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Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays

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A tetrazolium salt has been used to develop a quantitative colorimetric assay for mammalian cell survival and proliferation. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This method can therefore be used to measure cytotoxicity, proliferation or activation. The results can be read on a multiwell scanning spectrophotometer (ELISA reader) and show a high degree of precision. No washing steps are used in the assay. The main advantages of the colorimetric assay are its rapidity and precision, and the lack of any radioisotope. We have used the assay to measure proliferative lymphokines, mitogen stimulations and complement-mediated lysis.

Key words: *lymphokine assays – proliferation assays – colorimetric assay – tetrazolium – TCGF*

Introduction

Many biological assays require the measurement of surviving and/or proliferating mammalian cells. This can be achieved by several methods, e.g., counting cells that include/exclude a dye, measuring released ^{51}Cr-labeled protein after cell lysis, and measuring incorporation of radioactive nucleotides ([^{3}H]thymidine or [^{125}I]iododeoxyuridine) during cell proliferation. The radioactive method can be partially automated and can handle moderately large numbers of samples, but even with these methods, it is difficult to process thousands of assay points per day. In our current research we assay many samples of various lymphokines that induce cell proliferation, and so we required a rapid and quantitative assay capable of handling large numbers of samples.

Viable cells could be measured by using any of several staining methods, but we wished to avoid any washing steps that would increase processing time and sample variation. Multiwell scanning spectrophotometers (ELISA readers) can measure large numbers of samples with a high degree of precision, and so we investigated the possibility of using a color reaction as a measure of viable cell number. Ideally, a

colorimetric assay for living cells should utilize a colorless substrate that is modified to a colored product by any living cell, but not by dead cells or tissue culture medium. Tetrazolium salts are attractive candidates for this purpose, since they measure the activity of various dehydrogenase enzymes (Slater et al., 1963). The tetrazolium ring is cleaved in active mitochondria, and so the reaction occurs only in living cells.

We have developed a rapid colorimetric assay, based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), that measures only living cells and can be read on a scanning multiwell spectrophotometer (ELISA reader). This assay is versatile and quantitative, and we consider it a significant advance over traditional techniques for several commonly used proliferation and cytotoxicity assays.

Materials and Methods

Cell lines

The EL_4G^- mouse lymphoma cell line was obtained from G. Carlson, and subclone $EL_4.3$ was selected for growth in 6-thioguanine. Another subline of EL_4, designated EL_4E2, was obtained from V. Paetkau. The EL_4E2 subline produces large quantities of interleukin 2 when stimulated with phorbol myristate acetate (Farrar et al., 1980). A continuous line of mouse T cells, A70 13/13, was derived in the author's laboratory at the University of Alberta. All cells were grown in RPMI 1640 supplemented with 50 μM 2-mercaptoethanol and 5–10% fetal bovine serum, in a 6% CO_2 atmosphere.

Colorimetric MTT (tetrazolium) assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma catalog no. M2128) was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. At the times indicated below, stock MTT solution (10 μl per 100 μl medium) was added to all wells of an assay, and plates were incubated at 37°C for 4 h. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a Dynatech MR580 Microelisa reader, using a test wavelength of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.99 (or 1.00 if the samples were strongly colored). Plates were normally read within 1 h of adding the isopropanol.

Interleukin 2 assay

Interleukin 2 (IL2) was derived from phorbol-myristate-acetate stimulated EL_4E2 cells (Farrar et al., 1980). The IL2-dependent T cell line A70 13/13 was used as an indicator cell. Doubling dilutions of IL2 were prepared in 96-well trays using growth medium as diluent. T cells (2000 per well) were then added, with a final volume of 0.1 ml per well. At 48 h, proliferation was measured by the MTT colorimetric assay.

