EFFECT OF INTERCELLULAR CONTACT ON RADIATION-INDUCED DNA DAMAGE

By

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ABSTRACT

Chinese hamster V79-171B cells grown for about 24 hours in suspension culture display increased resistance to cell killing by ionizing radiation compared with cells grown as monolayers, an observation originally termed the "contact effect". More recently, development of that resistance was shown to be accompanied by changes in the conformation of the DNA which reduce its denaturation rate in high salt/weak alkali. These changes in DNA conformation, mediated by the cellular micro-environment, appear to be responsible for the contact effect.

The conditions necessary for the development of the effect are not, however, completely understood. In particular, when cells grown as monolayers on petri plates are suspended in spinner culture flasks, their growth characteristics change in three distinct ways. First, cells in suspension no longer have a solid substrate, so they remain round. Second, after several hours, they begin to aggregate to form "spheroids", so that three-dimensional intercellular cell contact develops. Third, cells in the stirred suspension cultures are not subjected to high local concentrations of metabolic by-products or surrounded by a zone depleted of nutrients, as are cells in monolayer culture. The studies described here were designed to determine how each of these factors influence changes in DNA conformation, as assayed using the alkali unwinding technique.

Our results indicated that a round shape may not be an essential requirement, since cells spread out on the surface of cytodex beads in suspension culture, and sparsely-seeded cells in monolayer culture demonstrated at least a partial contact effect. Three-dimensional intercellular contact does not always seem necessary for the development of the contact effect. Cells grown in a methyl cellulose matrix developed radioresistance, even though the cells formed only small clusters of less than five cells. Similarly, suspension culture cells which were prevented from aggregating by frequent exposure to trypsin, also developed the contact effect. There was no evidence that nutrient depletion plays a role in the failure of cells grown as monolayers to develop a contact effect. However, cells grown as spheroids in the presence of monolayer cells, or in monolayer cell-conditioned medium, did not display a full contact effect. This indicates a role for monolayer cell-produced factors (possibly extracellular matrix proteins) in <u>preventing</u> the development of the contact effect.

We conclude that changes in DNA conformation and the increase in radiation resistance, seen in V79-171b cells grown as spheroids, are not the result of intercellular contact or round shape of the cells. This radioresistance appears to be the result of an <u>absence</u> of monolayer cell-produced factors which could control both cell shape and DNA conformation.

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INTRODUCTION

A. The Problem

Chinese hamster V79 lung fibroblasts, grown for just 24 hours in suspension culture, are more resistant to damage by ionizing radiation than are the same cells grown as monolayers. The increased survival of the cells grown in suspension suggests an enhanced capacity for accumulation and for repair of sublethal radiation damage (17). It was thought that the three-dimensional contact which develops between cells grown in suspension in some way affects radiation sensitivity, and this increased radio-resistance was therefore termed the "contact effect".

Preliminary experiments, done in our laboratory using sparsely-seeded monolayer cultures, suggested that three-dimensional contact was not the only factor contributing to development of the contact effect. Hence, using the technique of alkali unwinding of the DNA to indicate the presence of the contact effect, my intentions were to determine:

a) whether changes in cell shape, preceding X-ray treatment, contribute to the radioresistance.

b) whether manipulation of growth environment alters

cellular radiosensitivity.

c) whether other cell types display a contact effect in terms of DNA unwinding kinetics.

The specific goals of this undertaking were to examine the relative importance of cell-to-cell contact, cell shape and metabolite exchange in the development of the contact effect.

B. The Rationale

Human cancer cells grown in vitro have been used to predict tumor radiosensitivity to anticancer therapy, but the results do not always take into account the role of the three-dimensional growth that occurs in solid tumors. Cells grown in close three-dimensional contact often differ in radiation sensitivity, perhaps as a result of a change in the ability of the cells to repair DNA damage (17, 24, 29). The radiocurability of human tumors varies widely and some of this variability may be related to the ability of different tumors and normal tissues to repair radiation damage, and not merely to the inherent radiosensitivity of the various tumors (58). Therefore, the rationale for this project was to develop a greater understanding of the factors influencing tumor response to therapy and to develop suitable models to study these factors.

The role of DNA conformation in radiation sensitivity is also emphasized in this thesis. The idea that cell sensitivity to damage is the result of the action of DNA repair genes is an obvious oversimplification. Increased radioresistance may be due to differences in DNA conformation (43). Changes in DNA conformation in response to changes in cell shape and growth substratum are believed to occur, but direct evidence linking DNA conformation to cell shape or extracellular environment is lacking.

C. History of the Contact Effect

More than a decade ago, Durand and Sutherland (17) first reported that Chinese hamster V79 cells, grown in suspension culture, form clusters of cells which are more resistant to killing by ionizing radiation than the same cells grown on petri plates as monolayers. Since the cells initially aggregate and then grow as "spheroids" in suspension culture, contact between the cells was presumed to play a role in the development of this increased resistance. Therefore, this resistance was termed the "contact effect". After 24 hours in suspension culture, there is no indication of differences, between spheroid and monolayer cells, in cell cycle distribution or rate of DNA synthesis, yet there is an increase in the fraction of cells which survive to form colonies after radiation exposure (see Figure 1). Thus, cell cycle and DNA synthesis cannot account for the increased radiation resistance of the spheroid cells. The small size

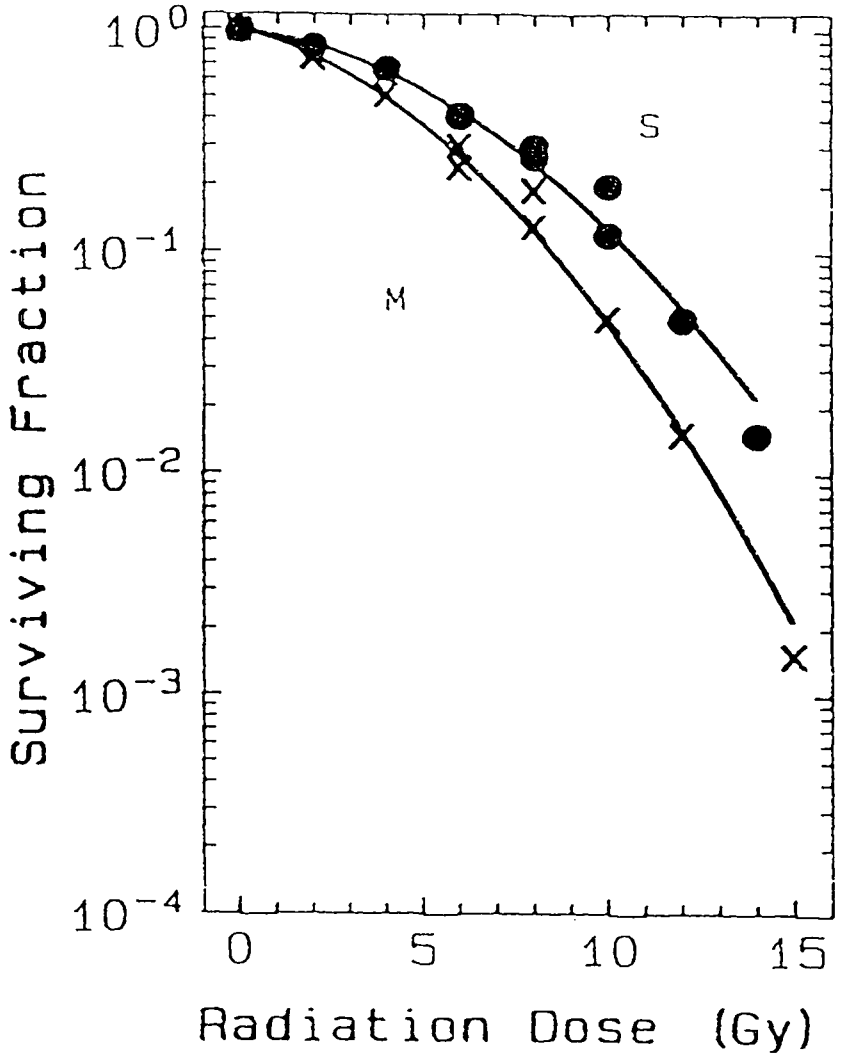


Figure 1. The Contact Effect. Survival curves of cells from day-old spheroid and monolayer cultures. These cells were grown as usual, then trypsinized to single cell suspensions and irradiated at room temperature in spinner flasks. The cells were then plated at pre-determined dilutions, in ten ml of growth medium and incubated for seven days, after which the surviving cells, which had formed colonies were counted. This figure displays the increased survival of the spheroid cells as compared with the monolayer cells. As evidenced by the wider shoulder, the spheroid cells are capable of increased accumulation and repair of sublethal radiation damage.

(20 to 50 cells in a cluster) of the spheroids eliminates complications that could be caused by hypoxic and non-cycling cells present in the larger spheroids. An increase in the shoulder of the radiation survival curve for spheroid cells is correlated with enhanced ability to repair sublethal cell damage. The role of cell-cell contact at the time of irradiation is unclear however. The spheroid cells demonstrate increased radioresistance even when separated before irradiation and exposed as single cells, and for several hours after dissociation. The effect is lost gradually over the time it takes the cells to spread out after plating as monolayers (17, 42). It seems that the history of growth of the cells is more critical to radioresistance than contact during radiation exposure.

Other investigators have since reported a similar "contact effect", in solid tumors as well as spheroids, that enhances resistance to radiation (see Table I). However, the effect varies considerably between cell lines and does not appear to be universal. Many different cell lines, including human (HeLa), hamster (V79, B14-FAF28), mouse (3T3, L) and rat (BICR/M1R-K), were used by Dertinger and Heulser (13). Their results indicated that the contact effect was a property acquired during three-dimensional growth under the influence of intercellular communication, at least in these cell lines. Guichard et al. (24) used four human tumor cell lines (Bell melanoma, HCT-8, HRT-18 and HT-29) growing as xenografts in athymic mice. Cells were isolated, plated as monolayers, and irradiated in vitro.

TABLE I: CELL LINES EXAMINED FOR THE "CONTACT EFFECT"

	Contact Effect	Electrically Coupled	Ref.
Chinese hamster			
V79-171b	++	?	Durand (17)
V79-M4	-	?	"
V79	+	+	Dertinger (13)
B14-FAF28	++	+	"
CHO	-	?	Durand, unpub.
Mouse			
3T3	++	+	Dertinger (13)
L	-	-	"
KHT	+	?	Hill (29)
S1 mammary Ca	-	?	Durand, unpub.
JM1 mammary Ca	+	?	"
Embryo cells	+	?	"
EMT6-RW	-	?	Rockwell (47)
Rat			
BICR/MIR-K	++	+	Dertinger (13)
P19	-	?	Olive, unpub.
9L	+	?	Wallen (55)
Mammary	+	?	Cathers (9)
Thyroid	+	?	Mulcahy (39)
Human			
Hela	+	+	Dertinger (13)
HT-29 colon Ca	++	?	Guichard (24)
HRT-18 colon Ca	++	?	"
Bell melanoma	++	?	"
Nall melanoma	-	-	"
HCT-8 colon Ca	+	?	"
Mammary	+	?	Cathers (9)
Skin fibroblasts	++	?	Durand, unpub.

These were compared with tumors irradiated in vivo. Three (all but HCT-8) showed increased radioresistance in vivo which could not be accounted for solely by the fact that the cells were hypoxic. Using EMT6-Rw mouse mammary cells, grown as solid tumors in BALB/c mice and as monolayers, then irradiated under conditions of uniform, maximal hypoxia, Rockwell (47) failed to find evidence for a contact effect. Also, no difference was observed in the radioresistance of cells plated from the solid tumors and irradiated at various times after plating. This indicated that there was no increase of radiosensitivity with time as would be seen with a contact effect. This was also true of the two human melanoma xenografts used by Rofstad and Brustad (48). Although some form of cell-to-cell communication may have been present, it did not enhance the radioresistance of these melanomas. Curtis and Tenforde (12) developed a model for the radiation response of the rhabdomyosarcoma tumor system in rats, using an in vitro system. Cell survival data indicated that oxygenated tumor cells in situ were less radiosensitive (or more readily repaired) than the same cells irradiated in suspension, indicating the presence of some type of contact effect.

In a study of the radiosensitivity of rat 9L subcutaneous tumor cells from tumors of various sizes, Wallen et al. (55) reported a contact effect when the cells were irradiated in vivo compared to exposure in vitro as single cell suspensions. This tumor does not develop hypoxia as do some other tumors and spheroids. Hypoxia causes radioresistance and can be a

complicating factor in contact effect studies. Cells irradiated as single cell suspensions showed only a slight increase in radioresistance compared with those grown as monolayer cultures, and this did not change with time. If the contact effect is defined as arising in cells grown together three-dimensionally and retaining a residual of the property of radioresistance for several hours, whether irradiated in contact or not, the effect is minimal in these cells.

KHT sarcoma cells were irradiated, in contact as small lung colonies in situ and as individual cells in vitro, by Hill et al. (29). The in vitro experiments were performed using cell suspensions which were irradiated as a function of time after excision of the tumors from the host animals. In agreement with results reported by Durand and Sutherland (17), cell survival decreased during the first cell cycle. In contrast with results reported by Durand and Sutherland, there was not a large initial difference in cell survival between the cells irradiated in contact as lung colonies and those irradiated within two hours after excision and separation into single cells.

Another effect that was seen with some cell types is that of increased cell survival if the cells are left in contact for some time after irradiation. Cathers and Gould (9) measured this effect with irradiated rat mammary glands. Mulcahy et al. (39) used normal rat thyroid cells and found that there was no difference in cell survival between cells irradiated in vivo and

removed immediately for assay, and those irradiated as single cells in vitro. However, an increase in cell survival was seen if the cells were irradiated and then left in situ for 24 hours before assaying. This in situ repair involves an increase in the shoulder of the survival curve, with no change in the slope. This is analogous to results obtained by Durand and Sutherland (18) with V79 spheroids which were irradiated and then plated intact. Survival was greater than for those dissociated and plated as single cells immediately after irradiation.

Several other observations have been made which may help elucidate the mechanism(s) of the contact effect. Durand and Sutherland (18) found that exposure of V79 cells to the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) resulted in the cloning of a cell subline which did not display a contact effect, suggesting that the contact effect may be mediated by genetic events. Hill et al. (29) observed that, with KHT sarcoma cells, the loss of the contact effect as a function of time after the tumors were dissociated did not require cell division. Cell survival declined at the same rate if the cells were held at 37°C or 22°C (when movement through the cell cycle would be slowed), but did not decline if the cells were held at 4°C between plating and irradiation. This suggested that a metabolic process was responsible. Along this same line, Edgren (19), using an experimental system of glutathione-deficient and -proficient human fibroblasts, observed that some part of the cellular rejoining process of DNA single-strand breaks required the

presence of glutathione or some other aminothiol. Sweigert and Alpen (51) reported that 9L rat gliosarcoma spheroid cells displayed an increased radiation-induced division delay that was significantly longer than for monolayer cells. They speculated that, like the contact effect, this division delay could be a consequence of growth of the cells in three-dimensional contact, or at least of the spherical shape. They suggested that enhanced radiation survival seen with spheroid cells might be a direct result of increased division delay, although other studies, using the V79 cell line, do not support this hypothesis (14, 37).

There are many cell types, normal and transformed, where three-dimensional growth results in greater cell survival at a given radiation dose. This radioresistance, or contact effect, is not universal. It manifests itself in various ways in different cell lines and the mechanism(s) is still unknown.

D. DNA as a Target of Radiation Damage

The biological effects of ionizing radiation are caused by the absorption of the radiation energy in cells. Absorption may lead to excitation, or at higher energies, to ionization when one or more orbital electrons are ejected from the atom. Free radicals and excited molecules can result. Free radicals are electrically neutral atoms or molecules having an unpaired electron in their outer orbitals. They are very reactive and can be electron acceptors or donors. In living material, which

consists of 70 - 90 percent water, most of the absorbed energy will be taken up by water molecules. Hydrated electrons, hydroxyl radicals and hydrogen atoms are formed, which are all highly reactive. This indirect action of ionizing radiation uses aqueous free radicals as intermediaries in the transfer of radiation energy to biological molecules. Interaction with a water molecule produces an ion radical and an electron:

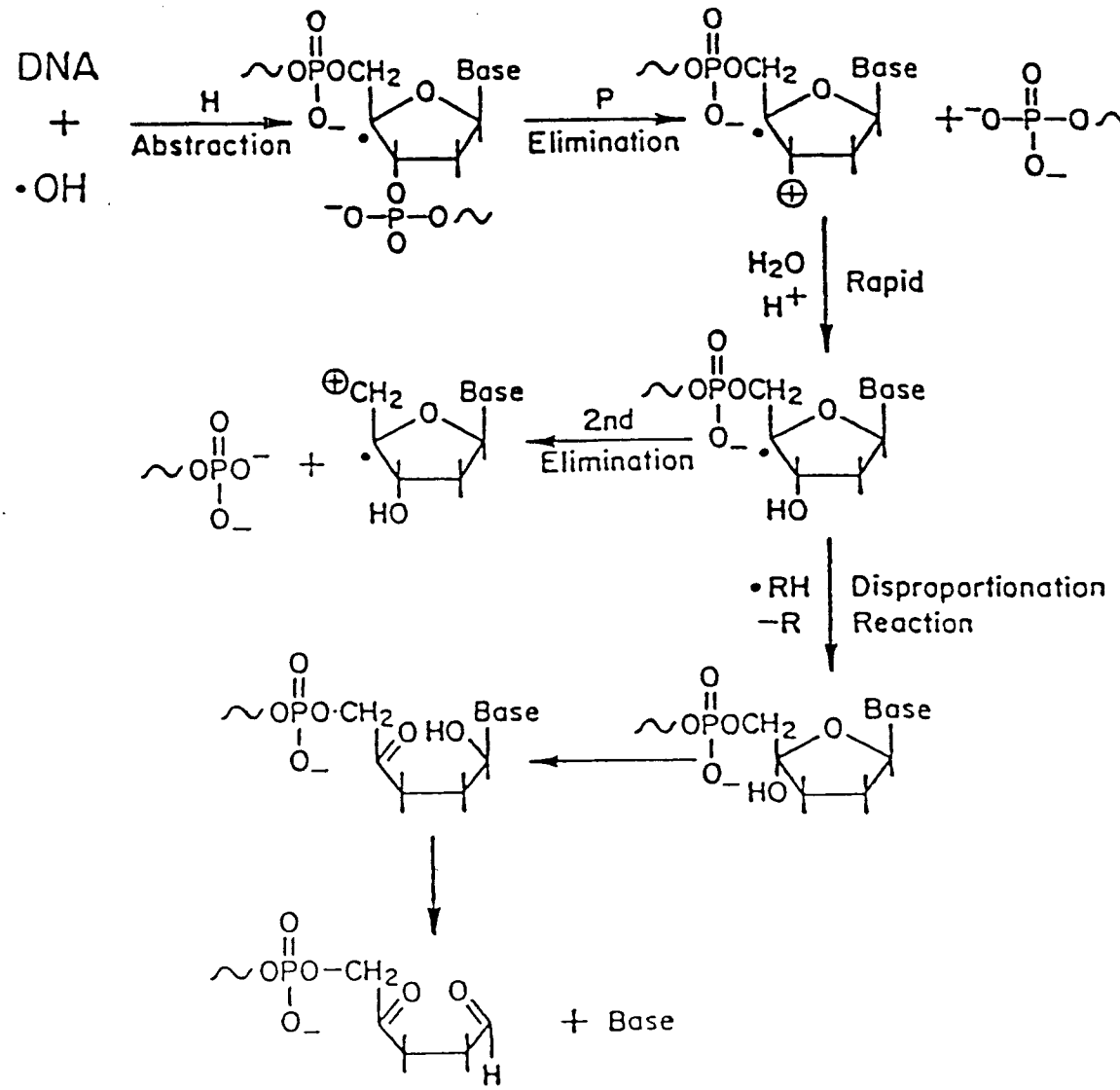
$$H_2O \text{ ------} \rightarrow H_2O^+ + e^-$$

The ion radical is highly reactive, but short-lived and reacts with another water molecule to produce an ion and a free radical:

$$H_2O^+ + H_2O \text{ ------} \rightarrow H_3O^+ + {}^{\cdot}OH$$

This hydroxyl radical is reactive and also has a relatively long lifetime and so is the major mediator of macromolecular damage in the cell. Perhaps 75 percent of all X-ray damage is mediated by the hydroxyl radical and damage occurs in all biological macromolecules. Less frequent is the direct action of ionizing radiation, which involves the simple interaction between the ionizing radiation and critical biological molecules. The events of greatest biological significance probably involve damage to DNA. Hydroxyl radicals cause strand breaks by attacking the sugar moiety of the phosphodiester backbone. This reaction probably results in two types of breaks (Figure 2); either the complete elimination of the sugar and associated base, leaving

Figure 2. Mechanisms of DNA Strand Breakage by the Hydroxyl Radical (31).



phosphoryl groups at both the 5' and 3' termini, or a break which leaves a phosphoryl group at the 5' end, but an open ring structure of the sugar at the 3' end.

Several types of DNA damage can result from ionizing radiation, including base damage, base loss, DNA-DNA and DNA-protein crosslinks and damage to the sugar-phosphate backbone, resulting in DNA single- and double-strand breaks. For low LET (linear energy transfer) radiation like X-rays, double-strand breaks occur much less frequently than single-strand breaks and when unrepaired, are thought to be the "lethal" lesion in some repair models (8, 61). Two single-strand breaks, very close together on opposite DNA strands, can also give rise to double-strand breaks. Therefore, the measurement of DNA strand breakage by ionizing radiation should provide information that is relevant to cell survival.

