EFFECTS OF AN L-GLUTAMINE ANALOGUE, 6-DIAZO-5-OXO-L-NORLEUCINE (DON), ON THE GROWTH PATTERNS OF TWO HUMAN CERVICAL CARCINOMA CELL LINES

by

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ABSTRACT

The objective of this study was to observe the effects of a potential chemotherapeutic drug and an inhibitor of glycosaminoglycan synthesis, 6-diazo-5-oxo-L-norleucine (DON), on the growth patterns of two human cervical carcinoma cell lines. In particular, the *in vitro* cell-shedding patterns, colony formation and aggregation of the cell lines were examined with DON treatment, to test the hypothesis that secretion of extracellular materials (ECM) by one cell line (C-4II) was responsible for its more dispersed and infiltrative growth pattern.

The cultured human cervical carcinoma cells used in this study were derived initially from the same biopsy specimen. These two morphologically different cell lines had distinctive patterns of growth both *in vivo* and *in vitro*. C-4I cells grew as round masses. *In vivo*, these cells did not infiltrate into host hamster tissues, while *in vitro*, few of these cells were shed from confluent cultures into culture medium. In contrast, C-4II cells did not grow as round cohesive masses, but in a dispersed manner. *In vivo*, these cells grew as small clumps and infiltrated into host tissues, while *in vitro*, such cultures shed many viable cells into culture medium. Previous ultrastructural analysis indicated that tumors with such dispersed growth patterns tend to have many features of secretory cells, with characteristics of glandular and basal cell differentiation.

In this study, an L-glutamine analogue, DON, was used to inhibit the secretion of ECM. DON has been shown to inhibit the formation of many glutamine-requiring metabolites in the cell, including glucosamine-6-phosphate, a metabolite essential for the formation of ECM. Hence, the

effects of DON on the growth and shedding patterns of the 2 types of human cervical cancer cells were examined.

This study showed that DON had numerous morphological effects on cultured C-4 cells, other than its well-known growth-inhibitory effect. In addition, results showed that the shedding patterns of C-4 cells were altered with DON treatment. By harvesting cells shed into culture medium, it was determined that DON induced shedding in C-4I cultures while it enhanced shedding in confluent C-4II cultures. This increased shedding was most likely caused by a decrease in cell-cell cohesion. Furthermore, the aggregation of C-4I cells, treated with DON prior to dissociation, was greatly inhibited. Decreased cell-cell cohesion might also account for the decrease in stratification of C-4I cultures, irregularly-shaped C-4I colonies and aggregates, and for the "holes" that appeared within both types of C-4 colonies treated with high doses of DON. In contrast, aggregation of similarly treated C-4II cells was not consistently inhibited.

Changes in colony forms, to more irregular shapes, were evident in both cell lines. By examining single cells growing on the substratum, it was determined that these changes might be due to DON decreasing cell-substratum adhesion in both cell lines.

DON-treated C-4 cells were significantly larger when projected two-dimensionally. This was due, at least in part, to increases in cell volume, as detected on the Coulter counter and size distribution analyzer.

The results of this study do not support the previous hypothesis that the more dispersed and infiltrative growth pattern of C-4II tumors, as compared to C-4I tumors, was due to the secretion of ECM. This hypothesis predicted a decrease in the shedding of viable cells and a change

from a dispersed to a compact growth pattern in DON-treated cultures. Instead, the results indicate the contrary. The results suggest that cells may be secreting DON-sensitive cell surface-associated ECM that are responsible for the cohesive nature of C-4I cells. C-4II cells may produce less of this cohesive material, resulting in less cohesive cultures and more dispersed growth. Hence, with DON treatment, C-4I cultures were altered, resembling C-4II cultures, in terms of their shedding patterns, aggregability and morphology of aggregates and colonies.

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INTRODUCTION

One of the major difficulties encountered in cancer research is that cancer is not one disease, but rather, consists of numerous diseases. This unique heterogenous nature of tumors has unfortunately complicated the treatment and prognosis of cancer patients. Since tumors may have different subpopulations of cells with different growth and invasion patterns and with different metastatic potentials (Fidler and Hart, 1981; Marx, 1982), any one treatment may not be effective in combating the tumorous growth and metastases (Marx, 1982).

Tumor heterogeneity has been observed in poorly differentiated human cervical invasive squamous carcinomas, which display a variety of *in vivo* and *in vitro* patterns of growth and invasion, and differentiation (Auersperg and Worth, 1966; Auersperg *et al.*, 1973). It has been shown that even though such tumors are histologically classified as being poorly differentiated, they still retain some ultrastructural traits of normal differentiation, which may influence their growth patterns. For example, tumors with spinous traits, such as glycogen accumulations, tonofibrils and desmosomes, have a compact growth pattern, central areas of necrosis and leucocytic responses. Tumors with glandular or basal traits, such as secretory vesicles, Golgi complexes, or basement membranes, have a diffuse growth pattern, or an infiltrative method of spreading (in small groups or strands of cells), no central necrosis and extensive invasion of the host connective tissues (Auersperg *et al.*, 1973).

Not only are tumors heterogenous in their traits of differentiation and in their growth patterns *in vivo*, but they are also heterogenous in

their growth patterns *in vitro*. A correlation study by Auersperg and Erber (1976) has shown that cervical carcinomas with spinous traits of differentiation grow cohesively *in vivo* as well as *in vitro*. Most carcinomas with basal or glandular traits have a dispersed growth pattern *in vivo*. However, they grow more variably in culture, producing completely and partially dispersed as well as cohesive cultures. Differentiated cultures of human invasive squamous cervical carcinomas are derived mainly from differentiated tumors, while poorly differentiated tumors produce mainly poorly differentiated cultures (Auersperg and Worth, 1966).

Tumor cell properties that may influence the spread of cancer cells are motility, and adhesion to other tumor cells and to host tissues. In addition, tumor cells interact with extracellular products of tumor or host origin (Skyvova *et al.*, 1973; Toole *et al.*, 1979; Roblin, 1981). Specifically, previous studies of carcinomas of the uterine cervix (Auersperg, 1969a, 1969b; Auersperg *et al.*, 1973; Auersperg and Erber, 1976) have shown that:

1) cultured cells which prefered to adhere to one another, rather than to substrata, grew as compact masses *in vivo* and *in vitro*. However, cultured cells that adhered preferentially to substrata grew more diffusely.
2) tumors that infiltrated into host tissues were associated with more basement membranes and amorphous extracellular materials than non-infiltrative ones.
3) tumor cells that grew diffusely *in vivo*, tended to shed more viable cells into culture medium. This shedding of cells was accompanied by an increase in intercellular spaces.

It was postulated that extracellular materials or secretory products of

tumor cells may aid in cell-cell separation and hence, in tumor cell invasiveness as well as in contributing to a diffuse growth pattern.

The above hypothesis was tested in the present study. Two human cervical carcinoma cell lines were used to represent two different types of poorly differentiated cervical carcinomas (Auersperg, 1969a, 1969b). The significance of using these two cell lines as a model was that certain in vivo tumor cell properties were reproduced in vitro.

Even though both cell lines were different in their in vivo and in vitro growth patterns and invasive patterns, metabolism and ultrastructural features, they originated from the same tumor biopsy.

The C-4I cell line represents tumors that are histologically cohesive. These cells have retained some traits of spinous differentiation--cells stratify and flatten apically, have a lower nuclear/cytoplasmic ratio than basal cells, lack organelle polarity, have abundant tonofibrils and tonofilament-associated desmosomes, wide intercellular spaces and glycogen granules. C-4I cells lack junctional complexes and, in vivo, have fewer areas with basement membranes than C-4II cells. In culture, C-4I cells grow as cohesive round masses and tend to overcome crowding by stratifying. In vivo (in hamster cheek pouches), the growth pattern correlates with that seen in vitro, that is, the tumors are cohesive, compact and have necrotic centers.

The C-4II cell line represents tumors with a diffuse growth pattern. The cells in culture are basically monolayered, less cohesive, and form irregularly-shaped colonies. C-4II cells have retained some traits of basal cell differentiation, such as a high nuclear/cytoplasmic ratio, organelle polarity, and secretory vesicles. Unlike C-4I cells, C-4II cells have few desmosomes, more complete and extensive basement membranes,

no glycogen, narrower intercellular spaces and have retained the capacity to form tight junctions and junctional complexes in an *in vitro* liquid environment. As C-4II cell cultures get crowded, flattened cells become more columnar, intercellular spaces widen, viable cells are shed into the culture medium, and domes form (which indicate secretory activity). Based on morphological studies of single subcultured cells and on adhesion studies of cultured colonies, C-4II cells showed preferential cell-substratum adhesion relative to cell-cell adhesion, in contrast to C-4I cells.

The initial aim of this study was to determine whether secreted extracellular materials aid in cell separation and dispersion, and whether they affect growth patterns of tumor cells in culture. Since 6-diazo-5-oxo-L-norleucine (DON) has been used in the past to inhibit glycoprotein, glycolipid and glycosaminoglycan (GAG) synthesis (Pratt *et al.*, 1973; Spooner and Conrad, 1975; Greene and Pratt, 1977; Hurmerinta *et al.*, 1979; Linsenmayer and Kochhar, 1979; Turley, 1980; Funderberg and Markwald, 1981; Hurmerinta and Thesleff, 1982), DON was used in this present study to determine its effects on the growth patterns of C-4I and C-4II cultures. DON (Fig. 1), being a glutamine analog, competes with L-glutamine in many L-glutamine requiring reactions in the cell, for example, it inhibits the enzyme L-glutamine-D-fructose-6-phosphate transamidase which forms glucosamine-6-phospate (Ghosh *et al.*, 1960), an essential precursor of glycoproteins, glycolipids and GAGs (Fig. 2). Examples of glucosamine-containing macromolecules are fibronectin and hyaluronic acid, both of which affect adhesion (Yamada and Olden, 1978; Mikuni-Takagaki and Toole, 1980; Culp, 1980; Mosher and Furcht, 1981).

Pratt *et al.*, (1973) have shown that DON-treated palates from

developing rats at the time of fusion produced less GAGs than untreated palates. In addition, Greene and Pratt (1977) have shown that not only did DON inhibit glycoprotein, glycolipid and GAG synthesis during rat palate formation, it also specifically inhibited the adhesion of cultured palatal shelves. This inhibition was counteracted by glucosamine addition. Therefore, DON could possibly affect adhesion in other systems by similar means.

Other researchers, such as Ekblom et al. (1979), Turley (1980) and Hurmerinta et al. (1979) have found that DON inhibited the synthesis of extracellular matrix as well as the in vitro differentiation of kidney tubules from mouse metanephric mesenchyme, adrenocortical cells and mouse embryonic tooth germs respectively. Ekblom et al. and Turley, but not Humerinta et al., could partially reverse DON's effects by the addition of glucosamine. Funderburg and Markwald (1981) and Turley (1980) found that DON also inhibited the motility of cultured heart cells and adrenocortical cells respectively. These effects were again only partially counteracted by glucosamine. Spooner and Conrad (1975) found that the motility of heart cells in vitro was not inhibited by DON treatment even though the synthesis of GAGs was decreased. Even though the addition of glucosamine to DON-treated cells only partially increased GAG synthesis, it had no effect on cell motility.

Not only does DON inhibit the synthesis of extracellular matrix, it also inhibits the synthesis of other L-glutamine-requiring macromolecules, for example, nucleic acids (DNA and RNA), proteins, and nicotinamide adenine dinucleotide (NAD) (Duvall, 1960) (Fig. 3). It also inhibits asparagine synthetase activity (Hiremagalur et al., 1976; Rosenbluth et

al., 1976), and competes with L-asparagine and L-glutamine uptake into cells (Cooney et al., 1976).

Since DON was first discovered and isolated from an unidentified Streptomyces culture in 1956 (Ehrlich et al., 1956), its properties have been characterized (Duvall, 1960; Pittillo and Hunt, 1967; Livingstone et al., 1970; Rando, 1975; Cabanillas, 1979; Catane et al., 1979). DON has been found to have some antibacterial, antifungal, teratogenic and anti-neoplastic effects. In many earlier trials, it was found that the effectiveness of DON in decreasing tumor size was only transient and occured only in a small proportion of cases studied. However, its anti-neoplastic effects are being re-investigated (Burchenal, 1979; Overjera, 1979; Rosenfeld and Roberts, 1981). Past and recent research has shown that DON has limited but definite anti-tumor activity in certain human cancers and in certain human tumor xenografts, implanted in nude mice (Overjera, 1979).

Figure 1: **Structures of DON (an L-glutamine analogue) and L-glutamine.**

DON L-GLUTAMINE

Figure 2: **Metabolic pathway for synthesis of aminosugars. (McGuire, 1972, p. 362)**

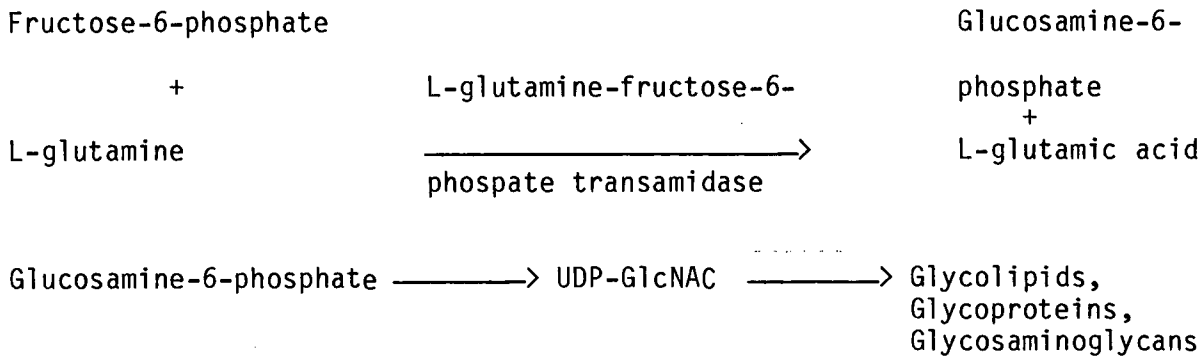
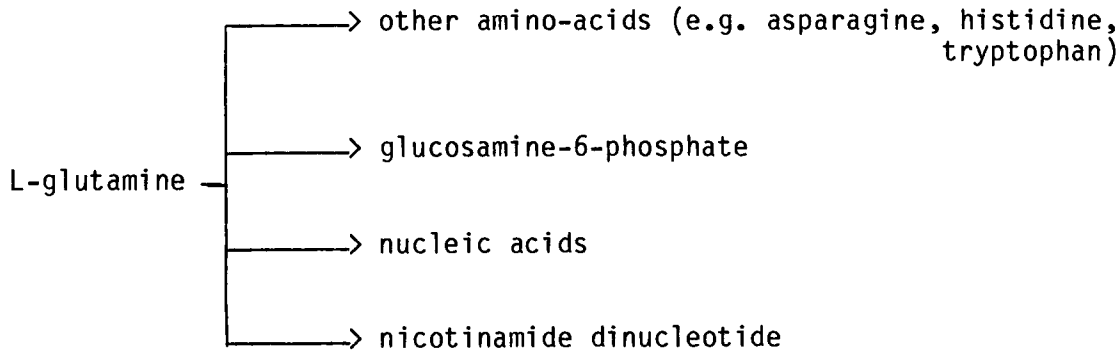


Figure 3: **Metabolic pathway of the amide nitrogen of L-glutamine**



MATERIALS AND METHODS

I. Cell culture

C-4I (passage 84) and C-4II (passage 97) cells were grown from frozen stocks, stored in liquid nitrogen. Stock cultures were maintained in 25 cm^2 tissue culture flasks (Corning), with 5 ml of Waymouth's medium (MB 752/1), supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 ug/ml of streptomycin. The cells were incubated in a dry incubator at 37°C. Medium of C-4I cultures was changed almost every day, while medium of C-4II cultures was changed every 2-3 days, since the rate of lactic acid accumulation was higher in C-4I than in C-4II cultures (Auersperg, 1972).

Confluent C-4I and C-4II cultures were subcultured every 1-2 weeks, using 0.125% trypsin in Ca^{++}, Mg^{++}-free Hanks' balanced salt solution (HBSS). Cells were centrifuged at 500-1000 rpm for 3-5 minutes and the cell pellet was taken up in 5 ml of culture medium. 20-25% of this cell volume in 5 ml of fresh culture medium was replated into a new flask. As C-4I cells were less sensitive to this form of dissociation than C-4II cells, C-4I cells were subcultured mainly as small to medium-sized clumps, while C-4II cells were subcultured mainly as single cells and small clumps. If dispersion into single cells was required, cultures were dissociated with 0.125% trypsin and 0.02% ethyleneglycol tetraacetic acid (EGTA) in Ca^{++}, Mg^{++}-free HBSS, since it was previously shown in C-4 cultures that cell-cell contacts were EGTA (Ca^{++} - chelator) sensitive, while cell-substratum contacts were trypsin sensitive (Auersperg, 1969b; Dembitzer et al., 1980a).

II. Fixation and staining

Cultures were fixed with 95% ethanol, after rinsing twice with Ca^{++}, Mg^{++}-free HBSS or with Waymouth's medium. After gradual rehydration, cultures were stained with 2% toluidine blue (aqueous).

III. Drug preparation and administration

DON was supplied as a dry powder (Calbiochem., Calif.), and prepared in 0.9% saline. Stock solutions of 0.001 and 0.0001 g/ml were filter-sterilized and stored frozen (with dessicant and in the dark).

Final dilutions were made from aliquots of stock solutions, and kept at 4°C for up to 2 weeks. In experiments, addition of 20 ul of DON stock solutions (0.001 and 0.0001 g/ml) to 2 mls of culture medium produced final concentrations of 10 ug DON/ml and 1 ug DON/ml of medium respectively. For convenience, such terms as "ug DON/ml medium" will be abbreviated "ug/ml DON". In all experiments, equal volumes of 0.9% saline were given to controls.

Glucosamine hydrochloride (GSA) (Sigma) was also dissolved in 0.9% saline and filter-sterilized. It was prepared immediately prior to treatment. Concentrations of GSA used were 10, 50, 100 and 200 ug/ml.

In all experiments, the time of subculture (cell dissociation and plating) was defined as day 0. In dose response experiments (page 11), the range of DON concentrations used was from 0.5 to 10 ug/ml. Subsequently, a DON concentration of 1 ug/ml, which did not appear to be too toxic to C-4 cells, was used in all further experiments. Single (added either on day 0, 1, or 2), double (added on days 1 and 2) and triple (added on days 1, 2 and 3) doses of 1 ug/ml DON were used. It was initially observed that adding a single dose one day after plating (or

day 1) produced little visible changes in cell growth and in culture morphology on day 2. However, if a second dose was added on day 2, clear changes in morphology could be observed in the treated cultures on day 3. Adding a third dose on day 3 produced even more evident results.

Single doses of 1 ug/ml were added at different times (days 0, 1, or 2) to examine effects of different exposure times to a single dose of the drug.

IV. Stability of DON at 37°C

1 ug/ml DON solutions in phosphate buffer (pH 7) (Fisher Scientific Co.) were incubated at 37°C in a humidified incubator (5% CO_2/air) for up to 11 days. The purpose of this experiment was to determine the stability of DON at 37°C and to compute the concentrations of DON in cultures given single, double or triple doses of 1 ug/ml DON.

In the first experiment, duplicate samples of DON solutions were frozen on days 1 to 4, 6, and 8 to 11. At the end of 11 days, thawed solutions were measured for DON stability by UV absorption at 274 nm (Dion et al., 1956) (Gilford spectrophotometer). In the second experiment, duplicate samples of DON were not frozen, but were measured on each of the above-mentioned days.

V. Cell growth on plastic surfaces

After centrifugation, subcultured cells were suspended in 5 ml of medium. In C-4I experiments, this volume of 5 ml was diluted with 30 to 50 ml of medium. In C-4II experiments, the 5 ml of cells in medium were

diluted with a smaller volume of medium (20 to 30 ml), since confluent, monolayered C-4II cultures reached a lower population density than confluent, highly stratified C-4I cultures. Two ml of cells and medium were dispensed into each tissue culture dish (35 mm, NUNC). Dishes were incubated in a humidified incubator at 37°C in 5% CO_2/air. DON treatments were given at various times as described in section III.

1. Dose-response

Cultures were grown with 0.5, 1, 5, and 10 ug/ml DON for 4 to 12 days. Fixed cultures were examined visually for any differences in amounts of growth between DON-treated and control cultures. A scale of "-" to "+++" was used, where "-" signified no growth inhibition, "+" slight but definite inhibition, "++" marked inhibition, "+++" extreme inhibition (little growth), and "±" borderline inhibition.

Cultures were also examined microscopically for changes in cell areas, colony forms, numbers and morphology of single cells, amount of cell debris, cell density, stratification, nuclear/cytoplasmic ratios, and intercellular contacts with increasing DON concentrations.

2. Colony form

Colonies, in fixed and stained cultures, were projected from an inverted Wild microscope onto the magnetic tablet of a Zeiss digital image analyzer, MOP 3, by use of a camera lucida. Colony forms were quantitated to determine changes in growth pattern with DON treatment.

Calculations of "form": This parameter was measured by MOP 3, which assigned a value of "1" for a perfectly round circle. Hence,

any irregular outline had a value of less than 1. Decreases in assigned value were proportional to increases in irregularity of colony forms. Forms of small colonies (more than 5 but less than 35 cells) and of large colonies (more than 50 cells) were determined separately. The data were analyzed by computer (Biological Sciences Data Center) using 1 way analysis of variance (ANOVA) and by using Newman-Keuls test, at a probability level, p, of less than 0.05.

3. Morphology of single adherent cells

In order to determine DON effects on cell-substratum adhesion (independently of cell-cell adhesion), the morphology of single cells was examined. Single cells adherent to plastic in fixed cultures were classified as being (i) flattened, (ii) irregularly-shaped, or (iii) spherical.

4. Cell area

The average projected area of individual cells was determined in small (5-35 cells) and large (more than 50 cells) colonies, since it appeared that DON-treated cells in large colonies were larger than in control colonies and in small DON-treated colonies.

In small colonies, the average cell area was derived at by dividing the total colony area (measured by MOP) by the number of cells in that colony. The latter was determined by counting the number of nuclei since it was difficult distinguishing individual cells.

Some large colonies were extremely stratified at the colony rims (edges). Hence, the average cell area was derived at by dividing the

area of the center part of the colony, by the number of cells present in the colony center.

Results were analyzed statistically using analysis of variance (ANOVA) test and Newman-Keuls test, at a probability level of less than 0.05.

5. Cell volume

To determine whether an observed effect of DON, the increase in cell area, was due to an increase in cell volume, cells were sized on a Coulter counter.

Cultures were dissociated into single cells, as described. In addition, cells were vigorously syringed with a 21 gauge needle. After centrifugation, cells were resuspended in 20 ml of isoton and sized on a Coulter counter. For a diameter range of 10-20 um, amplification=8, current=2, the number of cells sized was up to 1024/channel; and for a range of 20-40 um, amplification=8, current=32, the number of cells sized was up to 64/channel.

VI. Shedding of cells into the culture medium

1. C-4I and non-confluent C-4II cultures

Cells were plated into 6 flasks. On day 1, cells growing in 2 flasks were harvested by trypsin/EGTA treatment and counted with a hemocytometer, to determine the number of adherent cells per flask at the time of drug addition. The number and viability (by exclusion of 0.4% eosin) of cells shed were also determined. Also on day 1, 1 ug/ml DON or 0.9% saline was added to flasks. On days 2, 4, and 6,

medium was collected from the flasks, and numbers and viability of shed cells were determined. Cultures were fed new medium with DON or saline on days 2 and 4. On day 6, all cells adhering to the flasks were counted. Two and three separate experiments were completed for C-4I and C-4II cultures respectively.

2. C-4II (confluent) cultures

Cultures were grown until they were almost confluent, at which time (days 6 and 11 after subculture in 2 experiments) 1 ug/ml DON or 0.9% saline was added. On days 2, 4, and 6 after the day of initial DON addition, the cultures were treated as described above (Section VI.1). The reason for examining confluent C-4II cultures separately from non-confluent ones was that the shedding pattern of confluent and non-confluent C-4II cells differs (Auersperg, 1969a).

VII. Cell-cell aggregation

Cell-cell adhesion was examined in gyratory shaker culture. Two series of experiments were carried out: in one, the cells were pretreated with DON prior to dissociation and transferred to shaker culture; in the other, they were not pretreated.

1. Cells pretreated with DON prior to dissociation

C-4I cultures and C-4II cultures were grown in flasks, as described in section I, for 6 days. DON was added on days 1 or 5 (5 day and 1 day pretreatment respectively). On day 6, the cultures were

dissociated into single cells. Five ml of cells and medium ($0.9X10^6$ cells/ml) were added to 50 ml Erlenmeyer flasks, which were incubated at 37°C for up to 2 days on a gyratory shaker (New Brunswick Laboratory Rotator model G2), rotating at 70 rpm (revolutions per minute). At the end of the experiments the cells were fixed with formol-saline, and classified into single cells or clumps.

The aggregation of cells not pretreated with DON was also examined, to determine whether pretreatment for 1 and 5 day periods could have caused cell damage, thus inhibiting cell aggregation.

