An Improved Tissue Culture Assay

I. Methodology and Cytotoxicity of Anti-Tumor Agents*

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A technic for the determination of cytotoxicity of chemical compounds for mammalian cells *in vitro* has been described by Eagle and Foley (2). Their assay requires 7 days for completion, with renewal of medium every other day. An application of the Eagle-Foley technic, in which the total assay time is 5 days with medium changes at 1 and 3 days, has been worked out with fermentation products (12). Since the progress of fermentation extraction programs depends on the assay interval, it is advantageous to shorten assay time as much as possible. Recent studies in these laboratories have resulted in a simplified tissue culture assay which requires only 3 days for completion without intermittent medium changes. The improved assay is described in this publication.

MATERIALS AND METHODS

The cell line used throughout this work was Eagle's KB strain of human epidermoid carcinoma.[1] The culture was maintained in Roux bottles containing 100 ml. of Eagle's medium[2] (1) plus 10 per cent calf serum (penicillin-streptomycin at 100 μg/ml). Although horse and human sera had been used with KB in these laboratories, calf serum seemed to allow more reproducible and rapid growth over prolonged periods of time and was chosen for the standard medium. Stock bottles were planted with approximately 1 mg. of cell protein in 100 ml. of medium, which was renewed every 48 hours. Stock culture medium was always renewed 24 hours prior to use. Cell proliferation was measured by protein concentration (Folin-Ciocalteu reagent) as described by Oyama and Eagle (11). Maximum growth in stock bottles was kept below 20 mg. of cell protein by periodic subculturing to maintain growing cells.

To inoculate test tubes for an assay, a stock bottle of appropriate cell content was selected, the medium was decanted, and the cell sheet was scraped from the surface with a rubber scraper into 10 ml. of fresh medium. Cell clumps were broken by repeated pipetting. An aliquot of the cell suspension was centrifuged, washed with Earle's salts (minus bicarbonate)[2] by centrifugation, and the protein content was determined. The stock suspension was diluted to 10–20 μg. of cell protein/ml in Eagle's medium plus 10 per cent calf serum in an indented Erlenmeyer flask equipped with a mechanical stirrer. This suspension of cells was gassed with 50 per cent CO_2 in air and agitated. The pH (after the suspension was placed into culture tubes) was approximately 6.6 at 25° C., and it equilibrated at 7.1 in 15 minutes at 37° C. Four ml. of cell suspension were delivered via an automatic Cornwall pipette to 16 × 150-mm. culture tubes (10), which were then closed with silicone stoppers and placed in a stainless steel spring-loaded rack. Agents under test were added to the tubes in 0.1–0.2 ml. of water before the cell suspension was delivered. Water-insoluble compounds were dissolved in ethanol or dimethylformamide and diluted in water to give a final solvent concentration of less than 1000 μg/ml (solvents nontoxic at this level). The agents were added at twofold concentrations in triplicate tubes with four to five test levels per agent. Statistical analysis indicated that two tubes per concentration level were sufficient, and this is recommended for general use. After inoculation, the tube rack was agitated gently to give a uniform distribution of cells and was incubated at 37° C. at a 10° angle.

After 72 hours' incubation, the test tube rack was placed in a warm-air heater which maintained the cells at 37° C. during microscopic examination

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[1] Obtained through the courtesy of Dr. Harry Eagle.

[2] Purchased from Microbiological Associates, Inc., Bethesda, Md.

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and washing of the tubes. It was found that tubes kept at 37° C. could withstand washing procedures with no disturbance of the cell sheet. The tubes were examined microscopically to observe cell morphology and to estimate the cell content for subsequent protein determination. The medium in all tubes was decanted simultaneously by inversion of the entire test tube rack, and the cell sheets were washed twice with Earle's salts (minus bicarbonate) at 37° C.

The washing operation was carried out automatically with a Filamatic pipetting unit (National Instrument Company) modified to deliver fluid very slowly but to fill rapidly. Two 5-ml. washes were delivered into the tubes with this apparatus without disturbing the cell sheet. After each wash the inverted tubes were blotted with a towel to remove the last few drops of washing fluid. The tubes were allowed to drain for 15–20 minutes over a towel after the final rinse. A medium blank, containing neither cells nor test agent, was included in each assay after it was observed that a substantial amount of protein remained in the blank tubes after two washes.