The repair of radiation-induced DNA damage is assumed to be performed by enzymatic repair complexes. Repair may require several enzyme complexes that bind, operate on the template, and dissociate sequentially before the lesion(s) is removed and the chromatin structure restored. At a higher level of organization, the packing of DNA or chromatin must also be returned to its original configuration. While recent emphasis on DNA repair capacity has been directed towards the repair enzymes present within cells, it is important to remember that there may also be a complication of accessibility of the damage to the repair

process (26, 40). Some models of radiation action in mammalian cells make the assumption that the repair processes are dose-dependant. It is hypothesized that the shape of the mammalian cell survival curve is causally related to the saturation of a DNA repair process (28, 61).

Recent interest in isolation and characterization of DNA-repair deficient mutants (52, 62) as well as cloning of a human DNA repair gene (60), are the result of emphasis on repair enzyme activity as a prime controller of cellular repair capacity. However, the conformation of the template is also of critical importance in DNA repair. DNA sequencing methods have revealed that mutation "hot-spots" or genetic alterations do not always correspond to the areas where most chemical damage occurs, indicating the importance of what has been termed DNA "secondary" structure. Repair is also more efficient in transcribed as compared to non-transcribed DNA strands and in protein-matrix associated versus non-associated DNA (46). The nature of the damage appears to determine the type of mutation which results (for example, bulky lesions lead to frameshift mutations, while minor lesions cause base damage). While the complement of repair enzymes is critical to resistance to DNA damage, the structure of the target should not receive less attention than is merited.

E. A Role for DNA Conformation in the Contact Effect

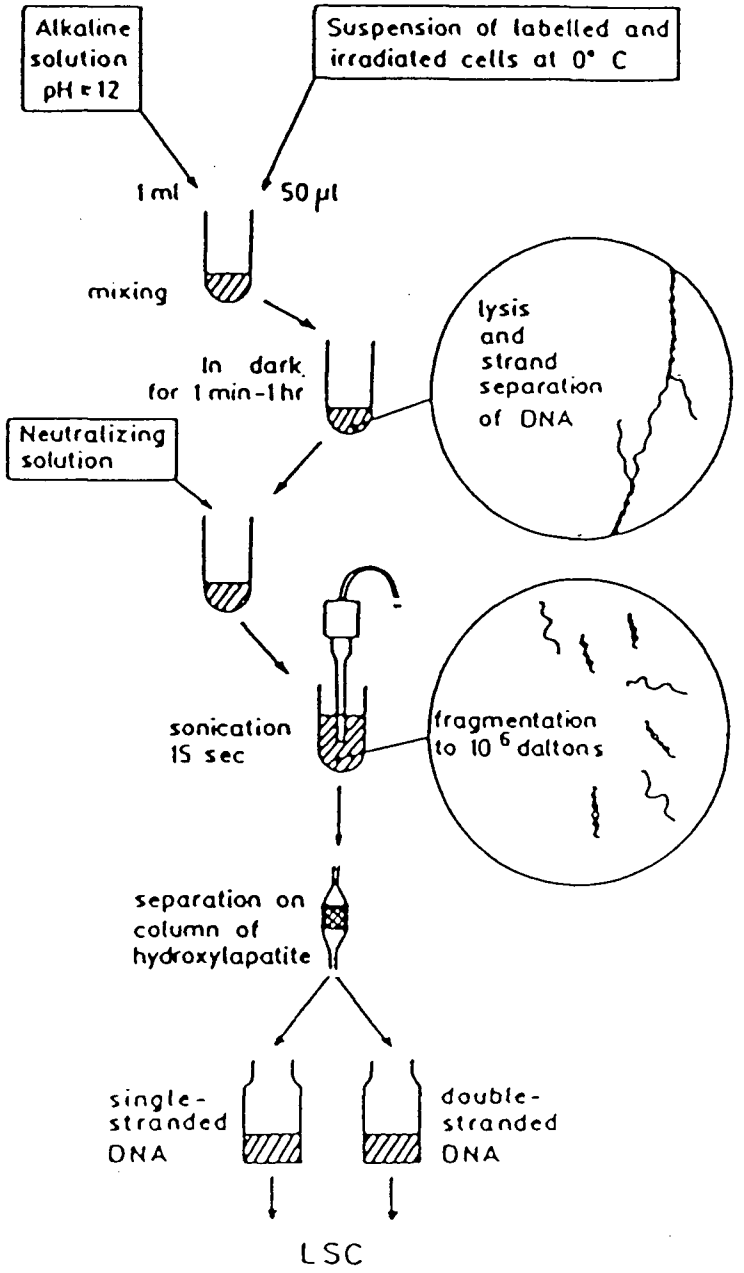
When cells are lysed in a mild alkali solution (pH 12.3),

the DNA undergoes denaturation and slow unwinding at sites of DNA strand-breakage. Most assays which measure single-strand breaks also measure double- and alkali-labile-strand breaks. Unwinding generally occurs at a constant rate for a given temperature and salt concentration. Under appropriate conditions, the amount of resultant double-stranded DNA is believed to be proportional to the number of strand breaks present. The assay was originally developed by Ahnstrom and Erixon (3) and is now in standard use to measure DNA strand breakage by ionizing radiation and genotoxic drugs. Figure 3 depicts the method of use in our laboratory.

Using this assay, Olive and Durand (42) reported a difference in the pattern of DNA unwinding in spheroid cells lysed in alkali after irradiation; DNA unwinding for monolayer cells was exponential while the rate of unwinding of spheroid cell DNA was exponential only for the first ten minutes. Explanations for this may include the presence of radiation-induced lesions which inhibit unwinding, differences in the interaction between DNA and other cell constituents or a difference in DNA conformation between the monolayer and spheroid DNA. Using the unwinding assay, the rejoining of strand breaks was observed to be 25 - 50 percent faster in spheroid cells (43), which may explain why the spheroid cells seem to have an advantage in the survival of radiation damage.

A difference in DNA conformation was also revealed using the

Figure 3. Schematic Representation of the Alkali Unwinding Assay.

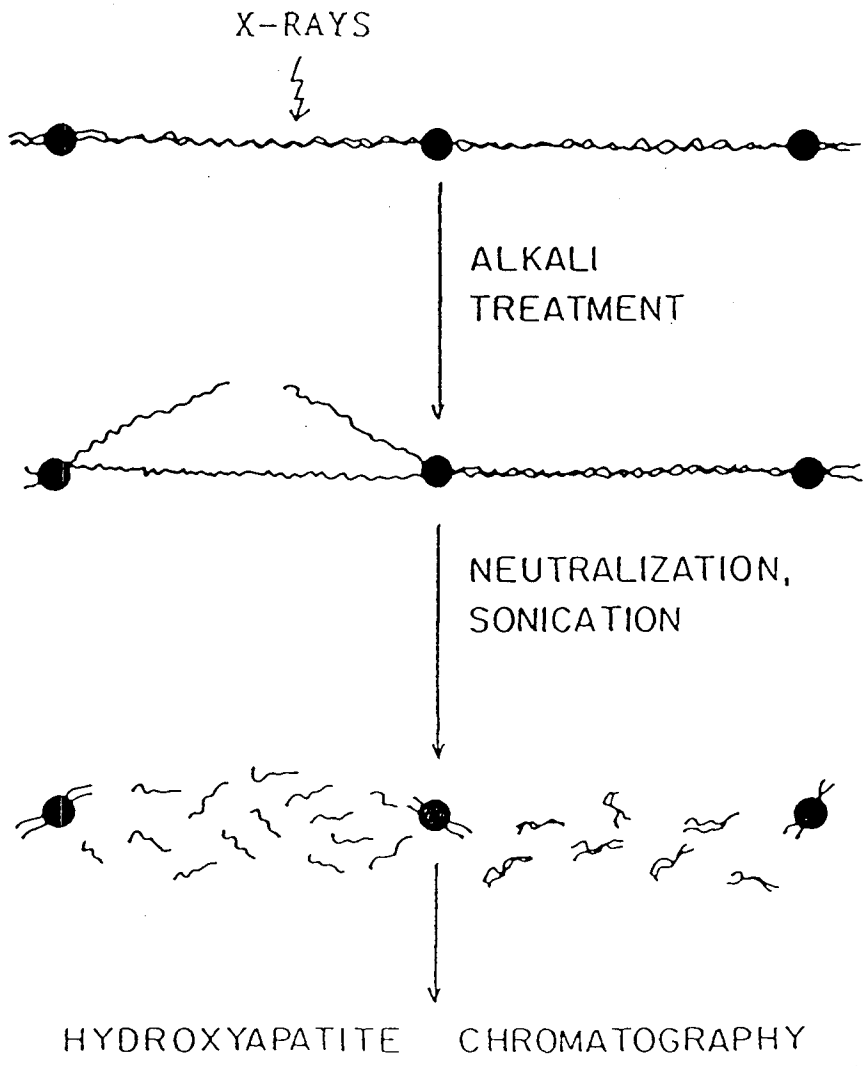


nucleoid sedimentation assay in which protein- and RNA-depleted nuclei, with the DNA still tightly associated, were sedimented on neutral sucrose gradients. These structures were more compact, and so sediment further, when less DNA damage was present. Nucleoids from spheroid cells sedimented twice as rapidly as those from monolayer cells. A possible explanation for this was that the spheroid DNA was more supercoiled than monolayer DNA, but this was shown to be untrue when ethidium bromide, a DNA intercalator, was used (43). A more likely explanation was that the "packaging" of the DNA differed. Olive et al. (43) suggested that when cells are grown in suspension, the packaging of the DNA is altered by the presence of constraints at regular intervals. A schematic of this model is shown in Figure 4. There may be similar constraints in monolayer cells, but they would be separated by much greater distances. These constraints may represent sites of covalent attachment of DNA to the nuclear matrix. If the constraints are closer together in spheroid cell DNA, it could explain the difference in unwinding kinetics and would allow the DNA to rejoin more quickly and perhaps more accurately after strand breakage. If DNA repair occurs in association with the nuclear matrix as do replication and transcription (7, 45, 56), more frequent attachment sites could increase DNA repair capacity.

F. The Approach

The main objective of this project was to investigate the

Figure 4. Proposed Model for the Presence of Constraints to DNA Unwinding in Cells Grown as Spheroids (43).



mechanism of the contact effect, specifically the role of cell shape and cell environment with relation to radioresistance. Since the spheroid cells grow in contact and remain round, the first task was to separate the two properties. To accomplish this, cells were grown on methyl cellulose which allowed them to remain round, but did not allow them to aggregate. Another method of preventing cell attachment and flattening was also used. Poly(2-hydroxyethyl methacrylate), a hydrophilic hydrogel of neutral charge, was applied to non-tissue culture dishes. This polymer has been shown to control the extent of cell spreading and attachment (21) so that cells can be grown in monolayer conditions, but cannot develop the usual flat or extended shape and so remain either more spherical or in suspension.

It was previously observed that sparsely seeded monolayer cells display a relative radioresistance and that their shape is somewhat different from confluent monolayer cultures (41). To investigate a possible role for the growth environment, the effects of different growth media and conditions were studied.

MATERIALS AND METHODS

A. Cell Lines and Culture Techniques

A variety of cell lines, other than the V79 line, was chosen to observe the generality of the contact effect as defined by DNA unwinding. Cells were selected for their ability to grow in monolayer as well as suspension cultures, either as aggregates or not.

a. V79-171B Cell Line

V79 is an established mammalian cell line of Chinese hamster lung fibroblasts. It is an extensively used line because of its high plating efficiency, rapid doubling time, discrete colony formation and ease of obtaining single cell suspensions. This line grows well in monolayer culture on petri plates, as well as in suspension, where many of its sublines grow as aggregates.

The spheroid-forming subline, V79-171b (originally obtained from Dr. Warren Sinclair at Argonne National Laboratories), was routinely grown in our laboratory and maintained as monolayer cultures, in exponential growth, by subculturing every two or three days on 100 mm plastic tissue culture plates (Falcon), in Eagles Minimal Essential Medium (Gibco) containing ten percent (v/v) fetal bovine serum (Gibco). The cells were incubated at

37°C in an humidified atmosphere of four percent CO_2 in air. Spheroids were initiated by seeding 2 x 10^6 exponentially growing cells into 200 ml of the above-mentioned medium in Bellco glass spinner culture flasks. The flasks were flushed with four percent CO_2 in air after adding the medium and were allowed to equilibrate at 37°C before the cells were added. After four to six hours, the cells aggregated spontaneously to form small clusters and divided about twice in a 24 hour period to produce spheroids containing 20 - 50 cells each. These cells were still in exponential growth so that the rate of DNA synthesis and the cell cycle distribution were identical with those of monolayers. The average cell cycle time for this subline is 11 - 12 hours.

Routine passages of monolayer cells were performed by twice rinsing and leaving a film of 0.1 percent trypsin (Gibco) in phosphate buffered saline. The plates were incubated for several minutes, then the cells were resuspended in medium by pipetting vigorously several times, counted and used to start subcultures.

b. SCCVII Cell Line

These murine squamous cell carcinoma cells grow as transplantable tumors in mice, but also adapt well to culture. The cell line we isolated grows as monolayers and in suspension, although the cells do not aggregate to form spheroids. The

doubling time of these cells is like that of the V79 cell line.

This line was passaged and maintained in the same manner as the V79 cell line and is routinely held in our laboratory.

c. RIF-1 Cell Line

The RIF-1 cell line originally arose as a murine tumor as a consequence of irradiation, and was observed to grow readily in culture (54). It was selected for its ability to grow in monolayer culture as well as in suspension as spheroids. The doubling time for this cell line is about 24 hours. The cells were passaged weekly using either 0.1 percent trypsin in citrate saline or 0.25 percent trypsin in phosphate buffered saline. After twice rinsing and then incubating the cells in a trypsin film for several minutes, they were resuspended and plated as usual, in medium containing ten percent fetal bovine serum.

d. WiDr Cell Line

This is a human colon adenocarcinoma cell line, obtained from American Type Culture Collection, which grows well in culture (59). In order to form uniform spheroids in spinner culture, the cells must first be permitted to aggregate in unstirred medium for 24 hours. These cells were passaged and maintained in the same manner as the RIF-1 cell line.

e. C-4 I, C-4 II Cell Lines

These human cervical squamous carcinoma cells originated from the same tumor biopsy and have been maintained as cell lines for many years. The cultures were kindly provided by Dr. Nelly Auersperg (Dept. of Anatomy, U. B. C.). Both lines grow as monolayers and in suspension. The C-4 I subline will grow in suspension as aggregates especially if initiated in unstirred medium in the same manner as the WiDR cell line. The C-4 II does not aggregate well nor grow considerably in suspension (4).

These cell lines were passaged and maintained in the same manner as the RIF-1 cell line.

B. Procedures

a. Preparation of Samples

There are some technical difficulties in the measurement of damage to DNA. Strand breaks caused by ionizing radiation are most easily detected by using radioisotopes incorporated into the DNA as tracer molecules. However, radioisotopes damage DNA, although this can be minimized by the use of low concentrations of the isotope. In addition, radiation-induced single-strand breaks in mammalian cells are rapidly rejoined in a half-time of only a few minutes, so the cells must be irradiated on ice or handled quickly and minimally.

For DNA unwinding experiments, the cells were radioactively labelled. This was done by plating 2 x 10^6 exponentially growing cells, which had been trypsinized and resuspended as previously described, into ten ml of five percent serum-supplemented medium containing a concentration of 0.74 KBq/ml [^{14}C]thymidine (specific activity, 2.2 GBq/mmole). These plates were then incubated at 37^{o}C for 24 hours and the cells were again suspended with trypsin and allowed to grow in unlabelled medium, in the conditions particular to the experiment.

Cells, radiolabelled as above, were plated at 3 x 10^4/cm^2 and 2.5 x 10^5 cells/ml medium in 100 mm tissue culture dishes or grown in suspension at 2 - 3 x 10^6 cells/200 ml medium in spinner flasks. These proportions were used in all experiments except where special conditions for density and cell crowding prevailed. Immediately prior to irradiation, spheroids were centrifuged to form a pellet, the medium was aspirated and the spheroids suspended in five ml of trypsin. The cells were incubated for five minutes after which at least five ml of medium was added to the tube to stop the trypsin activity. The cells were again centrifuged to a pellet, resuspended in two ml of cold medium, counted and adjusted to a concentration of 1 - 2 x 10^5 cells/50 ul, placed in a five ml capped polyethylene test tube and set in an ice-water bath. For monolayer cultures, the medium was aspirated, the plates were rinsed with five ml of trypsin and then incubated for five minutes in two ml of fresh

trypsin to more closely duplicate the exposure to trypsin of the spheroid cells. As with the cells from spheroids, medium was added to the plates containing cells and trypsin, the cells pipetted vigorously, transferred to tubes and centrifuged to a pellet. The medium plus trypsin was removed and the cells were resuspended and treated in the same manner as the spheroid cells.

b. DNA Strand Breakage Analysis

Cells suspended in medium on ice, as described above, were exposed to 250 kvp X-rays at a dose rate of 7.58 Gy/min. Triplicate samples of 50 ul each were taken and placed in five ml test tubes for each radiation dose exposure. For dose response analysis, this was done for successive exposures of 2.5 Gy each, up to an accumulated total of ten Gy. In the study of DNA unwinding kinetics, a single ten Gy dose was given. The samples remained on ice at all times to prevent the action of DNA repair enzymes. The 50 ul samples were lysed in one ml of freshly prepared, ice-cold solution containing 1 M NaCl and 0.03 M NaOH. The samples were then placed in the dark, to prevent subsequent UV light damage, and the DNA allowed to unwind. For dose response analysis, the samples were removed after 60 minutes, neutralized with 0.03 M NaH_2PO_4 to stop the unwinding process, and immediately sonicated for ten seconds using a Heat Systems Ultrasonic sonicator with microtip. This breaks DNA into small single- and double-strand fragments (10^6 daltons).

Approximately 50 ul of sodium dodecyl sulphate (SDS) was then added to each tube to reduce the association of DNA with protein. When DNA unwinding kinetics were analysed, the cells were allowed to lyse and the DNA to unwind for increasing amounts of time from 0.1 to 130 minutes, then treated as above.

c. Hydroxyapatite Chromatography

Hydroxyapatite chromatography was used to separate the resultant single- and double-stranded DNA. Small polyethylene columns, containing 150 mg of hydroxyapatite crystals (Bio-Gel HTP, Biorad Laboratories), were loaded into a column holder. The column holder and all solutions were held at 65^{o}C to melt imperfectly matched base-paired regions of the DNA. All washing and eluting solutions contained 0.40 percent SDS, to reduce the association of DNA with cellular protein. The columns were rehydrated with three ml of 0.012 M sodium phosphate buffer (pH 6.8) and were allowed to drain. The samples, warmed briefly to 65^{o}C, were poured onto the columns. After these had drained, the columns were once again washed with 0.012 M buffer. Single-stranded DNA was eluted with three ml of 0.12 M buffer (pH 7.4) and collected in liquid scintillation vials (Fisher Scientific). The double-stranded DNA was eluted, using 1.5 ml of 0.4 M buffer (pH 7.4), into liquid scintillation vials containing 1.5 ml of distilled water, to bring the volume to three ml. Five ml of scintillation cocktail (Hydrofluor, National Diagnostics) were added to each vial. The vials were then counted for two

minutes using a Beckman liquid scintillation counter with a window set for ^{14}C. The counting efficiency of the single- and double-stranded fractions was determined, and found to be identical. Results were expressed as counts per minute.

d. Quantifying DNA Damage

Results were expressed as the percentage of DNA in double-stranded form. The average and standard deviation were calculated for the triplicate samples and this is expressed as:

$$P = \frac{\text{cpm double-stranded DNA}}{\text{cpm double-stranded DNA + cpm single-stranded DNA}} \times 100$$

Dose response data were plotted on a logarthmic scale of percentage on the ordinate and a linear scale of dose on the abscissa. All experiments were repeated several times and presented one of two ways; (a) the mean and standard deviation of triplicate samples from one representative set of results is shown, or (b) results from two or three repeats of an experiment are represented by one curve (in all cases, triplicate samples were used for each data point in each separate experiment). The standard error for these combined experiments was calculated by dividing the standard deviation of each data point by the square root of n, the number of experiments per curve. In almost all cases, the standard error bars were smaller than the graph symbol sizes. Clarity was the determinant in choosing the

method used for presenting results from an individial experiment. Results were normalized for each experiment so that the unirradiated cell samples represented 90 percent double-stranded DNA. This value was chosen since the average value for ten experiments was found to be 90 $\pm$ 0.5 percent. Significant statistical differences were determined by calculating the 95 percent confidence limits for the slope of each curve defining log percent double-stranded DNA as a function of radiation dose. If the confidence limits of two slopes did not overlap, the results were considered to be significantly different. DNA unwinding kinetics analysis was plotted on a log/log scale of percentage double-stranded DNA versus time of lysis. Again, the experiments were repeated several times and a representative set of data presented.

C. Treatments

Unless otherwise indicated:

1) controls for all treatments consisted of the same cells as used in the treatments, grown as monolayers and spheroids, seeded and assayed as previously mentioned.

2) cells were incubated in minimal essential medium supplemented with five percent fetal bovine serum.

3) X-ray dose response experiments were performed.

a. Cell Crowding and Density

Monolayer and spheroid cells were compared for responses to ionizing radiation when grown at different cell packing densities. To observe the effects of crowding, the same number of cells (5×10^4) were plated in increasingly larger areas (1 - 80 square cm) on 100 mm plastic petri plates. The cells were allowed to attach for two hours in an incubator. Medium was added to each plate to bring the total volume to ten ml, and the plates were incubated for 24 hours. The cells were then trypsinized, counted for yield and assayed for percent double-stranded DNA as previously described.

