THE ROLE OF INTERCELLULAR COMMUNICATION IN CYTOTOXIC DRUG RESISTANCE

by

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ABBREVIATIONS.

ATP	adenosine triphosphate
BCF	base coated flask
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BDH	British Drug Houses
BSO	buthionine sulfoxamine
BSS	buffered salt solution
ß-gal	ß-galactosidase
cAMP	cyclic adenosine monophosphate
cDNA	copy deoxyribonucleic acid
CEA	carcinoembryonic antigen
CR	contact resistance
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunoassay
GSH	glutathione
GST	glutathione-S-transferase
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
LI	labelling index
LT	lymphotoxin
mAMSA	4 -(9-acridinylamino) methanesulfon-m-anisidide
MDR	multidrug resistance
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NSCLC	non-small cell lung cancer
PBS	phosphate buffered saline
RNA	ribonucleic acid
SCE	sister chromatid exchange
SCLC	small cell lung cancer
SD	standard deviation
SEM	standard error of the mean
T-B	touching minus background
T/B	touching divided by background
TCA	trichloroacetic acid
TNF	tumour necrosis factor
TPA	12-o-tetradecanoyl phorbol-13-acetate
topo II	topoisomerase II
UV	ultraviolet
X-gal	4-chloro-5-bromo-3-indoyl-ß-galactosidase

SUMMARY

The gap junction is an array of channels spanning the two membranes of adjacent cells and linking their cytoplasms. The aim of this thesis was to examine the role which intercellular communication through gap junctions might play in the development of cytotoxic drug resistance.

Increased junctional communication has been associated with resistance to radiation and to cytotoxic lymphokines. The explanation for this phenomenon is not clear, although it is possible that sharing of metabolic pools between coupled cells may allow them to withstand damage more effectively than isolated cells. To investigate whether this relationship between communication and resistance is a generalised one, applicable to conventional cytotoxic drugs, the strength of coupling of a panel of seven human NSCLC cell lines was measured and compared with the chemosensitivity of these lines to adriamycin.

Communication was assessed by autoradiographic detection of transfer of ^{3}H uridine nucleotides between cells. The strength of coupling varied widely between the cell lines and they could be separated into three groups: those which exhibit strong coupling, L-DAN and A549; those which exhibit weak coupling, SK-MES-1, Calu-3 and NCI-H125; and an intermediate group, WIL and NCI-H23. Adriamycin chemosensitivity of each cell line was

assessed in monolayer by both clonogenic and MTT assays. The range of IC_{50} values as measured by either assay was extremely narrow with no important differences between the cell lines. Thus, despite the wide spectrum of junctional communication observed in these lines, this did not correlate with their adriamycin resistance. Chemosensitivity of three dimensional spheroids of L-DAN and SK-MES-1 was also assessed by clonogenic assay but again this was not related to their communication.

Interaction between drug sensitive and resistant cell lines has been demonstrated in mixed culture. Nitrosourea resistance is transferred between cells in spheroids and ouabain resistance is transferred in monolayer culture by the rapid diffusion of K^+ and Na^+ ions through gap junctions. The membrane pumps in the ouabain resistant cells maintain physiological ion concentrations in the cytoplasms of both cell types. It seems possible, therefore, that other forms of drug resistance which are dependent on the cytoplasmic concentration of small ions or molecules should be transferred between cells joined by gap junctions.

The MDR phenotype has been related to the presence of a membrane pump, P-glycoprotein, which reduces intracellular drug concentration. The behaviour of normal (CHO-K1), resistant (Adr^r) and hypersensitive (ADR-6) Chinese hamster cell lines which have been shown to exhibit junctional communication was examined in mixed

culture. The CHO-K1 cells were genetically marked by transfection with a bacterial ß-galactosidase gene. Its enzyme produces a blue product with appropiate substrate and allows identification of the individual response of two cell types in mixed culture. Clonogenic assays were performed after exposure of mixed monolayer or spheroid culture to either adriamycin, mitozantrone or vincristine. In individual assays Adr^r is x100 more resistant to adriamycin and x20 more resistant to mitozantrone than CHO-K1, whereas ADR-6 is x2 more sensitive to vincristine. However, in mixed culture, the chemosensitivities of the cell lines were unchanged with no evidence of resistance transfer in this system.

CHAPTER 1 INTRODUCTION

1.1. Heterogeneity of cellular and tissue function

1.1.1. Heterogeneity of normal cellular function

Heterogeneity of cellular and thus of tissue function is the basis on which the activities of all complex multicellular organisms depend. Although each cell may have an identical genetic complement, the expression of a selected proportion of this allows the cell to produce a specific differentiated phenotype, appropriate to the function of that cell in the organism as a whole. Thus some cells produce contractile elements and function as muscle cells to endow the organism with motility; some develop suitable polar orientation and specialised membrane modifications to form epithelial tissues lining body surfaces; in others the manufacture of a specific product is dominant as in osteocytes which produce and secrete large amounts of extracellular matrix on which minerals are later deposited to provide structural rigidity to the skeleton.

1.1.2. Cell-cell communication

To coordinate the activity of the organism as a whole, however, it is necessary that some form of dialogue or cooperation is established between this large family of cells. Several mechanisms have evolved through which

such communication may occur in order that each cell does not behave in an independent manner and by which each cell is able to influence the activity of another.

These mechanisms can act between cells situated some distance apart through the production of circulating messengers or hormones as in the endocrine and paracrine systems. When cells are in close proximity mechanical factors become important and cell crowding and orientation may play a role in behaviour. One additional means of communication afforded by close contact is that of direct passage of molecules from the cytoplasm of one cell to that of its neighbours through transmembrane channels known as gap junctions. This last mechanism will be discussed in more detail in a section 1.2..

1.1.3. Tumour cell heterogeneity

Despite their presumed monoclonal origin from a single transformed cell, tumour cells have also been shown both *in vivo* and *in vitro* to exhibit considerable heterogeneity in a large number of phenotypic variables including morphology, growth rate, expression of specific markers, metastatic propensity and sensitivity to radiation and to cytotoxic drugs (Heppner and Miller, 1989). A number of explanations for this heterogeneity have been proposed including the underlying genetic instability associated with neoplasia (Nowell, 1976) and

the selective pressure of the cell's microenviroment within the tumour (Sutherland, 1988).

1.1.4. Interaction of tumour subpopulations

As in the organism as a whole, there is extensive evidence that these cellular subpopulations do not behave in an independent manner, but that the presence of one subpopulation can have considerable influence on the activities of another (Heppner, Miller and Miller, 1983).

Itaya et al. (1989) have recently demonstrated in murine colorectal carcinoma cells that, in contrast with the parent line, isolated clones of the cell line are often phenotypically unstable. They attributed the phenotypic stability of the parent line to interactions between the constituent subpopulations, and suggested that such interaction is necessary in order to allow the tumour cell population to retain its heterogeneity and allow numerous subpopulations to exist. The ability to isolate clones with markedly different growth rates from advanced tumours suggests that some interaction must be occuring otherwise the most rapidly growing and aggressive populations would soon come to dominate the tumour (Heppner, 1989).

It has been demonstrated by the use of mixtures with differing characteristics that the growth rate of one population can be altered by the presence of a second

population with a different growth rate (Miller et al., 1988; Tofilon, Buckley and Deen, 1984). Using a mouse mammary tumour model Miller et al. (1988) showed both in vivo and in vitro that in some cases one subpopulation is able to suppress the growth of another, and obtained evidence of a growth-inhibiting factor produced by the dominating cell line. Several groups have also shown that the interaction of mixed drug sensitive and resistant populations can lead to an alteration in drug response (Miller, Miller and Heppner, 1981; Tofilon, Buckley and Deen, 1984; Bradford et al., 1986; Tofilon, Arundel and Deen, 1987) (See section 1.7.). The mechanisms of the majority of these interactions remain obscure. In some cases interaction is dependent on host responses such as altered immune reaction and drug metabolism, although in other cases the interaction appears to occur directly between the tumour cells themselves (Heppner, 1989). The importance of the gap junction in intercellular communication and its possible role in mediating such interactions will now be discussed.

1.2. The gap junction

1.2.1. Metabolic cooperation

The description of metabolic cooperation by Subak-Sharpe, Burk and Pitts in 1966 was the first indication of the potential implications of cell-cell communication for altering tissue phenotype. They showed that mutant Syrian hamster fibroblasts lacking the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) enzyme, which were unable to incorporate hypoxanthine into nucleic acid when cultured alone, did so when grown in contact with wild-type cells. Cell-cell transfer of the enzyme or its messenger RNA (mRNA) were considered possible explanations of this phenomenon and Kolodny (1971) suggested that such transfer did occur. Subsequent work by Pitts and Simms (1977) showed that intercellular transfer of such macromolecules was not possible and that this metabolic cooperation was mediated by exchange of nucleotides between the mutant and wild-type cells, allowing the mutant cells to bypass the metabolic block caused by their lack of enzyme.

1.2.2. Gap junction structure

Further studies have shown that the route through which the nucleotides travelled from cell to cell was the gap junction (Gilula, Reeves and Steinbach, 1972). This is a channel spanning the two membranes of adjacent cells and

linking their cytoplasms. X-ray diffraction studies have shown the channel to be composed of six protein subunits radially arranged within the membrane and surrounding an aqueous central pore of approximately 2nm diameter (Makowski et al., 1977).

Gap junctions occur as close-packed arrays of channels which form in regions where the membranes of two cells come into close contact. At their extracellular face the junctional units protrude 1-2nm into the extracellular space with the result that a characteristic 2-4nm gap remains between opposing membranes and gives this structure its name. This is in contrast to the appearances of tight junctions which occlude the spaces between cells in epithelial sheets. In these regions the membranes are closely opposed and the intercellular cleft obliterated.

X-ray diffraction studies have shown that the channel protein subunits are arranged at a slight angle to each other and there is some evidence that a twisting movement in these subunits may result in closure of the central pore at its cytoplasmic end, thus acting as a mechanism for gating the junctional channel (Unwin and Ennis, 1984).

There has been some controversy over the identification of the proteins involved in forming gap junctions. However there is now evidence to suggest that a highly

conserved, tissue invariant 16 kilodalton (kD) protein is the core component of the junctional channel (Finbow et al., 1983; Buultjens et al., 1988), whilst one of a family of tissue specific proteins (the connexins) is also required for functional coupling. It is probable that specificity in formation of junctions between cells as reported in vitro by Pitts and Burk (1976) is dependent on the connexins. They observed that rat liver epithelial (BRL) cells showed extensive communication in pure culture and that C13 hamster fibroblasts also formed junctions among themselves, but in mixed culture there was little heterologous coupling between the two cell types.

This specificity of interaction, and the associated sorting-out of cell types during tissue differentiation, forms the basis for communication compartments in vivo, where a boundary effect results from the reduced frequency of heterologous relative to homologous junction formation (Pitts, Finbow and Kam, 1988).

1.2.3. Junctional permeability

With the use of a number of different fluorescent probes of defined size the permeability properties of junctional channels have been elucidated. For arthropod junctions the exclusion limit is 1500 daltons (Simpson, Rose and Loewenstein, 1977) whilst for mammalian junctions the limit is 900 daltons (Flagg-Newton, Simpson and

Loewenstein, 1979), which is broadly in keeping with estimates based on the diameter of the aqueous channel derived by X-ray diffraction.

In effect the junction acts as a sieve retaining macromolecules within the cell but allowing small molecules to pass freely without further selection. In practice there are few molecules of a size near to the critical limit whose ability to traverse the junction would be in doubt. Most cytoplasmic constituents are either much too large to pass, e.g. nucleic acids and proteins, or are relatively small, e.g. ions and nucleotides, and pass freely. The rate of movement through the junctions is very rapid. Pitts et al. (1985) have calculated that in metabolic cooperation studies with BHK cells more than 10^6 nucleotides must pass between each cell pair per second and it is likely that other small molecular weight components will be traversing the junctional channels at similar rates along diffusion gradients. The potential for dialogue between coupled cells is therefore immense.

It is generally believed that in the normal, resting state the junctional channel is open allowing maximal communication (Pitts and Finbow, 1986) but a number of modulators of communication have been identified. High intracellular free ionised calcium concentration (Rose and Loewenstein, 1975) and low pH (Turin and Warner, 1977) have been shown to uncouple cells. Extracellular

uncouplers include the tumour promotor 12-O-tetradecanoyl phorbol-13-acetate (TPA) (Yotti, Chang and Trosko, 1979), octanol (Johnston, Symon and Ramon, 1980), retinoic acid (Pitts et al., 1986) and procaine (Dertinger and Hulser, 1984). It has also been reported that cyclic adenosine monophosphate (cAMP) can promote junctional communication in poorly coupled cells (Azarnia, Dahl and Loewenstein, 1981). In addition, it has been demonstrated using a mouse skin model that TPA can increase the otherwise poor transboundary junctional communication between dermis and epidermis (Kam and Pitts, 1988).

1.2.4. Detection and function of gap junctions

Gap junctions are ubiquitous structures found in tissues of all metazoan animals. They are formed in virtually every cell type with the few exceptions where they are lost during the terminal stages of differentiation. Their appearance and disposition can be demonstrated by thin-section and freeze-fracture electron microscopy (Revel and Karnovsky, 1967). In addition to the previously described metabolic cooperation studies, their functional activity can be detected by measurement of electrical coupling (Loewenstein, 1979), by intercellular passage of dye following microinjection (Loewenstein and Kanno, 1964) or entry via a transient tear in the cell membrane (scrape-loading) (El-Fouly, Trosko and Chang, 1987) and by autoradiographic detection of ^{3}H labelled nucleotide transfer (Pitts and Simms, 1977).

The role of gap junctions in normal tissue function is widely debated. They are considered to be involved in the control of cellular growth and differentiation. The presence of coupling between cells in a tissue produces a partial syncitial state where all cells in the group are able to freely transfer their small ions and metabolites and thus integrate their metabolic behaviour. In addition to the reduced coupling at boundaries between heterologous cells in vitro (Pitts and Burk, 1976; Pitts and Kam, 1985), communication compartments have also been defined in a number of tissues in vivo (Kam, Melville and Pitts, 1986) and it has been proposed that gap junctions may regulate growth within the compartment by means of small diffusable molecules such as growth factor-induced second messengers whose concentration within the compartment would depend on the compartment size as defined by communication boundaries (Pitts and Finbow, 1986).

Examples of coupling of capillary endothelial cells to surrounding stromal cells have also been reported (Kam, Mellville and Pitts, 1986) suggesting that junctional communication may also play a role in the delivery of nutrients and metabolites into tissues.

1.2.5. The role of gap junctions in malignancy

The importance of gap junctions in the processes of growth and differentiation of normal tissues and the disruption of these controls associated with neoplastic transformation has led to extensive investigation of the relationship between intercellular communication and malignancy.

In the multistage model of carcinogenesis, several sequential events are involved in the malignant transformation of a cell. The first or initiating event must be followed by one or more promoting events before the cell is fully transformed. The observation that one of the most effective tumour promotors, the phorbol ester TPA, uncoupled cells in some situations led to the suggestion that loss of junctional communication between initiated and normal cells may play a part in allowing the initiated cell to escape from normal growth regulation (Yotti, Chang and Trosko, 1979).

A role for the gap junction in the malignant process was strengthened further by the report that a reduction in communication can also occur as a result of oncogene expression (Atkinson et al., 1981), whilst McNutt, Hershberg and Weinstein (1971) reported that the number of gap junctions was inversely correlated with the degree of dysplasia in cervical epithelium and that junctions were rare in frank invasive carcinoma. Conversely,

Brauner and Hulser (1987) presented a preliminary report using an *in vitro* model of tumour cell invasion indicating that the presence of junctions is positively correlated with invasive behaviour.

In 1971 a number of tumour cell lines with poor junctional communication were identified and it was suggested that loss of junctions was a precondition for malignancy (Azarnia and Loewenstein, 1971). Since then, however, a large number of communication competent tumour cell lines have been identified (Miller *et al.*, 1983; Morgan *et al.*, 1982). Thus the interrelationship between junctional communication and malignancy is clearly a complex one and much remains to be elucidated.

1.3. Tumour spheroids and contact resistance

1.3.1. Spheroids and radioresistance

The suggestion that gap junctional communication may play a role in the response of tumours to therapeutic intervention was prompted by study of the contact effect or contact resistance (CR). This phenomenon was first reported by Durand and Sutherland (1972) who found that tumour cells when cultured as three-dimensional multicellular spheroids were more resistant to the cytotoxic effects of ionising radiation than when the same cells were cultured as monolayer. Cells grown as spheroids but separated into a single cell suspension by

trypsinisation immediately before irradiation showed an intermediate survival, and this enhanced survival reduced with increasing time interval between trypsinisation and irradiation and had returned to the control level by 10 hours.

Thus, although three-dimensional contact appears important for the development of CR, direct cell-cell contact at the time of irradiation is not crucial for this effect. The cause of this difference was attributed to the altered environmental influences acting on the cells in view of the three-dimensional geometry within the spheroid and therefore related to the intimate intercellular contact afforded by this arrangement.

This spheroid model has since been widely adopted in an attempt to simulate in vitro the characteristics of the three-dimensional growth of tumours in vivo. The closest analogy is between spheroids and unvascularised micrometastases, although there are also similarities with poorly vascularised regions of larger solid tumours (Sutherland, 1986). The limitations of this model include the absence of tumour stroma or circulation, and of the influences of the host's metabolic processes.