The appropriate amount of biuret reagent was added to each washed tube to dissolve the cells. The tubes were restoppered with clean silicone stoppers and covered with a rubber-lined stainless steel top which encompassed the entire 150-tube rack. The closed rack was attached to a reciprocating shaker and agitated at approximately 100 strokes per minute for 10–15 minutes at 28° C. to affect complete solution of protein. When the cells were dissolved, 1-ml. aliquots were pipetted into colorimeter tubes for the protein assay. Protein was determined with the Folin reagent with standards of 25, 50, 75, and 100 μg. of crystalline bovine albumin/tube.

The per cent inhibition of protein synthesis was calculated from the following formula:

$$\text{Per cent I} = \frac{\text{Final protein content in controls} - \text{final protein content in experimental}}{\text{Final protein content in controls} - \text{initial protein content in controls}} \times 100.$$

Medium blanks which varied from 25 to 40 μg of protein/tube were subtracted where required. Zero-hour determinations were carried out as follows: Aliquots of the cell suspension were delivered into centrifuge tubes and the cells removed by centrifugation. The cells were washed twice with Earle's salts (minus bicarbonate), dissolved in biuret reagent, and the protein concentration was determined with the Folin reagent. Medium blanks were also used in this step to correct for residual protein. The dose for 50 per cent inhibition (ID_{50}) was calculated from a log-dose response curve. Agents with ID_{50} greater than 100 μg/ml were considered inactive, whereas compounds with ID_{50} less than 1 μg/ml were considered very active.

RESULTS

Inhibition by the streptovitacins and cycloheximide.—The streptovitacins are a family of cycloheximide derivatives (4, 9) which have been shown to inhibit experimental tumors *in vivo* (5–7) and mammalian cells *in vitro* (12). Streptovitacins A and B and cycloheximide[3] were used as models to develop this tissue culture assay. The activities of several members of this family of antitumor substances are shown in Table 1.

The standard error per ID_{50} determination with streptovitacins A and B and cycloheximide was 22 per cent, and per relative potency determination (streptovitacin A standard), 27 per cent. The data in Table 1 show that the activities of the various streptovitacins vs. KB cells in the simplified assay ranged from 0.035 μg/ml for A to 90 μg/ml for C_2. In the 5-day assay with intermittent feedings, the ID_{50} of streptovitacin A was 0.055 μg/ml (12). Thus, the simplified assay without intermittent feeding was somewhat more sensitive. The data in Table 1 further show good correlation between relative potency in tissue culture and in the Walker 256 tumor system with these agents. Representative dose-response curves for streptovitacins A and B and cycloheximide in the standard assay are shown in Chart 1.

The effect on the tissue culture assay of supplementing the growth medium with lactalbumin hydrolysate was investigated with streptovitacin A. The protocol described above was modified in that lactalbumin hydrolysate, at 3 mg/ml, was included in the medium. The ID_{50} for streptovitacin A was found to be 0.022 μg/ml (range: 0.019–0.026), and the standard error, 12 per cent. Supplementation with lactalbumin hydrolysate appears to increase the sensitivity and reproducibility of the KB assay with streptovitacin A.

The simplified assay also could be used to determine the streptovitacin A potency of fermentation extracts containing streptovitacins A and B. Table 2 shows the activities of crude extracts and a crystalline preparation in the more laborious 5-day assay and in the simplified 3-day assay. These data have previously been shown to correlate with the *in vivo* activities of these materials (12).

[3] β-[-2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide.

Growth of control cultures.—A large increase in cell mass during the assay interval was desired in order to decrease the likelihood of false negative results due to protein synthesis in the absence of cell division. Studies on the growth of KB cells in tissue culture under various conditions were carried out and will be the subject of another publication. Under the conditions described above, KB cells proliferated with a generation time of 15–24 hours, resulting in an increase in cell protein of eight- to twelvefold in 3 days. The total 3-day increase in cell mass was limited (*a*) by medium deficiency after 400–600 μg of cell protein per tube has been synthesized and (*b*) by a lag phase which was observed occasionally. Seeding with a low inoculum of cell protein proved to be critical, in order to insure that the controls would grow over the entire assay interval; e.g., between 40 and 70 μg of cell protein/tube, which allows an eight- to tenfold multiplication in the control tubes in 3 days.

DISCUSSION

The data presented above show that the simplified tissue culture assay with Eagle's KB cell line is a sensitive and reproducible method for the measurement of cytotoxicity of the streptovitacins and cycloheximide. The assay required 3 days for completion, with no intermittent medium changes, and allowed measurement of streptovitacin potency in crude extracts of fermentation beers. Recently, Eagle and Foley have reported in more detail on the use of tissue culture in the screening of compounds with potential antitumor activity (3, 8). The results of the present studies extend the utility of the tissue culture method through simplification of technic, and demonstrate a very good correlation between the inhibitory activities of various streptovitacins and cycloheximide *in vitro* and *in vivo*.