Varying numbers of cells were also seeded into the same sized area, in 100 mm petri plates. The cells were incubated overnight and treated as above. To further examine the differences between cells grown at high and low densities, the DNA unwinding kinetics were analysed as previously described.

Density effects were also analyzed for spheroids by seeding 2×10^6 cells into 20 ml of medium in a spinner flask and allowing the cells to incubate. The same number of cells was also plated into 20 ml of medium in a petri plate and analysed as a control.

b. Medium Flow

In spheroid cultures, the medium is constantly moving, while in monolayers, it is relatively static. To test the effects of continually moving medium on monolayer cultures, several experiments were devised.

Microcarrier beads (Cytodex 1, Pharmacia) were prepared as per manufacturer's instructions by autoclaving one gram of dry beads in 50 ml of phosphate buffered saline. A volume of beads and cells were then added to 200 ml of medium in a spinner flask, so as to provide a bead surface area to cell ratio that was equivalent to 3×10^4 cells/cm^2 (the same density as cells plated on petri plates). These beads provided tiny monolayer cultures which travelled through the moving medium of the spinner flask. As with plated cultures, cell crowding on the beads could be controlled by seeding flasks with varying cell concentrations. The cells were incubated for 24 hours and assayed for DNA damage caused by ionizing radiation.

A variation of this was to plate cells in 60 mm petri plates and allow them to attach for several hours, after which the plates were placed in spinner flasks. Then, 200 ml of medium was carefully added to the flasks, which had been gassed with four percent CO_2 in air, and they were permitted to spin in an incubator for 24 hours. This provided a continual movement of medium over the monolayer cells.

Cells were also placed in 60 mm tissue culture plates and allowed to attach for several hours. The plates were then placed in a moving platform incubator set at such a speed as to allow the medium to move gently back and forth over the monolayers. As a control, duplicate plates were placed in a non-moving incubator for 24 hours. Both were incubated and assayed for DNA damage following radiation exposure.

Another variation of this was to plate cells on 100 mm petri plates, allow them to attach for several hours, then to replace the medium every hour for 24 hours, while incubating.

c. Conditioned Medium

To further test the effects of medium, cells were grown as monolayers, low (3×10^3 cells /cm^2) and high (3×10^4 cells/cm^2) density and as spheroids, in medium which had been "conditioned". This medium was obtained from monolayer cultures which had been growing in it for 24 hours. The purpose of this was to observe whether medium with metabolic by-products or nutrient depletion had any effect on radioresistance.

To ensure that any effects seen were not due to some factor released into the medium from the plastic petri plates, medium was also conditioned in glass petri plates and the two compared. Cells were also grown in glass petri plates and assayed for the contact effect, as a control.

d. Poly(HEMA)

Poly(2-hydroxyethyl methacrylate) or poly(HEMA) is a polymer, which when applied to tissue culture dishes, reduces their adhesiveness and thus the extent that cells can spread or even attach. Poly(HEMA) crystals (Hydron Laboratories, New Jersey) were dissolved in 95 percent ethanol to a stock concentration of 12 percent (w/v). This mixture was turned slowly overnight at 37^{o}C and then centrifuged for 30 minutes to remove undissolved particles. The stock was then diluted to one-tenth the concentration with 95 percent ethanol, and two ml each were plated on 100 mm bacterial petri plates. Depending on the thickness of the poly(HEMA), the V79 cell line will adhere to a certain extent over a 24 hour period, with many cells left floating, although there were no cells attached at the concentration used in these experiments. These plates were allowed to dry overnight on a level bench free from vibrations.

Growth medium and cells were added to the poly(HEMA)-coated plates. The plates were then incubated for 24 hours, and the cells which remained floating in the medium as aggregates above the poly(HEMA) were examined for DNA damage following exposure to ionizing radiation. The thickness of the polymer was such that no cells attached to the surface of the plates. Cells attached to uncoated bacterial plates and the cells which floated above them were also assayed for comparison.

e. Trypsinization

To differentiate between the roles of cell shape and cell contact, exposure of the cells to trypsin was used to keep them from adhering to each other for any length of time, in the case of spheroids, or from fully elongating, in the case of monolayers.

Cells were seeded into spinner flasks with medium and incubated as usual except that they were removed from the flasks every four hours and centrifuged. The medium was aspirated and put back into the flasks. The cells were trypsinized by pipetting gently in three ml of 0.1 percent trypsin and incubated for four minutes in a 37^{o}C water bath, after which medium was added and the cells centrifuged again. The single cells were then resuspended in five ml of medium and added to the spinner flasks containing the previously aspirated medium.

Monolayer cultures were trypsinized every four hours by twice rinsing with 0.1 percent trypsin and incubated at 37^{o}C for four minutes in a film of trypsin. The cells were then suspended in ten ml of medium by vigorous pipetting. As controls, one plate received new medium every four hours but no trypsin, one received the trypsin treatment plus fresh medium, and another received the trypsin treatment but the same medium. Cells were then resuspended in medium, irradiated and examined for DNA damage.

f. Methyl Cellulose

To further study the effects of shape, cells were grown as monolayers in methyl cellulose. This viscous material prevents the cells from spreading and does not encourage aggregation, except as a result of cell division.

Eleven grams of methyl cellulose powder (4000 centipose) were added to 250 ml of distilled water (80 - 100^{o}C) and the mixture was stirred overnight at 4^{o}C. It was autoclaved and cooled slowly to 4^{o}C, after which 250 ml of double-strength minimal essential medium was added. This mixture was stirred overnight at 4^{o}C. Medium, containing ten percent fetal bovine serum, was added to bring the concentrations to 1.1 percent methyl cellulose and five percent fetal bovine serum. A single cell suspension (2 x 10^{6} cells in one ml) was added to ten ml of the methyl cellulose mixture, which had been warmed to 37^{o}C, in a 13 ml test tube and the tube gently rocked several times to distribute the cells. This was poured into a non-tissue culture plate, which was used to ensure that the cells could not spread out beneath the methyl cellulose medium, and incubated for one, two or three days. Cells were also incubated in the usual growth medium, in both types of plates, as controls.

RESULTS

The close three-dimensional cell contact which develops when Chinese hamster V79-171B cells are grown in suspension has been assumed to be a requirement for the development of the contact effect. However, preliminary experiments suggested that a similar effect could be observed in sparsely seeded monolayer cultures of these cells and in two other non-fibroblast cell lines grown as monolayers. These observations indicated that cells may not need intercellular contact to develop the contact effect. Two other likely explanations for the differences in DNA unwinding kinetics are the changes in cell shape which accompany growth in suspension and changes in nutrient delivery. In an attempt to discriminate between these three possible contributors to this effect, the experiments described below define the culture conditions under which the contact effect is observed. In all cases, the contact effect has been defined in terms of DNA unwinding kinetics using the alkali unwinding assay.

A. Culture Conditions Under Which the Contact Effect is Observed

Results obtained using V79 cells are summarized in Table II. This provides a guide for following the experiments and interpreting the data.

TABLE II. SUMMARY OF RESULTS FROM V79 CELL LINE EXPERIMENTS

Fig.	Experiment	Cell Shape	Cell:cell Contact	Medium Exchange[a]	Contact Effect[b]
6.	Low density monolayers	flat	-	+/-	++
8.	Monolayers in spinner flasks	flat	-	+	+/-[c]
9.	Dense monolayers with hourly medium renewal	flat	-	+	++
11.	Microcarrier beads in spinner flasks	flat	-	+	+/-[c,d]
12.	Spheroids in spinner flasks	round	+	+	++
13.	Dense monolayers trypsinized every 4 hr	round	-	+	++
14.	Spheroids trypsinized every 4 hr	round	-	+	++
15.	Cells in methyl cellulose	round	+/-	-	++
16.	Cells floating above poly(HEMA)	round	+	-	++
16.	Cells floating above monolayers	round	+	-	+[d]
18.	Spheroids in conditioned medium	round	+	-	+[d]
18.	Sparse monolayers in conditioned medium	flat	-	-	+[d]

a, refers to growth of cells in a large volume of medium or regularly renewed medium

b, measured using alkali unwinding assay

c, density dependent

d, partial contact effect, significantly different from monolayer or spheroid response at the 95% confidence level.

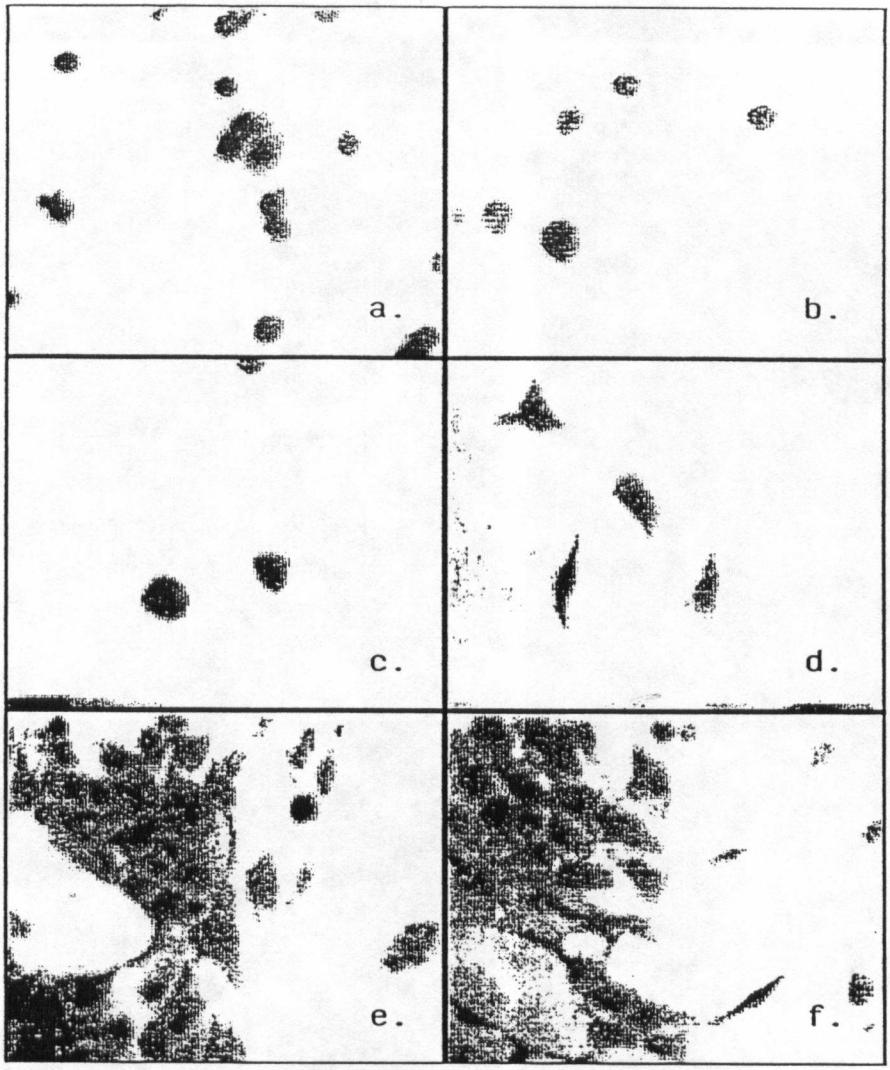


Figure 5. Microscopic appearance of cells using digitized images. Less extended cells are more radioresistant.
WiDr cells: (a) high density, (b) low density - equally radioresistant.
V79 cells: low density
(c) control, (e) conditioned medium - difference in shape, control more radioresistant.
V79 cells: high density
(d) control, (f) conditioned medium - no difference in shape or radioresistance.

a. Cell Crowding in Monolayer Cultures

Chinese hamster V79 cells were plated on the same surface area at various cell concentrations. Cells plated at concentrations of 3 - 6 x 10^4 cells/cm^2 and grown for 24 hours, resulted in the same radiosensitivity, i. e. a monolayer response (Figure 6). This concentration was used for the "high" density plating in all subsequent experiments. A tenfold decrease in density was used for "low" density plating, as it resulted in a spheroid-like, radioresistant response when examined. A response intermediate between that of monolayers and spheroids was observed for cells plated at intermediate densities.

Results in Figure 6 might be explained by differences in the degree of medium depletion, so to test this hypothesis, the same number of cells were plated into increasingly larger areas. The density of cells was chosen to avoid problems with severe overcrowding, which would have occurred in the smallest area, and prevented the cells from dividing.

The cells were seeded onto the plates and then medium was added to allow them to distribute to a predetermined area. The cells were allowed to attach for two hours, then medium was added to bring the total in each plate to ten ml. The cells remained in the area to which they had attached. After 24 hours of incubation, the cells were trypsinized to single cell

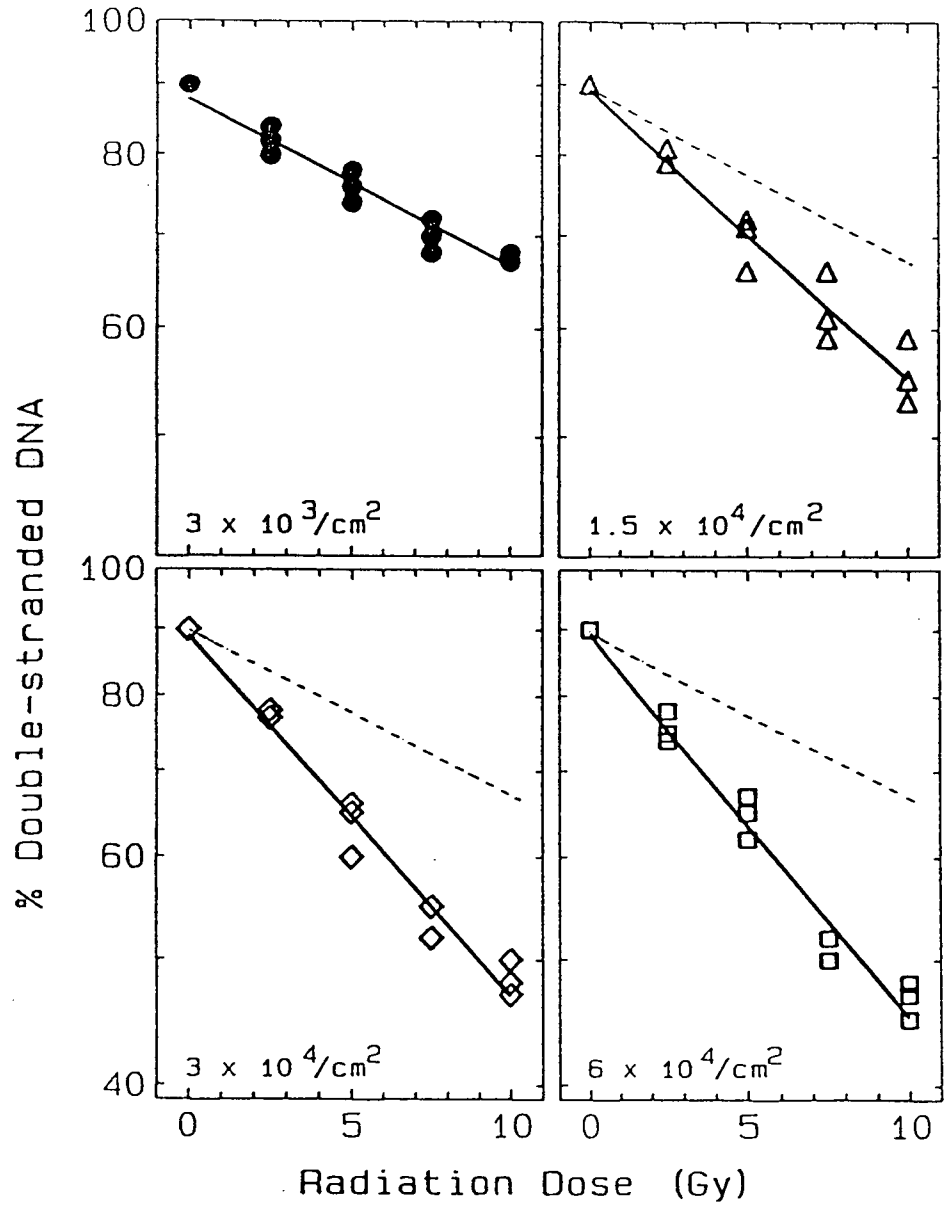


Figure 6. Effect of Cell Density - Varying Cell Number. X-ray dose response of monolayer cells grown at varying cell concentrations in the same sized area of 80 square cm. The dotted line represents the response seen with the lowest concentration.

suspensions, counted for yield, irradiated and assayed using the DNA unwinding assay. Cells in the more crowded conditions displayed a monolayer response, while as the cells became less crowded, more radioresistance, in terms of DNA unwinding kinetics, was apparent (Figure 7).

Figure 8 is the low density control graph, with cells grown and plated as mentioned above. As the more sparsely seeded cells displayed a more radioresistant dose response, it seemed prudent to examine another aspect of the contact effect, i. e. the DNA unwinding kinetics in alkali solution. A distiguishing trait of spheroid cells is that the DNA, once irradiated and left to unwind, will cease to do so after approximately ten minutes, while the densely growing monolayer cells will continue to unwind with time (43). This is one of the characteristics of spheroid cells that has led to speculation that there are some type of constraints within the DNA which will not permit further unwinding. Figure 9 indicates that sparsely grown cells show this same property. It was observed that low density monolayer cells show a less elongated shape than control cells, when observed with a microscope after 24 hours in culture (Figure 5).

b. Medium Replenishment in Monolayer Cultures

To test the effect of the exchange rate of medium on densely plated monolayer cultures, cells were permitted to attach in five ml of medium, to tissue culture plates. These

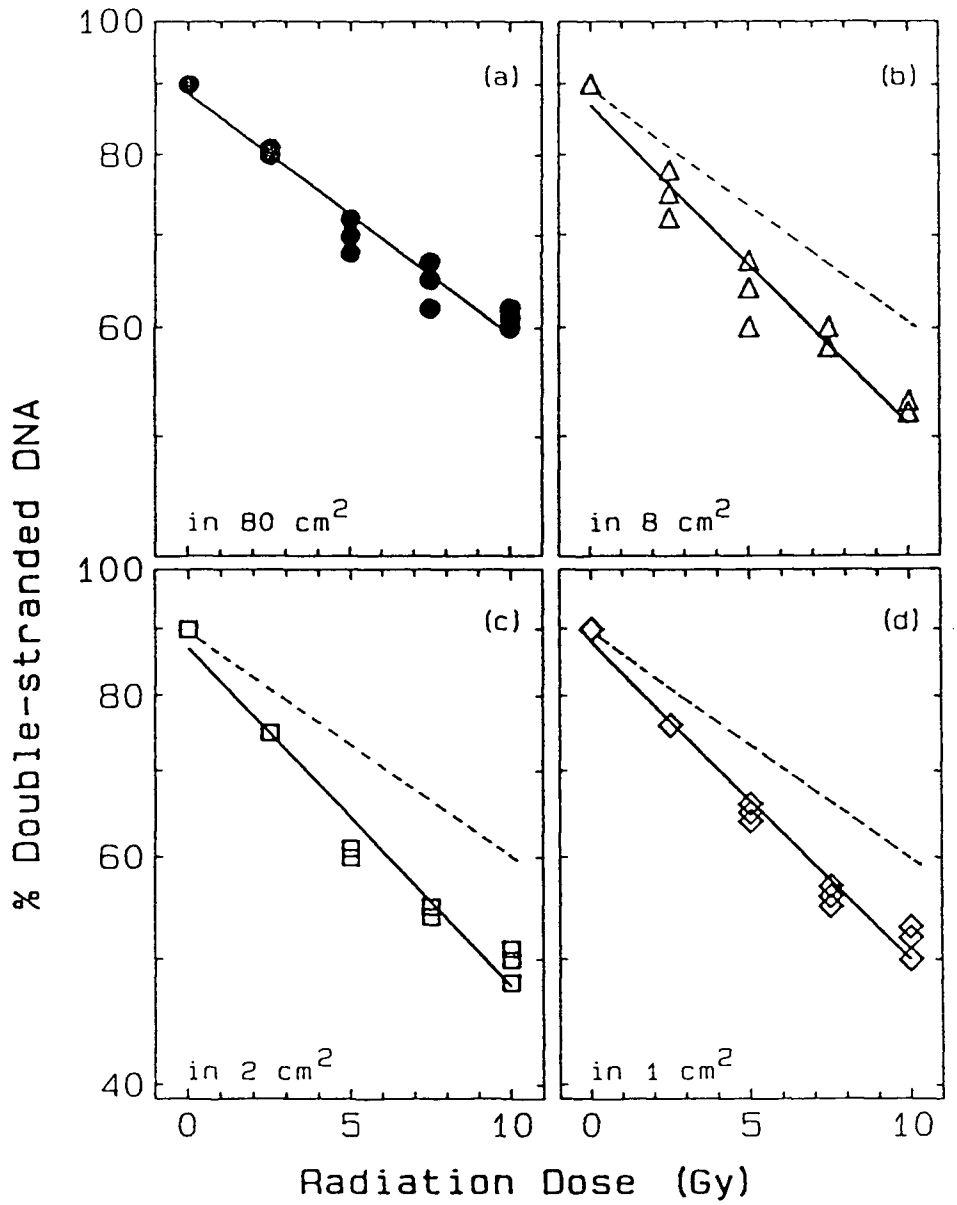


Figure 7. Effect of Cell Crowding on Monolayer Cells. X-ray dose response of monolayer cultures plated at the same cell number in different sized areas. The dotted line represents the response of the cells in the largest area. The slopes of the curves in panels (b), (c) and (d) are significantly different (at the 95 percent confidence level) from the slope of the curve in panel (a).
cell number = 5×10^4