1.3.2. The influence of spheroid microenvironment on response

Oxygen, glucose and other nutrients, hormones and growth factors reach the cells within the spheroid by diffusion from the surrounding medium resulting in the establishment of diffusion gradients between the outer and inner spheroid regions for these essential factors. Conversely a gradient for H^+, lactate and other metabolic products develops in the opposite direction (Sutherland, 1986). As a result, in the centre of larger spheroids necrotic regions develop, analogous to the appearances commonly seen <u>in vivo</u> when rapidly growing tumours outstrip their blood supply.

It has been proposed that the reduced radiosensitivity of spheroids could in part be attributed to the presence of viable, hypoxic and therefore radioresistant cells within the inner spheroid regions (Sutherland and Durand, 1984). There is a well established relationship between cellular hypoxia and radioresistance which is apparent when PO_2 < 10mmHg. Such levels have frequently been observed in human and rodent tumours and resistant hypoxic cells have been demonstrated in spheroids (Sutherland, 1986). Dertinger and Hulser (1981) however demonstrated that contact resistance can be detected in well-oxygenated outer spheroid cells and that the magnitude of CR is uniform regardless of the position of the cell within the spheroid.

Another major factor which may contribute to CR is reduced growth rate and altered cell cycle phase distribution of inner spheroid cells (Sutherland and Durand, 1984). Assessment of cell cycle phase distribution by flow cytometry has shown that the distribution of outer cells is similar to exponentially-growing monolayers whereas up to 90% of cells in the inner regions have a G1 phase DNA content (Freyer and Sutherland, 1980). However Hinz and Dertinger (1983) found that such kinetic influences were unable to explain CR. They reported that the radioresistance of outer proliferating cells was comparable to inner non-proliferating cells. Moreover in the original study of Durand and Sutherland (1972), very young spheroids composed of only 7-15 cells were used which therefore remained in exponential growth with a doubling time identical to that of single cells. Although Hinz and Dertinger found the decay of CR (when spheroid cells were irradiated at various time intervals after trypsinisation) to be comparable to that reported by Durand and Sutherland, their findings differed in that they also observed that CR occurred only in spheroids of more than 2mm diameter and that the radiosensitivity of very small spheroids was comparable to that of monolayers.

In summary, therefore, although there is some evidence that hypoxia and altered cell cycle phase distribution in the inner spheroid region may contribute to the

phenomenon of CR, they do not provide an adequate explanation in all cases, and it appears that some additional protection is afforded by the three-dimensional contact in this system.

1.3.3. Intercellular communication and contact resistance

Further light was thrown on this phenomenon by the observation of Dertinger and Hulser (1981) that CR was a property only of cell lines which displayed gap junctional communication and that the magnitude of CR was related to the extent of communication as measured electrophysiologically. They reported that there was no difference in radiosensitivity between monolayers or spheroids of uncoupled cell lines, and that blockage of communication by TPA could reverse the CR in spheroids of well-coupled lines.

It seemed possible, therefore, that the increased opportunity for intercellular communication when cells were in three-dimensional contact might explain CR if exchange of small signal molecules occurred between cells in coupled spheroids.

It has been suggested, however, that rather than the expected increase in opportunities for intercellular communication, cells within spheroids may, in fact, be less well coupled than when cultured as monolayer. Dertinger, Hinz and Jakobs (1982) reported a reduction in

coupling in the outer cells of spheroids with increasing age and this finding was confirmed independently by Sutherland (1986) who showed reduced spread of Lucifer Yellow in older spheroids produced from several different cell lines.

Other aspects of the CR phenomenon are inconsistent with an explanation based on metabolic cooperation. The increased survival of disaggregated spheroid cells is difficult to explain by intercellular transfer of any signal molecule, and such transfer would also be expected to occur to a significant extent in monolayer culture as indicated by the examples of metabolic cooperation.

There is some evidence however in support of a role for the tripeptide glutathione (GSH) in the development of CR. Cellular damage by indirectly ionising radiation, including the gamma irradiation in the above studies, is the consequence of the production of highly chemically reactive radical species and as GSH can act as an intracellular free radical scavenger and radioprotector (Biaglow et al., 1983; Edgren and Revesz, 1985), it may limit radiation induced damage.

The effect of GSH on radiation response has been studied by Edgren (1982) who reported that coculturing of cells deficient of GSH with normal cells increases the DNA repair capacity of the deficient cells, whereas the addition of medium conditioned by the normal cells had no

effect. Although junctional communication was not assessed in these cells, it seems possible that this interaction was the result of metabolic cooperation mediated by GSH transfer from the normal to the deficient cells. Kavanagh et al. (1988) have confirmed that GSH can be transferred from cell to cell through gap junctions.

In contrast, Dertinger and Hulser (1984) have attributed CR to differentiation occurring in spheroids. It has been shown that spheroids of human colonic adenocarcinoma will form pseudo-glandular structures which resemble the appearances of tumours in vivo and also express increased amounts of the tumour associated protein carcinoembryonic antigen (CEA) compared to monolayers (Sutherland, 1986), both of these features suggesting a degree of differentiation in spheroids above that encountered in monolayers.

1.3.4. In vivo models of contact resistance

Subsequent studies have shown that CR operates in vivo in rodent tumours (Hill, Warren and Bush, 1979) and in human tumour xenografts (Guichard, Dertinger and Malaise, 1983), suggesting that this phenomenon may make a significant contribution to the radiation response of human tumours. The previous correlation between CR and junctional communication was also manifest in this model. Dertinger, Guichard and Malaise (1984) studied this

relationship in four xenografts and reported that in three of the tumours which exhibited CR, junctional communication could be demonstrated (communication in these lines was assessed by electrical coupling *in vitro*). The other tumour which did not show CR also failed to show coupling.

By comparing the tumour radiosensitivity in mice asphyxiated in a nitrogen atmosphere for 10 minutes before irradiation with that in live air breathing mice, Guichard *et al*. (1983) have demonstrated that in this model system, as in spheroids, hypoxia was not an adequate explanation for the degree of CR observed.

1.3.5. Spheroid resistance to cytotoxic drugs

In parallel to the above series of experiments comparing radiosensitivity of spheroid to monolayer culture, the role of the three dimensional spheroid geometry in chemosensitivity has also been widely studied. Although there are some exceptions (Tofilon, Arundel and Deen, 1987), most studies show a survival advantage afforded to cells when cultured as spheroids for a number of cytotoxic drugs. In addition to the factors modifying radiation response of spheroids, the study of spheroid chemosensitivity is further complicated by alterations in drug accessibility, uptake and metabolism. Penetration barriers have been suggested to be at least partly responsible for spheroid resistance to vinblastine

(Nederman, Carlsson and Malmqvist, 1981; Nederman, 1984), adriamycin (Durand, 1976; Sutherland et al., 1979; Kerr et al., 1986) and 4'-(9-acridinylamino) methanesulfon-m-anisidide (mAMSA) (Wilson, Whitmore and Hill, 1981).

Increased spheroid drug resistance was first demonstrated to adriamycin. Durand (1976) has shown that even small spheroids of the Chinese hamster fibroblast line V79, composed of 5-15 cells, which would not be expected to present any penetration barrier, were more resistant to adriamycin than single cells. Subsequent studies with larger spheroids (Durand, 1981) confirmed the presence of a diffusion gradient of adriamycin into the spheroids, but showed that for a given intercellular adriamycin concentration spheroid cells had increased survival relative to monolayer cells, whilst cells from disaggregated spheroids had an intermediate survival.

Similarly, by selective trypsinisation of inner and outer cell layers, Sutherland and co-workers (1979) showed that cells in inner regions of EMT-6 mammary tumour spheroids were more resistant to adriamycin but that this difference could only partly be accounted for by a penetration barrier. They suggested that the increased resistance of the inner cells could result from other factors related to the three dimensional microenviroment of the cells, or alterations in the metabolism of the cells or of the drug itself.

Wibe (1980) subsequently extended these studies to the examination of the chemosensitivity of a human cervical carcinoma line to vincristine. A marked increase was seen in the resistance of spheroids compared to exponentially growing monolayers, although plateau-phase monolayers were even more resistant than spheroids. This result could be attributable to kinetic factors, as the spindle poison vincristine is inactive against non-cycling cells. However, in contrast to the earlier results with adriamycin, the sensitivity to vincristine was independent of distance from the spheroid surface, a surprising finding in view of the presumed reduction in cell cycling with increased spheroid depth.

By treating cells from spheroids with mAMSA at varying times following trypsinisation, Wilson, Whitmore and Hill (1981) showed a gradual loss of spheroid resistance to the drug with time, to reach a level comparable to that of exponentially growing monolayers after 28 hours, thus paralleling the decay of radiation CR. On this occasion the inner cells were more resistant than the outer layers although even the latter demonstrated improved survival over exponential cells.

Thus, there is a body of evidence which indicates that, as with radiation response, the three dimensional spheroid structure appears to provide some additional chemoresistance to cells beyond that which can be attributed to kinetic and penetration influences. Unlike

radioresistance, however, the contribution of gap junctional communication to this phenomenon has not yet been assessed.

1.4. Intercellular communication and lymphokine response

It has more recently been reported that the role of gap junctional communication in cellular resistance is not wholly dependent on the three dimensional structure of spheroids and xenografts nor limited to their response to radiation.

In monolayer culture Fletcher et al. (1987) demonstrated that communication-competent Chinese hamster ovary (CHO) cells were resistant to the cytolytic action of the lymphokines, tumour necrosis factor (TNF) and lymphotoxin (LT). Exposure of the cells to TPA or culturing at low density, both of which would be expected to reduce the frequency of intercellular communication, increased their sensitivity to the lymphokines. In contrast, two subclones of mouse L cells which do not communicate through junctions and which were TNF/LT-sensitive became less sensitive after promotion of coupling by elevation of their intracellular cAMP. In addition they studied the LA25 transformant of the NRK cell line which is infected with a temperature sensitive mutant of the Rous sarcoma virus and exhibits coupling and TNF/LT resistance at the restrictive temperature (39^{o}C) where the oncogene is not transcribed. Reduction to the permissive

temperature of 33^{o}C causes rapid uncoupling and the cells then became sensitive to the lymphokines. They suggested that modulation of the cellular response to these agents might be explained by the passage of small signal molecules from cell to cell in a coupled population.

There is conflicting evidence regarding the relationship between the resistance of cells to TNF and to members of the MDR group of drugs. Several studies (Dollbaum et al., 1988; Hofsli and Nissen-Meyer, 1989; Fruehauf, Mimnaugh and Sinha, 1991) have suggested cross-resistance between TNF and MDR drugs, but this finding is not universal (Matthews and Neale, 1989).

1.5. Mechanisms of cytotoxic drug resistance

1.5.1. Inherent and acquired resistance

In the clinical context the resistance of tumour cells to cytotoxic drugs is a major obstacle to effective treatment and eradication of the tumour. Cytotoxic drug resistance has conventionally been considered to occur in two separate settings. The first is that of inherent resistance, where from the outset of treatment the tumour is unresponsive to available cytotoxic drugs. The second form, acquired resistance, describes the situation where after an initial tumour response to a drug with shrinkage or even apparent disappearance of the tumour, the tumour

eventually regrows and fails to respond further to the drug.

Although this division is not absolute, both varieties of resistance are readily identifiable and distressingly common in the clinic. Tumours such as colorectal carcinoma, renal carcinoma and melanoma belong to the inherently resistant group whilst breast and small cell lung cancer display acquired resistance in the majority of patients.

The explanation for drug resistance can in general be considered to arise either from pharmacokinetic factors, where the achievement of an adequate intratumoural drug concentration is the problem, or from biochemical factors where the tumour cells possess or develop a mechanism which enables them to withstand attack by the cytotoxic drugs. It is not yet clear whether inherent and acquired resistance arise through different mechanisms.

A number of such biochemical mechanisms have been identified in resistant cells including reduced activation of prodrugs, increased drug inactivation and over production of target enzymes. One further resistance mechanism, whereby cells are able to reduce the amount of intracellular drug accumulation and presumably its delivery to its site of action, has been shown to play a major role in the resistance to a number

of cytotoxic drugs and will be considered in the following section.

1.5.2. The multidrug resistant phenotype

Although reduced intracellular drug uptake has been identified as contributing to methotroxate resistance by changes in a drug specific transport mechanism (Hill et al., 1979), and also to resistance to cisplatin (Bungo et al., 1990) and melphalan (Redwood and Colvin, 1980), the major interest in this field involves a common mechanism through which cells develop resistance to a group of structurally and functionally unrelated drugs.

This phenomenon was first described by Biedler and Rheim in 1970 who found that Chinese hamster lung cells and P388 leukemia cells which had been made resistant to actinomycin D by exposure to increasing concentrations of the drug displayed cross resistance to vinblastine and daunomycin. The degree of this cross resistance was proportional to that of actinomycin D itself. They reported that the resistant cells showed reduced accumulation of ^{3}H actinomycin D and proposed that this was the result of reduced membrane permeability to the drug.

This behaviour was termed pleiotropic or multidrug resistance (MDR) and similar findings have been obtained by other groups in a range of cell lines. The list of

drugs within the MDR group has been extended to include other anthracyclines and vinca alkaloids as well as mitomycin C and the epipodophyllotoxins (Kaye, 1988), although the precise pattern of cross resistance varies between the different reported lines.

1.5.3. P-glycoprotein

Ling and colleagues, using MDR Chinese hamster lung (V79) cells initially selected for resistance to colchicine, confirmed that MDR was associated with reduced intracellular drug accumulation and subsequently identified a plasma membrane associated 170kD glycoprotein in these cells which was not present in the parental sensitive line (Ling and Thomson, 1973; Juliano and Ling, 1976; Bech-Hanson, Till and Ling, 1976). The relative amount of this glycoprotein correlated with the degree of resistance and reduction in drug accumulation in independent clones with stepwise increases in resistance. They proposed that this glycoprotein reduced intracellular drug accumulation by modifying membrane fluidity and reduced the permeation rate of drugs entering the cell and suggested that it be called P-glycoprotein.

This group subsequently showed that P-glycoprotein was also present in MDR mouse and human cell lines and that it appeared to be highly conserved (Kartner, Riordan and Ling, 1983; Kartner et al., 1985). They were also able

to detect P-glycoprotein in much smaller amounts in the parent drug sensitive lines and suggested that the increased levels found in the resistant lines might arise from amplification of a gene present in the parent cells.

1.5.4. P-glycoprotein as an efflux pump

The action of P-glycoprotein in mediating the reduced intracellular drug accumulation associated with the MDR phenotype was clarified by the identification of extensive homology between P-glycoprotein and the Hyl-B protein, an *E. coli* membrane protein involved in the export of haemolysin (Gerlach *et al.*, 1986). Both protein sequences have multiple potential transmembrane segments consistent with channel forming functions. In addition the cytoplasmically located C-terminal regions of P-glycoprotein and Hyl-B are homologous to the nucleotide binding domains of four closely related bacterial ATP-binding proteins and it was proposed that P-glycoprotein functions in resistant cells as an energy dependent efflux pump to reduce intracellular drug levels.

The importance of P-glycoprotein in the MDR phenotype was supported by the observations that photoaffinity analogues of vinblastine will bind to P-glycoprotein and that this binding is competitively inhibited by anthracyclines and by vinblastine itself, although colchicine and actinomycin D do not compete for binding

suggesting that there may be more than one binding site on P-glycoprotein (Cornwell et al., 1986; Safa et al., 1986; Cornwell, Gottesman and Pastan, 1986).

It has also been reported that other agents including verapamil and quinidine will also compete for binding (Cornwell, Pastan and Gottesman, 1987), and have been shown to be capable of restoring sensitivity in some MDR lines (Tsuruo et al., 1983; Bellamy et al., 1988b). This finding has led to their introduction into clinical trials in conjunction with cytotoxic chemotherapy in an attempt to circumvent drug resistance (Ozols et al., 1987; Jones et al., 1990).

1.5.5. The mdr gene family

The MDR phenotype has been linked to increased expression with or without amplification of the gene encoding P-glycoprotein (Shen et al., 1986; Riordan, Deuchars and Kartner, 1985). Several classes of P-glycoprotein cDNAs have been identified in hamster (Endicott et al., 1987), mouse (Gros, Croop and Housman, 1986) and human (Van der Bliek et al., 1988) cells. It has been proposed that varying expression of these different genes may affect the pattern of drug resistance (Moscow and Cowan, 1988). However in five human MDR lines, Van der Bliek et al. (1988) have confirmed the central role of the mdr1 gene and found no over expression of other mdr genes to account for variations in cross resistance patterns.

They suggest that changes in the mdr1 gene itself, alternative splicing of mdr1 pre-mRNA or alterations in unlinked genes may be responsible for the observed resistance.

The most striking evidence of the importance of P-glycoprotein in MDR has come from studies where mouse (Gros, Croop and Housman, 1986) and human (Ueda et al., 1987) mdr1 cDNA sequences have been subcloned into expression vectors and transfected into drug sensitive cells. The expression of these genes in the recipient cells conferred the full MDR phenotype, and the relative drug resistance was found to have some correlation with the copy number of mdr1 cDNA in the transfected cell line.

1.5.6. Clinical relevance of mdr1 gene and P-glycoprotein

Using a DNA probe for the mdr1 gene isolated from an MDR human KB carcinoma cell line, Fojo et al. (1987) have measured levels of mdr1 mRNA in normal human tissues and in tumours. They found increased mdr1 expression in adrenal and kidney tissues and to a lesser extent in other tissues including lung, liver and colon, with levels of mdr1 mRNA in these organs being comparable to that of the MDR KB cell lines. When a series of predominantly untreated human tumours were probed it was noted that the majority of tumours arising from colon and adrenal also had high levels of expression, although in

some other tumours arising from these tissues, levels of mdr1 mRNA were no higher than that seen in the parent drug sensitive KB cell line which was used as a standard.

