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A New Non-radioactive Method for IL-2 Bioassay

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An oxidation-reduction (redox) indicator, alamarBlue, was used to measure the bioactivity of interleukin 2 (IL-2). This assay system has several advantages over other bioassays for measuring IL-2. It is a nonradioactive method unlike the conventional tritium-labeled thymidine ([3H]TdR) incorporation assay. The alamarBlue assay is also easier to use than other colorimetric methods, such as the MTT assay, because the alamarBlue assay does not depend on the extraction of insoluble formazan salt, which is time-consuming, error-prone, and cumbersome. Due to its solubility in culture medium and its nontoxicity to cells, alamarBlue provides an easy method to monitor cellular growth using either a fluorescence- or an absorbance-based instrument. The alamarBlue assay is not sample-destructive, unlike the thymidine incorporation and MTT methods. This adds another advantage to the alamarBlue method as the measurement of cellular growth by sample-destructive methods requires as many tubes as time points whereas the alamarBlue method requires only one tube for the entire growth period. In this study, alamarBlue was used to measure the proliferation of the IL-2-dependent cytotoxic T cell line, CTLL-2. The colorimetric change of alamarBlue at 570 nm compared to the reference wavelength, 600 nm, was proportional to the number of viable cells. The sensitivity of the IL-2 assay using alamarBlue was comparable to that of the [3H]thymidine incorporation method. These results demonstrate that the alamarBlue assay is valid for the IL-2 bioassay and that alamarBlue can replace the [3H]thymidine employed in the conventional proliferation assays.

Keywords: Fluorescence/Colorimetric Assay; IL-2 Assay; Redox Indicator.

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Introduction

Many cells require growth factors, which are not provided from media and sera. The growth of these cells is totally dependent on the availability of these supplemented factors. Therefore, proliferation of these cells has been used to measure the concentrations of those growth factors in the media. The CTLL-2 cell line was transformed from murine spleen cells, but remained dependent on exogenous growth factors such as human or murine IL-2 or murine IL-4. Bioassay protocols were developed utilizing this cell line to measure IL-2 and IL-4 in the culture medium (Bottomly *et al.*, 1992). The proliferation of CTLL-2 cells was monitored by [3H]TdR incorporation into the newly synthesized DNA. Although the [3H]TdR incorporation assay has been proven to be reliable and is still widely used, it is laborious and necessitates disposal of the radioactive waste. To circumvent these problems, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was developed by Mosmann (1983).

In the MTT-mediated proliferation assay, the yellow tetrazolium salt, MTT, is converted to the purple formazan crystals by mitochondrial enzymes. The formazan products are then dissolved with acidic isopropanol. This solubilization is an error-prone, time-consuming, and dangerous step. Moreover, it is often difficult to completely dissolve the formazan crystals, which need various modifications of the assay (Buttke *et al.*, 1993; Niks and Otto, 1990; Sladowski *et al.*, 1993; Stevens and Olson, 1993; Tada *et al.*, 1986). AlamaBlue is a fluorescence/colorimetric growth indicator that detects the metabolic activity of cells (Ansar Ahmed *et al.*, 1994). Specifically, cellular growth leads to reduction of the culture medium which in turn changes the indicator from blue to red. This redox potential change is proportional to the cell viability, and can be used as a barometer of cellular proliferation.

In this study, we developed a new colorimetric IL-2 assay method using alamarBlue. AlamarBlue was found to be soluble in the culture medium and non-toxic to the cells tested. The optical density of alamarBlue was proportional to the number of cells, validating this new dye as a cellular proliferation indicator. The sensitivity of the alamarBlue assay was similar to that of the conventional $^{[3H]}$TdR incorporation method. Here we report that alamarBlue is a valuable tool for cell proliferation assays and may provide a simple method for bioassays of cytokines and growth factors.

Materials and Methods

Cell lines and culture conditions The murine cytotoxic T lymphoma cell line, CTLL-2, was purchased from ATCC (Rockville, MD). Cells were cultured in JCM medium containing RPMI 1640 supplemented with heat-inactivated 10% bovine calf serum (Hyclone, Logan, UT), 2 mM glutamine (Gibco BRL, Rockville, MD), 0.1 mM nonessential amino acids (Gibco BRL), 100 U/ml penicillin (Sigma, St. Louis, MI), 100 μg/ml streptomycin (Sigma), 0.1 mM pyruvate (Gibco BRL), 25 mM HEPES (Gibco BRL), and 5.5×10^{-5} M beta-mercaptoethanol (Sigma) at 37°C, 5% CO_2. The CTLL-2 cells were maintained in JCM medium supplemented with 20 U/ml of recombinant human IL-2 (Genzyme, Cambridge, MA). AlamarBlue was supplied by Alamar Biosciences, Inc. (Sacramento, CA).