Mitogen-induced proliferation of spleen cells

BALB/c mouse spleen cells were stimulated in 0.1 ml at 10^6 cells/well with varying concentrations of *Salmonella typhosa* lipopolysaccharide (LPS; Sigma) or concanavalin A (Con A; Calbiochem) and assayed at 3 days for proliferation using both colorimetric MTT and [^{3}H]thymidine incorporation assays. For the radioactive assay, 0.001 mCi [^{3}H]thymidine was added to each well, and after 4 h at 37°C the cells were harvested using a PHD cell harvester (Cambridge Instruments, Cambridge, MA).

Computer processing

Readings from the Dynatech MR580 Microelisa reader were transferred directly to an Apple II computer, using a program that saved the results to a diskette and printed the OD values in a 96-well format that matched the original plate. Additional programs were written to process the data stored on diskettes. We now have programs to plot results, calculate and plot means and standard deviations, identify wells above a chosen threshold, and calculate units of growth factor. These programs are available on request.

Results

In preliminary experiments, we tested several tetrazolium salts by incubation with cells for several hours. The most promising reagent was MTT, a pale yellow substrate that produced a dark blue formazan product when incubated with live cells. The MTT formazan reaction product was only partially soluble in the medium, and so an alcohol was used to dissolve the formazan and produce a homogeneous solution suitable for measurement of optical density. Initially, ethanol was used for this purpose, but some precipitation of serum proteins occassionally occurred in the acid-alcohol mixture. Several other organic solvents were tested, and isopropanol was found to be the most suitable solvent. Normal tissue culture medium has a variable color due to pH changes and the red form of phenol red interfered at the wavelength most suitable for blue MTT formazan measurement. To minimize this interference, we converted the phenol red to the fully acidic, yellow form at the end of the assay.

Our final procedure was to add 0.01 ml MTT (5 mg/ml in phosphate-buffered saline) to 0.1 ml cells in growth medium. After 4 h at 37°C for MTT cleavage, the formazan product was solubilized by the addition of 0.1 ml 0.04 N HCl in isopropanol. Optical density was measured on a Dynatech MR 580 plate reader, using a reference wavelength of 630 nm and a test wavelength of 570 nm.

EL_4.3 lymphoma cells were used to test the relationship between cell number and the amount of MTT formazan generated. The results in Fig. 1 show that the absorbance is directly proportional to the number of cells. This linearity extends over almost the entire range tested, from 50,000 to 200 cells/well. In addition, these results indicate that the assay is capable of detecting very small numbers of living cells (e.g., 200). The actual cells do not absorb significantly, even at a concentration of 1×10^6 cells/ml.

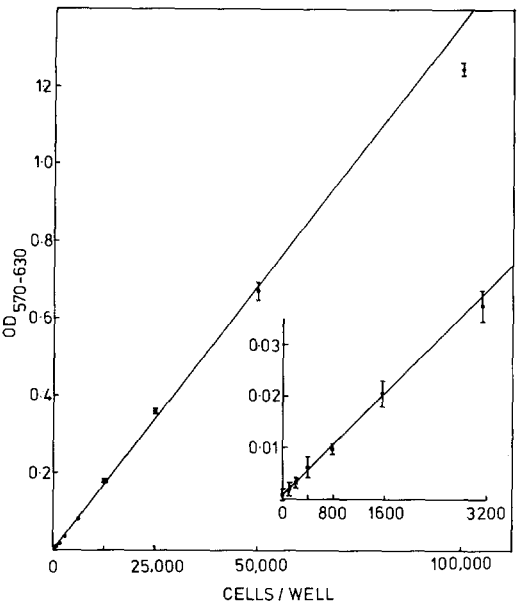

Fig. 1. Linearity of the MTT assay. $EL_4.3$ cells were plated out in doubling dilutions in 0.1 ml growth medium (RPMI 1640 + 10% fetal bovine serum) in 96-well flat-bottomed trays (Falcon), starting at 10^5 cells/well. MTT (0.01 ml of 5 mg/ml stock) was added immediately to all wells, and the plates were incubated at 37°C for 3 h, developed and measured. Each point shows the mean and standard deviation of 4 replicates. The straight line plotted is the best fit line calculated using all points from 100 to 50,000 cells/well.

