in cases of cell apoptosis, the assay loses its advantage since in these cases very little or no DNA is synthesized in the apoptotic cells. In addition, the thymidine incorporation method is laborious, potentially hazardous [16], and often difficult to automate largely due to the usage of radioactive materials. As alternatives, colorimetric assays have been developed including MTT, MTS, and XTT, which eliminate the use of radioactive materials. Nevertheless, their application is limited, largely because of their lower sensitivities when compared to traditional radioactive method. Other types of technologies have also been employed in the development of non-radioactive cell-based bioassays, including the recent use of b-DNA technology in neutralizing antibody assays, which measures changes in target gene expression [9]. CellTiter-Glo is another non-radioactive assay platform. It has been used primarily for determining the number of viable cells, including primary cells, cancer cells, engineered cells, and virus infected cells, by measuring the amount of ATP molecules present in the cells [17–22]. The number of ATP molecules is relatively consistent from cell to cell. Therefore the luminescent signal obtained from a cell culture that reflects the amount of ATP molecules exist in the culture is directly related to the number of healthy or metabolically active cells [23]. Unlike [^{3}H]-thymidine incorporation, CellTiter-Glo assay is a homogenous, one-step assay which involves addition of a single reagent. A number of experimental steps essential for assays using [^{3}H]-thymidine incorporation method, such as cell washing, removal of medium or multiple pipetting steps is eliminated in CellTiter-Glo, making the new assay much less prone to variations.

In this paper, we compare the CellTiter-Glo and [^{3}H]-thymidine incorporation assay platforms using two different experimental systems. We show that CellTiter-Glo offers many advantages over traditional [^{3}H]-thymidine incorporation and provides comparable sensitivities when cell proliferation and apoptosis are assessed. The biggest advantage of CellTiter-Glo over [^{3}H]-thymidine incorporation is the elimination of the use of radioactive materials, which impose potential health and environmental risks and are usually more costly. Additional advantages of using CellTiter-Glo® include the ease of automation, shortened assay times as well as the flexibility of reading the signal up to 5 h post addition due to its prolonged half-life.

2. Experimental

2.1. Materials

UT-7 and ME-180 cells were provided by Amgen Cell Bank (Thousand Oaks, CA). Cell culture reagents (media, FBS, trypsin, trypan-blue) were purchased from Invitrogen/Gibco BRL (Carlsbad, CA). Normal pooled human serum (Cat# HMSRM) was purchased from Bioreclamation, Inc. (Hicksville, NY). Granulocyte-macrophage colony-stimulating factor (GM-CSF), Erythropoietin (Epogen or EPO), and the polyclonal anti-interferon-gamma neutralizing antibody were provided by Amgen (Thousand Oaks, CA). Recombinant human interferon-gamma (Cat# 285-IF-100) was purchased from R&D Systems (Minneapolis, MN). Tritiated thymidine (Cat# 24059) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). CellTiter-Glo reagents (Cat# G7570) were purchased from Promega Corporation (Madison, WI).

2.2. Maintenance of cell cultures

UT-7, a human megakaryoblatic cell line, was cultured in RPMI 1640 (Gibco BRL) supplemented with 10% heat-inactivated FBS (Gibco BRL), and 1% penicillin/streptomycin (Gibco BRL) 10 ng/ml recombinant human GM-CSF (UT-7 growth medium). ME-180, a human cervix carcinoma cell line was cultured in McCoy's 5A medium (Gibco BRL) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin (ME-180 growth medium). All cells were cultured in incubators set at 37 °C, 5% CO_2, and 90% humidity.

2.3. Human serum tolerance of UT-7 and ME-180 cells

UT-7 cells to be used for EPO experiments growing at an exponential phase were washed 3 times with GM-CSF-free UT-7 growth medium and re-suspended and incubated in the same medium at 37 °C, 5% CO_2 and 90% humidity for 16–24 h. The next day, the cells were counted, re-suspended in the same medium at a density of 400,000 cells/ml, and 50 μl of the cell suspension was seeded in each well of 96 well plates. UT-7 cells were plated at 20,000 cells/well (50 μl/well). Recombinant human EPO was diluted to 2.5 ng/ml (2× of final assay concentration) with GM-CSF free UT-7 growth medium containing 2× of the final concentrations of pooled human serum (ranging from 0% to 20%, see Fig. 1A). Equal volume of each of these dilutions (50 μl) was added to the cells in triplicate wells. The cells were incubated at 37 °C, 5% CO_2 and 90% humidity for 3 days with EPO. At the end of the experiment, the cells in each well were treated with 100 μl of CellTiter-Glo reagent and incubated at room temperature for 10 min. The luminescence was then determined using a Victor^2 multi-probe plate reader from PerkinElmer, (Wellesley, MA).