2. Cells not pretreated with DON prior to dissociation

The following is a flow diagram of the procedure used:

confluent cultures → dissociate → single cells in flasks → rapid shaking (200 rpm) 24 hours → size cell clumps → slow shaking (70 rpm) for 24 hours → size cell clumps

Drugs were added during this period

Confluent cultures were dissociated into single cells as described. Five ml of medium (450,000 cells/ml) were dispensed into 50 ml Erlenmeyer flasks, which were incubated at 37°C for 24 hours on a gyratory shaker, shaking at 200 rpm. The purpose of this rapid shaking was to allow for recovery of trypsinized cell surface components while minimizing cell-cell aggregation (Takeichi, 1977). DON was added at various times during this period of rapid shaking, that is, 0, 6, 12, 18 and 24 hours after subculture. This period of rapid shaking was followed by another 24 hour period of shaking at 70 rpm. The purpose of the slow period was to allow aggregation to occur, after repair of trypsinized cell surface components. Live clumps were

sized at (i) the end of 24 hours at 200 rpm and (ii) the end of 24 hours at 70 rpm (or a total of 48 hours of incubation), on a 100 u aperture Coulter counter (model Z_f), P_{64} size distribution analyzer, and an xy recorder (number 4) (Coulter Electronics, Hialeah, Florida). In addition, large C-4I clumps were sized on an Artek counter (model 980, Artek Systems, N.Y.) after being fixed (2% glutaraldehyde in Millonig's buffer) and stained (0.4% eosin).

3. Reversal of the DON effect

GSA (10, 50, 100 and 200 ug/ml) was added simultaneously with DON, during aggregation experiments in an attempt to reverse the effect of DON.

RESULTS

I. Variations in growth pattern of C-4 cell lines

Treatment of C-4 cultures with trypsin, an endopeptidase, produced suspensions that consisted mainly of clumps. When such cell suspensions were plated, C-4I colonies that grew were round while C-4II colonies were irregular (Figs. 4a and 4b).

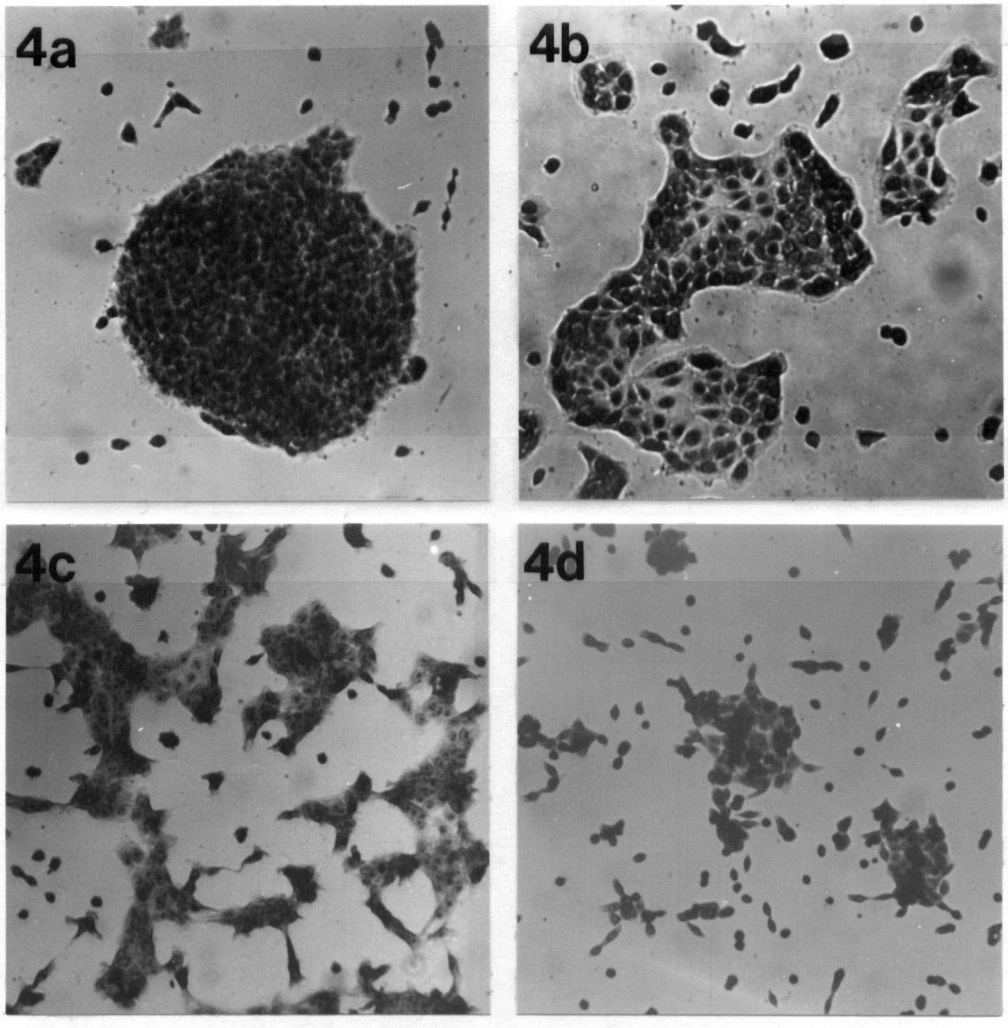
In the course of this study, it was observed that the growth pattern of C-4I cells could be altered by the combined use of trypsin and EGTA. Such dissociation treatment produced suspensions that consisted mainly of single cells. When these suspensions were plated, C-4I and C-4II colonies were both irregularly-shaped (Figs. 4c and 4d).

These observations indicated that the growth pattern of C-4I cells depended on the maintenance of cell-cell contacts, in contrast to that of C-4II cells. C-4II cells grew relatively independently of their cell-cell contacts.

II. DON Stability

UV absorption of DON, at 274 mm, is thought to be proportional to its biological activity (Dion *et al.*, 1956).

After an initial 24 hours of incubation at 37°C, the UV absorption of 1 ug/ml of DON in pH 7.0 phosphate buffer decreased by 26-42% (average of 34%) (Fig. 5). Storage of frozen samples for up to 10 days did not change the results. These results were consistent with these of Dion *et al.*, (1956), who reported a decrease in stability of 32 ug/ml DON of about 30% at 30°C after 18 hours. From days 2 to 7, there was little change in UV absorption. However, from days 8 to 11, there was a slight increase of 0.05 to 0.10 ug/ml of DON (Fig. 5).



C-4I C-4II

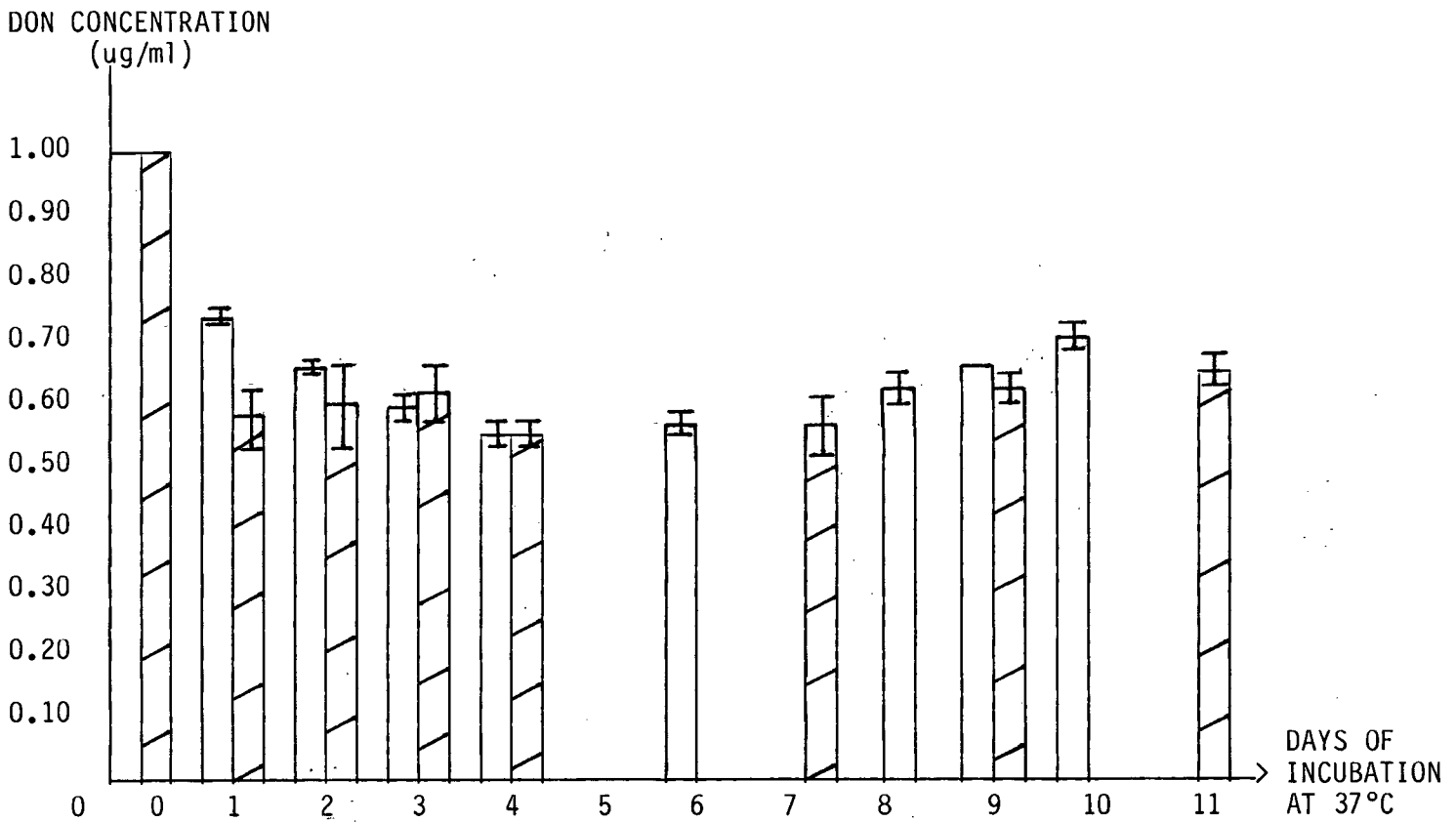
Figs. 4 a-d. Growth patterns of C-4 cell lines. Cultures were stained with 4% aqueous toluidine blue, x 90.

Figs. 4a and 4b. C-4I and C-4II cultures, respectively. These cultures were grown from large clumps.

Figs. 4c and 4d. C-4I and C-4II cultures, respectively. These cultures were grown from single cells.

Figure 5

STABILITY OF DON WITH TIME, AT 37°C[a]



Key:

[a] As determined by UV absorption of 1 ug/ml DON (diazoketones) at 274 nm.

I Range of values

☐ Expt 1: Measured aliquots each day.

▨ Expt 2: Froze aliquots each day. Measurements determined on last day of experiment.

Table 1

STABILITY OF DON AT 37°C[a]

(calculated from Fig. 5)

TREATMENT[b]	DON CONCENTRATIONS (ug/ml)[c]		
	day 4	day 7	day 11
Expt #1			
single dose	0.62	0.56	0.62
double dose	1.21	1.10	1.24
triple dose	1.78	1.65	1.84
Expt #2			
single dose	0.59	0.57	0.59
double dose	1.24	1.12	1.33
triple dose	1.98	1.67	1.93

key:

[a]Computed from Fig. 5, where duplicate samples of 1 ug/ml DON were incubated in phosphate buffer, pH 7.0, at 37°C.

[b]Number of doses of 1 ug/ml DON as used in cultures: a single dose would be added on day 1, a double dose on days 1 and 2, and a triple dose on days 1, 2, and 3. The calculations are based on DON samples measured each day in expt #1 or at the end of the expt (day 11) in expt #2 (see Fig. 5).

[c]Computed DON concentrations in cultures of various ages.

Therefore, it was assumed throughout this study that double doses of 1 ug/ml of DON added to cultures on the first and second days after subculture were equivalent to approximately 1.2 ug/ml on day 4 and 1.1 ug/ml on day 7 (Table 1). If triple doses of DON were given, concentrations of DON were assumed to be equivalent to approximately 1.9 ug/ml on day 4 and to 1.7 ug/ml on day 7.

III. Dose response of C-4 cells

To find an effective but relatively nontoxic dose of DON, the following 3 sets of experiments were carried out.

1. Growth inhibition

In both cell lines, there was increased growth inhibition with increasing concentrations of DON (Figs. 6a and 6b). In C-4II cultures, no inhibition by 0.5 ug/ml DON was visually observed. This dose was not tested in line C-4I (Table 2). Inhibition of cell growth using a single dose of 1 ug/ml DON was visually observed in only a minority of cultures (Fig. 6, Table 2). Inhibition by multiple doses of 1 ug/ml DON was greater than by single doses. Inhibition by 5 and 10 ug/ml was clearly evident in both cell lines (Fig. 6, Table 2).

Even though little inhibition was observed visually with 1 ug/ml DON treatment (Table 2), actual cell counts showed that there was growth inhibition. This growth inhibition was proportional to the number of doses of DON applied (Table 3).

Thus, DON, a potential anti-tumor drug, decreased cell growth even at a dose of 1 ug/ml. The discrepancy between visual observations and cell counts could be due to DON decreasing stratification of

cultures, and/or increasing cell surface area so that there was a lower number of cells covering the same amount of substratum. Both of these changes were introduced by DON, as shown below.

2. Morphology

Changes in morphology were observed with increasing concentrations of DON. A dose of choice for subsequent experiments was chosen which had minimal effect on growth and yet produced some morphological effects, which could be quantitated.

In both cell lines, DON-treated cells showed the following changes:

i) increase in projected cell surface area, increase in flatness of cells and decreased cell density (Figs. 7, 8, 9, 10, 11),

ii) increased irregularity in colony form with higher DON concentrations (Figs. 7, 8, 9, 11),

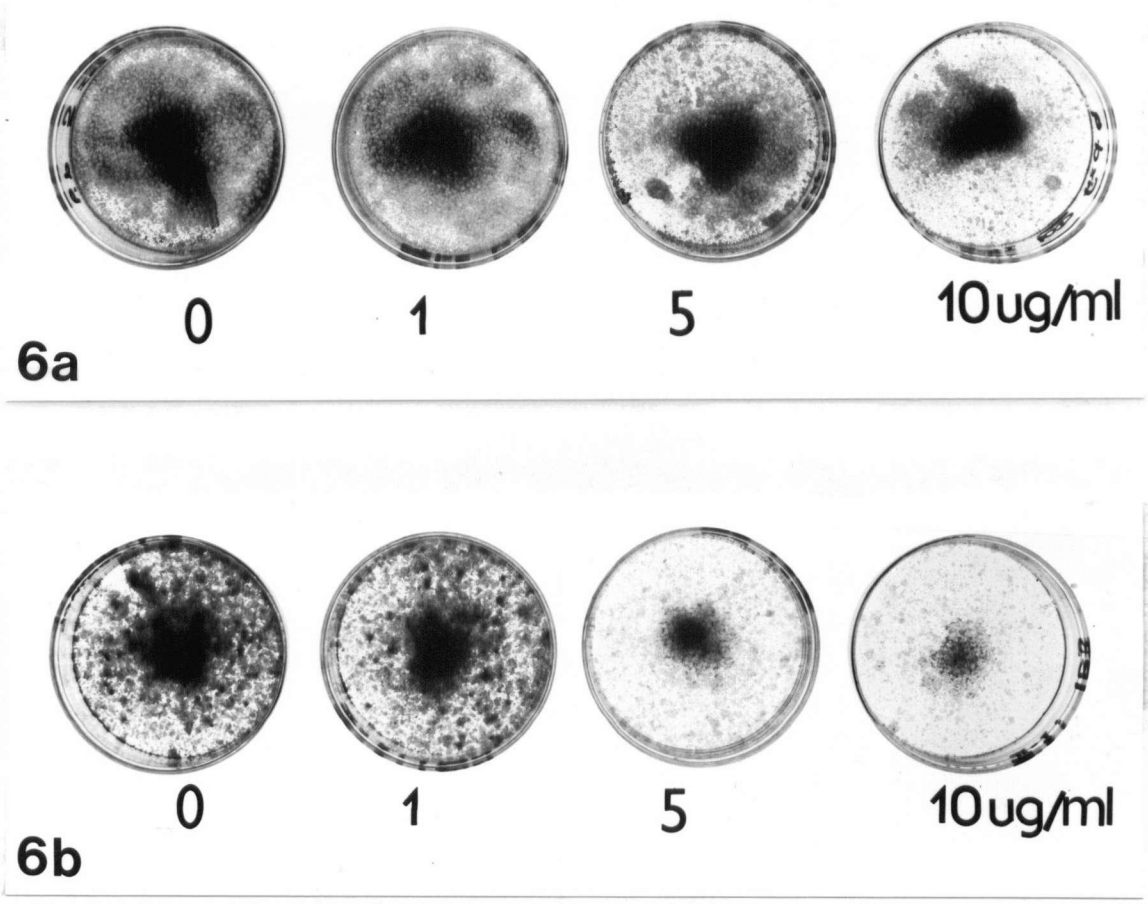
iii) increase in numbers of single cells which were spherical rather than flat (Fig. 11),

iv) increased cell debris in the culture medium,

v) decreased nuclear/cytoplasmic ratios (Figs. 7, 8, 9, 10, 11),

vi) decreased stratification in C-4I cultures, with little nuclear overlap (Fig. 7, 8), and

vii) decreased cell-cell contacts, with appearance of "holes" within colonies (Fig. 8). In certain areas, cells pulled away from each other (Figs. 7, 8, 9).



Figs. 6a-b. Effects of DON on growth. Ethanol fixation, toluidine blue staining, x1.

Fig. 6a. C-4I culture (11 day). DON was added to cultures 1 day after plating.

Fig. 6b. C-4II culture (12 day). DON was added to cultures at time of plating.

Table 2

INHIBITION OF CELL GROWTH[a]

C-4I

dose in ug/ml (No. of treatments)	age of cultures (days) 4-5	6-9	no. of dishes
1 (S.D.[b])	- ($\pm$ $^{4}/_{14}$)	-	27
1 (M.D.[c])	-,$\pm$	+	13
5 (S.D. & M.D.)	+	+	8
10 (S.D. & M.D.)	+	++	10
		Total =	58

C-4II

dose in ug/ml (No. of treatments)	age of cultures (days) 4-5	6-8	9-12	no. of dishes
0.5 (S.D. & M.D.)		-		8
1 (S.D.)	$\pm$ ($^{8}/_{27}$)	-	-	51
1 (M.D.)	+ ($\pm$ $^{4}/_{16}$)	+ ($\pm$ $^{7}/_{17}$)	+	34
5 (S.D. & M.D.)	+,++	$\pm$,++	+,++	12
10 (S.D. & M.D.)	++	+++	+,++	11
			Total =	116

Key:

[a] Dishes were examined visually for growth.
C-4I: based on 8 expts.
C-4II: based on 17 expts.

[b] Single dose of DON given at various days (0, 1, and 2) after plating.

[c] Multiple doses of DON. Double doses were given on days 1 and 2. Triple doses were given on days 1, 2 and 3.

Scale of inhibition:

-...no inhibition
$\pm$...borderline
+...definite
++...marked
+++...extreme (little growth)
Superscripts indicate the number of cultures showing the various degrees of inhibition over the total number of cultures.

Table 3

EFFECT OF DON ON GROWTH

CELL LINE	DON TREATMENT[a]	% GROWTH INHIBITION WITH DON TREATMENT[b] EXPT 1[c]	EXPT 2[d]
C-4I	single dose	70.9 (68.4, 73.3)	48.8 (28.6, 69.0)
	double dose	--	76.2 (64.3, 88.1)
C-4II (non-confluent)	single dose	57.9 (55.6, 78.7, 39.4)	41.9 (40.4, 43.4)
	double dose	--	73.8 (80.8, 66.7)
C-4II (confluent)	single dose	26	--

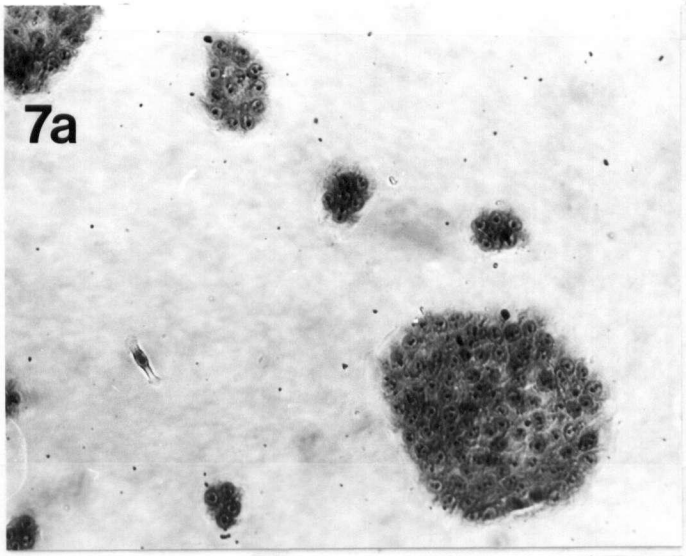
Key:

[a] Single dose of 1 ug/ml DON was given on day 1.
Double dose of 1 ug/ml DON was given on days 1 and 2.

[b] Values are the average of all experiments; values in brackets are the means of each experiment.

[c] Based on 2 C-4I and 3 C-4II experiments. Final cell number was determined on 6 day old duplicate cultures, except for confluent C-4II cultures which were day 17 cultures.

[d] Based on 2 separate experiments for each cell line. Final cell number was determined on 4 day old duplicate cultures.



Figs. 7a-c.
Effects of varying concentrations of DON on C-4I cultures (day 4). DON was added 1 day after plating. Ethanol fixation, toluidine blue staining, x 55.

Fig. 7a. Control colonies. Note: Round colonies, with uniform cell areas.

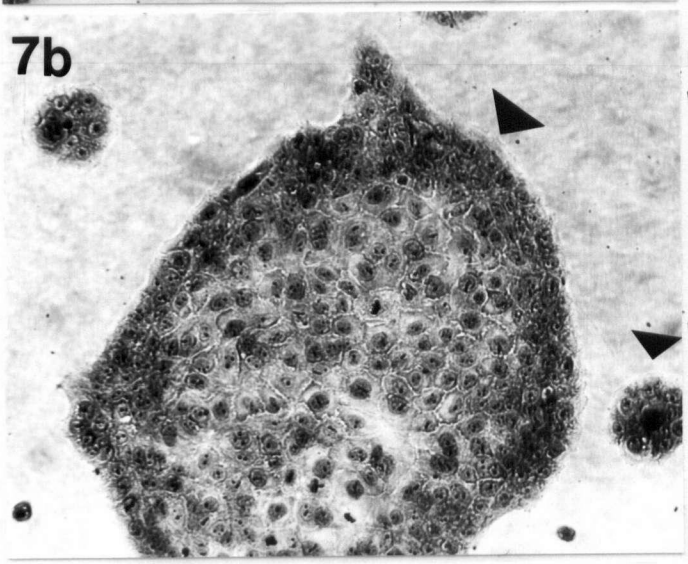


Fig. 7b. Colonies treated with 1 ug/ml DON. Note: Small colony with small cells (small arrowhead). Large colony with small stratified cells at the rim (large arrowhead) and large flat cells in the center, with a low nuclear/cytoplasmic ratio and decreased nuclear overlap.

