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A RAPID MICRO METHOD FOR COUNTING CELLS "IN SITU" USING A FLUOROGENIC ALKALINE PHOSPHATASE ENZYME ASSAY

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SUMMARY

A new method has been developed to count cells "in situ", based on a fluorogenic enzyme assay that measures the activity of alkaline phosphatase. Increasing cell number was shown to correlate closely with alkaline phosphatase activity and this relationship did not change with time in culture. The alkaline phosphatase assay (ALP assay) was able to estimate relative cell numbers over a range from about 10^4 to 5×10^5 for many cell types, including Hep-2, a derivative of HeLa, several human colorectal cell lines SW1222, SW837, LS174T and HT29, a normal human diploid cell strain MRC5 and a rodent line NIH-3T3. The ALP assay is rapid and efficient, making it a useful method for studying growth assays.

Key words: alkaline phosphatase enzyme assay; tissue culture.

INTRODUCTION

The rate of growth of mammalian cells in culture can be measured by several methods, namely haemocytometer, Coulter counter, thymidine labeling (1) or the MTT assay which uses a tetrazolium dye (2). These techniques can be laborious and time consuming when handling large numbers of cultures, as well as being inaccurate at low cell densities.

We have developed a rapid and accurate method to assess cell growth based on a sensitive fluorogenic enzyme assay (3,4). The number of cells is estimated by measuring the activity of alkaline phosphatase (ALP), a mostly membrane bound enzyme found in nearly all human cell types (5). There are three main forms of this enzyme which are found respectively in placenta, intestine and liver, bone or kidney, of which the latter is by far the most common in different cell types (6).

Under appropriate conditions for the assay, the non-fluorogenic substrate 5-methylumbelliferyl-phosphate will react with all forms of the enzyme to produce a free phosphate and 5-methyl umbelliferylerone which is a highly fluorogenic compound. The relative fluorescence can then be measured on a Dynatech microfluor reader on cells "in situ", which gives a measure of the total enzyme activity that is directly proportional to cell number.

MATERIALS AND METHODS

Cell lines and cultures. Cell cultures used in this study were MRC5 at passage 26, [a human fetal lung fibroblast cell strain (7)], Hep-2 a derivative of HeLa and a series of human colorectal lines, HT29 (8), LS174T (9), SW837 and SW1222 (10), NIH-3T3 mouse fibroblasts were also studied, which were kindly provided by Dr. Harmut Land of this Institute.

All cultures were routinely maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) at 37° C in 10% CO_2 in air at 100% humidity, with the exception of SW837 which was grown in RPMI-1640 medium containing 10% FCS in 5% CO_2 in air at 100% humidity. The cultures were routinely screened for mycoplasma contamination by growing cell culture fluid in specifically prepared nutrient agar as described by House & Waddell (11). All were found to be negative.

The cells were harvested using a trypsin-versene mixture (0.25% trypsin and 0.01% EDTA) and then plated at appropriate cell densities onto 96 well trays (Falcon Microtest III Testplate, Becton, Dickinson Labware, USA) using a multipipetter. (Titertek USA).

Counting cells. a) Coulter counter — an aliquot of a trypsinized and well dispersed cell suspension was taken to calibrate the cell number using a Coulter counter (Model Z B1 Coulter Electronics Ltd., Harpenden, UK). b) ALP assay-cell number was scored 'in situ'. The cells were washed 2 times in phosphate buffered saline Dulbecco-PBS "A", drained and then 200 μl of freshly

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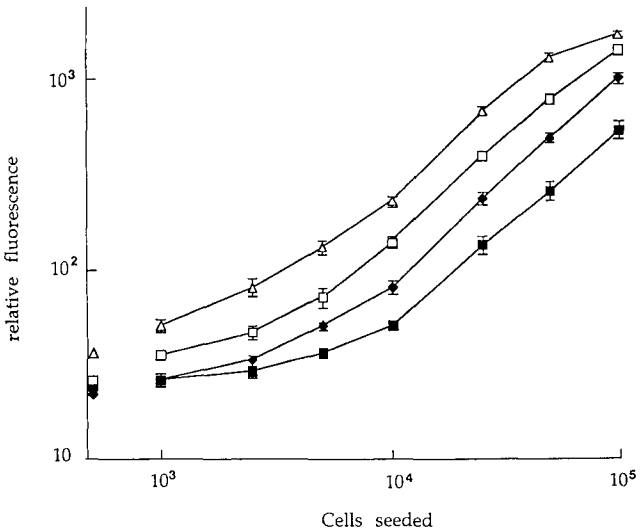

FIG. 1. Alkaline phosphatase activity of SW1222 cells measured at various time intervals ■ - 1 hour, ◆ - 2 hours, □ - 4 hours and △ - 8 hours. The average relative fluorescence and standard deviation was calculated for each cell density, based on data from 8 wells.

made assay solution at 37° C [0.2*M* boric acid, 1 m*M* $MgCl_2$, 1.2 m*M* 4-methyl umbelliferyl phosphate (Sigma Chemical Co., UK)] was added to each well. After incubating the tray for various times up to 8 hours at 37° C, the relative fluorescence was measured using a Dynatch Fluor Tm reader (Dynatech Products).

