

A Simple Colorimetric Method for the Evaluation of Growth and
Chemosensitivity of Tumor Cells in Soft Agar Culture

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(Received, March 3, 1986)

SUMMARY

A simple colorimetric method (color assay) was developed in order to evaluate the *in vitro* growth and survival of tumor cells in soft agar cultures. The assay detects the color changes in the medium caused by the accumulation of acidic metabolites at the end point of the culture. The chemosensitivity of two human tumor lines to various cytotoxic agents determined by the color assay correlated well with that determined by conventional colony count in bilayer soft agar cultures. The results suggested that the color assay can be used as a supportive method for the assessment of human tumor clonogenic assay (HTCA).

INTRODUCTION

Since Salmon and Hamburger reported the application of the bilayer soft agar culture system to the *in vitro* chemosensitivity tests of primary human tumors^{1,2)}, the human tumor clonogenic assay (HTCA) has been proved to have some clinical benefits³⁾. However, HTCA has some technical limitations, such as poor plating efficiency and difficulty in obtaining single cell suspensions⁴⁾.

Though enzymatic disaggregation with collagenase, and DNase is commonly used to obtain single cell suspensions from solid tumor specimens in HTCA, complete removal of cell aggregates by this procedure is usually difficult. Presence of cell aggregates considerably disturbs the colony count and may cause both false-negative and false-positive interpretations of the sensitivity tests⁵⁾.

We recently developed a simple colorimetric method for the evaluation of the *in vitro* growth and survival of tumor cells. The method is free of the artifacts caused by the presence

of cell aggregates. The color assay utilizes the change in medium pH produced by the accumulation of acidic metabolites in the culture medium. The change in medium pH is reflected by the change of the color of phenol red usually contained in ordinary culture media. Here, we compared this method to the conventional colony assay using two human tumor cell lines to assess the applicability of this assay to the present system of HTCA.

MATERIALS AND METHODS

Cells and Reagents

McCoy 5A medium (Gibco Laboratories, Grand Island, N. Y.) supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin was used as the culture medium. The human colon adenocarcinoma cell line (HT29, ATCC HTB38) was kindly provided by Dr. Donald L. Morton, Division of Surgical Oncology, John Wayne Clinic, UCLA School of Medicine, and human renal cell carcinoma cell line (NC65)⁶⁾ was a kind gift of Dr. Osamu Yoshida, Department of Urology, Kyoto University. Both cell lines were maintained with medium containing 10% fetal calf serum (FCS, Flow Laboratories Inc., McLean, VA.) as monolayers in a humidified 5% CO₂-air atmosphere at 37°C. Cells were passaged at 3 to 4 day intervals. All experiments described below were performed with cells dissociated by 0.25% Trypsin-0.02% EDTA at the subconfluent stage. All drug solutions were made freshly immediately before each experiment.

Monolayer Culture Assay

On Day 0, cells were suspended in the culture medium supplemented with 10% FCS and plated in 16 mm culture dishes (Falcon Plastics, Oxnard, CA) at a density of 5×10^4 cells/well. Cultures were maintained at 37°C, 5% CO₂-air and 100% relative humidity. On day 1, each well was washed three times with phosphate-buffered saline (PBS) and the medium supplemented with 10% FCS and the various concentrations of cytotoxic agents were added in a volume of 2 ml. On day 7, after the medium was collected for the color assay described below, each well was washed with PBS and allowed to dry. Protein content of the remaining cells were determined by Lowry's method⁷⁾. Drug sensitivity was calculated as sensitivity (%) = $(1 - \text{Protein drug} / \text{Protein control}) \times 100$ (%). Each assay was run in quadruplicate.