Salmon et al. (1989) have suggested that the presence of P-glycoprotein in human tumours does predict intrinsic cellular drug resistance. Using monoclonal antibodies to detect P-glycoprotein in 26 human tumour samples, they found that only 3 of 14 P-glycoprotein-negative tumours showed in vitro resistance to adriamycin, in contrast to all of 12 tumours which stained positively for P-glycoprotein

The role of mdr1 and P-glycoprotein in normal tissues remains uncertain, although it has been suggested that in tissues such as colon, kidney and liver which are exposed to high levels of naturally occurring enviromental toxins, P-glycoprotein may have a protective function by promoting the efflux of these toxins from the cell (Kartner and Ling, 1989). The proposal is that this natural defence mechanism has been adopted to provide protection for cells against these cytotoxic drugs, all of which are naturally derived products.

1.5.7. Exocytosis as a drug efflux mechanism

There is recent evidence implicating increased exocytic/endocytic activity in the MDR phenotype (Sehested et al., 1987a, 1987b). Using an

ultrastructural morphometric technique to measure membrane area and cell compartmental volumes, a significantly increased membrane traffic between the plasma membrane and endosomal system has been observed in four MDR Erlich ascites tumour lines and a MDR P388 murine leukemia line, compared to the sensitive parent lines.

It was proposed that this observation could account for the increased drug efflux in MDR cells by the trapping of drug in the endosomal system and subsequent exocytosis into the extracellular compartment. This hypothesis was supported by the finding that verapamil, which has been shown to inhibit active drug efflux in these cell lines (Kessel and Wilberding, 1985; Friche, Skovsgaard and Nissen, 1987), inhibited this increased plasma membrane traffic. They suggested that protonation of these basic drugs could account for drug trapping and concentration in the acid endosomal compartment.

This model of MDR does not, however, exclude a role for P-glycoprotein in this efflux mechanism. Cornwall, Gottesman and Pastan (1986) have reported that the binding of vinblastine by plasma membrane and endocytotic vesicles was increased in resistant KB cell lines and that this binding could be reduced by trypsin. Their conclusion was that drug binding to a protein receptor within the vesicles may be responsible for their drug

accumulation. P-glycoprotein would clearly be a suitable candidate.

1.5.8. Evidence for other resistance mechanisms

Although the evidence supporting a major role for the mdr1 gene and P-glycoprotein in resistance to this group of drugs is clear, a number of cell lines have been identified which display the classical MDR phenotype but show no increase in P-glycoprotein or reduction in drug accumulation (Danks, Yalowich and Beck, 1987; Marsh and Center, 1987). In addition, several studies have reported a lack of correlation between adriamycin accumulation and resistance (Kessel and Corbett, 1985; Marsh, Sickieri and Center, 1986).

Kessel and Wilberding (1985) showed that when sensitive and MDR P388 leukemia cells were incubated at different extracellular daunorubicin concentrations designed to produce identical intracellular drug concentrations, the MDR cells remained more resistant than the sensitive cells to a given intracellular daunorubicin concentration.

Similarly, in their study of sensitive and MDR human myeloma lines, Bellamy et al. (1988a) have shown a direct relationship between intracellular drug accumulation and formation of single and double strand breaks and DNA-protein cross-links. However, at least at low adriamycin

concentrations, there was no correlation between drug accumulation and cytotoxicity as assessed by clonogenic assay and they suggested that this might be explained by prolonged retention of drug by sensitive cells compared to resistant cells with a consequent increase in the amount of damage sustained by the sensitive cell. In a subsequent report (Bellamy et al., 1988b), they showed the addition of verapamil blocked drug efflux in resistant cells and restored sensitivity.

It now seems likely that a number of other resistance mechanisms are possible and that in many cases the development of resistance is multifactorial in origin. These additional mechanisms would be expected to vary among the MDR group of drugs, depending on their particular mode of action.

It is worth considering at this point the proposed mechanisms of action of adriamycin, which is the main agent studied in this thesis. These include DNA intercalation, where the amino sugar portion of the drug binds to the sugar phosphate backbone of DNA, blocking synthesis of DNA, RNA and hence protein, and the interaction of the drug with the enzyme topoisomerase II (topo II). A further mechanism is via the reduction of the drug by microsomal P_{450} reductase to a semiquinone free radical, which in turn produces highly reactive oxygen species which may be responsible for DNA strand breaks and damage to cytoplasmic constituents (Begleiter

and Blair, 1984). Using adriamycin coupled to an insoluble agarose support, it has also been demonstrated that adriamycin can also exert its cytotoxic effect without entering cells (Tritton and Lee, 1982), and it is possible that membrane damage can contribute to cytotoxicity.

1.5.9. Glutathione and related enzymes

The glutathione-S-transferases (GST) are a family of enzymes involved in drug detoxification by the conjugation of the tripeptide, glutathione (GSH), to electrophilic compounds. GSH also detoxifies oxygen-induced free radicals, a reaction catalysed by glutathione peroxidase. Increased intracellular levels of GSH, the substrate for both GST and glutathione peroxidase, are associated with resistance to adriamycin, as well as to melphalan and cisplatin (Green et al., 1984). Depletion of intracellular GSH levels with Buthionine sulfoxamine (BSO), which inhibits its synthesis has been shown to reverse adriamycin resistance in V79 cells (Russo and Mitchell, 1985).

Further evidence of the importance of this group of enzymes has come from the work of Mimnaugh et al. (1989), who have shown that MDR MCF-7 human breast tumour cells have increased levels of enzymatic defences against reactive oxygen species. This group have subsequently reported that treatment with BSO caused an 80-90%

depletion in intracellular GSH content and resulted in a partial reversal of adriamycin resistance in this line, associated with an increase in drug-induced hydroxyl radical production (Dusre et al., 1989).

1.5.10. Topoisomerases

Topoisomerases are nuclear enzymes which catalyse changes in the secondary and tertiary structure of DNA. Most interest is centred on topoisomerase II (topo II) which catalyses transient double strand breaks to allow two double stranded DNA segments to pass during DNA replication or transcription. There is evidence that a number of drugs including anthracyclines and epipodophyllotoxins interact with topo II to stabilise the cleavable complex between the enzyme and DNA and thus increase the strand breaks produced (Tewey et al., 1984). Increased drug resistance may therefore result from either reduction of cellular topo II levels or from an enzyme mutation resulting in reduced drug affinity of the enzyme. Altered topo II activity has been reported in an MDR CHO cell line (Glisson et al., 1986) and an adriamycin resistant P388 leukemia line (Deffie, Batra and Goldenberg, 1989), whilst Ferguson et al. (1988) have shown that resistance to etoposide in an MDR KB cell line appeared to result both from reduced accumulation of drug and from reduced levels of topo II.

1.6. Resistance models

1.6.1. MDR and clinical response

There is considerable debate over the relevance of many of the models of drug resistance to the undoubtedly real problem of resistance as encountered in the clinic. Increased expression of mdr1 or P-glycoprotein has been detected in a number of human tumours (Fojo *et al.*, 1987; Salmon *et al.*, 1989; Goldstein *et al.*, 1989). Although clarification of the extent of the relationship between mdr1 and clinical response awaits further information, there is some evidence that high levels of mdr1 expression may be associated with poor prognosis in neuroblastoma (Bourhis *et al.*, 1989) and soft tissue sarcoma (Chan *et al.*, 1990).

The *in vitro* phenomenon of MDR certainly mirrors a frequently observed clinical situation where on the recurrence of tumour following previous drug treatment, the tumour is then refractory, not only to further treatment with the same drug but is also cross resistant to other drugs. Therefore, for any tumour type, the probability of a second response to cytotoxic chemotherapy following relapse is very much less than that to the first line treatment.

Resistant cell lines are most commonly derived by *in vitro* exposure to levels of drug never encountered in the

therapeutic context, and can result in cell lines with greater than 100 or even 1000 fold resistance compared to the parent line. However, because toxicity to normal tissues limits the clinical use of these agents to a narrow dose range, even a two or three fold increase in resistance may potentially be sufficient to result in a failure of response in the clinical setting. Therefore the relevance of these highly resistant models to the clinical situation is uncertain. This consideration has been appreciated by several groups who have concentrated their efforts on the study of low levels of resistance (Hill, 1986; Lemont, Azarnia and Gross, 1988).

Although it is not yet clear whether the distinction between high and low levels of MDR is generally one of degree, there is evidence that the mechanism of resistance to methotrexate differs depending on the magnitude of resistance expressed (Hill, 1986). In addition, the method used to induce drug resistance can also affect the mechanism. It has been demonstrated that resistance to vincristine in a murine lymphoma cell line can be induced by prior exposure either to the drug itself or to radiation, and that both these methods resulted in independently derived lines which exhibited approximately 50-fold resistance to vincristine (Hill, Whelan and Bellamy, 1984). Although the drug exposed cell line displayed the characteristic MDR phenotype of reduced accumulation and cross resistance to a number of drugs, the radiation exposed line showed no change in

drug accumulation and a different pattern of cross resistance.

1.6.2. Prediction of drug response

In their mathematical model of drug resistance Goldie and Coldman (1983) related the development of resistance in a tumour to the mutation rate of its stem cell compartment. The higher the mutation rate or the larger the tumour, the greater the probability that it would contain resistant cells. The model also predicts that tumours with a small stem cell compartment, and therefore slower growth, will have accumulated more resistant mutations in those stem cells at a given tumour size than tumours with a larger stem cell compartment. The model also supports the policy of earlier diagnosis or use of adjuvant chemotherapy, since the probability of the existence of resistant cells is lower, and therefore the likelihood of therapeutic success is higher in these smaller tumours, although if chemotherapy is unsuccessful, the mutagenic effects of the drugs may result in the increased production of new variants by survivors.

It is widely assumed that the presence of one or more resistant cells within a tumour is sufficient to result in ultimate therapeutic failure. It is proposed that following exposure to cytotoxic drugs the sensitive cells are killed resulting in tumour shrinkage. However the resistant cells survive and continue to divide and as a

result of this selection come to dominate the tumour so that the tumour becomes progressively more resistant with time (Kartner and Ling, 1989). The existence of multiple subpopulations with varying sensitivities to different treatment provides the rationale for the use of combination therapies either of drugs or of mixed treatment modalities such as chemotherapy and radiotherapy.

This model assumes that sensitive and resistant cells within the tumour behave independently and that the drug response of any individual cell is not affected by the presence of its neighbours. However several groups have suggested this may not be the case but that interaction between sensitive and resistant populations within the tumour may alter the ultimate drug response.

1.7. Interaction of sensitive and resistant cells

1.7.1. Transfer of drug resistance

The most striking evidence of such an interaction was reported by Tofilon and colleagues who studied the response to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) of rat brain tumour cells grown as spheroids (Tofilon, Buckley and Deen, 1984; Tofilon, Arundel and Deen, 1987). They found that when measured by cell survival and spheroid growth delay assays, spheroids composed of mixed BCNU sensitive and resistant cells were more resistant

than that predicted from the sensitivities of purely sensitive or resistant spheroids.

To identify the individual drug response of the two subpopulations in mixed spheroids they used the number of drug-induced sister chromatid exchanges (SCE) within the cells both as an index of DNA damage and cytotoxicity, and also to identify the resistant and sensitive populations. Fewer SCEs were sustained by resistant compared to sensitive cells when exposed to drug individually, and this difference between the two groups allowed them to be distinguished in mixed culture. They found a proportionate decrease in the number of SCEs sustained by the sensitive cells as the proportion of resistant cells within the mixed spheroids was increased. However, when the spheroids were disaggregated into single cells before drug exposure, there was no decrease in the number of SCEs induced in the sensitive cells, the level instead remaining comparable to that found in spheroids grown from sensitive cells alone.

In contrast, when the two cell types were grown in mixed monolayer culture there was no resistance transfer and they suggested that the interaction was dependent on the three dimensional contact afforded by spheroid geometry. However Bradford et al. (1986) did not find a three dimensional relationship essential for the occurrence of this phenomenon. In a preliminary report they described a similar interaction between vincristine/vindesine

resistant and sensitive clones of a spontaneous murine astrocytoma in monolayer culture. They found that a mixed culture produced from equal numbers of sensitive and resistant cells adopted the chemosensitivity of the more resistant line, suggesting possible resistance transfer.

1.7.2. Transfer of drug sensitivity

Miller, Miller and Heppner (1981) have demonstrated that interaction between sensitive and resistant cells does not even always require cell to cell contact. By culturing methotrexate sensitive and resistant mouse mammary tumour lines on separate cover slips within the same dish they observed reduced survival of the resistant cells following drug exposure. They also showed that the simultaneous presence of the sensitive cells in the dish was required to produce this interaction as growth of the resistant line in medium conditioned by the sensitive line had no effect. They suggested that this implicated the transfer of a labile mediator in this phenomenon. A second resistant line, however, failed to show any interaction.

In the same report this group also demonstrated interaction of mammary tumour lines in vivo. Following injection of syngeneic mice on opposite flanks with cyclophosphamide sensitive and resistant cells they found that drug induced growth delay in the resistant line was

increased by the presence of the sensitive tumour line on the opposite flank but not by a second resistant tumour. On this occasion the proposed mechanism of interaction was by increased drug activation in mice bearing the sensitive line.

It is therefore evident that, depending on the cell types, model system and drug studied, the interaction between populations can produce a shift towards either sensitivity or resistance. Although these studies describe interactions between only two subpopulations they may be considered representative of the multiple interactions possible between the large number of subpopulations present in a heterogenous tumour (Itaya et al., 1989).

1.7.3. Junctional communication in interactions

It is not clear in the earlier examples of interaction involving direct cell contact whether intercellular communication through gap junctions may be involved. The property of metabolic cooperation within a coupled population has been shown to be responsible for transfer of drug sensitivity or resistance in other situations.

Intercellular transfer of nucleotides is responsible for interaction between normal and HGPRT-negative cells with respect to 6-thioguanine sensitivity. Fujimoto et al. (1971) demonstrated that HGPRT-negative fibroblasts

established from skin biopsies from patients with the Lesch-Nyhan syndrome were resistant to the drug when cultured on their own, but in mixed culture with normal (HGPRT-positive) cells, the transfer of thioguanine nucleotides via gap junctions from normal to HGPRT-negative cells conferred sensitivity on the mutant line.

Ouabain exerts its cytotoxic effects by inhibition of Na^+/K^+ ATPase, thus blocking Na^+/K^+ fluxes across the membrane necessary to maintain cellular ionic balance. Rodent cells are inherently more resistant to the effects of ouabain on account of a reduced affinity of their enzyme for the drug. Corsaro and Migeon (1977) have shown that when a mixed culture of human and mouse fibroblasts were exposed to a ouabain concentration adequate to inhibit division of human but not mouse cells on their own, the human cells continued to proliferate. They proposed that this protection of the human cells by mouse cells was the result of intercellular transfer of Na^+ and K^+ along diffusion gradients between the cytoplasms of the two cells. Avoidance of cell to cell contact by growing the two cells lines on separate cover slips within the same dish, or the use of mouse L cells which do not communicate through gap junctions abolished this effect, indicating the fundamental role of junctional intercellular communication in this phenomenon.

1.7.4. A model of interaction between sensitive and MDR cell lines

As the above example demonstrates, where the cytotoxicity of a drug is dependent on the cytoplasmic concentration of a small molecule or ion, the ability of this molecule to pass freely from cell to cell along diffusion gradients enables equilibrium to be approached within the coupled population. Consequently the tendancy towards homogeneity afforded to the population by the junctional channels is translated into a more uniform response to the drug.

In general, therefore, if there were resistant cells within the population whose ability to survive drug exposure was dependent on their capacity to regulate the cytoplasmic concentration of this molecule within tolerable limits, then the coupling of these cells to adjacent sensitive cells which did not posess this regulatory capacity might enable the resistant cells to rescue the sensitive cells by means of their shared pools of small cytoplasmic molecules.

An analogy can be drawn between the mechanism of ouabain resistance and that of the MDR phenotype mediated by P-glycoprotein. In both instances resistance is dependent on the adequate functioning of a membrane pump. In the case of MDR it is the intracellular concentration of the drug itself rather than of ions or intermediary

metabolites which appears to be the crucial factor governing cytotoxicity. The molecular weight of many drugs is small enough to allow them to pass freely through gap junctions, which suggests the possibility of rescue of a sensitive cell by an adjacent resistant cell if the P-glycoprotein pump in the resistant cell was able to reduce the intracellular drug concentration of both.

1.8. Summary and objectives

Intercellular communication through gap junctions may affect cellular response to radiation, cytokines or cytotoxic drugs in a number of ways.

There is good evidence that junctional communication affects radiation and lymphokine response, although there is no present data on its possible role in the increased resistance to cytotoxic drugs observed in spheroid compared to monolayer culture. The mechanism through which junctional communication may influence resistance to these different modalities is unknown. One possibility is that if random damage from either radiation, lymphokines or cytotoxic drugs produces a variety of metabolic defects within different cells, then in a coupled population some form of mutual support of defective cells such as that shown in the metabolic cooperation studies of Subak-Sharpe *et al*. (1966) could be responsible.

There is also evidence that intercellular communication is involved in some examples of interactions between drug sensitive and resistant cells. As discussed in the above model (section 1.7.4.), it is possible that interactions of sensitive and MDR populations may occur via junctional transfer of drug.

The principal objectives of this present study are:

1. To determine whether the correlation between junctional communication and cellular resistance to radiation and the lymphokines, lymphotoxin and tumour necrosis factor, can be extended to include conventional cytotoxic drugs.

