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# Letter to the Editor

## ACID PHOSPHATASE: ENDPOINT FOR IN VITRO TOXICITY TESTS

Dear Editor:

Several methods are available for assessing toxicity of drugs and other chemicals to cells in culture. These include cell counting; dye exclusion, cloning in semisolid media, image analysis and incorporation of radioactive precursors of DNA, RNA and protein. Succinate dehydrogenase and ATP levels have also been used (1). These more traditional tests however, have some limitations, They require quite high cell densities, and are labor-intensive and time-consuming. This limits the number of drugs and other parameters that can be examined and becomes particularly relevant, for example, when using primary cultures of limited replicative potential. There is consequently considerable interest in development of assays which can be miniaturized and semiautomated, while providing an accurate measure of relative cell number after treatment with toxic agents. Several of such assays have been developed, including dye elution (2), MTT (3), XTT (4), neutral red (5) assays, and a fluorometric alkaline phosphatase assay (6). These assays have proved to be very useful in toxicity testing.

Connolly et al. (7), reported that cellular acid phosphatase (AP) activity could be used to measure relative numbers of endothelial cells in culture. We report here that a slightly modified version of this procedure can be applied to a variety of cell types, can be used for in vitro toxicity testing, and may have certain advantages, over the methods listed above. The method is described in the legend to Figure 1.

In our study we determined the relationship between cell number and acid phosphatase activity for 6 human epithelial cell lines (RPMI-2650, Hep-2, SCC-9, SK-MES-1 and DLKP — all squamous cell carcinomas, and the lung adenocarcinoma, SK-LU-1) and for 1 rat fibroblastic cell line (NRK). All lines were obtained from the American Type Culture Collection, Rockville, MD (full details are given in the ATCC catalogue); except for DLKP, a human squamous cell carcinoma of the lung, which was established in this laboratory. We also carried out similar determinations using MTT, Neutral Red and Dye Elution assays on replicate plates, following procedures described elsewhere (2,3,5).

The relationship between optical density and cell number was approximately linear for each of these lines (RPMI-2650 and NRK shown in Figure 1). Cell number of 5000 to 300 000 per well (and lower, e.g. for NRK) can be readily measured using the standard 2 hour incubation assay. Thus the linear range of this assay (when cell number is plotted against O.D.) is comparable to, and in some cases more extensive than the other methods mentioned (5,6,8). Unlike the other assays, however, sensitivity can be increased (and therefore lower cell densities measured) by re-incubation of the cells, due to the non destructive endpoint of the assay. Activity of the enzyme gives a linear response with time over the range 0–6 hours at least. Alternatively, sensitivity can be increased by termination of the assay with 1N NaOH (10 μl/well).

