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The Effect of Canavanine on Cultured Mouse Cells[1] (38145)

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The requirement of arginine for the replication of polyoma virus in mouse cells is essential (1), however, the arginine biosynthetic pathway (2) in polyoma infected mouse cells is nonfunctional (3).

In an effort to understand the arginine requirement of microbial and mammalian systems, theeffect of canavanine, an arginine analogue, has been investigated (4–17). Miller and Consigli (18) recently demonstrated that canavanine could not functionally substitute for arginine during polyoma infection of mouse cells, and that canavanine was a potent inhibitor of viral DNA synthesis and virion assembly.

The present communication reports the effect of canavanine on growing uninfected mouse cells and indicates an essential arginine requirement of mammalian cells.

Materials and Methods. Primary mouse embryo cells were seeded (1.2×10^6 cells/ml) in 60 × 15 mm plastic Falcon dishes, and allowed to attach overnight. The medium utilized was Eagle's minimal essential medium (MEM), containing 5% fetal calf serum (FCS) and supplemented with antibiotics: penicillin, 200 U/ml; streptomycin, 0.2 mg/ml; and kanamycin, 0.01 mg/ml. The cultures were then washed once with phosphate buffered saline (PBS, 0.01 *M* sodium phosphate buffer, pH 7.2, 0.15 *M* sodium chloride) and the cells maintained in Eagle's MEM containing 5% dialyzed FCS with or without arginine and/or canavanine (lot 840293; Calbiochem, San Diego, California 92112). The arginine content of the Eagle's MEM used in these experiments was 0.6 m*M* unless otherwise stated. Cell proliferation was quantified by cell enumeration in a hemocytometer and by protein determination (19).

Release of cellular arginine due to canavanine treatment was investigated by radioisotopically prelabeling cellular proteins with ^{3}H-arginine. TCA soluble ^{3}H-arginine present in the medium was considered an index of cellular proteins, but more specifically arginine, released into the medium. This was accomplished by allowing mouse cells to reach monolayers in Eagle's MEM (0.3 m*M* arginine) supplemented with 5% dialyzed FCS and containing 0.25 μCi/ml of ^{3}H-arginine. After a 48 hr exposure to the radioactive label, the medium was removed, cells were washed twice with PBS and fresh, unlabeled Eagle's MEM returned to the culture for an additional 24 hr to empty cellular pools of ^{3}H-arginine. Cells were washed with PBS and fersh Eagle's MEM containing the various concentrations of canavanine was added back to the cultures. At designated times, medium from the respective cultures was removed, precipitated with TCA and the soluble fractions obtained and assayed for radioactivity (counts per min) as an index of cellular protein release.

The effect of canavanine on macromolecular synthesis in mouse cells was determined by the incorporation of radioisotopic precursors into cellular constituents. Cultures treated with canavanine were exposed to ^{3}H-

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thymidine (1 μCi/ml), ^{3}H-uridine (1 μCi/ml) or ^{3}H-valine (1 μCi/ml) to isotopically determine cellular DNA, RNA and protein synthesis, respectively. The cells were harvested at various times after labeling; the medium containing the radioisotopic precursors was removed, the cells were washed once with PBS, and then cold TCA (5% final concentration) was added and the cultures stored overnight at 4°. The cultures were washed 3 times with cold 5% TCA to remove unincorporated radioactive precursors and then dissolved in hot 1 *N* NaOH (19). The samples were asayed for radioisotopic incorporation (counts per min) in a Beckman model LS-233 scintillation counter and protein was determined by the method of Lowry *et al.* (20), usin bovine albumin as the standard.

Results and Discussion. Experiments were designed to compare the effects of various concentrations of canavanine on proliferation of mouse cells during a 72 hr growth period. The growth of cells maintained in 0.06 m*M* canavanine was inhibited 30%, in 0.6 m*M* canavanine by 65%, and in 6.0 m*M* canavanine by greater than 100% (Fig. 1). The calculations were obtained by considering the normal arginine system as uninhibited and the arginine deprived system as 100% inhibited. The higher concentration of canavanine (6.0 m*M*) did not cause visible cytotoxic effects through the initial 48 hr of treatment, although some toxicity was observed thereafter as determined microscopically.

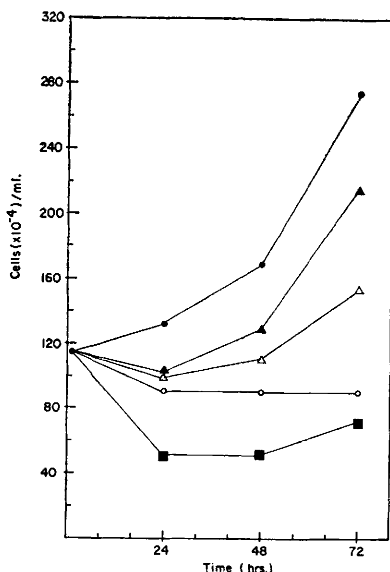

FIG. 1. Effect of various concentrations of canavanine on the proliferation of mouse cells maintained in Eagle's medium ●—●; Eagle's medium containing 0.06 m*M* canavanine ▲—▲; 0.6 m*M* △—△; 6.0 m*M* canavanine ■—■; and Eagle's medium deprived of arginine ○—○.