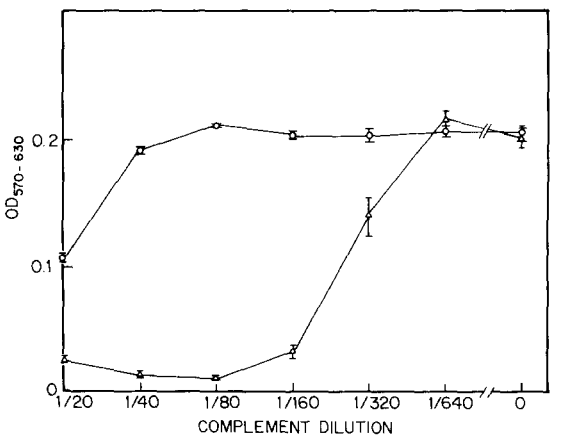

Fig. 2. Living cells are required for MTT cleavage. $EL_4.3$ cells were treated with anti-Thy1.2 (Cedarlane), and then treated cells and untreated controls were added to rabbit complement dilutions in a 96-well tray. After 30 min incubation at 37°C, MTT was added to all wells, and after another 4 h the plates were developed and read. Each point shows the mean and standard deviation of 4 replicates. △, anti-Thy1.2-treated; ○, untreated.

In many assays, dead cells will be present, and so it was important to determine if recently killed cells were positive or negative in the assay. Fig. 2 shows that only live cells actively cleave MTT, while dead cells are almost completely negative even immediately after complement-mediated lysis. These results suggested that living cells with active mitochondria are required to generate a strong signal, and raised the possibility that the amount of formazan generated per cell would depend on the level of energy metabolism in the cell. To test this, we measured formazan generation by metabolically inactive cells (red blood cells), resting cells (spleen cells) and activated cells (concanavalin A-stimulated lymphocytes). Fig. 3a shows that neither chicken nor sheep red blood cells cleave MTT to a significant extent, and neither red cell type interferes significantly in the assay, up to concentrations of 2×10^6 cells/ml. Fig. 3b shows that Con A-activated lymphocytes produce approximately 10 times as much formazan per cell as their normal counterparts.

A continuous line of interleukin 2 (IL2)-dependent T cells (A70 13/13), previously established in the author's laboratory at the University of Alberta, was used as

