Use of a Soluble Tetrazolium/Formazan Assay for Chicken Cells
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ABSTRACT. We evaluated a soluble tetrazolium/formazan assay using 2, 3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) for chicken cell growth. Fifty microliter of solution containing 1 mg/ml of XTT and 0.025 mM phenazine methosulfate was added to the cells in a well of 96-well microplate. After 4 h incubation at 37°C, the absorbance was measured at 490 nm. Under this condition, absorbances were well correlated with cell number of Marek's disease tumor cells and chicken embryo fibroblasts. Proliferation of chicken lymphocytes stimulated with mitogens was also effectively measured. The formazan of XTT is water-soluble and can be quantitated in culture medium without the necessity for extraction with organic solvents. Thus XTT assay is simple and useful for the quantity assay with chicken cells.—KEY WORDS: chicken cell, colorimetric assay, XTT.


Mitochondrial dehydrogenases in living cells reduce tetrazolium salt, such as 3(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), to colored formazan. Previously, Mosmann applied this principle to a rapid and simple colorimetric assay [5]. MTT assay was used to study the in vitro effects of lymphokines [3, 5] and chemosensitivity on human tumor cell lines [1, 2]. In veterinary medicine, the assay has been applied to various functional assays of cytomolgus monkey lymphocytes [9]. It was also used to detect the tumor-associated antigens on bovine leukemic cells [11]. MTT assay is usually performed in 96-well microplates and analyzed with a scanning multiwell spectrophotometer. Thus large number of samples can be analyzed simply and rapidly. However, MTT assay requires the solubilization of formazan with organic solvents. This step complicates the assay and lengthens the time required to complete it. Recently, the new tetrazolium reagent, 2, 3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT), was designed to yield an orange-colored, water-soluble and non-toxic formazan upon metabolic reduction by viable cells [6]. It has been applied to chemosensitivity assays of human tumor cells [7]. The formazan of XTT, in contrast to MTT formazan, is water-soluble and thus can be quantitated in culture medium without the necessity for extraction with organic solvents. We examined advantage of XTT assay over MTT assay using several kinds of chicken cells. This paper describes the usefulness of XTT assay to estimate the proliferation of Marek's disease (MD) tumor-derived cell lines, chicken lymphocytes, and chicken embryo fibroblasts.

Five MD tumor cell lines; MDCC-MSB1, HP1, HP2, JP1 and RP1, were used in the study. The cells were cultured in the same manner as described previously [4]. Chicken spleen lymphocytes were collected from adult chickens by Ficoll-Conray centrifugation method [8]. Concanavalin A (Con A) or phytohemagglutinin (PHA) was added at a different concentration to PRMI 1,640 medium containing 10% fetal calf serum. A hundred thousand of chicken spleen cells per well were incubated for 72 h in a humidified atmosphere of 5% CO2-95% air at 37°C. Chicken embryo fibroblasts were prepared in the routine manner and different numbers of cells were seeded to the microplate. The cells were incubated for 72 h in a humidified atmosphere of 5% CO2-95% air at 37°C.

MTT assay was performed by the method of Denizot and Lang [3] with a slight modification. Briefly, the cells were cultured in 96-well microplate and the plate was centrifuged at 800 × g for 5 min after the cultivation. The supernatant of each well was removed by inverting the plate, and 50 μl of a 1 mg/ml MTT solution (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added. The plate was gently shaken and incubated for 3 h in a humidified atmosphere of 5% CO2-95% air at 37°C. Then the plate was centrifuged again and the non-reactive MTT was removed by inverting the plate. Fifty microliter of isopropanol was added to each well and the plate was then vigorously shaken to insure solubilization of the blue formazan. Absorbance of each well was measured with the ELISA reader ETY-96 (Oriental Instruments Ltd., Tokyo, Japan) at 570 nm test wavelength and 630 nm reference wavelength. The mean and standard deviation (SD) were determined from triplicate samples.

XTT assay was performed by the method of Scudiero et al. [7] with a slight modification. XTT (Polysciences Inc., Warrington, PA, U.S.A.) was prepared at 1 mg/ml in prewarmed Eagle's minimum essential medium (37°C) without serum. Phenazine methosulfate (PMS) (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added at various concentrations to the XTT solution to prepare PMS-XTT solution. The cells were cultured in 96-well microplate. After the cultivation, the plate was centrifuged and the supernatant was removed. Fifty microliter of PMS-XTT solution was added to each well and incubated for 4 h in a humidified atmosphere of 5% CO2-95% air at 37°C or 41°C. After incubation, the plate was vigorously shaken and the absorbance was measured with the ELISA reader ETY-96. Absorbance of the test well was subtracted by that of the well containing only PMS-XTT solution. The mean and SD were determined from triplicate samples.

