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Estimation of Cell Number by Neutral Red Content

Applications for Proliferative and Survival Assays

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Abstract

A simple photometric method for estimating viable cell number in culture is described. When cultured cells are allowed to internalize 0.005–0.01% neutral red for 1 h, the amount of accumulated dye is directly proportional to cell number. The linear relationship holds for adherent and suspended cell lines. Thus, dye content reflects cell number. Since dye content is easily measured by instruments that photometrically scan microtiter trays, proliferative and survival (cytotoxic) responses can be easily quantitated.

Index Entries: Cell counting; dye uptake; neutral red; survival assay; proliferative assay; red dye uptake.

Introduction

Present methods for measuring cell number, such as light scatter counting or incorporation of a radiolabeled substance, may be tedious when measuring many samples. These methods involve dislodging adherent cells or other multistep procedures. Here we report a simple method for measuring viable cell number.

The method is based on a well-established phenomenon: viable cells internalize and concentrate weak bases (see Discussion). One such substance, the dye neutral red (Color Index #50040), has been used as a vital stain for many years (*1*). For example, it has been used to quantitate the cytopathic effects of viruses on cells (*2*).

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Here we describe conditions by which dye accumulation is directly proportional to cell number. The availability of instruments that photometrically scan microtiter trays makes our method practical for the quantitation of substances that influence cell number. Examples of the measurement of a proliferative and a cytotoxic activity are illustrated.

Materials and Methods

The dye uptake measurement procedure is detailed in the legend to Fig. 1. The L-M murine tumorigenic fibroblast line, CCL 1.2, and the CPAE bovine endothelial cell line, CCL 209, were obtained from the American Type Culture Collection, Rockville, MD. The HL-60, a human promyelocytic leukemia cell line (*3*), was a generous gift to our laboratory from Dr. Robert Gallo. J774.1, a murine macrophage-like line, was a generous gift from Dr. Yancy Gillespie. L-M cells were cultured in Eagle's minimum essential medium with Earle's salts (MEM†, GIBCO, Grand Island, NY), 5% fetal bovine serum (FBS, Sterile Systems, Logan, UT). CPAE cells were cultured in MEM, 10% FBS. HL-60 and J774.1 cells were cultured in RPMI 1640 (GIBCO), 10% FBS. Cells were grown in tissue culture grade plasticware (Becton Dickinson, Oxnard, CA, and Corning Glass Works, Corning, NY) in a humidified incubator, 37°C, in an atmosphere of air, 5% CO_2. Lines were free of mycoplasma by transmission electron microscopy evaluation. Adherent cells were dislodged by brief treatment with 0.05% EDTA and/or 0.05% VMF trypsin (Millipore Corp., Bedford, MA) in 140 m*M* NaCl, 16 m*M* Na_2HPO_4, 4 m*M* KH_2PO_4, pH 7.4. Cell concentrations were determined with a hemocytometer. Neutral red was obtained from Maufacturing Chemists, Norwood, OH. A 2% (w/v) stock solution was prepared in 25% ethanol.

An ethanol extract of Walker 256 rat carcinoma was prepared as described elsewhere (*4*). The Walker tumor was a generous gift from Dr. Alan Fenselau. Macrophage cytotoxin, also called tumor necrosis factor (*5*), was partially purified from a murine macrophage-like cell line (manuscript in preparation) by methods similar to those reported elsewhere (*6*, *7*).

Results

Figure 1 compares dye content as a function of cell number for three unrelated cell types. Included are three adherent types (L-M, J774.1, and CPAE) and one suspension type (HL-60). The amount of accumulated dye is directly proportional to cell number for each type. The cell concentrations examined (number/cm^2) ranged from very sparse to the point where cells were crowded. The linear relationship shown in Fig. 1 appears to be universal. We have examined dye uptake in a variety of established cell lines. Regardless of tissue or species of origin, the cells accumulate dye (data not shown). However, the extent of accumulation among cell types

†Abbreviations used: MEM, minimum essential medium; FBS, fetal bovine serum.