Soft Agar Culture Assay

Soft agar culture was performed using a modification of the method of Hamburger and Salmon¹⁾. The medium supplemented with 15% FCS was used for the soft agar cultures. Layers of 1 ml 0.5% agar in the medium were prepared in 35 $\times$ 10 mm dishes (Linbro 76-247-05; Flow Laboratories). Cells to be tested were suspended in 1 ml of the medium with 0.3% agar and poured over the bottom layers. For the drug sensitivity tests, 5×10^4 cells were plated in each well. Cultures were maintained at 37°C, 5% CO₂-air, 100% relative humidity. On day 10, 1 ml of 0.1% 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) solution was added in each well. On day 11, the colonies were counted using a Handex CP-2000 Automatic Particle Counter (Shiraimatsu, Osaka). Cell clusters larger than 60 μ m were counted as colonies. Drug sensitivity was calculated as sensitivity (%) = $(1 - \text{Colonies drug} / \text{Colonies control}) \times 100$ (%). Each assay was run in triplicate.

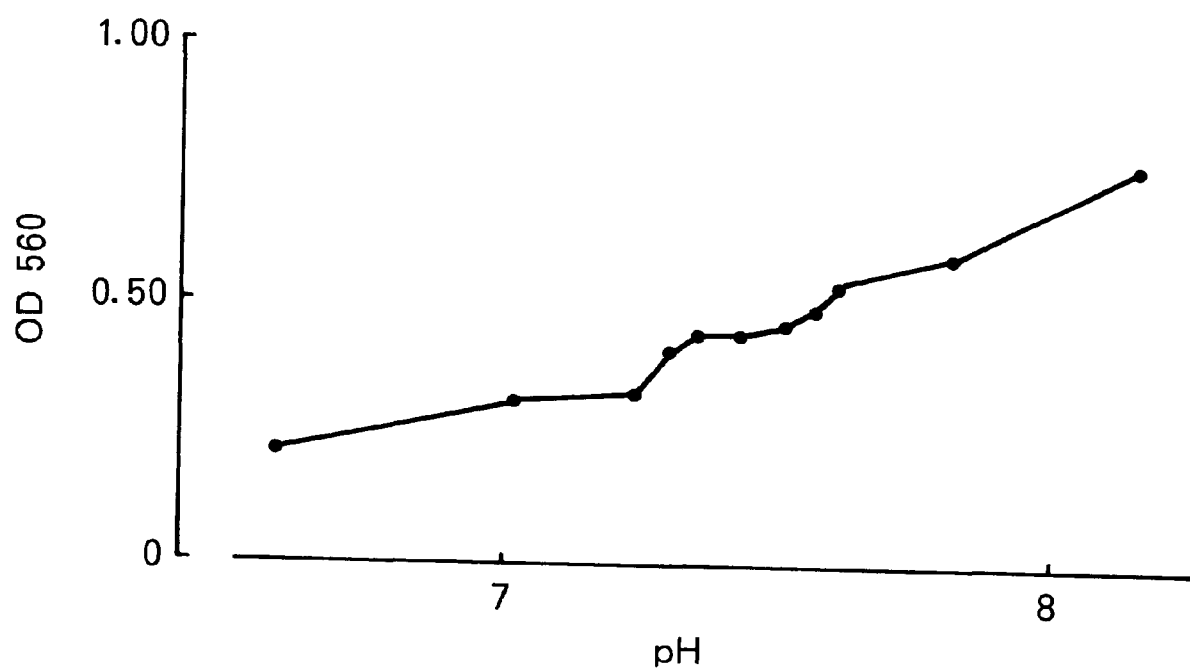


Fig. 1 Relationship between pH and OD₅₆₀ of McCoy 5A medium. pH and OD₅₆₀ were determined after various concentrations of NaOH and HCl were added to an equal volume of McCoy 5A medium, supplemented with 10% FCS.