ME-180 cells in near-confluent plates were trypsinized and washed once with ME-180 growth medium. The cells were re-suspended at a density of 200,000 cells/ml in the same medium and 50 μl of the cell suspension were seeded in each well of 96-well plates. Recombinant human interferon-gamma was diluted to 20 ng/ml (2× of final assay concentration) with ME-180 medium containing 2× the final concentrations of pooled human serum (ranging from 0% to 20%, see Fig. 1B). Equal volume of each of these dilutions (50 μl) was added to the cells in triplicate wells. The cells were incubated at 37 °C, 5% CO_2 and 90% humidity for 7 days. The cells in each well were treated with

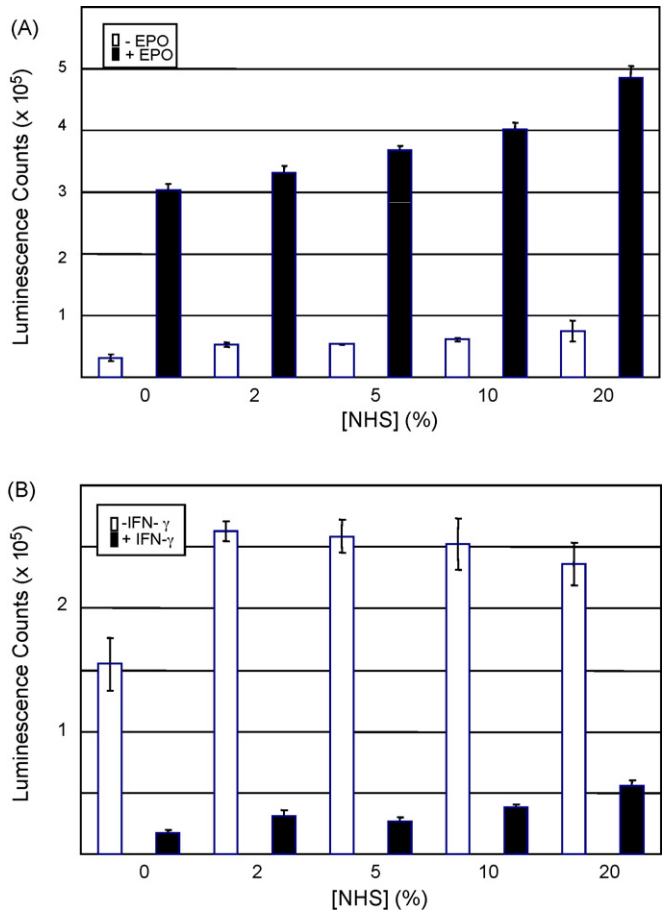

Fig. 1. Effects of various concentrations of pooled normal human serum on the growth of UT-7 (A) and ME 180 (B) cells measured by CellTiter-Glo method. Filled bars depict EPO (A) treated UT-7 cells, and interferon-gamma (B) treated ME 180 cells. Open bars represent untreated controls. Error bars represent standard deviations of three replicates.