The standard $^{[3H]}$TdR incorporation assay for IL-2 IL-2 activity was measured by DNA synthesis 24 h after addition of 1 μCi of $^{[3H]}$TdR (Amersham, Arlington Heights, IL) into each well of a 96-well plate (Bottomly *et al.*, 1992). The IL-2 standard was 5-fold serially diluted in 50 μl of JCM medium in a 96-well microtiter plate. CTLL-2 cells were harvested in active log-phase growth and washed with PBS three times to remove residual IL-2. Cells were resuspended in JCM medium at 1×10^5 cells/ml and added to a 96-well plate, which contained 50 μl of serially diluted IL-2 standard. The CTLL-2 cells were cultured for 24 h in a 37°C, 5% CO_2 humidified incubator. One μCi of $^{[3H]}$TdR was added into each well and incubated for an additional 24 h. The cultures were harvested on a cell harvester (Skatron Inc., Sterling, VA) and the amount of $^{[3H]}$TdR incorporated into DNA was measured by liquid scintillation counting (Beckman, Irvine, CA).

The IL-2 bioassay using alamarBlue The IL-2 standard was 5-fold serially diluted in 50 μl of JCM medium in a 96-well microtiter plate. CTLL-2 cells were harvested in active log-phase growth and washed with PBS three times to remove residual IL-2. The cells were resuspended in JCM medium at 4×10^5 cells/ml and 50 μl were added to each well of a 96-well plate which contained 50 μl of serially diluted standard IL-2. The cells were cultured for 44 h in a 37°C, 5% CO_2 humidified incubator. Fifty μl of 30% (v/v) alamarBlue, which was diluted with JCM medium, was added to each well and further incubated for 4 h in a 37°C, 5% CO_2 humidified incubator. The plates were subsequently read on a Microplate Autoreader (Bio-Tek Instruments, Inc., Winooski, VT) at 570 nm (measurement) and 600 nm (reference). The specific absorbance values were calculated from the following equation; the specific $OD_{(570-600)}$ = sample $OD_{(570-600)}$ - media $OD_{(570-600)}$.

Statistical analysis Statistical analysis was performed with the student *t* test using the StatView software (Abacus Concepts, Inc., San Francisco, CA).

Results and Discussion

Assessment of toxicity of alamarBlue for CTLL-2 cells To determine whether alamarBlue is cytotoxic, proliferation of CTLL-2 cells in the presence of alamarBlue was monitored. The CTLL-2 cells were at a concentration of 4×10^6 cells in 1 ml of JCM medium containing 30 U/ml IL-2, 10 μCi/ml of $^{[3H]}$TdR, and 0–20% (v/v) of alamarBlue. An aliquot of 100 μl of this cell suspension was added into each well of a 96-well plate and incubated in a 37°C, 5% CO_2 humidified incubator. Every 4 h the cultures were harvested on a cell harvester and the amount of $^{[3H]}$TdR incorporated into DNA was measured by liquid scintillation counting. AlamarBlue had very little effect on the growth of CTLL-2 cells (Fig. 1). The incorporation of $^{[3H]}$TdR by CTLL-2 cells in the presence of either 5% or 10% alamarBlue for up to 24 h, or 20% of alamarBlue for 4 h, was not significantly different from that of the control (without alamarBlue). Therefore, the presence of either 5% and 10% alamarBlue up to 24 h or 20% of alamarBlue for 4 h is non-toxic to CTLL-2 cells.

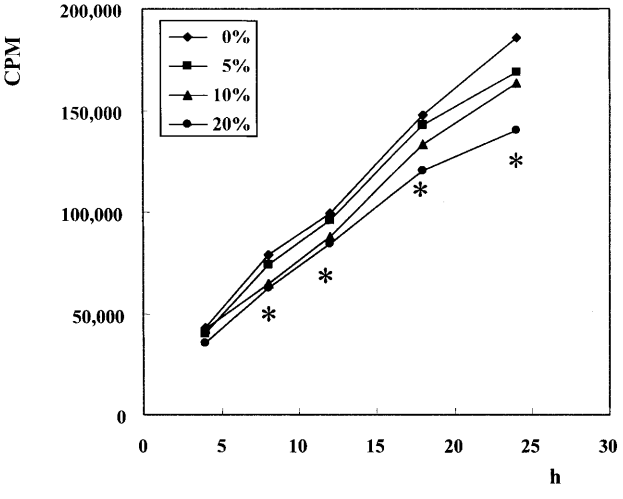

Fig. 1. The cytotoxicity of alamarBlue was evaluated. CTLL-2 cells were cultured with various concentrations of alamarBlue and 1 μCi/200 μl of $^{[3H]}$TdR for 4–24 h. The proliferation of cells was measured as $^{[3H]}$TdR incorporation. Average data without standard error bars from three independent experiments are presented. Incubation with 20% of alamarBlue reduced CTLL-2 cell growth significantly for more than 8 h (*).