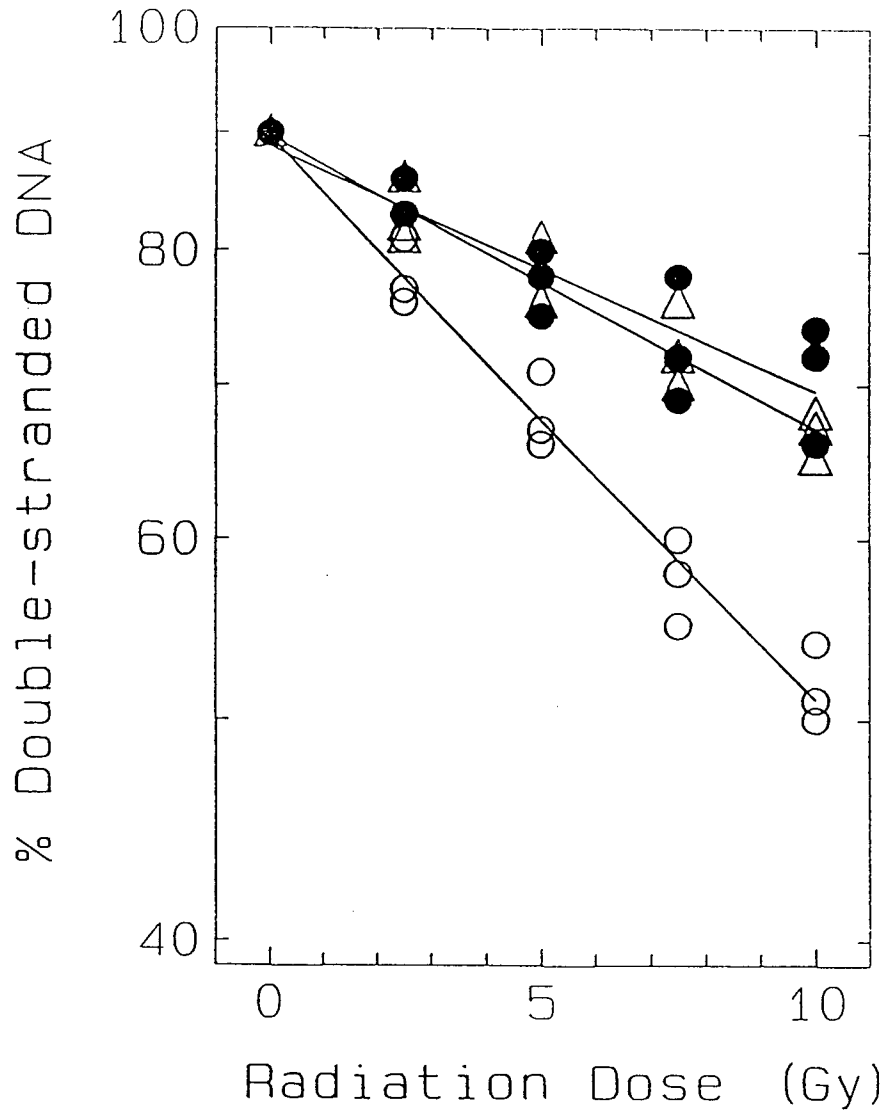


Figure 8. Effect of Cell Density - X-ray Dose Response. Cells grown as spheroids, low and high density monolayers. low (Δ), high (o), spheroids (•)

high = 3 x $10^4/cm^2$ low = 3 x $10^3/cm^2$

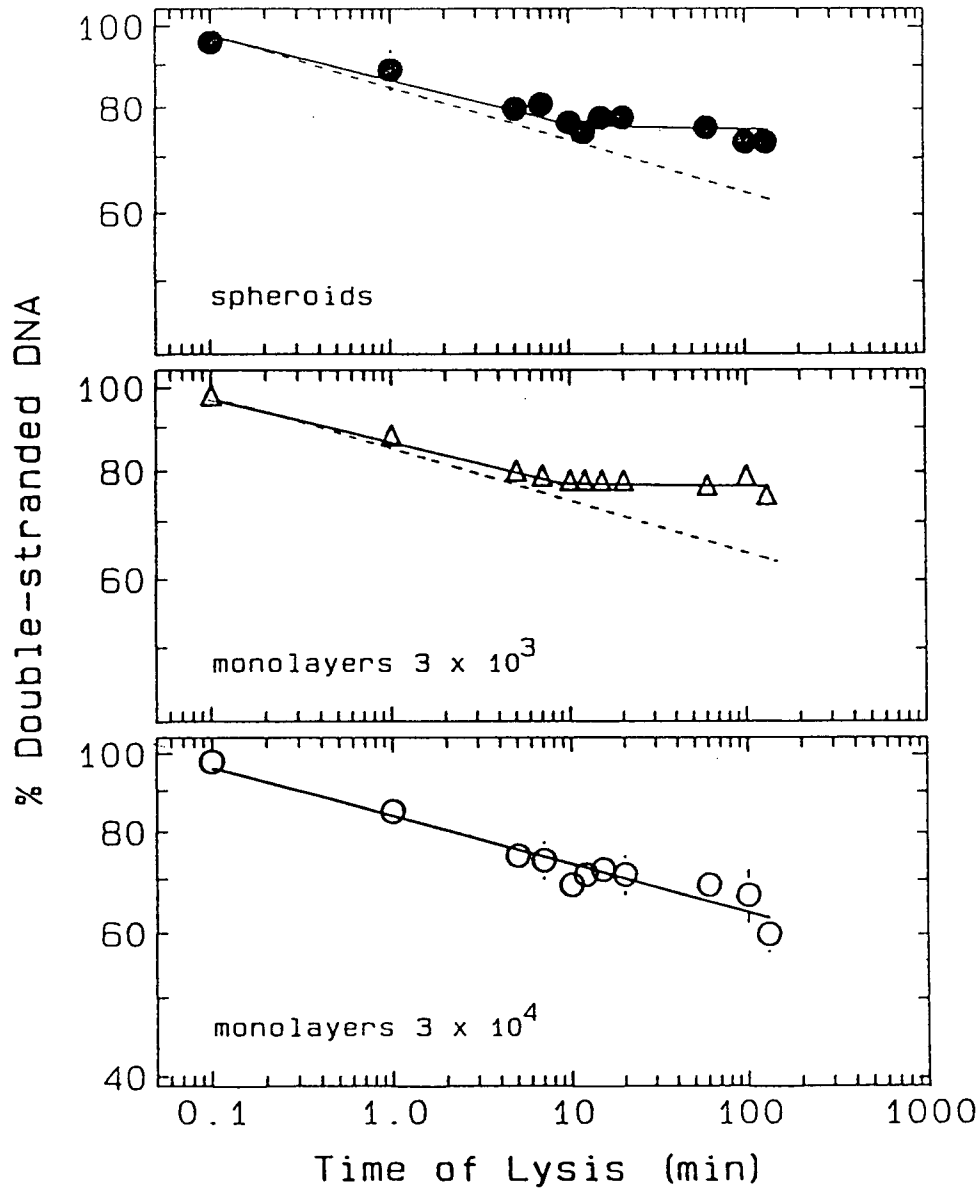


Figure 9. Effect of Cell Density - DNA Unwinding Kinetics. Cells grown as spheroids, low and high density monolayers (cells/square cm). The dotted line represents the densely-grown monolayer response.

were placed in spinner flasks to which 200 ml of medium was carefully added. The cultures were allowed to spin overnight. These cells also displayed radioresistance (Figure 10) and when observed under a microscope, had a more spherical shape than cells grown in static medium as controls.

Thus it would seem that there are two effects; the physical movement of the medium and the sweeping away of by-products and replenishment of nutrients. To distinguish between the two, densely seeded monolayers were grown in ten ml of medium which was renewed every hour during a 24 hour incubation period. Figure 11 shows that this resulted in relative radioresistance. This population of cells was assayed for DNA unwinding kinetics and the results are shown in Figure 10. Again the DNA unwinding kinetics are similar to those of spheroid cells, inferring a similarity in DNA conformation.

Monolayer cells were also grown as above except that the medium was renewed every four hours (Figure 15). Results from the assay of this culture indicated an intermediate radio-resistance compared with Figure 11, where the culture displayed a full contact effect.

c. Growth of Cells on Microcarrier Beads

Using cells attached to microcarrier beads, which are placed in suspension in spinner flasks, provided another method

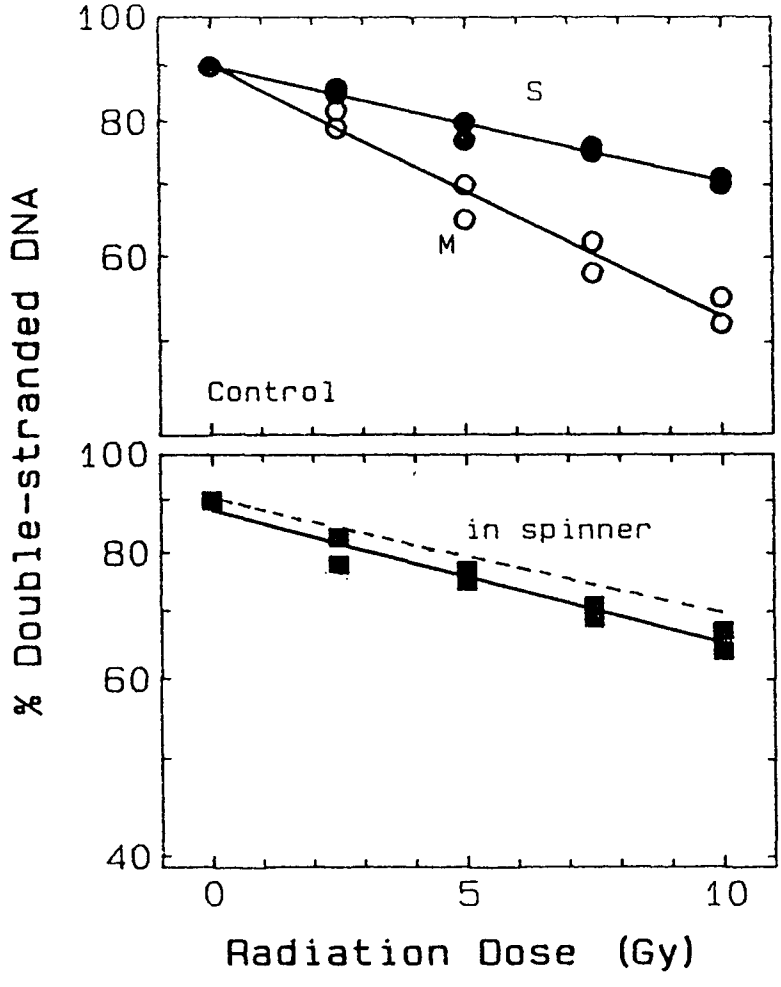


Figure 10. Monolayer Cultures in Moving Medium of Spinner Flasks. X-ray dose response of cells grown on plates inside spinner flasks, bathed by 200 ml of continually moving medium. The dotted line represents the spheroid control response. S = spheroids, M = monolayers

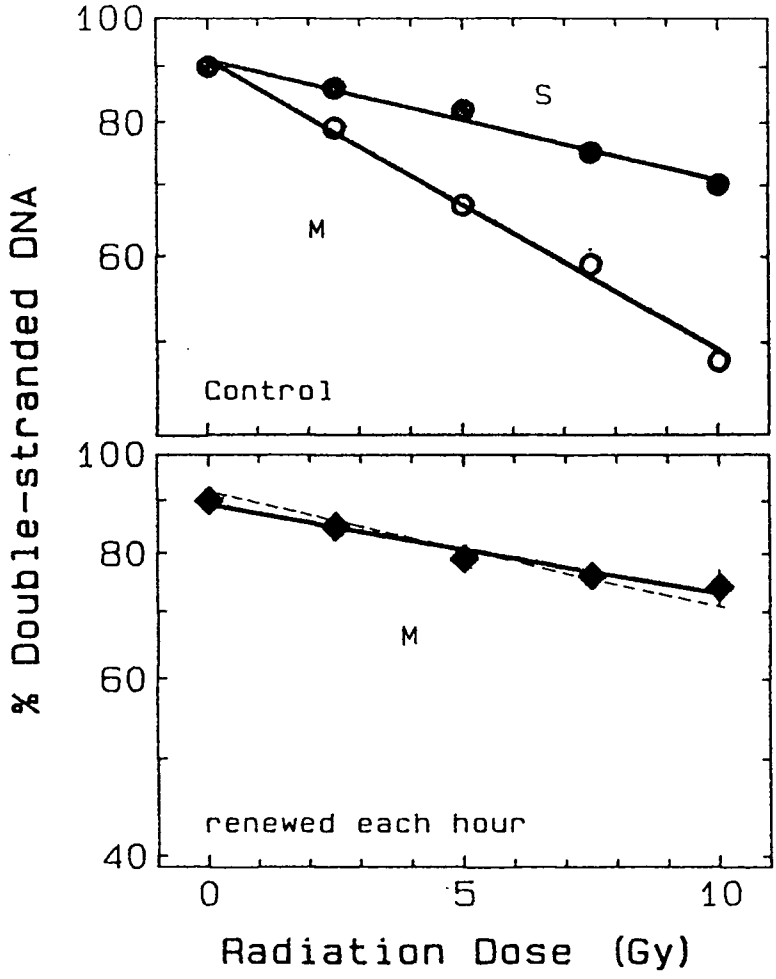


Figure 11. Monolayer Cultures in Renewed Medium - X-ray Dose Response. Cells grown in medium which was replaced hourly for 24 hours. The dotted line represents the spheroid control response.
S = spheroids, M = monolayers

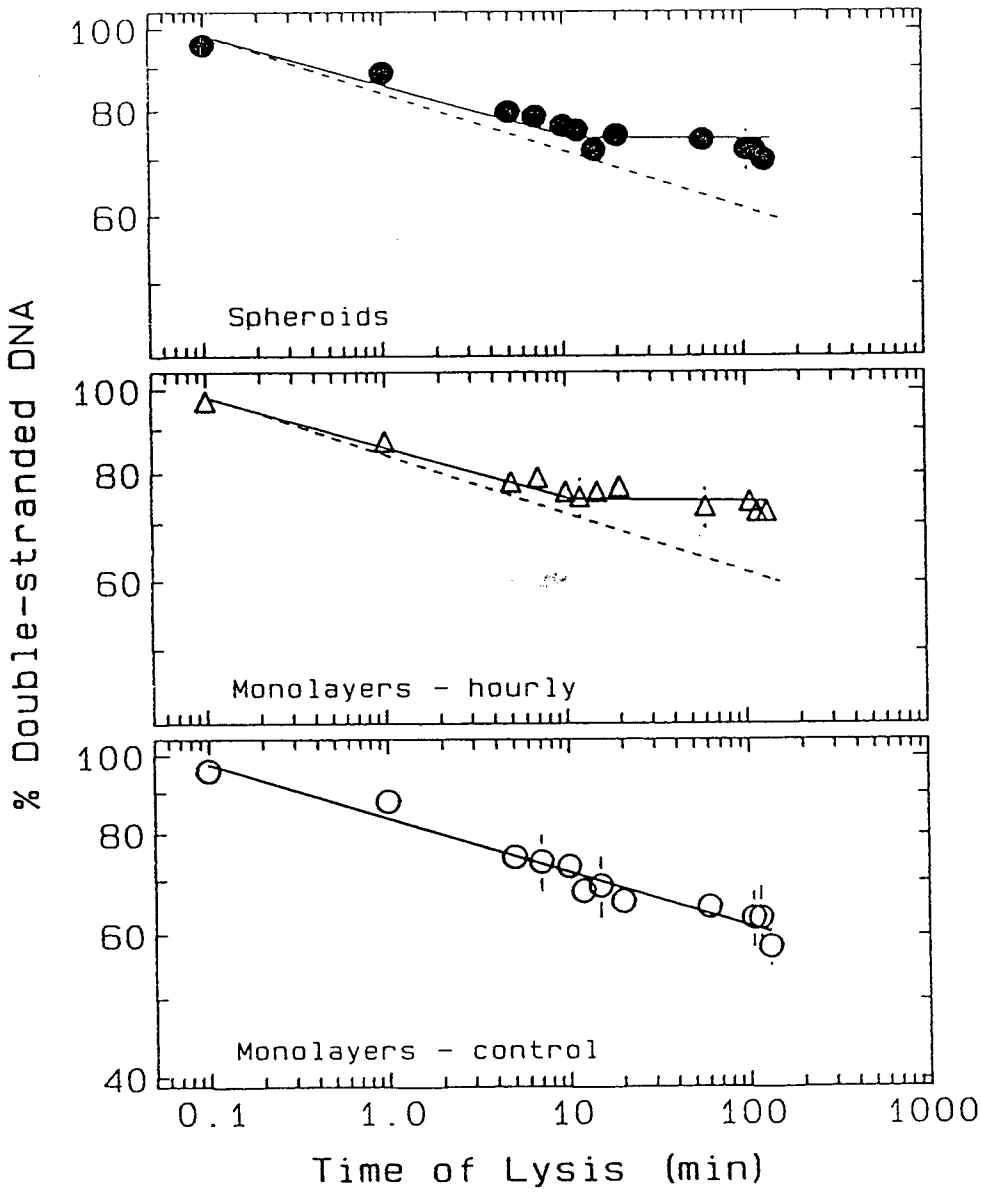


Figure 12. Monolayer Cultures in Renewed Medium - DNA Unwinding Kinetics. Cells grown in medium which was replaced hourly for 24 hours. The dotted lines represents the monolayer control response.

to prevent local gradients from forming. When the cells do not completely coat the beads, the radioresistance of these cells is like that of spheroid cells as can be seen in Figure 13. However, when the beads are fully coated with cells, less radioresistance is observed.

In all the experiments with medium moving over monolayers, the medium itself was assayed for cells, as the more radiosensitive mitotic cells tend to detach and this could influence the results. Very few cells were seen when this medium was observed under a microscope. This medium was incubated in plates for one week and assayed for colonies, which would have grown from these cells. Very few colonies were observed.

d. Growth of Cells in Suspension Culture

Spheroids are usually grown in a much larger volume of medium than are monolayers. To investigate the possibility that this may have some effect, spheroids and monolayers were both grown in 20 ml of medium. Both cultures showed indentical cell yields after 24 hours, so growth was comparable. However, the spheroid cells continued to display a contact effect, as seen in Figure 14. There may be a role for movement. Movement may prevent local gradients from forming, and the stress of medium moving over the surface of the cells may keep them in a spherical conformation. Also, it is known that cell attachment to a surface induces the synthesis of cell spreading factors,

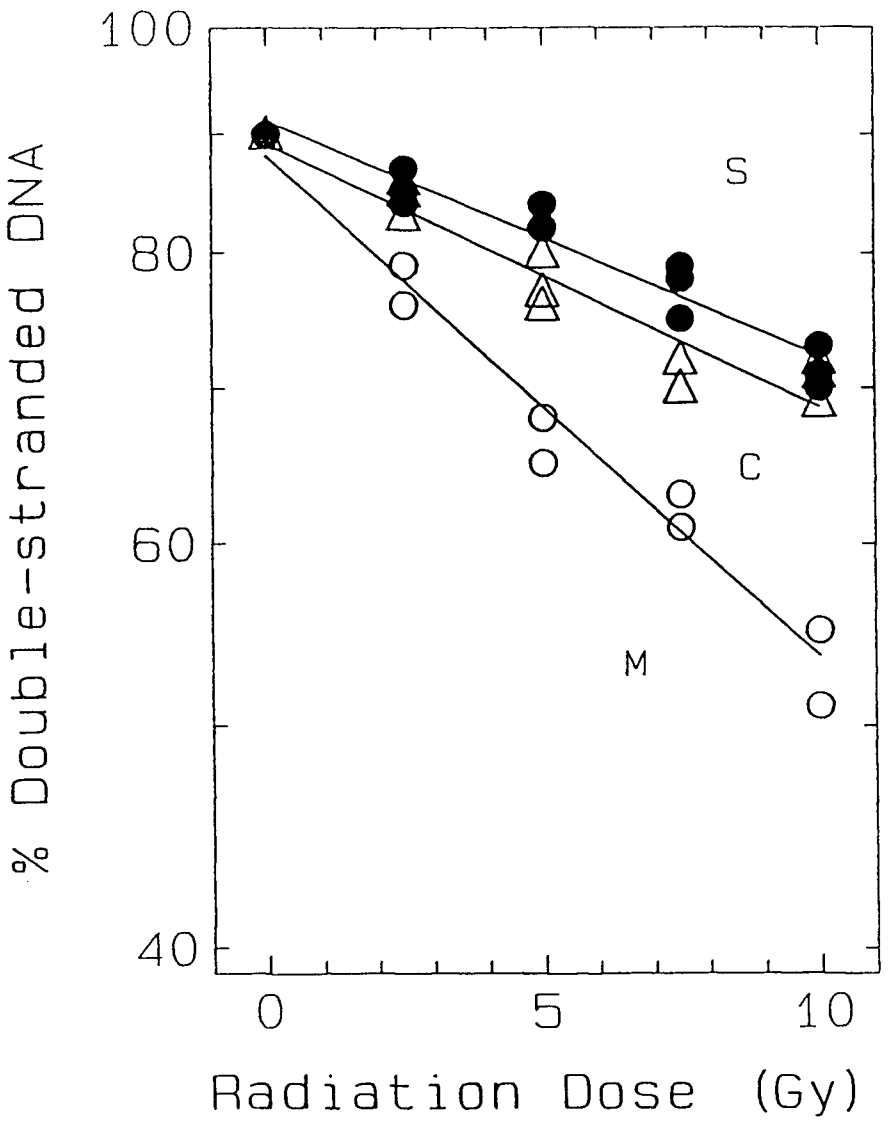


Figure 13. Monolayer Cultures on Microcarrier Beads. X-ray dose response of cells grown at intermediate density on microcarrier beads in spinner flasks.
S = spheroids, M = monolayers, C = microcarriers

