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IN VITRO EFFECTS OF AN EXTRACT OF CHARA
GLOBULARIS ON THE GROWTH OF
JENSEN SARCOMA AND NORMAL RAT KIDNEY CELLS

A Thesis
Presented to the
Faculty of
California State
University, San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

by
Carl R. Inman
March 1986

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ABSTRACT

The effects of exposure to an organic extract prepared from the green alga, Chara globularis Thuill., were studied in vitro to determine if compounds present in the extract differentially affect the growth rates of Jensen sarcoma cells vs normal rat kidney (NRK-52E) cells. The extract was added to the culture medium at 5 and 10 volumes-percent and the resulting Chara conditioned medium used to expose experimental cultures for 24, 48, 72 and 96 hours. Additional cultures received continuous exposure to the extract at each concentration. Control cultures were treated similarly, but with medium amended with double distilled water instead of extract. Growth was followed daily using negatives of photomicrographs to enumerate cells.

At a concentration of 10 volumes-percent, a steady and similar increase in growth delay for both cell types is proportional to the length of exposure up to 96 hours. Continuous exposure led to death of both cell types. At 5 volumes-percent a similar increase in growth delay occurred through 72 hours of exposure, but the extract had no further effect at longer exposures. The similar growth delays suggest that inhibition is non-specific.

DEDICATION

To my very special Mother and Father

Walter and Irene Inman

and of course, to my dearest Friend and cherished Love

Pauline

ACKNOWLEDGEMENT

I wish to express my sincere appreciation to Dr. Dalton Harrington, educator, advisor and friend, whose guidance and patience helped me through this often tenuous project.

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INTRODUCTION

Excretion products from the freshwater alga Chara, order Charales, have a history of scientific investigation beginning with Caballero (1919), who attributed mosquito larvicidal properties to the plant. This was later confirmed by other investigators (Vasconcelos 1923; Pardo 1923; MacGregor 1924; Barber 1924; Buhot 1927; Matheson 1928) and the plant was subsequently used by Matheson (1928) in biological control studies. More recently, Amonkar (1969) prepared an organic effluent from the plant to be used for the same purpose in metropolitan areas. In addition, the plant has been shown to exhibit allelopathic and allelocatalytic properties (Harrington 1969) and to inhibit the aquatic stages of certain blood flukes (Remusat 1962). Harrington (1969) showed that nutrient agar supplemented with Chara oospore conditioned medium inhibits the growth of certain bacteria.

The nonproteinaceous nature of the compounds effecting allelocatalysis was first demonstrated by Harrington (1969), who found that the distillate of oospore conditioned medium inhibited Chara oospore germination. Later, DeLaurie (1971), using a steam distillate prepared from whole plant material, observed adverse affects on the growth and morphology of the unicellular alga, Chlorella vulgaris. Chlorella cultures exposed to the volatiles in the distillate developed giant, multinucleate cells, two to five

times larger than control cells, and they were reminiscent of the mitotically senescent internodal cells normally found in Chara (Harrington 1969).

These later findings prompted research on the effects of the volatiles isolated from Chara on mammalian cells. Most notably, Sherif (1980), who suspected that the extract might contain carcinogens, found instead that a distillate derived from Chara globularis Thuill. contained compounds that caused in vitro cultures of embryonic rat heart cells transformed by polyoma virus to exhibit, uncharacteristically, contact inhibition. Further studies revealed that the extract caused in vitro agglutination of transformed polyoma cells, but not of normal fibroblasts. Since it had been previously demonstrated (Oliver-Janet 1975; Sharon 1977) that certain plant lectins cause agglutination of transformed cells by modifying the mobility patterns of membrane receptor sites, Sherif (1980) concluded that the extract contained a lectin-like substance similar to concanavalin A and/or wheat germ agglutinin but which was more effective since exposure to Chara promoted a more rapid agglutination.

Dramatic anti-neoplastic effects by the Chara extract have been demonstrated in vivo. Sherif (1980), in an extension from his in vitro research, found 100 percent remission of polyoma tumors in rats given a single intra-tumor injection of Chara extract. In addition, tumors failed to develop in an experimental group of rats provided the

undiluted crude extract as a drinking water substitute. Other investigators have obtained comparable results. Gallo (personal communication) showed complete remission of tumors in rats bearing a right hind limb Jensen sarcoma while Ellefson (1985) was able to reduce tumors in hamsters carrying J.C. virus-induced gliomas. Both of the latter investigators gave multiple intra-tumor injections of an organic extract derived from the distillate. Less dramatic results were obtained by Su and Staba (1973), who reported a modest delay in the appearance of melanoma tumors in hamsters following a single, pre-transplantation injection of a crude extract of Chara vulgaris.