2. To investigate whether transfer of drug resistance can be demonstrated between sensitive and multidrug resistant cells in mixed culture.

Chapter 2 MATERIALS AND METHODS

2.1. Materials

2.1.1. Radiochemicals

^{3}H uridine (1mCi/ml) and ^{3}H thymidine (1mCi/ml) were purchased from Amersham International plc.

2.1.2. Drugs

Adriamycin (doxorubicin) (Farmitalia Carlo Erba Ltd.), mitozantrone (Lederle Ltd.), vincristine and vindesine (Eli Lilly and Co. Ltd.) were solubilised according to manufacturers' instructions for injection and then stored as frozen aliquots at -20^{o}C until required.

2.1.3. Fluorescent dyes

Lucifer yellow and rhodamine isothiocyanate-dextran were obtained from Sigma Chemical Company. Adriamycin for microinjection studies was also obtained from Sigma Chemical Company. Stock solutions were prepared in PBS and stored in darkness at 4^{0}C until required.

2.1.4. Chemicals

Potassium ferricyanide, potassium ferrocyanide, 4-chloro-5-bromo-3indolyl-ß-galactosidase (X-gal), 3-(4,5-

dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Company.
Trichloracetic acid (TCA) was obtained from Koch-Light Laboratories.
Ilford L4 emulsion gel and Hypam fixative were obtained from Ilford Ltd. D19 developer solution was obtained from Kodak Ltd.
All other chemicals were obtained from British Drug Houses (BDH), Analar grade.

2.1.5. Tissue culture media

Hams F10 medium, PBS, BSS and all supplements were obtained from Gibco Ltd. RPMI 1640 medium, Glasgow modified Eagle's medium and Foetal calf serum were obtained from Northumbria Biologicals Ltd.

2.1.6. Tissue culture dishes

$25cm^2$ and $75cm^2$ flasks were obtained from Sterilin Ltd.
3.5cm, 6cm and 10cm diameter Petri dishes were obtained from Becton Dickinson and Company, and 24 and 96 well plates from Costar Ltd.
Glass microscope slides and coverslips were obtained from Chance Propper Ltd.

2.1.7. Cell lines

The following cell lines were studied:

1. Human non small cell lung cancer (NSCLC).
Seven different lines were studied, kindly provided by Dr. R.I. Freshney, Glasgow. Histological and other details are listed in table 1.

2. Murine astrocytoma.
These lines were kindly provided by Dr. J. Darling, London. They comprised two clonal lines, designated C12 and F1, derived from the P497 cell line, originally isolated from a spontaneous murine astrocytoma (Bradford et al, 1986).

3. Chinese hamster ovary (CHO).

CHO-K1	originally derived as a sub clone from parental CHO initiated from a biopsy from the ovary of an adult Chinese hamster.
Adr^r	multidrug resistant line produced by growth of CHO-K1 in adriamycin containing medium.
ADR-6	drug hypersensitive line obtained following exposure of CHO-K1 to the mutagen ethyl methanesulphonate.

These lines (Chatterjee and Harris, 1990; I. Hickson, unpublished) were kindly provided by Dr. I. Hickson, University of Newcastle upon Tyne.

Cell line	Pathology/Source
A549	Bronchio-alveolar carcinoma American Type Culture Collection, USA
Calu-3	Adenocarcinoma American Type Culture Collection, USA
L-DAN	Squamous carcinoma Medical Oncology, Glasgow, UK
NCI-H23	Adenocarcinoma National Cancer Institute, USA
NCI-H125	Adenocarcinoma National Cancer Institute, USA
SK-MES-1	Squamous carcinoma American Type Culture Collection, USA
WIL	Adenocarcinoma Ludwig Institute, Sutton, UK

Table 1
Characteristics of NSCLC cell lines

Adapted from Merry et al., 1987

CHO-K1-BG marked line following transfection of parent line (CHO-K1) with ß-gal (section 4.3.4.).

4. Virus producer lines.

These lines were kindly provided by Dr. R. Brown, Glasgow. They comprised G4-12.2-YZ (Price, Turner and Cepko, 1987) and PZ-NC1 (Brown and Mee, unpublished).

5. Rat liver epithelial cell line (BRL).

This cell line was kindly provided by Dr. J.D. Pitts, Glasgow .

2.1.8. Vectors for DNA transfection

These recombinant vectors were kindly provided by by Dr. R. Brown, Glasgow. They comprised BAG (Price, Turner and Cepko, 1987) and $pLGV_1$ (Debenham and Thacker, unpublished). Both contain the ß-galactosidase gene, together with neo, which codes for resistance to the aminoglycoside G418, to allow selection of the marked line.

2.1.9. Solutions

1. Formol saline

The solution comprised 0.08M NaCl, 0.2M Na_2SO_4 in 4% w/v formaldehyde in water.

2. ß-galactosidase staining solution

This solution comprised 1mg/ml X-gal, 5μM potassium ferricyanide, 5μM potassium ferrocyanide and 2mM $MgCl_2$ in PBS.

2.2. Tissue culture methods

All cell lines were maintained in monolayer culture at 37^{o}C. Stock cultures were maintained in exponential growth in plastic tissue culture flasks. The growth medium was replenished every 3-4 days and the cells were subcultured every 5-10 days depending on growth rate by trypsinising (section 2.2.3.) the monolayers to yield a single cell suspension and transferring an aliquot of this suspension to a new flask containing fresh growth medium. After 3 months of continuous growth, cells were discarded and culture was restarted from cells stored in liquid nitrogen. Cell lines were periodically monitored for mycoplasma infection By staining with Hoescht 33258 stain and were consistently negative.

2.2.1. NSCLC lines

NCI-H23 was grown in RPMI 1640 medium, supplemented by 2mM glutamine, 1M pyruvate, non-essential amino acids, Na bicarbonate and 10% foetal calf serum. All other cell lines were grown in Hams F10 medium supplemented by 2mM glutamine, 1M pyruvate, 20mM Hepes buffer, non-essential amino acids, Na bicarbonate and 10% foetal calf serum.

Cells were grown in air in routine culture but for nucleotide transfer and clonogenic assay experiments they were transferred to a humidified atmosphere of 5%CO_2/95%air, and for MTT assays to an atmosphere of 2%CO_2/98%air.

2.2.2. Murine astrocytoma and CHO lines

All lines were grown in Hams F10 medium supplemented by 2mM glutamine, 1M pyruvate, 20mM Hepes buffer, non-essential amino acids, Na bicarbonate and 10% foetal calf serum in a humidified atmosphere of 5%CO_2/95%air.

2.2.3. Trypsinisation

Following removal of growth medium by suction, 0.25% trypsin solution was added (3ml to a 25cm^2 flask; 5ml to a 75cm^2 flask) for 30 seconds and then removed by suction. The flask was then incubated at 37^oC until the cells were seen to round up, and detach from the surface of the dish. Fresh medium was then added and the culture dispersed into a single cell suspension by repeated pipetting. The cell density of the suspension could be assessed by measurement of cell number on an automatic counter (model "Industrial D", Coulter Electronics Ltd., Luton, UK).

2.2.4. Preparation of base-coated flasks

1g agar was added to 20ml distilled H_2O in a glass universal container and boiled until agar was completely dissolved. This solution was then added to 60ml growth medium (preheated to 37^{o}C) and gently mixed. 5ml aliquots were immediately pipetted to cover the base of 25cm^2 flasks. On cooling at room temperature, the agar would set in approximately 5 minutes.

2.2.5. Preparation of spheroids

Stock cultures growing as monolayer were trypsinised and counted as above. 5 x 10^5 cells were added to each base-coated flask (BCF) in a total volume of 5ml medium and incubated in a humidified atmosphere of 5% CO_2 / 95% air at 37^{o}C. Within 2-3 days small floating aggregations of cells were visible which grew steadily to form spheroids. For most experiments 6-7 day old spheroids were used. There was often considerable variation in size, shape and compactness of the spheroids at this stage , but with practise and the aid of a dissecting microscope spheroids of reasonably uniform diameter of 200-300nm could be selected by eye and picked off from the dish with a Pasteur pipette.

2.2.6. Autoradiography

5ml Ilford L4 emulsion gel was melted in a waterbath at 47°C and diluted to 15ml with distilled H_2O. Microscope slides mounted with coverslips were dipped in the emulsion, drained to remove excess emulsion and dried in a stream of cold air. The slides were placed in a light-tight box and stored at 4°C for the appropriate period of exposure. They were then immersed in D19 developer solution for 5 minutes, washed briefly in H_2O and fixed in Hypam for 4 minutes. After washing in running tap water for 5 minutes, the slides were stained in 5% Giemsa for 1 minute, then washed again with tap water and finally with distilled H_2O. They were dried in air and glass coverslips were mounted with DePeX mountant.

2.2.7. Nucleotide transfer

This technique is a modification of the method originally described by Pitts and Simms (1977) and involves measurement of the transfer of ^3H uridine nucleotides from labelled donor cells to unlabelled recipient cells. For each cell type, three 35mm petri dishes were prepared. Two dishes contained 4 sterile 13mm diameter plastic coverslips (recipient dishes) whilst one was empty (donor dish). Cells were plated (10^5 or 2 x 10^5 cells/dish) in 2ml medium into each dish. After 24 hours incubation the donor cells were labelled with 20μCi ^3H uridine for 2 hours and then washed 4 times with medium

to remove excess ^{3}H uridine. They were then trypsinised and resuspended in 1ml medium and 400μl aliquots were added to each of the recipient dishes. One of the resulting mixed dishes was incubated for 1.5 hours and the other for 3 hours. The medium was then removed, the dishes were washed 4 times in PBS, once in formol-saline and then fixed in formol-saline for 30 minutes. The coverslips were removed, placed in racks, washed twice in ice-cold 5% TCA, twice in ice-cold H_2O and rinsed in ethanol. Once dry, the coverslips were mounted, cells uppermost, on microscope slides with DePeX and processed for autoradiography (section 2.2.6.). After development and staining a second coverslip was mounted on top with DePeX.

For quantitative analysis of the extent of incorporation of transferred labelled nucleotides, the coverslip was randomly scanned and the number of autoradiographic grains were counted over 30 consecutively observed recipient cells in contact with donor cells (touching cells) and over 30 recipient cells clearly separated from donor cells (background cells). The microscopical images from a Leitz Orthoplan microscope were displayed on a television monitor and grain counts over the selected cells obtained using a Micromeasurements Ltd System III image analyser.

2.3. NSCLC chemosensitivity assays

2.3.1. Clonogenic assay: monolayer

2.3.1.1. 24 hour drug exposure

Exponentially growing cells were trypsinised and resuspended in medium to a concentration of 5 x 10^4 cells/ml. 5 x10^3 cells/well (100μl) were seeded into a 96 well microtitration plate and incubated overnight at 37^{o}C. The medium was then removed and replaced with fresh medium containing serial dilutions of adriamycin. For each cell type there were two control wells without drug. After incubation for 24 hours the medium and drug were removed, the wells were washed twice with 100μl medium and 50μl/well trypsin was added. After 10 minutes a further 150μl/well of medium was added to neutralise the trypsin and the cells were separated to a single cell suspension by repeated pipetting. 20μl aliquots from each well were then added to 25cm^2 flasks, each containing 5ml medium. After incubation for 11-28 days depending on the growth rate of the cell line the resulting colonies were fixed in methanol and stained with 10% Giemsa. Colonies > 50 cells were counted and expressed as a percentage of control. The concentration of drug which reduced the number of surviving colonies to 50% of control (IC_{50}) was determined graphically.

2.3.1.2. 72 hour drug exposure

The method was identical to that above with the exceptions that NCI-H23 were seeded at 2 x 10^3 cells/well and L-DAN at 10^3 cells/well. Triplicate wells were prepared at each drug concentration. The duration of drug exposure was 72 hours with replenishment of medium and drug at 24 and 48 hours.

2.3.2. MTT assay

Exponentially growing cells were trypsinised and resuspended in medium. 10^3 cells/well in 200μl medium were seeded into 96 well microtitration plates (increased to 2.5 x 10^3 and 5 x 10^3 cells/well for NCI-H125 and Calu-3 respectively in view of their slower growth rate). The plates were then incubated at 37^0C for 3 days.

On the fourth day the medium was removed and replaced with fresh medium containing serial adriamycin dilutions. There was a control column without drug at each end of the plate, whilst the first and last columns contained medium alone and were used to blank the plate reader. All drug concentrations and standards were tested on 8 replicate wells on each plate. After 24 hours incubation the medium and drug were removed and replaced with fresh medium. the cells were also fed with fresh medium on each of the next two days.

On the fourth day following drug exposure the cells were again fed with fresh medium, on this occasion with the addition of 10mmol hepes buffer. 50μl/well of MTT was then added (4.5mg/ml of a solution prepared in PBS), the plates were wrapped in tin foil to protect them from light and they were incubated for a further 4 hours. The medium was then removed and the MTT formazan crystals dissolved in 200μl dimethyl sulphonate (DMSO). 25μl of 0.1M tris buffer was added to each well and the plates were read on an ELISA plate reader at an absorbance of 570nm. The concentration of drug which produced half the absorbance of the control wells (IC_{50}) was determined graphically for each cell line.

2.3.3. Clonogenic assay: spheroid

In view of their differing growth characteristics, in order to produce speroids of equivalent size 5-6 day old L-DAN spheroids and 6-8 day old SK-MES-1 spheroids were used in assays.

1.25ml medium containing serial adriamycin dilutions was added to 25cm^2 base-coated flasks (BCF) containing 5ml medium and spheroids. Medium alone was added to a control flask. Following 24 hours incubation at 37^oC the medium and spheroids from each flask were transferred by pipette to 60 mm diameter petri dishes. Using a dissecting microscope 10 spheroids of 200-300nm diameter were selected from each dish by eye, picked off with a

pasteur pipette and transferred to one well of a 24 well microtitration dish. 0.5ml of 0.25% trypsin solution was added to each well and the dish was incubated at 37°C for 10 minutes. The spheroids were disaggregated to a single cell suspension by repeated pipetting and 1.5ml of medium added to each well to neutralise the trypsin. 200µl aliquots of this suspension were then added in triplicate to new 25cm^2 flasks containing 4ml medium and incubated at 37°C. The resulting colonies were fixed in methanol and stained with 10% Giemsa. Colonies > 50 cells were counted and expressed as a percentage of control. The IC_{50} was calculated as before.

2.4. Murine astrocytoma cell lines

2.4.1. Clonogenic assay

Exponentially growing cells were trypsinised and resuspended in medium. 2 x 10^4 or 10^5 cells/well in 2ml medium were added to a 24 well microtitration plate. After 24 hours incubation at 37°C, the medium was removed and and replaced with fresh medium containing dilutions. One set of wells contained medium alone and acted as control.

Following a further 24 hours incubation, the medium and drug were removed and 200µl 0.25% trypsin solution was added to each well. After 10 minutes incubation 1.8ml/well of medium was added to neutralise the trypsin

and the cells separated to a single cell suspension by repeated pipetting. 400µl aliquots from each well were then counted on a Coulter counter and, after dilution in medium, 5 x 10^2 cells/well were plated into triplicate 60mm Petri dishes each containing 4ml medium.

The dishes were incubated at 37°C for 8 days, fixed in methanol and then stained with 10% Giemsa. Colonies > 50 cells were counted and expressed as a percentage of control.

2.4.2. Genetic marking with ß-galactosidase

Exponentially growing recipient lines were trypinised and resuspended in medium. For each cell line, 5 x 10^5 cells were plated into each of four 75cm^2 flasks in 15ml medium. Concurrently the medium was removed from a 75cm^2 flask containing a subconfluent monolayer of virus producer cells and replaced with 15ml fresh medium. After 24 hours incubation at 37°C medium was removed from both virus producer and recipient flasks and a mixture of 470µl fresh culture medium, 30µl of 0.8mg/ml solution of polybrene in PBS and 500µl medium from virus producer cell flasks (after passing through a 0.2µm filter) were added to each recipient flask. After incubation at 37°C for two hours a further 6ml of non selective medium was added to each flask.

After 72 hours incubation and growth, medium was removed and cells were trypinised and resuspended in medium. Selection of infected cells was commenced by plating 5 x 10^5 cells from each flask into each of two 10cm Petri dishes containing 15ml medium with 1mg/ml G418. The viability of cells was also checked by plating 2 x 10^2 cells into 10cm dishes containing 15ml non selective medium. The plates were incubated at 37^{o}C for 2-3 weeks with weekly replenishment of medium and G418 to allow growth of colonies of infected cells.

2.5. CHO cell lines

2.5.1 Clonogenic assay

Exponentially growing cells were trypsinised and resuspended in medium to a concentration of 5 x 10^4 cells/ml. 5 x 10^3 cells (100μl) were then added to each well of a 96 well microtitration plate. After overnight incubation at 37^{o}C the medium was removed and replaced with fresh medium containing serial adriamycin dilutions in triplicate. For each cell type there were also triplicate control wells without drug. The plates were incubated for 24 hours then the medium was removed and 50μl/well of trypsin added. After a further 10 minutes incubation 150μl medium was added to neutralise the trypsin and the cells were separated to a single cell suspension by repeated pipetting. 20μl aliquots from each well were then added to 25cm^2 flasks, each

containing 4ml medium. The flasks were incubated for 7 days at 37°C, fixed in methanol and stained with 10% Giemsa. Colonies > 50 cells were counted and expressed as a percentage of control. The IC_{50} was calculated as before.