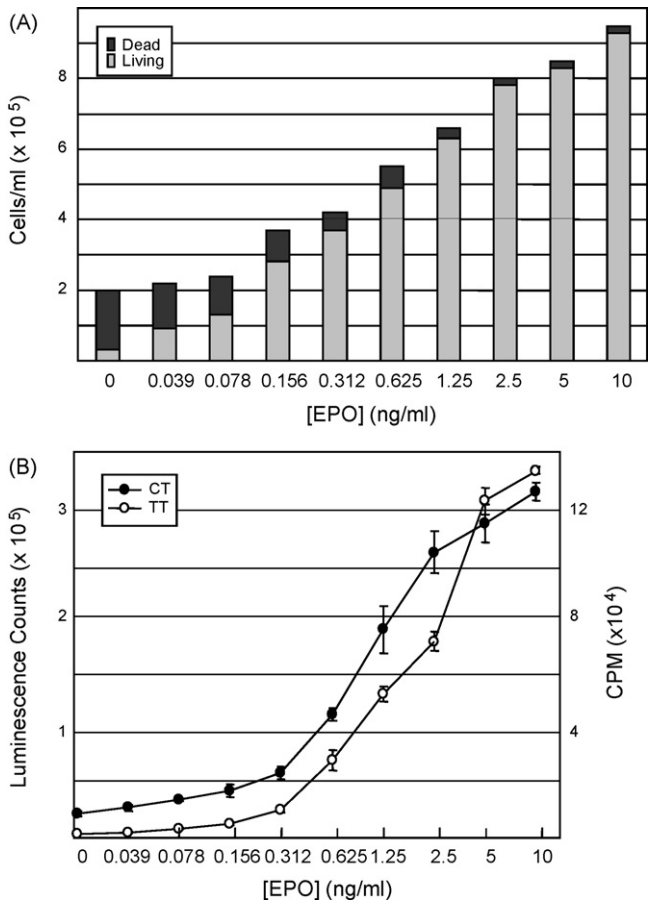

Fig. 2. Numbers of UT-7 cells growing in media supplemented with various concentrations of EPO for 3 days were determined by trypan-blue exclusion method (A). The growth of the UT-7 in 10% pooled human serum supplemented with various concentrations of EPO was also measured by the CellTiter-Glo (CT) and [^{3}H]-thymidine incorporation (TT) methods (B). Error bars represent standard deviations of three replicates.

100 μl of CellTiter-Glo reagent and incubated at room temperature for 10 min. The luminescence was then determined using a Victor^2 multi-probe plate reader.

2.4. Treatment of UT-7 cells with EPO in the absence or presence of anti-EPO neutralizing antibodies

UT-7 cells growing at an exponential phase were washed three times with GM-CSF-free UT-7 growth medium, and re-suspended and incubated in the same medium at 37 °C, 5% CO_2 and 90% humidity for 16–24 h. The next day, the cells were counted, re-suspended in the same medium at a density of 400,000 cells/ml, and 50 μl of the cell suspension was seeded in each well of 96-well plates. Recombinant human EPO was diluted with the same medium supplemented with 20% pooled human serum to 2× of the final concentrations (ranging from 0 to 20 ng/ml, 2× of final assay concentrations, see Fig. 2). Each of these dilutions (50 μl) was added to cells in triplicate wells to reach a 10% final concentration of pooled human serum and concentrations of EPO ranging from 0 to 10 ng/ml (Fig. 2). The cells were incubated at 37 °C, 5% CO_2 and 90% humidity for 3 days. For experiments using CellTiter-Glo method, the cells in each well were treated with 100 μl of CellTiter-Glo reagent and incubated at room temperature for 10 min. The luminescence was then determined using a Victor^2 multi-probe plate reader. For experiments using the [^{3}H]-thymidine incorporation method, 2 μCi of [^{3}H]-thymidine was added to cells in each well after the 3 days of incubation. The cells were further incubated for 4 h and then harvested onto filter mats using a Filtermate harvester from PerkinElmer (Wellesley, MA). The filter mats were air-dried, and the radioactivity associated with the filter mats was determined using a Matrix 9600 Beta counter (PerkinElmer, Wellesley, MA).

To determine the number of cells in each well, 10 μl of cells were removed from a replicate well after 3 days of growth and treated with 10 μl of trypan blue solution (Fig. 2A).

For experiments with anti-EPO neutralizing antibodies, the same procedure was followed, except 2.5 ng/ml EPO (2× of final assay concentrations) was pre-incubated with various concentrations of anti-EPO antibodies (ranging from 0 to 500 ng/ml, 2× of final assay concentrations, see Fig. 3) for 30 min at room temperature before adding to the cells.