While Sherif (1980) proposed a model involving a primary response of the tumor cells to the components of the Chara extract, a plausible alternative may involve a secondary response, such as immunostimulation, on the part of tumor-bearing animal in reaction to substances in the Chara extract. For example, a hot water extract of the unicellular green alga, Chlorella vulgaris, was shown to inhibit the growth of Meth-A tumors in mice via T-cell and macrophage modulation (Tanaka et al 1984). Other investigators have shown that bacteria and bacterial products, e.g., Bacillus Calmetti-Grecin (BCG) (Baldwin and Pimm 1978; Mathe et al 1973; Mathe et al 1974; Old et al 1961), and Corynebacterium parvum (Day et al 1983), can also act as immunostimulants affecting the growth of spontaneous and transplanted tumors. These results provide supporting evidence for the possibility

that a similar mechanism may occur relative to treatment with Chara extract of tumor bearing animals as well.

Toxicity studies in rats (Sherif 1980) and mice (Gallo, personal communication) indicate that the mixture of volatile compounds isolated from Chara globularis is nontoxic. During a three month trial, no deaths occurred among 12 experimental mice when the undiluted, crude extract was substituted for drinking water. The non-toxic nature of Chara extract is further illustrated by evidence from earlier work using Chara vulgaris (Su and Staba 1973), in which no change occurred in either prothrombin time or partial thromboplastin time following multiple injections of mice with whole extract.

Few studies have been undertaken to identify the volatile compounds isolated from Chara globularis. Table 1 (Harrington and Gallo 1985) lists the known compounds present in the steam distillate. Because of recent interest in certain sulphur compounds as anti-neoplastic agents (Miller 1983), the sulphur compounds found in Chara (Anthoni et al 1980) may be of some importance. In addition, one of the compounds, undecanal, has been shown to be anti-neoplastic (Higashikaze 1981), but also exhibits a high degree of toxicity in mice. Dihydroactinodioidide has been shown to inhibit root length elongation and seed germination in aquatic plants (Stevens and Merrill 1980), 6,10-dimethyl-2-undecanone is an active component in a commercial insecticide (Kusiak et al 1978) and at least two of the compounds, 3-

TABLE 1

COMPOUNDS ISOLATED FROM THE STEAM DISTILLATE OF CHARA
GLOBULARIS (MODIFIED AFTER HARRINGTON AND GALLO, 1985)

<u>COMPOUND</u>	<u>USES AND CHARACTERISTICS</u>
BUTYLISOPROPYLPHTHALATE	NOT CHARACTERIZED
3-CHLORODECANE	CARCINOGEN
$C_4H_8S_3$, $C_4H_8S_4$	INHIBITS PHOTOSYNTHESIS
DECANOIC ACID	NOT CHARACTERIZED
DIHYDROACTINIDIOLIDE	INHIBITS ROOT ELONGATION AND SEED GERMINATION IN AQUATIC PLANTS
6,10-DIMETHYL-5, 9-UNDECADIEN-2-ONE	NOT CHARACTERIZED
6,10-DIMETHYL-2-UNDECANONE	INSECTICIDE
DIOCTYLPHTHALATE	NOT CHARACTERIZED
1-ETHYLIOENEOCTAHYDRO -7A-METHYL-(12, 3A <u>A</u> : 7 <u>AB</u>) -IH-INDENE	CARCINOGEN
OCTACOSANE	NOT CHARACTERIZED
PHTHALIC ACID	NARCOTIC IN HIGH CONCENTRATIONS
2,6,10,14-TETRAMETHYLHEPTADECANE	NOT CHARACTERIZED
4-(2,2,6 TRIMETHYL-7-OXABICYCLO [4.1.0]-HEPT-1-YL)-3-BUTENE-2-ONE	NOT CHARACTERIZED

chlorodecane and the indene compound are generally considered carcinogenic. The other components found in the extract have not been characterized, relative to their biological activity.