RESULTS

a) *Development of the ALP assay.* The cell line SW1222 was used to standardize the assay. A range of cell densities was plated onto 96 well plates and the relative fluorescence measured after incubating the cells in the assay solution for various time intervals (Fig. 1). There is a direct linear relation between cell number seeded and relative fluorescence for cells in the range of 10^4 to 10^5 per well at all time points up to 4 hours incubation. After 4 hours or more the ALP assay begins to distinguish 5 × 10^3 cells per well. However for longer periods of incubation, such as 8 hours the relation between cell number and relative fluorescence was no longer linear at high cell densities, presumably because the substrate was exhausted. An incubation time of 3 hours has been chosen for routine assays.

Several other cells were also shown to exhibit a strict linear relation between cell number and relative fluorescence. These were HT29, LS174T, SW837, all human colorectal cell lines, Hep-2, the diploid cell strain MRC5 and the mouse fibroblast line NIH-3T3. Illustrations for NIH-3T3 and Hep-2 are shown in Fig. 2. The relationship between cell number and fluorescence is linear at least over a range of about 10^4 to 5 × 10^5, though the slope of the line may vary from one line to another.

b) *ALP assay for long term growth in culture.* As an example of the application of the assay we have used experiments on the growth of the cell line SW1222 in different concentrations of FCS. The growth of SW1222 over several days was scored using a Coulter counter and the ALP assay in parallel to measure cell number. The results, illustrated in Fig. 3 show that the growth curves in different FCS concentrations have a similar profile when cell number above about 10^4 was measured by both methods. This indicates that the alkaline phosphatase activity per cell does not change during growth under those conditions, over a more than tenfold range of cell concentrations.

Similar long term growth experiments were done with cultures, of HT29, LSI74T, Hep-2, MRC5 and NIH-3T3 cell lines. In each case the same growth curves were obtained using the ALP assay and the Coulter counter (data not shown).

DISCUSSION

We have shown that cell number can be rapidly and efficiently estimated 'in situ' by measuring the activity of

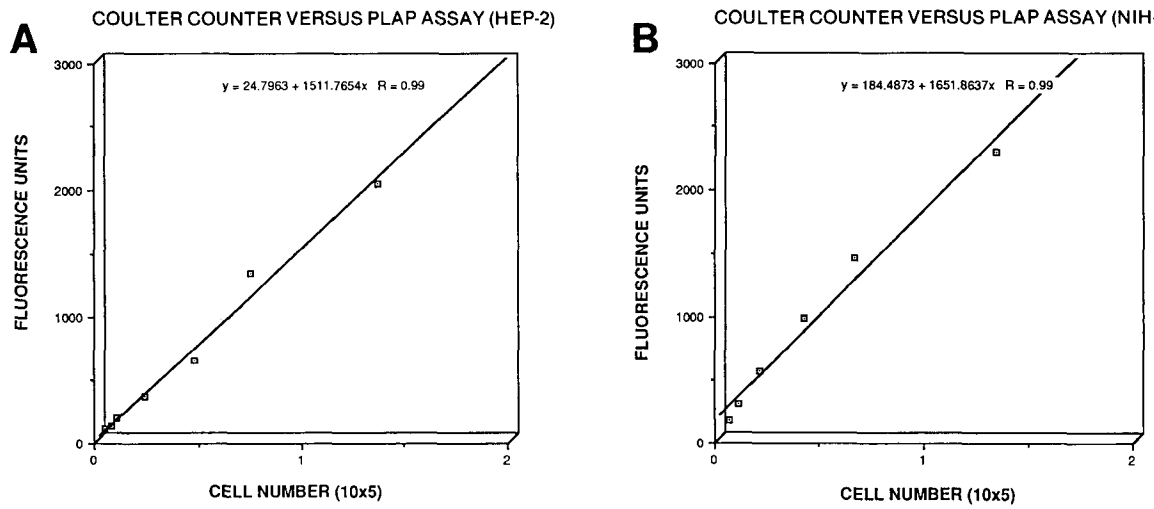

FIG. 2. The relationship between Coulter counter cell count and fluorescence using the ALP assay and a 3 hour incubation. Experimental procedures as in Fig. 1. Hep-2 (a) and NIH-3T3 (b).

