An Improved Tissue Culture Assay

III. Alternate Methods for Measuring Cell Growth*

JOSEPH E. GRADY, WILLIAM L. LUMMIS, AND CHARLES G. SMITH

(Research Laboratories, The Upjohn Company, Kalamazoo, Mich.)

SUMMARY

Simplified methods were described for the measurement of growth of human carcinoma cells in tissue culture: (*a*) determination of color intensity in lysates of cells stained with crystal violet (CV method), (*b*) determination of the reduction of 2,6-dichlorophenol indophenol, and (*c*) determination of total organic solids (dichromate method).

The three methods gave comparable measurements of relative cell growth and correlated well with the more laborious protein assay. The CV method was especially suitable for assaying large numbers of samples and was shown to measure the cytotoxicity in several compounds in a reproducible and precise manner. Standard errors per assay were comparable between protein and CV assays (approximately 20 per cent).

The previous publications (12, 13) of this series described a simplified tissue culture assay and its application in cytotoxicity studies with antibiotics, chemicals, and solvents. A protein determination was used to measure cellular growth in the above tissue culture system. If the search for new cytotoxic agents in fermentation beers is to proceed at a rapid pace, a method for the measurement of cell proliferation which is more efficient than the protein determination is needed.

Three simplified assay methods for cell measurement have been studied in these laboratories, and their use in tissue culture investigations is the subject of this paper.

MATERIALS AND METHODS

The human epidermoid carcinoma cell (KB) originally isolated by Eagle (4) was used throughout this study. The details of culture maintenance and test tube assay procedures have been described previously (12).

Crystal violet method for growth determination.—Three days after inoculation of the test tube assay, the stoppers were removed from all culture tubes (15 × 100 mm.) including medium blanks (uninoculated medium), and 1 ml. of an 0.025 per cent aqueous solution of crystal violet was added to each tube (containing 4 ml. of medium) while they were inclined and warmed at 37° C. (12). Dye addition was made to that side of the culture tubes opposite the cells. The tube rack was then reversed and placed at an angle of 5° so that the entire cell sheet was covered and the tubes were incubated for 15 minutes to stain the cells. The medium was then decanted (over the side of the tubes opposite the cell sheets), and 10 ml. of 0.9 per cent saline maintained at 37° C. was added to each tube to remove excess dye. The tubes were rotated gently, the saline wash was decanted, and the inverted tubes were allowed to drain for 5–10 minutes on a Turkish towel. At this point, 6 ml. of aqueous 0.05 per cent trypsin was added to all tubes, which were then stoppered. The tubes were shaken in a horizontal position for 15 minutes on a reciprocal shaker (12), during which the cells became detached from the glass and began to lyse. After removal from the shaker, the addition of 1 drop of 0.01 N HCl completed lysis which liberated the bound dye. Optical densities (OD) of the solutions were then determined at 570 mμ in the Beckman Model B spectrophotometer. The instrument was adjusted to zero with a reagent blank which had been incubated with 4 ml. of medium but no cells and

* This work was supported by Contract No. SA-43-ph-1933, Cancer Chemotherapy National Service Center, National Cancer Institutes, National Institutes of Health.

Received for publication March 1, 1960.

had been treated with dye, trypsin, and dilute acid.

Measurement of growth of KB cells by reduction of 2,6-dichlorophenol indophenol.—After 3 days of growth, the medium was decanted from the tube cultures, and the cell sheets were washed once with 5 ml. of Earle's salts (minus bicarbonate at 37° C.). To each tube 6 ml. of a solution of 2,6-dichlorophenol indophenol (90 μg/ml) in saline was added. The tubes were then stoppered and shaken for 30 minutes at 28° C. on a reciprocal shaker. The optical densities of the culture tubes were determined against the appropriate blank at 600 mμ in a Beckman Model B spectrophotometer.

Measurement of growth of KB cells by organic solids determination.—This method is a modification of the procedure developed by Johnson (7) to measure total organic solids during enzyme purification. The single change instituted for the measurement of tissue culture growth was a 1.5-fold increase in the amount of dichromate reagent used. This change gave a Δ OD_{440} (sample minus reagent blank) which was a measure of relative cell concentrations among tubes.