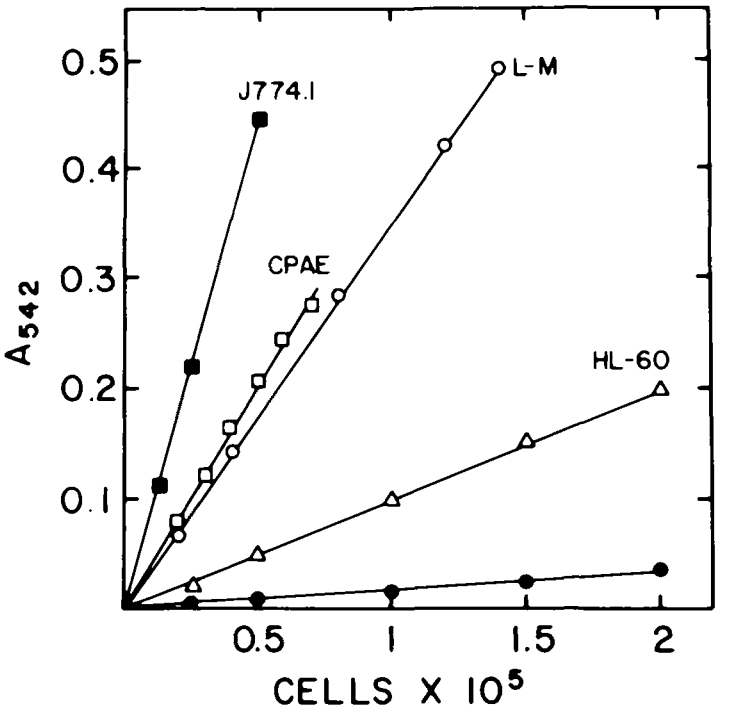

Fig. 1. Dye content as a function of cell number. Stock solutions of cell suspensions were diluted to give the indicated number of cells/200 μL culture medium. This volume was seeded into wells of 96-well flat bottom microtiter trays (0.3 cm^2). J774.1, CPAE, and L-M cells were allowed to attach by incubating for 4 h at 37°C. Fifty μL of 140 m*M* NaCl, 16 m*M* HCl, and 0.036% neutral red were added to each well, and the cultures were incubated for 1 h. The HL-60 tray (suspended cells) was centrifuged for 1 min at 500*g*. Non-internalized dye was removed from the wells by suction of the supernatants. Wells were washed once with MEM. The HL-60 tray was centrifuged as before. The solid circles show HL-60 cells treated identically, but without dye. The cells were lysed with 200 μL of a solution of equal parts (v/v) of ethanol and 100 m*M* NaH_2PO_4, pH 4.5. A_{452} was determined with a Titertek Multiskan (Flow Laboratories, Bethesda, MD.). Points are the mean of at least four determinations subtracted from the mean blank (no cell) value (~0.04) and the mean cell (no dye) value. Standard deviations for each point ranged from 0.015 to 0.04. Figure 2 is a composite of determinations performed for each cell line on different days. The results for each line are representative of at least two trials.

varies as can be seen by comparing the *slopes* in Fig. 1. J774.1, a macrophage-like line, readily internalizes dye. HL-60, a promyelocyte line, is less capable. Many cell lysates (without dye) have a modest A_{542} ($<0.03/10^5$ cells). Some cell types, such as melanomas, may have a more substantial absorbance. The A_{542} of HL-60 cells without dye is shown in Fig. 1. It is also proportional to cell number, and it is representative of results obtained with the other lines used in Fig. 1.

The linear relationship (dye content versus cell number) suggests that the rate of dye accumulation per cell is independent of cell density. This possibility is examined with L-M cells in Fig. 2. Figure 2 shows neutral red accumulation as a function of time for two cell densities, sparse ($0.5 \times 10^5/cm^2$) and in one with com-

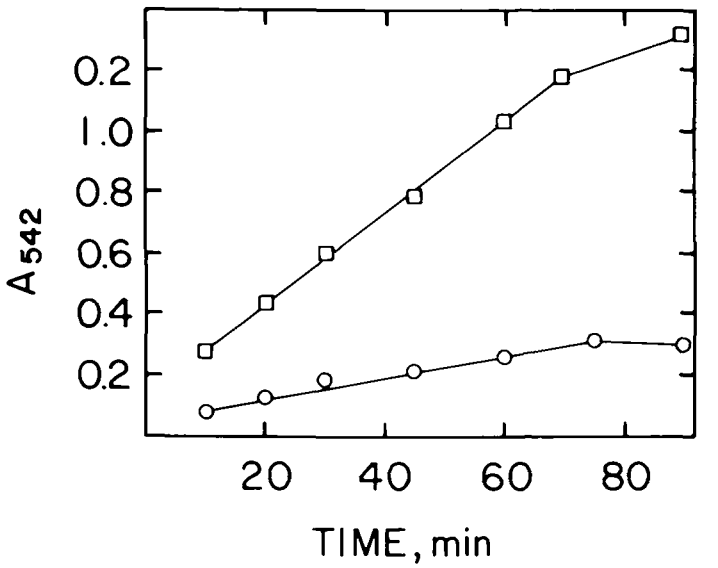

Fig. 2. Time course of neutral red accumulation in L-M cells. L-M cells were seeded into wells of 24-well trays (2 cm^2) in 1 mL culture medium. Cells were allowed to attach by incubating for 4 h at 37°C. 100 μL of 0.1% neutral red in 140 m*M* NaCL, 16 m*M* HCl were added to each well, and the cultures were incubated for the indicated times. Supernatants were removed by suction, and the wells were washed with MEM. The cells were lysed with 1 mL as described in the legend to Fig. 1. A_{542} was determined spectrophotometrically. Points are the mean of four determinations. The SEM for each point was approximately 0.01 and did not exceed 0.05. Results are representative of two separate trials. (□), 400,000 cells/well; (○), 100,000 cells/well.

plete cell–cell contact ($2 \times 10^5/cm^2$). The rate of accumulation per cell is similar for both densities during the linear portion of the curve (0.036 A_{542} U/min/10^5 cells). At more crowded cell densities ($>3 \times 10^5/cm^2$) the linear rate described above declines (data not shown).