Jensen sarcoma cells (ATCC 1741) have been used by Beltz (Beltz et al 1983) to study modulation of the uptake of 5-fluororacil both in vitro and in vivo. The cells are characteristically large (30-50 μm in diameter), have distinct borders and have heavily granulated cytoplasm. In addition, Jensen sarcoma cells exhibit contact inhibition and form only monolayers in vitro (Beltz et al 1983). These characteristics make the cells easily identifiable using a compound light microscope, thus allowing growth (division rate) to be easily monitored.

While Chara extract has been shown to be effective in causing remission of polyoma tumors in rats, (Sherif 1980) glioblastoma tumors in hamsters (Ellefson 1985) and Jensen sarcomas in rats (Gallo, personal communication), the mechanism of remission has not been determined (Harrington, personal communication). Sherif (1980) suggested that the mechanism of remission engendered by Chara extract depended upon re-establishment of contact inhibition via a lectin-like action. Since this cannot be the case in Jensen sarcoma cells, it is likely that the extract operates through some other direct, or indirect mechanism leading to remission of all tumors studied to this point (Harrington, personal communication).

Is tumor remission a direct result of a tumor-specific primary effect of the extract upon the tumor cells, or does remission require a secondary response, such as immunostimulation, mediated by the intact organism?

Statement of Purpose

The purpose of this research is to answer the first part of the preceding question by studying the effect of an organic extract prepared from a distillate of Chara globularis Thuill. on the growth of Jensen sarcoma cells in vitro. An attempt has been made to discriminate between non specific effects that may be expected following exposure of tumor and non-tumor cells to a heterogeneous extract and specific effects which would be expected to target tumor cells only.

MATERIALS AND METHODS

Materials

Powdered medium and serum were purchased from Irvine Scientific, Santa Ana, California. All chemicals, including buffers, were obtained from Cal Biochem-Behring, Cleveland, Ohio. Antibiotics and antimycotics were purchased from Grand Island Biological Company, Grand Island, New York. Culture flasks and dishes were obtained from Corning, Cleveland, Ohio, and cellulose membrane filters were bought from Millipore Corporation, Bedford, Massachusetts. Kodak Tripan film, ester base, obtained from Eastman Kodak, Rochester, New York, was used for taking photomicrographs.

Culture Media

McCoy's 5a medium (McCoy et al. 1959) with glucose and vitamin B-12, as in the formulation of Iwakata and Grace (1964) was prepared without serum, but with the addition of 10mM each of HEPES, BES and TES buffers (based on the general suggestions of Eagle 1971) and stored at -20°C . Complete medium was prepared by mixing freshly thawed McCoys 5a with heat-inactivated new born calf serum (10%) and an antibiotic-antimycotic solution (1%) containing penicillin-G (10,000 units per ml), streptomycin sulphate (10,000 ug per ml) and amphotericin B (25 μg per ml) and sterilized by positive pressure filtration through a 0.22 μm cellulose filter. The pH of the complete medium in air at 22°C was 7.1.

Cell Cultures

Normal rat kidney cells (NRK-52E) were purchased from American Type Culture Collection (ATCC 1571). Jensen tumor cells were the kind gift of Dr. Richard Beltz, Loma Linda University, Loma Linda, California.

Stock cultures of both cell types were prepared as follows: Monolayers cultured in polystyrene, flat-bottomed, 25cm² flasks were harvested prior to reaching confluence by treatment with 0.25% trypsin in 0.2% glucose dissolved in 50mM EDTA as devised by Merchant et al (1964) at 37°C for four minutes. The detached cells were suspended in 1 ml complete growth medium, pelleted by centrifugation, resuspended in complete medium containing 10% glycerol, frozen according to the method of Paul (1974), and stored at -80°C.

Preparation of Chara Extracts

All extracts of Chara globularis (provided by D. Gallo, M.S., of California State University, San Bernardino) were prepared using 2 kg (wet weight) of freshly collected plant material grown in the Biology Pond at the University. The plants were washed thoroughly with double-distilled water and drained. The plant material was weighed and an equal weight of double-distilled water was added. The mixture was homogenized in a Waring blender operating at full speed, using 15-second bursts, until homogeneity was attained. The resulting homogenate was distilled at 105°C for approximately

three hours until a thick sludge remained in the distillation flask. The effluent vapor was condensed at approximately 15°C, the temperature of the tap water flowing throughout the condensor jacket. Approximately one liter of condensate was collected in a 1-liter Erlenmeyer flask. An organic fraction was prepared by extracting 500 ml of the crude distillate three times with 100 ml of freshly distilled chloroform. The chloroform extracts were combined and taken to near-dryness at room temperature under nitrogen. The oily residue was first suspended in 10 microliters of absolute methanol and the volume then increased to 100 ml with double-distilled water. The extract was stored in 10 ml aliquots at -80°C for up to six months. Thawed aliquots were stored at 4°C for no more than two weeks. A single extract preparation was used throughout the study.