2.5.2. Growth rate

Exponentally growing monolayers were trypsinised and resuspended in medium. For each cell type 10^4 cells were plated into each of 14 replicate 35mm diameter Petri dishes in a total volume of 3ml medium/dish and incubated at 37°C to allow growth of cells. The total cell number per dish was estimated at regular intervals by trypsinisation of paired dishes at each time point and counting the resulting single cell suspension on a coulter counter. After the third day of growth the medium was replenished every 24 hours. The doubling time was calculated as the time taken for the cell number to increase two-fold at maximum growth rate.

2.5.3. ^3H thymidine incorporation: coculture of CHO-K1-BG and Adr^r

Exponentially growing cells were trypsinised and resuspended in medium. They were then seeded into 35mm diameter Petri dishes, each containing 3 x 13mm sterile glass coverslips. Three sets of dishes were prepared: one containing Adr^r cells alone, one with CHO-K1 cells

alone and one with a 50:50 mixture of the two cell types. Cell density in a series of experiments varied from 10^5 to 10^6 cells per dish in 2ml medium. Following 2 hours incubation at 37°C to allow attachment of the cells to the dish, 1ml medium containing serial dilutions of adriamycin at 3 times the final concentration was added. A set of control dishes without drug were included. After a further 24 hours incubation the drug and medium were removed and replaced with fresh medium.

The dishes were labelled for 24 hours with ^3H thymidine (200μCi/ml in PBS, 50μl/dish) either concurrently with the period of drug exposure on the first day of the assay, or alternatively, following a further period of incubation, on either the second, third or fourth day of the assay. Following labelling the medium was removed, the dishes washed twice with PBS, once with formol-saline and then fixed in formol-saline for 30 minutes. The coverslips were then transferred to racks, washed twice with ice-cold 5% TCA, twice with ice-cold H_2O and rinsed in ethanol. The coverslips were then used either for autoradiography or scintillation counting (see below).

2.5.3.1. Autoradiography

One of the coverslips from each dish was mounted (cells uppermost) on microscope slides with DePeX and processed for autoradiography (section 2.2.6.). After 1-2 days exposure the slides were developed, fixed and stained

with 10% Giemsa, and a second coverslip mounted on top with DePeX. The coverslips were examined microscopically. 500 cells consecutively observed on randomly scanning the coverslip were studied and the number of cells exhibiting photographic grains over their nucleus indicative of incorporation of label were counted. The labelling index (LI) was calculated as follows:

$$LI = \frac{\text{number of labelled cells} \times 100}{\text{total number of labelled and unlabelled cells}}$$

2.5.3.2. Scintillation counting

Coverslips from each dish, selected as having the most even cell distribution, were added individually to scintillation vials each containing 5ml scintillant and counted for 1 minute on a liquid scintillation counter (model 1217 Rackbeta, LKB Wallac). The counts for each coverslip were expressed as a percentage of control for each set of dishes, Adr^r alone, CHO-K1 alone and mixed, and plotted against adriamycin concentration. In the majority of experiments there were two coverslips at each drug concentration and the mean of the two counts was plotted.

In later studies, to avoid any variability caused by uneven distribution of cells between dish and coverslips, and in order that the incorporation of label over the

entire dish could be assessed, the cells were seeded into dishes without coverslips. The dose of label on this occasion was 50μl of 40μCi/ml ^{3}H thymidine in PBS. Following TCA extraction the cells were solublised in 2ml 0.1M NaOH for 15 minutes, then acidified with 0.3ml 1M HCl, added to scintillation vials with 5ml scintillant and counted as before.

2.5.4. Genetic marking of cells with ß-galactosidase

2.5.4.1. DNA transfection

Exponentially growing recipient cells were trypsinised and resuspended in medium. For each cell line 10^6 cells were added to each of two $75cm^2$ flasks in 15ml medium. After incubation for 20 hours the medium was removed and replaced with fresh medium.

5 μg of plasmid DNA and 15μg of carrier DNA (55μl volume) were added to 380μl distilled H_2O and 60μl of 2.5M $CaCl_2$ and mixed on ice. This was then added dropwise to 500μl of 2 x Hepes buffered saline in a Falcon 2058 tube and mixed by air bubbling. The mixture was left for 60min at room temperature, then 500μl was added to each recipient cell flask and mixed gently to distribute evenly.

After incubation at $37^{o}C$ for 72 hours, the medium was removed from the flasks, and the cells trypsinised and resuspended in medium. Selection of transfected cells

was commenced by plating 5 x 10^5 cells from each flask into each of two 10cm Petri dishes containing 15ml medium with 1mg/ml G418. The viability of cells was also checked by plating 2 x 10^2 cells into 10cm dishes containing 15ml non selective medium. The dishes were incubated at 37°C for 2-3 weeks with weekly replenishment of medium and G418 to allow growth of colonies of transfected cells.

2.5.4.2. Retroviral Infection.

Exponentially growing recipient lines were trypinised and resuspended in medium. For each cell line, 5 x 10^5 cells were plated into each of four $75cm^2$ flasks in 15ml medium. Concurrently the medium was removed from a $75cm^2$ flask containing a subconfluent monolayer of virus producer cells and replaced with 15ml fresh medium. After 24 hours incubation at 37°C medium was removed from both virus producer and recipient flasks and a mixture of 470μl fresh culture medium, 30μl of 0.8mg/ml solution of polybrene in PBS and 500μl medium from virus producer cell flasks (after passing through a 0.2μm filter) were added to each recipient flask. After incubation at 37°C for two hours a further 6ml of non selective medium was added to each flask.

After 72 hours incubation and growth, medium was removed and cells were trypinised and resuspended in medium. Selection of infected cells was commenced by plating 5 x

10^5 cells from each flask into each of two 10cm Petri dishes containing 15ml medium with 1mg/ml G418. The viability of cells was also checked by plating 2 x 10^2 cells into 10cm dishes containing 15ml non selective medium. The plates were incubated at 37°C for 2-3 weeks with weekly replenishment of medium and G418 to allow growth of colonies of infected cells.

2.5.4.3. G418 Titration

For each cell line, exponentially growing cells were trypinised and resuspended in medium and 2 x 10^2 cells plated into 25cm^2 flasks containing 5ml medium with serial dilutions of G418. One flask contained medium alone and acted as control. Flasks were incubated at 37°C for 7-10 days, then medium removed, fixed in methanol, stained with 10% Giemsa and colonies > 50 cells counted.

2.5.4.4. ß-galactosidase staining

Medium was removed from monolayer cultures and dishes rinsed with PBS. Cells were then fixed for 10 mins at 4°C in a solution of 2% formaldehyde and 0.2% glutaraldehyde in PBS. Fixative was then removed, cells were rinsed in PBS and staining solution (section 2.1.9.) added (3ml for 60mm dish or 25cm^2 flask). Dishes were incubated overnight at 37°C, staining solution removed, rinsed in PBS then in distilled H_2O and dried in air.

2.5.5. CHO monolayer coculture

Exponentially growing cells were trypsinised and resuspended in medium. 10^5 cells/well were added to a 24 well microtitration plate in 2ml medium. Three sets of wells were prepared: one containing each cell line on its own and one set containing a 50:50 mixture of the appropriate two cell types (CHO-K1-BG and Adr^r for adriamycin and mitozantrone assays; CHO-K1-BG and ADR-6 for vincristine assays).

The plate was incubated at 37°C for 24 hours, then the medium was removed and replaced with fresh medium containing serial dilutions of drug. For each set of wells, one well contained medium alone and acted as control. Following a further 24 hours incubation, the medium and drug were removed and 200µl 0.25% trypsin solution was added to each well. After 10 minutes incubation 1.8ml/well of medium was added to neutralise the trypsin and the cells separated to a single cell suspension by repeated pipetting. 400µl aliquots from each well were then counted on a Coulter counter and, after dilution in medium, 5 x 10^2 cells/well were plated into triplicate 60mm petri dishes each containing 4ml medium.

The dishes were incubated at 37°C for 7 days and then stained for ß-galactosidase (section 2.5.4.4.). Colonies > 50 cells were counted and expressed as a percentage of

control. Marked CHO-K1-BG cells could be readily identified by their blue staining and in those dishes derived from mixed culture the number of marked and unstained colonies could be counted separately. In dishes derived from CHO-K1-BG alone, the number of colonies showing positive staining for ß-gal as a fraction of the total number of colonies could also be calculated.

2.5.6. CHO spheroid coculture

2.5.6.1. Assessment of optimum initial ratio of CHO-K1-BG and Adr^r to produce mixed spheroids

Base coated flasks were prepared with 5 x 10^5 cells as before to initiate spheroid formation. Duplicate flasks were prepared containing CHO-K1-BG cells alone and with ratios of CHO-K1-BG : Adr^r of 1:1, 1:4, 1:8 and 1:12 and incubated at 37°C for 6 days to allow spheroid formation and growth.

The medium and spheroids from each flask were then transferred to 60mm Petri dishes. Under a dissecting microscope 10 spheroids of 200-300nm diameter were selected from each dish by eye, picked off with a Pasteur pipette and transferred to one well of a 24 well microtitration plate. 0.5ml of 0.25% trypsin solution was then added to each well and the plate was incubated at 37°C for 10 minutes. The spheroids were disaggregated

to a single cell suspension by repeated pipetting and 1.5ml of medium added to each well to neutralise the trypsin. 50μl aliquots of this suspension were then added in triplicate to new 60mm Petri dishes each containing 4ml medium.

After incubation at 37^{o}C for 7 days the cells were fixed and stained for ß-gal (section 2.4.4.4.) and the number of colonies > 50 cells counted. Those colonies staining positively for ß-gal were counted separately from the unstained colonies.

2.5.6.2. Confirmation of heterogeneity within individual spheroids

Six day old spheroids initiated from a 1:5 ratio of CHO-K1-BG to Adr^{r} cells were picked off individually from a petri dish with a Pasteur pipette and each transferred to a single well of a 24 well microtitation dish. 0.5ml of 0.25% trypsin solution was then added to each well and the dish was incubated at 37^{o}C for 10 minutes. The spheroids were disaggregated to a single cell suspension by repeated pipetting and 1.5ml of medium added to each well to neutralise the trypsin. 200μl from each well were then added to new 60mm Petri dishes each containing 4ml medium.

After incubation at 37^{o}C for 7 days the dishes were fixed and stained for ß-gal and the number of marked and

unstained colonies > 50 cells counted separately. The proportion of positively staining colonies derived from individual mixed spheroids was therefore assessed.

2.5.6.3. CHO spheroid coculture

Three sets of base coated flasks were seeded with 5 x 10^5 cells. Two of the sets contained CHO-K1-BG or Adr^r cells on their own, and the other contained a 1:5 mixture of CHO-K1-BG and Adr^r. Following incubation of the flasks at 37°C for 5 days to allow spheroid initiation and growth, 1.25ml medium containing serial adriamycin dilutions was added to each flask, whilst 1.25 ml medium alone was added to control flasks. The flasks were incubated at 37°C for 24 hours and then medium and spheroids from each flask were transferred to 60mm Petri dishes. Under a dissecting microscope 10 spheroids of 200-300nm diameter were selected from each dish by eye, picked off with a Pasteur pipette and transferred to one well of a 24 well microtitration dish.

0.5ml of 0.25% trypsin solution was then added to each well and the dish was incubated at 37°C for 10 minutes. The spheroids were disaggregated to a single cell suspension by repeated pipetting and 1.5ml of medium added to each well to neutralise the trypsin. 20μl aliquots of this suspension were then added in triplicate to new 60mm petri dishes each containing 4ml medium and incubated at 37°C for 7 days. The dishes were then fixed

and stained for ß-gal (section 2.5.4.4.) and the number of colonies > 50 cells were counted and expressed as a percentage of control. In those dishes derived from mixed spheroids, the number of colonies staining positively for ß-gal were counted separately from the unstained colonies.

2.6. Microinjection of cells

2.6.1. Preparation of microelectrodes

Glass capillary tubes of 1.2mm outside diameter (Clark Electromedical Instruments) were pulled into microelectrodes and back filled with 5μl of a 4% aqueous solution of Lucifer Yellow or 1% solution of adriamycin.

2.6.2. Injection procedure

Exponentially growing cells were trypsinised and resuspended in medium. 10^6 cells were seeded into 35mm Petri dishes each containing three 13mm diameter sterile glass coverslips in 2ml medium and incubated at 37^{o}C for 24 hours. For microinjection the coverslips were transferred to a 60mm Petri dish containing 3ml hepes buffered medium. The injection was monitored on a Leitz Orthoplan microscope with U.V. epi-illumination and visible (phase contrast) light sources. A suitable cell was selected and the electrode inserted using a micromanipulator. The cell was injected for one minute

with hyperpolarising current pulses of 500ms duration , 1Hz frequency and up to 10nA amplitude for Lucifer Yellow injections. A positive pressure injection system (Picospritzer II, General Valve Corporation, USA) was employed for adriamycin injections. Dye spread to surrounding cells was monitored during and after injection and after 5 minutes the number of cells in which dye could be observed was recorded as an index of the extent of dye transfer.

2.7. Scrape loading

Exponentially growing BRL cells were trypsinised and resuspended in medium. 3×10^5 cells in 3ml medium were seeded into 60mm Petri dishes each containing six 13mm diameter sterile glass coverslips. After 24 hours incubation the coverslips were rinsed briefly in hepes buffered medium then transferred to dye-containing medium and immediately scored with a scalpel. After one minute the coverslip was removed from the dye-containing medium, washed twice in medium, twice in BSS/hepes and mounted cells downmost on a microscope slide for immediate examination. The dye concentrations used were: 1. 0.5% lucifer yellow; 2. 0.5% lucifer yellow / 0.1% rhodamine dextran; 3. 0.05% adriamycin.

Chapter 3 STUDY OF THE RELATIONSHIP BETWEEN INTERCELLULAR COMMUNICATION AND DRUG RESISTANCE

3.1. Introduction

As discussed in chapter 1, there is evidence that the ability of cells to communicate through gap junctions is associated with resistance to radiation when cells are cultured in a three-dimensional arrangement in tumour spheroids or xenografts, and with resistance to TNF and lymphotoxin in monolayer culture. Studies with spheroids have also shown that three-dimensional culture confers a survival advantage following cytotoxic drug exposure, beyond that which might be explained by penetration limitations or cell kinetic factors. There have, however, been no reports investigating whether gap junctional communication might also contribute to this enhanced resistance.

This study was therefore designed to investigate whether the ability to communicate through gap junctions could be correlated with the extent of tumour cell resistance to conventional cytotoxic drugs. The tumour type selected for this purpose was human lung cancer.

For clinical purposes, human lung cancer is usually considered to consist of two major types: the first comprising those tumours defined on morphological grounds as small cell lung cancer (SCLC), and the second type,

non- small cell lung cancer (NSCLC), which encompasses all other histological categories including squamous carcinoma, adenocarcinoma and large cell or anaplastic carcinoma. The relevance of this classification is shown in the distinct biological and clinical behaviour of the two types.

Although up to 80-90% of cases of SCLC are initially responsive to a number of cytotoxic drugs, including most of those included in the MDR group, acquired resistance develops in the majority of patients, either during the initial phase of chemotherapy or on relapse. Less than 10% of patients with metastatic disease achieve long term disease-free survival and potential cure. In NSCLC in contrast most tumours are inherently drug resistant. The majority of cases are unresponsive to cytotoxic chemotherapy and those responses which are seen are usually partial and short-lived.

The cell lines used to study the relationship between intercellular communication and drug resistance were a panel of seven human NSCLC cell lines previously studied in the Department of Medical Oncology, University of Glasgow (see table 1 for list).

Earlier work with these cell lines (Merry et al., 1987), none of which had been previously exposed to cytotoxic drugs in vitro, had shown this group of lines to exhibit cross resistance to members of the MDR group of drugs

including adriamycin, vincristine and etoposide. Assessment of the chemosensitivity of these cell lines was obtained using an assay based on measurement of cell viability by means of their ability to incorporate ^{3}H-leucine into protein following drug exposure. Their results showed a considerable range in chemosensitivity observed between the different cell lines. This was particularly marked for adriamycin, where there was a 10^{5} fold difference in resistance between the most and least resistant lines (Table 2).

It was hypothesised that if cytotoxic drug resistance was related to the extent of gap junctional communication within a population, then in view of the magnitude of the chemosensitivity differences between the lines, correspondingly substantial differences in communication could be expected. It was proposed, therefore, to investigate the gap junction forming abilities of these cell lines to determine whether this also showed variation and if so, whether such variation might be related to their adriamycin resistance.

