adult rats (1 year old) has been compared with that of young growing rats (2 months old) by balance studies and tracer calcium. 2. In the adult rats the mass of exchangeable calcium, bone anabolism, bone catabolism, intestinal absorption and balance are reduced; but urinary excretion of calcium remains unchanged. 3. Similarities were noted between the effects of aging and those previously reported for hypophysectomy.

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In vitro Test Systems for Cancer Chemotherapy. II. Correlation of *in vitro* Inhibition of Dehydrogenase and Growth with *in vivo* Inhibition of Ehrlich Ascites Tumor.* (28685)

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The desirability of simpler methods for finding cancer chemotherapeutic agents or for determining their effectiveness has led to the development of a variety of *in vitro* procedures. Mammalian cell culture techniques, although less expensive and less laborious, have a number of innate disadvantages which are only partially understood. The difficulties include extrapolation to the *in vivo* situation, where the drug may be so modified, altered, metabolized or excreted as to render it more or less toxic, or more or less effective than originally observed in the *in vitro* assay. Development of host resistance may also make correlation between *in vitro* and *in vivo* tests difficult and at times unimpressive. Recently DiPaolo(1) has shown that compounds of proven clinical value or wide effectiveness in animal tumor systems are inhibitory in tissue culture; the exact conditions of the *in vitro* test may determine, in many instances, the extent of inhibition caused by any one compound, ranging from inactivity to high inhibition.

The present investigation utilizes Ehrlich ascites tumor cells which have been in continuous culture for 33 months and which have been used in a variety of *in vitro* tests. The results of these tests are compared with the results obtained when cells from the same cell line are grown in Swiss mice.

Materials and methods. An established

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TABLE I. Effect of a Variety of Compounds on Cultured Ehrlich Ascites Tumor Cells in Swiss Mice.

Compound	Dose, mg/kg/day	% extent survival	Results*
Colchicine	.50	112	+
Methylbis(B-chloroethyl)amine	.75	107	+
Actinomycin-D	.05	102	+
5-Fluorouracil	10	89	+
Methotrexate	1.50	71	+
Ethyl-N-bis-(2,2-dimethylethylenimido)-phosphorocarbamate	500	44	±
Benzyl[bis-(1-aziridinyl)phosphinyl]-carbamate	10	42	±
Nitromin	40	41	±
5-Mercaptouracil	50	14	—
Sodium arsenite	10	13	—
Hydrocortisone	100	13	—
6-Mercaptopurine	100	8	—
N,N′,N″-triethylenethiophosphoramide	2	1	—
Diethylstilbestrol	20	− 2	—
Thalidomide	500	−17	—

* — = 0-19%; ± = 20-50%; + = 51-125%.

strain of Ehrlich ascites tumor cells which has been in continuous culture for 33 months was used for *in vitro* studies. The cells are grown in the medium of Earle's salts supplemented with 20% fetal calf serum and 20% bovine amniotic fluid(2). Although the original tumor was hyperdiploid, the cultured cells have had a chromosome mode of 67-75 after the first 6 months of *in vitro* culturing. Inoculation of 2 — 3 × 10^6 cells results in a mean survival time of 20.65 ± 0.83 days. The virulence of the cultured cells is very similar to that of the original tumor which causes death in approximately 18.5 days.

The drugs (Table I) were tested for their ability to inhibit growth or dehydrogenase activity of these cells. Tests were carried out using overlaid monolayers with completely defined and semi-defined medium in agar. In addition, growth inhibition was noted with cells placed on coverslips and exposed to drugs at concentrations based on the accepted mouse dose and on the assumption that all the drug was in the circulatory system. In this test cells were exposed to the drugs for one hour, except to methylbis(B-chloroethyl)amine (HN_2) for 8 minutes. The reduction of 0.05% methylene blue solution was used to indicate cytotoxicity or inhibition of dehydrogenase activity. Details of the cells used, and procedures have been described(3,4).