We have employed dye content for the quantitation of both proliferative and survival (cytotoxic) activities. Examples are illustrated in Figs. 3 and 4, respectively. Figure 3 shows the proliferative effects of FBS and a tumor extract (*4*) on endothelial cell proliferation. A 7.5% quantity of FBS caused a 1.5-fold increase in the amount of dye (number of endothelial cells) after a 3-d period. Tumor extract (0.1 A_{260} units) caused a similar stimulation. These values are in good agreement with values obtained elsewhere (and confirmed by us) for a 1.5-fold increase in cell number as measured by direct cell counting or incorporation of [^{3}H]-thymidine into acid-precipitable material (*4*).

Figure 4 illustrates the decline in the amount of dye (number of L-M cells) as a consequence of exposure to cytotoxic macrophage protein. A 50% survival was achieved with 125 ng protein. A similar sigmoidal response has been observed with the putative macrophage cytotoxin purified from tumor necrosis serum (*8*). Probit transformation of the dilution curve yields a straight line from which accurate quantitations of the cytotoxic activity ± 6% can be obtained (see ref. *6*).

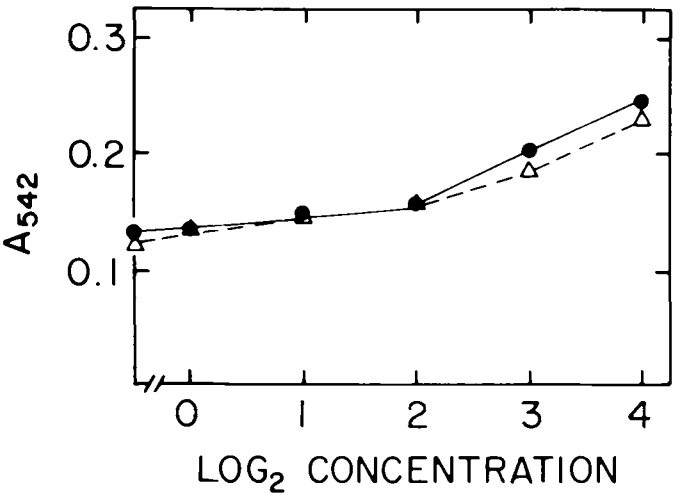

Fig. 3. The effect of FBS and tumor extract on endothelial cell proliferation. CPAE cells were seeded at 4×10^3/well/200 μL MEM, 5% FBS into 96-well flat-bottom microtiter trays. Trays were incubated overnight. The culture medium was replaced with RPMI 1640, 2% dialyzed FBS. Twofold concentrations of test material diluted in 50 μL of RPMI 1640 were added to wells in quadruplicate. The first concentration (Log_2 0) of nondialyzed FBS was 0.625%). The first concentration of tumor extract was 0.0125 A_{260} units/mL. Cultures were incubated for 3 d. Dye content was determined as described in the legend to Fig. 1. Points are the mean of four determinations, SD < 0.04. Results are representative of at least three separate trials. (△), FBS; (●), tumor extract.

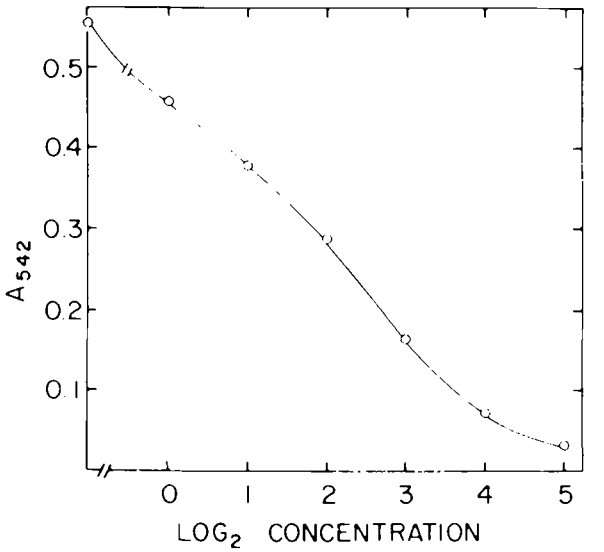

Fig. 4. The effect of macrophage protein on L-M cell survival. L-M cells were seeded at 1.5×10^4/well/200 μL MEM, 2% FBS into 96-well flat-bottom microtiter trays. Trays were incubated overnight. Twofold concentrations of test material, diluted in 50 μL MEM, were added to duplicate wells. The first concentration (Log_2 0) of macrophage protein was 31 ng/mL. Cultures were incubated overnight. Dye content was determined as described in the legend to Fig. 1. Results are representative of many separate trials.