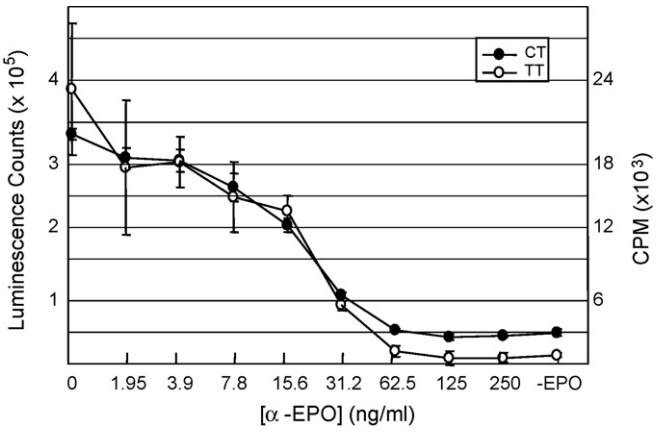

Fig. 3. Anti-EPO neutralizing antibody inhibits EPO induced UT-7 cell growth. The proliferation of UT-7 cells treated with 1.25 ng/ml of EPO and various concentrations of anti-EPO antibody in the presence of 10% pooled human serum were measured by CellTiter-Glo (CT) and tritiated thymidine incorporation (TT) methods. Error bars represent standard deviations of three replicates.

2.5. Treatment of ME-180 cells with interferon-gamma in the absence or presence of anti-interferon gamma neutralizing antibodies

ME-180 cells in near-confluent plates were trypsinized and washed once with ME-180 growth medium. The cells were re-suspended at a density of 200,000 cells/ml in the same medium and 50 μl of the cell suspension were seeded to each well of 96-well plates one day before the experiment. Recombinant human interferon-gamma was diluted with the same medium supplemented with 10% pooled human serum to 2 × of the final concentrations (ranging from 0 to 500 ng/ml, 2× of final assay concentrations, see Fig. 4). Each of these dilutions (50 μl) was added to cells in triplicate wells to reach a 5% final concentration of pooled human serum and concentrations of interferon-gamma ranging from 0 to 250 ng/ml (2× of final assay concentrations) (Fig. 4). The cells were incubated at 37 °C, 5% CO_2 and 90% humidity for 7 days. For experiments using the CellTiter-Glo method, the cells were treated with CellTiter-Glo reagent and the luminescence was measured as described above. For experiments using the [^{3}H]-thymidine incorporation, 2 μCi of [^{3}H]-thymidine was added to each well on day 7. The cells were further incubated for 4 h. The media containing radioactive materials was removed, the cells were trypsinized and harvested onto filter mats using a Filtermate harvester. The radioactivity was measured as described above.

For experiments with anti-interferon-gamma neutralizing antibodies, the same procedure was followed, except 10 ng/ml interferon-gamma was pre-incubated with various concentrations of anti-interferon-gamma antibodies (ranging from 0 to 10,000 ng/ml, see Fig. 5) for 30 min at room temperature before adding to the cells.

To determine the number of cells in each well, cells in a separate well were trypsinized on day 7 and the number of cells was determined as described above (Fig. 4A).