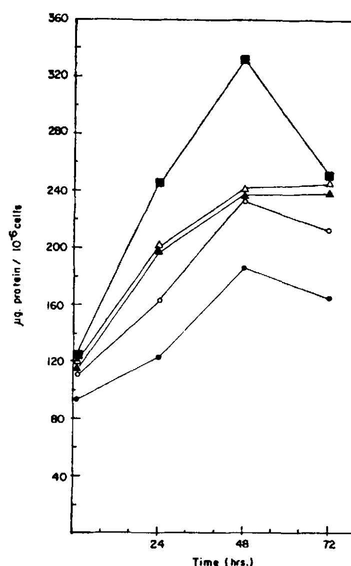

FIG. 2. Effect of various concentrations of canavanine on protein content in mouse cells maintained in Eagle's medium ●—●; Eagle's medium containing 0.06 m*M* canavanine ▲—▲; 0.6 m*M* △—△; 6.0 m*M* canavanine ■—■; and Eagle's medium deprived of arginine ○—○.

The cultures treated with the various concentrations of canavanine demonstrated reduced cell proliferation but an increased quantity of protein per cell (Fig. 2). It was notable that cells maintained in 6.0 m*M* canavanine demonstrated a two-fold increase in protein content per cell, although cell proliferation was totally inhibited (Fig. 1).

To determine whether *de novo* protein synthesis was occurring in cells treated with canavanine, the incorporation of ^{3}H-valine was followed into canavanine treated cells over a 48 hr period. It was found that the low concentration of canavanine (0.06 m*M*) did not affect protein synthesis and the high concentration of canavanine (6.0 m*M*) reduced protein synthesis by only 25% as com-

pared to the untreated cells (Fig. 3). The ability of the canavanine treated cells to undergo protein release is further indication that these cells are capable of protein synthesis. Release of cellular arginine was evident in the canavanine treated cells but not in the cells maintained in Eagle's MEM totally deprived of arginine where the release of cellular arginine was suppressed (Fig. 4).

Macromolecular synthesis of RNA and DNA was also investigated in mouse cells with canavanine (6.0 m*M*) and compared to cells maintained in Eagle's MEM lacking the analogue. The cells treated with canavanine were capable of RNA and DNA synthesis although at a slightly reduced level (20–30%) when compared to untreated mouse cell cultures (Fig. 5A and B).

These findings indicate that cells maintained in high concentrations (6.0 m*M*) of canavanine allow protein synthesis without cell division. This observation is in accord with the previous report of Miedema and Kruse (11) demonstrating *de novo* protein synthesis and inhibition of cell division by canavanine in normal and transformed human cells derived from amnion tissue (FL and WISH) and epidermoid carcinoma tissue (HEp-2), respectively.

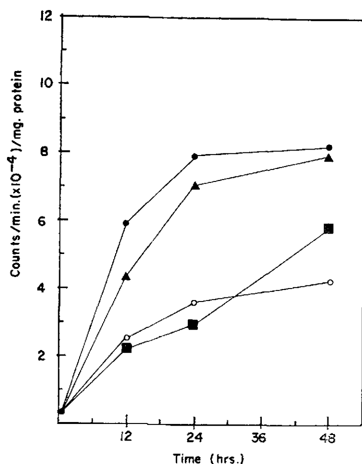

FIG. 3. Effect of canavanine on the incorporation of ^{3}H-valine into mouse cell protein. Mouse cells maintained in Eagle's medium ●—●; Eagle's medium containing 0.06 m*M* canavanine ▲—▲; 6.0 m*M* canavanine ■—■; and Eagle's medium deprived of arginine O—O.

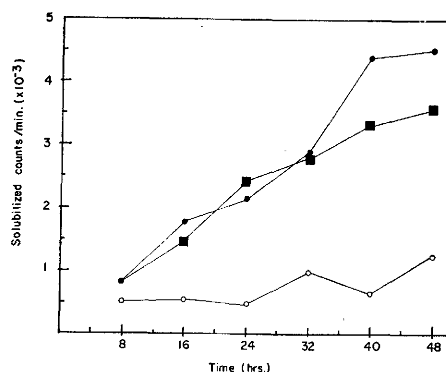

FIG. 4. Effect of various concentrations of canavanine on the solubilization of mouse cell arginine. Mouse cells maintained in Eagle's medium ●—●; Eagle's medium containing 6.0 m*M* canavanine ■—■; and Eagle's medium deprived of arginine O—O.