The procedure used was the following: The reagent contained 5 gm. of $Na_2Cr_2O_7$, 2 H_2O in 20 ml. of water diluted to 1 liter with 95 per cent H_2SO_4. Tissue culture tubes at harvest were rinsed twice with 5 ml. of saline to remove extracellular organic solids, and 1.5 ml. of the reagent and 0.4 ml. of H_2O were syringed down the side of the tube containing the cells. This mixture was heated for 20 minutes at 100° C., cooled, and 10 ml. of water was added. Enough Na_2SO_3 was added to one blank to reduce the dichromate, and this tube was set to zero. OD_{440} was determined on both the unreduced blank and the samples. OD unreduced blank − OD sample is a measure of the relative concentrations of organic solids in the tubes.

RESULTS

Comparison of methods.—To determine whether a correlation existed among the "simplified" assay methods described above and the protein assay, the growth of KB cells in replicate tubes was determined by the various procedures, and the results are presented in Chart 1. These data show that a direct correlation existed among the three "simple" methods of cell determination and the more laborious protein assay. The agreement between the crystal violet and protein methods was good over the cell concentration range generally encountered in the tube assay (100–800 μg/tube).

Additional studies were carried out on the crystal violet assay, since it seemed to be most easily adaptable to large scale testing. The organic solids method involved the heating of dichromate in culture tubes and therefore constituted a hazard for continued use of the tubes for cell growth because of the cytotoxicity of dichromate ion (12). The dichlorophenol indophenol and the crystal violet methods, however, involved small concentrations of relatively innocuous reagents. The crystal violet method, in addition, offered the advantage of a proportional response over a wide range of cell growth (see Chart 1), and the color intensity was stable indefinitely.

Crystal violet method.—The cytotoxicities of some known inhibitors were determined in a series of experiments with (*a*) the standard protein assay

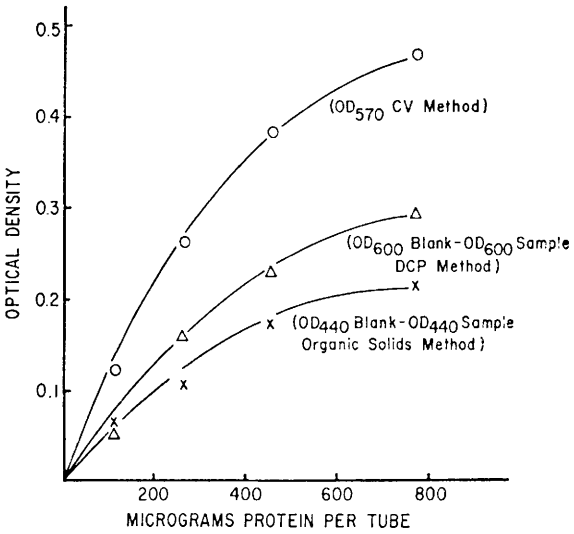

CHART 1.—Determination of KB cell growth by three "simple" assay methods.

for cell growth (12) and (*b*) the crystal violet assay (CV). Agents which represented a wide range of cytotoxicities and mechanisms of action were selected for this comparison study. It has been found convenient to designate degree of inhibition in the CV assay as OD_{570} treated/OD_{570} untreated × 100.

The cytotoxicities of selected compounds, determined by CV and protein assays, are given in Table 1. These data show that the results obtained with the CV assay are entirely comparable to those found with the protein assay. Good correlations have also been observed between these assay methods when various fermentation beers were tested for cytotoxicity.

Standard error of methods.—To determine the precision of the CV assay, six replicate samples of streptovitacin A and iodoacetic acid were assayed on each of 2 days. The same samples were also assayed by the protein method, and the results

are presented in Table 2. These data show a high degree of reproducibility in both CV and protein assays with the above compounds. The fact that the CV-ID_{50} values are consistently higher than the protein values is in some part due to the manner in which the CV-ID_{50} is calculated; the contribution of the inoculum to the final optical density is not considered as it is in the protein assay (12). Per cent standard errors for the two assays were calculated from the data of Table 2 and are shown in Table 3. The standard error of the protein assay with streptovitacin A had been calculated to be 22 per cent in a previous series of experiments (12).