Proportionality of cell number to the optical density of alamarBlue An experiment was carried out with alamarBlue to test whether this reagent can be used as an indicator of cell proliferation. Various numbers of CTLL-2 cells were cultured with 10% alamarBlue for 4 h in the presence of excess IL-2 (30 U/ml) at 37°C and 5% CO_2. The specific absorbance (OD_{570}–OD_{600}) was linear in a range of cell numbers between 1.6×10^4 and 2.0×10^6 cells/ml (Fig. 2).

Comparison of accuracy and sensitivity for the IL-2 assay The $^{[3H]}$TdR incorporation assay has been employed to measure the concentration of IL-2 by using cells that are dependent on IL-2 for proliferation. In this study, the use of alamarBlue for the IL-2 bioassay was developed with CTLL-2 cells with a hope to replace the conventional radioactive assay. CTLL-2 cells were cultured with IL-2 and $^{[3H]}$TdR or alamarBlue and then processed as described in **Materials and Methods**. The accuracy of the IL-2 assay result by the alamarBlue method was compared to that of the conventional $^{[3H]}$TdR incorporation assay. The four different amounts of IL-2 were measured by both methods. The measured IL-2 concentrations were compared to the applied IL-2 concentrations (Fig. 3). Four hundred U/ml of IL-2 was used as the standard for the calculation (Gearing and Bird, 1987). The IL-2 concentrations measured by both the conventional and alamarBlue methods were very close to the applied IL-2 concentration. This indicates that both methods are equally reliable for the IL-2 bioassay between 16 U/ml and 2,000 U/ml of IL-2.

To evaluate the sensitivity of the alamarBlue assay verses the $^{[3H]}$TdR incorporation assay, CTLL-2 cells were cultured with serially diluted IL-2 and processed according to either the $^{[3H]}$TdR incorporation or the alamarBlue assay as described in **Materials and Methods**. The lowest concentration of IL-2 activity that could be determined by the alamarBlue assay and the $^{[3H]}$TdR incorporation assay was 4.8 ± 0.9 U/ml and 6.1 ± 0.8 U/ml, respectively (Fig. 4). The minimum assay limits of these two methods were not different significantly ($p = 0.4101, n = 3$). This indicates that the sensitivity of the alamarBlue method was comparable to that of the conventional $^{[3H]}$TdR incorporation assay.

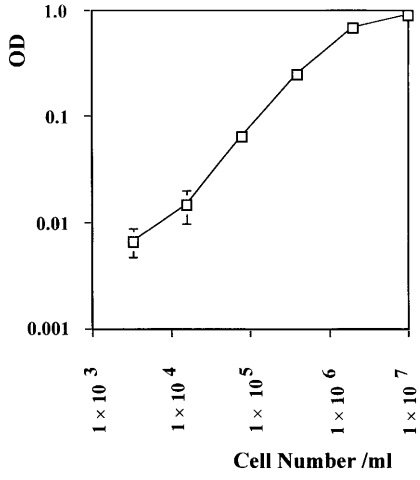

Fig. 2. The optical density of alamarBlue is proportional to cell number. Different numbers of CTLL-2 cells were incubated with 10% alamarBlue for 4 h and measured for optical densities at 570 and 600 nm. The specific absorbance (OD_{570}–OD_{600}) of cellular growth was calculated from the following equation. Specific OD = Sample OD – Media OD. The specific absorbances are presented as the mean ± the standard deviation of three independent experiments. The OD value versus cell number was plotted on a log scale vs log scale graphic sheet.