3.2. Assessment of cell-cell communication

3.2.1. Transfer of ^{3}H uridine nucleotides

The extent of intercellular communication via gap junctions was assessed by a modification of the

	ID_{50}(nM)		
	Adriamycin	Vincristine	Etoposide
A549	204-225	27-28	3600
Calu-3	2.7	1.6	23
L-DAN	34-70	17	920-1100
NCI-H23	0.002-0.005	1.9	330
NCI-H125	9.0	40	3810
SK-MES-1	48-53	13-22	810-1200
WIL	168	136	700-830

Table 2
NSCLC chemosensitivity: ^{3}H leucine incorporation assay

Adapted from Merry et al., 1987

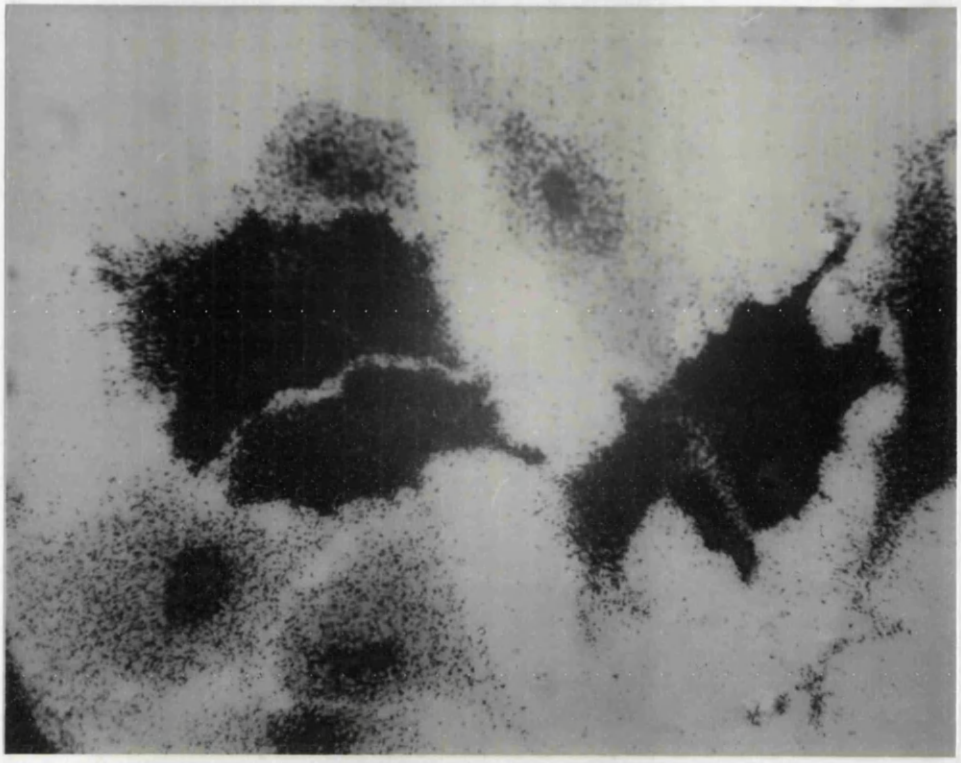
nucleotide transfer technique originally described by Pitts and Simms (1977) (section 2.2.7.). The principle underlying this technique is based upon uptake of ^{3}H uridine by the growing donor cells and its conversion to labelled uridine nucleotides which are then incorporated into RNA. Washing with unlabelled medium removes the remaining free ^{3}H uridine, leaving the labelled nucleotides and RNA. Coculturing donor and recipient cells allows the formation of junctions between the two groups of cells and the labelled uridine nucleotides are small enough to pass between donor and recipient cells in contact. The labelled nucleotides are then incorporated into the recipient cells' RNA and this incorporation can be assessed autoradiographically following TCA extraction to remove remaining nucleotides. If the cells form junctions, transfer of labelled material can be seen between donor and recipient cells in contact, whilst those recipient cells which are not in contact with the donor cell and have therefore been unable to form junctions remain unlabelled (fig.1).

3.2.2. Autoradiography

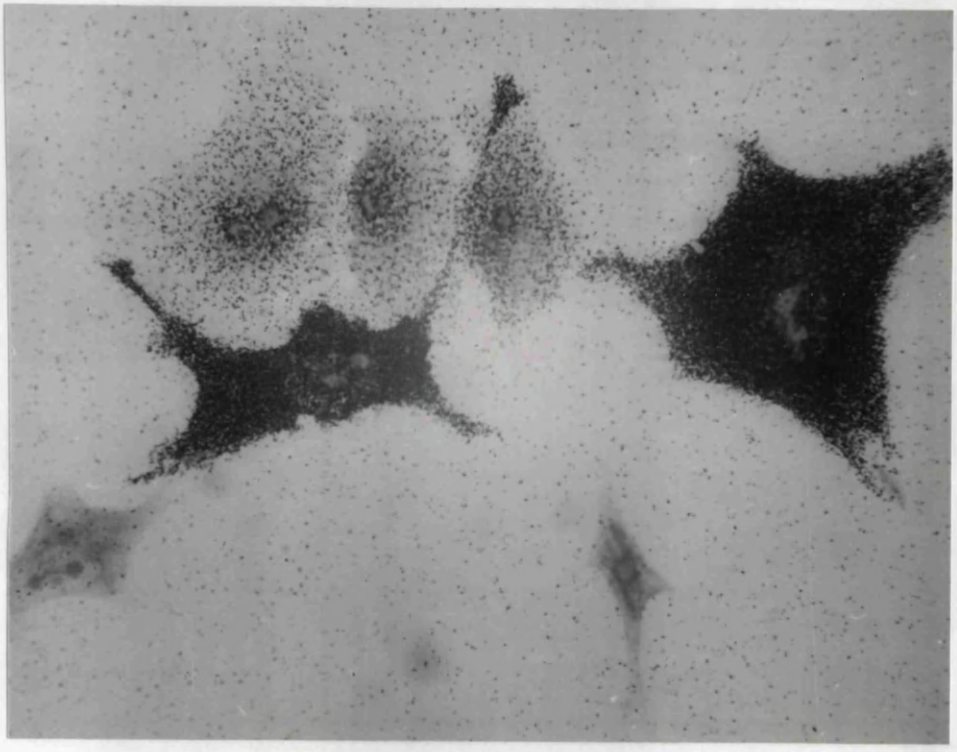
Autoradiographs for each experiment were developed following exposure times of 1-4 days. Thus for each experiment, a series of autoradiographic slides was obtained, each with a different combination of duration of coculture (1.5 or 3 hours) and of exposure. The

Figure 1
Intercellular communication in NSCLC lines: transfer of ^{3}H uridine nucleotides between donor and recipient cells

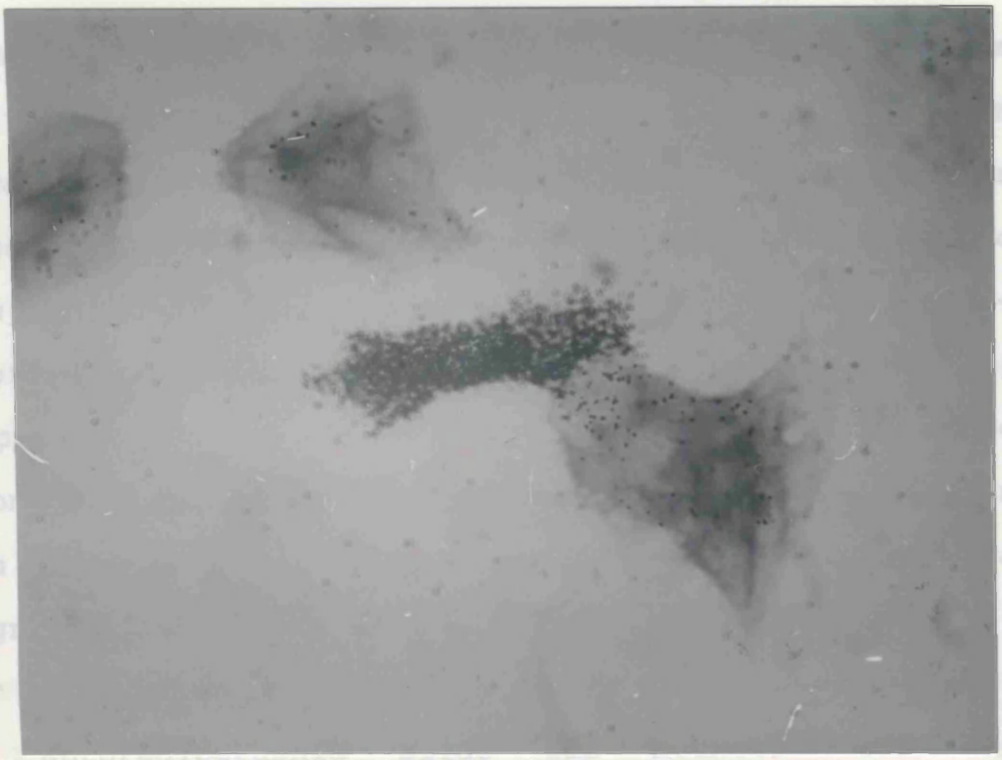
10^{5} cells/dish were seeded into each of two 35mm dishes, one with (recipient dish) and the other without (donor dish) 13mm coverslips . After 24 hours incubation the donor cells were labelled with 20μCi ^{3}H uridine for 2 hours and then washed with medium, trypsinised and resuspended in 1ml medium. A 400μl aliquot was then added to the recipient dish. After 3 hours incubation, the coverslips were washed in PBS, fixed in formol-saline, washed in ice-cold 5% TCA, H_2O and ethanol and processed for autoradiography (section 2.2.6.). (a) A549; (b) L-DAN; (c) Calu-3; (d) WIL.



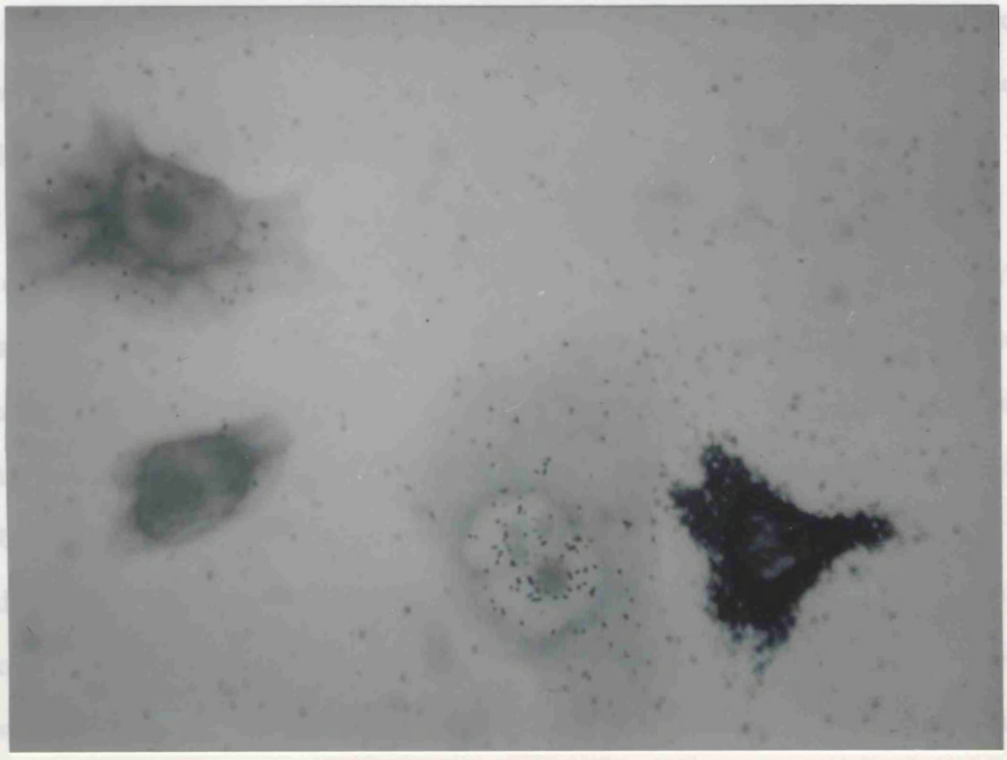
(a) A549



(b) L-DAN



(c) Calu-3



(d) WIL

slides were examined microscopically to determine for each experiment which combination produced optimum autoradiographs for grain counting. The factors influencing this selection were suitable cell density to allow identification of adequate numbers of donor-recipient pairs and of isolated cells on each coverslip; an appropiate grain count over recipient cells (a dense or confluent grain distribution would result in errors in grain counting using the image analysis system); and a background grain count as low as possible (see below).

Each autoradiographic slide was mounted with one coverslip from each of the cell lines used in the experiment so that when the optimum slide was selected for grain counting the exposure and coculture conditions were identical for each cell line. This allowed a valid comparison to be made between the junction forming abilities of the different cell lines.

For each cell line the number of grains over recipient cells in contact with donor cells (touching cells) could be compared to that over those clearly separated from donor cells (background cells). A small background grain count was always present and may result from a combination of the photographic background and medium mediated transfer of label. Increasing the number of washes, either with medium or TCA, employed in the experiment had no discernible effect on the background grain count.

For each of the seven cell lines studied, some increase was observed in the grain counts over those recipient cells in contact with donor cells, in comparison to background cells. This was indicative of intercellular transfer of ^{3}H uridine nucleotides from the donor cells, although the extent of transfer showed considerable variation between the cell lines (figs 1 and 2).

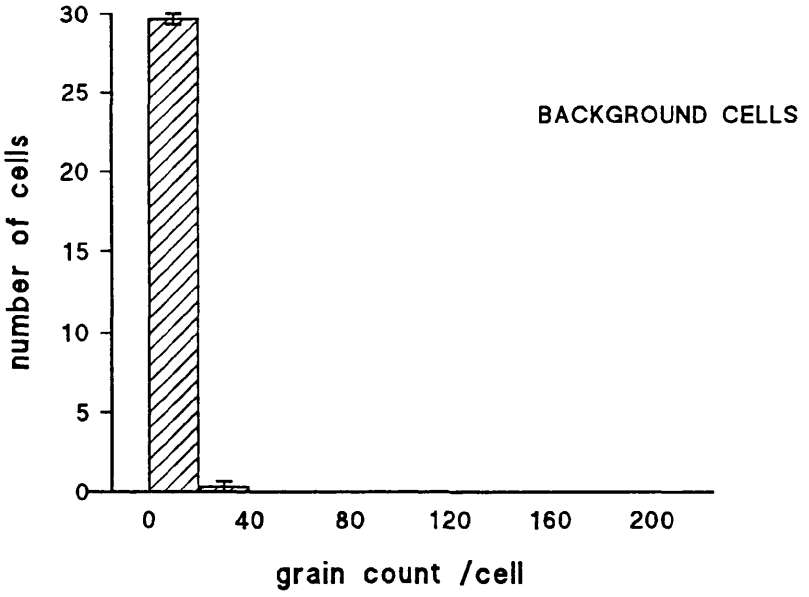
3.2.3. Quantification of gap junctional communication

In order to differentiate between those cell lines which form many junctions and are well coupled and those which form relatively few and are poorly coupled it was necessary to quantify the extent of intercellular transfer. Several indices were examined for this purpose including the ratio of mean grain count over touching cells to that over the background cells (touching/background ratio), and the mean touching cell grain count minus the mean background count.

Although the background grain count was usually of the order of only a few grains per cell, it was apparent that a small change in background, as a result of one of the factors discussed above, would have a disproportionately large influence on the touching/background (T/B) ratio. The results from a series of experiments with the A549 cell line illustrate that such an index would result in an apparent six-fold difference in coupling ability of this cell line between different experiments (table 3).

Figure 2
Intercellular communication in NSCLC lines: grain counts over recipient cells

Method as described for figure 1. For each cell line autoradiographic grains were counted over 30 recipient cells in contact with donor cells (touching cells) and over 30 recipient cells clearly separated from donor cells (background cells). For detail see section 2.2.7. (a) A549; (b) Calu-3; (c) L-DAN; (d) NCI-H23; (e) NCI-H125; (f) SK-MES-1; (g) WIL.