Preparation of Chara Conditioned Complete Medium

Medium containing Chara extract was prepared just prior to use as follows: An exact volume of complete medium was carefully transferred into a sterile beaker containing a magnetic stir bar. An appropriate volume of the extract was added so that the final concentration was 5% or 10% (vol/vol) depending on experimental conditions. The resulting Chara conditioned complete medium was stirred at a moderate speed for five minutes to ensure homogeneity, then warmed to 37°C in a constant temperature bath. The pH at 37°C in air was

7.3. Medium to be used with control cultures (water conditioned complete medium) was prepared in a similar manner except that sterile double-distilled water was used in place of Chara extract.

Exposure to Chara Extract

Polystyrene flasks of 25 cm² were inoculated with 1.2×10^5 cells in 3.0 ml complete medium from rapidly thawed cell stocks of either Jensen sarcoma or normal NRK-52E cells to produce monolayers which grew at a logarithmic rate after three days of incubation at 37°C in a humidified, 5% CO₂ - air atmosphere. The cells were then harvested, suspended in complete medium, counted using a hemacytometer and diluted to 5,000 cells per ml. Aliquots (1.0 ml) from the stirred cell suspension were pipetted into each well of three, flat-bottomed, polystyrene multi-well plates (24 wells per plate). Each well had been previously scored in order to identify a relocatable field. Following three hours of incubation under the same conditions as described above, unattached cells were removed by washing with 0.5 ml of complete medium. The wash medium was aspirated and 1.0 ml of 5% or 10% Chara conditioned complete medium was pipetted into each well designated as an experimental culture. Cultures to be used as controls received 1.0 ml of water conditioned complete medium.

Experimental cultures were exposed to Chara conditioned complete medium for 24, 48, 72, or 96 hours.

Additional cultures were subjected to continuous exposure of Chara conditioned complete medium. Control cultures were exposed only to water conditioned complete medium for the duration of an experiment. All media were changed at 24 hour intervals. When exposure times were completed, experimental cultures were washed three times with 0.5 ml of complete medium. One (1.0) ml of water conditioned complete medium at the appropriate concentration (5% or 10%) was pipetted into each well following the last wash. Control cultures were washed in a similar manner following the three hour incubation period previously described.

Quantitation of Growth

Quantitation of growth was based on cell multiplication factors and comparisons between Jensen sarcoma and normal NRK-52E cells were based on growth delay factors following the method of Beltz et al (1983). Following three hours of incubation, time zero photomicrographs were taken of each of the scored fields using a Nikon 35 mm camera mounted to an American Optical inverted stage microscope. The photographed cells in each field were enumerated on projections of photographic negatives. Photomicrographs were repeated at 24 hour intervals immediately following the daily media change previously described. Daily cell counts were then tabulated for each field and used to compute cell multiplication factors. The cell multiplication factor represents a quantitation of the

cell population in each field relative to its time zero population and, thus, is the quantitative ratio of the number of cells in each field at a selected time, after time zero, to the number of cells in that field at time zero. Mathematically it is expressed as:

Cell Multiplication Factor (CMF) =

$$\frac{\text{number of cells at time } t}{\text{number of cells at time zero}}$$

Each well contained a culture that was considered as an independent population for which cell multiplication factors were followed. Cultures were photo-micrographed and cells enumerated until a cell multiplication factor of 15 had been exceeded. The time for each culture to attain CMF-15 was used as a reference point for which all data could be compared. It was calculated by rearranging the exponential growth equation and solving for time,

$$t_{\text{CMF-15}} = k \ln 15,$$

where k was determined experimentally from the time interval containing CMF-15. Each exposure time, including the control, was represented by 12 cultures. Thus, 12 time values were computed to derive each experimental data point.

