A Simple Radiochemical Assay of Inhibition by Chemotherapeutic Agents of Precursor Incorporation into Biopsy Samples, Effusions, and Leukocyte Preparations

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Summary. An in vitro test for measuring the inhibition by various chemotherapeutic agents of the uptake of radioactive precursors into nucleic acids, proteins, and lipids is described. Because only biopsy-size tissue volumes are required and the assay can be completed in three to four hours, it seems feasible for clinical use. With appropriate modifications normal and neoplastic cells from the same patient can be included, thereby increasing the relevance of the technic to clinical problems.

Introduction. The steady expansion of chemotherapeutic agents useful in the treatment of advanced or inoperable malignancy has been hampered by the absence of a simple test which will predict the best agent to use in individual patients. While certain agents have been found useful in some categories of malignancy (26), it is not otherwise possible to determine which agent to employ except by clinical trial. There has been much interest in developing in vitro chemotherapeutic sensitivity tests (1, 2, 4, 8, 14, 17, 21-26). In general, the usefulness of these procedures has been limited by a requirement for in vitro multiplication of the cells (e.g. Ref. 1), by the indirect nature of the assay (e.g. Ref. 10), or by a requirement for meticulous preparation and rather large amounts of tumor material (4, 24). In the present communication, a simple assay system based on short-term culture (two hours) of biopsied cells is described. It utilizes the direct paper disc assay technic (5, 6), is quantitative, needs only microscopic amounts of cells, and allows the simultaneous processing of hundreds of samples including normal cells from the same patient.

Materials and Methods. Three animal cell systems were employed to examine the quantitative aspects of this procedure. As a model system for solid tumors, the mouse C-1300, Jackson Laboratory, round-cell tumor line was used. Pieces weighing one gram or less were removed and minced in Hank’s solution (19) containing 0.01 mg/ml of penicillin and 0.01 mg/ml of streptomycin sulfate. The suspension was then filtered through a piece of flame-sterilized 80-mesh wire gauze, and a portion of the resulting suspension of cells was used directly for assay purposes.

As a model system for the evaluation of malignant effusions, log phase Ehrlich ascites tumor cells were used. These were diluted by the addition of one-tenth volume of Hanks’ solution containing ten times the antibiotic concentrations cited above and were used without further treatment. Microscopic examination showed the ascitic fluid to consist largely of single cells.

To determine the effectiveness of the assay on blood cells (as a model for malignant leukemic leukocyte preparations), whole blood was obtained from mature New Zealand white rabbits and immediately heparinized. The blood (about 20 ml) was then allowed to sediment for 60 minutes at 29°C, and the concentrated buffy coat was removed by gentle pipeting. This suspension was diluted by the addition of an equal volume of Hank’s solution containing twice the concentration of antibiotics cited above. Stained preparations revealed erythrocytes with about 1% white blood cells.

Amino acids-14C (1 mc/m mole), thymidine-3H (10 c/mmole), uridine-3H (10 c/mmole), and glycerol-3H (200 mc/mmole) were added to the medium in the amounts indicated in the legend to Chart 1. Incubations were in an air atmosphere at 37°C. Suitable zero time controls were subtracted from the final values obtained. In the experiments designed to determine the quantitative and statistical aspects of the assay, samples were removed and processed after labeling for two hours with either uridine-3H or amino acids-14C. The work-up and counting of such samples was otherwise the same as the technic we have described for microbial systems (5, 6). After scintillation counting the results were recorded, averaged, and the zero time controls subtracted. Quenching proved so uniform that no correction was necessary.

The cancer chemotherapeutic agents were freshly prepared weekly in distilled water and stored at freezer temperature. Light sensitive agents (such as actinomycin D) were stored in black bottles. The concentrations used are described in the specific experiments and in the legend to Fig. 1; the agents were placed in the labeling wells or flasks prior to the addition of the cell suspension. In general, the sequence which has evolved is to prepare the suspension, examine it microscopically to ensure the absence of excessively large clumps of cells, add the label, remove zero time controls, and then place the mixture in contact with the relevant agents. This sequence was followed in all experiments except where specifically noted.

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Inhibition of Precursor Incorporation

We currently employ an assay board prepared by drilling 1 cm-deep wells (diameter, 1 cm) in a lucite board 6 x 6 x 1 inch. This apparatus will include as many as 64 separate samples.

Results. There is a progressive uptake of labeled amino acids, uridine, thymidine, and glycerol for at least sixty minutes in all three types of cell suspensions (Chart 1). Use of too small a sample volume (less than 300 µl of incubation volume) led to a reduction in count obtained, as did failure to agitate the assay board prior to taking each sample. Under optimum conditions the standard deviation for 100 identical samples was 2%. In other experiments it was found that the amount of uptake of exogenous label was a linear function of cell number for cultures up to 16,000 cells/ml and that the inhibitory capacity of added antimetabolites was a logarithmic function of the drug dose.