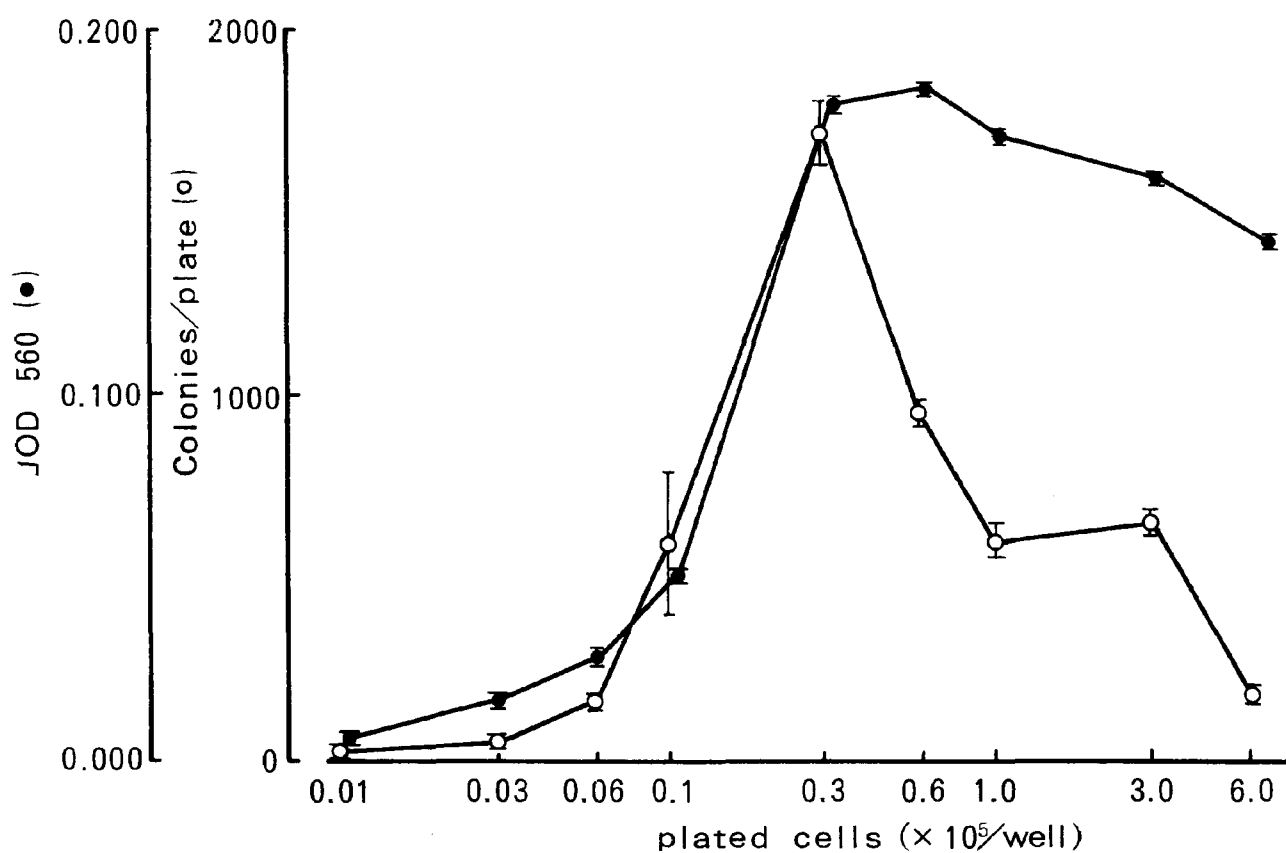


Fig. 2 Correlation between ΔOD_{560} and colony count at different plated cell densities of NC65 in soft agar culture. Colony count (○), OD₅₆₀ (●).

Color Assay

In each experiment, a set of cell-free dishes was used as negative controls and treated in the same manner as the cell-containing dishes. With soft agar cultures, 1 ml of distilled water was added to each well after colony count, and dishes were left to stand at room temperature overnight. Liquid above the agar was collected by gentle aspiration with Pasteur pipettes the next day. With monolayer cultures, medium was collected from each well at the end of the culture and was kept at room temperature for 24 hours. Samples were centrifuged at 3,000 rpm for 10 minutes. Optical density of the supernatant at 560 nm was determined using a spectrophotometer. ΔOD_{560} was defined as the difference between OD_{560} of the samples from cell-free dishes and OD_{560} of those from cell-containing dishes. Drug sensitivity was calculated as sensitivity (%) = $(1 - \Delta OD_{560, \text{drug}} / \Delta OD_{560, \text{control}}) \times 100(\%)$.