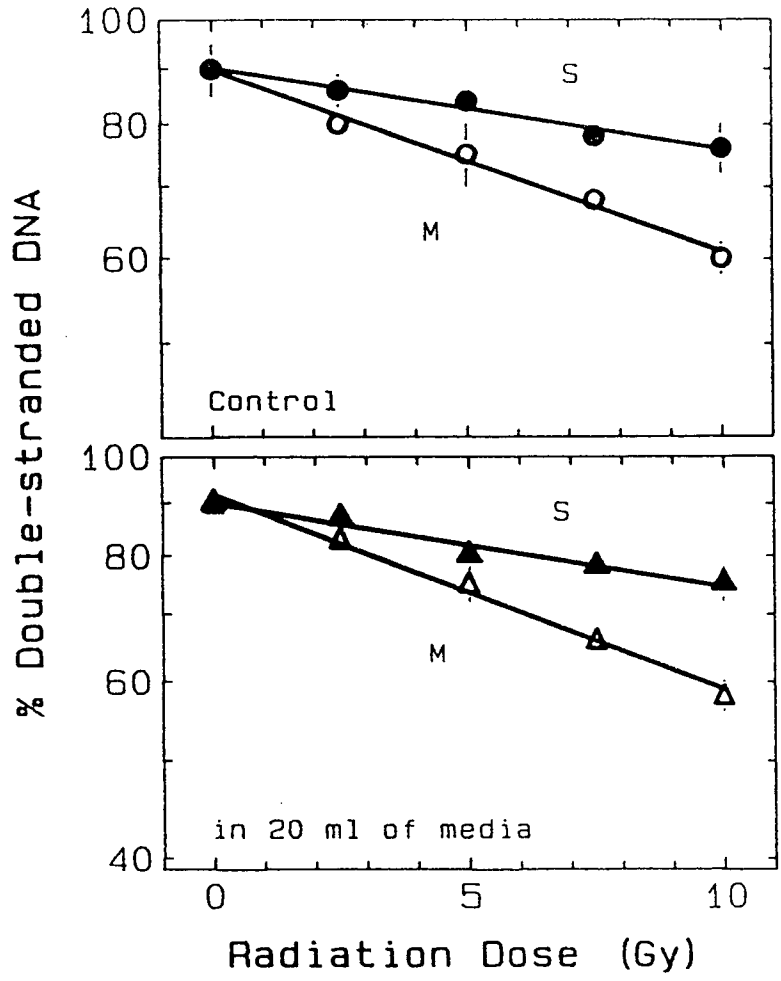


Figure 14. Effect of Cell Crowding on Spheroid Cells. X-ray dose response of spheroids cells grown at the same density in either 20 or 200 ml of medium. S = spheroid, M = monolayer

and perhaps these factors cause the radiosensitive response of the cells grown as monolayers.

e. Effect of Trypsinizing Cell Cultures

Monolayer cells were plated at a high density and allowed to attach. Every four hours, they were treated, either by merely replacing the medium with fresh, or by trypsinizing and replacing the medium, or trypsinizing and adding the same medium back to the plates. Four hours was chosen as the optimum time for cells to retain most of their rounded conformation, and yet be subjected to as few trypsin treatments as possible. To prevent excessive cell loss due to centrifugation and handling, the cells were trypsinized in a film rather than the usual two ml, and were resuspended in the same plate in which they had been growing. The amount of trypsin used will affect the the DNA unwinding kinetics of the cells, but this is not a large effect (Olive, unpublished). Radioresistance seen in these cultures is slightly higher than if the cells had been trypsinized as usual. Figure 15 seems to indicate that trypsin and new medium result in more radioresistance than either treatment alone. The individual treatments result in an intermediate amount of radioresistance. Perhaps this was a result of some factor which was present in two forms; soluble, which was removed by the changing of the medium, and attached to the cells, which was removed by the trypsin treatment.

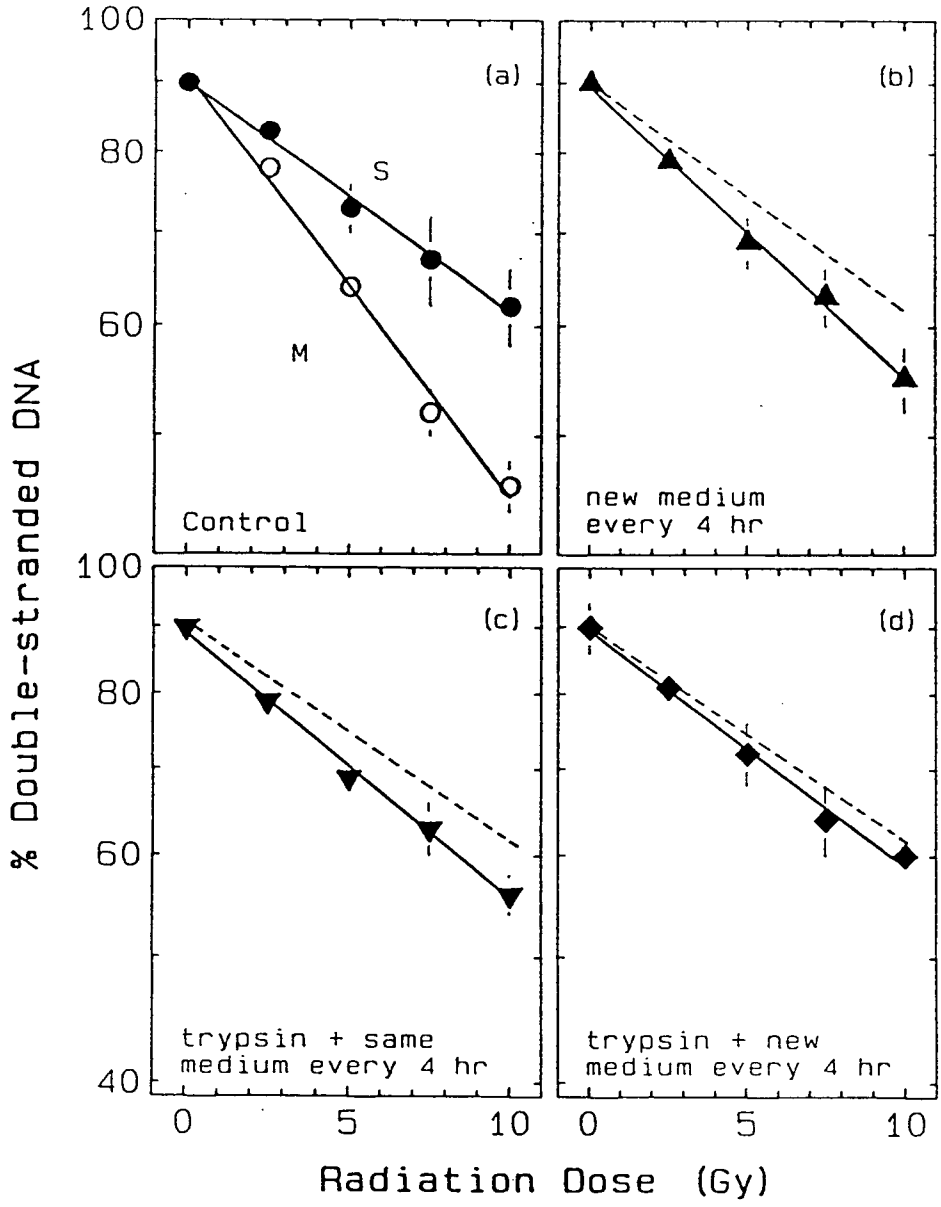


Figure 15. Trypsin Treatment of Monolayers. X-ray dose response of cells treated with trypsin to allow them to maintain a spherical conformation. The dotted line represents the spheroid control response. The slopes of the curves in panels (b) and (c) are significantly different (at the 95 percent confidence level) from the slope of the spheroid control response.
S = spheroids, M = monolayers

To separate the effect of contact from that of shape, spheroid cells were trypsinized every four hours. The cultures were observed before each trypsin treatment and it was seen that they had not formed many aggregates and those that had formed were in groups of two or four. Over 60 percent of the population was in single cell form. The cells in suspension treated with trypsin maintained the radioresistance of cells in contact, and the small decrease in the effect is probably due to toxicity of the trypsin treatments (Figure 16).

f. Growth of Cells in Methyl Cellulose

To further investigate the role of cell shape, cells were grown in methyl cellulose. The viscosity of methyl cellulose prevents cells from elongating or forming aggregates other than those formed when the cells divide. Cells were plated in methyl cellulose and incubated for one, two or three days. When assayed using the alkali unwinding technique, it was observed that these cells developed the contact effect slowly over three days (Figure 17). It has been observed that cells must grow and divide to display the contact effect, so the methyl cellulose cultures were assayed for rate of growth using the radioisotope tritiated thymidine which is incorporated into the DNA of dividing cells. The rate of growth for these cells was shown to be approximately half that of the control spheroid and monolayer cells (data not shown). Therefore, these results may be due to a difference in growth rate.

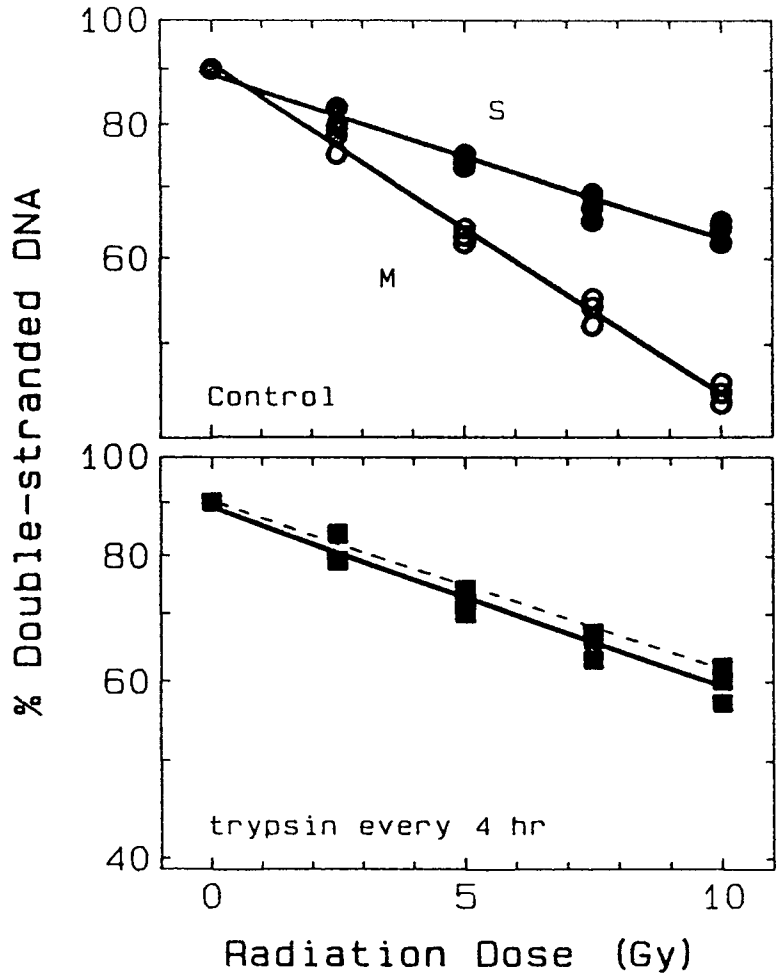


Figure 16. Trypsin Treatment of Spheroids. X-ray dose response of spheroid cells treated with trypsin to prevent aggregation. The dotted represents the spheroid control response. S = spheroids, M = monolayers

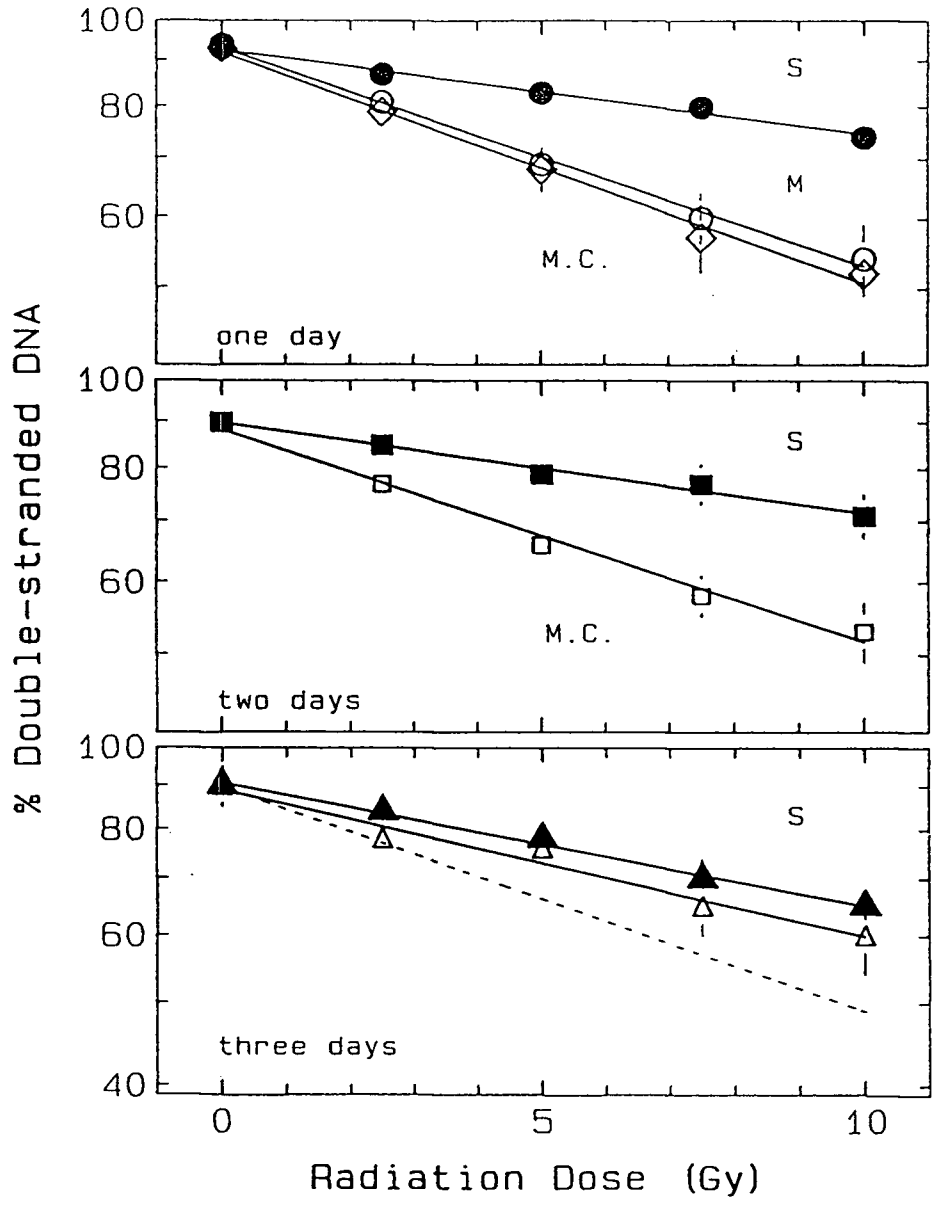


Figure 17. Monolayer Cultures Grown in Methyl Cellulose. X-ray dose response of cells grown for the indicated times in this medium. The dotted line represents the monolayer control response. The slopes of both curves in the third panel are significantly different (at the 95 percent confidence level) from the slope of the monolayer control response. S = spheroids, M = monolayers, M.C. = methyl cellulose

g. Growth of Cells above Poly(HEMA)

To understand the role of cell contact, a method was devised to grow cells in suspension under monolayer conditions, i. e. in ten ml of unstirred growth medium. Thus, the cells could float and aggregate, but the medium would not be moving.

The nature of V79 cells is such that, in the presence of serum, they will attach and flatten even on petri plates that are not designed to allow this. Therefore, a relatively thick layer of the polymer poly(HEMA) was applied to these plates to prevent any cells from attaching and assuming a flattened conformation. Attached and elongated cells exude different metabolites, including extracellular matrix proteins (57), into the medium and these would not necessarily be found in the spheroid cultures with which these cells were to be compared.

When the cultures were assayed using the alkali unwinding assay, there was a difference in radioresistance (Figure 18). Control cells attached to plates displayed a monolayer response as expected, but the cells floating spherical and aggregated in the same plates, exhibited only a little less radiosensitivity. Cells floating in the growth medium of the poly(HEMA)-coated plates though, displayed almost as much radioresistance as control spheroid cells growing in spinner flasks. There were no attached cells in the poly(HEMA) plates to exude monolayer-produced factors, suggesting that this may play some role.

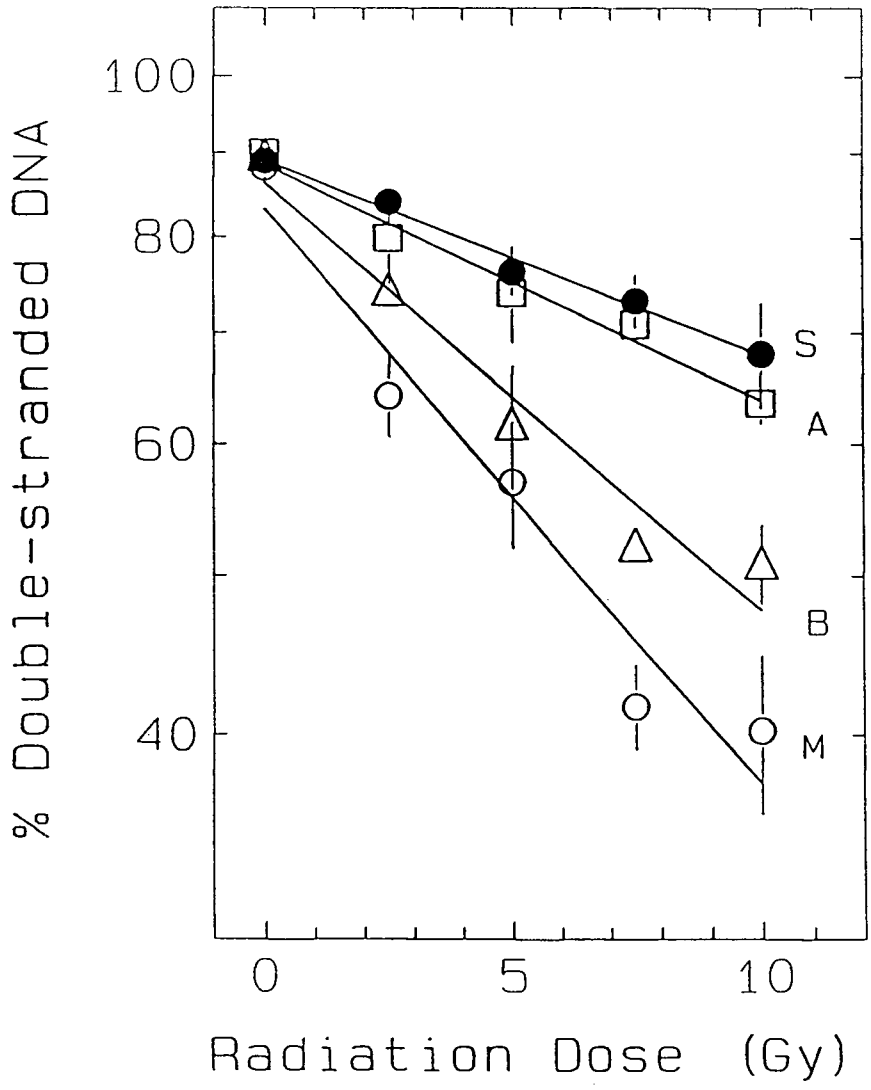


Figure 18. Cells Grown with Poly(HEMA). X-ray dose response of cells grown in monolayer conditions without the ability to attach. S = spheroids, M = monolayers attached to non-tissue culture plastic plates
A = Cells in suspension above poly(HEMA)
B = Cells in suspension above monolayers

B. Factors Inhibiting Development of the Contact Effect

a. Inhibition of Cell Growth and Division

It is known that cells require approximately 24 hours in suspension culture to display the full contact effect (17). For this reason it is important to ensure that the cells are growing normally, either by counting the cells for yield or using the incorporation of tritiated thymidine into the DNA as was done with the relatively slowly growing methyl cellulose cultures.