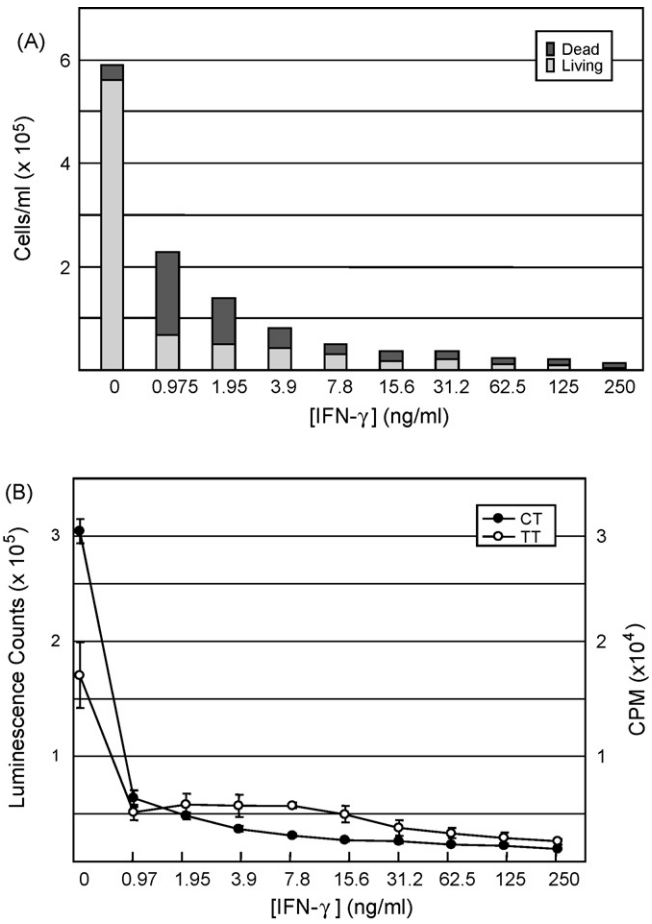

Fig. 4. Numbers of ME-180 cells growing in media supplemented with various concentrations of interferon-gamma for 7 days were determined by trypan-blue exclusion method (A). The apoptosis of the ME-180 cell induced by various concentrations of Interferon-gamma in the presence of 5% pooled human serum was also measured by the CellTiter-Glo (CT) and [^{3}H]-thymidine incorporation (TT) methods (B). Error bars represent standard deviations of three replicates.

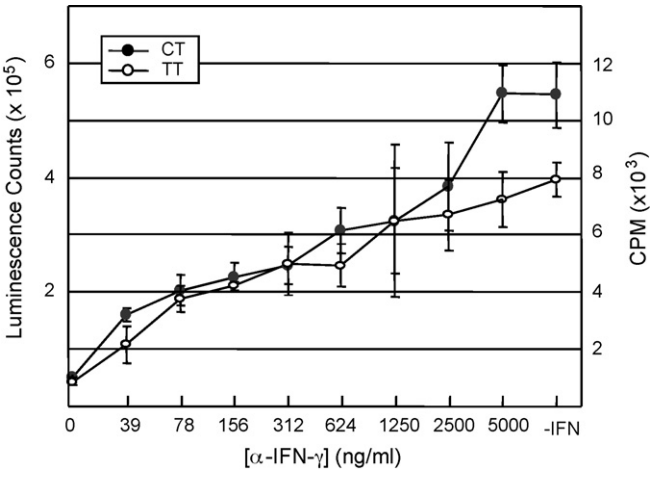

Fig. 5. Anti-interferon-gamma neutralizing antibody blocks interferon-gamma induced apoptosis of ME-180 cells. The effects of various concentrations of anti-interferon-gamma antibodies on the apoptosis of ME-180 cells treated with 10 ng/ml of interferon-gamma in the presence of 5% pooled human serum were measured by CellTiter-Glo (CT) and tritiated thymidine incorporation (TT) methods. Error bars represent standard deviations of three replicates.

3. Results

3.1. Matrix influence of human serum

The matrix influence of human serum on EPO induced UT-7 cell proliferation and interferon-gamma induced ME-180 cell apoptosis was tested by treating the cells with and without corresponding cytokines in the presence of various concentrations of pooled normal human serum. As shown in Fig. 1A, either in the presence or absence of EPO, higher concentrations of human serum caused a slight increase in the metabolic activities of UT-7 cells. EPO stimulated increase of metabolic activities in the presence of 0%, 2%, 5%, 10%, and 20% serum was 9.48-, 6.25-, 6.82-, 6.55-, and 6.47-fold, respectively (Fig. 1A). Fig. 1B depicted the influences of increased concentration of human serum on interferon-gamma induced ME-180 cell apoptosis. As shown in the figure, the metabolic activities of the ME-180 cells treated with interferon-gamma in the presence of 0%, 2%, 5%, 10%, and 20% serum decreased 8.66, 8.38, 9.56, 6.58, and 4.24 folds compared to that of the cells treated with the same concentrations of serum but not interferon-gamma (Fig. 1B).

3.2. Measurement of EPO-induced dose-dependent proliferation of UT-7 cells

In the presence of increased concentrations of EPO, the numbers of UT-7 cells increased in a dose-dependent manner after 3 days of growth (Fig. 2A). EPO-induced UT-7 cell proliferation was measured by either CellTiter-Glo or [^{3}H]-thymidine incorporation methods. As shown in Fig. 2B, the metabolic activity of UT-7 cells and the cellular incorporation of [^{3}H]-thymidine both increased following the increase of the EPO concentrations. The shapes of the two curves are very similar. The EC_{50} value obtained from the CellTiter-Glo curve is 1.09 ng/ml, while the same from the tritiated thymidine incorporation curve is 2.48 ng/ml.