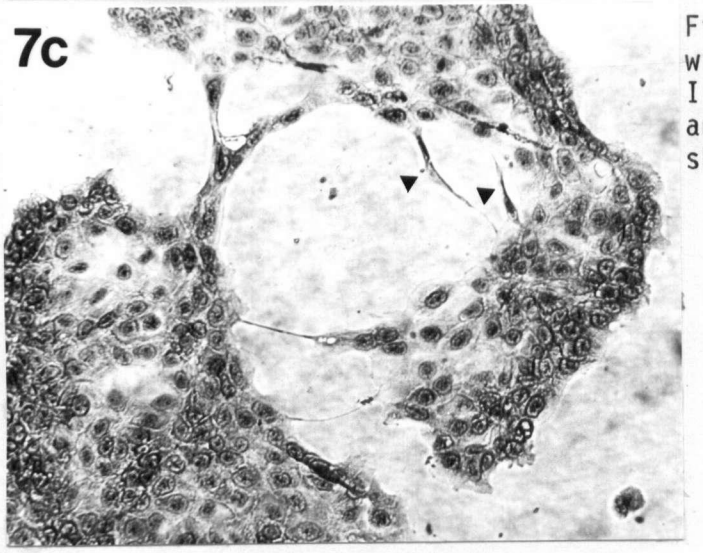
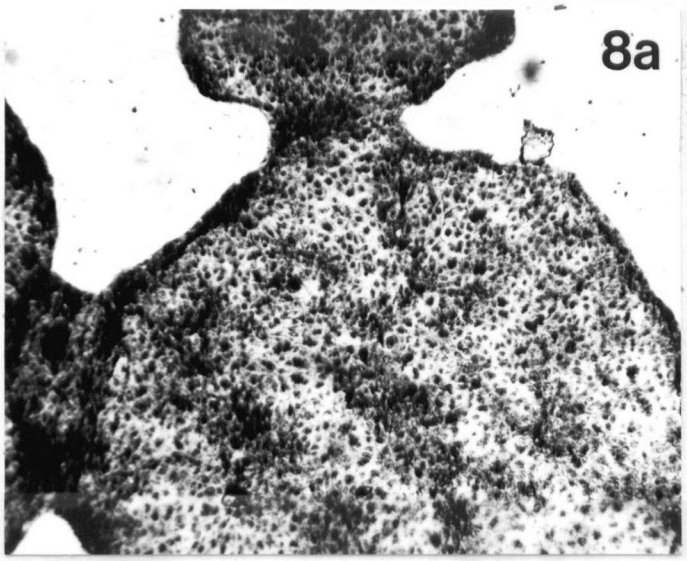
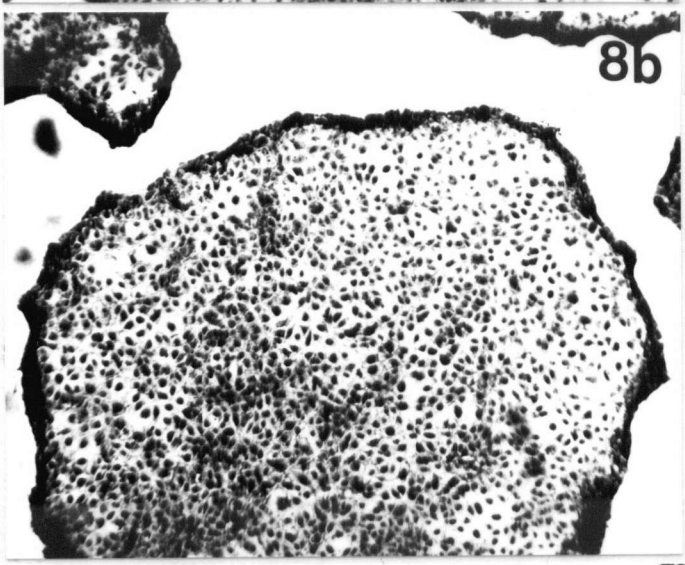
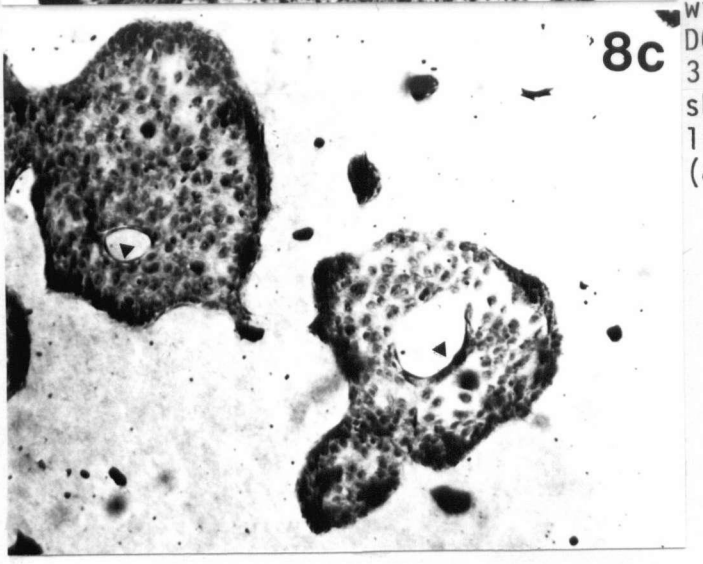


Fig. 7c. Colonies treated with 5 ug/ml DON. Note: Irregularly-shaped colonies and the presence of spindle-shaped cells (arrowheads).







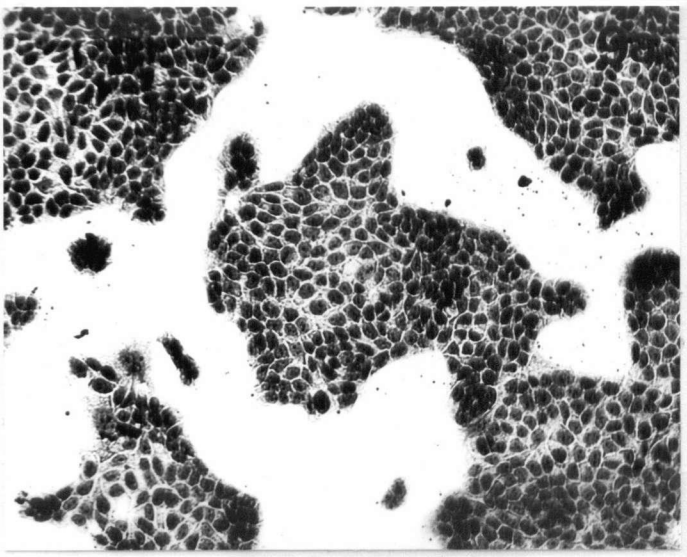
Figs. 8a-c.
Effect of multiple doses of 1 ug/ml DON on C-4I cultures. 7 day old cultures. Ethanol fixation, toluidine blue staining, x 55.

Fig. 8a. Control colonies. Note: Evident nuclear overlap and stratification.

Fig. 8b. Colonies treated with double doses of 1 ug/ml DON (added days 1 and 2). Note: Evident decreased nuclear overlap, decreased stratification, decreased nuclear/cytoplasmic ratio, and increased colony flatness.

Fig. 8c. Colonies treated with triple doses of 1 ug/ml DON (added days 1, 2 and 3). Note: Irregularly-shaped colonies with holes, lined by retracted edges (arrowhead).

Figs. 9a-c. Effects of varying concentrations of DON on C-4II cultures (day 8). DON was added 1 day after plating. Ethanol fixation, toluidine blue staining, x 55.



Figs. 9a. Control colonies.
Note: Irregularly-shaped colonies, with pavement-like (columnar) cells that have a high nuclear/cytoplasmic ratio.

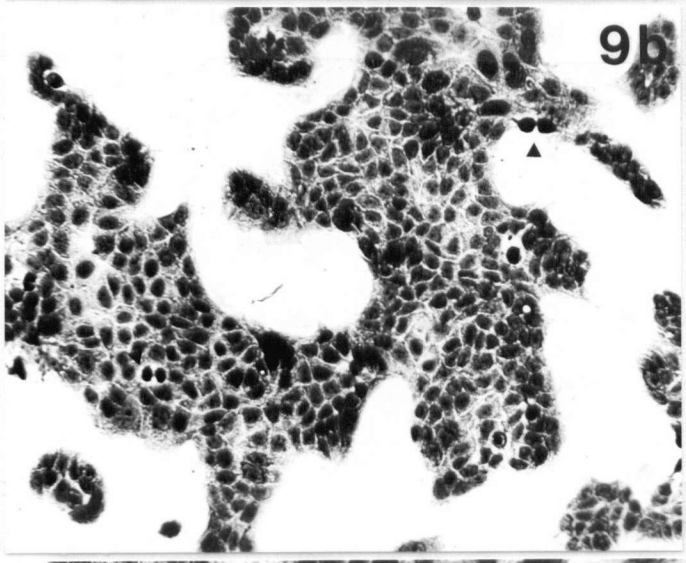


Fig. 9b. Colonies treated with 5 ug/ml DON.
Note: Irregular-shaped, but flatter-looking colonies. Cells are less pavement-like (less columnar) and have a lower nuclear/cytoplasmic ratio. Mitotic figures are seen (arrowheads).

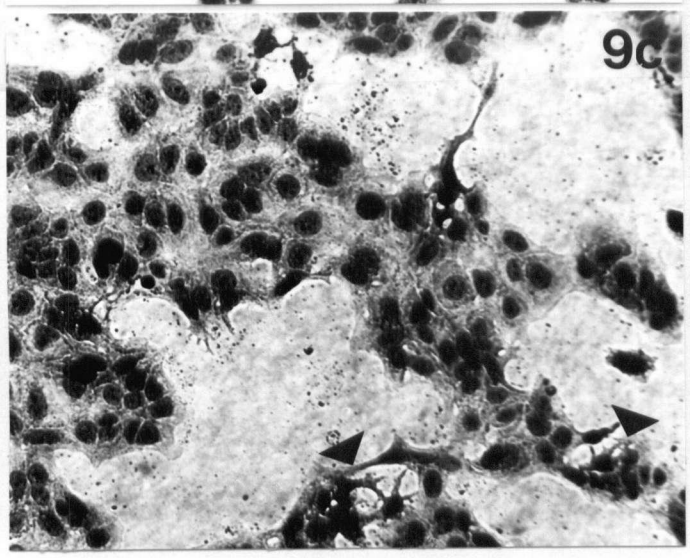


Fig. 9c. Colonies treated with 10 ug/ml DON.
Note: Colonies are very irregular-shaped, with retracted edges. Cells are flat and have a low nuclear/cytoplasmic ratio. Cells are separated from adjacent cells by "holes", but are still in contact by thin cytoplasmic processes (arrowheads).

Figs. 10a-b.
Effects of multiple doses of 1 ug/ml DON on C-4II cultures. 4 day old cultures. Ethanol fixation, toluidine blue staining, x 55.

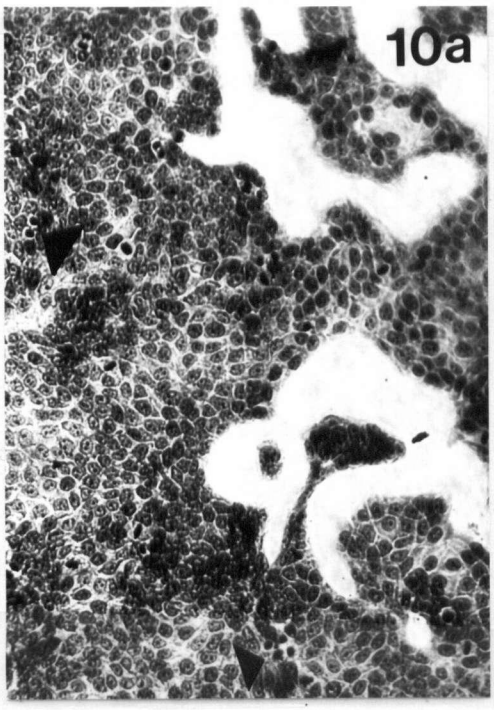


Fig. 10a. Control culture.
Note: Mitotic figures (arrowheads).

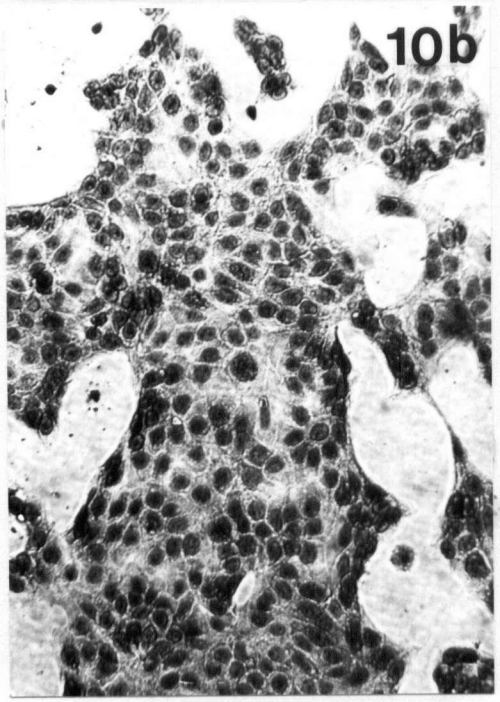
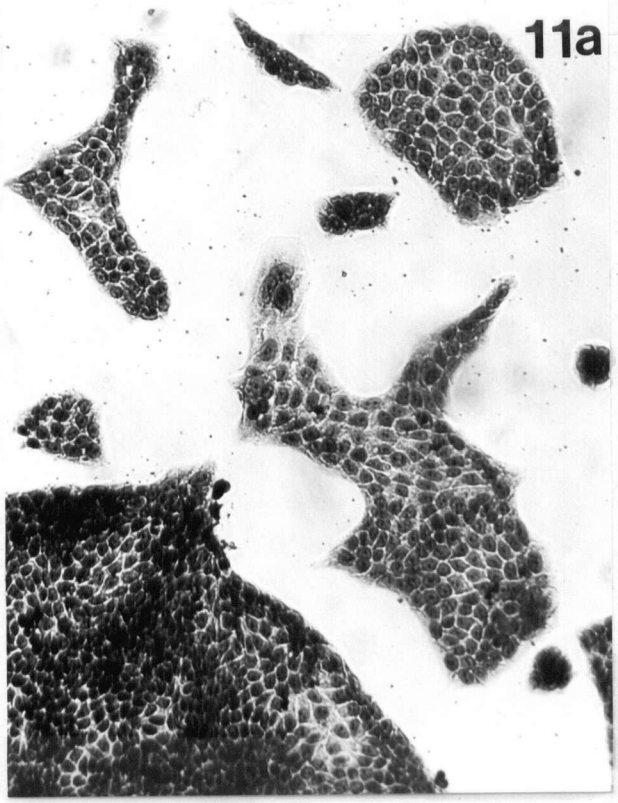
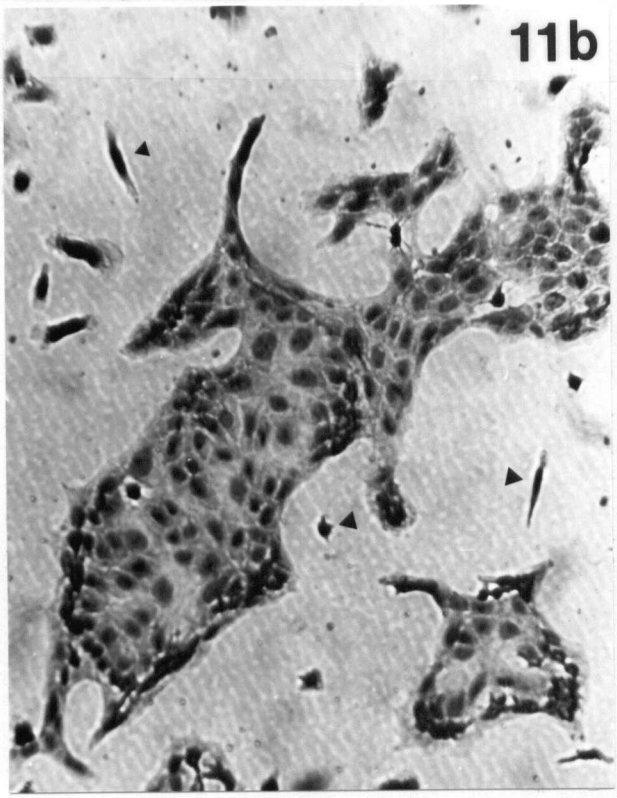


Fig. 10b. Cultures treated with double doses of 1 ug/ml DON (added days 1 and 2).
Note: Cells are flatter, less pavement-like, less columnar, and have a lower nuclear/cytoplasmic ratio.





Figs. 11a-b.
Effects of multiple doses of 1 ug/ml DOn on C-4II cultures.
4 day old cultures. Ethanol fixation, toluidine blue staining, x 55.

Fig. 11a. Control culture.
Note: There are few single cells on the substratum.

Fig. 11b. Cultures treated with triple doses of 1 ug/ml DON (added days 1, 2 and 3).
Arrowsheads: Numerous single cells, many of which are spindle-shaped (irregularly-shaped) or spherical.

In both cell lines, all or a combination of these changes were obvious in cultures treated with high concentrations of DON (5 and 10 ug/ml) and multiple doses of 1 ug/ml DON. Single doses of 1 ug/ml DON produced less obvious changes, while 0.5 ug/ml caused no change in any cultures.

3) Cell-substratum adhesion

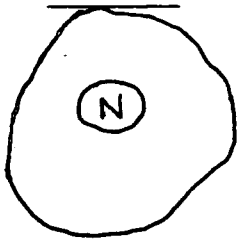
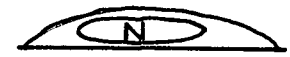
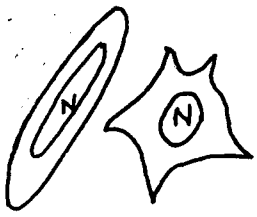
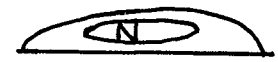
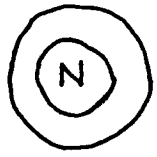
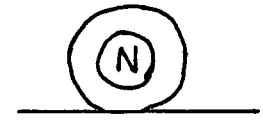
Effects of increasing DON concentrations on cell-substratum interactions, as represented by changes in morphology of single cells adhering to the substratum, were quantitated. Single cells adhering to the substratum were classified into 3 groups (Figs. 12a-c). Cells that were flattened were considered to be most interactive with the substratum (Fig. 12a). Cells that were irregularly-shaped were only less interactive (Fig. 12b), while spherical cells were least interactive with the substratum (Fig. 12c).

In both cell lines, the percentage of single cells that were fully spread and flattened on the substratum decreased with increasing DON concentrations (Fig. 13). In the C-4I cell line, the decrease in flattened cells was accompanied by increases in the percentage of spherical cells (Fig. 15). In contrast, in the C-4II cell line, the decrease in percentage of flattened cells was accompanied mainly by an increase in the percentage of irregularly-shaped cells (Fig. 14).

Statistical tests using analysis of variance (Petkau and Crapeau, 1983) indicated that DON treatments in both cell lines produced statistically significant differences in the proportions of flattened cells.

Figs. 12a-c.

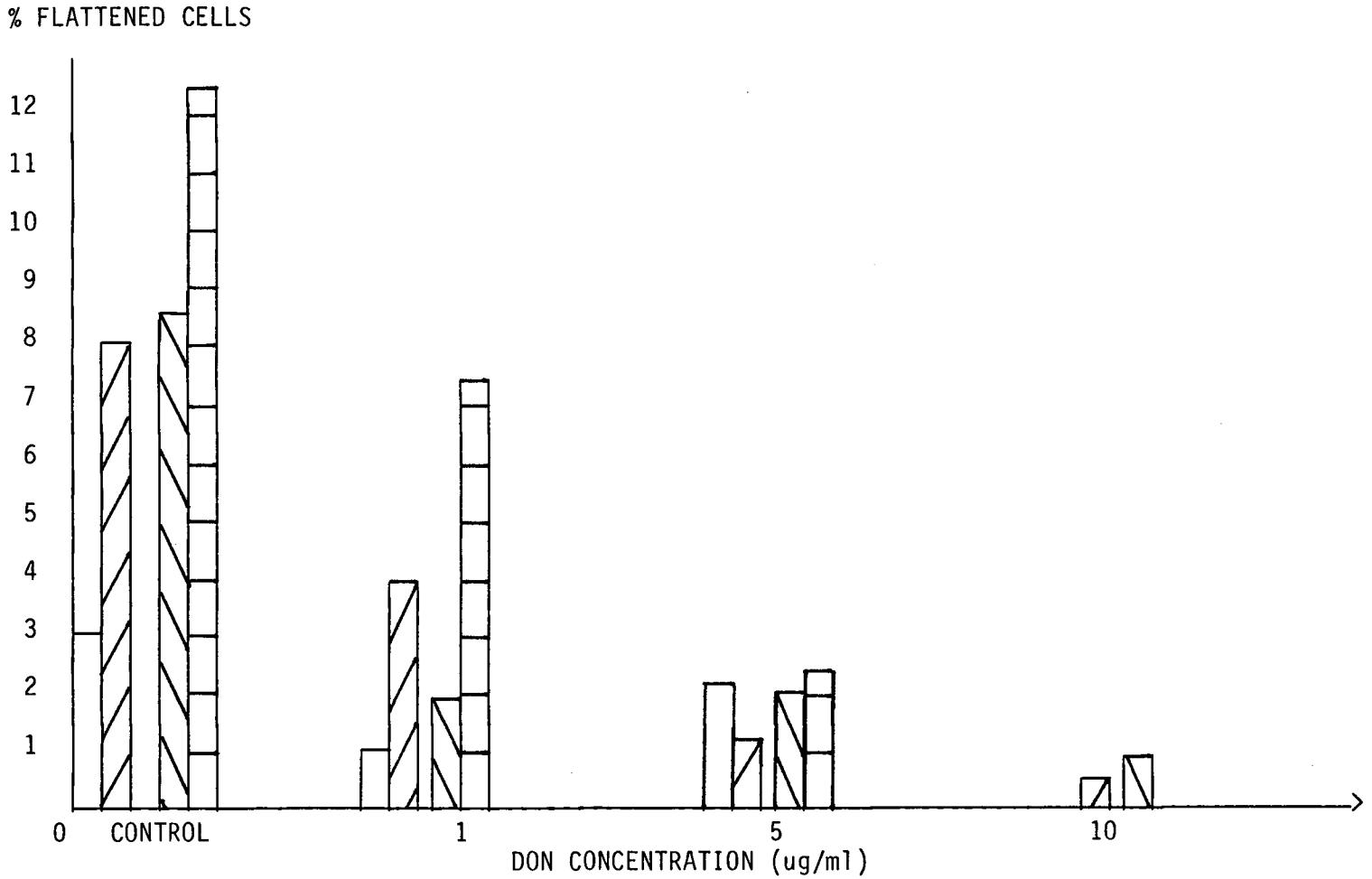
Morphologies of single cells
(indicative of cell-substratum interactions)

TOP VIEW	MORPHOLOGY	CROSS-SECTION
	Fig. 12a. Flattened (round and spread) cell (most cell-substratum interactive)	
	Fig. 12b. Irregularly-shaped cell (partly cell-substratum interactive)	
	Fig. 12c. Spherical (round and unspread) cell (least cell-substratum interactive)	

N Nucleus

Figure 13

EFFECT OF DIFFERENT CONCENTRATIONS OF DON ON THE PERCENTAGE OF FLATTENED CELLS[a]



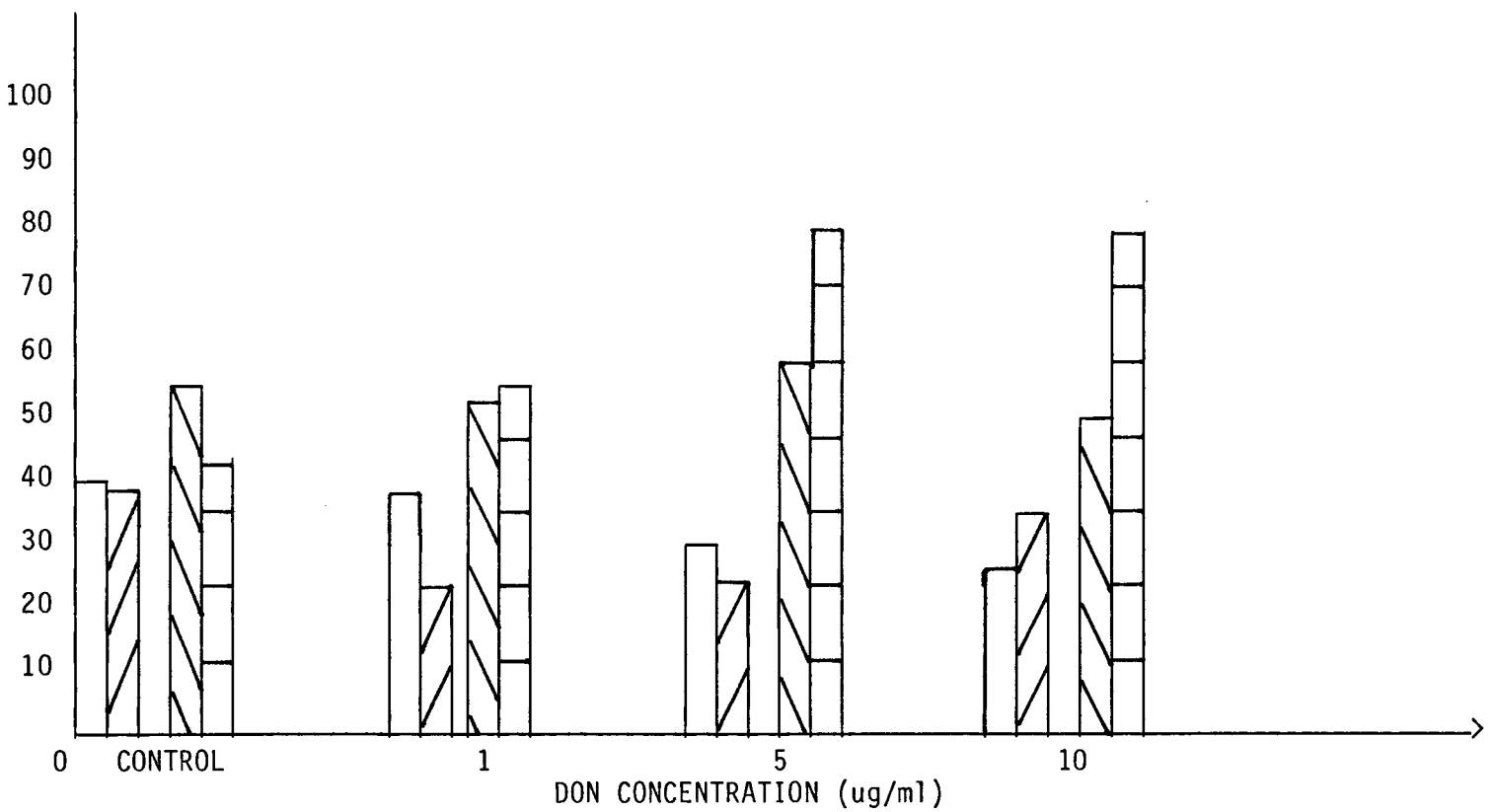
Key:

[a] Cultures were 4 days old. Duplicate cultures/treatment. DON was added on day 1 after plating. Total number of cells counted was 2,473 C-4I cells and 1,139 C-4II cells.