Cells in spinner flasks, incubated overnight at room temperature, showed no division and did not display a contact effect. These cells were grown under the same conditions, except temperature, that spheroids displaying the contact effect were grown. However, cells grown for 24 hours in suspension at $37^{o}C$, then incubated overnight at room temperature, did not grow but did maintain the radioresistance.

b. Growth of Cells in Monolayer Culture

It has been observed that cells in densely seeded monolayer culture do not develop a contact effect. Whether this is due to the lack of three-dimensional cell contact, cell shape or medium constituents has not been established. Medium movement over monolayer cells in spinner flasks resulted in development of radioresistance (Figure 10), but these cultures were grown in a

large amount of medium (200 ml). It was important to determine if it was the volume of medium or the movement of it over the cells that permitted the exhibition of the contact effect. To differentiate between the medium and movement, cells were densely seeded in plates with ten ml of medium. The cells were allowed to attach and then the plates were placed on a moving platform incubator. This allowed the medium to move gently over the cells. The radiosensitivity of these cells was not significantly different from control monolayer cells (Figure 19). These cells were also observed to be in the more elongated conformation of the control cells.

c. Use of Conditioned Medium

To more closely observe the effects of the medium, studies were done using conditioned medium. This was produced by exposing the usual growth medium to high density monolayer cultures for 24 hours. This medium was used to grow spheroid, low and high density cultures. Conditioned medium did not affect the response of the high density cultures, and the medium was not so depleted as to affect the growth of cells, but it did reduce the radioresistance of the spheroid and low density cultures (Figure 20). It can only be speculated whether this was the result of nutrient depletion or the presence of metabolic by-products which affect the development of the contact effect. Perhaps some substance released from monolayer cells, such as soluble extracellular matrix proteins, would

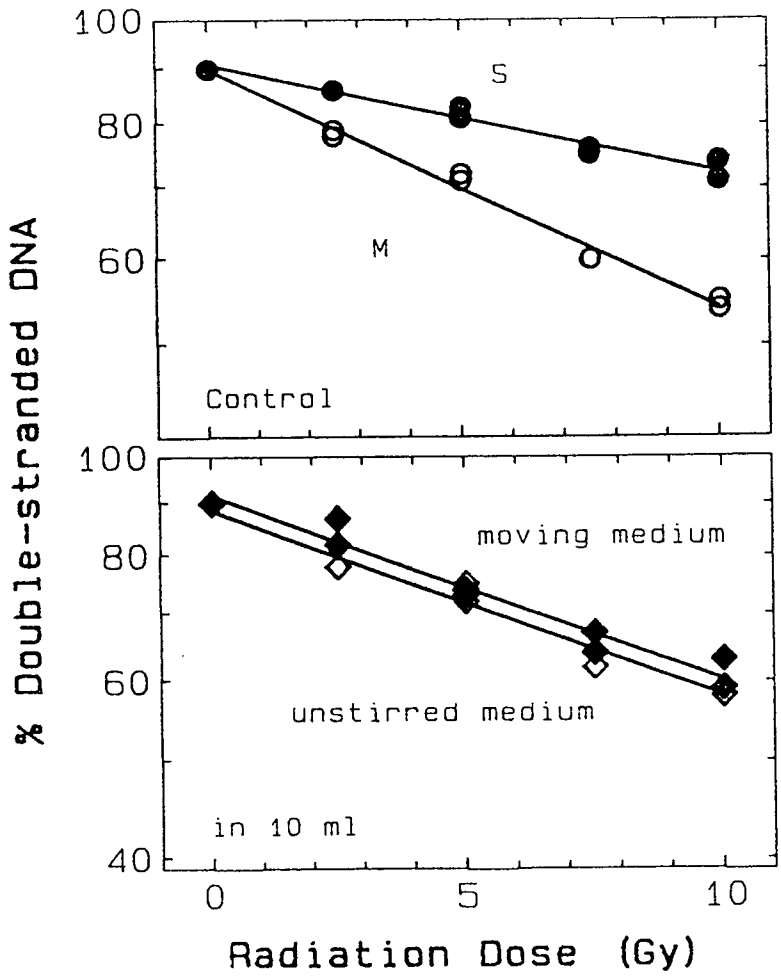


Figure 19. Monolayer Cultures in Moving Medium on Moving Platform Incubator (mixer). X-ray dose response of cell grown in ten ml of continually moving medium. S = spheroids, M = monolayers

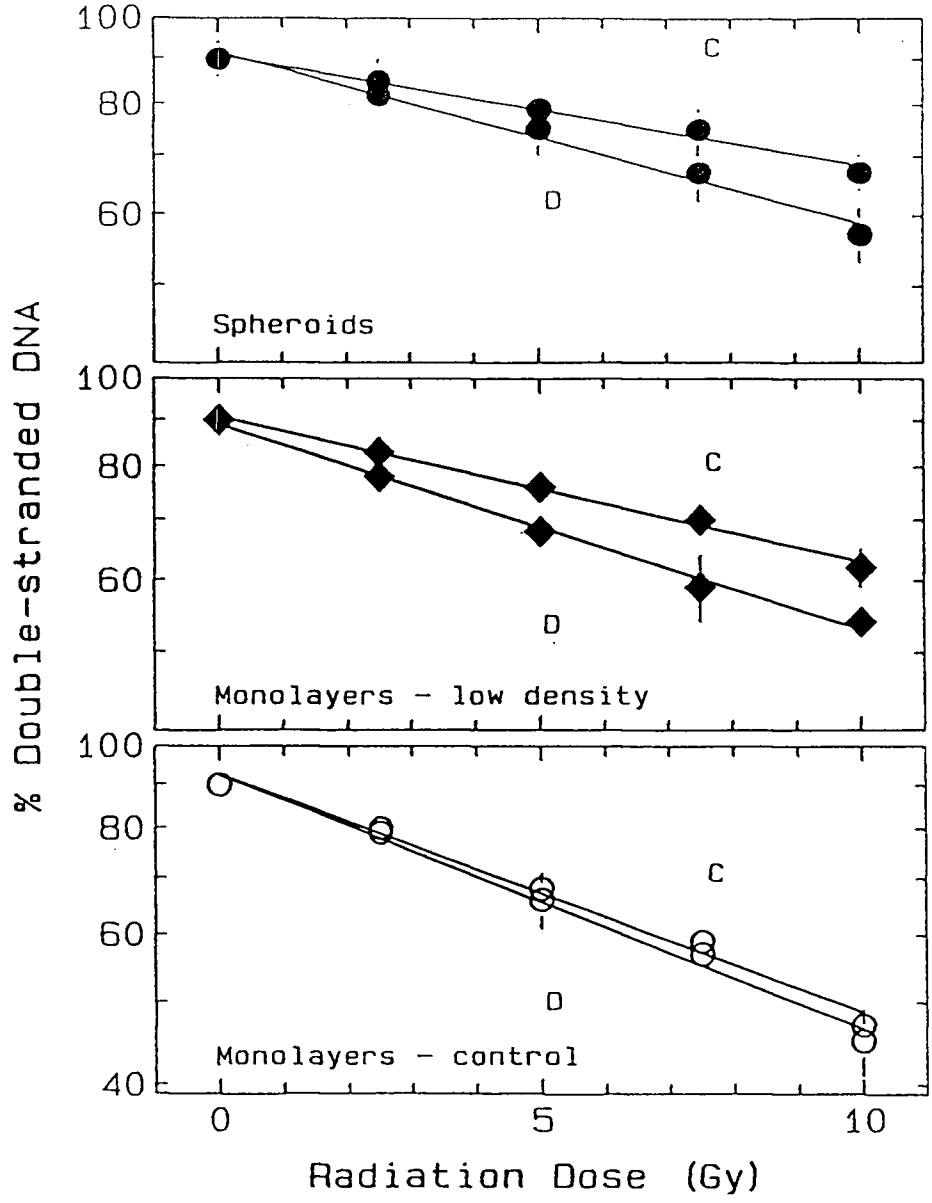


Figure 20. Cells Grown in Conditioned Medium - X-ray Dose Response. Effect of growing cultures in medium which has been conditioned by monolayer cells for 24 hours. The slopes of the curves in the top two panels are significantly different (at the 95 percent confidence level) from the slopes of their respective control responses.
C = cells grown in normal medium,
D = cells grown in conditioned medium

affect the DNA of the spheroid cells. The low density cells, treated with conditioned medium, were observed to be less rounded than the same cells grown in control medium (Figure 5).

To ensure that these results were not caused by toxicity from the plastic of the petri plates, cells were also grown on glass plates and assayed for the contact effect. There was no difference in radiosensitivity as compared with cells grown on plastic plates (Figure 21). Medium was then conditioned on plastic and glass plates and used to culture spheroid and monolayer cells. Figure 22 shows that there was no difference resulting from the type of conditioned medium used. The spheroid cells continued to display the lowered radioresistance seen when the cells are grown in conditioned medium, and the results were identical whether glass- or plastic-conditioned medium was used.

C. Other Cell Lines Examined for the Contact Effect

To establish the generality of the effect described for Chinese hamster V79-171b cells, other cell lines were examined for the presence of the contact effect. Cell lines were chosen for their ability to grow in suspension, as aggregates or not, as well as in monolayer culture.

The SCCVII cell line was intriquing because, although it grows well in suspension, cell aggregates do not form. When

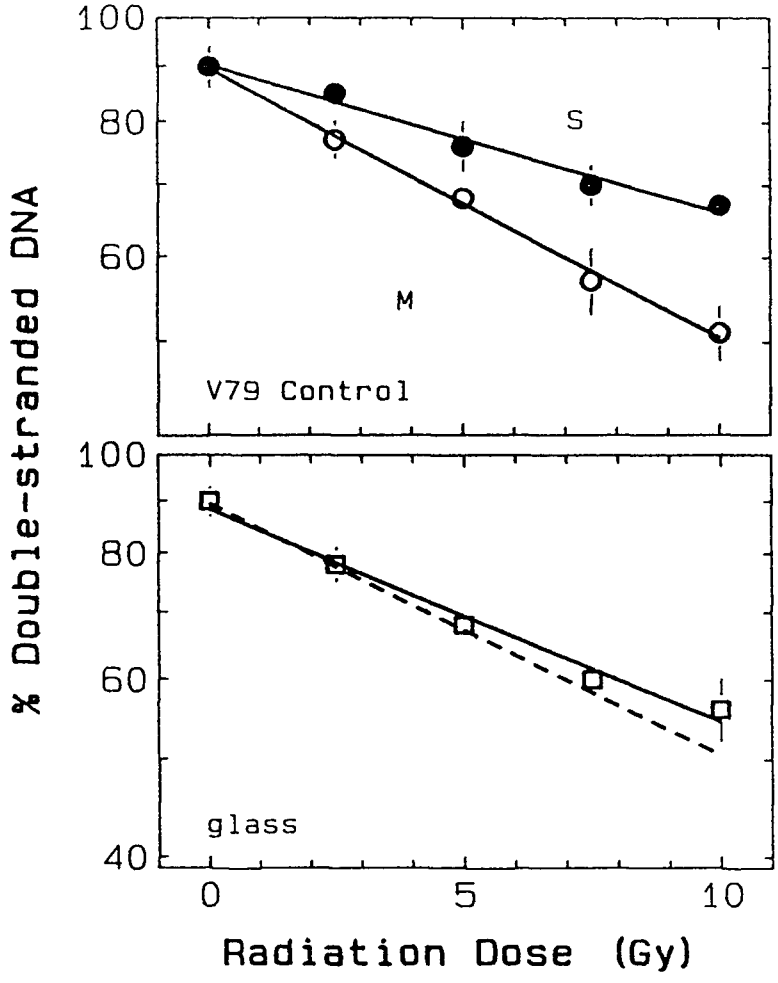


Figure 21. Conditioned Medium Control - Growth on Glass Petri Plates. X-ray dose response comparison between monolayers grown on glass or plastic. The dotted line represents the monolayer control response. S = spheroids, M = monolayers

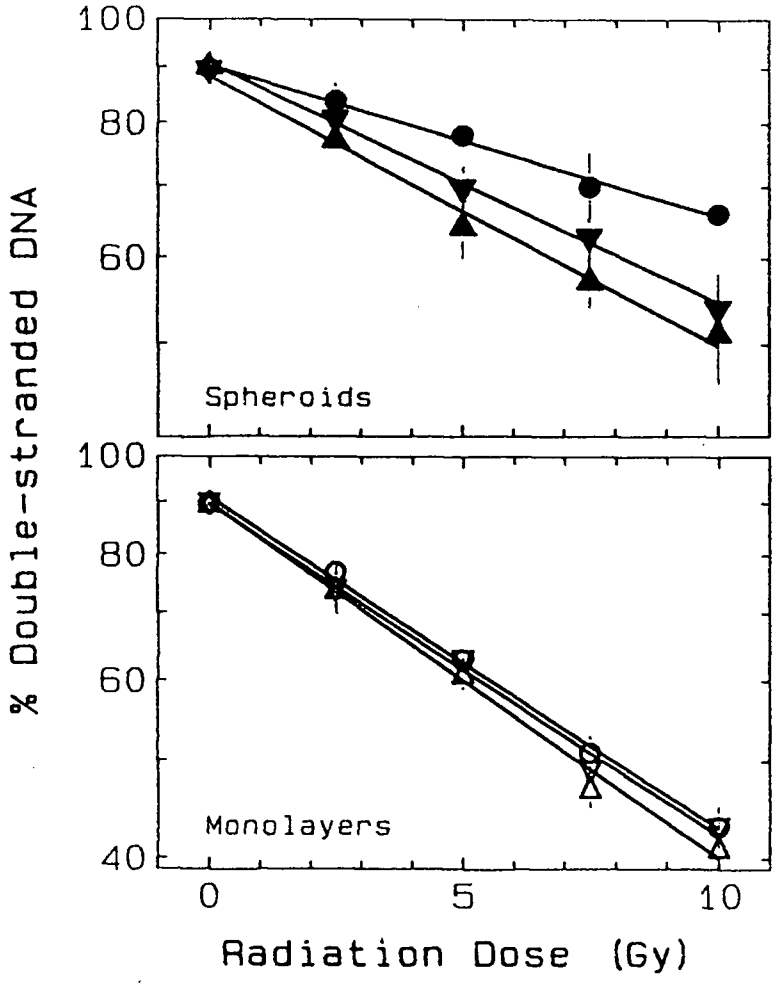


Figure 22. Conditioned Medium Control - Medium Conditioned in Glass or Plastic. Comparison of cultures grown in medium conditioned by growing monolayer cultures for 24 hours in either plastic or glass plates. control (●,○), glass (▼,▽), plastic (▲,△)

assayed for dose response (Figure 23), and DNA unwinding kinetics (Figure 24), results characteristic of V79 cells were observed. This supports the theory that cell contact does not play a significant role, although other properties of this cell line may provide the explanation.

The RIF-1 cell line grows in monolayer culture and as aggregates in suspension much like the V79 line, except that the doubling time is longer. The X-ray dose response and DNA unwinding kinetics indicated the presence of a contact effect (Figures 25 and 26).

The WiDr cell line grows as spherical cells in discrete colonies in monolayer culture (see Figure 5), and as aggregates in suspension. When DNA damage was assayed (Figure 27) both monolayers and spheroid cells were more radioresistant than V79 monolayer cultures. This could support the cell shape theory, but it must be noted that until more studies are done with these cells, it may be a property of inherent radioresistance and could represent a different mechanism altogether.

The C-4 I and C-4 II cell lines were also assayed for the contact effect (Figure 28). The C-4 I cell line grows as spherical cells in discrete colonies like the WiDR cells, and forms aggregates in suspension. An intermediate response was seen for the monolayer cells and the spheroid response was like that of the V79 cells. The C-4 II line grows in a more

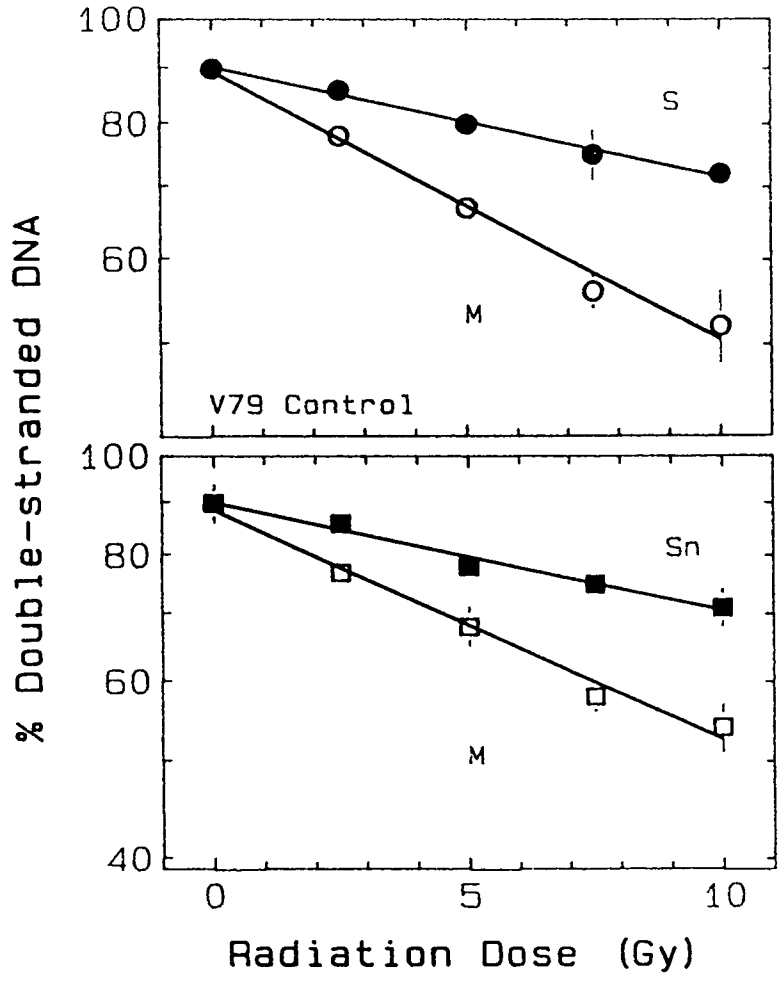


Figure 23. SCCVII Cell Line and the Contact Effect - X-ray dose response. Cells were grown in suspension culture and as monolayer cultures for 24 hours. S = spheroids, Sn = in suspension, M = monolayers

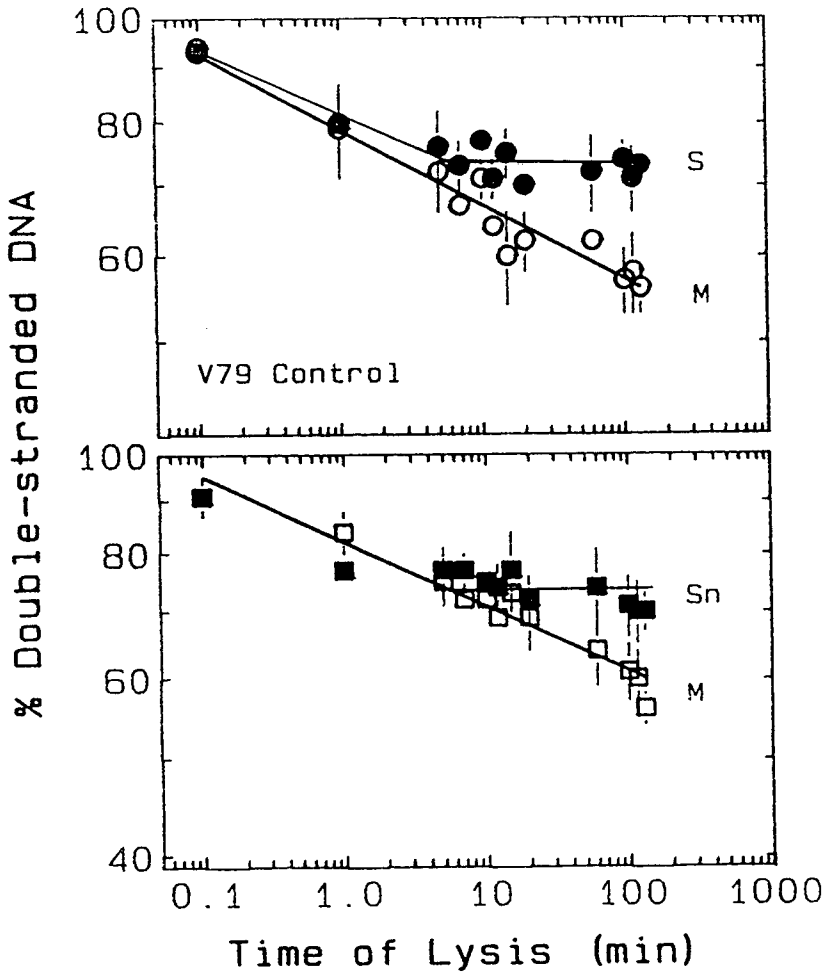


Figure 24. SCCVII Cell Line and the Contact Effect - DNA Unwinding Kinetics. Comparison with V79 cell line unwinding kinetics. S = spheroids, M = monolayers Sn = in suspension

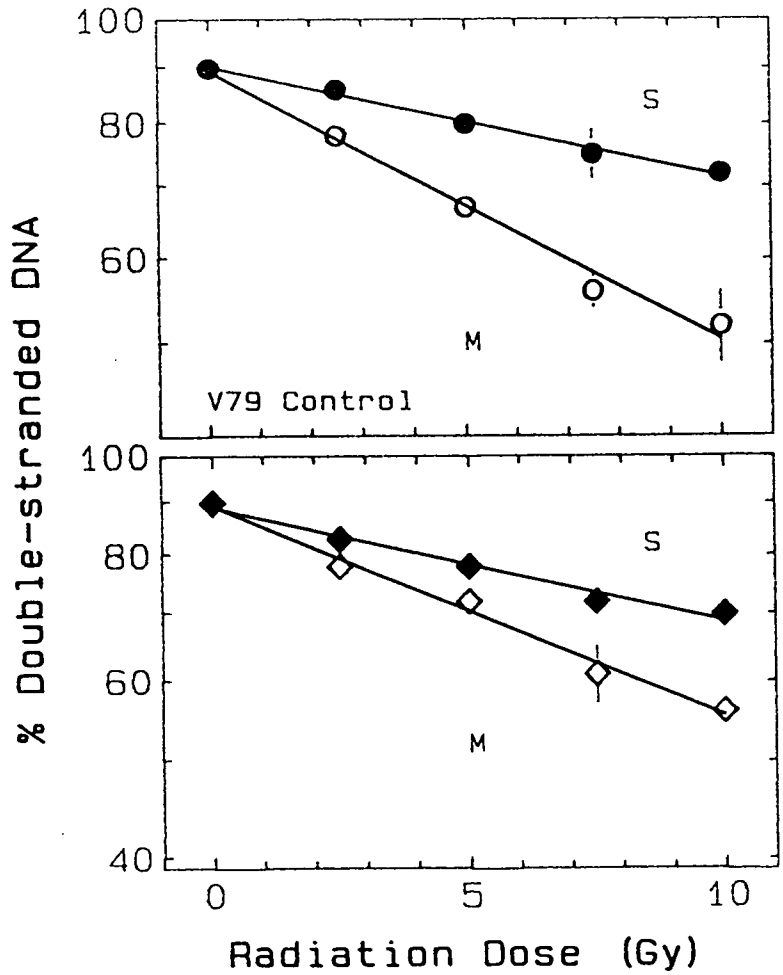


Figure 25. RIF-1 Cell Line and the Contact Effect - X-ray Dose Response. Cells were grown as monolayers and spheroids for two days.
S = spheroids, M = monolayers