The data derived by calculating the time to attain CMF-15 for each culture was normalized to allow statistical comparisons of growth delay between Jensen sarcoma and normal NRK-52E cells. Growth delay factors (GDF) were computed as

the ratio of the time intervals required for Chara treated and control cell populations to increase 15 fold:

$$\text{GDF} = \frac{T_{\text{CMF-15}} \text{ (experimental)}}{T_{\text{CMF-15}} \text{ (control)}}$$

The time for control cultures to reach CMF-15 was taken as the average of 12 determinations. Growth delay factors were then calculated for each experimental culture and, thus, 12 growth delay factors were used to compute an average and a standard deviation for each experimental point. The normalized results obtained for each cell type were statistically compared using the two sample t test with 22 degrees of freedom at a level of significance of 0.05.

RESULTS

Effect of Chara Conditioned Complete Medium (10%) on Jensen Tumor and NRK-52E Cells

No differences in population growth between the cancerous Jensen cells and the normal NRK-52E cells could be discerned when Chara extract was present in the complete medium at a concentration of 10 volumes-percent. Figure 1 shows the delay in growth resulting from various exposure times of 24, 48, 72, 96 hours and continuous exposure (until cell death occurred). Cultures exhibited a slight but steady cell loss during exposure periods, a trend that was reversed in all cases 24 hours after the removal of the extract. A 48 hour lag period was noted for each 24 hours of exposure, up to a 96-hour exposure time. However, neither cell type was able to recover after 192 hours (8 days) of continuous exposure at which time all cells had died.

Effect of Chara Conditioned Complete Medium (5%) on Jensen Tumor and NRK-52E Cells

Owing to the diverse nature of the compounds in the extract (Table 1), it was decided to repeat the previous experiment at a reduced Chara concentration. This was done to determine if non-specific growth delay effects associated with the extract at 10 volumes percent could be decreased while retaining any specific effects at five-volumes percent that would reflect differential growth delay of Jensen cells versus NRK-52E cells; that is, specific targeting of tumor

cells by some compound or compounds, in the extract. Figure two shows the delay in growth resulting from exposure to Chara conditioned complete medium at 5-volumes percent. Both cell types (Jensen cells and NRK-52E cells) exhibited an increasing delay in growth associated with increased exposure times through 72 hours of exposure. No additional inhibition was noted for exposure times longer than 72 hours.

Growth Delay Factors (GDF) Associated with Chara Exposure

Growth delay factors were computed for both experiments and plotted for comparison in Figure 3. Following exposure at 10 volumes-percent, the resulting growth delay factor curves are superimposable (Fig. 3B). Through 96 hours, a constant increase in growth delay was noted for each 24 hours of exposure. It is intuitively obvious that the extract at this concentration does not discriminate with respect to growth delay. The maximum growth delay factor for both Jensen and NRK-52E cells was 4.2. At 5 volumes-percent, a slight discrimination is noted: at 0.05 level of significance, the two-sample t test indicates that the delay in growth associated with exposure is greater for NRK-52E cells than for Jensen sarcoma cells. However, a constant increase in growth delay was noted for both cell types through 72 hours of exposure, after which time, no further increase occurred and a maximum growth delay was attained (Fig. 3A). The maximum growth delay factor of NRK-52E cultures was 1.85 and that of Jensen sarcoma cultures was 1.54.

Figure 1. Growth curves of : (A) NRK-52E (kidney) and (B) Jensen sarcoma cells, following exposure to Chara conditioned complete medium at 10 volumes-percent. Exposure times are indicated parenthetically. Each condition was monitored until growth attained a cell multiplication factor of at least 15 (dotted line), or until cell death occurred. Each curve represents the average of 12 experiments. Where standard deviation lines are not shown, the symbol used to represent the average enclosed more than one standard deviation.