C-4I Expt #1 C-4I Expt #2 C-4II Expt #1 C-4II Expt #2

Figure 14

EFFECT OF DIFFERENT CONCENTRATIONS OF DON ON THE PERCENTAGE OF IRREGULARLY-SHAPED CELLS[a]



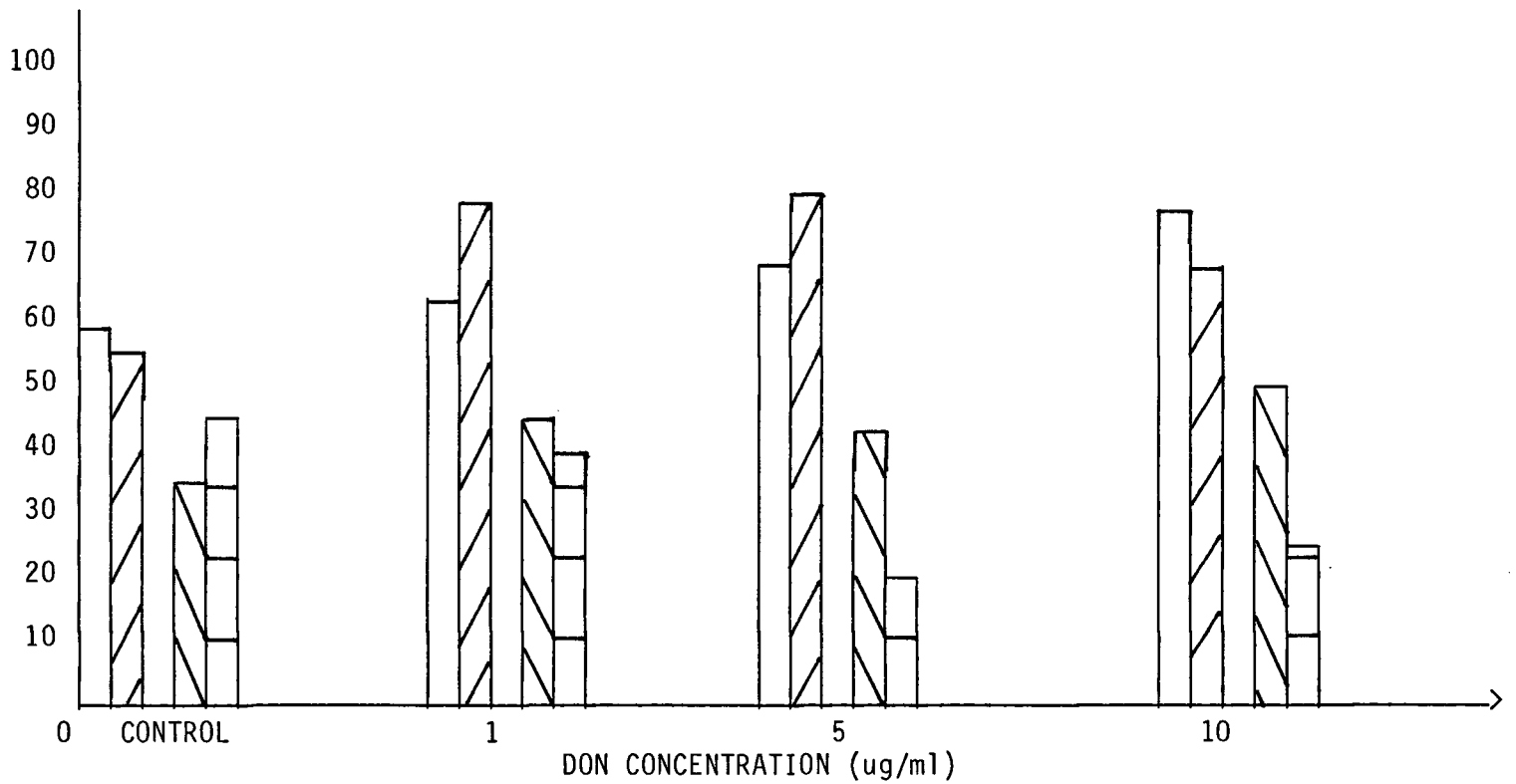
Key:

[a] Cultures were 4 days old. Duplicate cultures/treatment. DON was added on day 1 after plating. Total number of cells counted was 2,473 C-4I cells and 1,139 C-4II cells.

C-4I Expt #1 C-4I Expt #2 C-4II Expt #1 C-4II Expt #2

Figure 15

EFFECT OF DIFFERENT CONCENTRATIONS OF DON ON THE PERCENTAGE OF SPHERICAL CELLS[a]



Key:

[a] Cultures were 4 days old. Duplicate cultures/treatment. DON was added on day 1 after plating. Total number of cells counted was 2,473 C-4I cells and 1,139 C-4II cells.

C-4I Expt #1 C-4I Expt #2 C-4II Expt #1 C-4II Expt #2

Conclusion

On the basis of the dose response in terms of growth, morphology and cell-substratum adhesion, 1.0 ug/ml DON was chosen for all subsequent experiments, since its effects were definite, but less severe than those of 5.0 and 10.0 ug/ml.

IV. Effects of 1 ug/ml of DON on the growth patterns of C-4I and C-4II lines

Colony form

It was observed that DON at high doses caused increased irregularity in colony outlines (Figs. 7a, 7c, 8a, 8c, 9a-c and 11a-b). These changes in colony form were quantitated by use of a Zeiss MOP-3 image analyzer and by computer statistical analysis. Forms of small colonies (between 5 and 35 cells per colony) were initially determined. However, since it was later observed that small and large colonies differed in their response to DON treatment in cell area and colony forms, large colonies (more than 50 cells per colony) were also quantitated.

In C-4I cultures (Table 4), most small colonies were rounder than control colonies if they were treated with one dose of DON. In contrast, colonies treated with multiple doses tended to be more irregularly-shaped than control colonies (Table 4). The changes were significant with a single dose on day 4 and with a double dose on days 6 to 7.

In small C-4II colonies (Table 5), there were no consistent changes in response to DON. One day 4 experiment showed increased roundness of colonies while another similar experiment showed significantly increased irregularity with DON treatment. On day 6-7, the colonies showed

increased irregularity but only the change in the colonies treated with triple doses was statistically significant.

In large C-4I colonies (Table 4), the majority of treated colonies was more irregular than that of controls. These changes were significant in cultures treated with multiple doses and for longer culture periods.

In most large C-4II cultures (Table 5), the form of treated colonies became significantly more irregular.

The variances in colony form were determined for DON-treated and control colonies (Appendix I). In large colonies of both cell lines, variances in colony form of DON-treated cultures were mainly larger than those of control colonies. However, in small colonies, variances in colony form were generally smaller in DON-treated C-4I and more variable in DON-treated C-4II cultures. Considering control colonies only, small colonies in both cell lines were more variable in colony form than large colonies.

CONCLUSIONS

1. In small C-4I colonies, the response to DON was a biphasic one: a low dose of DON resulted in increased roundness, while a higher dose resulted in increased irregularity. The response of C-4II colonies was variable.
2. In large colonies in both cell lines, DON treatment resulted in colonies becoming more irregular (these changes were more significant in line C-4II). As a result, the irregular DON-treated C-4I colonies resembled control C-4II colonies.
3. In both cell lines, small colonies responded to DON treatment differently than large colonies. In addition, in both cell lines, the form

Table 4

C-4I: CHANGE IN FORM OF COLONIES TREATED WITH 1 UG/ML OF DON[a]

Treatment[b]	Time of DON Addition[c] (days after plating)	Form of Small Colonies[d] Age of Cultures day 4	days 6-7
Control		0.805	0.847
Single Dose	2	0.853*	0.862
Single Dose	1	0.863*	0.868
Single Dose	0	0.885*	0.822
Double Dose	1 + 2	0.826	0.746*
Triple Dose	1, 2 + 3	0.782	0.790

Total number of colonies = 871

Treatment[b]	Time of DON Addition[c] (days after plating)	Form of Large Colonies[d] Age of Cultures day 4	days 6-7 Expt (i)	Expt (ii)
Control		0.885	0.807	0.889
Single Dose	2	0.886		0.845
Single Dose	1	0.865		0.878
Single Dose	0	0.866	0.807	0.856
Double Dose	1 + 2	0.893		0.833*
Triple Dose	1, 2 + 3	0.881		0.834*

Total number of colonies = 381

[a] Based on 4 experiments. Colony form was measured on an image analyzer, MOP 3 (Zeiss). A numerical value of colony form of 1.00 represented the form of a perfectly round circle. Deviations from this roundness (i.e. irregularity) were represented by values less than 1.00. Differences between control and treated colonies were tested for significance at a probability level of 0.05, by an analysis of variance and Newman-Keuls test. In each experiment, the variance among and within treatments were analyzed to determine if differences were significant. Hence, among experiments, deviations from control values were of different magnitudes for differences to be significant.

[b] Total number of doses of 1 ug/ml of DON.

[c] Days after plating. Time of plating was defined as day 0.

[d] Small colonies had 5 to 35 cells. Large colonies had more than 50 cells.

* Changes were significantly different, at a probability level of 0.05.

Table 5

C-4II: CHANGE IN FORM OF COLONIES TREATED WITH 1 UG/ML OF DON[a]

Treatment[b]	Time of DON Addition[c] (days after plating)	Form of Small Colonies[d] Age of Cultures day 4 Expt (i)	day 4 Expt (ii)	days 6-7
Control		0.669	0.688	0.79
Single Dose	2	0.721		0.77
Single Dose	1		0.581*	0.77
Single Dose	0	0.715	0.615	0.76
Double Dose	1 + 2	0.714	0.585*	0.75
Triple Dose	1, 2 + 3		0.580*	0.68*

Total number of colonies = 1,110

Treatment[b]	Time of DON Addition[c] (days after plating)	Form of Large Colonies[d] Age of Cultures day 4 Expt (i)	day 4 Expt (i)	days 6-7 Expt (ii)
Control		0.469	0.600	0.776
Single Dose	2	0.478		0.739*
Single Dose	1		0.540*	0.709*
Single Dose	0	0.492	0.454*	0.713*
Double Dose	1 + 2	0.430	0.627	0.641*
Triple Dose	1, 2 + 3		0.493*	0.541*

Total number of colonies = 591

For legend, see Table 4.

of small control colonies was more variable than the form of large control colonies, and DON-treated large colonies were more variable than corresponding control colonies.

These results indicated that DON altered the growth pattern of the two cell lines differently and in a complex fashion. The following experiments were undertaken to determine whether these alterations in growth pattern were due to effects of DON on cell-cell and/or on cell-substratum adhesion.

A. Cell shedding

It was previously shown that confluent C-4II cultures shed more cells, and a higher proportion of live cells, into the culture medium than do C-4I cultures (Auersperg, 1969a). It was postulated that secretion of ECM may facilitate cell shedding in the less cohesive C-4II cell line, but not in the cohesive C-4I line. The following experiment was to determine if DON had any effect on cell shedding.

In C-4I cultures, DON increased cell shedding, and in contrast to control cultures, most of the shed cells were live (66-89%, as compared to 45-54% for controls) (Table 6). This may have accounted for the decreased stratification in DON-treated cultures (Figs. 7a-b and 8a-b). In non-confluent C-4II cultures, DON caused no increase in the absolute number of cells shed and approximately a 2-fold increase in relative numbers. In contrast, the drug caused an increase in shedding in all confluent C-4II cultures, both in absolute and relative numbers, with the relative increase being about 5-fold (2.0 vs. 10.7 on day 0-6, and 0.8 vs. 4.1 on day 5-6) (Table 6). In addition, the

proportion of live cells from DON-treated cultures was high (87-88%), comparable to that of control cultures (87%).

These results indicated that DON induced cell shedding in C-4I cultures and also in C-4II cultures, particularly when they were confluent. DON did not cause increased cell shedding by causing cell death, since the proportions of shed cells that were live in most treated cultures was as high or higher than in control cultures. Thus, DON likely increased cell shedding by decreasing cell-cell adhesion.

B. Cell-cell aggregation

Since DON probably increased cell shedding by decreasing cell-cell adhesion, it seemed possible that it would also decrease cell aggregation. The following experiments were carried out to determine what effects DON had on cell aggregation in the two cell lines.

1. Aggregation of cells pretreated with DON prior to trypsin/EGTA dissociation

C-4I:

After rotation for 1 day at 70 rpm in the absence of DON, the aggregation of cells that were pretreated with DON for 5 days was greatly inhibited (84% single cells) in contrast to that of controls (46% single cells) (Table 7). Aggregation of cells that were pretreated with DON for 1 day was also inhibited but less so (67% single cells). In addition, control clumps that resulted were round and compact with flat cells on the surface (Fig. 16a), while treated clumps

Table 6

ABSOLUTE AND RELATIVE NUMBER AND VIABILITY OF SHED CELLS PER CULTURE[a]

	C-4I		C-4II (non-confluent)		C-4II (confluent)	
	Control	DON	Control	DON	Control	DON
Day 0-6						
Number of shed cells (x 10^4):						
absolute	6.4	20.8	13.4	9.4	30.4	48.0
relative[b]	0.5	3.1	1.2	2.4	2.0	10.7
% viability[c] of shed cells	54	66	82	73	87	88
Day 5-6						
Number of shed cells (x 10^4):						
absolute	2.0	5.8	6.4	3.7	13.2	20.6
relative	0.1	0.9	0.6	0.9	0.8	4.1
% viability of shed cells	45	89	87	81	87	87

[a] Average total shed cells (x 10^4) per culture; based on 2 C-4I, 3 non-confluent C-4II and 2 confluent C-4II experiments. In each experiment, treatments were duplicated.

[b] Absolute number of shed cells relative to the number of cells growing in the culture flasks.

[c] Percentage of total cells shed into the culture medium that were live (determined by dye exclusion test).

were smaller, less compact, irregularly-shaped and all cells remained more or less spherical (Figs. 16b and 16c).

After rotation for 2 days at 70 rpm, DON-pretreated cultures had more clumps than after 1 day of rotation, but still fewer than control cultures.

C-4II:

In 2 separate experiments, after either 1 or 2 days of rotation, there were only slight differences between control and DON-pretreated cultures, except for a limited decrease in aggregation of one set of cultures pretreated with DON for 5 days (Table 8, experiment 1).

The clumps that formed from both control and DON-pretreated cells were mainly irregular and small (Figs. 17a and 17b).

Summary of results and conclusion:

In the C-4I cell line, DON pretreatment, whether for 1 or 5 days, caused a definite decrease in cell-cell aggregation and in cell spreading or cell deformation. Pretreatment with DON for 5 days caused a greater inhibition of aggregation than pretreatment for 1 day. In the C-4II cell line, DON pretreatment, whether for 1 or 5 days, caused only an inconstant decrease in cell-cell aggregation if cultures were rotated for 2 days. There was no difference in morphology of clumps formed from control and DON-pretreated cells. In both cases, the clumps were irregularly-shaped and small. To conclude, DON clearly decreases cell adhesion in C-4I but not in C-4II clumps.

Table 7

C-4I: AGGREGATION OF CELLS PRETREATED WITH DON[a]

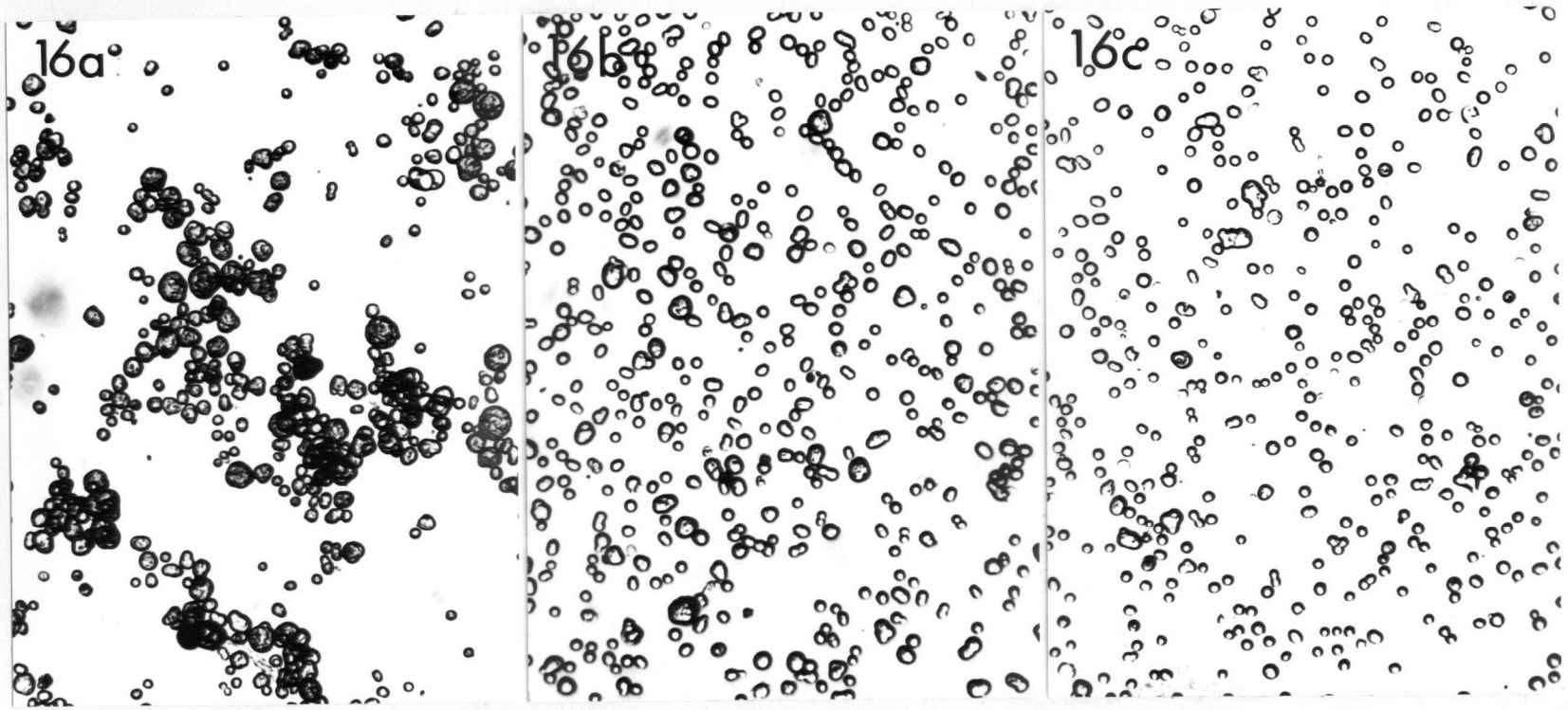
Treatment[b]	% of Cells and Clumps[c]		Total Number Counted
	% Single Cells	% Clumps	
Control	46	54	1,232
1 day DON pretreatment	67*	33*	1,923
5 day DON pretreatment	84*	16*	1,326

[a] Based on 2 experiments; results presented here are representative of 1 experiment only. All treatments were duplicated.

[b] DON (1 ug/ml) was added to cultures for 1 or 5 days prior to dissociation into single cells. No DON was added while cultures were rotated for 1 day at 70 rpm.

[c] Samples were sized (hemocytometer slide) and classified either as single cells or as clumps (with 2 or more cells).

* Controls values were significantly different from treated values, as determined by analysis of variance and Newman-Keuls tests, at a probability level of 0.05.



Figs. 16a-c. C-4I: Aggregation of cells pretreated with DON (1 ug/ml). Fixed 1 day after shaking at 70 rpm. No DON was added during shaking, x 90.

Fig. 16a Control culture.

Fig. 16b 1 day DON-pretreated culture.

Fig. 16c 5 day DON-pretreated culture.

Table 8

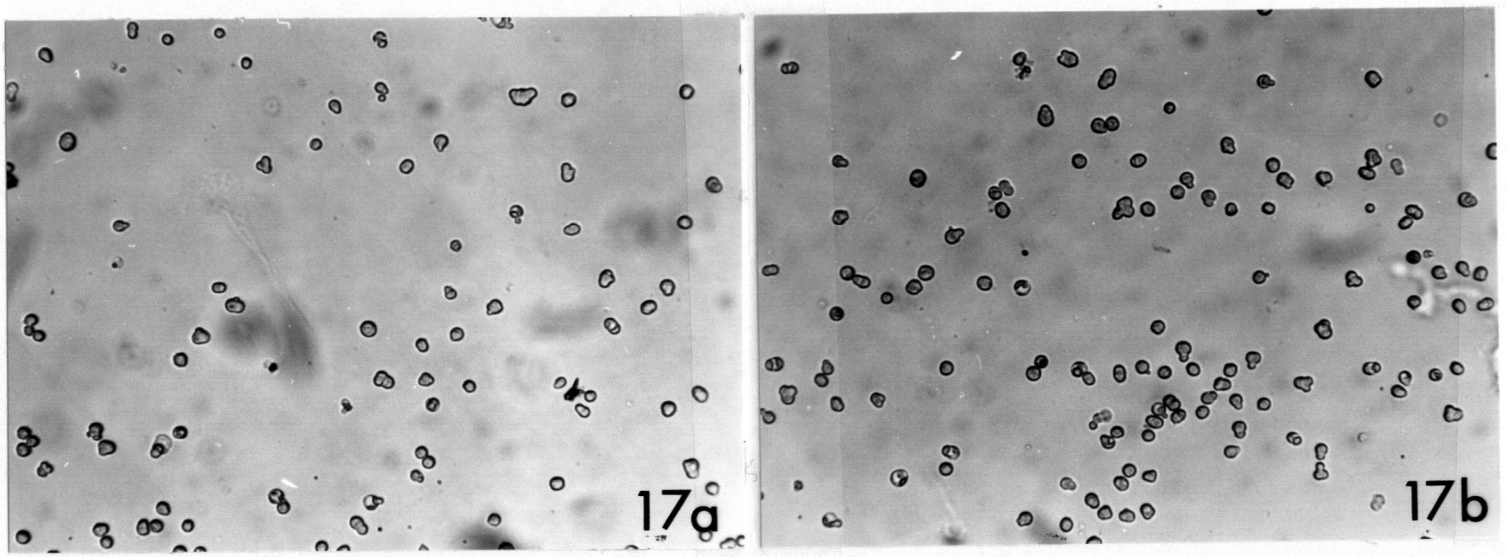
C-4II: AGGREGATION OF CELLS PRETREATED WITH DON[a]

Treatment[b]	% of Cells and Clumps[c]		Total Number Counted
	% Single Cells	% Clumps	
Expt #1			
1 day of shaking:			
control	78.9	21.2	586
1 day DON pretreatment	79.6	20.4	567
5 day DON pretreatment	68.4	31.7	451
2 days of shaking:			
control	5.4	94.6	813
1 day DON pretreatent	10.6	89.5	1,008
5 day DON pretreatment	23.0	77.1	789
Expt #2			
1 day of shaking:			
control	49.0	51.0	635
1 day DON pretreatment	42.3	57.7	442
5 day DON pretreatment	46.5	53.5	1,752
2 days of shaking:			
control	27.9	71.9	583
1 day DON pretreatment	19.2	80.7	666
5 day DON pretreatment	20.6	79.5	1,446

[a] Based on 2 experiments. In expt #2 only, the dissociated cells were syringed with a 21 gauge needle in an attempt to dissociate the clumps more completely prior to shaking.

[b] DON (1 ug/ml) was added to cultures for 1 or 5 days prior to dissociation into single cells. No DON was added while cultures were rotated for up to 2 days at 70 rpm.

[c] Samples were sized (hemocytometer slide) and classified either as single cells or as clumps (with 2 or more cells).



Figs. 17a-b C-4II: Aggregation of cells pretreated with DON (1 ug/ml). Fixed 2 days after shaking at 70 rpm. No DON was added during shaking, x 90.

Fig. 17a Control culture.

Fig. 17b 5 day DON-pretreated culture.

2. Aggregation of cells treated with DON after trypsin/EGTA dissociation (Cells not pretreated with DON prior to dissociation).

Any DON-induced inhibition of aggregation in the DON-pretreated shaker cultures could have resulted from a slower rate of recovery of adhesive cell surface components following trypsinization, due to depletion of glycosaminoglycan precursors stored during DON-pretreatment. It could also have been influenced by cell damage due to long-term DON pretreatment. To reduce the influence of these possibilities, the aggregation experiment was modified so that freshly trypsinized cells had a recovery period, in which trypsin-sensitive cell surface components were regenerated (see flow diagram, p. 15). During this recovery period, shaker cultures were maintained at a relatively fast speed (200 rpm) so that there was minimal cell-cell aggregation while cell surfaces were being repaired (Takeichi, 1977). The recovery period was assumed to be 24 hours long since recent literature on cell surface regeneration indicated that cells were fully adhesive and had regenerated their cell surface components within 24 hours after trypsinization (Steinberg et al., 1973; Dembitzer et al., 1980b). After this recovery period at a rapid speed, the cells were allowed to aggregate at the relatively slow speed of 70 rpm.

In addition to the above modification, cultures were not treated with DON prior to trypsinization. This eliminated the possiblity that decreased aggregation was due to the impaired abilities of damaged cells to aggregate. Instead, cultures were only treated with DON for a shorter period of time after trypsinization, during the cells' recovery from the effects of trypsin. It was previously observed that

there was a lag period (of about 1 day) between the time of addition of DON treatment and the time that changes were visually observed. Hence, DON was added to cells in shaker cultures during their recovery from trypsinization, so that DON-induced effects would be evident soon after the onset of maximal aggregation at 70 rpm. Hence, ideally, at 24 hours of recovery with minimal aggregation at 200 rpm, i.e. at the onset of maximal aggregation at 70 rpm, control and DON-treated cells in shaker cultures would be maximally adhesive but still equally as unaggregated. The effects of DON should become apparent subsequently. The differences in aggregation of control and DON-treated trypsin-recovered cells were quantitated 24 hours after the onset of maximal aggregation (70 rpm), i.e. 48 hours after trypsinization.