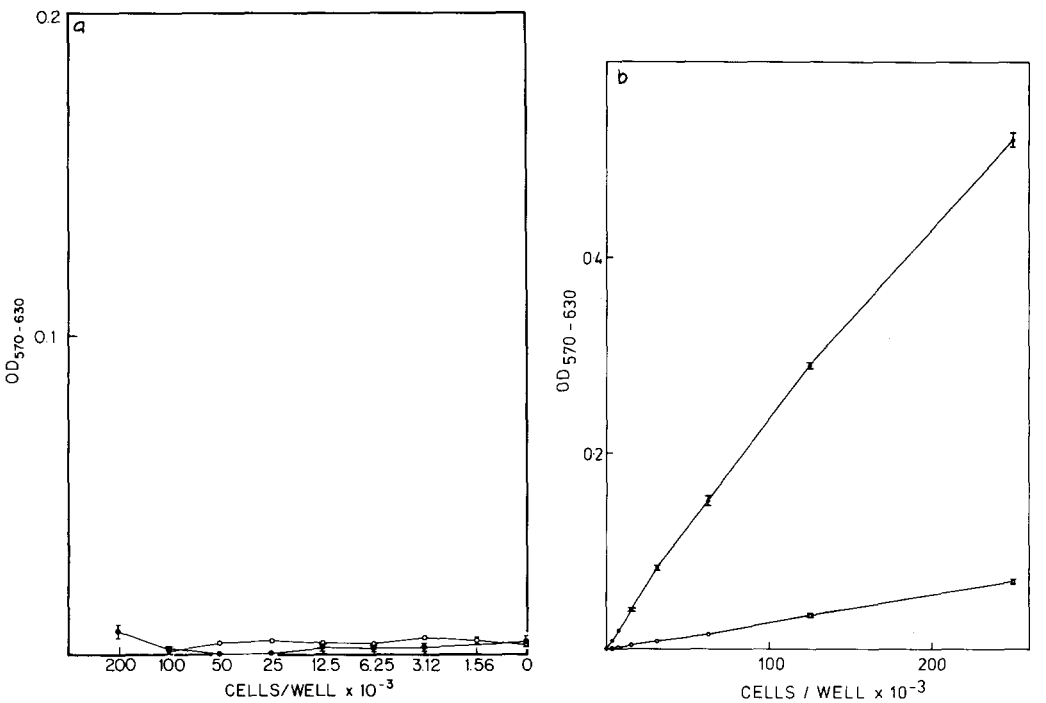

Fig. 3. MTT cleavage by erythrocytes and normal and activated lymphocytes. a: chicken and sheep erythrocytes were incubated in 0.1 ml medium with MTT for 3 h at 37°C. The plates were then developed and read. Means and standard deviations of 3 replicates per point are shown. ●, chicken erythrocytes; ○, sheep erythrocytes. b: mouse spleen cells were stimulated with 2 μg/ml concanavalin A for 48 h, and then the Con A-activated lymphocytes and normal lymphocytes were plated in doubling dilutions in 96-well flat-bottomed trays. MTT was added immediately, and after 2 h, the plates were developed and read. The means and standard deviations of 3 replicates per point are shown: ●, Con A-activated lymphocytes; ○, normal lymphocytes.

the target cell for an IL2 assay. This assay was used to test 4 parameters of the colorimetric reaction – the length of exposure of cells to IL2, the duration of MTT treatment, the concentration of MTT used, and the number of test cells added to the assay.

Fig. 4 shows the effect of varying the time of incubation with MTT. The signal increased almost linearly from 1/2 to 2 h, but increased at a lesser rate from 2 to 4 h. In similar experiments, the concentration of MTT and cell number were optimized for the cell lines used in our studies (results not shown). The formazan generated was approximately proportional to the MTT concentration at low concentrations, and reached a plateau at about 0.45 mg/ml MTT. The formazan generated was also proportional to the number of cells at high IL2 concentrations, but the amount of factor required to produce 50% stimulation was increased at higher target cell concentrations. The assay could be read at 1, 2 or 3 days, but the apparent titer of the IL2 declined with increasing incubation time, probably due to depletion of the growth factor during cell growth. Our optimum values for these 4 parameters may need modifying for other assays, but in general, we have found that widely differing cell lines require only minimal changes.

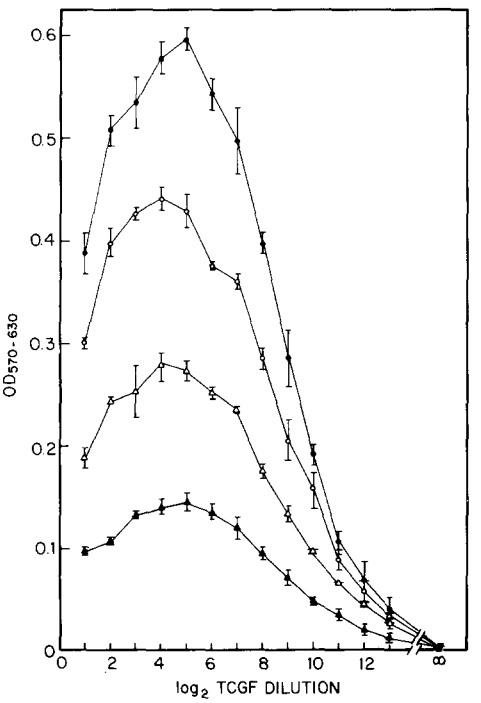

Fig. 4. Duration of MTT incubation. Interleukin 2 dilutions were assayed on A70 13/13 T cells. MTT was added at 44, 46, 47 and 47½ h, to sets of 3 rows each, and at 48 h, all wells were developed and read. Optical density readings were measured relative to control wells containing medium, cells and MTT but no growth factor. The means and standard deviations of 3 replicates per point are shown. ▲, 1/2 h; △, 1 h; ○, 2 h; ●, 4 h.