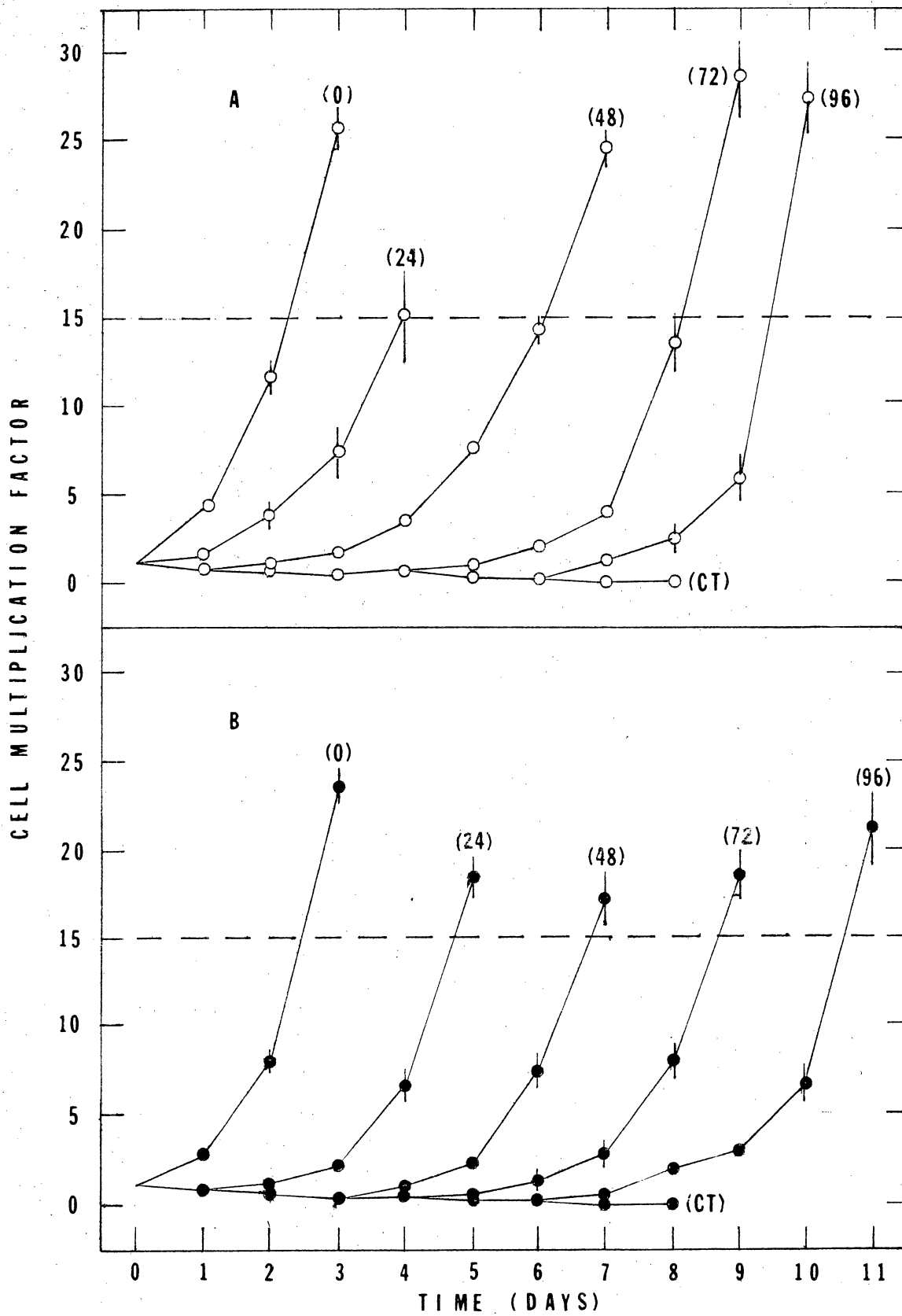


Figure 2. Growth curves of : (A) NRK-52E and (B) Jensen Sarcoma cells following exposure to Chara conditioned complete medium at 5 volumes-percent. Exposure times are indicated parenthetically. Each curve represents the average of 12 experiments and where standard deviation lines are not shown, the symbol used to represent the average enclosed more than one standard deviation.