The optimal time of DON addition was worked out initially with the C-4II cell line, as shown below, and later applied to the C-4I line.

C-4II:

In three experiments, DON was added to shaker cultures immediately after dissociation. In all three experiments, there was some indication that DON-treated cultures were less aggregated than control cultures already after 24 hours of rapid shaking and also after a further 24 hours of shaking at 70 rpm (Fig. 18; Table 9, part i).

In one experiment, DON was added to cultures 6 or 12 hours after dissociation. DON-treated cells were again less aggregated than control cells at 24 hours after rapid shaking, and also after 48 hours of fast and

slow shaking (Table 9, part ii). Hence, DON decreased cell aggregation after an incubation period of only 18 hours, and possibly 12 hours.

In five experiments, DON was added 18 hours after dissociation. Four experiments indicated that treated cells (shaken at 200 rpm in the presence of DON for 6 hours) were as aggregated as control cells (Fig. 19). However, at the end of a total 48 hours of shaking, there was some indication that control cells were slightly more aggregated than treated cells (Fig. 19; Table 9, part iii).

In one experiment, DON was added to shaker culture after 24 hours of rapid shaking. At the end of 24 hours of slow shaking, there was little difference between treated and control cultures (Table 9, part iv).

The clumps found in control and DON-treated cultures were irregularly-shaped. To summarize, when DON was added to cultures which were recovering from effects of trypsinization, there were only borderline differences detected between control and treated cultures.

C-4I:

In all experiments, DON was only added to cultures 18 hours after trypsinization, while the cultures were recovering from the effects of trypsinization as they were rotating at 200 rpm. Results generally indicated that DON caused a small, insignificant inhibition of cell-cell aggregation into clumps, with diameters which ranged from 10 to 20 um (Table 10) and with diameters greater than 20 um (Table 11). There was no difference detected between the morphologies of control and treated clumps.

Table 9

C-4II: AGGREGATION OF CELLS TREATED WITH DON AFTER TRYPSIN/EGTA DISSOCIATION[a]

Time of DON Addition[b] (Hrs after Dissociation)	Incubation Time (Hours) with DON[c]	% of Cells and Clumps with Diameter Range (um)[d]					
		12-14	>14-16	>16-18	>18-20	>20-25	>25
Part i							
control	0	11.9	34.6	30.9	14.9	6.0	1.7
DON, 0 hours	24	19.1	36.7	25.4	12.0	5.6	1.2
Part ii							
control	0	20.9	38.1	28.9	12.2		
DON, 6 hours	18	31.6	39.9	20.1	8.4		
DON, 12 hours	12	37.0	37.7	18.2	7.1		
Part iii							
control	0	17.4	28.6	29.5	16.5	6.0	2.2
DON, 18 hours	6	14.9	33.0	31.2	14.9	5.1	0.8
Part iv							
control	0	14.8	33.2	29.5	16.6	5.2	0.6
DON, 24 hours	0	22.1	38.1	25.9	13.8	6.7	0.9

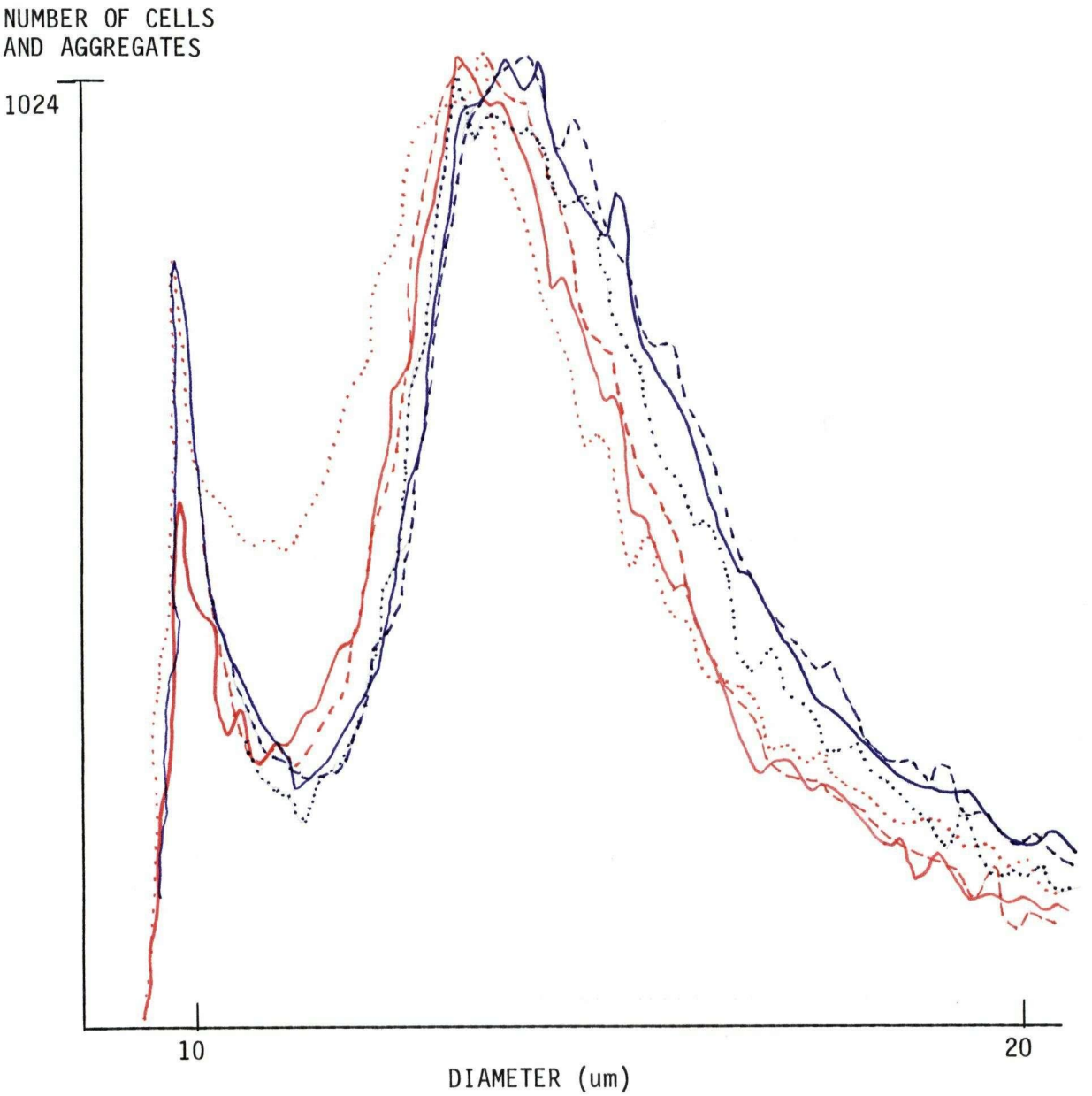
[a] Single cells were shaken for 24 hours at 200 rpm, and then for another 24 hours at 70 rpm.
[b] Treatments (1 ug/ml DON) were added at various times after subculture (trypsin/EGTA dissociation), while cultures were shaken at 200 rpm.
[c] Incubation time (hours) in the presence of DON, at the end of 24 hours of shaking at 200 rpm.
[d] All samples were sized live on a Coulter counter at the end of the experiment (i.e. 24 hours at 200 rpm followed by another 24 hours at 70 rpm).

Parts:
i...representative of 2 additional experiments
ii,iv...this experiment was not duplicated
iii...representative of 4, out of 5, additional experiments

Fig. 18

C-4II: AGGREGATION OF CELLS TREATED WITH DON AFTER TRYPSIN/EGTA DISSOCIATION[a]

(after 24 hours shaking at 200 rpm, and another 24 hours at 70 rpm.)
(Tracing of coulter counter sizing)



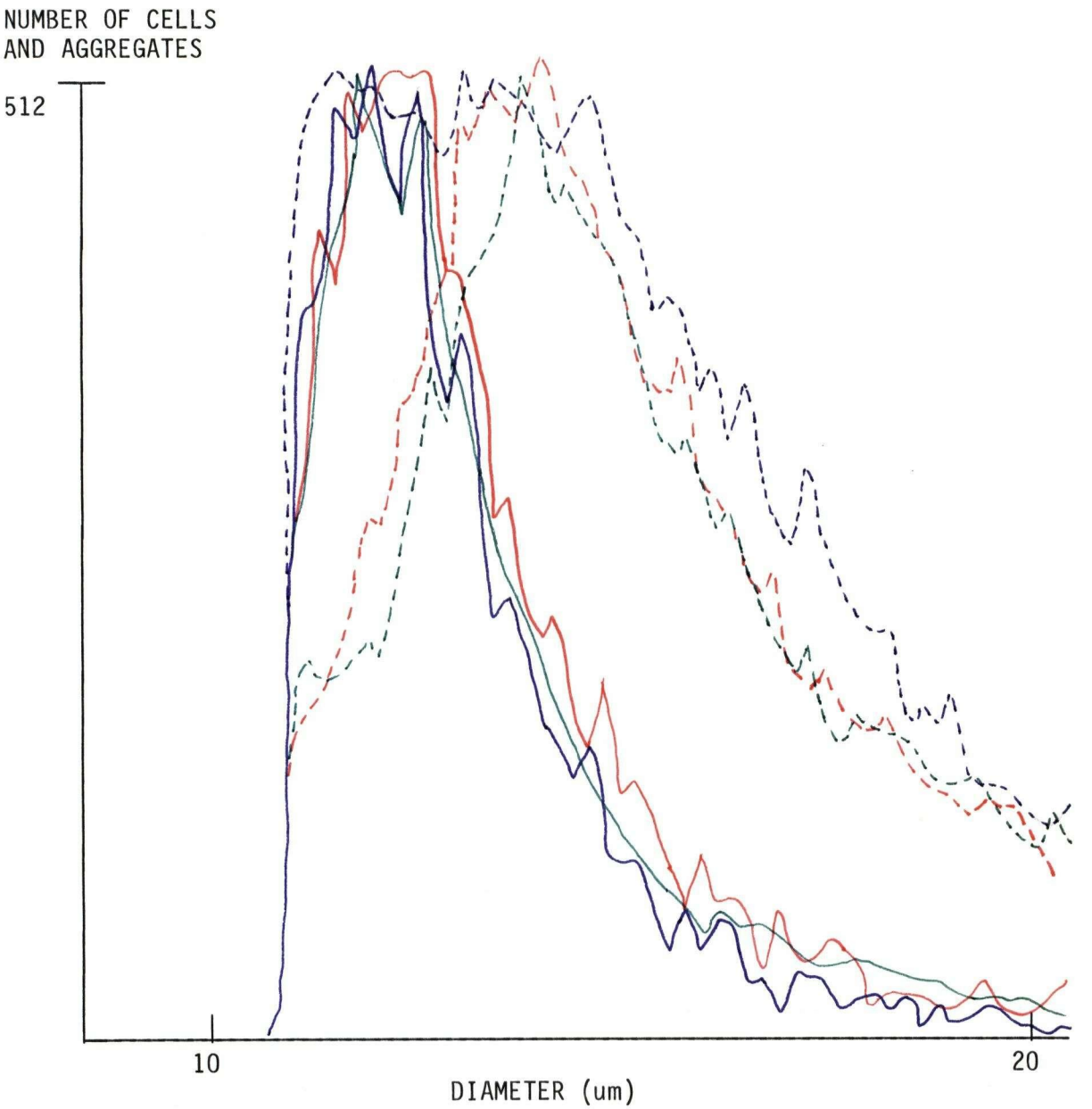
Legend:

[a] 1 ug/ml of DON was added immediately after dissociation (see Table 9, part i).

Control sample 1
Control sample 2
Control sample 3
DON-treated sample 1
DON-treated sample 2
DON-treated sample 3

Fig. 19

C-4II: AGGREGATION OF CELLS TREATED WITH DON OR DON PLUS GSA AFTER TRYPSIN/EGTA DISSOCIATION[a]
(Tracing of coulter counter sizing)



Legend:

[a] 1 ug/ml of DON (see Table 9, part iii) and/or 100 ug/ml GSA was added 18 hours after dissociation. After 24 hours shaking at 200 rpm:

——— Control sample
——— DON-treated sample
——— DON + GSA sample

After 24 hours shaking at 200 rpm, followed by another 24 hours at 70 rpm:

- - - - Control sample
- - - - DON-treated sample
- - - - DON + GSA sample

Table 10

C-4I: AGGREGATION OF CELLS TREATED WITH DON AND/OR GSA AFTER TRYPSIN/EGTA DISSOCIATION[a]

Treatment[b]	% of Cells and Clumps with diameter range (um)[c]				
Expt #1	10-12	>12-14	>14-16	>16-18	>18-20
control		28.6	32.4	26.3	12.6
DON		32.9	38.1	20.1	8.9
DON + GSA		30.9	39.5	20.5	9.1
Expt #2					
control	8.3	29.3	25.9	22.7	13.9
DON	7.5	31.8	26.8	22.6	11.4
DON + GSA	10.6	40.0	25.6	16.3	7.5
Expt #3					
control	7.9	30.9	30.2	21.9	9.1
DON	7.9	32.8	31.4	18.9	8.9
DON + GSA	5.7	26.6	33.4	23.2	11.0

[a] Based on 3 experiments.

[b] 1 ug/ml DON with or without 100 ug/ml glucosamine (GSA) added 18 hours after subculture while shaking at 200 rpm.

[c] Samples sized live on a Coulter counter, at the end of 24 hours at 200 rpm and another 24 hours at 70 rpm. Diameters (12-16)um represent mainly single cells.

Table 11

C-4I: EFFECT OF GSA ON AGGREGATION OF CELLS TREATED WITH DON AFTER TRYPSIN/EGTA DISSOCIATION

(% clumps of more than 20 um diameter)

Treatment	% Sized in the Diameter Range (um)[a]		
	(20)-(<43) um	(43)-(<78) um	>78 um
Expt. A			
control	76.2	22.2	1.6
DON[b]	77.5	21.3	1.3
DON + 100 GSA[c]	72.7	24.9	2.5
DON + 200 GSA	70.4	26.4	3.3

Expt. B	<20 um	(20)-(<43) um	(43)-(<78) um	>78 um
control	67.5	24.9	4.1	3.5
DON	76.6	20.8	1.8	0.9
DON + 100 GSA	46.5	47.6	3.6	2.3

[a] Based on 2 experiments, duplicate or triplicate samples per treatment. Cells and clumps were sized on an Artek counter; area setting = 482, sensitivity of 730 and 590, at least 8 fields of 4 mm^2 each were counted. Samples were sized fixed (2% glutaraldehyde in Millonig's buffer) and stained (4% eosin). Diameter range settings were set using standard calibration particles (Coulter Electronics).

[b] 1 ug/ml DON was added 18 hours after trypsinization.

[c] ug/ml GSA (glucosamine) added, 18 hours after trypsinization.

Conclusion:

With DON present only during recovery from trypsinization, there was a borderline effect on cell aggregation after 24 hours of slow shaking in both cell lines, irregardless of the duration of DON treatment.

3. Effects of glusosamine on aggregation of cells treated with DON after trypsin/EGTA dissociation

In vitro, DON had been shown to inhibit adhesion of rat palatal shelves (Greene and Pratt, 1977), to decrease the synthesis of sulfated glycosaminoglycans by embryonic chick heart fibroblasts (Spooner and Conrad, 1977), and to decrease migration of embryonic heart cells (Funderburg and Markwald, 1981). These authors also used glucosamine (GSA) to counteract these inhibitory effects of DON, since DON inhibits the synthesis of glucosamine-6-phosphate. Their results indicated that glucosamine either partially or fully reversed the effects of DON.

In this study, it was previously concluded that in both C-4 cell lines, DON had a borderline inhibitory effect on the aggregation of trypsin-recovered cells (Figs. 18 and 19 showed that in C-4II cells, this inhibitory effect was small but consistent). Hence, in this study, GSA was added simultaneously with DON to shaker cultures to determine what effects, if any, GSA had on cell aggregation in the absence or presence of DON.

C-4I:

Shaker cultures were sized live at the end of each experiment on a Coulter counter (Tables 10 and 11). These results showed that for clumps less

than 20 um in diameter, 100 ug/ml of GSA had no effect in 2 experiments, but increased aggregation beyond that of control cells or DON-treated cells in experiment #3 (Table 10; ~~Fig. 19~~). Since many C-4I clumps were as large as 78 um in diameter, and the Coulter counter best sized particles less than 40 um in diameter, the large clumps of the cultures were sized on an Artek counter after they were fixed and stained. Of a total of 3 experiments, clump sizes in only two experiments were determined (Table 11), as one experiment contained too much debris. These results indicated that there was either partial reversal (Table 11, experiment B) of inhibition of aggregation in DON + GSA cultures, or that the addition of GSA increased aggregation of cells beyond that observed in control or DON-treated cultures (Table 11, experiment A).

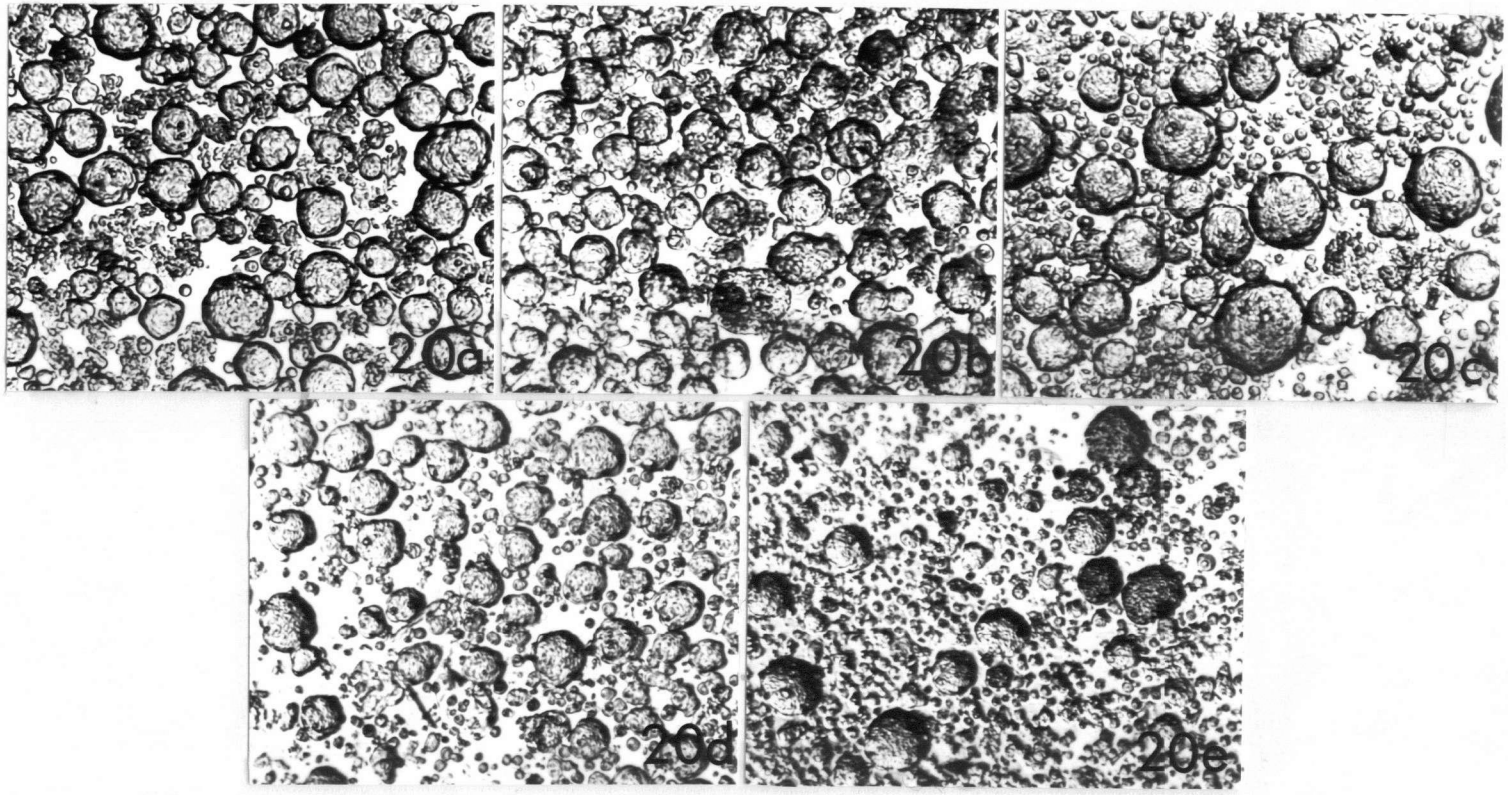
In other experiments where 10, 50 and 200 ug/ml of GSA were used, there was no consistent trend indicating that GSA increased aggregation.

However, it was observed that all clumps, irregardless of treatment, were round and large (Figs. 20a-e), with the possible exception of those treated with DON only, which appeared to have a more irregular outline.

C-4II:

In six experiments in which 100 ug/ml GSA was added concurrently with DON, there was no clearcut indication that GSA increased aggregation (Fig. 19).

Of three experiments in which control cultures were compared to GSA-treated cultures (10, 100 and 200 ug/ml), only one experiment indicated that at the end of 24 hours of rapid shaking in which cultures were treated with GSA for 6 hours, the GSA-treated cultures were more aggregated than the control cultures.



Figs. 20a-e. C-4I: Aggregation of cells pretreated with DON after trypsin/EGTA dissociation. Drugs were added 18 hours after trypsinization. Cells were fixed with 2% glutaldehyde in Millonig's buffer and stained with 0.4% eosin. x 90.

Fig. 20a Control culture

Fig. 20b 1 ug/ml DON

Fig. 20c 1 ug/ml DON + 10 ug/ml GSA

Fig. 20d 1 ug/ml DON + 50 ug/ml GSA

Fig. 20e 1 ug/ml DON + 100 ug/ml GSA

Conclusion:

There was no clear cut indication that GSA, when added with DON to C-4I and C4II cultures, increased aggregation of cells beyond that which occured in cultures treated with DON only except, possibly, for large C-4I clumps; however, GSA reverted the effect of DON on the shape of C-4I aggregates and thus, presumably, on the deformability and/or ability to spread of C-4I cells.

V. Morphology of single adherent cells treated with 1 ug/ml DON

Among controls in both cell lines, the C-4II cell line had a higher percentage of single fully spread cells, which indicated that C-4II cells were more interactive with the substratum (Auersperg, 1969a).

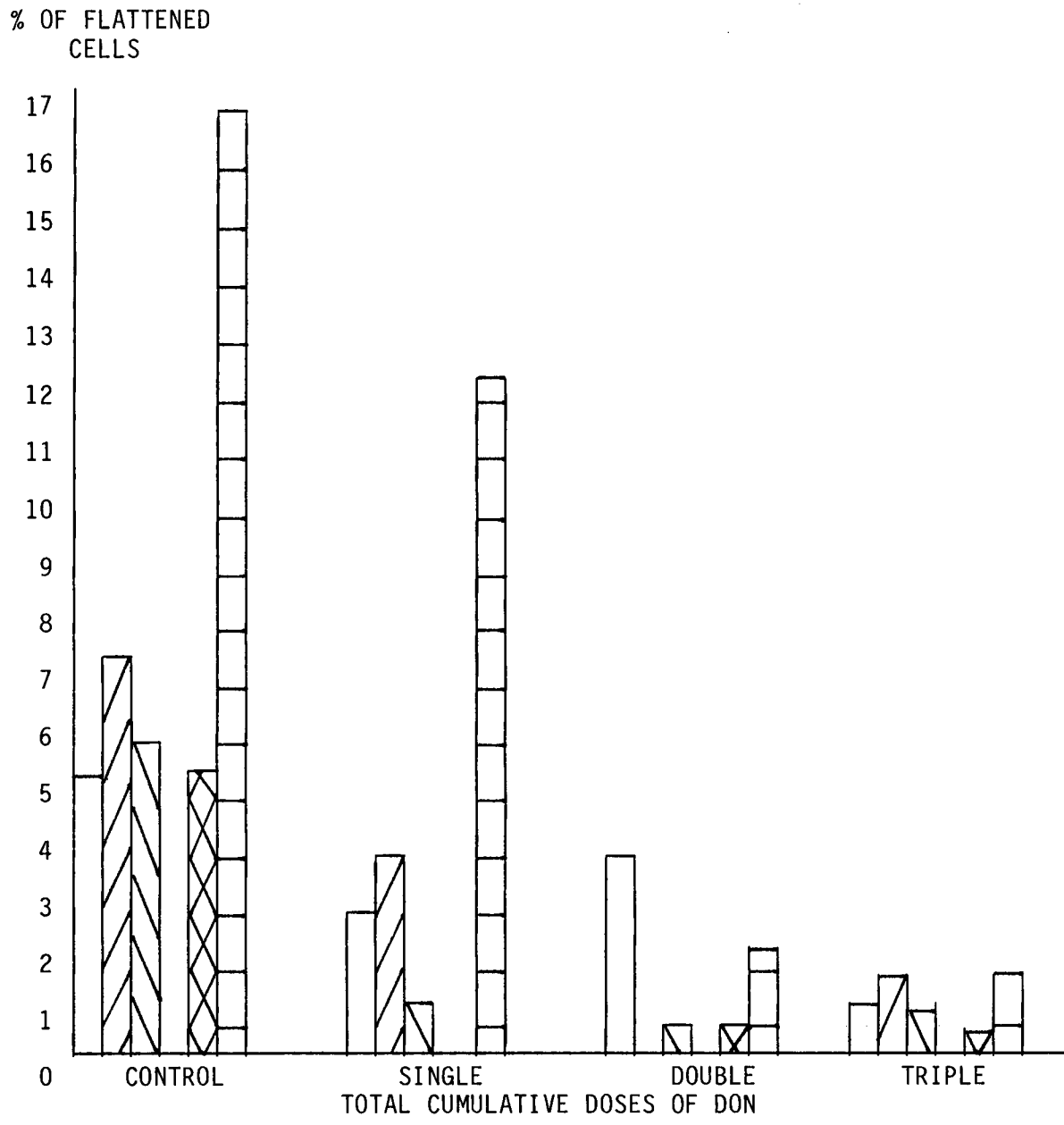
To determine the effect of DON on cell-substratum interactions, the morphologies of single cells adhering to the plastic substratum were quantitated; since cell morphology was indicative of substratum adhesiveness and of cell-substratum interactions (Auersperg, 1969a; Harris, 1973). Single cells were classified into three types of morphologies (Fig. 12), of which flattened cells were most substratum interactive, irregularly-shaped cells were less interactive, and spherical cells were least interactive.