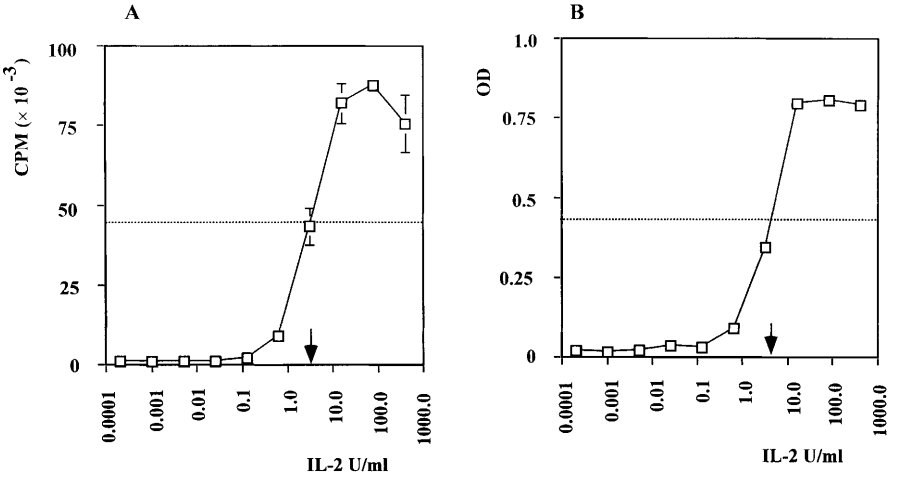

Fig. 3. The sensitivity of alamarBlue assay is comparable to that of the $^{[3H]}$TdR incorporation assay. CTLL-2 cells were cultured with the indicated concentrations of IL-2. The growth of cells was determined by $^{[3H]}$TdR incorporation (**A**) or the alamarBlue (**B**) assay using the same conditions as described in **Materials and Methods**. Values are given as the mean ± standard deviation of triplicates. Typical data from three independent experiments are presented. The dotted line is 50% of the maximum. The arrows show the minimal concentrations of IL-2 that can be calculated.

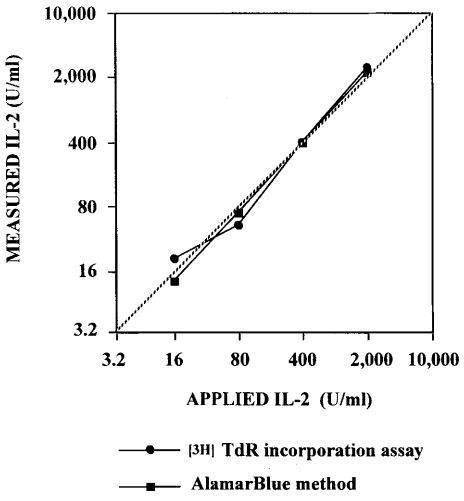

Fig. 4. The accuracy of the alamarBlue assay is comparable to that of the $^{[3H]}$TdR incorporation assay. CTLL-2 cells were cultured with the indicated concentrations of IL-2 and IL-2 activity was measured by the alamarBlue method (rectangle) and the $^{[3H]}$TdR incorporation assay (circle). The IL-2 activity was calculated using an arbitrary standard (400 U/ml). The dotted line is a theoretical IL-2 assay result. Typical data from three independent experiments are presented.

We describe above a simple, accurate, quantitative assay for IL-2 bioactivity using the redox indicator alamarBlue and based on the method described by Ansar Ahmed *et al.* (1994). This new method has several advantages over other proliferation assays that are commonly used. One principal advantage is that this assay does not employ any radioactive reagent.

Another advantage of the alamarBlue method is that the data collection step is not sample-destructive. The plate can be read at several different times without affecting the samples. This feature reduces the cost in kinetic studies that simultaneously measure dose and time functions when compared to earlier assays. This is a helpful aspect when a wide range of concentrations of growth factors are tested for their effect on cell proliferation. The activities of samples that are below the detection limit at an early time point can be detected by reading the plate again after further incubation. This eliminates the requirement inherent in the $^{[3H]}$TdR incorporation and MTT assays for preliminary experiments to set the range of IL-2 concentrations for the standard or the experimental samples, or for the incubation time of cells with the testing reagents. In the $^{[3H]}$TdR incorporation assay, if the incubation time is too short or too long, the IL-2 concentration of the test samples that gives 50% maximum incorporation of $^{[3H]}$TdR will fall above or below, respectively, the useful range for the IL-2 standard.

In addition, alamarBlue is soluble in standard media, nontoxic to the cells, and the assay is applicable to diverse cell culture systems. This method is easy to change to from the conventional $^{[3H]}$TdR incorporation or MTT assay system, if a microplate reader is available.

We successfully utilized the alamarBlue assay to measure the bioactivity of IL-2. This method was found valid for the IL-2 assay with CTLL-2 cells. The accuracy and sensitivity and the required length of time of this method were comparable to those of the conventional $^{[3H]}$TdR incorporation assay.

The alamarBlue method can be extended to other assay systems for measuring concentrations of other cytokines and cellular growth or inhibitory factors.

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