Effect of Canavanine on Proliferation and Metabolism of Human Cells *in vitro*. (31010)

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There is much evidence in the literature demonstrating the growth inhibition by canavanine on bacteria and viruses(1-3). Some evidence has also been presented that canavanine can inhibit growth of mammalian cells cultured *in vitro*(4). A mechanism explaining its inhibitory effect on cellular proliferation was suggested in a previous report(5) showing that canavanine was incorporated into the protein of Walker carcinosarcoma 256 cells. Subsequently Allende and Allende(6) demonstrated incorporation of canavanine-C^{14} into rat liver protein by partially purified preparations of arginyl-RNA-synthetase.

In addition to proliferation inhibition, the guanidinoxy compound has been shown to interfere with the formation of certain bacterial enzymes(2). Structurally related guanidine compounds also increased glucose utilization and lactic acid production in KB cell cultures(7). The present work was undertaken to clarify further the nature of canavanine inhibition and to extend previous studies to include other cells of human origin derived from normal and malignant tissues.

Materials and methods. The WISH and HEp-2 epithelial-like cells of human origin were obtained from the American Type Culture Collection Cell Repository, Rockville, Md. Dr. J. Fogh, Sloan-Kettering Institute for Cancer Research, kindly supplied the FL human amnion cells. Periodic examinations of stock cultures for mycoplasma and other contaminants were negative. Experiments were initiated from stock cultures grown in Medium 7a plus 10% whole calf serum fortified with penicillin and streptomycin(8). Initial cell inocula of 2-4 $\times$ 10^5 cells/T-15 flask were maintained throughout for all cell types. Twenty-four hours after subculture, the sticking cells were replenished with fresh Medium 7a or 7a plus 0.2, 0.4, and 0.8 mM canavanine (sulphate). Cultures were taken in duplicate at 0, 48, 72, and 96 hours for determination of cell numbers, protein content, and glucose utilization, the remaining cultures in each group being replenished with fresh medium (control or test) at 48 and 72 hours. Cell numbers were determined by visual counting in a hemocytometer. Glucose was determined by the anthrone method for reducing sugars (9). Protein was assayed by the Lowry method as adapted for cell culture by Oyama and Eagle(10). Lysine utilization from the culturing medium and purification and incorporation of lysine-C^{14} into cell protein were determined with a Technicon amino acid analyzer equipped with a flow cell (Packard Instrument Co.) for scintillation counting. Specific activity measurements of the lysine-C^{14} were made on a Nuclear-Chicago Co. windowless gas flow counter. Proliferation data are expressed as percent of controls (= 100) for comparative purposes among the cell types. Control cultures went through at least two doublings of the cell populations. In all cases experiments were carried out in duplicate or triplicate.

Results and discussion. Initial experiments, Fig. 1 (A and B), compared the effects of canavanine on proliferation of 2 human amnion cell lines, WISH and FL. Both were derived from normal amnion; however, the FL cells were shown to have undergone a neoplastic transformation(11). WISH cells cultured in the presence of canavanine (0.2 mM) showed little or no inhibition of proliferation through 72 hours, whereas the same concentration caused over 50% inhibition of the FL cells. Although proliferation of FL cells was completely inhibited by 0.8 mM canavanine within 48 hours, proliferation of WISH cells was arrested only 44%.

The proliferation inhibition studies were extended to include another epithelial-like cell line, HEp-2, derived from malignant tissue. The same marked toxicity was apparent here, Fig. 1 (C), as seen previously with the FL cells (*cf* Fig. 1 B). Again 0.8 mM canavanine caused over 90% repression of cellular proliferation within the first 48 hours of exposure.

To clarify further the nature of this inhibi-

tion, growth recovery experiments were conducted (Fig. 2). Replicate cultures of HEp-2 were exposed to 0.8 mM canavanine for 24 and 48 hours and subsequently replenished with control medium to test the reversible or irreversible nature of response. From these results it appeared that partial recovery was possible from only 24 hours exposure to canavanine but that inhibition was irreversible after 48 hours.

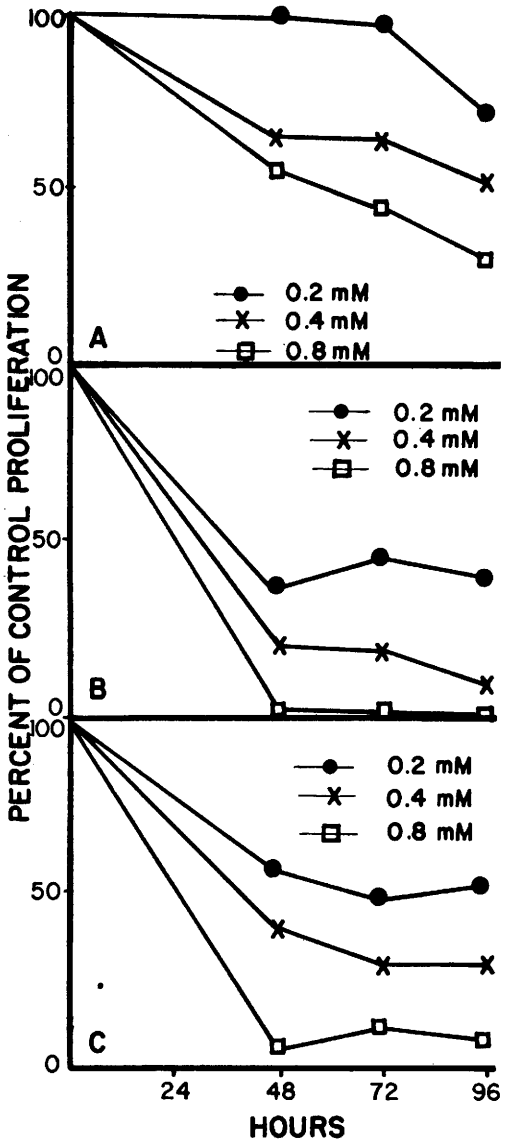

FIG. 1. Effect of canavanine on proliferation of WISH (A), FL (B), and HEp-2 (C) cells. Media changed at 48 and 72 hr; cells counted in a hemocytometer.