In both cell lines, increasing concentrations of DON caused proportional decreases in the percentage of cells that were flattened (Fig. 21). ANOVA analysis (Petkau and Crapeau, 1983) showed that these decreases were statistically significant.

The decrease in percentage of cells that were flattened in response to 1 dose of 1 ug/ml DON treatment was greater in C-4II cultures than in C-4I cultures (Fig. 21). Most of the DON-treated C-4II cells remained partly

Fig. 21

EFFECT OF DON ON THE MORPHOLOGY OF SINGLE CELLS



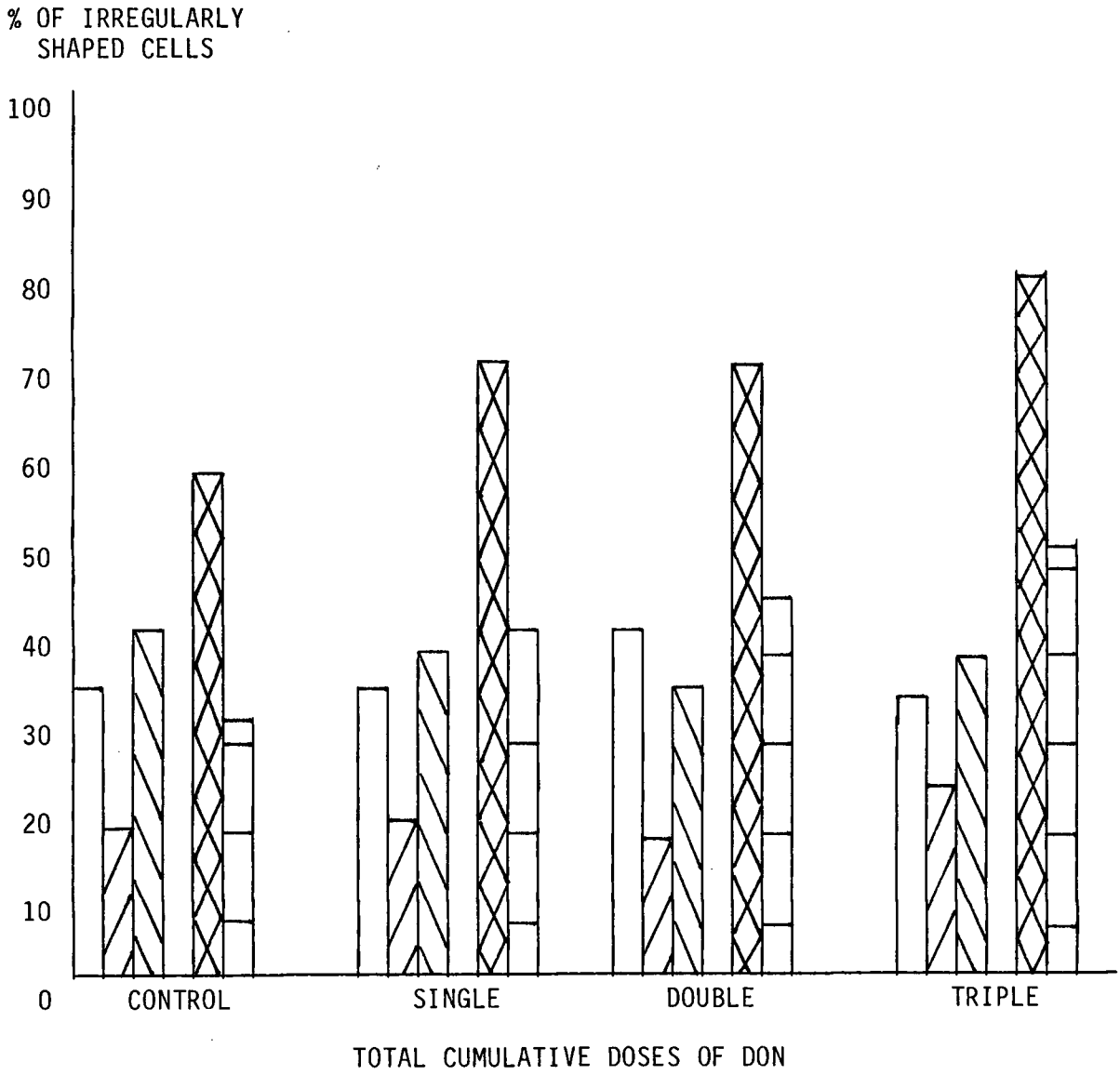
Key:

- C-4I EXPT. 1
- C-4I EXPT. 2
- C-4I EXPT. 3
- C-4II EXPT. 1
- C-4II EXPT. 2

Each dose was equivalent to 2 ug of DON (i.e. 1 ug/ml). The first dose was added on day 1 after plating. The second dose was added on day 2, while the third dose was added on day 3. Cultures were fixed on days 4-5.

Fig. 22

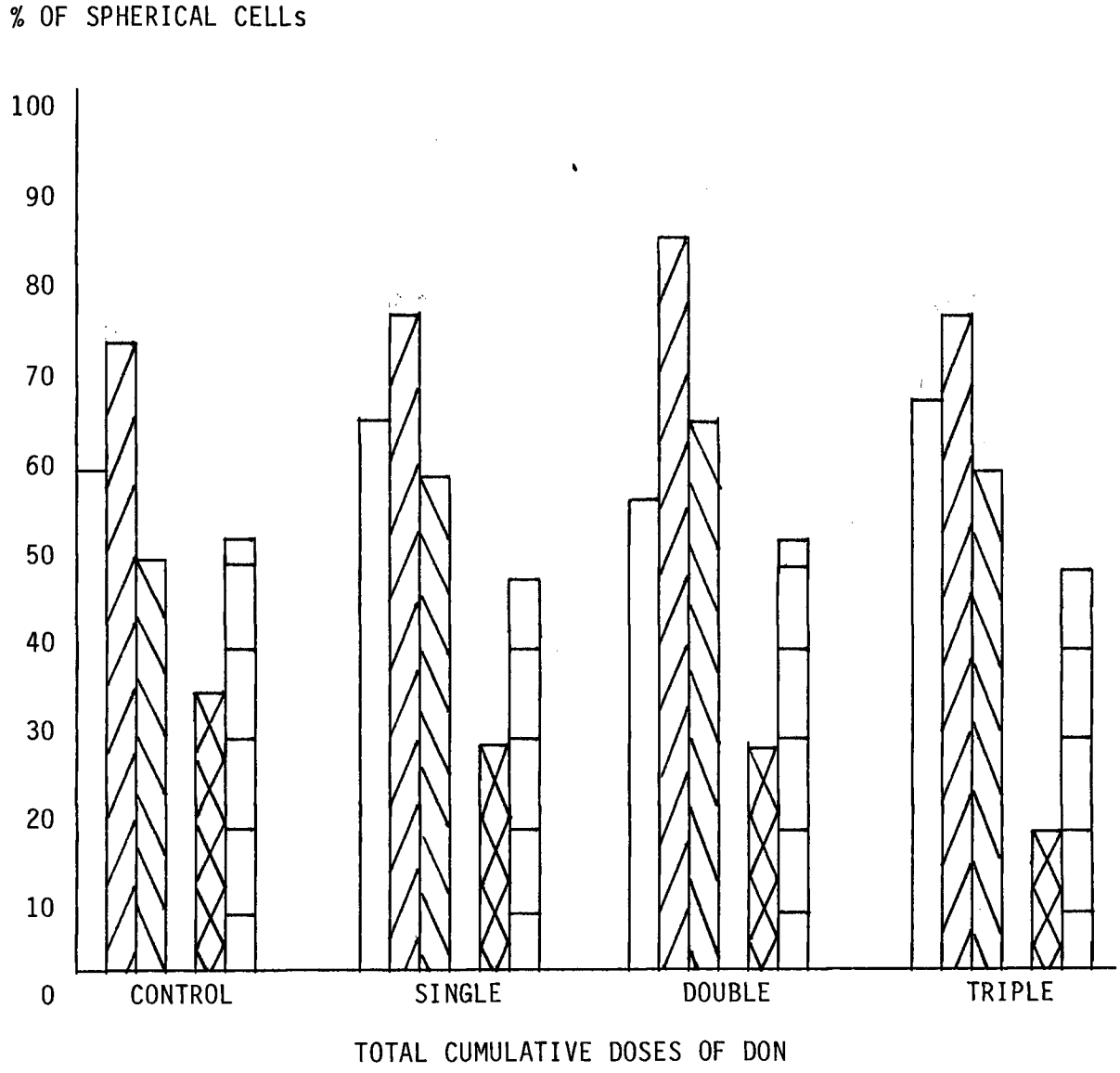
EFFECT OF DON ON THE MORPHOLOGY OF SINGLE CELLS



Each dose was equivalent to 2 ug of DON (i.e. 1 ug/ml). The first dose was added on day 1 after plating. The second dose was added on day 2, while the third dose was added on day 3. Cultures were fixed on day 4-5.

Fig. 23

EFFECT OF DON ON THE MORPHOLOGY OF SINGLE CELLS



Each dose was equivalent to 2 ug of DON (i.e. 1 ug/ml). The first dose was added on day 1 after plating. The second dose was added on day 2, while the third dose was added on day 3. Cultures were fixed on days 4-5.

adherent or irregularly-shaped (Fig. 22) and did not round up completely (Fig. 23). In contrast, the decrease in percentage of flattened C-4I cells was accompanied by an increase in percentage of spherical cells (Fig. 23).

Conclusion:

DON significantly decreased the spreading and adhesion of cells in both cell lines. However, C-4II cells showed a greater decrease in percentage of flattened cells.

If DON decreased cell-substratum adhesion and if C-4II cells were more cell-substratum interactive, then DON should affect adhesion of C-4II cells more than C-4I cells. The results of these experiments support this conclusion.

VI. Cell area

In addition to changes in colony form, there were also changes in projected cell areas (projected two dimensionally) in cultures treated with DON (Figs. 7a-b, 8a-b, 9a-c, 10a-b and 11a-b).

In small C-4I and C-4II colonies, the changes in areas of DON-treated cells were variable, although in general, DON-treated cells tended to be larger than control cells (Tables 12 and 13).

DON-treated large C-4I colonies tended to have stratified rims or edges (Figs. 7a-b) and flatter colony centers (Figs. 7a-b and 8a-b). Therefore, the average cell area of large colonies was calculated by determining the area of the center of the colony only and dividing this area by the number of nuclei (cells) in that area.

Table 12

C-4I: CHANGE IN CELL AREA[a]

Total number of doses of 1 ug/ml DON added	Time of DON addition[b] (days after plating)	Average cell area in small colonies ($x10^{-3}$ mm^2) day 4 cultures	day 6-7 cultures
control	-	2.51	2.20
1	2	2.37	2.16
1	1	2.48	2.46
1	0	2.48	2.50
2	1 + 2	2.60	1.94
3	1, 2, 3	2.64	2.46
Total number of doses of 1 ug/ml DON added	**Time of DON addition[b] (days after plating)**	**Average cell area in large colonies ($x10^{-3}$ mm^2) day 4 cultures**	**day 6-7 cultures**
control	-	2.19	1.93
1	2	3.22*	2.69*
1	1	2.74*	2.59*
1	0	2.70*	2.49*
2	1 + 2	3.42*	3.02*
3	1, 2, 3	3.65*	3.34*

[a] Projected cell areas ($x10^{-3}$ mm^2). Using a camera lucida, colonies were projected and analyzed by a digital image analyzer (Zeiss MOP 3). In small colonies (5-35 cells), the entire colony area was projected and the number of cells (nuclei) counted. In large colonies (more than 50 cells), the cell area was calculated by dividing the area of centers of colonies by the number of cells (nuclei) in the center (since some colonies had a stratified edge or rim, the cells in the colony edge or rim were omitted).

[b] Doses of 1 ug/ml of DON were added at various days after plating or subculture; day 0 was the day of plating.

* The difference between the average cell area of treated cells and that of control cells was statistically significant. The data was analyzed using analysis of variance and Newman-Keuls tests, at probability levels of 0.05.

Table 13

C-4II: CHANGE IN CELL AREA[a]

Total number of doses of 1 ug/ml DON added	Time of DON addition[b] (days after plating)	Average cell area in small colonies ($x10^{-3}$ mm^2) day 4 cultures		day 6-7 cultures
		Expt. (i)	Expt. (ii)	
control	-	2.04	0.99	1.46
1	2	2.73*		1.33
1	1		1.67*	1.44
1	0	2.31*	1.18	1.47
2	1 + 2	3.17*	2.19*	1.70*
3	1, 2, 3		1.88*	2.00*
Total number of doses of 1 ug/ml DON added	**Time of DON addition[b] (days after plating)**	**Average cell area in large colonies ($x10^{-3}$ mm^2) day 4 cultures**		**day 6-7 cultures**
		Expt. (i)	Expt. (ii)	
control	-	1.76	1.29	1.17
1	2	2.87*		1.28
1	1		2.10*	1.36
1	0	2.07*	1.40	1.42
2	1 + 2	3.99*	2.84*	1.72*
3	1, 2, 3		2.74*	1.95*

[a] Projected cell areas ($x10^{-3}$ mm^2). Using a camera lucida, colonies were projected and analyzed by a digital image analyzer (Zeiss MOP 3). In small colonies (5-35 cells), the entire colony area was projected and the number of cells (nuclei) counted. In large colonies (more than 50 cells), the cell area was calculated by dividing the area of centers of colonies by the number of cells (nuclei) in the center (since some colonies had a stratified edge or rim, the cells in the colony edge or rim were omitted).

[b] Doses of 1 ug/ml of DON were added at various days after plating or subculture; day 0 was the day of plating.

* The difference between the average cell area of treated cells and that of control cells was statistically significant. The data was analyzed using analysis of variance and Newman-Keuls tests, at probability levels of 0.05.

Results indicated that in the centers of large C-4I colonies, all DON-treated cells were significantly larger than control cells, irregardless of length of culture or treatment (Table 12). In large C-4II colonies, DON-treated cells in all cultures were also larger than control cells, but the changes were significant mainly in cultures treated with multiple doses of DON (Table 13).

VII. Cell volume

Previous results on cell area indicated that DON, when added to C-4 cultures, increased the projected surface area of cells. Most of these changes were statistically significant in large colonies.

To determine if DON increased the surface area of cells by increasing cell volumes, cells were sized on a Coulter counter. C-4 cultures were treated with DON one day after plating. On the fourth day after plating, the cultures were dissociated using trypsin/EGTA. Previous results, as well as the results of Dembitzer et al. (1980a), indicated that such treatments produced mainly single cells. In this study, only 1.3% of control C-4I particles sized were greater than 20 um in diameter (Table 14), and only approximately 1% of control C-4II particles counted were of that size (Table 15).

In both cell lines, DON increased cell volume. This increase was generally proportional to the number of doses of DON given (Tables 14 and 15: see modal diameters and volumes). Also in both cell lines, cells in DON-treated cultures were not normally distributed, but instead, they were positively skewed.

Coulter counter plots of dissociated C-4 cells not only indicated the shapes of the cell distributions, but also indicated the modal diameters

Table 14

C-4I: EFFECT OF DON ON CELL VOLUME

Diameter range (um)	Percentage of all cells sized[a]					
	Control		DON treatment[b]			
			1 dose		2 doses	
	(i)	(ii)	(i)	(ii)	(i)	(ii)
<8-10	3.6	2.0	3.0	2.5	3.6	4.4
>10-12	11.9	9.5	4.9	6.6	4.5	6.4
>12-14	23.3	28.6	16.2	21.4	15.0	13.9
>14-16	33.4	31.6	33.7	31.2	32.4	23.9
>16-18	19.6	20.5	26.2	27.0	27.3	30.9
>18-20	8.3	7.7	15.9	11.4	17.1	20.5
Modal diameter[c] (um)(± S.D.)	13.60 (±0.57)	13.80 (±0.36)	14.65 (±0.22)	14.60 (±0.28)	14.65 (±0.22)	16.10 (±0.92)
Modal volume (um^3)	1308	1367	1652	1618	1652	2170
% counts greater than 20um	-	1.3	-	1.9	-	4.3

[a] Duplicate cultures per treatment (except for DON treatment, 2 doses, experiment (ii). Four day cultures were sized on a Coulter counter, after being dissociated (with trypsin/EGTA) into single cells.

[b] Cultures were treated with DON at a concentration of 1 ug/ml. 1 dose-treated cultures were treated with DON 1 day after plating; while 2 dose-treated cultures were treated with 1 dose of DON 1 day after plating and with a second dose of DON 2 days after plating.

[c] Modal diameter as determined from plots of cell sizes, on a Coulter counter. ± S.D. or ± standard deviation. Modal volumes were determined from modal diameters.

(i), (ii) ... Two separate experiments.

Table 15

C-4II: EFFECT OF DON ON CELL VOLUME

Diameter range (um)	Percentage of all cells sized[a]					
	Control		DON treatment[b]			
			1 dose		2 doses	
	(i)	(ii)	(i)	(ii)	(i)	(ii)
<8-10	2.7	4.4	5.6	4.2	6.0	6.2
>10-12	4.4	8.7	4.2	4.9	3.0	3.7
>12-14	27.0	34.7	20.6	26.6	9.5	9.4
>14-16	38.7	32.9	38.7	36.6	26.7	26.5
>16-18	21.1	14.9	19.9	21.0	33.5	32.7
>18-20	6.1	4.4	11.0	6.7	21.3	21.5
Modal diameter[c] (um)(± S.D.)	14.0 (±0.18)	13.7 (±0.02)	14.8 (±0.02)	14.1 (±0.06)	15.9 (±0.02)	16.0 (±0)
Modal volume (um^3)	1,440	1,330	1,700	1,470	2,120	2,130
% counts greater than 20um	0.9%	1.0%	1.3%	1.5%	2.5%	2.7%

[a] Duplicate cultures per treatment. Four day cultures were sized on a Coulter counter, after being dissociated (with trypsin/EGTA) into single cells.

[b] Cultures were treated with DON at a concentration of 1 ug/ml. 1 dose-treated cultures were treated with DON 1 day after plating; while 2 dose-treated cultures were treated with 1 dose of DON 1 day after plating and with a second dose of DON 2 days after plating.

[c] Modal diameter as determined from plots of cell sizes, on a Coulter counter. ± S.D. or ± standard deviation. Modal volumes were determined from modal diameters.

(i), (ii) ... Two separate experiments.

as represented by the peaks of graphical plots. In C-4I cultures (Table 14), the average modal diameter of control cells was approximately 13.70 um. However, in C-4II cultures (Table 15), the average modal diameter of control cells was approximately 13.85. Thus, the results indicated that the majority of C-4II cells were slightly larger and less variable than C-4I cells.

Comparing modal diameters of single-dose treated cells, C-4I treated cells had a diameter of approximately 14.63 um, which was similar to that of C-4II cells of 14.45 um. However, when cells were treated with 2 doses, C-4II cells had a diameter of approximately 15.95 um, while C-4I cells had a smaller diameter of about 15.38 um.

VIII. Summary of the properties of control C-4 cells used in this study

The properties of control C-4 cells are summarized and presented in Table 16.

1. The growth pattern of C-4I cultures depended on the dissociation method, or on the maintenance of cell-cell contacts.
2. The growth patterns of C-4 cells were substratum-independent. If C-4I cell sheets were suspended in culture medium after trypsinization, they tended to retract into round and cohesive clumps (Figs. 24 a-c). In contrast, C-4II cell sheets remained generally irregular and only retracted to a limited extent (Figs. 25 a-c).
3. C-4II colonies, irregardless of size, were always more irregular than C-4I colonies. In general, large C-4II colonies were more irregular than small colonies. In contrast, large and small C-4I colonies were equally round.

4. C-4I cultures shed fewer cells (50% were live) than C-4II cultures (over 80% were live). Among C-4II cultures, confluent cultures shed more than non-confluent ones.
5. Shed C-4I cells were less proliferative than shed C-4II cells (Auersperg, 1969a).
6. Aggregated C-4II clumps were small and irregular (Fig. 17a), while C-4I clumps were larger, round and compact (Fig. 16a).
7. Single C-4I cells were less flattened on a plastic substratum than C-4II cells. In contrast, in the C-4II cell line, there were fewer spherical, more-irregularly shaped and more flattened cells. Hence, C-4II cells were more interactive with the substratum than C-4I cells.
8. In both cell lines, cells in small colonies had larger projected cell surface areas than those in large colonies.
9. Cell diameter and volume of C-4I cells were slightly smaller than that of C-4II cells.

IX. Summary of the effects of DON treatment on C-4I and C-4II cells

The effects of DON treatment on C-4I and C-4II cells are summarized and presented on Tables 17 and 18 respectively.

Briefly, both C-4 cells showed qualitatively similar effects on treatment with DON. For example:

1. Colonies were generally more irregular,
2. More cells were shed into culture medium,
3. Cells were less cohesive as shown by "holes" within colonies,
4. Single cells were less spread/interactive on plastic substratum, and
5. Cells were larger, both in projected cell surface area and volume.

In addition, properties of DON-treated C-4I cells resembled those of C-4II cells. For example:

1. C-4I colonies grew irregularly,
2. C-4I cultures shed more viable cells,
3. C-4I cell in shaker cultures aggregated less, and
4. Aggregated C-4I clumps (when treated prior to dissociation) were irregular and small.

Table 16

SUMMARY OF PROPERTIES OF CONTROL C-4I AND C-4II CELLS

		C-4I	C-4II
1.	Colony form if seeded as:		
	(i) clumps	round	irregular
	(ii) single cells	irregular	irregular
2.	Cell sheets (clumps) hours after trypsinization	round-up	remain irregular
3.	Colony form* of:		
	(i) small colonies	0.85 (.81-.91)	0.74 (.67-.79)
	(ii) large colonies	0.85 (.81-.89)	0.66 (.47-.78)
4.	Cell shedding	0.5% shed (50% live)	non-confluent: 1.2% shed (82% live) confluent: 2.0% shed (87% live)
5.	Shed cell proliferation	little	more
6.	Cell-cell aggregates	round, large, compact	irregular, smaller, less compact
7.	Morphology** of single cells (percentage of total cells):		
	(i) spherical	50.2-72.8	34.1-51.7
	(ii) irregularly-shaped	19.6-43.3	31.2-60.3
	(iii) flattened	5.4-7.6	5.6-17.1
8.	Cell area* ($x10^{-3}$ mm^2) in:		
	(i) small colonies	2.7 (2.2-3.1)	1.6 (1.0-2.0)
	(ii) large colonies	2.4 (1.9-3.1)	1.4 (1.2-1.8)
9.	Cell diameter (um)	13.6-13.8	13.7-14.0
	(±standard deviation)	(±0.57-±0.36)	(±0.02-±0.18)
	Cell volume (um^3)	1308-1367	1330-1440

* Mean of MOP data of all experiments. Range of values are presented within brackets.