BACKGROUND CELLS
number of cells
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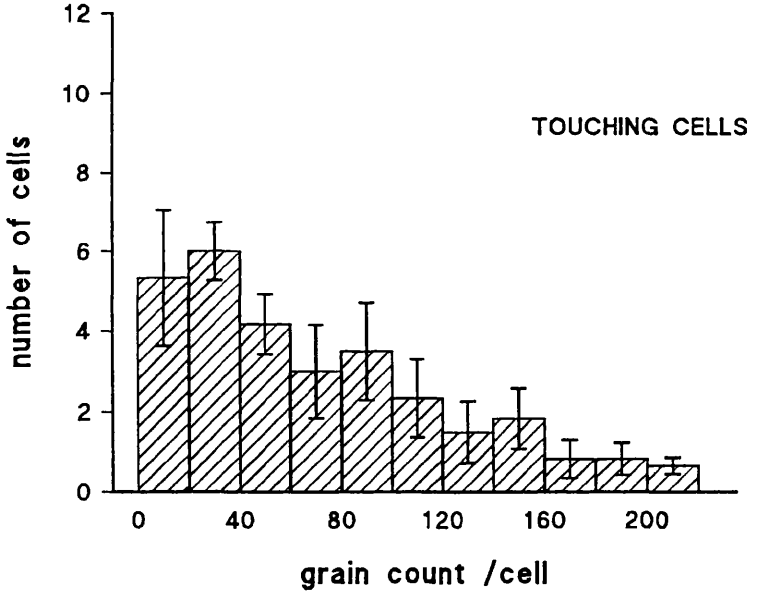
25
20
15
10
5
0
0
40
80
120
160
200
grain count /cell


TOUCHING CELLS
number of cells
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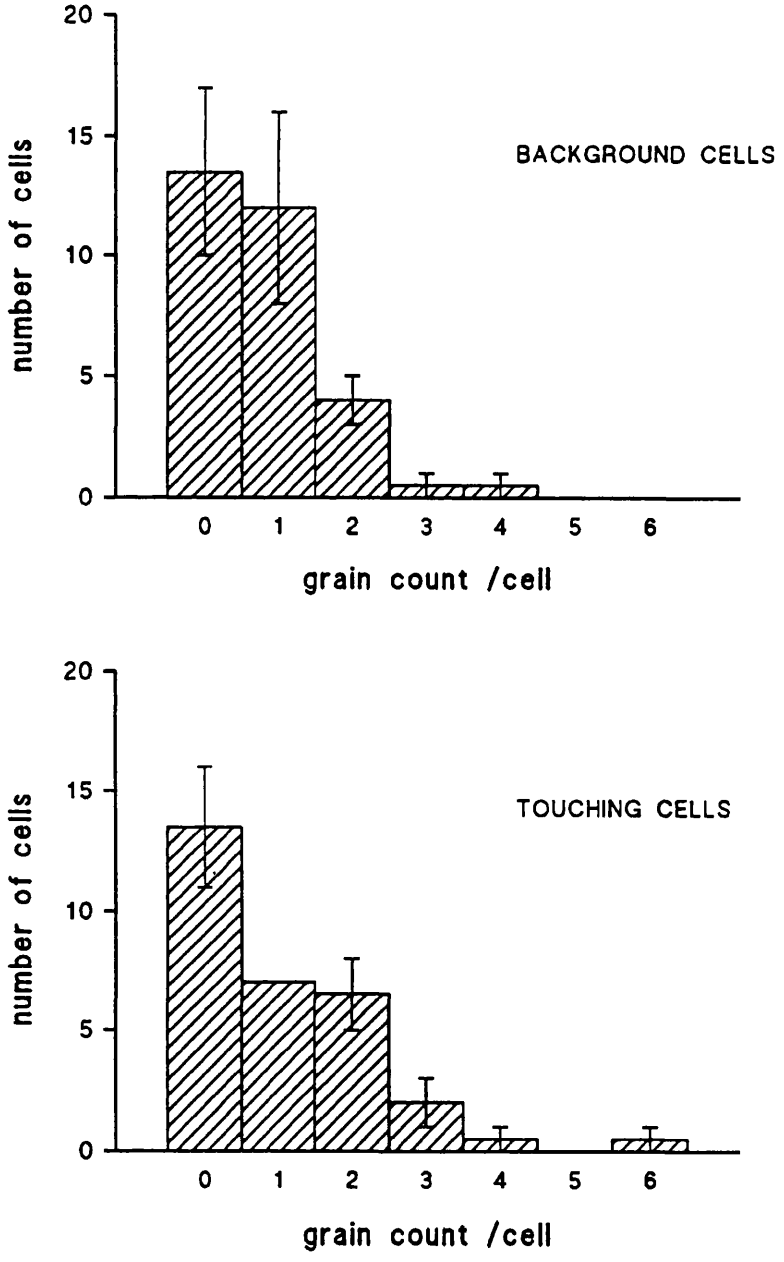
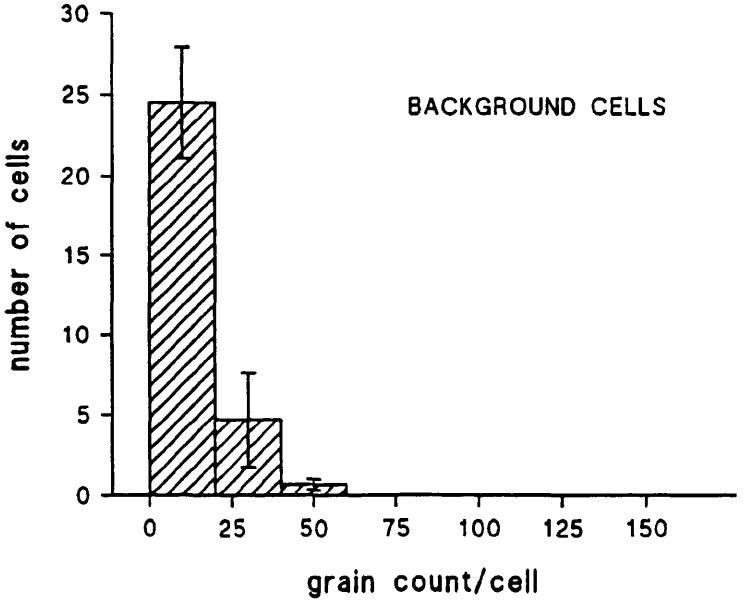
10
8
6
4
2
0
0
40
80
120
160
200
grain count /cell

(a) A549


20
15
10
5
0
number of cells
BACKGROUND CELLS
0 1 2 3 4 5 6
grain count /cell
20
15
10
5
0
number of cells
TOUCHING CELLS
0 1 2 3 4 5 6
grain count /cell

(b) Calu-3


BACKGROUND CELLS
number of cells
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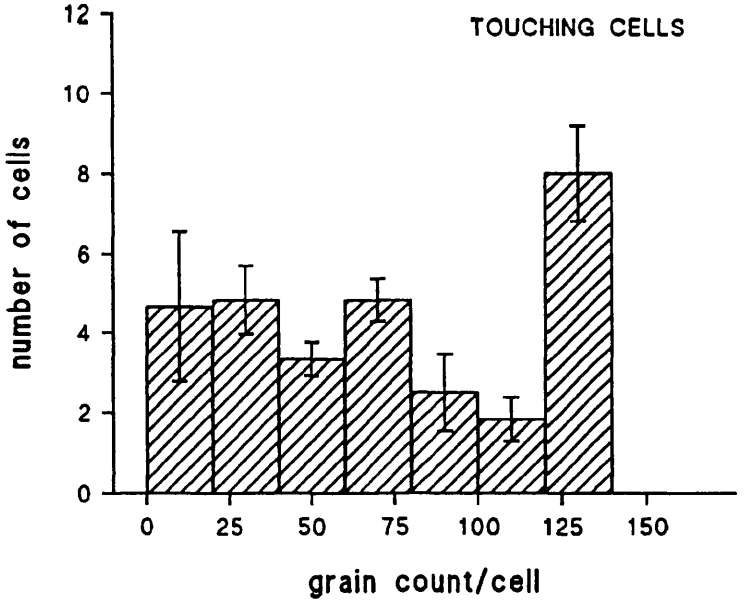
25
20
15
10
5
0
0
25
50
75
100
125
150
grain count/cell


TOUCHING CELLS
number of cells
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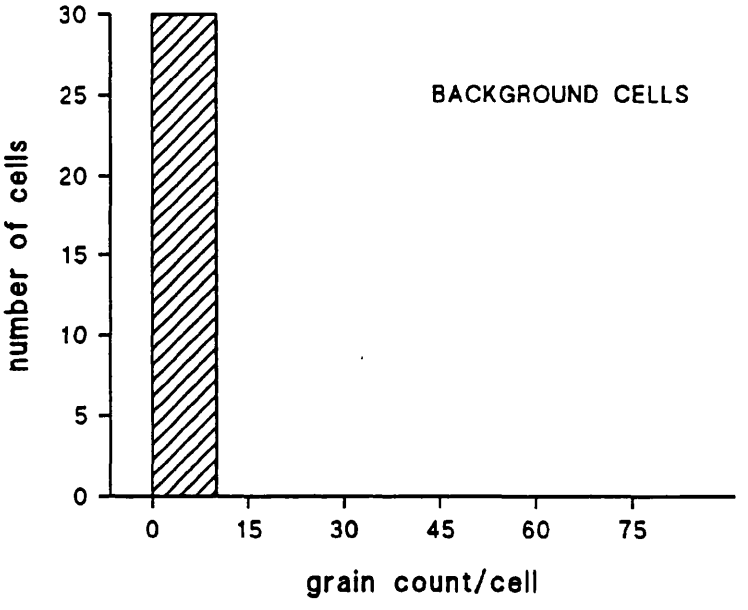
10
8
6
4
2
0
0
25
50
75
100
125
150
grain count/cell

(c) L-DAN


BACKGROUND CELLS
number of cells
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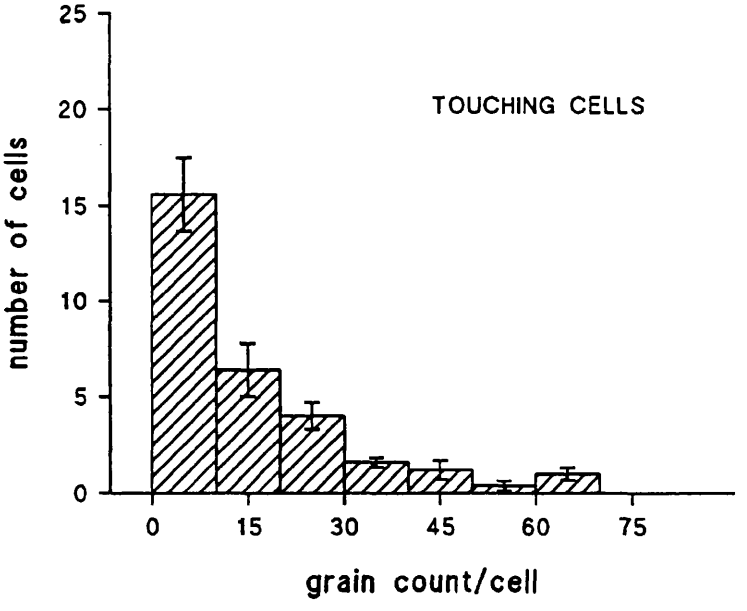
20
15
10
5
0
0
15
30
45
60
75
grain count/cell


TOUCHING CELLS
number of cells
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15
10
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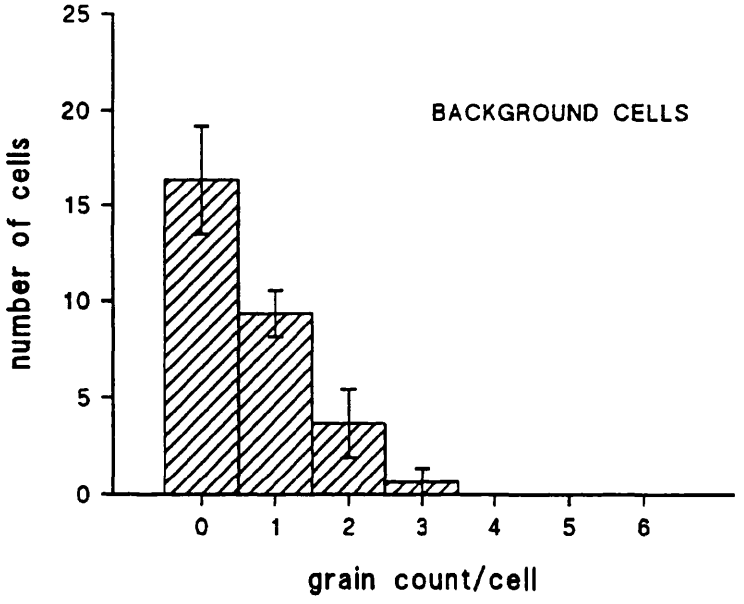
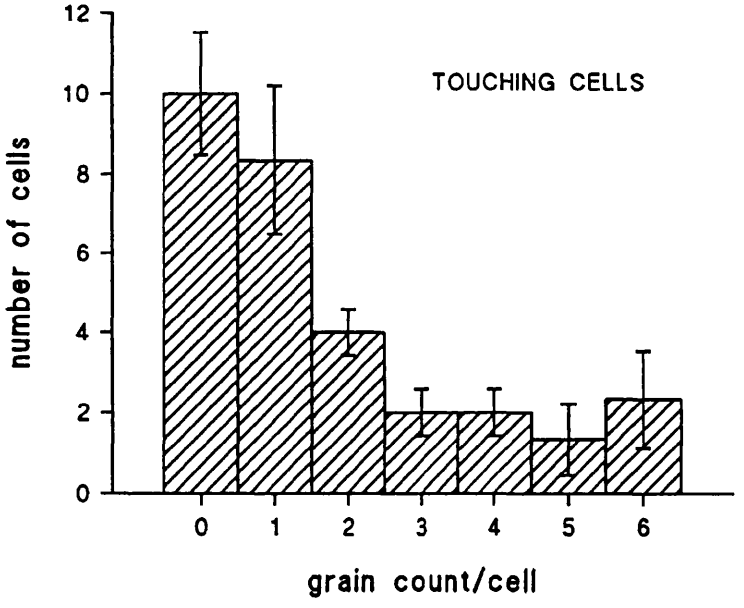
15
30
45
60
75
grain count/cell

(d) NCI-H23


BACKGROUND CELLS
number of cells
25
20
15
10
5
0
0 1 2 3 4 5 6
grain count/cell


TOUCHING CELLS
number of cells
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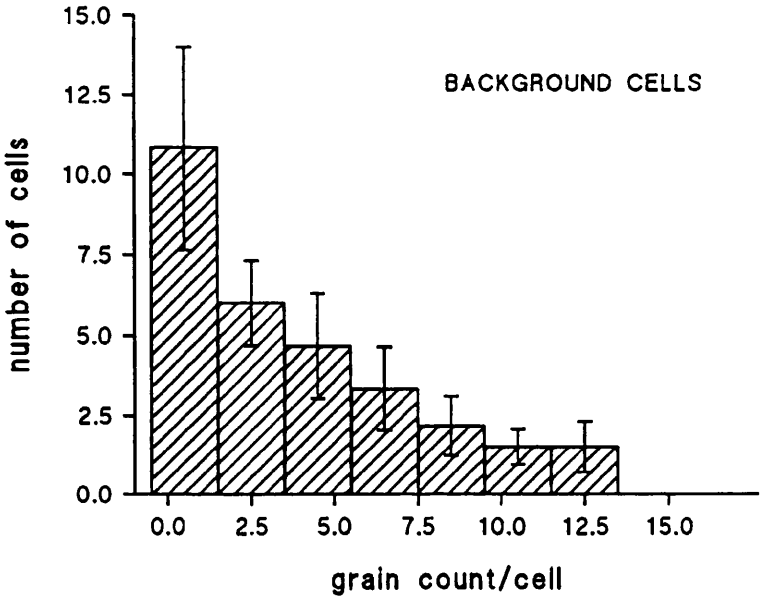
10
8
6
4
2
0
0 1 2 3 4 5 6
grain count/cell

(e) NCI-H125


BACKGROUND CELLS
number of cells
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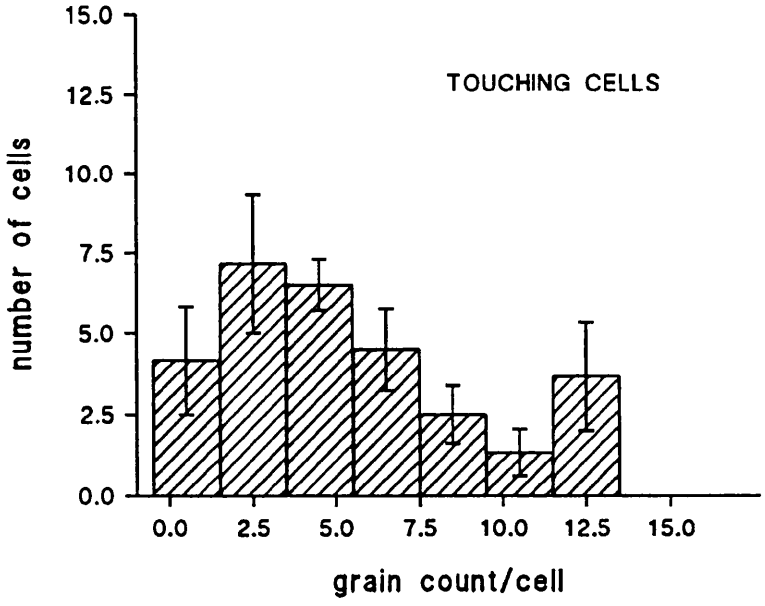
12.5
10.0
7.5
5.0
2.5
0.0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
grain count/cell


TOUCHING CELLS
number of cells
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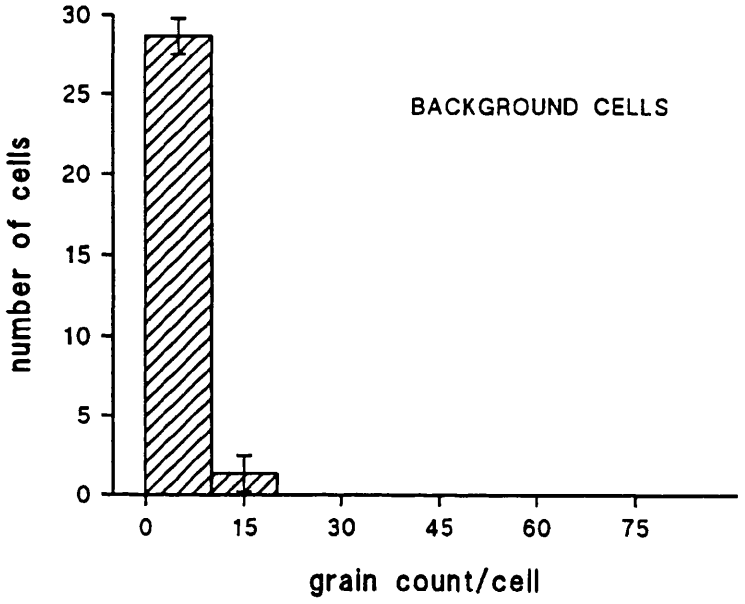
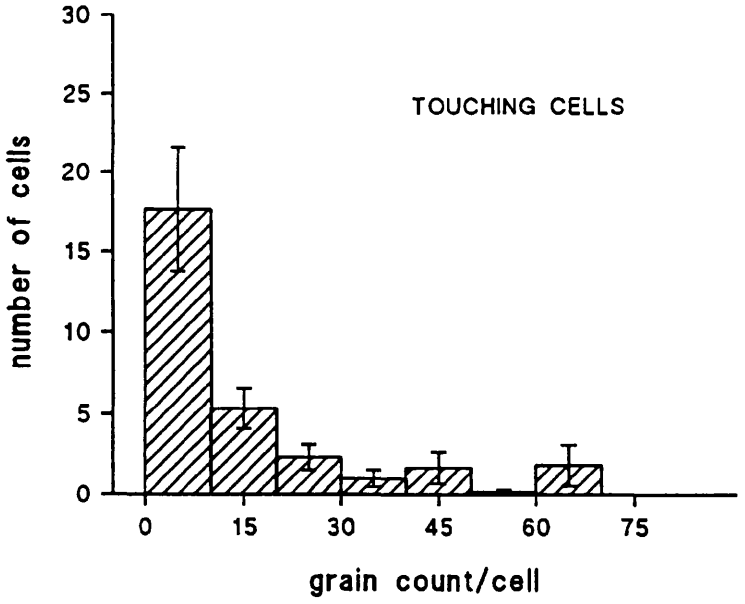
12.5
10.0
7.5
5.0
2.5
0.0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
grain count/cell

(f) SK-MES-1


BACKGROUND CELLS
number of cells
30
25
20
15
10
5
0
0
15
30
45
60
75
grain count/cell


TOUCHING CELLS
number of cells
30
25
20
15
10
5
0
0
15
30
45
60
75
grain count/cell

(g) WIL

Expt.	mean grain count/cell T	B	T/B	T-B	% coupling
1	93	7	13.5	86	87
2	58	5	11.4	53	70
3	76	1.3	58.4	74.7	97
4	44	1	40.3	43	87
5	84	4	23.3	80	90
6	69	5	15.1	64	93

Table 3
Intercellular communication in A549: effect of different indices of coupling

^{3}H uridine nucleotide transfer performed as described in Figs.1 and 2. Results of 6 separate experiments. Mean grain count/cell over touching (T) and background (B) cells. T/B represents ratio of mean grain count over T cells to that over B cells. T-B represents mean grain count over B cells subtracted from mean grain count over T cells. % coupling calculated as percentage of T cells where grain count was greater than mean +3 SD of background grain count.