The *in vivo* studies were done using 7 to 8 week old Swiss female ICR/HA mice weighing between 23 to 28 g, which had been given intraperitoneal injections of 2 − 3 × 10^6 tissue culture cells suspended in 0.5 ml of physiological saline. The test drugs were dissolved in physiological saline or suspended in 0.5% carboxymethylcellulose (CMC) dissolved in saline. An accepted dosage (Table I) was administered intraperitoneally 24 hours after tumor implantation and continued for 7 consecutive days. Groups of 8 mice were used for each drug tested and controls consisted of 20 mice for each experiment. Compounds which gave doubtful or negative results were rescreened. Evaluation of the therapeutic effect of the drug was obtained by comparing survival time of treated animals with control solvent treated animals and was expressed as extension of survival time.

Results. The responsiveness of these cells when returned to mice (Table I) is identical to that obtained with the parent tumor ELD hyperdiploid Ehrlich ascites. Of the variety of compounds examined, 8 were positive, and 7 were negative.

A variety of responses obtainable by different *in vitro* techniques with the same drugs is summarized in Table II. In all test systems used approximately 50% of the drugs were inhibitory. It is impossible to classify the drugs as effective or non-effective by class. For example, in the animal studies, cytotoxic agents such as HN_2, actinomycin-D, and nitromin were inhibitory while N, N′, N″ - triethylenethiophosphoroamide (TSPA) was ineffective. Specific purines and pyrimidines were both effective and ineffective in the animal studies. The dehydrogenase test utilizing *in vitro* cells revealed the largest number of agents as being inhibitory compounds (12/15). Of the 8 compounds that extended the survival time of mice bearing tumor cells, colchicine failed to

TABLE II. Comparison of Results Obtained with *in vitro* Screens and Animal Tests.

Compound	Tissue culture cells *in vivo*	Screen I	Screen II	Screen IIA	Screen III
Colchicine	+	—	+	±	+
Methylbis(B-chloroethyl)amine	+	+	+	+	+
Actinomycin-D	+	+	+	+	+
5-Fluorouracil	+	+	—	+	±
Methotrexate	+	+	—	+	+
Ethyl-N-bis-(2,2-dimethylethylenimido)-phosphorocarbamate	±	±	—	±	+
Benzyl[bis-(1-aziridinyl)phosphinyl]-carbamate	±	+	—	+	+
Nitromin	±	+	+	+	—
5-Mercaptouracil	—	+	—	—	+
Sodium arsenite	—	—	+	+	Not done
Hydrocortisone	—	—	+	+	—
6-Mercaptopurine	—	±	—	+	—
N,N′,N″-triethylenethiophosphoramide	—	+	+	±	+
Diethylstilbestrol	—	—	+	+	+
Thalidomide	—	—	—	—	+

Screen I: Dehydrogenase test *in vitro* cells. Screen II: Monolayer agar diffusion semi-defined medium. Screen IIA: Monolayer agar diffusion defined medium. Screen III: Coverslip test.

Screens I, II & IIA: + = zone of inhibition size (diameter) greater than 15 mm; ± = 11-14 mm; — = 0-10 mm.

Screen III: + = minimum 50% growth inhibition and cell damage (nuclear swelling, cytoplasmic vacuoles, or decreased cell size); ± = fewer mitotic figures, indicating partial inhibition; — = no cell damage.