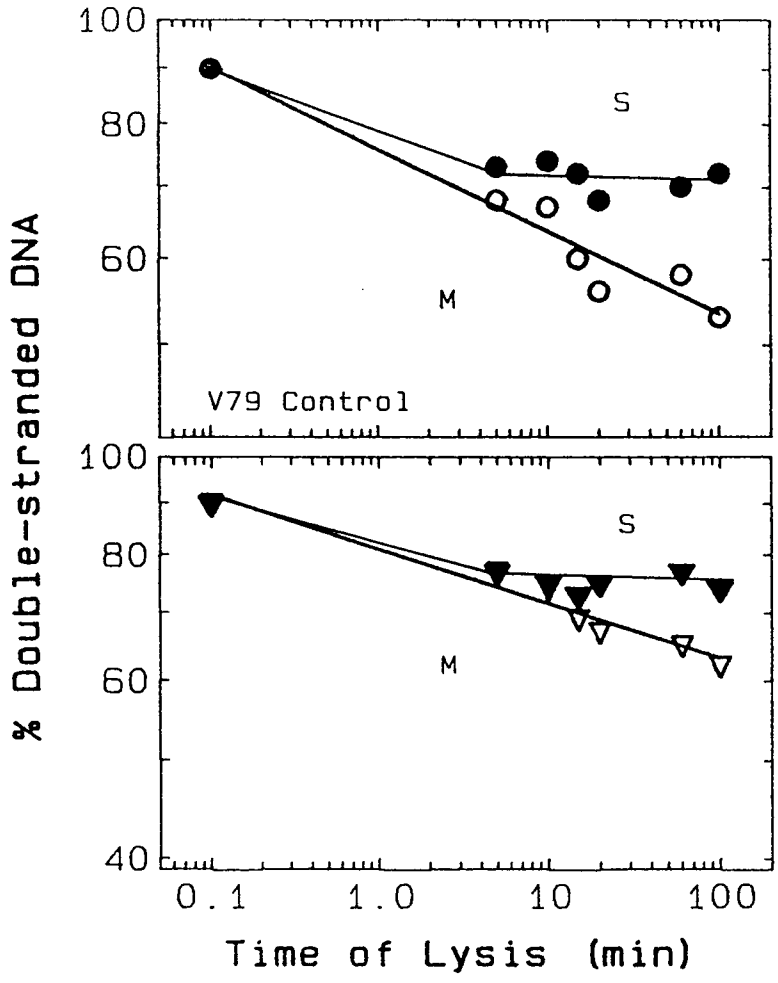


Figure 26. RIF-1 Cell Line and the Contact Effect - DNA Unwinding Kinetics. Comparison with V79 cell line unwinding kinetics. S = spheroids, M = monolayers

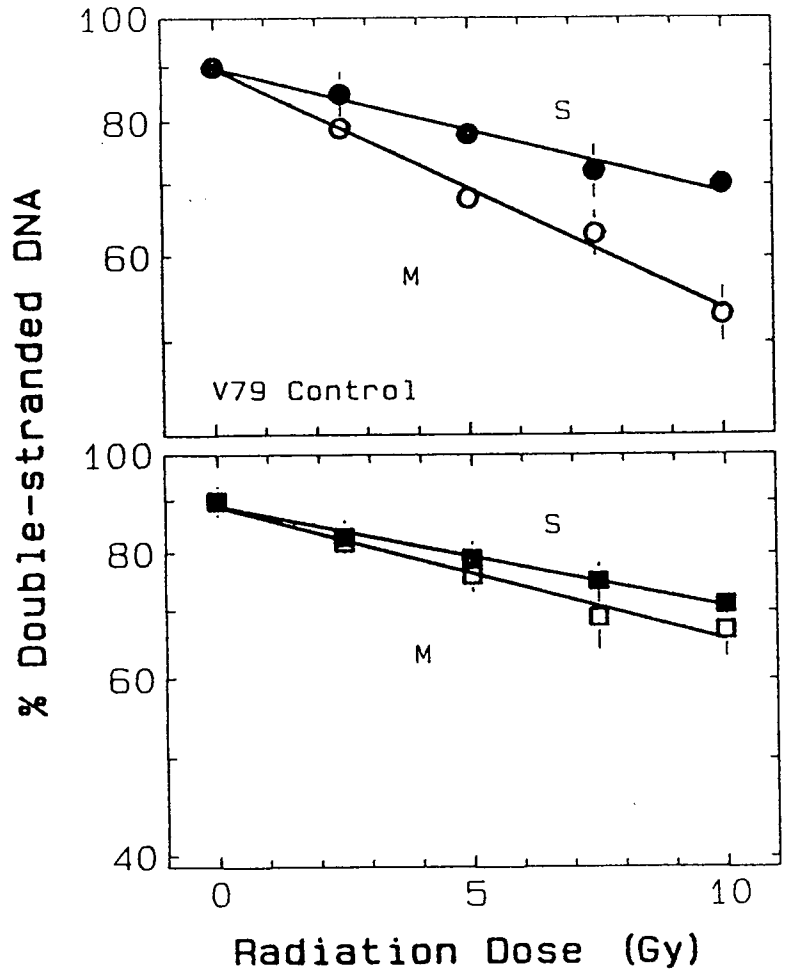


Figure 27. WiDr Cell Line and the Contact Effect. Cells were as monolayers and spheroids for two days. S = spheroid, M = monolayer

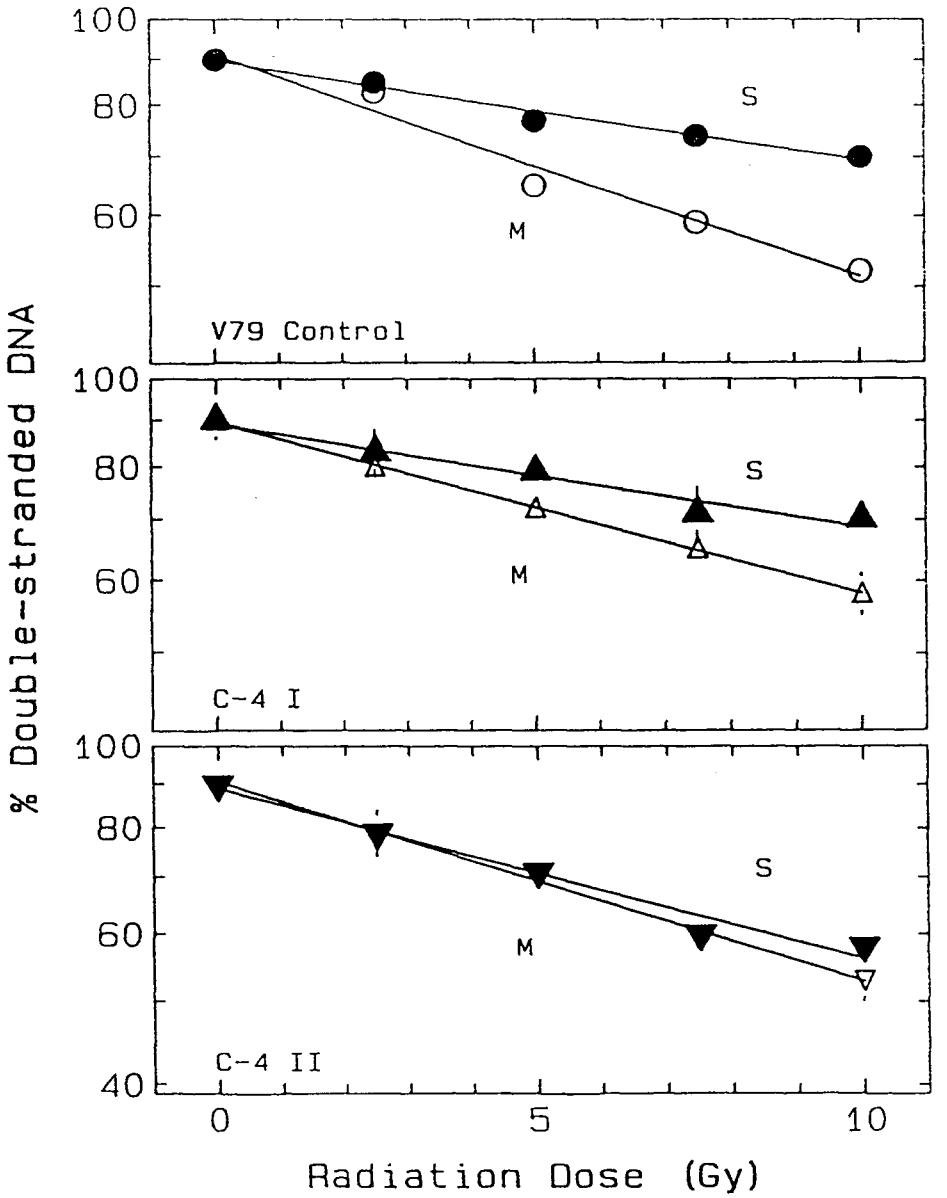


Figure 28. C-4 I and C-4 II Cell Lines and the Contact Effect. Cells were grown as spheroids and monolayers for two days. The dotted line represents the control spheroid response. S = spheroids, M = monolayers

flattened conformation and does not aggregate in suspension, where it does not grow very well. DNA damage in the unirradiated cells caused some difficulty in interpreting the results. The resultant double-stranded DNA was approximately 75 percent in these cells, as compared with about 90 percent for the control V79 cells. So, although both growth conditions produce a partial radioresistance, it is difficult to ascertain if this is a property of contact or not. Table III summarizes the results using tumor cell lines.

TABLE III: SUMMARY OF RESULTS FROM TUMOR CELL LINE EXPERIMENTS

Fig.	cell line	forms aggregates	presence of constraints to unwinding	
			suspended	monolayers
	Mouse			
21.	SCCVII Ca	-	++	-
23.	RIF-1 Sarcoma	+	++	-
	Human			
25.	WiDR colon Ca	+	++	++
26.	C-4 I cervical Ca	+	++	+
26.	C-4 II cervical Ca	-	-	-

DISCUSSION

The main objective of this project was to ascertain whether three-dimensional cell contact is always a requirement for the exhibition of the contact effect, or if other factors, such as cell shape and growth environment, play a role. Table II (see Results, p. 36) lists the key experiments and results, which are more fully discussed below. While it was not possible to state conclusively that intercellular contact was not required for the contact effect, it is clear that factors in addition to cell contact contribute to, if not control, the development of resistance to DNA unwinding. A likely hypothesis is that cell spreading factors and extracellular matrix proteins control the development of constraints to DNA unwinding characteristic of the contact effect.

A. Intercellular Contact and Cell-to-Cell Communication

Cells grown in contact may have the advantage of intracellular differences that allow for greater repair of DNA damage. These differences may involve changes in DNA conformation or in the intracellular environment itself. Passage of small molecules such as glucose, ATP and others, through gap junctions, may alter energy levels or modify radiosensitivity. However, gap junctions also exist between cells grown as monolayers. Cells grown in vivo or as spheroids in vitro are exposed to a continually renewing environment which removes

metabolites and replenishes nutrients, whereas monolayer cultures are not.

It has been suggested that communication via gap junctions may be important in modulating the radiation response of cells grown as spheroids (13, 17). Gap junctions are the most common type of cell junction and are widely distributed in animal tissues. They allow small (1000 - 1500 daltons) water soluble molecules (sugars, inorganic ions, amino acids, nucleotides, vitamins) to pass directly from the cytoplasm of one cell to the cytoplasm of another (1). Durand and Sutherland (17) implicated a role for bioelectrical and biochemical communication between cells, possibly with an exchange of repair molecules. Dertinger and Huelser (13) observed that, with the cell lines they examined, only electrically coupled cells exhibited a contact effect (Table I), although it was suggested that growth of cells in three-dimensional shape is also required. They hypothesized that the contact effect depends on the exchange of certain substances, probably molecules related to DNA repair (for example ATP, cAMP). Though gap junctions may play a role, it does not appear to be directly related to the repair process, as several investigators have separated spheroid cells before irradiation and found that the cells still retained radio-resistance (29, 43). In fact, all the experiments reported here used single cell suspensions, trypsinized prior to irradiation. Also, cells grown sparsely seeded in monolayer culture displayed a contact effect and these cells could not be exchanging repair

molecules, to any great extent, via gap junctions.

It seems that the close three-dimensional contact is not always necessary for observing radioresistance, as evidenced by the experiments using methyl cellulose (Fig. 17) and spheroids trypsinized every four hours (Fig. 16). In the viscous methyl cellulose, the cells remained spherical and could not aggregate except in groups of two or four probably resulting from cell division. Also, spheroids which were trypsinized every four hours only formed aggregates caused by cell division and even then these were dissociated every four hours by the trypsin treatment. Even cells grown as monolayers exhibited a contact effect as long as certain conditions prevailed. These conditions included a constantly renewed source of growth medium, either by replacement at regular intervals (Figure 11) or by maintaining a high medium to cell ratio (Figures 8, 10, 13). Monolayers which were trypsinized every four hours also displayed a contact effect (Figure 15). This may have occurred because the cells maintained a more spherical conformation, either due to the trypsin treatment itself or because the trypsin removed any adhesion-promoting proteins, such as fibronectin or vitronectin, which were attached to the cells (see section C.).

Although SCCVII cells do not aggregate in suspension, they displayed the same radioresistance and DNA unwinding kinetics as V79 cells, which aggregate in suspension (Figures 23, 24). With

SCCVII cells, the property of a contact effect may represent a different mechanism from that of V79 cells. Alternately, it supports the hypothesis that cell contact is not necessary for the development of the contact effect.

B. DNA Conformation and Cell Shape

It is agreed that DNA is a primary target for radiation-induced cell killing, and the contact effect represents an increase in radioresistance, so the elucidation of the mechanism may be found in DNA-related events.

Using two assays for DNA damage, alkaline sucrose gradient sedimentation and alkali elution, Durand and Olive (16) found no difference in the numbers of radiation-induced single-strand DNA breaks produced in monolayer and spheroid cells, and no difference in DNA rejoining kinetics. However, Koerner et al. (33) observed that rejoining of single-strand DNA breaks after irradiation, takes place more quickly and more completely in spheroid cells, and using the more sensitive alkali unwinding assay, Olive and Durand (42) reported similar findings. One explanation for these results may be that there is a difference in DNA conformation between monolayer and spheroid cells. If the DNA conformation is affected by the shape of the nucleus and the nuclear shape is related to the cytoskeleton, then it may follow that the DNA "packaging" of spherical cells is different from that of more flattened cells. The difference in DNA

unwinding characteristics does not appear to be a result of a decrease in the number of DNA strand breaks produced in these cells by ionizing radiation, so there must be another explanation.

One possibility may be that crosslinks, either DNA-DNA or DNA-protein, brought on by a conformational change of the DNA, form constraints to DNA unwinding. Olive et al. (43) suggested that in cells which show radioresistance, the packaging of the DNA is altered and that there are constraints at regular intervals. These constraints could explain the difference seen in unwinding kinetics after DNA strand breakage. They also may allow the DNA of these cells to rejoin more quickly and/or more accurately. Expression of particular genes are specific to cell and DNA conformations. Crosslinks in radioresistant DNA may be the result of protein expressed by these cells, although there is no evidence for this. The conformation of DNA itself could result in DNA-DNA crosslinks via the action of topoisomerases. This class of enzymes can control and modify the topological states of DNA by breaking the strand(s) and catalyzing inter-conversions between duplex molecules. Topoisomerase II has been found to affect many vital biological functions, including the replication, and possibly the repair, of DNA (38, 56).

Cell shape is known to cause nuclear changes. The monolayer cells may produce substances, such as extracellular matrix proteins which influence nuclear shape via the cyto-

skeleton, and which indirectly prevent the DNA from forming the more radioresistant conformation. This was suggested by the decreased radioresistance of spheroids grown either in plates containing attached cells or in medium which had been conditioned by exposure to attached cells. If this substance was removed, such as when monolayer cells were trypsinized frequently or when the medium was replenished, the cells showed the same DNA response as control spheroid cells (Figure 12).

C. Cell Attachment and Spreading Factors

Cellular adhesion to a substratum and cell spreading are thought to play an important role in many biological processes. A number of glycoproteins have been isolated that mediate cell attachment and spreading in culture. These include fibronectin, vitronectin, laminin and others. Fibronectin is the most thoroughly characterized of these. It exists as a soluble protein in plasma and in insoluble form (attached to receptors) at cell surfaces. Vitronectin, which has been more recently characterized (27), has some of the same properties, such as promoting cell attachment and interacting with glycosamino-glycans and proteoglycans, but has a somewhat different tissue distribution. Cellular receptors recognize the short amino acid sequence -arginine-glycine-aspartic acid- (-Arg-Gly-Asp-) in fibronectin, vitronectin and some other cellular recognition proteins, and this sequence is required for the binding of cell receptors to the proteins (44). Despite this similarity,

however, the cell surface receptors which recognize fibronectin and vitronectin are different.

Experiments with differing growth conditions have shown that the growth medium may play a larger role in the contact effect than previously assumed, and this may be due to cell spreading factors. Cells grown densely as monolayers, but in an environment with a large volume of stirred medium, such as on microcarrier beads or petri plates in spinner flasks, exhibit at least a partial contact effect. Of course it is not possible to distinguish between some cells showing a full effect while some show none, and all cells showing a partial effect. High cell density cultures, attached to petri plates and grown in unstirred medium, do not exhibit a contact effect. This may be due to a high local concentration of by-products, such as cell attachment and spreading factors, produced by these cells in response to their substratum.

Fetal bovine serum, which is used in growth medium, contains spreading factors, including fibronectin and vitronectin. Some cells may use these components to attach and spread on the substrate. However, V79 cells make their own fibronectin in response to attachment to tissue culture plates. After a few hours in monolayer culture, V79 cells appear to be fully spread. It seems likely that attachment factors or extracellular matrix proteins made by the cells themselves are involved in the development of the contact effect. Otherwise,

monolayers grown at low cell packing or in constantly renewed medium should have better access to serum spreading factors than monolayers grown at high density.

A high concentration of these factors may also prevent the cells from maintaining the cell shape that is conducive to radioresistance (see section D.). The radiosensitivity may be the result of some substance produced by the monolayer cells, and not merely due to the relatively small volume of medium in which the cells are grown. This theory is supported by the results from experiments where cells were grown in a small amount of medium, in suspension (Figure 14) or above poly(HEMA)-coated dishes (Figure 18). These cells exhibited a full contact effect.

Cells grown at a density ten times sparser than "normal" do exhibit a contact effect. This may be the result of reduced levels of these factors as all of the cells are further apart from each other. Conditioned medium experiments seem to support the theory that some substance released by the monolayer cells interferes with the acquisition of the contact effect. Very dense monolayer cultures in large amounts of moving medium may only show partial radioresistance because some of this factor attaches to the cells before the moving medium can sweep it away. The attachment of this factor to cells may then be responsible for changes in the cytoskeleton which in turn bring about changes in the nucleus.

D. Role of Cell Shape and Extracellular Matrix

With the possible exceptions of cell shape and cell-to-cell communication, no differences have been found in the physical properties (cell cycle distribution, plating efficiency, rate of DNA and RNA synthesis) of V79 cells grown as spheroids and monolayers. One of the most intriguing observations of the contact effect is that cells from spheroids can be disaggregated prior to irradiation and still display relative radioresistance. Therefore, the history of the cell with respect to growth conditions must play a critical role in the exhibition of the contact effect. Several lines of evidence suggest that the substratum upon which the cell grows plays an important role in determining cell shape, rate of DNA synthesis, response to growth factors and susceptibility to cytotoxic agents (21, 38). Recent results suggest that the extracellular matrix formed by a cell affects the number and/or distribution of extracellular matrix receptors in the plasma membrane. This influences the interaction of polymerized actin with the membrane, and changes in the actin assembly cause changes in cell shape (57). It is possible that alterations in cell shape or intracellular architecture, resulting from differences in substratum, are responsible for the increased resistance to ionizing radiation.

The importance of cell shape to cell function was examined by Ben-Ze'ev et al. (6). Using anchorage-dependant mouse fibroblasts, attachment to a surface and the effects of cell

shape were studied. Changes in cell shape and lack of attachment were seen to cause the cessation of certain metabolic functions in some cell types. These functions were recovered when the appropriate conditions were restored. The relationship between cell shape and recovery of macromolecular metabolism was examined. Their results suggest that protein synthesis recovery is induced by cell attachment to a surface, while nuclear functions, such as recovery of mRNA production, rRNA and DNA synthesis require extensive cell spreading. Fibroblasts respond to shape changes by regulating growth, but other cell types have different responses. Chondrocytes and epithelial cells have been shown to change their spectrum of synthesized proteins when cell shape is altered (6).