RESULTS

As shown in Fig. 1, the relationship between pH and OD_{560} of McCoy 5A medium was almost linear. When various densities of NC65 cells were cultured in soft agar, the colony count

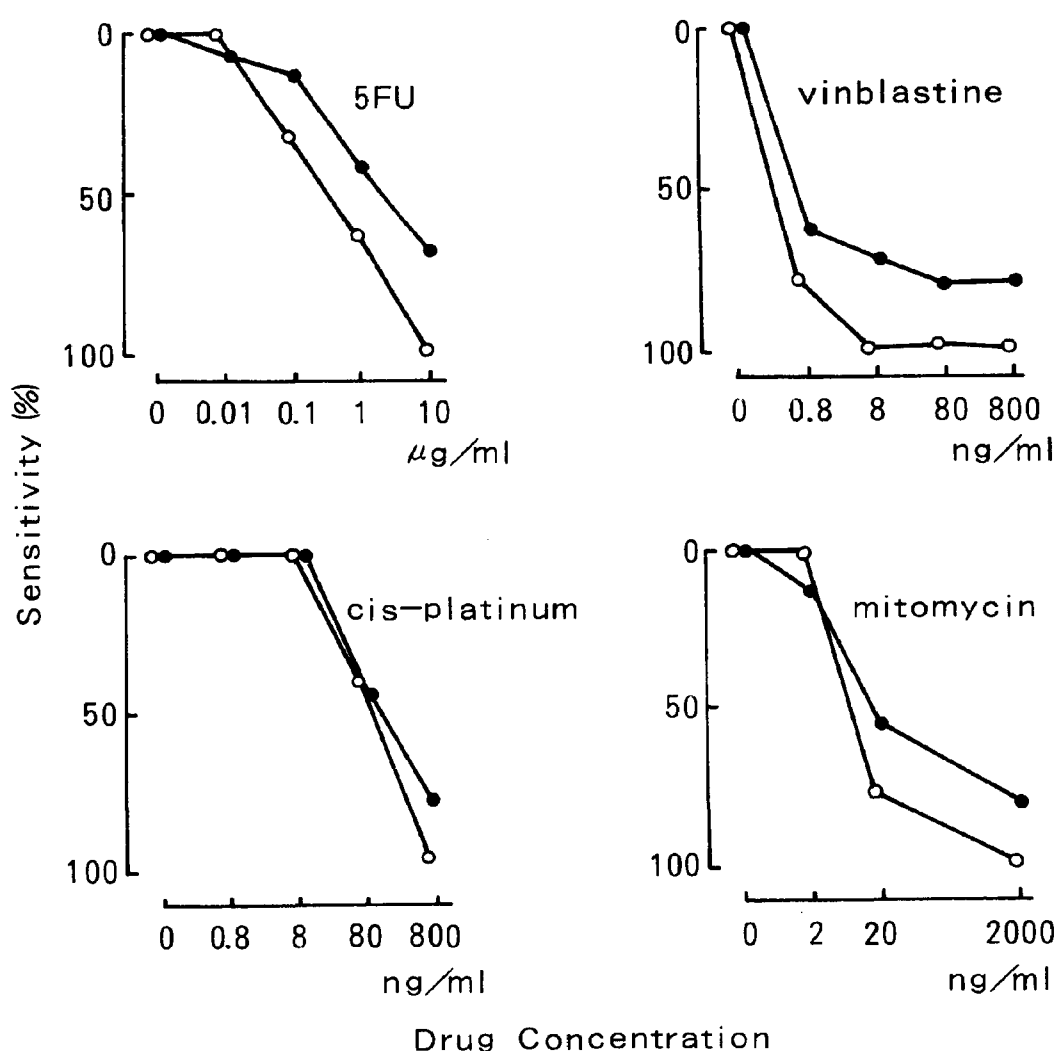


Fig. 3 Chemosensitivities of NC65 in monolayer culture. Sensitivity was determined by the color assay (●) and protein assay (○).