** Values are range of percentage of separate experiments (Figs. 21-23).

Figs. 24 and 25. C-4I and C-4II cell clumps, hours after trypsinization, respectively:

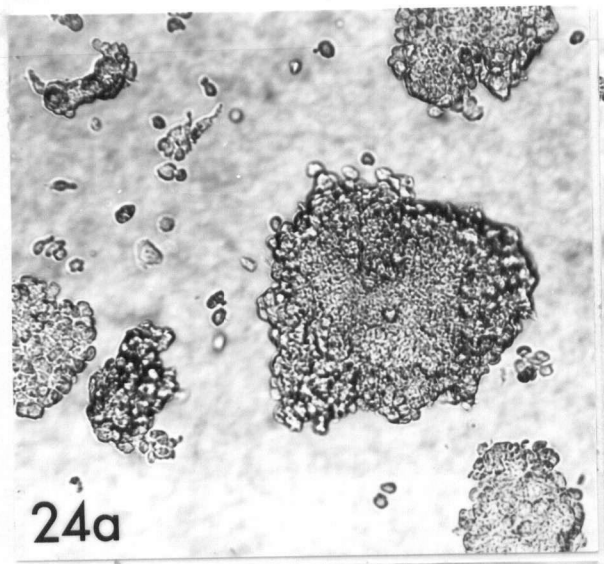
Figs. a. 0 hour after trypsinization, x 135

Figs. b. 1 hour after trypsinization, x 225

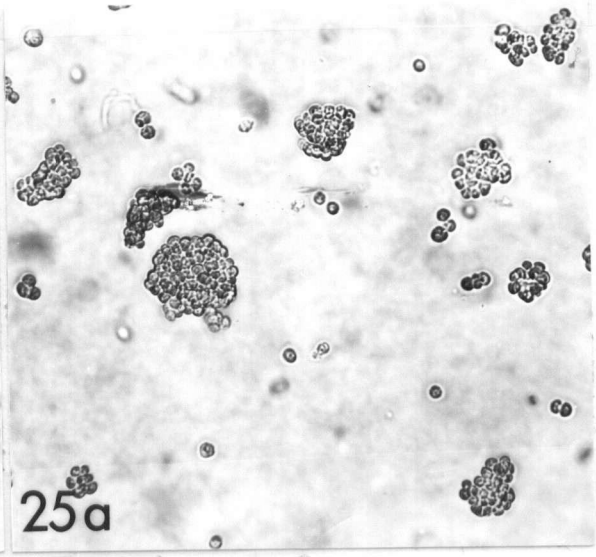
Figs. c. 3 hours after trypsinization, x 225

C-4I

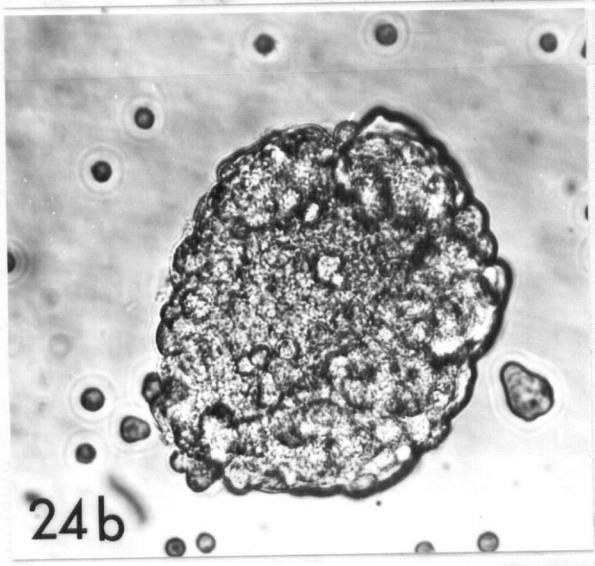
C-4II



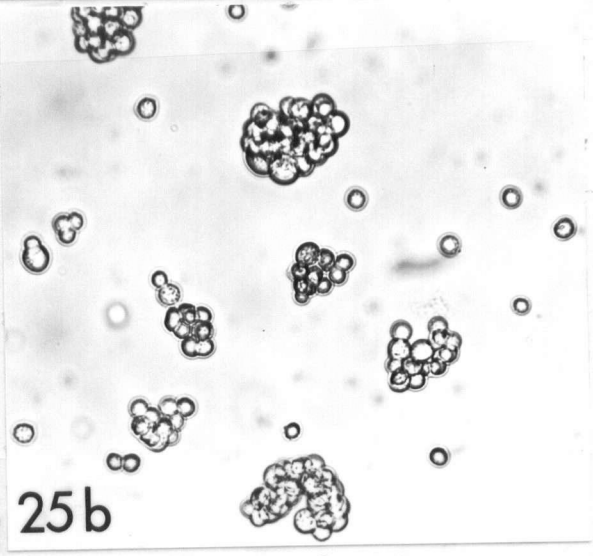
24a



25a



24b



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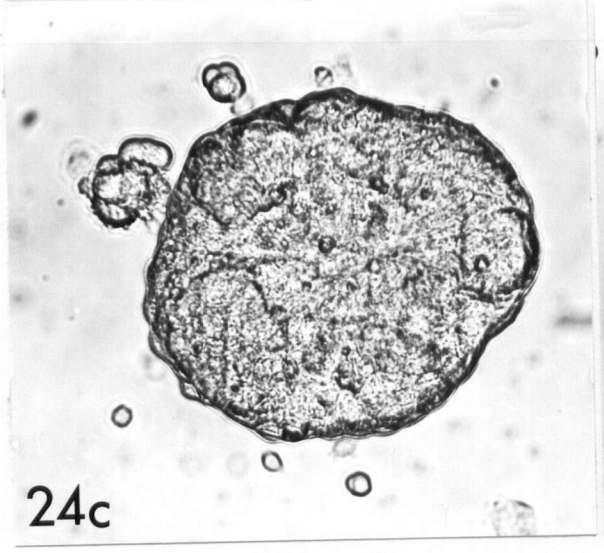
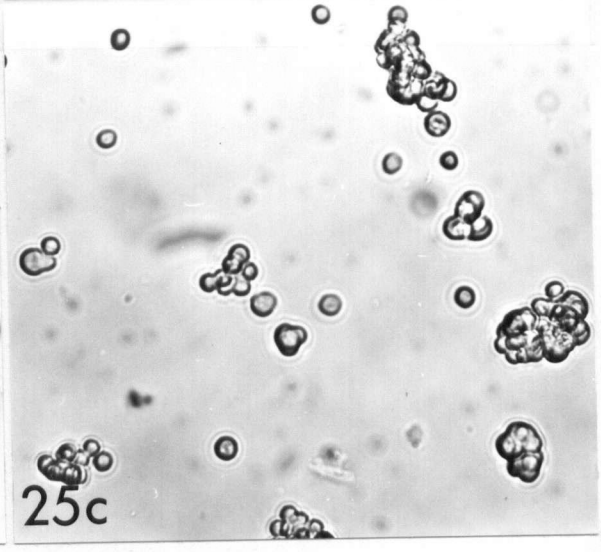


24c



25c

Table 17

SUMMARY OF THE EFFECTS OF DON TREATMENT ON C-4I CELLS

	CONTROL	DON	
		LOW DOSE	HIGH DOSE
Growth inhibition	-	+	++
Colony form* of:			
(i) small colonies	round (.81-.91)	rounder (short-term)/ irregular (long-term)	irregular
(ii) large colonies	round (.81-.89)	more irregular	most irregular
Cell shedding	little; 50% live	6 fold increase; 78% live	
Aggregation of cells treated with DON:			
(i) prior to dissociation	aggregation; round clumps	less aggregation; irregular clumps	
(ii) after dissociation	aggregation; round clumps	less aggregation; round clumps	
Morphology** of single cells (Percentage of total number of cells):			
(i) spherical	61% (50.2-72.8)	more (57.6-77.6)	most (54.5-84.3)
(ii) irregularly-shaped	33% (19.6-43.3)	less (17.9-39.8)	less (15.7-41.5)
(iii) flattened	6% (5.4-7.6)	less (0-4.5)	least (0-4.1)
Cell area* ($x10^{-3}$ mm^2) in:			
(i) small colonies	2.7 (2.2-3.1)	variable, generally larger	generally larger
(ii) large colonies	2.4 (1.9-3.1)	all significantly larger	all significantly larger
Cell volume (um^3)	1,308-1,367	22% larger	43% larger

* Mean of MOP data of all experiments. Range of values are presented within brackets.

** Range of values of separate experiments are presented within brackets.

Table 18

SUMMARY OF THE EFFECTS OF DON TREATMENT ON C-4II CELLS

	CONTROL	DON	
		LOW DOSE	HIGH DOSE
Growth inhibition	-	+	++
Colony form* of:			
(i) small colonies	irregular (.67-.79)	inconclusive	most irregular
(ii) large colonies	irregular (.47-.78)	more irregular	most irregular
Cell shedding of:			
(i) non-confluent cultures	1.2% shed; most live	2 fold increase; most live	
(ii) confluent cultures	2.0% shed; most live	5 fold increase; most live	
Aggregation of cells treated with DON:			
(i) prior to dissociation	aggregation; irregular clumps	aggregation; irregular clumps	
(ii) after dissociation	aggregation; irregular clumps	less aggregation (borderline); irregular clumps	
Morphology** of single cells (Percentage of total number of cells):			
(i) spherical	43% (34.1-51.7)	more (28.5-65.1)	less (19.0-51.9)
(ii) irregularly-shaped	46% (31.2-60.3)	more (27.7-71.6)	most (45.8-80.7)
(iii) flattened	11% (5.6-17.1)	less (0-12.4)	least (0.9-2.3)
Cell area* ($x10^{-3}$ mm^2) in:			
(i) small colonies	1.6 (1.0-2.0)	variable, generally larger	all significantly larger
(ii) large colonies	1.4 (1.2-1.8)	all larger	all significantly larger
Cell volume (um^3)	1,330-1,440	13% larger	51% larger

*, ** See legend of Table 17.

DISCUSSION

Since its discovery in 1956 (Ehrlich et al., 1956), DON has been characterized and its biological activities analyzed (Dion et al., 1956; Maxwell and Nickel, 1957). However, there have been few reports on the effects of DON on the morphology of cultured cells. This study examines the effects DON has on two morphologically different types of cultured human cervical cancer cells, in an attempt to understand the basis for their different growth patterns. However, prior to examining the effects of DON, the properties of the control C-4I and C-4II cells will be discussed.

I. Control C-4I and C-4II cells (Table 16)

The growth patterns of certain cell types depend on the presence of calcium in the culture medium (Auersperg, 1969b; Hennings and Holbrook, 1983). In C-4I cultures, the maintenance of Ca^{++}-dependent cell-cell contacts greatly influenced their growth patterns. In contrast, C-4II cultures grew relatively independently of their cell-cell contacts. The cohesiveness of C-4I cells could be due to the presence of numerous desmosomes and microvilli (Auersperg, 1969a), which were less numerous in the less cohesive C-4II cell line.

The growth patterns of these cell lines were substratum-independent. The greater ability of C-4I cell sheets to retract into round clumps could, again, be due to the more numerous intercellular contacts, while the relative absence of intercellular contacts in C-4II cells could account for their relative inability to retract as cohesive units (Figs. 24 and 25). The forms of small and large colonies suggest that C-4II cells tend to exist relatively independently of each other as the colonies

grow, since large C-4II colonies were more irregular than small ones. In contrast, both small and large C-4I colonies tend to grow cohesively, since small colonies were as round as large ones.

Shedding patterns were different between the C-4 cell lines. Shedding of C-4I cells might be limited by their numerous cell-cell contacts. In contrast, C-4II cells have fewer intercellular contacts and this may be the reason why they shed more readily into the culture medium.

C-4II cells were more cell-substratum interactive, which might have accounted for the greater ability of shed C-4II cells to reattach and to proliferate. Many factors, such as serum factors and serum concentration, low molecular weight nutrients, plating density and anchorage dependence, regulate cell proliferation in culture (Lanks and Kasambalides, 1980). It has been documented that most cells in culture do not proliferate unless they can attach and spread on a surface (Stoker et al., 1968; Maroudas, 1973).

In both cell lines, cells in small colonies generally had larger projected cell surface areas (computed as area of colony/number of nuclei) than those in large colonies (Tables 12 and 13). This could be explained by the fact that in large C-4I colonies, cultures were more stratified (with more overlap of nuclei) while in C-4II colonies, cells in large colonies were more columnar with genuinely smaller apical cell surface areas. Since C-4I is a highly stratified cell line, the cell surface area was greatly underestimated because of multi-layering and overlapping of nuclei. In contrast, the estimated cell surface area in C-4II cultures was more valid since stratification in these cultures is limited. In small and large colonies, C-4I projected cell surface areas were always

larger than C-4II cell areas, suggesting that C-4I cells were also flatter than C-4II cells. This would relate well with previous observations (from vertical sections) that the morphologies of confluent C-4I cells ranged from flattened to polygonal, while the morphologies of confluent C-4II cells ranged from cuboidal to columnar (Auersperg, 1969a).

The volume of C-4I cells was slightly smaller than that of C-4II cells. Since the projected cell area of C-4I cells was larger than that of C-4II cells, C-4I cells also must have been flatter.

To summarize, the growth patterns of the two cell lines are influenced by both cell-cell and cell-substratum adhesions. However, the C-4I cell line is more influenced by cell-cell than by cell-substratum interactions, while the reverse is true for the C-4II cell line.

Multiple mechanisms of adhesion have been documented in many types of normal, embryonic and tumor cells and cell lines (Takeichi, 1977; Magnani et al., 1981; Hayashi and Ishimaru, 1981; Fischer and Schachner, 1982). Many cell types display different mechanisms of adhesion depending on the method of dissociation. Takeichi (1977) showed that cells with a calcium independent mechanism of adhesion formed less tightly adhering clumps with decreased intercellular contacts. Hayashi and Ishimaru (1981) isolated a cell adhesive factor from island-forming rat ascites hepatoma cells, but not from hepatoma cells that grew singly and did not form islands. When the latter cells were incubated with this adhesive factor, stable clumps with junctional complexes formed. Dembitzer (1980a) and Auersperg (1969b) showed that cell-cell and cell-substratum adhesions in C-4I cultures were qualitatively different; the former was EDTA (ethylene dinitrilotetraacetic acid, a cation chelator) sensitive and trypsin resistant, while the latter was trypsin sensitive and EDTA resistant.

II. DON Stability

In this study, UV absorption of 1 ug/ml DON in pH 7 phosphate buffer at 274 nm decreased by about 20% over 18 hours or 26% over 24 hours and by 25% over 18 to 90 hours at 37°C (Fig. 5, experiment 1). These results were not inconsistent with the results of Dion et al. (1956). He reported that UV absorption of 32 ug/ml DON in pH7 phosphate buffer decreased by about 26% over 18 hours, and by 46% over 18 to 90 hours at a lower temperature of 30°C.

There was a slight increase of absorption (about 3%) over 90 to 168 hours in this study (Fig. 5), while Dion reported a small decrease of 5% over the same time period (unfortunately, he did not measure beyond 168 hours). In the present study, the slight increase in UV absorption by DON could possibly be due to formation of DON breakdown products, which absorb UV rays. According to Dion (1982), there had never been any investigation of DON breakdown products. However, he assumed that DON degradation resulted in loss of the diazo group with subsequent replacement by a hydroxyl group, which did not absorb UV radiation between 220-320 nm.

Dion et al. (1956) reported that the decrease of UV absorption by DON in 0.1N alkali was accompanied by a decrease in activity against Torulopsis albida. Hence, it was assumed by Dion and in this study that UV absorption was equivalent to DON biological activity.

III. DON-induced changes in growth pattern of C-4 cells, as represented by changes in colony form.

With DON treatment, small C-4I colonies showed a biphasic response: single doses produced rounder colonies while multiple doses produced more

irregular ones. In contrast, the results with small C-4II colonies were inconsistent. Large colonies in both cell lines became more irregular, with more significant changes in C-4II colonies.

Increase in the roundness of colonies could be due to factors such as:

(i) a uniform inability of colonies to spread so that colonies remained round and unspread (possibly caused by DON decreasing cell-substratum adhesion), or

(ii) increased intercellular cohesion within the colony. In this study, since DON probably decreased cell cohesion, factor (ii) is unlikely. Hence, it is possible that colonies, especially small ones, have difficulty adhering to and spreading onto the substratum; this difficulty may be even more enhanced in the presence of DON.

Increase in irregularity of colonies could be due to:

(i) cells in a colony spreading non-uniformly,

(ii) an uneven retraction of colony edges due to an inability of the cells to adhere, or

(iii) decreased intercellular cohesion so that cells in a colony do not act as part of a cohesive unit.

Changes in colony form were complex since they were influenced by many factors such as colony sizes, cell-cell and cell-substratum interactions, and the number of doses of DON added.

Small control colonies in both cell lines were always more variable in form than large colonies (Appendix I). This, again, suggests that small colonies adhered and spread less readily than large colonies since small colonies had smaller substratum adhesion areas than large colonies. In addition, small colonies, unlike large colonies, had a higher

proportion of cells around the colony edge compared to cells in the center of the colony. In addition, since small C-4I colonies were less stratified, they were probably less influenced by cell-cell, than by cell-substratum, interactions. Unlike C-4I colonies, C-4II colonies were less cohesive as a unit, the cells grew more independently of each other as colonies enlarged and there were no morphologic distinctions observed between rim/edge and center cells.

IV. Effect of DON on cell shedding.

In this study, it was shown that DON decreased cell-cell adhesion in C-4I and in confluent C-4II cultures. This resulted in increased numbers of shed cells, of which the majority were live, and in a change to less stratified C-4I and to less columnar C-4II cultures.

Cell shedding or cell detachment is a phenomenon associated with tumor necrosis (Weiss, 1977a and 1978). Lysosomal enzymes from necrotic sites have been shown to facilitate cell detachment from tumors. Thus, it is possible that metastasis is facilitated by lysosomal enzymes acting on tumors (Weiss, 1977a and 1978) and/or host tissues such as basement membranes (Liotta et al., 1980). Such enzymes have been thought to be of both tumor and non-tumor (e.g. host macrophage) origin. In this study, one suggestion was that necrotic materials and degradative enzymes produced by damaged DON-treated cells could have increased cell shedding. This possibility was not very likely, since in vivo, the presence of necrotic centers in C-4I tumors grown in hamsters was not associated with increased cell detachment, or with a dispersive or infiltrative growth pattern. Similarly, C-4II tumors grown in hamsters had an infiltrative growth pattern even though they did not have necrotic centers (Auersperg, 1969a).

Even though DON increased cell shedding in both C-4 cultures, it was not determined if these DON-treated shed cells were proliferative. Even if they were less proliferative in culture, they might still be able to form metastases *in vivo*. Tumor cells from a single tumor have heterogenous proliferative and metastatic potentials (Weiss, 1977b; Fidler, 1978; Liotta *et al.*, 1980; Fidler and Hart, 1981; Easty *et al.*, 1981). Hence, metastatic potential is not necessarily directly related to the number of cells detached or to the cell's proliferative capacity *in vitro*. Instead, metastatic potential has been correlated with enzymatic degradation of collagen in basement membranes (Liotta *et al.*, 1980; DeVore *et al.*, 1980; Alhadeff and Holzinger, 1981), with cell surface properties (Yogeeswaran and Salk, 1981; Alhadeff and Holzinger, 1981), with increased GAG content in tumor parenchyma and capsule (Toole *et al.*, 1979) and with ability to inhibit embryonic cell aggregation (Maslow *et al.*, 1976; Weiss and Maslow, 1980).

DON is currently being investigated as a potential anti-tumor drug. This study showed that DON increased cell shedding *in vitro*. However, further experiments will have to be conducted to determine if DON does increase cell shedding *in vivo*, causing increased metastasis. Obviously, the treatment of cancers with such chemotherapeutic drugs, which increase metastasis, would be detrimental to the cancer patient.

V. Cell Aggregation.

DON pretreatment caused a significant decrease in cell-cell aggregation and a decrease in cell spreading or cell deformation within C-4I clumps in suspension culture. However, similar pretreatment in C-4II cultures did not produce consistent decreases in cell-cell aggregation.

Control and pretreated C-4II aggregates were similar morphologically.

In C-4I cultures, the DON-induced decrease in aggregation could have been due to several factors, such as:

(i) a decrease or alteration of cell surface adhesive components,

(ii) a decreased rate of recovery of adhesive trypsin-sensitive and DON-sensitive cell surface components, or

(iii) cell damage, due to long-term DON pretreatment.

Since pretreated cultures after 48 hours of shaking had more clumps than cultures after 24 hours of shaking, the first two factors could possibly be true. If DON pretreatment caused a depletion of glycosylated stores intracellularly, then:

(i) the cell surfaces would be depleted of glycosylated adhesive cell surface components (which would be consistent with the observation that DON-treated cultures were always more easily dissociated with trypsinthan control cultures), and

(ii) the regeneration of glycosylated trypsin-sensitive and DON-sensitive components would be slower in DON-pretreated than in control cultures.

The irregularity and decreased compactness of DON-treated clumps could be due to a decrease in adhesion, in cell deformability or cell re-arrangement between DON-pretreated cells. Cell re-arrangement within an aggregate occurs in cell sorting, a phenomenon that occurs after heterogenous cells have adhered to form mixed aggregates. Cells within mixed aggregates tend to sort out, so that like cells that form the inner sphere are different from those that form the outer sphere. Many theories attempt to explain this sorting-out phenomenon. One is that desmosomes influence cell-sorting. Cells which form more desmosomes tend to sort out internally, in contrast to cells of the exterior that have fewer

desmosomes (Overton, 1977; Wiseman and Strickler, 1981).

Desmosomes are adherens-type junctions (Staehelin and Hull, 1978) that rapidly form in culture. Overton (1977) showed that in embryonic cells, desmosomes formed as early as 3 hours. Dembitzer et al. (1980b) demonstrated that C-4I cells with repaired cell surfaces reformed desmosomes within 90 minutes. In this study, C-4I re-arrangement (like desmosome formation) was also demonstrated to be a rapid process. Fig. 24b showed that cell re-arrangement had already begun within 1 hour, while fig. 24c showed that cell re-arrangement was complete within 3 hours. It is likely therefore, that desmosomes play a role in cell re-arrangement in C-4I cultures.

Unlike the C-4I cell line, the C-4II line is a relatively desmosome-free one. Hence, even with DON pretreatment of C-4II cells, the clumps that resulted remained irregular (like control clumps, with no cell re-arrangement), and the DON-induced inhibition of aggregation was inconstant and insignificant. Therefore, the results suggest that desmosomes are one factor affected by DON pretreatment.

Intercellular glycoproteins of desmosomes have been isolated (Gorbsky and Steinberg, 1981). It was found that desmosome cores were rich in glycoproteins of specific molecular weights (100,000; 115,000; 150,000 daltons). It is possible that DON, which inhibited glycosylation, also inhibited desmosomal glycoprotein synthesis.

In both cell lines, DON which was added 18 hours after trypsinization decreased cell-cell aggregation only slightly. This could be due to the fact that during the 18 hour recovery period, glycosylated stores and cell surface components had already been restored. It is also possible that cultures had not been treated with DON for a sufficient period necessary

to produce clearly observable differences between control and treated cultures.

In the C-4II cell line, 6 hours of DON treatment was insufficient for differences in aggregation between control and treated cultures to be detected. However, longer periods of DON treatment (18 and possibly 12 hours) produced a small inhibition of aggregation. Thus, there was a lag period between the time DON was added to cultures and the time DON-induced effects were sufficient to be quantitated. However, the lag period, which was more than 6 hours but less than 18 hours, was less than that previously assumed on the basis of visually detected differences in colony and cell form and growth which were observed on the second day after DON addition. The lag period likely represents the time taken to deplete intracellular glycosylated stores and/or the time of turnover of DON-sensitive cell surface materials.

Considering all experiments in both cell lines, the effects of GSA on aggregation of DON-treated cells were not entirely clear. However, in some experiments, there were indications that DON plus GSA cultures were more aggregated than DON-treated cultures, and similar to control cultures. Similarly, the addition of GSA alone to C-4II cultures produced inconclusive results. Of 3 experiments, only 1 experiment indicated that addition of GSA to shaking cultures increased cell-cell aggregation. Since the effects of DON on trypsin-recovered cells were small, it was difficult to determine if these small DON-induced effects could be prevented or diminished by the concurrent addition of GSA. Perhaps, the addition of GSA to DON-pretreated cells would have been preferable as results in those experiments were more evident and any changes due to GSA might have been more easily detected.

Previous autoradiography studies by Lee (1978) showed that over 90% of labelled GSA in C-4II cultures was extracellular, irregardless of whether labelling was for 3 hours or 8 days after cultures were subcultured or after they reached confluency. In all cultures, most extracellular label was found between cells. More label was basally- than apically-located. In addition, cultures labelled after they were confluent had a higher total number of grains per cell and a higher percentage of grains labelled intercellularly, than cultures labelled after they were subcultured. This observation lends further support to the original hypothesis that the secretion of GSA-containing extracellular materials might influence the shedding of cells into culture medium; since both phenomena were more prominent in confluent cultures. My studies did not prove conclusively that DON-treated cells aggregated more in the presence of GSA or that DON decreased GAG synthesis. However, other researchers have shown that DON decreased labelled GSA incorporation into cells (Greene and Pratt, 1977) and labelled sulfate incorporation into GAGs (Pratt et al., 1976; Spooner and Conrad, 1977; Green and Pratt, 1977; Hurmerinta and Thesleff, 1982). Since GSA-containing macromolecules have adhesive intercellular functions (Pessac and Defendi, 1972; Yamada and Olden, 1978; Mosher and Furcht, 1981), their depletion (presumably due to DON) would be expected to cause increased cell shedding from confluent C-4I and C-4II cultures and less from non-confluent C-4II cultures [since the latter have less GSA-label (Lee, 1978) and hence, less GSA-containing components intercellularly]. This could well explain this project's results on cell shedding. With regards to Lee's work on C-4II cultures, one could attempt to explain his observations, that cultures labelled after reaching confluency had more label intercellularly than

cultures labelled after subculture, by considering the changes in morphology of C-4II cells as they grew from a non-confluent to a confluent state. Non-confluent cells were usually flattened against the substratum; hence, the number and percentage of grains located intercellularly would be low. In contrast, confluent cells, being cuboidal or columnar, might have more grains located intercellularly. Hence, the percentage of grains located intercellularly over the total number of extracellular grains would also be higher.

VI. Morphology of single adherent cells.

Cell morphology was indicative of cell-substratum adhesiveness and interactions (Harris, 1973). According to Harris, cells accumulated on substrata according to a hierarchy of cell-substratum affinity which corresponded to the order of relative wettability of the substratum. In addition, the morphology of cells altered with changes in the substratum. Cells were flattened on more wettable substratum, but were rounder and more easily detached on less wettable substratum.