3.3. Measurement of the inhibition of anti-EPO neutralizing antibodies on EPO-induced cell proliferation

Pre-incubation of EPO with anti-EPO neutralizing antibodies prevented EPO from interacting with UT-7 cells and stimulating further cellular responses. In the presence of anti-EPO neutralizing antibodies, EPO-induced increase in cellular metabolic activity and incorporation of [^{3}H]-thymidine in UT-7 cells, as measured by CellTiter-Glo and [^{3}H]-thymidine incorporation methods, were both abolished (Fig. 3). As shown in Fig. 3, the IC50s obtained from the CellTiter-Glo and tritiated thymidine curves are 17.58 ng/ml and 16.34 ng/ml, respectively. A complete inhibition of the biological effect of EPO (at 1.25 ng/ml) was seen at 62.5 ng/ml of anti-EPO neutralizing antibodies (Fig. 3) when measured with both methods.

3.4. Measurement of interferon-gamma-induced apoptosis of ME-180 cells

ME-180 cells underwent cell death when treated with human interferon-gamma. Fig. 4A shows the increased death of ME-180 cells induced by increased concentrations of interferon-gamma as measured by counting the cells using a trypan-blue exclusion method. After 7 days of growth, the numbers of living ME-180 cells in cultures not treated with interferon-gamma increased from 100,000 cells/ml to 560,000 cell/ml. In contrast, the number of living ME-180 cells in the interferon-gamma-treated cultures decreased although the total number of the cells (including the living and the dead cells) increased slightly in the cultures treated with low concentrations of interferon-gamma (Fig. 4A). The trend of both the living and the total cell number decrease closely correlated with the dose of interferon-gamma used in the experiment. In the presence of high concentrations of interferon-gamma (250 ng/ml) the number of live cells decreased to ~3000 cells/ml.

The effects of interferon-gamma-triggered ME-180 cell death were assessed by both [^{3}H]-thymidine incorporation and CellTiter-Glo methods, and the results are shown in Fig. 4B. Both the metabolic activity, and the amount of [^{3}H]-thymidine incorporated into the interferon-gamma-treated ME-180 cells decreased following the increase of interferon-gamma concentrations. As shown in Fig. 4B, a significant decrease in either the metabolic activity or the incorporation of [^{3}H]-thymidine in ME-180 cells was triggered by 1–2 ng/ml of interferon-gamma. Interferon-gamma concentrations higher than 2 ng/ml, do not significantly increase cell death (Fig. 4B).

3.5. Measurement of the inhibition of anti-interferon-gamma neutralizing antibodies on interferon-gamma-induced cell death

Pre-incubation of interferon-gamma with anti-interferon-gamma neutralizing antibodies protected the ME-180 cells from interferon-gamma-induced cell death. When ME-180 cells were treated with 10 ng/ml of interferon-gamma and increased amount of anti-interferon-gamma antibodies, increased numbers of living cells were seen, reflected by an increasing amount of cell metabolic activities and [^{3}H]-thymidine incorporations (Fig. 5). As shown in Fig. 5, a complete inhibition of the interferon-gamma activity was detected at ~2500 ng/ml of anti-interferon-gamma neutralizing antibodies. The IC50s obtained with both methods are virtually the same (Fig. 5).

4. Discussion

Experimental analysis of serum samples for the presence of neutralizing antibodies against therapeutic proteins requires the assay system to be able to tolerate the presence of serum in the assay matrix. Such a tolerance means that the presence of a certain amount of serum in the assay system will not significantly affect the readout of the assay, either positively or negatively. In general, higher assay sensitivity can be reached by using higher concentrations of serum samples. However, higher concentration of serum will also bring about higher matrix effect, which may affect the quality, sometimes even the sensitivity of the assay. For each cell line used in a particular assay, an appropriate serum concentration needs to be carefully chosen to ensure a balance between a desired assay sensitivity and an