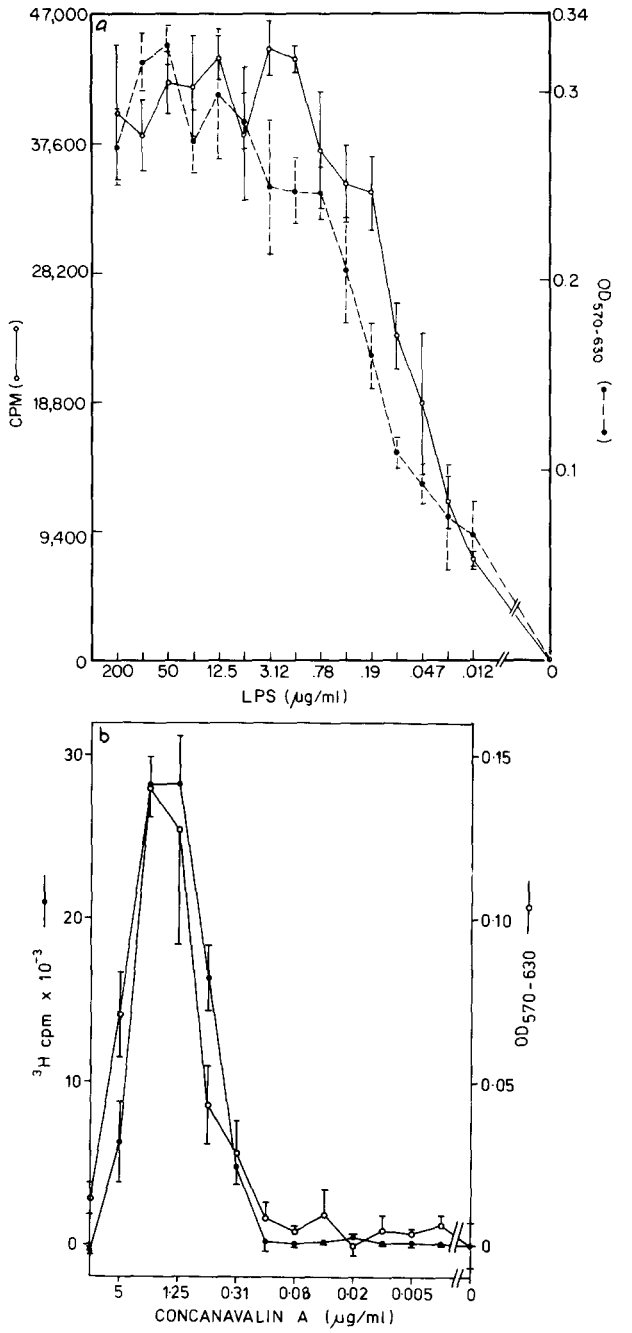

Fig. 5. Mitogen-induced proliferation of spleen cells. Spleen cells were stimulated for 3 days with varying concentrations of LPS and Con A and proliferation was measured using both colorimetric and radioactive assays. Results are shown as the means and standard deviations of 4 replicates per point. Background values, obtained from wells with cells but no mitogen, were subtracted from all points. a: LPS stimulation. b: Con A stimulation.

The results of the colorimetric assay with cloned cell lines were very encouraging, and so we explored the utility of the assay in more complex systems: the lymphocyte proliferative responses to the mitogens Con A and LPS. Since many cell types are present in the cell populations normally used for such proliferations, it was conceivable that certain cell types would generate abnormally large or small signals. Accordingly, we compared the colorimetric assay to a [^{3}H]thymidine incorporation assay for both Con A and LPS responses of normal mouse spleen cells.