inhibit the dehydrogenase activity of the same cells when placed in suspension. A possible reason for this might be that colchicine has its greatest effect on the mitotic spindle of the growing population of cells and consequently would be expected to be ineffective when used on a static cell population. Inhibition of dehydrogenases was obtained using 5-mercaptouracil (5-MU), 6-mercaptopurine (6-MP), and TSPA, all of which failed to extend the survival time of tumor-bearing animals. Of these 3 compounds, 5-MU is not considered a cancer chemotherapeutic agent, but it does potentiate methotrexate (AM) and 5-fluorouracil (5-FU) inhibition(5,6). When growth inhibition is the criterion of effectiveness of a compound the number of effective drugs decreases from 12 to 7, one less than the number of compounds which cause inhibition in the animal tests; however, the drugs which are ineffective with this test differ to some extent from the drugs which were effective or ineffective with the dehydrogenase inhibition tests. For example, colchicine causes inhibition of growth when cells are in a monolayer. To establish that this is true growth inhibition rather than the result of variation in drug absorption time, the dehydrogenase test was repeated allowing colchicine to be in contact with the agar surface for the same period of time as in the monolayer test. Even under these circumstances colchicine failed to inhibit the dehydrogenases of the tumor suspension. Three compounds effective in the animal tests, AM, ethyl-N-bis-(2,2-dimethylethylenimido)phosphorocarbamate (AB-132) and benzyl[bis-(1-aziridinyl)phosphinyl]carbamate (AB-103), were not inhibitory in the monolayer test under standard conditions. Neither was 6-MP which caused inhibition of dehydrogenases but was ineffective in the *in vivo* experiments. A second compound which caused inhibition of dehydrogenases but failed to cause growth inhibition of the monolayer was 5-MU. However, this compound also failed in the animal test. When the growth medium for the monolayer test was altered by eliminating serum and lactoalbumin hydrolysate and supplementing with additional folic acid, the compounds which had been negative in the original monolayer test with the exception of 5-MU and thalidomide, now caused inhibition of monolayer growth.

Among the various methods, use of the coverslip tests indicated the largest number of inhibitory drugs. Drug concentrations were based on the premise that all of the drug was in the circulatory system. Under these circumstances the concentration of drug/ml of medium in most instances exceeded the minimum dosages required to produce inhibition of these cells in liquid medium(1). The inhibition was probably due to nonspecific toxicity of excess amount of drug. With the exception of nitromin, which failed to cause inhibition in this coverslip test, 5 compounds, 5-MU, hydrocortisone, TSPA, diethylstilbestrol, and thalidomide, must be considered false positives when compared to the animal data.

Discussion. Not all drugs used in experimental animals or in clinical chemotherapy would be expected to have a positive *in vitro-in vivo* correlation. Since the advantages of *in vitro* assays are obvious, it is imperative that attempts to develop techniques at the cellular level take into account the problems of extrapolation to the *in vivo* situation.

Probably the most important question to be answered is: Does the action of a drug on a cell of mammalian origin, growing in tissue culture, bear a relationship to the action of these drugs on a tumor *in vivo*? A comparison of the various *in vitro* screens which have been currently used in our work shows that the largest number of compounds is found to be inhibitory when the estimation of the degree of cell dehydrogenase inhibition is the indicator of drug effectiveness. This test also had the largest positive correlation with the animal tumor study. However, it failed to select colchicine which is effective against Ehrlich *in vivo* and it did select 6-MP and TSPA which, although known cancer chemotherapeutic agents, are considered ineffective against the Ehrlich ascites in animals. Two other interesting compounds, hydrocortisone and diethylstilbestrol both ineffective in animal experiments, were selected by the monolayer test.

When the concentration of drugs was increased to that which might exist in the animal's circulatory system, practically all drugs gave a positive response. These results seem to indicate that a dose-response relationship established *in vitro* probably does not give any indication of the dose equivalence to be used in humans. Possibly in instances where toxic side effects limit the maximum dosage, the *in vitro* or tissue culture methods would give an indication of the toxicity-therapeutic ratio. This might be done by simultaneous testing of various concentrations of drugs on both the tumor cells and normal cells *in vitro*.

Any attempt to establish an animal-*in vitro* correlation must be done with some sort of control situation, *e.g.*, the use in animals of drugs not recommended by the *in vitro* screening procedure. Without one, there is no surety that the animal experimental results would not be achieved with drugs showing no *in vitro* selectivity. The same argument applies in trying to increase the level of correlation by increasing the clinical drug dosage. Until a 1:1 relationship between *in vitro* and experimental *in vivo* dosages can be established, it may be possible to get a clinical response by increasing the dosage of the compound which shows no toxicity at the corresponding *in vitro* dosage.

Summary. Chemotherapeutic compounds of clinical values as well as those of wide effectiveness in animal tumor systems are shown to give varying responses *in vitro*. The extent of inhibition caused by any one compound *in vitro* ranges from none to significant, depending upon the exact *in vitro* conditions. The dehydrogenase enzyme inhibition test was the most successful on the basis of correlation with extension of survival time of mice bearing the same tumor used in the *in vitro* studies.

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