acceptable matrix impact. Our data demonstrated that when UT-7 and ME-180 cells were used in a CellTiter-Glo and thymidine incorporation based neutralizing antibody assays, both cell lines could tolerate reasonably high concentrations of human serum (Fig. 1). This was a big advantage that enabled us to design and develop an assay using relatively high concentrations of the testing samples (less sample dilutions needed) and therefore increase the final sensitivity of the assay. For UT-7 cells, EPO induced cell proliferation in the presence of 2–20% of human serum was relatively stable (6.25–6.82 folds), suggesting that these concentrations of serum samples might be used in a real assay without introducing un-desired matrix effect. For the ME-180 cells, however, interferon-gamma induced cell apoptosis was relatively consistent only in the presence of 2–5% of human serum (8.36–8.59 folds). This activity dropped very quickly when higher concentrations of human serum were included in the assay (6.58–4.24 folds for 10–20% of human serum), largely due to decreased apoptosis inducing activities of interferon gamma in the higher concentrations of human serum (Fig. 1B). For this reason, only 2–5% of serum samples were used in ME-180-based anti-interferon gamma assays.

UT-7 is a factor-dependent human megakaryoblatic cell line that relies on the presence of cytokines GM-CSF or EPO for survival and proliferation. Treatment of the UT-7 cells with EPO or GM-CSF resulted in an increase in cell numbers. ME-180, a human cervix carcinoma cell line, can be induced into apoptosis by interferon-gamma [24]. As shown in Fig. 4A, treatment with low concentrations of human interferon-gamma triggered significant death and therefore a dramatic decrease in the numbers of the ME-180 cells. Using these two experimental systems, we evaluated the suitability and relative sensitivity of the CellTiter-Glo method in detecting neutralizing antibodies against therapeutic proteins and compared that to the same of the [^{3}H]-thymidine incorporation method.

The suitability of the CellTiter-Glo-based bioassay in detecting neutralizing antibodies against therapeutic proteins was judged by whether the measurement obtained with the assay truly reflected the change of the cellular status stimulated by the therapeutic proteins. [^{3}H] thymidine incorporation is a widely used method that monitors the proliferation activity of EPO or the apoptotic activity of interferon-gamma by measuring the amount of [^{3}H] thymidine incorporated into the cells in response to the stimulation of the cytokine. Different from the [^{3}H] thymidine incorporation method, CellTiter-Glo assesses the activities of the cytokines by measuring the levels of the cellular metabolic activities in the cytokine-treated cells. Our data demonstrates that CellTiter-Glo-based assays are suitable for detecting neutralizing antibodies against the therapeutic proteins since the dose-response curves of EPO-induced UT-7 cell proliferation, and interferon-gamma triggered ME-180 cell death produced by this method, were not only parallel with that of the [^{3}H] thymidine incorporation method, but also parallel with the trends of the cell number changes triggered by the cytokines (Figs. 2 and 4).

The sensitivity of the CellTiter-Glo-based assay was estimated by the value of the EC50 and IC50 derived from the dose-response curves (Figs. 2 and 4). Our data indicates that the sensitivity of the CellTiter-Glo platforms was comparable to that of the [^{3}H] thymidine incorporation method in the interferon-gamma assay and more sensitive in the EPO assay as determined by EC50 and IC50.

In summary, we demonstrated that CellTiter-Glo technology is a simple, reliable, and non-radioactive alternative to the [^{3}H]-thymidine incorporation method. When proliferation or apoptosis activities are analyzed by [^{3}H]-thymidine incorporation and CellTiter-Glo, both methods provide valid measurements of the activities and similar sensitivities. The major advantage of CellTiter-Glo over the tritiated thymidine method is the elimination of radioactivity usage, which makes this platform more versatile, less hazardous, less laborious, and simpler to automate. The second advantage is its homogeneity; this assay is performed in one plate without the need for cell harvesting or any other processing. This makes the assay less tedious to perform and the results less variable. Since there is only short incubation required with CellTiter-Glo reagent, shortened assay time is another advantage of this method. A disadvantage of CellTiter-Glo, at least in some proliferation assays, could be the relatively higher background compared to that of tritiated thymidine incorporation method. This often results in lower fold response in cells treated with therapeutic proteins compared to that of untreated cells.

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