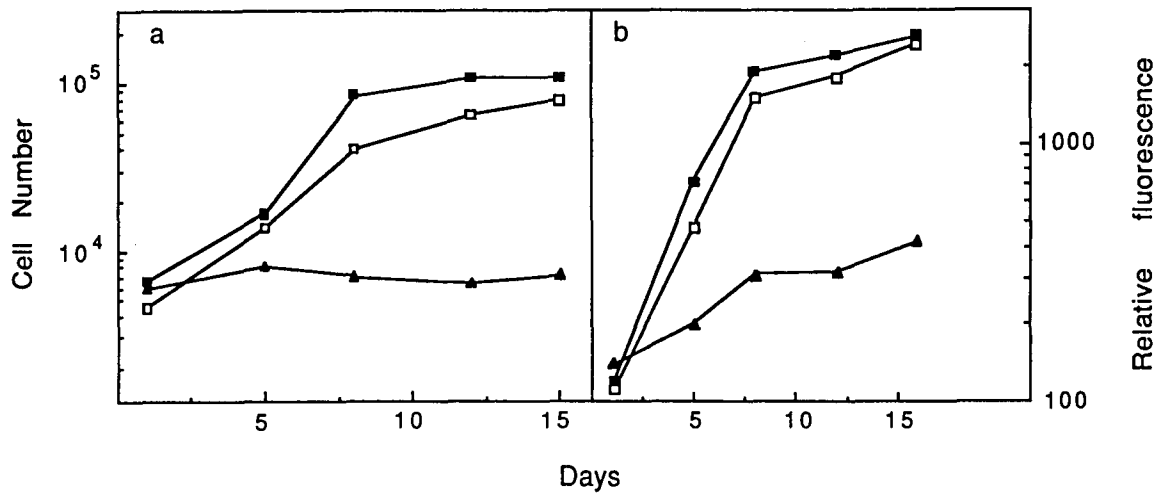

FIG. 3. Growth curves of SW1222 cells in different concentrations of sera ▲ - 0.1% FCS, □ - 2% FCS and ■ - 10% FCS. As measured by a Coulter counter (a) or ALP assay (b). 10^4 cells were plated in each well. The average cell number was calculated from 3 wells for the Coulter counter and 9 wells for the ALP assay for each time point.

alkaline phosphatase using a fluorogenic enzyme assay. The activity of alkaline phosphatase increased proportionally with cell number and this relationship did not change with time in culture. Dead cells are usually almost completely removed by the washing procedure before assay. The ALP assay could also successfully estimate population size for many cell types, such as Hep-2 and human colorectal tumour lines, SW1222, SW837, LS174T and HT29, a normal human diploid cell strain MRC5 and NIH-3T3 a rodent cell line. Other assays for ALP may be used, but are likely to be less satisfactory than the fluorogenic assay, which is more sensitive.

A limitation of the ALP assay is that it cannot be utilized to count cells when chemical agents have been used that may modulate the expression of alkaline phosphatase activity. For instance alkaline phosphatase activity has been shown to be altered in HT29 cells when grown in galactose containing medium (12).

One of the main advantages of the ALP assay described here is the speed with which cell growth from a large number of cultures can be estimated, thus alleviating the tedium of counting cells using the standard methods, such as haemocytometer, Coulter counter or thymidine labeling. The ALP assay has been successfully used by this laboratory to characterize serum-free growth conditions of various human colorectal cell lines. These experiments were otherwise very laborious and time-consuming, since they required many cultures to be set up with different combinations of growth factors. The ALP assay should also be useful for selecting batches of serum for special cell cultures.

Another advantage of the ALP assay is its sensitivity since as few as 10^4 cells can be accurately estimated. Other methods of scoring cell number are in general not so sensitive, the lower limit for a haemocytometer count being at least 5×10^5 cells. The Coulter counter and MTT assays also have lower limits of about 10^4 cells. The ease and sensitivity of this method, has, for example, proved useful for cell attachment assays. [Pignatelli and Bodmer (12).]

We have thus developed a new method for counting cells based on a fluorogenic enzyme assay that measures the alkaline phosphatase activity, which is rapid and sensitive, being able to estimate reliably between 10^4 and 5×10^5 cells in the wells of a 96 well microtitre tray.

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EDITOR'S STATEMENT

This paper describes a quick method for quantitation of cell number in microcultures. Such procedures are valuable for the many situations in which minimizing cells and medium volume is desirable, although somewhat specialized equipment is required for the procedure. An alternative procedure for quantitation of cells in microtiter culture appeared previously in this journal (McCaffrey, et al., 24:247-252).