First, the test wavelength of XTT assay was determined. Five hundred thousand MDCC-MSB1 cells per well were incubated with PMS-XTT solution, containing 0.025 mM of PMS, and the absorbance was measured at 450, 474, 490 and 577 nm. Mean and SD of the absorbance at each
Table 1. Effect of phenazine methosulfate (PMS) concentration and incubation temperature

<table>
<thead>
<tr>
<th>Cell</th>
<th>Incubation temperature</th>
<th>PMS (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCC-MSB1</td>
<td>0</td>
<td>0.092±0.011</td>
</tr>
<tr>
<td>37</td>
<td>0.107±0.028</td>
<td>0.360±0.031</td>
</tr>
<tr>
<td>MDCC-RP1</td>
<td>0.036±0.034</td>
<td>0.405±0.025</td>
</tr>
<tr>
<td>37</td>
<td>0.066±0.046</td>
<td>0.344±0.069</td>
</tr>
<tr>
<td>MDCC-JP1</td>
<td>0.196±0.022</td>
<td>0.377±0.007</td>
</tr>
<tr>
<td>37</td>
<td>0.226±0.035</td>
<td>0.306±0.007</td>
</tr>
</tbody>
</table>

a) Absorbance at 490 nm, subtracted by the absorbance of control, Mean±standard deviation

Fig. 1. Absorbance of various numbers of Marek's disease tumor-derived cell lines in XTT and MTT assays.

wavelength were 0.601±0.114, 0.660±0.121, 1.138±0.042, 0.417±0.053, respectively. The absorbance at 490 nm was highest. Similar results were obtained by using other MD cell lines. Therefore we measured absorbances at 490 nm, though Scudiero et al. measured the absorbances at 450 nm in the previous XTT assay [7].

Next, the effects of concentration of PMS and incubation temperature were examined. Eighty thousand cells were incubated with PMS-XTT solution, containing different concentration of PMS. The plate was incubated at 41°C, an optimum temperature for MD tumor cell lines, or 37°C. After 4 hr incubation, the absorbance was measured. The results are shown in Table 1. The addition of PMS resulted in a marked increase in absorbance as previously reported with human tumor cell lines [7]. The absorbance of the samples incubated at 41°C was a little higher than those incubated at 37°C. However, control of 41°C had 1.2-fold the absorbance of that of 37°C (data are not shown). Therefore, the assay was performed using 0.025 mM PMS and 37°C incubation, that was the same method as described previously [7].

Since XTT-formazan resembles phenol red in color, the effect of phenol red in the medium was examined. No significant difference was observed between PMS-XTT solutions with or without the phenol red (data are not shown).

Absorbance of various numbers of MD tumor cell lines is shown in Fig. 1. Various numbers of cells were distributed to each well of the microplates. Then, XTT and MTT assays were compared. A range of cell number that gave a detectable and relatively linear range of absorbances was 1×10⁵ - 5×10⁶ in XTT assay, while that was 1×10⁴ - 5×10⁶ in MTT assay. These results indicate that XTT assay can estimate a smaller number of cell than MTT assay. Furthermore, from the results shown in Fig. 1, less number of cells might be assayed by the XTT assay.
COLORIMETRIC ASSAY FOR CHICKEN CELLS

Fig. 2. Absorbance of stimulated chicken spleen lymphocytes.

Besides the lymphoid tumors, we also examined the proliferation of chicken lymphocytes by XTT assay. As shown in Fig. 2, proliferation of chicken lymphocytes stimulated with Con A or PHA was effectively measured by XTT assay.

Next, we examined the proliferation of chicken embryo fibroblasts by XTT assay. Figure 3 shows that a nearly linear relationship is observed between cell number and absorbance when the cell number is between $5 \times 10^5$ and $1.5 \times 10^6$.

A colorimetric assay, such as MTT assay, offers major advantages in speed, simplicity, cost and safety over conventional assays using the uptake of radiolabelled compounds. However, the main problem of MTT assay is the difficulty of solubilizing the formazan product. Thus, XTT, a second generation tetrazolium salt, was synthesized [6]. Since then, XTT has been evaluated for feasibility of use in high-flux in vitro antitumor screening against a broad panel of human tumor cell lines [7].

In this paper, we describe the application of XTT assay to chicken tumor cells, lymphocytes and fibroblasts. From the present results, absorbances were well correlated with cell number or cell proliferation. Thus, XTT assay can be applied not only to MD tumor cell lines but also to primary chicken lymphocyte culture and fibroblasts.

Previously, Scudiero et al. indicated that the addition of PMS, an electron-coupling agent, increased the complexity of the cellular reduction environment [7]. They also indicated the occasional formation of crystalline material in the XTT assay, which caused erratic absorbance measurements. Recently, Vistica et al. reported that crystal formation was significant at pH 7.8 to 8.0 and was attributed to reaction of PMS with the cellular nucleophile glutathione [10]. Though we did not experience such an injurious influence of PMS in this study, it should be considered in the experiments. Because of its simplicity and safety, tetrazolium-based assays may widely be used in veterinary medicine. More applications using many kinds of cells are expected and the assay condition should be established to each experiment.

REFERENCES