The AP assay is simpler to perform than the Neutral Red assay,

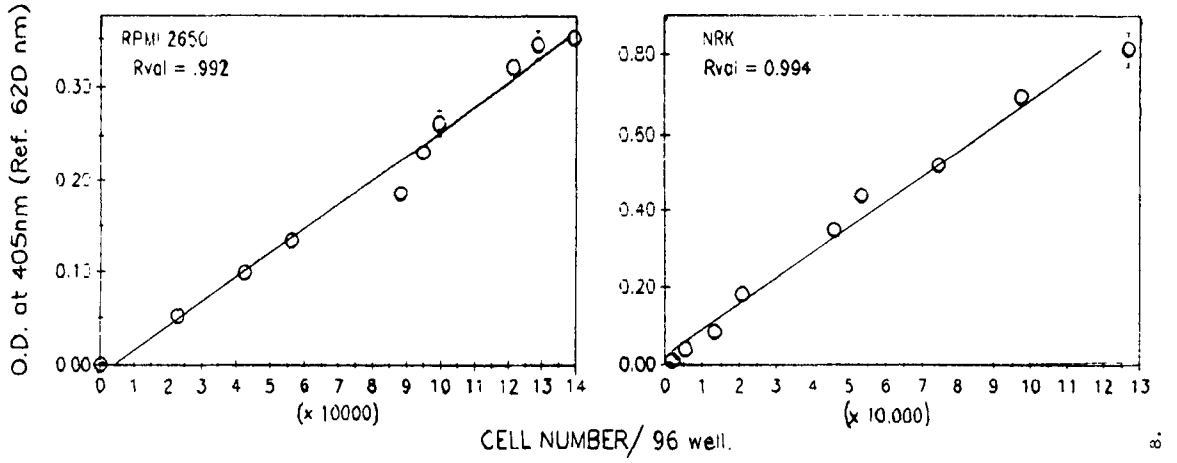


FIG. 1. Linearity of the Cellular Acid Phosphatase assay over a range of cell densities for NRK and RPMI-2650 (best fit lines shown). Each point is the mean value of 8 replicates. The R value is the regression coefficient. Cells were plated in 0.1 ml growth medium in 96 well dishes at densities from 100 to 100 000 cells/well. After 1 day (NRK shown here) and again after 4 days (RPMI-2650 shown here) cell number in each well was determined by Haemocytometer and Coulter Counter and the standard 2 hour Acid Phosphatase assay was performed: medium was removed and wells were rinsed with 0.1 ml PBS. 0.1 ml Buffer (0.1 *M* sodium acetate and 0.1% Triton X100, pH 5.5) containing 10 m*M* p nitrophenyl phosphate (Sigma C104, add fresh from frozen aliquots) was added to each well. After incubation (37° C/5% $CO_2$) plates were read on an ELISA plate reader at 405 nm (reference 620 nm).

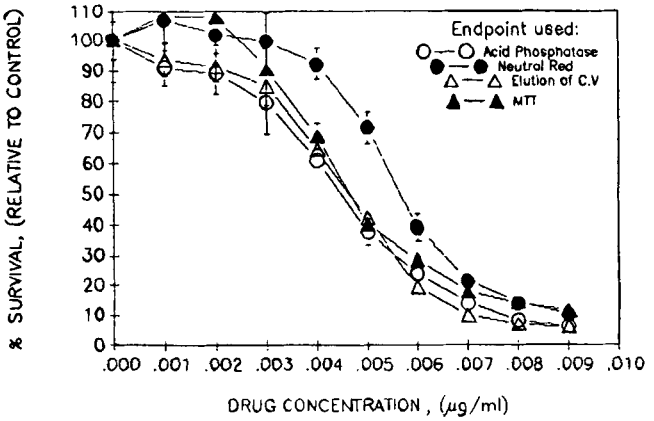


FIG. 2. Dose-response curve showing Vinblastine toxicity to RPMI-2650. Cellular Acid Phosphatase is compared to Neutral Red, MTT and Dye Elution methods. Cells plated in 96 well dishes were allowed to attach (24 h) before addition of drug dilutions. Plates were then incubated for 6 days before analysis.

as it involves fewer steps and use of fewer reagents. It is also more convenient than the MTT assay because of the inherent problem of removal of the medium from the insoluble crystals. Twentyman and Luscome (3) report that up to 40μl of medium remaining in the wells will not significantly affect the absorption of MTT, but nevertheless, removal of the medium without disturbing the crystals is often difficult and requires careful manipulation. The AP assay is technically easier to perform and the plate, once removed from the incubator, is ready for reading without need of standing, shaking or mixing until the solvent has solubilized the endproduct, as is the case with other assays.

It is important to emphasize that both the sensitivity of the assay and the range of the linear relationship between AP activity and cell number may vary between different cell types, and may also vary under different culture conditions for any particular cell type. For example, the standard curve of cell number vs. acid phosphatase activity is quite different for some cell lines when measured 4 days after cell seeding as opposed to 1 day after cell seeding. It is therefore necessary to investigate these parameters for the cell type and culture conditions being used, if the precise relationship between cell number and acid phosphatase activity is important in the investigation. These considerations also apply to other similar assays (6,9).