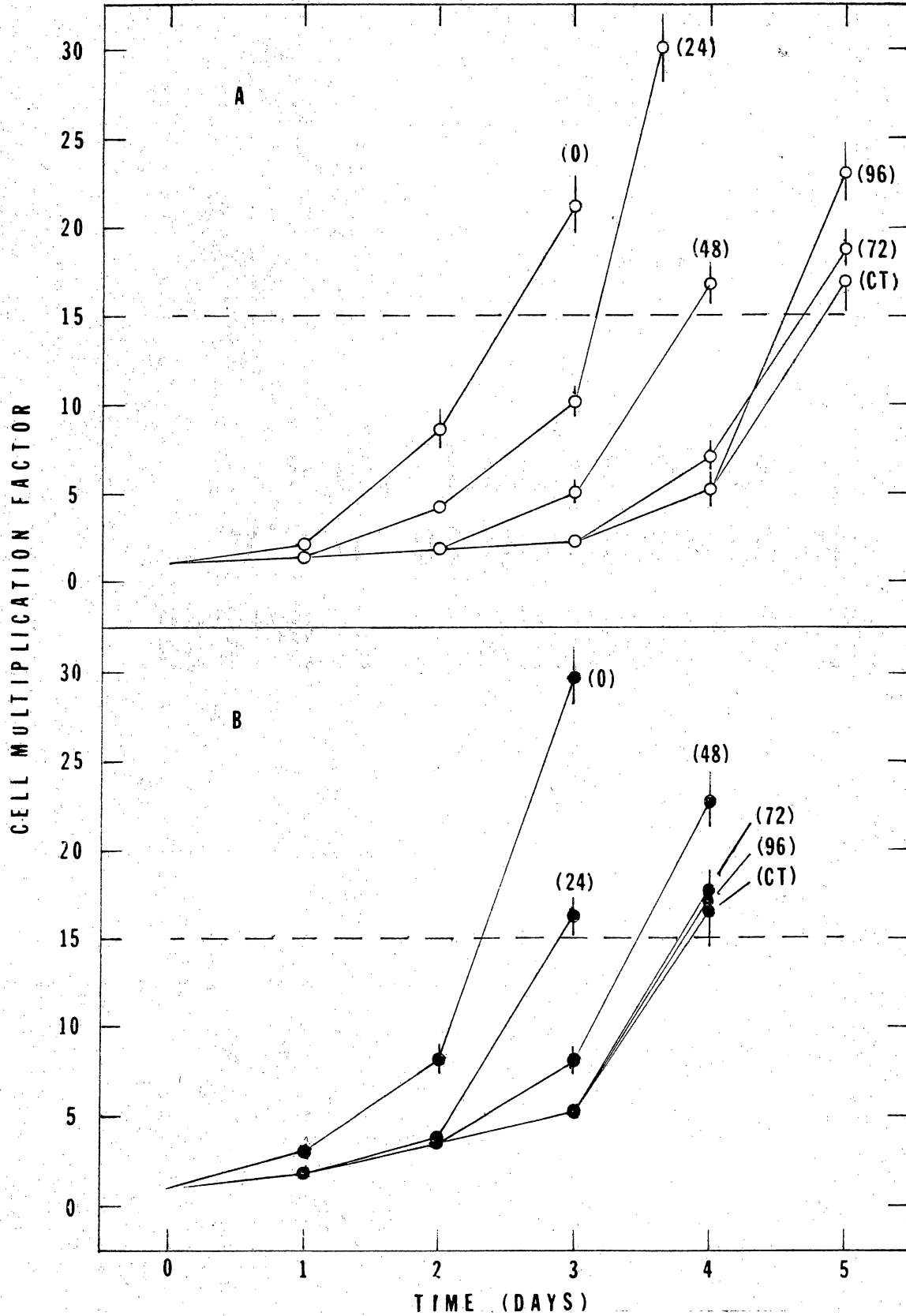
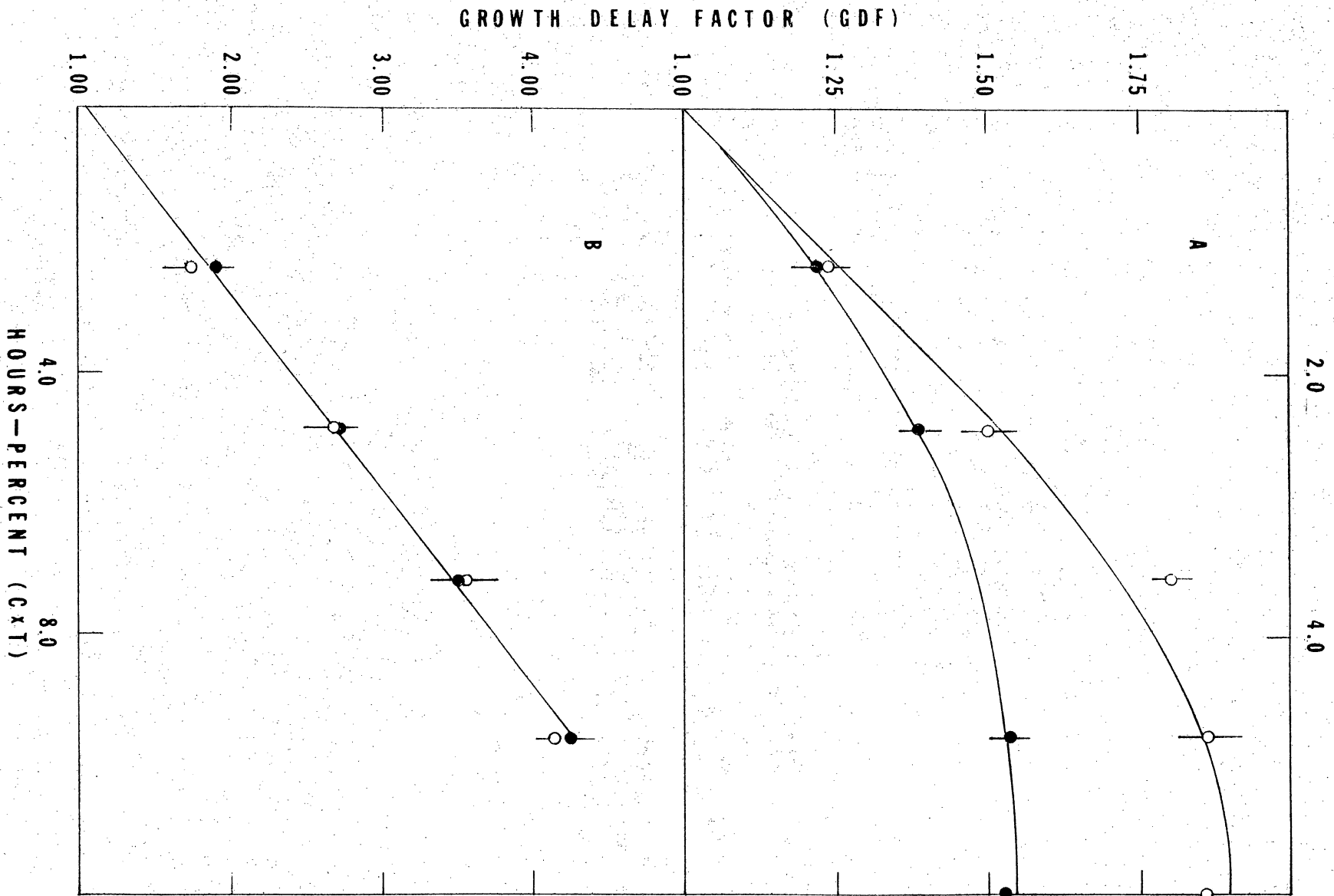