DISCUSSION

In the intensive search for cancer chemotherapeutic agents, diverse approaches and screening methods are desirable. The potential significance of data obtained in tissue culture systems has been discussed by Eagle and Foley (6) and Smith *et al.* (13). The investigation of fermentation beers as potential sources of antitumor agents with mammalian cell culture as the indicator of biological activity offers an interesting new empirical approach in the search for carcinolytic compounds. The approach requires reproducible, sensitive, and quantitative assay systems, however, to proceed from crude beers to crystalline agents in a reasonable period of time.

Modifications in tissue culture methods have been forthcoming since the early report of Eagle

TABLE 1

COMPARISON OF CYTOTOXICITIES OF TEN AGENTS TO KB CELLS DETERMINED BY PROTEIN AND CRYSTAL VIOLET ASSAYS

AGENTS	NSC NO.*	ID_{50}, μG/ML	
		Protein	Crystal violet
Azaguanine	749	0.055(2)†	0.035(2)
Azaserine	742	1.7 (1)	1.5 (1)
Colchicine	754	0.0012(2)	0.002(2)
Deoxypyridoxine	3063	45 (2)	21 (2)
Sodium fluoroacetate		>175 (2)	>200(2)
Iodoacetic acid	2125	0.30 (2)	0.35 (2)
Malonic acid		>375 (2)	>450(2)
Puromycin aminonucleoside	3056	2.0 (2)	2.7 (2)
Sodium arsenate (·$7H_2O$)		20 (2)	16 (2)
Streptovitacin A	39147	0.032 (2)	.042(2)

* These compounds were supplied by Dr. S. A. Shepartz, CCWSC.

† Numbers in parentheses indicate the number of assays represented. Each assay consisted of five levels of drug run in duplicate or triplicate.

TABLE 2

COMPARISON OF ASSAY RESULTS OBTAINED WITH CRYSTAL VIOLET AND PROTEIN ASSAYS ON TWO AGENTS

AGENT	ID_{50}, μG/ML			
	CV		Protein	
	Exp. 1*	Exp. 2	Exp. 1	Exp. 2
Streptovitacin A	.019	.030	.013	.017
	.022	.025	.010	.011
	.023	.035	.012	.014
	.025	.022	.017	.014
	.023	.033	.014	.014
	.028	.054	.014	.013
Iodoacetic acid	0.50	0.84	0.66	0.54
	0.54	0.90	0.59	0.58
	0.92	0.84	0.54	0.50
	0.80	0.89	0.66	0.62
	0.80	0.88	0.66	0.52
	0.80	0.90	0.74	0.62

* Each experiment represents six replicate samples weighed on a single day.

TABLE 3

PER CENT STANDARD ERRORS OF ASSAY METHODS

ASSAY METHOD	PER CENT STANDARD ERROR*	
	Streptovitacin A	Iodoacetic acid
Protein	16.7	16.9
Crystal Violet	22.0†	15.4

* SE of ID_{50} value based on one sample in one assay.

† If one extreme value (ID_{50} = 0.054 μg/ml) is included, the standard error is 36 per cent.

and Foley (1, 3, 5, 8–12, 14, 15). With the rapid, sensitive assay methods described in this paper, beers or synthetic chemicals can be tested for cytotoxic activity on a large scale. When growth of KB cells is determined by the protein assay as described previously (12), an accurate and reproducible determination of cytotoxicity is possible. In this procedure, however, the investigator must dilute the protein in each tube in order to bring the final concentration into the usable range of the Folin-Ciocalteau method (25–100 μg/tube). This necessitates (*a*) estimation of the cell content of individual tubes, (*b*) washing each tube to remove extracellular protein, (*c*) addition of an appropriate volume of biuret reagent to insure that aliquots of the resulting solution will contain a concentration of protein which is within the limits of the Folin assay, and (*d*) development of color with the Folin-Ciocalteau reagent. Each of the simplified methods described above obviates the estimation of cell growth and the dilution before assay. In the case of the crystal violet assay, the initial washing of cells is also unnecessary, as is the biuret solution step, and this method actually reduces the time required per assay sample approximately twofold. The crystal violet assay method is particularly suitable for large-scale screening operations. A similar technic has been used for the determination of dead yeast cells (2).