In the AP assay the reproducibility between replicate wells was excellent and in many cases was found to be better than the Neutral Red assay and the MTT assay. Replicate wells for 6 experiments showed standard deviations which averaged 7.1% for acid phosphatase (range 2.6% to 10.7%) 8.5% for Dye Elution (range 3.5% to 17.1%), 13.6% for MTT (range 7.8% to 16.7%) and 11.2% for Neutral Red (range 6.3% to 13.3%). Presumably this is a reflection of the number of washing steps involved, and the technical ease in carrying out the assay.

In toxicity assays, the AP assay gave broadly (but not exactly) similar results to other commonly used assays such as MTT, Neutral Red and Dye Elution (Figure 2).

The AP assay (like other similar assays) does not, of course, readily distinguish between cytostatic and cytocidal effects. In its use as a cytotoxicity assay, the constraints on experimental design that have been discussed elsewhere for the MTT assay (8), remain relevant. For example, optimal conditions for plating cell density and assay duration will have to be established for each cell line, to ensure, for example, that control wells are still in exponential growth at the time of endpoint determination.

In summary, the use of the AP assay in cytotoxicity testing described here has given results comparable to the other three assays mentioned. It is a useful addition to the range of tests already available, since it appears to be more rapid, simpler to perform and more accurate than the other commonly used assays. Further work will be necessary to evaluate the appropriate areas of application of the AP assay and to compare it with other recently described techniques such as the SRB method (10). This should identify any limitations, such as those associated with the MTT assay, which, for example underestimates the growth inhibitory effects of interferons (11).

## REFERENCES

1. Maehara, Y.; Anai, H.; Tamada, R., et al. The ATP assay is more sensitive than the succinnate dehydrogenase inhibition test for predicting cell viability. Eur. J. Cancer Clin. Oncol. 23:173–176; 1987.
2. Matthews, N.; Neale, M. L. Cytotoxicity assays for tumour necrosis factor and lymphotoxin. In: Clemins, M. J.; Morris, A. G.; Gearing, A. J. H., eds. Lymphokines and interferons: a practical approach. Oxford, Washington D.C.; IRL Press: 1987:222–225.
3. Twentyman, P. R.; Luscome, M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br. J. Cancer 56:279–285; 1987.
4. Scudiero, D. A.; Shoemaker, R. H.; Paull, D. K., et al. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res. 48:4827–4833; 1988.
5. Borenfreund, E.; Puerner, J. A. A simple quantitative procedure using monolayer cultures for cytotoxicity assays. J. Tissue Culture Methods 9:7–9; 1983.
6. Huschtscha, L. I.; Lucibello, F. C.; Bodmer, W. F. A rapid method for counting cells 'in situ' using a fluorogenic alkaline phosphatase enzyme assay. In Vitro Cell. Devel. Biol. 25:105–108; 1989.
7. Connolly, D. T.; Knight, M. B.; Harakas, N. K., et al. Determination of the number of endothelial cells in culture using an acid phosphatase assay. Anal. Biochem. 152:136–140; 1986.
8. Carmichael, J.; De Graff, W.; Gazdar, A. F., et al. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res. 47:936–942; 1987.
9. Hertz, F. Caution about assay of alkaline phosphatase to quantitate cell numbers. In Vitro Cell. Devel. Biol. 25:865; 1989.
10. Jabbar, S. A. B.; Twentyman, P. R.; Watson, J. V. The MTT assay underestimates the growth inhibitory effects of interferons. Br. J. Cancer 60:523–528; 1989.
11. Skehan, P.; Storeng, R.; Scuderio, D., et al. New colorimetric cytotoxicity assay for anticancer-drug screening. Journal of the National Cancer Institute 82:1107–1112; 1990.

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