Stimulation with both mitogens was measured effectively by both assays (Fig. 5). LPS stimulated cells over an extended concentration range, whereas the titration curve for Con A showed a narrow optimum, with little or no proliferation at high or low concentrations. The colorimetric and radioactive assays showed excellent agreement for Con A stimulations, and showed a small difference between the endpoint of LPS stimulations.

Activated macrophages produce more formazan product from nitroblue tetrazolium than do non-activated macrophages (Baehner et al., 1976), and so we measured MTT formazan production after LPS activation of a macrophage-like cell line, P388D1 (Lachman et al., 1977). No increase in MTT formazan production was seen after stimulation with a wide range of LPS concentrations, and P388D1 cells did not produce an unusual amount of MTT formazan (results not shown).

Discussion

The cleavage of MTT has several desirable properties for assaying cell survival and proliferation. MTT is cleaved by all living, metabolically active cells that we have tested, but not by dead cells or erythrocytes. The amount of formazan generated is directly proportional to the cell number over a wide range, using a homogeneous cell population. Activated cells produce more formazan than resting cells, which could allow the measurement of activation even in the absence of proliferation. These properties are all consistent with the cleavage of MTT only by active mitochondria.

The main advantage of the colorimetric assay is the speed with which samples can be processed. The substrate does not interfere with measurement of the product, and we have found conditions in which components of the medium do not interfere. This allows the assay to be read with no removal or washing steps, which increases the speed of the assay and helps to minimize variability between samples. The final stages of the assay (adding the MTT, reading the plate and printing the data) take much less time than setting up the assay (mixing cells and growth factor dilutions). The assay can be read a few minutes after the addition of acid-isopropanol, and the color is stable for a few hours at room temperature. The results are also apparent visually, which is very useful if rapid qualitative results are required.

The colorimetric assay measures the number and activity of living cells at the end of the assay, whereas [^{3}H]thymidine incorporation measures the number of cells synthesizing DNA during the last few hours of the assay. So the colorimetric assay correlates well with visual examination of the cells at the end of the assay (Kappler

et al., 1981) but these 2 assays can potentially differ from radioactive nucleotide incorporation methods. This should be kept in mind for specific applications, e.g., distinguishing between death, survival and proliferation. In practice, we have not seen large differences between the colorimetric assay, radioisotope assay or visual inspection of the wells.

The only additional reagents used in the assay are MTT, isopropanol, and HCl. No radioisotopes are used, and no scintillation counter or gamma-counter is needed. This advantage is partially offset by the requirement for a plate reader, but the high scanning rate of typical machines (e.g., $1\frac{1}{2}$ min per 96 wells) allows a single plate reader to handle very large numbers of samples.

The colorimetric assay shares with the radioisotope assays the advantages of precise quantitation and compatibility with computer analysis programs. Since the colorimetric assay is so rapid, large amounts of data can be generated, and some form of computer processing is very desirable. We have set up programs for calculating means and standard deviations, plotting curves, and calculating units of growth factor in the original sample (using a linear interpolation to calculate the exact dilution at which stimulation is a preset value, e.g., 25% of the maximum plateau stimulation). These programs are written for an Apple II computer, and are available on request.

The reduction of MTT to a formazan product appears to be carried out by all the cell types we have examined. These include mitogen stimulated T and B cells, myeloma, T lymphoma and macrophage-like tumor cell lines, as well as various IL2-dependent T cell lines. This suggests that the colorimetric MTT assay may have very wide applicability for measuring survival and/or proliferation of various cells and can potentially be applied to any assay in which living cells must be distinguished from dead cells or a lack of cells. The results in Fig. 2 show that dead cells are unable to cleave MTT within 30 min of complement-mediated lysis. This indicates that the assay also has potential value for quantitative and rapid measurement of cell death, e.g., in HLA typing. The MTT assay may also be applicable to the assay of cytotoxic T lymphocytes, although the signal generated by the CTL population could mask the signal from the target population at high effector : target ratios.

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