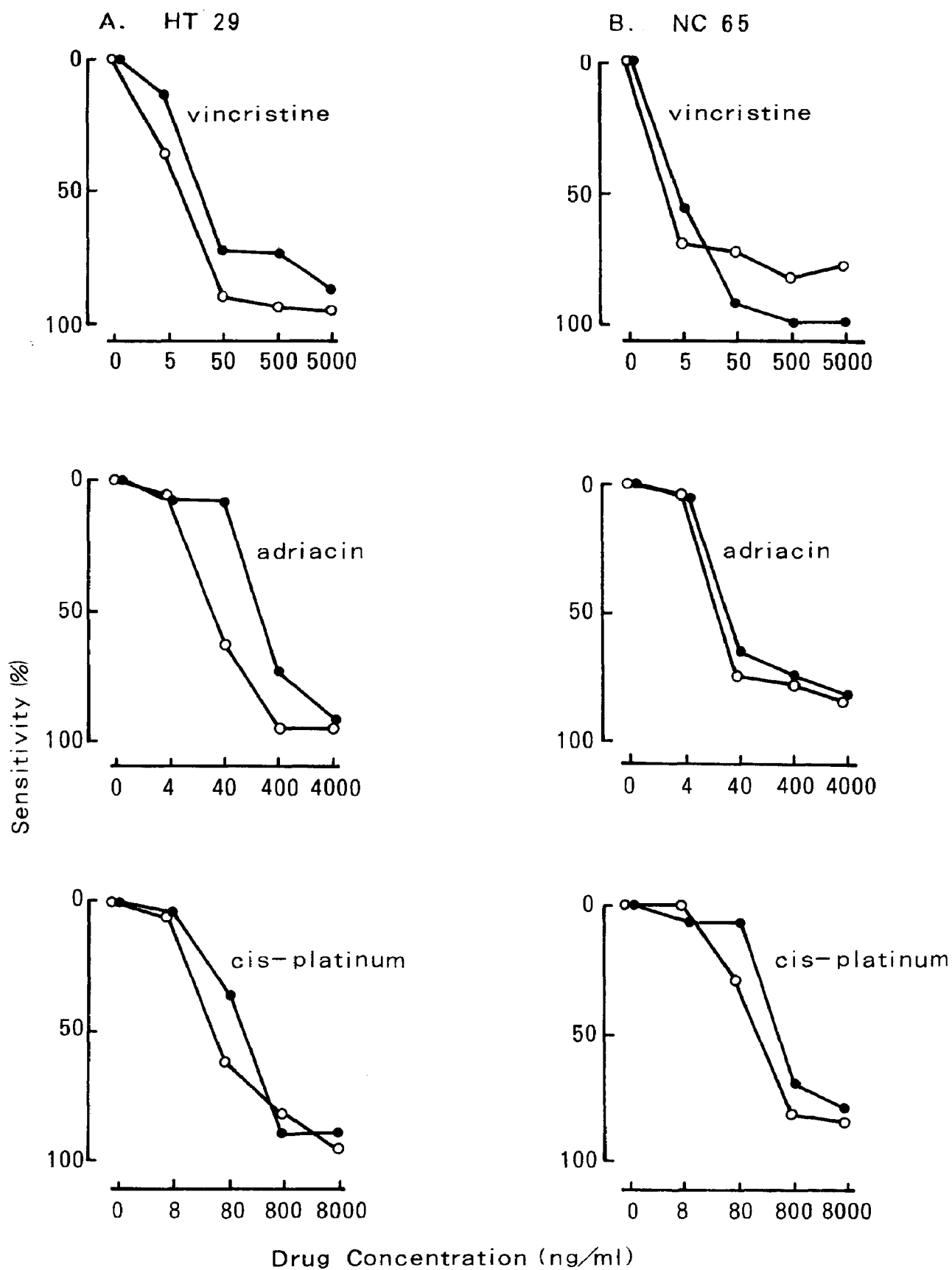


Fig. 4 Chemosensitivities of HT29 (A) and NC65 (B) in soft agar culture. Sensitivity was determined by color assay (●) and colony assay (○).

and ΔOD_{560} increased almost in parallel up to the peak at the density of 3×10^4 cells/well (Fig. 2). The correlation coefficient between colony count and ΔOD_{560} up to this peak was 0.995. Though the colony count decreased sharply after this peak, the ΔOD_{560} still remained high.

Fig. 3 shows the results of chemosensitivity tests of NC65 cells in monolayer culture. Sensitivity determined by the color assay and that by the protein assay gave similar profiles. Correlation coefficients were 0.976 for 5-fluorouracil, 0.989 for cisplatinum, 0.861 for vinblastine and 0.993 for mitomycin.