An "appropriate" cell shape appears to be critical for DNA synthesis since, as untransformed cells become round, their rate of DNA synthesis decreases. In contrast, a spherical shape has only minimal effect on DNA synthesis in tumor cells. Lotan et al. (38) plated a variety of untransformed (human skin fibroblasts, mouse embryo Swiss 3T3 fibroblasts) and neoplastic (human cervical carcinoma HeLa-S3, osteosarcoma Hs791, and murine melanomas B16-F1, S91-C2, S91-C154) cell lines on tissue culture plates coated with increasing concentrations of poly(HEMA). Both normal cell lines and the neoplastic Hs791 line showed a marked shape dependant decrease in DNA synthesis, whereas the other neoplastic lines were only slightly affected. It is interesting to note that WiDr human colon carcinoma cells

and C-4 I human cervical carcinoma cells showed constraints to unwinding even when grown as monolayers (Figures 25, 26). Like other malignant cells, these cells probably do not synthesize extracellular matrix molecules (57), and this could account for the results.

Kulesh and Greene (20) also observed that cellular proliferation in normal cells can be regulated by cell shape, but not in malignant cells. However, shape-dependant proliferation in certain malignant cells could be restored by treatment of the cells with specific amounts of interferon. Lotan noticed this same effect with retinoic acid (18). This suggests that malignant cells retain the potential for shape-dependant proliferation. Also, the induction of the shape-dependant regulatory mechanism did not require the continuous presence of interferon, suggesting that an alteration in gene expression was involved.

As shown by the experiments with trypsin, renewed medium (or sparse seeding), and medium flow, there appears to be a role for cell shape in the formation of the contact effect. It appears that the cell must actually grow and pass through the cell cycle at least twice for the effect to be observed.

Folkman and Moscona (21) observed that for nontransformed mammalian cells, the shape of the cell is tightly coupled to DNA synthesis. They used the hydrophilic hydrogel, poly(HEMA), to

reduce the adhesiveness of plastic tissue culture dishes in a graded manner. The extent of cell spreading was controlled so that the cell could be held at any one of a graded series of quantitated shapes. Cell incorporation of ^{3}H-thymidine was found to be inversely proportional to the height of the cell. Thus, spreading, which involves reformation of the cytoskeletal framework composed of microtubules, microfilaments and intermediate filaments (57), appears to be required for these cells, for reactivating DNA and RNA synthesis, mitosis and cell division. The V79 cells used in this study are able to divide regardless of cell shape, but substrate could influence DNA conformation. In this laboratory, attempts to grow V79 cells on graded concentrations of poly(HEMA) were unsuccessful since, during the 24 hours required for the development of the contact effect, the cells either synthesized sufficient fibronectin or partially dissolved the poly(HEMA) layer and this allowed attachment and spreading of the cells. However, a high concentration of poly(HEMA) was used to coat petri plates and completely prevented cell attachment to these plates (Fig. 18).

Olive et al. (43) suggested that there is a difference in the packaging of DNA in spheroid cells. The round shape of the cells, which brings about a different conformation for the nucleus and DNA, may allow for constraints to form relatively close together on the DNA. It is also possible that the lack of a solid substrate means that the DNA of cells grown in suspension requires additional physical support which is

supplied in the form of these constraints.

The cells in continually moving medium may develop an altered organization of actin fibers, or perhaps an increased number of these fibers as do the endothelial cells in high blood flow areas (22), and this may cause nuclear changes. However, this cannot be a factor in the sparsely seeded cultures, although when observed under a microscope, the cells are much less extended than control monolayers (41). At low cell packing, less spreading could be due to a lower local concentration of extracellular matrix proteins. The lack of contact effect in the moving platform incubator experiments (Figure 19) could be due to the fact that the medium was not moving with enough force to cause this effect or, perhaps because of a high concentration of V79 cell-produced spreading factors in the relatively small amount of medium. Lack of cell spreading factors may explain the radioresistance of cells growing above the poly(HEMA)-coated plates in unstirred medium. These cells displayed a contact effect perhaps because no attached cells were present to produce spreading factors. It may be that one of two effects can dominate. If movement of the liquid medium over the surface of a cell is strong enough, stress may cause an alteration in the cytoskeleton and nucleus. Alternately, if the medium is free of high concentrations of monolayer cell-produced by-products, then the cells will grow in the spherical conformation and display radioresistance.

There is increasing recognition that cytoskeletal elements mediate and regulate a variety of cellular processes. Stevenson et al. (50) observed that certain cells grown in vitro as monolayers, were more radiosensitive than those grown in a rounded conformation. These rounded cells were grown both in contact with other cells and as individual cells. When these rounded cells were exposed to specific cytoskeletal poisons, which affected microtubules and microfilaments, the radiosensitivity increased, whether the cells had been grown in contact or not. Cells grown in spherical conformation have a different morphology than those grown as monolayers and that the radioresistance observed was interpreted to be due to "improved" cytoskeletal (including the nuclear matrix) function, and not due to cell-to-cell contact.

Results from experiments with other cell lines can be seen in Table III. Differences in cell shape are reflected in radioresistance. The RIF-1 and SCCVII cell lines both grow in an elongated shape as monolayers and yet remain spherical in suspension, much like the V79 cells. All three of these cell lines show quantitatively similar radiation responses (Figures 23, 25). Even though the SCCVII cells do not aggregate in suspension, a contact effect was observed. Alternatively, with the WiDr and C-4 I cell lines, the cells grow in a more rounded conformation as monolayers, which may explain why the monolayer cultures of these cells show more radioresistance than the V79 monolayer cells. Monolayer cells of the WiDr cell line have

also been shown to have a similar radioresponse to the WiDR spheroid cells when assayed for cell survival (59). The C-4 II monolayer cells have a more extended cell shape, but they do not grow well as spheroids so it is difficult to draw conclusions about them.

In addition to cell growth and division, cell shape seems to exert a profound influence on a variety of cellular and nuclear functions, including protein synthesis (6, 21), RNA synthesis (7), and actin gene expression (20). Cell shape appears to play a role in the acquisition of the contact effect. This theory seems reasonable in that cell shape, via the cytoskeleton, is intimately related to nuclear conformation.

SUMMARY AND CONCLUSION

The contact effect appears to be functionally related to constraints to DNA unwinding which develop when cells are grown as spheroids. Elucidating the growth conditions that promote this difference was the purpose of this project.

Three-dimensional cell contact is not always necessary for the development of the contact effect in V79-171B cells. Similarly, a "round" shape alone does not always determine radioresistance, as evidenced by the fact that under certain conditions elongated monolayer cells displayed a contact effect while round cells did not. However, cells grown in suspension, in medium which had been exposed to attached cells, displayed a decreased radioresistance. This led to the hypothesis that the monolayer cells were synthesizing some factor, possibly an extracellular matrix protein, which inhibited the development of the contact effect. These proteins can affect the cytoskeleton and indirectly, the nucleus. This hypothesis could be further investigated by:

a) blocking receptors for extracellular matrix proteins, in monolayer cells, using synthetic arg-gly-asp peptides (44).

b) growing V79 cells as spheroids in medium conditioned by spheroids rather than by monolayers.

c) growing WiDR monolayer cells, which do not produce spreading factors and do display radioresistance, in medium conditioned by V79 monolayers, or on petri dishes coated with extracellular matrix proteins.

Overall, it would appear that cell shape, cell-to-cell contact and growth medium are interrelated in promoting the development of the contact effect in V79 cells. Cells grown in suspension culture may develop a different cytoskeletal structure which could lead to a change in DNA "packaging". A change in DNA conformation could enhance DNA repair capabilities, perhaps by allowing better access of repair enzymes to damaged sites in DNA. This greater repair capacity would then be reflected in the higher cell survival of spheroid cells exposed to ionizing radiation.

REFERENCES

1. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J. D. (1983) Molecular Biology of the Cell. Garland Publishing, Inc., New York and London. pp. 682-691.

2. Acker, H., Carlsson, J., Durand, R. and Sutherland, R. E. (1984) Spheroids in Cancer Research. In P. Rentchnick (Ed.) Recent Results in Cancer Research, Vol. 95. Springer-Verlag Berlin, Heidelberg, New York, Tokyo.

3. Ahnstrom, G. and Erixon, K. (1973) Radiation Induced Strand Breakage in DNA from Mammalian Cells. Strand Separation in Alkaline Solution. Int. J. Radiat. Biol., 23(3):285-289.

4. Auersperg, N. (1969) Histogenic Behavior of Tumors. I. Morphologic Variation In Vitro and In Vivo of Two Related Human Carcinoma Cell Lines. J. Nat. Cancer Inst., 43:151-173.

5. Ben-Ze'ev, A. (1984) Differential Control of Cytokeratins and Vimentin Synthesis by Cell-Cell Contact and Cell Spreading in Cultured Epithelial Cells. J. Cell Biol., 99:1424-1433.

6. Ben-Ze'ev, A., Farmer, S. R., Penman,S. (1980) Protein Synthesis Requires Cell-Surface Contact while Nuclear Events Respond to Cell Shape in Anchorage-Dependant Fibroblasts. Cell, 21:365-372.

7. Benecke, B-J., Ben-Ze'ev, A. and Penman, S. (1978) The Control of RNA Production, Translation and Turnover in Suspended and Reattached Anchorage-Dependant Fibroblasts. Cell, 14:931-939.

8. Blocher, D. and Pohlit, W. (1982) DNA Double Strand Breaks in Erlich Ascites Tumour Cells at Low Doses of X-Rays. II. Can Cell Death be Attributed to Double Strand Breaks? Int. J. Radiat. Biol., 42:333-338.

9. Cathers, L. E. and Gould, M. N. (1983) Human Mammary Cell Survival Following Ionizing Radiation. Int. J. Radiat. Biol., 44(1):1-16.

10. Coggle, J. E. (1973) Biological Effects of Radiation. In N. Mott, G. R. Noakes, W. B. Yapp (Eds.) The Wykeham Science Series, Vol. 14. Wykeham Publications (London)

11. Corn, B. W., Liber, H. L. and Little, J. B. (1987) Differential Effects of Radical Scavengers on X-Ray-Induced Mutation and Cytotoxicity in Human Cells. Radiat. Res., 109:100-108.

12. Curtis, S. B. and Tenforde, T. S. (1980) Assessment of

Tumor Response in a Rat Rhabdomyosarcoma. Br. J. Cancer, 41(Suppl.IV):266-270.

13. Dertinger, H. and Huelser, D. (1981) Increased Radioresistance of Cells in Cultured Multicell Spheroids. I. Dependance on Cellular Interaction. Radiat. Environ. Biophys., 19:101-107.

14. Dertinger, H. and Lucke-Huhle, C. (1975) A Comparative Study of Post-Irradiation Growth Kinetics of Spheroids and Monolayers. Int. J. Radiat. Biol., 28:255-265.

15. Durand, R. E. (1979) Relevence of Radiobiological Data on Mammalian Cells in Culture for the Prediction of Tumor Responses to Fractionated Irradiation: Effects of Intracellular Contact. In M. Moore (Ed.), Advances in Medical Oncology, Research and Education. Pergamon Press Oxford and New York, pp. 173-181.

16. Durand, R. E. and Olive, P. L. (1979) Radiation-Induced DNA Damage in V79 Spheroids and Monolayers. Radiat. Res., 78:50-60.

17. Durand, R. E. and Sutherland, R. M. (1972) Effects of Intercellular Contact on Repair of Radiation Damage. Exptl. Cell Res., 71:75-80.

18. Durand, R. E. and Sutherland, R. M. (1975) Intercellular Contact: Its Influence on the Dq of Mammalian Cell Survival Curves. In T. Alper (Ed.) Cell Survival After Low Doses of Radiation. Wiley, New York., pp. 237-249.

19. Edgren, M. (1982) Intercellular Co-operation in Repairing Radiation-Induced Single-strand DNA Breaks. Int. J. Radiat. Biol., 41(5):589-593.

20. Farmer, S. R., Wan, K. M., Ben-Ze'ev, A. and Penman, S. (1983) Regulation of Actin mRNA Levels and Translational Response to Changes in Cell Configuration. Mol. Cell Biol. 3:182-189.

21. Folkman, J. and Moscona, A. (1978) Role of Cell Shape in Growth Control. Nature, 273:345-349.

22. Franke, R.-P., Graefe,M., Schnittler, H., Seiffge, D., Mittermayer, C. and Drenckhahn, D. (1984) Induction of Human Vascular Endothelial Stress Fibres by Fluid Shear Stress. Nature, 307:648-649.

23. Frankenberg-Schwager, M., Frankenberg, D. and Harbich, R. (1987) Potentially Lethal Damage Repair is Due to the Difference of DNA Double-Strand Break Repair under Immediate and Delayed Plating Conditions. Radiat. Res., 111:192-200.

24. Guichard, M., Dertinger, H. and Malaise, E. P. (1983) Radiosensitivity of Four Human Tumor Xenografts. Influence of Hypoxia and Cell-Cell Contact. Radiat Res., 95: 602-9.

25. Hall, E. J. (1978) Radiobiology for the Radiobiologist, 2nd Edition. Harper & Row, New York.

26. Harless, J., Hittelman, W., Meyn, R. and Hewitt, R. (1983) Chromatin Factors Affecting DNA Repair in Mammalian Cell Nuclei. In, Cellular Response to DNA Damage. Alan R. Liss, Inc., New York, pp. 183-193.

27. Hayman, E. G., Pierschbacher, M. D., Ohgren, Y. and Rouslahti, E. (1983) Serum Spreading Factor (Vitronectin) is Present at the Cell Surface and in Tissues. Proc. Natl. Acad. Sci., 80:4003-4007.

28. Haynes, R. H. (1966) The Interpretation of Microbial Inactivation and Recovery Phenomena. Radiat. Res., supp. 6:1-29.

29. Hill, R. P., Ng, R., Warren, B. F. and Bush, R. S. (1979) The Effect of Intercellular Contact on the Radiation Sensitivity of KHT Sarcoma Cells. Radiat. Res., 77:182-192

30. Hinz, G. and Dertinger, H. (1983) Increased Radioresistance of Cells in Cultured Multicell Spheroids. II. Kinetic and Cytogenic Studies. Radiat. Environ. Biophys., 21:255-264.

31. Jorgensen, T. J. (1984) DNA Damage in Mammalian Cells Caused by Low Doses of Ionizing Radiation. Ph.D. Thesis., Johns Hopkins University, Baltimore, Maryland.

32. Jorgensen, T. J., Olive, P. L. and Durand, R. E. (1987) DNA Strand Breakage in Chinese Hamster V79 Cells Caused by Low Levels of Incorporated [3H] and [14C] Thymidine. Int. J. Radiat. Biol., 51(4):673-680.

33. Koerner, I. J., Kopp, J. and Malz, W. (1978) Radiation-Biological Investigations with Multicellular Spheroids in an In Vitro Tumor Model. Studia Biophysica 68:161-2.

34. Kulesh, D. A. and Greene, J. J. (1986) Shape-Dependant Regulation of Proliferation in Normal and Malignant Human Cells and Its Alteration by Interferon. Cancer Res., 46:2793-2797.

35. Lett, J. T., Klucis, E. S., Sun, C. (1970) On the Size of the DNA in the Mammalian Chromosome. Biophys. Journal, 10:277-292.

36. Lotan, R., Stolarsky, T., Lotan, D. and Ben-Ze'ev, A. (1984) Retinoic Acid Restores Shape-Dependant Growth

Control in Neoplastic Cells Cultured on Poly(2-Hydroxy-ethyl methacrylate)-Coated Substrate. Int. J. Cancer, 33:115-121.

37. Lucke-Huhle, C., Blakely, E. A., Ngo, F. Q. H., Chang, P. Y. and Tobias, C. A. (1980) Survival and Kinetic Response of V79-Spheroids after Exposure to Heavy-Ion Beams. Int. J. Radiat. Biol., 37:483-492.

38. Lui, L. F., Lui, C-C. and Alberts, B. M. (1980) Type II Topoisomerases: Enzymes that can Unknot a Topologically Knotted DNA Molecule via a Reversible Double-Strand Break. Cell, 19:697-707.

39. Mulcahy, R. T., Gould, M. N. and Clifton, K. H. (1980) The Survival of Thyroid Cells: In Vivo Irradiation and In Situ Repair. Radiat. Res., 84:523-528.

40. Oleinick, N. L., Chiu, S.-M. and Friedman, L. (1984) Gamma Radiation as a Probe of Chromatin Structure: Damage to and Repair of Active Chromatin in the Metaphase Chromosome. Radiat. Res., 98:629-641.

41. Olive, P. L. (1981) Influence of Cell Crowding on Toxicity of Nitroheterocycles. Chem.-Biol Interactions, 35:285-296.

42. Olive, P. L. and Durand, R. E. (1985) Effect of Inter-cellular Contact on DNA Conformation, Radiation-Induced DNA Damage, and Mutation in Chinese Hamster V79 Cells. Radiat. Res., 101:94-101.

43. Olive, P. L., Hilton, J. and Durand, R. E. (1986) DNA Conformation of Chinese Hamster V79 Cells and Sensitivity to Ionizing Radiation. Radiat. Res., 107:115-124.

44. Pytela, R., Pierschbacher, M. D. and Rouslahti, E. (1985) A 125/115-kDa Cell Surface Receptor Specific for Vitronectin Interacts with the Arginine-Glycine-Aspartic Acid Adhesion Sequence Derived from Fibronectin. Proc. Natl. Acad. Sci., 82:5766-5770.

45. Ramanathan, B. and Smerdon, M. J. (1986) Changes in Nuclear Protein Acetylation in U. V.-Damaged Human Cells. Carcinogen., 7(7):1087-1094.

46. Razin, S. V., Chernokhvostiv, V., Roodyn, A. V., Zharsky, I. B. and Georgiev, G. P. (1981) Proteins Tightly to DNA in Regions of DNA Attachment to the Skeletal Structures of Interphase and Metaphase Chromosomes. Cell, 27:65-73.

47. Rockwell, S. (1986) Absence of Contact Effects in Irradiated EMT6-Rw Tumors. Radiat. Res., 107:375-381.

48. Rofstad, E. K. and Brustad, T. (1983) Intercellular Contact and the Radiation Response of Cells of Human Melanoma Xenografts. Int. J. Radiat. Oncol. Biol. Phys., 9:191-195.

49. Spiro, I. J., Kennedy, K. A., Stickler, R. and Ling, C. C. (1985) Cellular and Molecular Repair of X-Ray-Induced Damage: Dependence on Oxygen Tension and Nutritional Status. Radiat. Res., 101:144-155.

50. Stevenson, A. F. G., Ostashevsky, J. and Lange, C. S. (1987) The Cytoskeleton and Cellular Radiosensitivity. Proc. 8th Int. Congress of Radiat. Res., Edinburgh, Abst. No. C51-13P.

51. Sweigert, S. E. and Alpen, E. L. (1987) Radiation-Induced Division Delay in 9L Spheroid versus Monolayer Cells. Radiat. Res., 110:473-478.

52. Thompson, L. H., Mooney, C. L., Burkhart-Schultz, K., Carrano, A. V. and Siciliano, M. J. (1985) Correction of a Nucleotide Excision Repair Mutation by Chromosome 19 in Hamster-Human Hybrid Cells. Somatic Cell Mol. Genet., 11:87-92.

53. Todorova, M. and Russev, G. (1984) Random Distribution of Mammalian Replication Origins in Matrix and Total Nuclear DNA. Biochim. Biophys. Acta, 783:36-41.

54. Twentyman, P. R., Brown, J. M., Gray, J. W., Franko, A. J., Scoles, M. A. and Kallman, R. F. (1980) A New Mouse Tumor Model System (RIF-1) for Comparison of End-Point Studies. J. Natl. Cancer Inst., 64(3):595-604.

55. Wallen, C. A., Michaelson, S. M. and Wheeler, K. T. (1980) Evidence for an Unconventional Radiosensitivity of Rat 9L Subcutaneous Tumors. Radiat. Res., 84:529-541.

56. Wang, J. C. (1985) DNA Topoisomerases. Ann. Rev. Biochem. 54:665-697.

57. Watt, F. M. (1986) The Extracellular Matrix and Cell Shape. TIBS, 11:482-485.

58. Weichselbaum, R. R., Epstein, J., Little, J. B. and Kornblith, P. L. (1976) In Vitro Cellular Radiosensitivity of Human Malignant Tumors. Eur. J. Cancer, 12:47-51.

59. West, C. M. L. and Sutherland, R. M. (1987) The Radiation Response of a Human Colon Adenocarcinoma Grown in Monolayer, as Spheroids, and in Nude Mice. Radiat. Res., 112:105-115.

60. Westerveld, A., Hoeijmakers, J. H. J., van Duin, M.,

de Wit, J. Odijk H., Pastink, A., Wood, R. D. and Bootsma, D. (1984) Molecular Cloning of a Human DNA Repair Gene. Nature, 310:425-429.

61. Wheeler, K. T. and Nelson, G. B. (1987) Saturation of a DNA Repair Process in Dividing and Nondividing Mammalian Cells. Radiat. Res., 109:109-117.

62. Willis, A. E. and Lindahl, T. (1987) DNA Ligase I Deficiency in Bloom's Syndrome. Nature, 325:355-357.

PUBLICATIONS

1. P. L. Olive, A. P. S. Chan, C. S. Cu and S. H. MacPhail (1988) Extending the Range of Sensitivity for Detecting DNA Strand Breaks. Brit. J. Radiol., 61:69.

2. S. H. MacPhail and P. L. Olive (1988) Cell Shape, Intercellular Contact, Metabolite Exchange and the "Contact Effect". Thirty-Sixth Annual Meeting of the Radiation Research Society, Philadelphia, Abst. No. Ej-3.

3. P. L. Olive and S. H. MacPhail (1988) Involvement of Cell Spreading and DNA Conformation in the Contact Effect. Fourth International Congress of Cell Biology, Montreal.

4. S. H. MacPhail and P. L. Olive Intercellular Contact and Development of the Contact Effect. (in preparation)