The possible potential of this procedure in clinical situations is illustrated by a study of a patient with malignant ascites from a cervical epidermoid carcinoma. The cell suspension from this patient prior to treatment consisted of a dense, partially clumped suspension of pleomorphic cells (Fig. 1, upper left). Due to its high cell content, it was not concentrated prior to use. The cells were placed in medium containing thymidine-3H (1.0 µc/ml) and a variety of commonly used chemotherapeutic agents. Inhibition of thymidine uptake indicated that the cells were partially sensitive to several agents (Fig. 1, lower left). The patient was accordingly treated with oral cytoxan (150 mg/day) and quinacrine intraperitoneally (200 mg). After ten days the remaining ascites fluid was again sampled and assayed. The cell content was greatly reduced (Fig. 1, upper right) and the patient felt somewhat improved. The cells were still capable of thymidine-3H uptake (Fig. 1, lower right). Sensitivity to the various chemotherapeutic agents appeared insignificantly altered except for an increase in resistance to cytoxan. The patient was then begun on prednisone and, in addition, received a second intraperitoneal injection of quinacrine (200 mg). The ascites cleared considerably for a month but has since recurred.

Discussion. This communication describes a sample radiochemical assay which may be useful in monitoring the in vitro inhibitory potential of chemotherapeutic cancer drugs as more data is obtained. However, numerous problems remain to be solved before this type of assay can be generally applied. The relationship of inhibition of macromolecule synthesis in vitro to inhibition of cancer cell multiplication in vivo remains to be clarified and is subject to many adventitious phenomena, some of which are apparent in the data presented here. For example, incorporation of label to all cell fractions in vitro decays during the relatively brief period of cultivation (Chart 1), indicating cellular deterioration unrelated to any antimetabolite effect.

Furthermore, the best precursor or precursors to use in this type of study has yet to be established. Although labeled thymidine would seem a reasonable choice, the control of DNA synthesis may not always accurately reflect the cells' potential for subsequent multiplication (15). The control and turnover of unstable RNA fractions makes the data obtained from experiments using labeled uridine especially difficult to

Chart 1. Applicability of the assay to different cell types and different precursors. Ehrlich ascites, suspension of solid C-1300 round cell mouse tumor, and normal rabbit leukocytes (heme) were labeled with thymidine-3H, 10 µc/ml (-○-); uridine-3H; 10 µc/ml (-□-); amino acids-14C, 2 µc/ml (-Δ-); and glycerol-3H, 10 µc/ml (-△-). Each culture was sampled in duplicate at the times indicated by removing 75 µl aliquots and placing them on discs of Whatman 3-mm filter paper. The wet disc was then dropped immediately into ice-cold 5% trichloracetic acid. The subsequent details of processing for liquid scintillation counting have been described (5, 6). The kinetics of uptake into trichloracetic acid-insoluble material are plotted against time. Absolute reduction in cpm are indicated in the parentheses in each panel.

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interpret (7, 18, 20). Studying protein or structural lipid synthesis partially avoids these pitfalls, but the mode of action of most of the currently useful chemotherapeutic agents lies far from these biosynthetic pathways.

Another major obstacle lies in the distinction between inhibition of specific synthetic pathways and general inhibition of cellular multiplication. For example, the data from Fig. 1 might suggest that neither Methotrexate nor 5-fluorouracil would be useful in the treatment of the ascites condition in the patient under study. However, it seems more likely that these results stem more from the mode of action of these compounds (reducing endogeneous thymidine formation) than from a real lack of inhibitory potential (4, 16).

Another question is the dosage level to be used in vitro for each agent. What must be approximated is that dose which will be a true measure of the in vitro potential of each drug. To make a preliminary estimate of the amounts to use in the assay, we assumed that the maximum recommended daily dose was given as a single injection and diluted equally throughout the body fluids (taken arbitrarily as 35 liters). This concentration of many of the agents produced insignificant in vitro inhibition. We therefore arbitrarily increased this concentration five-fold. The validity of these doses must be established by further studies.

As more experience is obtained with various precursors, antimetabolites, and culture technics, it is anticipated that trends will emerge suggesting correlations between the data and the actual clinical results. Given sufficient refinement, the technic should eventually yield accurate results.

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References


Fig. 1. A clinical application of the assay. An ascites cell suspension from the patient with metastatic epidermoid carcinoma was obtained (upper left). The assay was conducted as described in the text using a 120 minute incubation time, with the results indicated (lower left). The patient was then placed on cytoxan and received a single i.p. injection of quinacrine. The ascites persisted at a lower level and another sample was removed eleven days later. Microscopic examination revealed a reduced cell number (upper right). These cells were reassayed, with the results shown (lower right). Details in the text. Drug concentrations (in μg/ml): 5-fluorouracil (5-FU), 430; Methotrexate, 1.4; chlorambucil, 3.5; vincristine, 1.5; thio-TEPA, 4.0; actinomycin D (Act D), 0.4; prednisone, 28.5; sarcolysin, 2.9; myleran, 2.3; vinblastine 300.0; cytoxan, 85.7; quinacrine, 51.0; 6-mercaptopurine (6-MP), 52.6. All photos × 200.
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