Microscopic examination of stained preparations of mouse cells maintained for 36 hr in various concentrations of canavanine revealed that increasing concentrations of canavanine caused increased binucleation of cells. The mouse cells maintained in 6.0 m*M* canavanine exhibited a four-fold increase in binucleation. Similar multinucleated cells have been induced in cell systems after treatment with cytochalasin B which causes breakdown of the microfilaments involved in cell division (21) and by colchicine which inhibits microtubule formation (*22*, *23*). The possibility exists since canavanine has been shown to be incorporated into protein in place of arginine (14) that canavanine treatment of mouse cells could prevent synthesis of, or cause the synthesis of aberrant proteins involved in microfilament or microtubule formation and consequently inhibit cell division but not nuclear division.

In microbial systems, canavanine caused irreversible damage and cell death (9). In various mammalian cells (WISH, HEp-2 and FL cells) however, it has been shown that the inhibitory effect of canavanine is reversible (11). Thus, it was considered essential to determine if the inhibitory effect of 6.0 m*M* canavanine observed in mouse cells was reversible. It was found that when mouse cells were maintained in Eagle's MEM containing 6.0 m*M* canavanine for 72 hr, cell proliferation was inhibited while protein synthesis

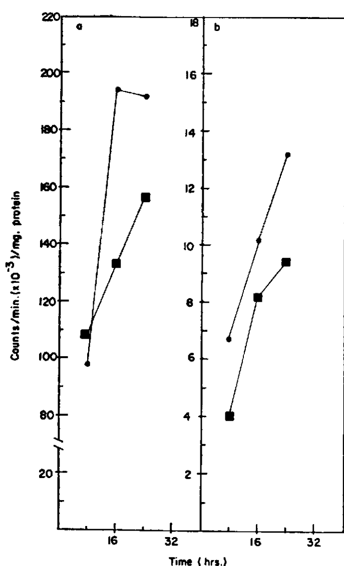

FIG. 5. Effect of canavanine on the incorporation of radioisotopic precursors into mouse cells: (a) DNA (^{3}H-thymidine); and (b) RNA (^{3}H-uridine). Mouse cells maintained in Eagle's medium ●—●; and Eagle's medium containing 6.0 m*M* canavanine ■—■.

continued (Figs. 1, 2, 6A and B). When these cells were maintained in canavanine for 24 or even 48 hr and then returned to Eagle's MEM lacking canavanine, it was found that these cells were still capable of proliferating. Cells exposed to canavanine for 24 hr required 48 hr to recover while cells exposed for 48 hr required only 24 hr to recover from the effects of the analogue. This finding is unexplained at this time but certain cell functions may have to accumulate in the canavanine treated cells to allow cell division. Following removal of canavanine in these cells, protein synthesis recovers prior to recovery of cell proliferation (Fig. 6B).

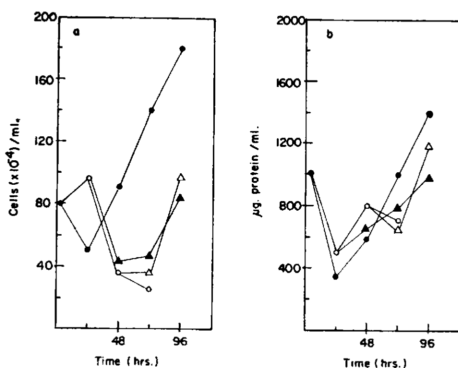

FIG. 6. Recovery after exposure to canavanine for 24 or 48 hr, (a) cell number, (b) protein content. Cells maintained in Eagle's medium ●—●; and Eagle's medium containing 6.0 m*M* canavanine ○—○. Canavanine containing Eagle's medium removed and Eagle's medium replaced on the cultures at 24 hr ▲—▲; and 48 hr △—△.

It has been previously demonstrated for the microbial system (12, 13) and suggested for the mammalian system (15, 16) that canavanine inhibition is by the formation of a canavanyl-protein-DNA-membrane complex. Recent findings have demonstrated that mammalian cell DNA replication occurs in association with nuclear membranes (24, 25). Thus, it is possible that the inhibitory effect of canavanine on growing mouse cells may ultimately be associated with the membrane-protein complex involved in DNA replication as previously suggested for the microbial and mammalian system.

Summary. Canavanine (6.0 m*M*) reversibly inhibited cell division and reduced cellular protein, DNA and RNA synthesis. Treatment of mouse embryo cells with canavanine was characterized by increased binucleation and cellular protein content, with inhibition of cell division but not nuclear division.

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