Figure 3. Growth delay factor (GDF) curves for NRK-52E (o) and Jensen Sarcoma (●) cells resulting from exposure to Chara conditioned complete medium at: (A) 5 volumes-percent and (B) 10 volumes-percent.



DISCUSSION

The close correlation in growth delay between Jensen sarcoma and normal NRK-52E cells is suggestive of non-specific inhibition (Dipalma 1976; Goodman and Geiman 1975; Levine 1973), with both cell types responding essentially equally to the extract. The data indicate that, irrespective of the Chara concentration, even for exposure as long as 96 hours, both cell types eventually resume growth similar to that of their respective controls. Thus, an extract of Chara present in the growth medium for as long as 96 hours at a concentration as high as 10 volumes-percent is shown only to delay cell growth, but not to cause cell death.

While discrimination is shown between Jensen sarcoma and normal NRK-52E cells when treated at an extract concentration of five volumes-percent, the maximum growth delay exhibited (1.54 and 1.85, respectively) is not deemed effective in reducing in vivo Jensen tumors (Beltz, personal communication). Furthermore, the fact that a greater increase in growth delay is demonstrated for NRK-52E cells versus Jensen cells is not consistent with previous in vivo experimental results (Gallo, personal communication). Finally, cell death does not occur at a concentration of five volumes-percent regardless of the length of exposure. Thus, the discrimination noted between cell types at this concentration is probably insignificant.

These findings indicate that Chara extract does not

exert anti-tumor effects directly on Jensen cells, as suggested by Sherif (1980) for polyoma cells. Since cancer specific effects in vivo have been shown to occur (Sherif 1980; Ellefson 1985; Gallo, personal communication), it must be suspected that a compound or compounds, in the Chara extract are likely to mediate a secondary response by biochemical and/or cellular processes that occur in the intact animal, but not in vitro. However, even though such a secondary response may be the mechanism of effective action in the case of Jensen's sarcoma, the findings reported here do not necessarily imply a similar mechanism for polyoma or glialneuroma tumors. Further study is necessary in order to resolve the effects of Chara extract toward the tumors investigated thus far.

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