The results of this study indicated that in both cell lines, DON significantly decreased the spreading and adhesion of cells to plastic substratum, as indicated by the decrease in the proportion of flattened single cells. However, DON-treated C-4II cells showed a larger decrease in percentage of fully spread or flattened cells than DON-treated C-4I cells. This could be due to the fact that the C-4II cell line was the more cell-substratum interactive cell line of the two. These treated C-4II cells detached incompletely, became partly adherent and irregularly-shaped. In contrast, since C-4I cells were less adhesive to the substratum, C-4I cells detached more readily and completely with DON treatment

and became spherical or minimally spread. The percentage of least adhesive spherical C-4I and C-4II cells might even have been higher since some of these cells might have detached and floated off into the medium. In addition to results on cell shedding in this study, a review of past literature has shown that DON also increased absolute numbers and the percentage of detached cells in fibroblast cultures (Spooner and Conrad, 1975).

The observations that C-4II cells were more cell-substratum interactive were also supported by studies by Auersperg (1969a and 1969b) who showed that C-4I clumps adhered preferentially to C-4I colonies rather than to the substratum, in contrast to C-4II clumps which adhered preferentially to the substratum rather than to C-4II colonies.

VII. Effect of DON on projected surface areas of cells within colonies.

In both cell lines, there was a trend towards an increase in projected cell surface areas with DON treatment. This increase was significant in all large C-4I and in most large C-4II colonies.

The increase in cell area in both cell lines was due, at least in part, to an increase in cell volume and an observed increase in colony flatness (Figs. 8a-b, 9a-b, and 10a-b), but other factors may have been involved. A decrease in stratification in the C-4I cell line might account partly for an apparent increase in cell area. However, this decrease in stratification would not account for the increase in cell area of C-4II cells since stratification of C-4II cultures was limited. Instead, the increase in cell area of DON-treated C-4II cultures might have been due to an increase in colony flatness associated with a decrease in cell density.

VIII. Effect of DON on cell volume.

This study conclusively showed that in both cell lines, the increase in cell area with DON treatment was partially due to an increase in cell volume.

This increase in cell volume could be due to several factors, one of which was damage to the cell, resulting in cell swelling. Another factor could be an inhibition of cell division. DON has been shown to inhibit DNA synthesis by inhibiting glycine and formate incorporation into nucleic acids (Maxwell and Nickel, 1957). Hence, inhibition of DNA synthesis by DON treatment might have inhibited cell division and growth in the C-4 cell lines. In this study, even though mitotic figures were occasionally observed in DON-treated cultures, the possibility that inhibition of DNA synthesis resulted in an increase in cell volume cannot be ruled out.

Erlinger and Saier (1982) found that kidney epithelial cells from a less dense culture had larger volumes than those from dense cultures. Hence, it is also possible that C-4 cells might display similar properties. Further experiments would determine if the volume of C-4 cells is inversely proportional to cell density.

IX. Relationship between colony form and size, cell size, and dose of DON. C-4I

Low doses of DON increased the roundness of small colonies. This might be due to an inability of colonies to spread, as a result of a decrease in cell-substratum adhesion. When small colonies were treated with higher doses, the decrease in cell-substratum adhesion caused not only an inability of colonies to spread, but also an uneven retraction of colony edges that had initially been adhering to the substratum. Decreased

intercellular cohesion might also have caused cells to spread as part of a less cohesive unit (forming irregular colonies) and to shed in increased numbers (forming less stratified, flatter colonies and giving the impression that cells had larger projected surface areas). These reasons might also account for the increase in irregularity and flatness of large DON-treated colonies.

C-4II

Low doses of DON decreased both types of adhesions in this less cohesive cell line. Thus, DON-treated cells in small colonies became more like DON-treated single cells which were less adherent and either became more irregular or rounder.

A high dose also decreased cell-substratum adhesion since treated single cells and treated colonies were mainly irregular (partly adherent). Cells that rounded up might have detached from the substratum, causing increased shedding. A high dose also decreased cell-cell adhesion causing cells in a colony to be less cohesive and more independent, holes to appear between cells within colonies, and the colonies to be significantly more irregular.

X. Conclusion.

DON not only inhibited the growth of C-4I and C-4II cells, but it also changed their colony morphology, degree of stratification, shedding patterns, cell areas and volumes, and spreading on plastic substratum. In addition, treatment of C-4I cells with DON prior to cell dissociation resulted in their decreased aggregation in shaker cultures and in an altered morphology of aggregates, resembling that of C-4II clumps.

This study and that of Conrad and Spooner (1975) indicate that DON increased the shedding of cells that were grown *in vitro*. It is presently unknown if DON would increase cell shedding *in vivo*, possibly resulting in increased metastasis. Recent literature has reported that some drugs, with anti-tumor activity, also enhance metastasis (Heppner *et al.*, 1978). Hence, if DON has such properties, it may prove disadvantageous to treat tumors in patients with DON. Further experiments would resolve this question.

Results of this study suggest that DON decreased synthesis of DON-sensitive extracellular materials (ECM) that were responsible for the cohesiveness of C-4I cells. Cohesiveness of C-4II cells was also decreased, but to a lesser extent. From the results of this study, it is unlikely that cell shedding or dispersion of C-4II cells *in vitro* is a result of secretion of ECM. If this was the case, then treatment of C-4II cells with DON would presumably decrease ECM synthesis resulting in a decrease in cell shedding. The opposite effect of DON was observed.

In the course of this study, it was observed that C-4I cells, with DON treatment, came to resemble C-4II cells with regards to their colony form, shedding pattern, decreased aggregation in shaker cultures and clump morphology. An interpretation of these observations is that C-4II cells may be less cohesive because they produce less DON-sensitive, cohesive ECM. In contrast, C-4I cells may be producing more of such ECM, resulting in cultures that grow cohesively and which do not shed much. Hence, with DON treatment, C-4I cells would be less cohesive--they would tend to produce more viable shed cells, fewer aggregated clumps which are also smaller and more irregular, and also, irregular colonies.

SUMMARY

1. DON, a potential chemotherapeutic drug, was shown *in vitro* to inhibit the growth of C-4I and C-4II cells, two cell lines that were originally derived from one human cervical carcinoma biopsy specimen. However, in addition to its growth inhibitory activity, DON also had numerous other effects on their growth and shedding patterns, and morphology.
2. On visual observation, DON-treated C-4 cultures showed changes in colony form, cell size, degree of stratification and degree of cell flattening, cell-cell contacts, nuclear/cytoplasmic ratios, and number and morphology of single adhering cells. Some of these changes were quantitated.
3. By quantitating projected colony form and cell sizes, it was found that in general, large colonies in both cell lines became significantly more irregular and cells were significantly larger in surface areas. However, results from small colonies were less prominent and more variable. Changes in growth pattern, or changes in colony forms, could have resulted from changes in cell-cell and/or cell-substratum adhesions. Increase in cell areas was determined to be due to an increase in cell volume and, possibly, an increase in colony flatness.
4. Shedding patterns of DON-treated C-4I and confluent C-4II cultures indicated that DON induced shedding in C-4I cultures, while shedding was enhanced in C-4II cells. Most DON-treated shed cells were live as determined by dye exclusion tests. Increased shedding was probably due to decreased cell-cell cohesion, which probably also

accounted for the observed "holes" (or areas of decreased cell-cell contact) within colonies.

5. When C-4I cells (from cultures treated with DON prior to dissociation into single cells) were shaken in gyratory shakers, the clumps that formed after 24 hours were small, irregularly-shaped (resembling C-4II clumps) and significantly fewer than those from control cultures. It was clear that cell-cell cohesion was greatly decreased in C-4I cultures. In contrast, C-4II cells from DON-treated cultures did not show a significant decrease in aggregation.
6. In both cell lines, if single cells were treated with DON while they were recovering from the effects of dissociation, an incubation period with DON of 18 hours was sufficient to inhibit cell aggregation. An incubation period of 6 hours had no effect. However, if cells were slowly shaken in the presence of DON for a further 24 hours, a small but consistent decrease in aggregation was noted in treated cultures. Attempts to reverse DON effects by glucosamine addition gave inconclusive results.
7. DON also decreased cell-substratum adhesion, by decreasing the spreading of single cells on the substratum in both cell lines.
8. To conclude, since DON did not decrease cell dispersion or shedding <u>in vitro</u>, the process of cell shedding did not seem to result from synthesis and secretion of ECM. On the contrary it suggests that ECM is necessary for adhesion. The basis for the difference in growth patterns between the two cell lines may be that cell-cell cohesion was dependent on the synthesis and secretion of DON-sensitive cell surface-associated ECM. If C-4I cells (being more cohesive) produced

more of this cohesive ECM than less cohesive C-4II cells, then DON-treated C-4I cells would resemble C-4II cells. Therefore, the results of this study tend to support the alternate hypothesis that the synthesis and secretion of DON-sensitive, cohesive cell surface-associated extracellular material is associated with a compact growth pattern of tumors, and that a decrease in the production of such ECM may lead to a dispersed or infiltrative pattern of growth. Examples of such extracellular macromolecules include hyaluronate and fibronectin, both of which bind to cell-surfaces and have cohesive functions.

9. The investigation of the tumor-inhibitory activity of potential chemotherapeutic agents should also include a study of other effects of the agent on the tumor. For example, the effects of the agent on the tumor's growth pattern should also be considered. This study showed that DON increased cell shedding from cultured tumor cells. The pertinent question is whether it would have similar cell dispersion - enhancing effects in vivo.

BIBLIOGRAPHY

Alhadeff, J.A., and Holzinger, R.T. (1981). Sialyltransferase, sialic acid and sialoglycoconjugates in metastatic tumor and human liver tissue. Int. J. Biochem. 14:119-126.

Auersperg, N. (1969a). Histogenetic behavior of tumors. I. Morphologic variations in vitro and in vivo of two related human carcinoma cell lines. J. Nat. Cancer Inst. 43:151-173.

Auersperg, N. (1969b). Histogenetic behavior of tumors. II. Roles of cellular and environmental factors in the in vitro growth of carcinoma cells. J. Nat. Cancer INst. 43:175-190.

Auersperg, N. (1972). Histogenetic behavior of tumors. III. Possible relationships to patterns of glycolysis. J. Nat. Cancer Inst. 48:1589-1596.

Auersperg, N., and Worth, A. (1966). Growth patterns in vitro of invasive squamous carcinomas of the cervix - A correlation of cultural, histologic, cytogenetic and clinical features. Int. J. Cancer 1:219-238.

Auersperg, N., Erber, H., and Worth, A. (1973). Histologic variation among poorly differentiated invasive carcinomas of the human uterine cervix. J. Nat. Cancer Inst. 51:1461-1477.

Auersperg, N., and Erber, H. (1976). Effects of tissue culture environment on growth patterns and ultrastructure of poorly differentiated human cervical carcinomas. J. Nat. Cancer Inst. 57:981-994.

Burchenal, J.H. (1979). Antitumor effects of azaserine and DON. Cancer Treat. Rep. 63:1031-1032.

Cabanillas, F. (1979). DON. Drugs of the Future 6:572-576.

Catane, R., Daniel, D.V.H., Glaubiger, D.L., and Huggia, F.M. (1979). Azaserine, DON, and Azotomycin: Three diazo analogs of L-glutamine with clinical antitumor activity. Cancer Treat. Rep. 63:1033-1038.

Cooney, D.A., Hiremagalur, N., Jayaram, Milman, H.A., Homan, E.R., Pittilo, R., Geran, R.I., Ryan, J., and Rosenbluth, R.J. (1976). DON, CONV and DONV - III. Pharmacologic and toxic studies. Bioc. Pharm. 25:1859-1870.

Culp, L.A., Ansbacher, R., and Domen, C. (1980). Adhesion sites of neural tumor cells: Biochemical Composition. Biochem. 19:5899-5907.

Dembitzer, H.M., Schermer, A., Herz, F., and Koss, L.G. (1980a). A preliminary fine structural analysis of cell:cell and cell:substrate adhesion in C4I. 38th Ann. Proc. Electron Microscopy Soc. Amer.: 770-771.

Dembitzer, H.M., Herz, F., Schermer, A., Wolley, R.C., and Koss, L.G. (1980b). Desmosome development in an in vitro model. J. Cell Biol. 85:695-702.

DeVore, D.P., Houchens, D.P., Ovejera, A.A., Dill, Jr., G.S., and Hutson, T.B. (1980). Collagenase inhibitors retarding invasion of a human tumor in nude mice. Expl. Cell Biol. 48:367-373.

Dion, H.W. 1982. Personal Communication.

Dion, H.W., Fusari, S.A., Jakubowski, Z.L., Zora, J.G., and Bartz, Q.R. (1956). 6-diazo-5-oxo-L-norleucine, a new tumor-inhibitory substance. IV. Isolation and characterization. J. Am. Chem. Soc. 78: 3075-3077.

Duvall, L.R. (1960). 6-dizao-5-oxo-L-norleucine. Cancer Chemo. Rep. 7:86-98.

Easty, D.M., Easty, G.C., Carter, R.L., Monaghan, P., and Butler, L.J. (1981). Ten human carcinoma cell lines derived from squamous carcinomas of the head and neck. British J. Cancer 43:772.

Ehrlich, J., Coffey, G.L., Fisher, M.W., Hilligas, A.B., Kohberger, D.L., Machamer, H.E., Rightsel, W.A., and Roegner, F.R. (1956). 6-diazo-5-oxo-L-norleucine. A new tumor-inhibitory substance. Anti. and Chemo. 6:487-497.

Ekblom, P., Lash, J.W., Lehtonen, E., Nordling, S., and Saxen, L. (1979). Inhibition of morphologic cell interactions by 6-diazo-5-oxo-norleucine (DON). Expl. Cell Res. 121:121-126.

Erlinger, S., and Saier, M.H. (1982). Decrease in protein content and cell volume of cultured dog kidney epithelial cells during growth. Importance for transport measurements. In Vitro 18:196-202.

Fidler, I.J. (1978). Tumor heterogeneity and the biology of cancer invasion and metastasis. Cancer Res. 38:2651-2660.

Fidler, I.J., and Hart, J.R. (1981). The origin of metastatic heterogeniety in tumors. Europ. J. Cancer 17:487-494.

Fischer, G., and Schachner, M. (1982). In vitro reaggregation of dissociated mouse cerebellar cells. I. Demonstration of different aggregation mechanisms. Expl. Cell Res. 139:285-296.

Funderburg, F.M., and Markwald, R.R. (1981). Decreased synthesis and secretion of matrix glycosaminoglycans (GAG) accompanies altered formation and migration of endocardial cushion tissue (ECT) cells in the embryonic chick. J. Cell Biol. abstracts 91:160a.

Ghosh, S., Blumenthal, H.J., Davidson, E., and Roseman, S. (1960). Glucosamine metablolism. V. Enzymatic Synthesis of glucosamine-6-phosphate. J. Biol. Chem. 235:1265-1273.

Gorbsky, G., and Steinberg, M.S. (1981). Isolation of the intercellular glycoproteins of desmosomes. J. Cell. Biol. 90:243-248.

Greene, R.M., and Pratt, R.M. (1977). Inhibition by DON of rat palatal glycoprotein synthesis and epithelial cell adhesion in vitro. Expl. Cell Res. 105:27-37.

Harris, A. (1973). Behavior of cultured cells on substrata of variable adhesiveness. Expl. Cell Res. 77:285-297.

Hayashi, H., and Ishimaru, Y. (1981). Morphological and biochemical aspects of adhesiveness and dissociation of cancer cells. Int. Rev. of Cytology 70:139-215.

Heaysman, J.E.M., and Pegrum, S.M. (1973). Early contacts between fibroblasts. An ultrastructural study. Expl. Cell Res. 78:71-78.

Hennings, H., and Holbrook, K.A. (1983). Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture. An ultrastructural study. Expl. Cell Res. 143: 127-142.

Heppner, G.H., Dexter, D.L., DeNucci, T., Miller, F.R., and Calabresi, P. (1978). Heterogeneity in drug sensitivity among tumor cell subpopulations of a single mammary tumor. Cancer Res. 38:3758-3763.

Hiremagalur, N. Jayaram, Cooney, D.A., Milman, H.A., Homan, E.R., and Rosenbluth, R.J. (1976). DON, CONV and DONV - I. Inhibition of L-asparagine synthetase in vitro. Bioc. Pharm. 25:1571-1582.

Hurmerinta, K., Thesleff, I., and Saxen, L. (1979). Inhibition of tooth germ differentiation in vitro by diazo-oxo-norleucine (DON). J. Emb. Expl. Morpho. 50:99-109.

Hurmerinta, K., and Thesleff, I. (1982). Diazo-oxo-norleucine (DON)-induced alterations in the extracellular matrix of the mouse tooth germ. Cell Diff. 11:107-113.

Lanks, K.W., and Kasambalides, E.J. (1980). Factors that regulate proliferation of normal and transformed cells in culture. Pathobiol. Ann. 10:35-50.

Lee, H.C. (1978). The role of calcium and extracellular materials in the vitro growth and contractility of carcinoma cells. M.Sc. thesis, UBC, pp. 101-102.

Linsenmayer, R.F., and Kochhar, D.M. (1979). In vitro cartilage formation: Effects of 6-diazo--5-oxo-L-norleucine (DON) on glycosaminoglycan and collagen synthesis. Dev. Biol. 69:517-528.

Liotta, L.A., Tryggrason, K., Garbisa, S., Hart, I., Foltz, C.M., and Shafie, S. (1980). Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 284:67-68.

Livingstone, R.B., Venditti, J.M., Cooney, D.A., and Carter, S.K. (1970). Glutamine antagonists in chemotherapy. Adv. Pharm. and Chemotherapy 8:57-120.

Magnani, J., Thomas, W.A., and Steinberg, M.S. (1981). 2 distinct adhesion mechanisms in embryonic neural retina cells. I. A Kinetic analysis. Dev. Biol. 81:96-105.

Maroudas, N.G. (1973). Growth of fibroblasts on linear and planar anchorages of limiting dimensions. Expl. Cell Res. 81:104-110.

Marx, J.L. (1982). Tumors: A mixed bag of cells. Sci. 215:275-277.

Maslow, D.E., Mayhew, E., and Minowada, J. (1976). Differential inhibition of embryonic cell aggregation by cultured human cells with "malignant" or "normal" characteristics. Cancer Res. 36:2707-2709.

Maxwell, R.E., and Nickel, V.S. (1957). 6-diazo-5-oxo-L-norleucine, a new tumor-inhibitory substance. V. Microbiologic studies of mode of action. Anti. and Chemo. 7:81-89.

McGuire, E.J. (1972). A possible role for carbohydrates in cell-cell adhesion. In: Membrane Research, pp. 347-368.

Mikuni-Takagaki, Y., and Toole, B.P. (1980). Cell-substratum attach ment and cell surface hyaluronate of rous sarcoma virus-transformed chondrocytes. J. Cell Biol. 85:481-488.

Mosher, D.F., and Furcht, L.T. (1981). Fibronectin: Review of its structure and possible functions. J. Invest. Derm. 77:175-180.

Overjera, A.A., Houchens, D.P., Catane, R., Sheridon, M.A., and Muggia, F.M. (1979). Efficacy of 6-diazo-5-oxo-norleucine and N-(N-o-glutamyl-6-diazo-5-oxo-norleucine) -6-diazo-5-oxo-norleucine against experimental tumors in conventional and nude mice. Cancer Res. 39:3220-3224.

Overton, J. (1977). Formation of junctions and cell sorting in aggregation of chick and mouse cells. Dev. Biol. 55:103-116.

Pessac, B., and Defendi, V. (1972). Cell aggregation: role of acid mucopolysaccharides. Sci. 175:898-900.

Petkau, J., and Crapeau, H. (1983). Department of Mathematics, UBC. Personal communication.

Pittillo, R.F., and Hunt, D.E. (1967). Azaserine and 6-diazo-5-oxo-L-norleucine (DON). In: Antibiotics I, pp. 481-493, Springer-Verlag, New York.

Pratt, R.M., Goggins, J.F., Wilk, A.L., and King, C.T.G. (1973). Acid mucopolysaccharide synthesis in the secondary palate of the developing rat at the time of rotation and fusion. Dev. Biol. 32:230-237.

Rando, R.R. (1975). On the mechanism of action of antibiotics which act as irreversible enzyme inhibitors. Bioc. Pharm. 24:1153-1160.

Roblin, R. (1981). Contributions of secreted tumor cell products to metastasis. In: Cancer Biology Reviews. Vol. 2:59-94. Marcel Dekker, New York.

Rosenbluth, R.J., Cooney, D.A., Hiermagalur, N. Jayaram, Milman, H.A., and Homan, E.R. (1976). DON, CONV and DONV. II. Inhibition of L-asparagine synthetase in vivo. Bioc. Pharm. 25:1851-1858.

Rosenfeld, H., and Roberts, J. (1981). Enhancement of antitumor activity of glutamine antagonists 6-diazo-5-oxo-L-norleucine and Acivicin in cell culture by glutaminase-asparaginase. Cancer Res. 41:1324-1328.

Spooner, B.S., and Conrad, G.W. (1975). The role of extracellular material in cell movement. I. Inhibition of mucopolysaccharide synthesis does not stop ruffling membrane activity of cell membrane. J. Cell Biol. 65:286-297.

Skyvova, M., Kocent, A., and Vermouseki, I. (1973). Host-tumor relationship. XXXI. Acid glycosaminoglycans in the plasma and in the ascitic fluid of rats during experimental tumor growth. Neoplasma 20:181-188.

Staehelin, L.A., and Hull, B.E. (1978). Junctions between living cells. Sc. Amer. 238:141-152.

Steinberg, M., Armstrong, P.B., and Granger, R.E. (1973). On the recovery of adhesiveness by trypsin-dissociated cells. J. Membr. Biol. 13:98-128.

Stoker, M., O'Neill, C., Berryman, S., and Waxman, V. (1968). Anchorage and growth regulation in normal and virus-transformed cells. Int. J. Cancer 3:683-693.

Takeichi, M. (1977). Functional correlation between cell adhesive properties and some cell surface proteins. J. Cell Biol. 75: 464-474.

Toole, B.P., Biswas, C., and Gross, J. (1979). Hyaluronate and invasiveness of the rabbit V2 carcinoma. Proc. Natl. Acad. Sc. 76:6299-6303.

Turley, E. (1980). The control of cytodifferentiation by extracellular matrix. Diff. 17:93-103.

Weisman, L.L., and Strickler, J. (1981). Desmosome frequency: experimental alteration may correlate with differential cell adhesion. J. Cell Sci. 49:217-223.

Weiss, L. (1977a). Tumor necrosis and cell detachment. Int. J. Cancer 20:87-92.

Weiss, L. (1977b). A pathobiologic overview of metastasis. Seminars in Oncology 4:5-17.

Weiss, L. (1978). Some mechanisms involved in cancer cell detachment by necrotic material. Int. J. Cancer 22:196-203.

Weiss, L., and Maslow, D.E. (1980). In vitro studies on the interactions of tumor and non-tumor cells. In: Tissue culture in medical research (II), pp. 117-124. Pergamon, N.Y.

Yamada, K.M., and Olden, K. (1978). Fibronectins - adhesive glycoproteins of cell surface and blood. Nature 275:179-184.

Yogeeswaran, G., and Salk, P.L. (1981). Metastatic potential is positively correlated with cell surface sialylation of cultured murine tumor cell lines. Sci. 212:1514-1516.

APPENDIX I

CHANGE IN VARIANCE IN COLONY FORM ($\times 10^{-3}$).

Treatment	Time of DON addition (days after plating)	C-4I small colony day 4	C-4I small colony day 6-7	C-4II small colony day 4	C-4II small colony day 6-7
single dose	2	$L^{10.0}$	$S^{9.7}$	$S^{19.2}$	$S^{22.0}$
single dose	1	$S^{10.0}$	$S^{9.7}$; $S^{9.7}$	$L^{24.7}$	$S^{22.0}$
single dose	0	$S^{10.0}$	$L^{7.4}$; $S^{9.7}$	$S^{19.2}$; $L^{24.7}$	$S^{22.0}$
double dose	1 + 2	$L^{10.0}$	$S^{4.0}$; $S^{9.7}$	$S^{19.2}$; $L^{24.7}$	$L^{22.0}$
triple dose	1, 2 + 3	$S^{10.0}$	$S^{9.7}$	$S^{24.7}$	$L^{22.0}$

Treatment	Time of DON addition (days after plating)	C-4I large colony day 4	C-4I large colony day 6-7	C-4II large colony day 4	C-4II large colony day 6-7
single dose	2	$L^{1.7}$	$S^{2.2}$	$L^{16.4}$ $L^{12.5}$	$L^{13.7}$
single dose	1	$L^{1.7}$	$L^{7.4}$ $L^{2.2}$		$L^{13.7}$
single dose	0	$L^{1.7}$	$L^{3.7}$ $L^{2.2}$	$L^{16.4}$; $L^{12.5}$	$L^{13.7}$
double dose	1 + 2	$L^{1.7}$	$L^{2.9}$ $L^{2.2}$	$L^{16.4}$; $L^{12.5}$	No diff.
triple dose	1, 2 + 3	$L^{1.7}$	$L^{2.2}$	$L^{12.5}$	$L^{13.7}$

Superscripts...variance of colony form of control cultures ($\times 10^{-3}$).
S ...variance is smaller than that of the corresponding control variance.
L ...variance is larger than that of corresponding control variance.
No diff. ...variance is the same as that of corresponding control variance.