Discussion

We describe conditions whereby the amount of neutral red accumulated by cells in culture is directly proportional to their number. Thus, dye content gives a convenient measure of the number of viable cells, and activities that influence cell number can be easily quantitated. The availability of instruments that photometrically scan microtiter trays makes our method suitable for the rapid analysis of multiple concentrations of test materials and with replicates sufficient for statistical evaluation. We have found the method useful for quantitating some physiocochemical (*6*) and mechanistic (*8*) properties of a cytotoxin, for drug in vitro toxicity tests, and for the quantitation of growth promoting activities.

The conditions we employ appear to be useful for measuring cell number in a variety of established cell lines. The dye concentrations is fourfold greater than that employed for virus titration (*2*). Higher dye content is necessary to measure low numbers of cells, but too much dye generates high background. Dye is added to the cells in acidic conditions, because neutral red tends to precipitate at neutral pH. The following conditions also bear consideration. First, measurements should be made during the time when accumulation is linear (10–75 min). Second, the linear relationship (dye content vs cell number) fails for cells at densities that are too high. The initial and later rates of accumulation appear to be related to cell density (Fig. 2). These rates may be influenced by the area of exposed plasma membrane. Cells seeded at low density spread more than those seeded at high density. Ideally, conditions should be examined for individual lines.

Active sequestering mechanisms are believed to underlie the capacity of living cells to concentrate the dye (*11*). The internal accumulation of neutral red appears to be a general cellular phenomenon whose rate is a combination of internalization, compartmentalization, and exocytosis. In fibroblasts, neutral red is internalized into autophagic vesicles and concentrated in lysosomes (*10*). We find no evidence (in the literature or in our experience) that other cell types behave differently. We do observe differences between different cell lines in the quantity of dye accumulated (Fig. 1), which is to be expected. The variables contributing to the kinetics of accumulation have been discussed elsewhere (*10*). In summary, uptake of neutral red is similar to other lysosomotropic agents (*12, 13*). These agents pass freely (or actively) through the plasma membrane. They are protonated and trapped inside lysosomes, where they may change the pH by two log units. Intracellular concentrations may reach 100- to 1000-times the extracellular concentration. When neutral red is removed from the culture fluid, macrophages exocytose the dye at a rate that is equivalent to that of its entry (*14*). Neutral red stains subcellular structures such as mitochondria (*9*) and RNA (*10*); however, the dye is not concentrated to the same degree. We employ a washing step that removes nonadherent cell debris before measuring dye content.

Acknowledgments

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References

1. Conn, H. J. (1969), *Biological Stains*, 8th ed., Williams and Wilkins, Baltimore, MD, pp. 269–270.
2. Finter, N. B. (1969), *J. Gen. Virol.*, **5**, (3), 419.
3. Collins, S. J., Gallo, R. C., and Gallagher, R. E. (1977), *Nature* **270**, 347.
4. Fenselau, A., Watt, S., and Mello, R. J. (1981), *J. Biol. Chem.* **256**, 9605.
5. Matthews, N. (1978), *Br. J. Cancer* **38**, 310.
6. Kull, F. C., Jr., and Cuatrecasas, P. (1981), *J. Immunol.* **126**, 1279.
7. Männel, D. N., Moore, R. N., and Mergenhagen, S. E. (1980), *Infect. Immun.* **30**, 523.
8. Kull, F. C., Jr., and Cuatrecasas, P. (1981), *Cancer Res.* **41**, 4885.
9. Dell'Antone, P., Colonna, R., and Azzone, G. F. (1979), *Eur. J. Biochem.* **24**, 553.
10. Bulychev, A., Trouet, A., and Tulkens, P. (1978), *Exptl. Cell Res.* **115**, 343.
11. Ohkuma, S., and Poole, B. (1978), *Proc. Natl. Acad. Sci. USA* **75**, 3327.
12. Ohkuma, S., and Poole, B. (1981), *J. Cell Biol.* **90**, 656.
13. de Duve, C., de Barsy, T., Poole, B., Trouet, A. Tulkens, P., and Van Hoof, F. (1974), *Biochem. Pharm.* **23**, 2495.
14. Hammond, M. E., Goodwin, J., and Dvorak, H. F. (1980), *J. Reticuloendothel. Soc.* **27**, 337.