Results of the sensitivity tests of both HT29 cells and NC65 cells in soft agar culture are presented in Fig. 4. Correlation coefficients between the color assay and the colony assay were 0.991 and 0.823 for vincristine, 0.826 and 0.994 for adriacin and 0.933 and 0.955 for cisplatinum with HT29 and NC65 respectively.

DISCUSSION

Though HTCA has become an important tool in the treatment of cancer patients⁸⁾, HTCA has some basic problems that must be resolved. One of them is how to evaluate the *in vitro* growth and survival of tumor cells without being disturbed by artifacts such as those caused by contamination of cell aggregates. Complete removal of cell aggregates is one possibility to escape this error in the colony count. However, vigorous treatment of tumor specimens by the nylon-mesh or thin-needle method may cause other errors in the interpretation of the chemosensitivity, such as considerable loss of tumor cells and unpredicted cell damage. Development of an evaluation system independent of colony count is another possibility, and such methods have been reported by Tanigawa *et al.*⁹⁾ and by Freedman *et al.*¹⁰⁾ However, as these methods utilize tritium thymidine incorporation, they are too costly and complex for routine use.

The system of colony counting itself has not been fully established yet. In addition to the fact that the cutoff point of colony size is arbitrarily determined by each laboratory, the adequacy of INT treatment to exclude errors caused by the presence of "dead" cell aggregates¹¹⁾ and the necessity of positive controls such as sodium azide or abrin in the assay system⁸⁾ are still controversial. Visual counting of the colonies by an inverted microscope is an easy and simple method but is also time-consuming and painstaking. The introduction of the computer-assisted image analyzing system made non-biased colony count feasible¹²⁾. However, the high cost of the device has prevented the widespread use of the system.

Our color assay reported here detects the change in medium pH by measuring the change of the color of phenol red. As the growth of cells proceeds, acidic metabolites increase and pH of the medium is lowered. Actually, the pH of the medium correlated well with the OD_{560} of the medium as shown in Fig. 1. Thus, by determining the difference in OD_{560} between cell-containing and cell-free dishes, the growth and the survival of cultured cells could be determined.

In this study the growth and chemosensitivities of two tumor cell lines in soft agar culture determined by the color assay correlated well with those determined by the colony assay. The color assay also gave quite similar results to those obtained by the protein assay in the evaluation of chemosensitivity of NC65 cells in monolayer culture.

Our assay is extremely simple and adds almost no cost to the present HTCA system. The

assay is free of the artifacts caused by the presence of cell aggregates and can be run even after INT treatment. In addition to these merits, the color assay can be performed without altering the present HTCA system except that preparation of cell-free controls is required.

Meyskens *et al.*¹³⁾ reported that colony formation is inhibited when “too many” cells are plated. Even under these circumstances, Color Assay may still be used since ΔOD_{560} still remained high in this “over seeding” range (Fig. 2). Actually such cases of poor colony formation were not uncommon with primary specimens in which only the color assay could be performed (data not shown).

Whether the cell populations evaluated by the color assay differ from those evaluated by the colony assay is unclear. However, Alley *et al.*¹⁴⁾ reported that computer-assisted volume analysis is better for the interpretation of the overall growth profile of the primary cultures than the conventional colony counting method based on the arbitrary cutoff of the colony size. In this sense, the color assay may reflect the overall profile of the growth better than the colony assay.

Our assay can not be used to determine sensitivity to agents that greatly alter the pH of the culture medium, such as extremely high concentrations of 5-fluorouracil or sodium azide, which are strongly alkaline. The color assay is less sensitive than the colony assay when plated cell density is relatively low and overall metabolism poor. If these conditions are properly controlled, the color assay can be performed with the same dishes prepared for colony count.

We believe that the color assay is a simple and useful method that supports the present system of HTCA. A comparative study between the color assay and colony assay with primary specimens is now in progress.

ACKNOWLEDGEMENT

We wish to thank Dr. Nobuo Maekawa, Professor Emeritus, Kyoto University, for his support and encouragement throughout this study.

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