The modified tissue culture assay has been used to determine cytotoxic activity of both chemicals and crude fermentation beers and preparations. In addition to ID_{50} data, the tissue culture assay furnished, in some instances, an indication of the type of inhibition from the slope of the dose-response curve. Agents with the same mechanism of action should give dose-response curves with the same slope. Thus, in fractionation studies, if one follows the same inhibitor from one fraction to the next, although the ID_{50} changes with purification, the slope should remain essentially constant. In some cases, the presence of a second inhibitor has been suspected because of a change in the slope of the dose-response curve. It is also possible that some fermentation products might stimulate the growth of KB cells and thus complicate the results obtained.

Lactalbumin hydrolysate did not stimulate and

TABLE 1

ACTIVITIES OF STREPTOVITACINS AND CYCLOHEXIMIDE VS. KB CELLS

AGENT	ID_{50} μG/ML		No. EXPERIMENTS	RELATIVE POTENCY*	
	Median	Range		KB	WAC† in vivo
Cycloheximide	0.10	0.07 –0.13	13	35	40
Streptovitacin A	0.035	0.024–0.047	20	100	100
Streptovitacin B	0.48	0.28 –0.82	13	7	1
Streptovitacin C_2	90	60–150	3	0.04	< 10
Streptovitacin D	0.06	0.02 –0.09	6	60	100

* Ratio of ID_{50} values.

† Walker 256 assay of Dr. J. S. Evans (to be published).

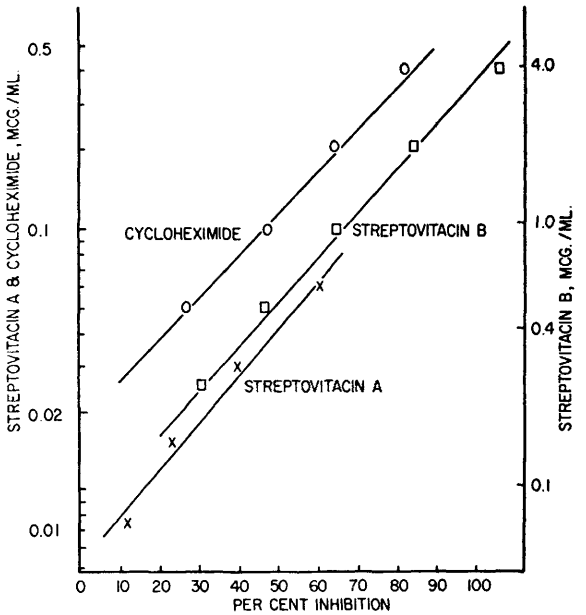

CHART 1.—Dose-response curves for streptovitacins A and B and cycloheximide.

occasionally decreased the rate of KB growth in this test system. The medium was more nutritionally efficient when lactalbumin hydrolysate was added, however, as evidenced by an increased yield of total cell protein beyond 400–600 μg/tube. In the presence of lactalbumin supplementation, the KB assay appeared to be more reproducible for streptovitacin A. Further evaluation of medium supplementation is in progress with other inhibitors.

TABLE 2

ACTIVITIES OF STREPTOVITACIN PREPARATIONS

PREPARATION	ID_{50}, μG/ML		RELATIVE POTENCY*	
	5-day assay (12)	3-day assay	5-day assay	3-day assay
Crystalline A	0.055	0.037	100	100
Crude preparation	0.10	0.085	55	44
Crude preparation	0.85	0.55	7	7

* Ratio of ID_{50} values.

SUMMARY

1. A simplified tissue culture assay has been developed with the KB strain of human epidermoid carcinoma cells *in vitro*, which requires 3 days and no intermittent medium changes for completion.

2. With streptovitacins A and B and cycloheximide, the simplified assay was shown to be approximately as sensitive and reproducible as more laborious technics. The doses for 50 per cent inhibition (ID_{50}) with streptovitacins A and B and cycloheximide were 0.035, 0.48, and 0.10 μg/ml, respectively. The standard error per assay was 22 per cent, and per relative potency determination it was 27 per cent.

3. With streptovitacin A, supplementation of Eagle's medium with lactalbumin hydrolysate (3 mg/ml) resulted in an ID_{50} of 0.02 μg/ml and a standard error of 12 per cent. Supplementation appears to increase the reproducibility of the assay with this antitumor agent.

4. The ID_{50} values for streptovitacins C_2 and D were 90 and 0.06 μg/ml, respectively. The inhibitory activities of streptovitacins A, B, C_2, and D and cycloheximide *in vitro* correlated well with those observed *in vivo*.

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