ACKNOWLEDGMENTS

The authors are indebted to Mr. J. I. Northam for statistical analyses and gratefully acknowledge the excellent technical assistance of Mrs. Lorna Schroeder, Mrs. Anita Krivis, Mrs. Wilma Newbre, and Miss Marilyn Norman.

REFERENCES

1. ARAI, T., and SUZUKI, M. A Rapid Agar Dilution Technique for the Estimation of Antitumor-Cell Activity. J. Anti. (Jap.), **9**:169–71, 1956.
2. BORZANI, W., and VAIRO, M. L. R. Quantitative Adsorption of Methylene Blue by Dead Yeast Cells. J. Bact., **76**:251–55, 1958.
3. DIPAOLO, J. A., and MOORE, G. E. An Evaluation of Ascites Tumor Cell Plating for Screening Chemotherapeutic Agents. Antibiotics & Chemother., **7**:465–70, 1957.
4. EAGLE, H., Propagation in a Fluid Medium of a Human Epidermoid Carcinoma, Strain KB. Proc. Soc. Exper. Biol. & Med., **89**:362–64, 1955.
5. EAGLE, H., and FOLEY, G. E. The Cytotoxic Action of Carcinolytic Agents in Tissue Culture. Am. J. Med., **21**:739–49, 1956.
6. ———. Cytotoxicity in Human Cell Cultures as a Primary Screen for the Detection of Antitumor Agents. Cancer Research, **18**:1017–25, 1958.
7. JOHNSON, M. J. A Rapid Micromethod for Estimation of Non-Volatile Organic Matter. J. Biol. Chem., **181**: 707–11, 1949.
8. MCALLISTER, R. M.; GRUNMEIER, P. W.; CORIELL, L. L.; and BLAKEMORE, W. S. A Quantitative Technique for the Measurement of *in Vitro* Cytotoxicity of 5-Fluoro-2′-Deoxyuridine (FUDR), Nitrogen Mustard (HN) and Other Antitumor Agents. Cancer, **12**:938–43, 1959.
9. MCINTIRE, F. C., and SMITH, M. F. A New Chemical Method for Measuring Cell Populations in Tissue Culture. Proc. Soc. Exper. Biol. & Med., **98**:76–79, 1958.
10. MIYAMURA, S. A Determination Method for Anti-Cancer Action of Antibiotics by the Agar Plate Diffusion Technique. Antibiotics & Chemother., **6**:280–82, 1956.
11. MIYAMURA, S., and NEWAYAMA, S. An Agar Plate Diffusion Method Using HeLa Cells for Antitumor Screening. Antibiotics & Chemother., **9**:497–500, 1959.
12. SMITH, C. G.; LUMMIS, W. L.; and GRADY, J. E. An Improved Tissue Culture Assay. I. Methodology Studies; Cytotoxicities of Streptovitacins and Cycloheximide. Cancer Research, **19**:843–46, 1959.
13. ———. An Improved Tissue Culture Assay. II. Cytotoxicity Studies with Antibiotics, Chemicals, and Solvents. *Ibid.*, pp. 847–53.
14. TOPLIN, I. A Tissue Culture Cytotoxicity Test for Large-Scale Cancer Chemotherapy Screening. Cancer Research, **19**:959–66, 1959.
15. YAMAZAKI, S.; MITTA, K.; HIKIJI, T.; NOGI, M.; TAKEUCHI, T.; YAMAMOTO, T.; and UMEZAWA, H. Cylinder Plate Method of Testing the Anti-Cell Effect. J. Anti. (Jap.), **9**:135–40, 1956.