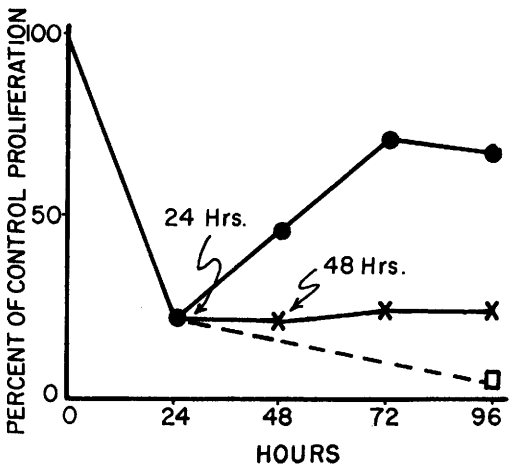

FIG. 2. Recovery of HEp-2 cells after exposure to canavanine (0.8 mM). Media changed every 24 hr; cells counted in a hemocytometer.

The effect of canavanine on glucose uptake of cultured human cells is shown in Table I. The FL and HEp-2 cells showed a markedly increased uptake of glucose (78-279%) with 0.8 mM canavanine, whereas the WISH cells were affected much less (only 33%). With 0.2 mM canavanine, wherein virtually no proliferation inhibition was observed, the WISH cells were not significantly affected in their uptake of glucose. The greatest increased uptake occurred in the FL cell cultures which also showed the strongest proliferation inhibi-

TABLE I. Effect of Canavanine on Glucose Uptake by Human Cells *in vitro*.

Canavanine conc (mM)	% of control*		
	HEp-2	FL	WISH
.2	170	143	93
.4	125	227	102
.8	178	379	133

* Glucose uptake was determined on spent media by anthrone method. Cells cultured in Medium 7a plus canavanine for 96 hr.

TABLE II. Effect of Canavanine on Protein Content of Cultured Human Cells.

Canavanine conc (mM)	% of control*		
	HEp-2	FL	WISH
.2	123	155	140
.4	121	199	160
.8	175	190	201

* Protein determined as $\mu g/10^6$ cells bovine serum albumin equivalents. Cells cultured in Medium 7a plus canavanine for 96 hr.

TABLE III. Effect of Canavanine on Lysine-C^{14} Incorporation into HEp-2 Cell Protein.

	Total cell protein (mg)	Total cpm in protein	Spec. act. (cpm/μM lysine)	Rel. spec. act. (cpm/mg new cell protein)
Control	9.25	41,466	58,788	5183
Canavanine	16.26	27,638	35,162	1841
% of control	178	67	60	35

Cells were cultured in Medium 7a $\pm$ 0.8 mM canavanine for 50 hr. Protein acid hydrolysates (representing 19.9 $\times$ 10^6 cells from control cultures and 20.1 $\times$ 10^6 cells from canavanine cultures) were chromatographed on an Auto-Technicon amino acid analyzer. Specific activity was determined in a windowless gas flow counter. All counts are corrected for self-absorption and counting efficiency.

tion by canavanine (Fig. 1). Guanidine compounds reportedly stimulate glycolysis(7) and act as inhibitors of oxidative phosphorylation (12). The above results are probably due to one or both of these phenomena in the canavanine-treated cultures.

Although canavanine markedly inhibited proliferation, the protein content of the cells was increased (Table II). Here 0.8 mM canavanine caused the same end result in all 3 cell lines, increasing the protein content about 2-fold over control cultures. Similar effects on the protein content of human Chang liver cells by aflatoxin B_1 were recently reported by Gabliks *et al*(13). Lysine-C^{14} incorporation experiments, summarized in Table III, also were run to determine if the elevated protein content of the cells reflected synthesis of new protein or a block in cell protein turnover. From these results, showing a 30-35% decrease of total lysine-C^{14} incorporation compared to control cultures, it would appear that protein synthesis was partially blocked by canavanine. However, the relative specific activities of lysine-C^{14} based on new cell protein synthesized suggested that turnover of cell protein may be blocked to a much greater degree than synthesis.

Summary. Canavanine inhibited the proliferation of WISH, FL, and HEp-2 cells *in vitro*. HEp-2 cells of malignant origin and FL cells derived from normal tissue but later shown to be malignant were most strongly inhibited. Growth recovery experiments with HEp-2 indicated that proliferation inhibition was irreversible after 48 hours exposure to canavanine. Glucose uptake was increased to a greater extent in the FL and HEp-2 cells than in WISH cells. The protein content of all cells was increased about 2-fold by canavanine. Lysine-C^{14} incorporation into the cell protein of HEp-2 cells suggested that the increased protein content of this cell line might be due to a block in cell protein turnover.

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