Although the use of touching minus background (T-B) as an index reduces this variability, the differences in absolute grain count between experiments caused by variation in exposure duration also limit its usefulness.

To rank the cell lines with respect to their junction forming ability the variability in rate of junction formation was therefore utilised. Even in cell lines which exhibit extensive communication, over the time course of the experiment not every donor-recipient pair has formed sufficient gap junctions to show nucleotide transfer and the percentage of donor-recipient pairs which display coupling could therefore be used as an index of the strength of communication.

Coupling was defined as positive when the recipient cell had a grain count above that which could reasonably be attributed to "background factors". A cut-off grain count value of > mean + 3SD of the background grain count was employed. The use of this method avoided the problems associated with the other indices of coupling and removed much of the interexperimental variation caused by extraneous factors such as duration of exposure and variability in background grain count (see table 3).

In a series of experiments, a marked variation in junction forming ability between the different cell lines was observed (table 4). These experiments were performed sequentially over a period of 2-3 months. Not all cell

	% coupling						
Expt.	1	2	3	4	5	6	mean
A549	87	70	97	87	90	93	87
Calu-3					13	3	8
L-DAN	97	83	73	67	80	70	78
NCI-H23		57	67	57	43	67	58
NCI-H125			43		3	30	25
SK-MES-1	0	10	13	3	3	20	8
WIL	63	73	60	27	27	17	44.5

Table 4
Intercellular communication in NSCLC cell lines

^{3}H uridine nucleotide transfer performed as described in Figs. 1 and 2. Results and mean of 6 separate experiments. In some experiments not all cell lines were included.
% coupling calculated as percentage of touching cells where grain count was greater than mean +3 SD of background grain count.

lines were available for initial experiments. One feature of note is the apparent reduction in coupling ability of WIL over the time course of the study. No corresponding reduction was observed in the other cell lines and the observation may represent a genuine phenotypic change in WIL with continued culture, although this phenomenon was not explored further.

This index of coupling also had some limitations. Although the mean percentage coupling values of for example A549 at 87% and SK-MES-1 at 8% show that there is an 11 fold difference in frequency of coupling between the two cell lines, they do not take account of the extent of communication between each donor-recipient pair, which is much greater for A549, as indicated by the considerably higher touching cell grain count observed in A549 compared to SK-MES-1 (see figs 2a and 2f). This suggests that even the considerable differences in coupling frequency which have been obtained with this method may substantially underestimate the functional differences in extent of coupling between the cell lines.

Although the remaining interexperimental differences limited the precision of an exact ranking, several groups could clearly be distinguished. The cell lines could be divided into those with a very high frequency of communication, L-DAN and A549; those with a very low frequency, SK-MES-1, Calu-3 and NCI-H125; and an intermediate group, WIL and NCI-H23.

Therefore in addition to the wide spectrum of adriamycin sensitivity previously reported by Merry and colleagues (table 2), these cell lines also exhibited a marked difference in junction forming ability. However when these sets of data are compared no correlation between the two is observed (Spearman's rank correlation coefficient, r_s= 0.51, $p>0.05$).

3.3. Cytotoxicity assays

3.3.1. Introduction

In order to validate the chemosensitivity data, and in view of the apparent instability of the WIL gap junctional communication phenotype observed above, it was decided to perform further cytotoxicity assays to confirm whether the values obtained from the ^3H leucine incorporation assay could be repeated in this laboratory using different assays of chemosensitivity.

Chemosensitivity of the cell lines was assessed by both clonogenic and MTT assays which have previously been shown to correlate well (Carmichael et al., 1987). As this study was concerned with the possible effects of intercellular communication within a coupled cell population on its response to cytotoxic agents, the methodological differences between clonogenic and MTT assays may be relevant.

In a clonogenic assay the cells are necessarily separated and plated out at low density following the period of drug exposure, thus removing any effect of cell-cell contact from that time. In contrast, the MTT assay allows cells to continue to grow together following drug exposure before the enzymatic reduction of the tetrazolium salt MTT to a coloured formazan product is used as an index of cell number (Cole, 1986). The extended period of culture in the MTT assay might therefore be expected to offer increased opportunity for and may maximise any effects of potential interactions.

3.3.2. Clonogenic and MTT assays: 24 hour drug exposure

For clonogenic assay, survival curves following 24 hour exposure to adriamycin show a marked similarity between the 7 cell lines (fig.3). The mean IC_{50} of 2-3 separate experiments shows only a 3-fold variation between the least and most sensitive cell lines (table 5).

Survival curves derived from the MTT assay also show little difference between cell lines (fig.4) and produce a similar scatter of IC_{50} values (table 5). Although the ID_{50} values for the MTT assay are in general one order of magnitude greater than those obtained by clonogenic assay, this is largely a consequence of the very shallow dose-response curve seen with the MTT assay (Carmichael et al., 1988a), which also accounts for much of the variability in IC_{50} between plates.

Figure 3
NSCLC chemosensitivity: clonogenic assay

For each cell line 5 x10^3 cells/well in 100μl medium were seeded into a 96 well plate, incubated overnight at 37^{o}C, then the medium was replaced with fresh medium containing serial dilutions of adriamycin. After incubation for 24 hours, the wells were washed twice with 100μl medium and 50μl/well trypsin was added. After 10 minutes a further 150μl/well of medium was added and the cells were separated to a single cell suspension. 20μl aliquots from each well were then added to 25cm^2 flasks, incubated for 11-28 days and the resulting colonies were fixed in methanol and stained with 10% Giemsa. Colonies > 50 cells were counted and their survival expressed as a percentage relative to control wells without drug. Each point represents the mean of 2 experiments.

FIGURE 3
NSCLC chemosensitivity: clonogenic assay

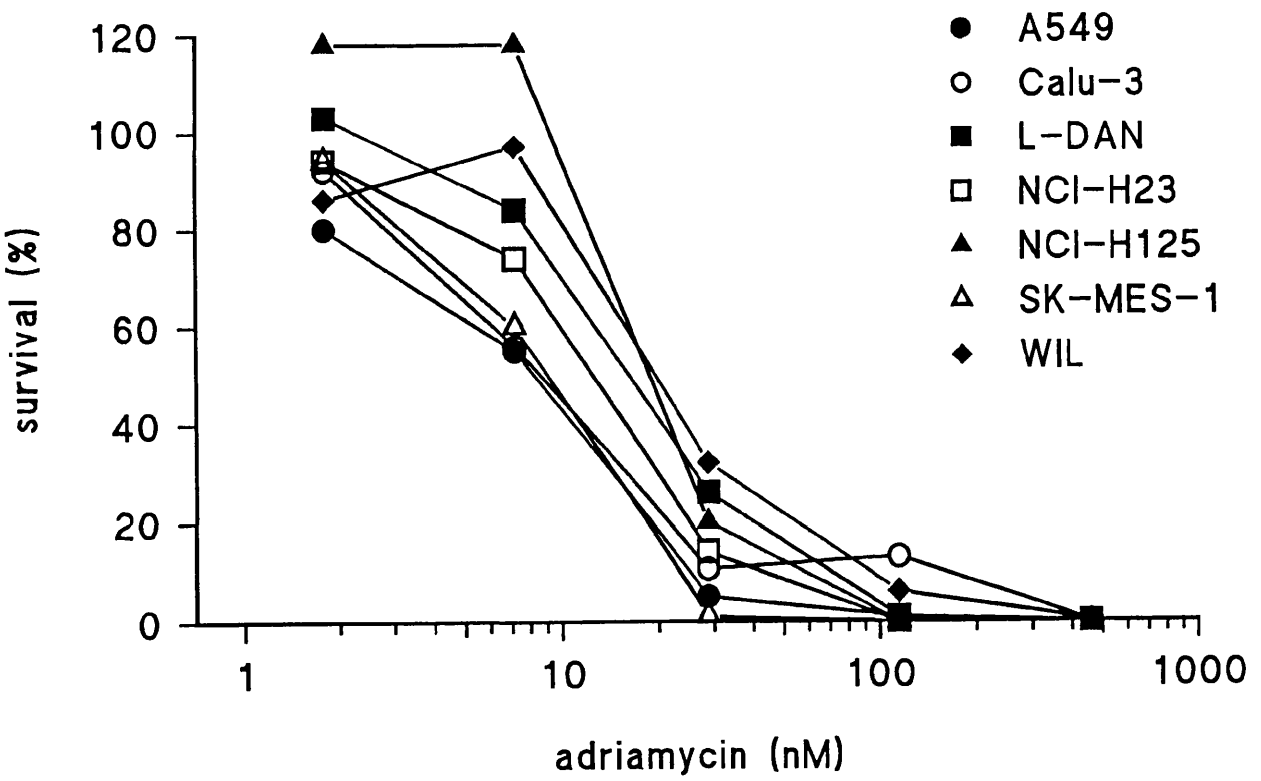
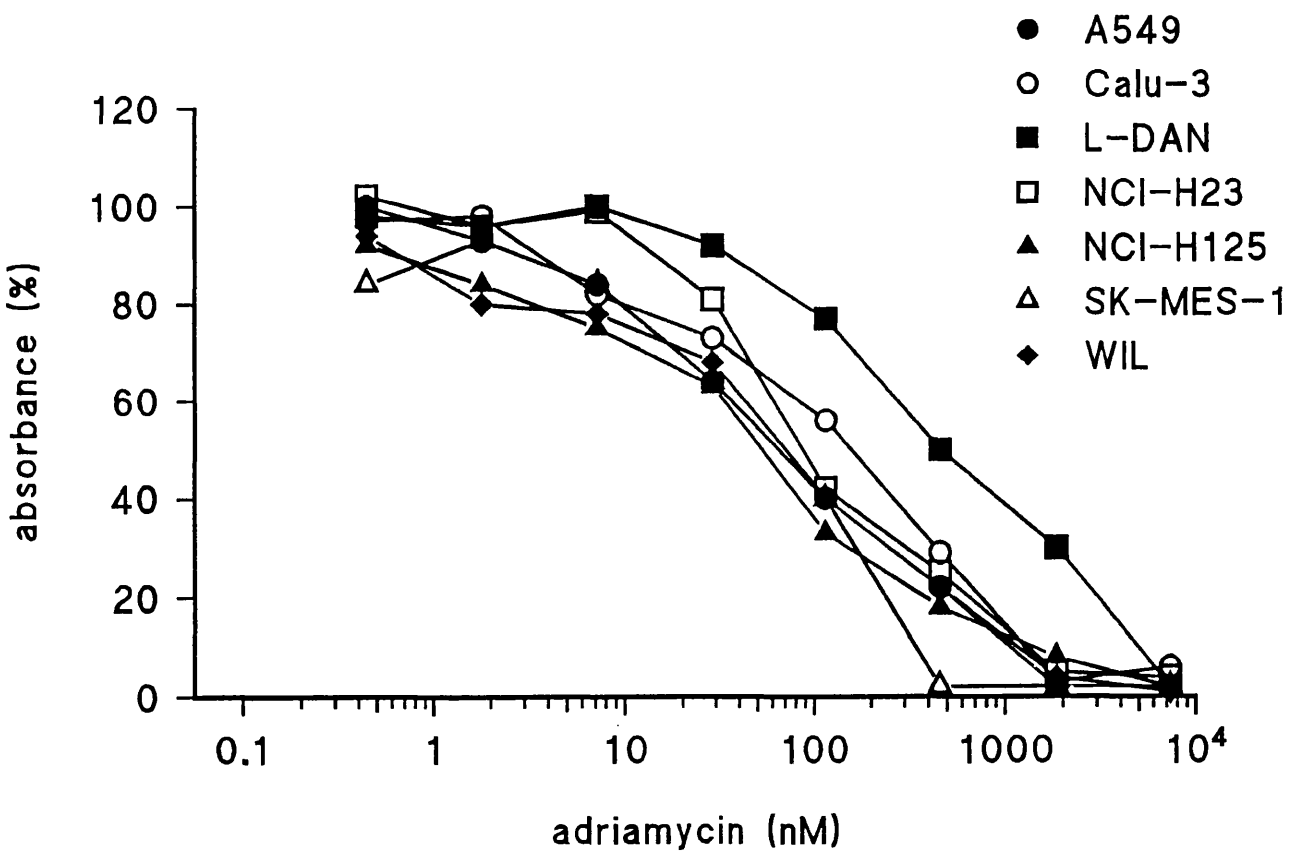


Figure 4
NSCLC chemosensitivity: MTT assay

10^3 (A549, NCI-H23, L-DAN and WIL), 2.5 x 10^3 (NCI-H125) or 5 x 10^3 (Calu-3) cells/well in 200μl medium were seeded into 96 well plates and incubated at 37^0C for 3 days. The medium was then replaced with fresh medium containing serial adriamycin dilutions and reincubated for a further 24 hours. The medium and drug were removed and replaced with fresh medium then and on each of the next two days. On the fourth day following drug exposure the medium was replenished with the addition of 10mmol hepes buffer. 50μl/well of 4.5mg/ml MTT was then added and the plates were reincubated in darkness for a further 4 hours. The medium was then removed and the MTT formazan crystals dissolved in 200μl DMSO. 25μl of 0.1M tris buffer was added to each well and the plates were read on an ELISA plate reader at an absorbance of 570nm and their absorbance calculated as a percentage relative to control wells without drug. Each point represents the mean of 8 wells.

FIGURE 4
NSCLC chemosensitivity: MTT assay



	IC_{50}(nM)	
	Clonogenic assay	MTT assay
A549	7.7 (1.4)	207
Calu-3	8.7 (3.3)	138
L-DAN	14.7 (1.9)	129
NCI-H23	13.5 (1.0)	94
NCI-H125	21.5 (5.3)	50
SK-MES-1	8.0 (1.4)	66
WIL	19.0 (2.1)	74

Table 5
NSCLC chemosensitivity: comparison of clonogenic and MTT assays

Assays performed as described in Figs. 3 and 4. IC_{50} determined graphically as drug concentration which reduced survival of colonies (clonogenic assay) or absorbance (MTT assay) to 50% of that obtained from controls in the absence of drug. Values for clonogenic assay represent mean (+SD) of 2-3 separate experiments.

The narrow range of IC_{50} values from either assay, and the consequent discrepancy between the two methods in ranking of chemosensitivity based on these values (Spearman's rank correlation coefficient, r_s= -0.607, p>0.05), suggests that they are unlikely to represent important differences in chemosensitivity between the seven cell lines.

3.3.3. Clonogenic assay: 72 hour drug exposure

The reason for the discrepancy between chemosensitivity results obtained in this study and the broad spectrum of chemosensitivity previously reported for these lines by Merry et al. (1987) is not clear. In the ^3H leucine incorporation assay of the earlier report the period of drug exposure employed was 72 hours, and it was possible that longer periods of drug exposure might reveal chemosensitivity differences not apparent following 24 hour drug exposure used in the MTT and clonogenic assays described here.

To investigate this possibility, the most sensitive cell line on the basis of ^3H leucine incorporation, NCI-H23, and the least sensitive line, A549, were studied further. If duration of drug exposure was critical, changes in chemosensitivity resulting from variation in exposure time should be most readily apparent in those lines at opposite ends of the chemosensitivity range.

A clonogenic assay was therefore performed for each of these two cell lines using a 72 hour period of adriamycin exposure. In comparison to the 24 hour exposure, this resulted in a shift of the survival curves of both cell lines to the left (fig.5). This reduced the IC_{50} for NCI-H23 from 13.5 to 2.8nM and that for A549 from 7.7 to 5.5nM. Therefore, although increasing the duration of adriamycin exposure does appear to have a more marked effect on NCI-H23 than on A549, it does not seem likely to significantly affect the range of chemosensitivity seen with these lines. The discrepancy between chemosensitivity results obtained with ^{3}H leucine and clonogenic assays can not therefore be explained on the basis of differences in duration of drug exposure.

3.3.4. Spheroid clonogenic assay

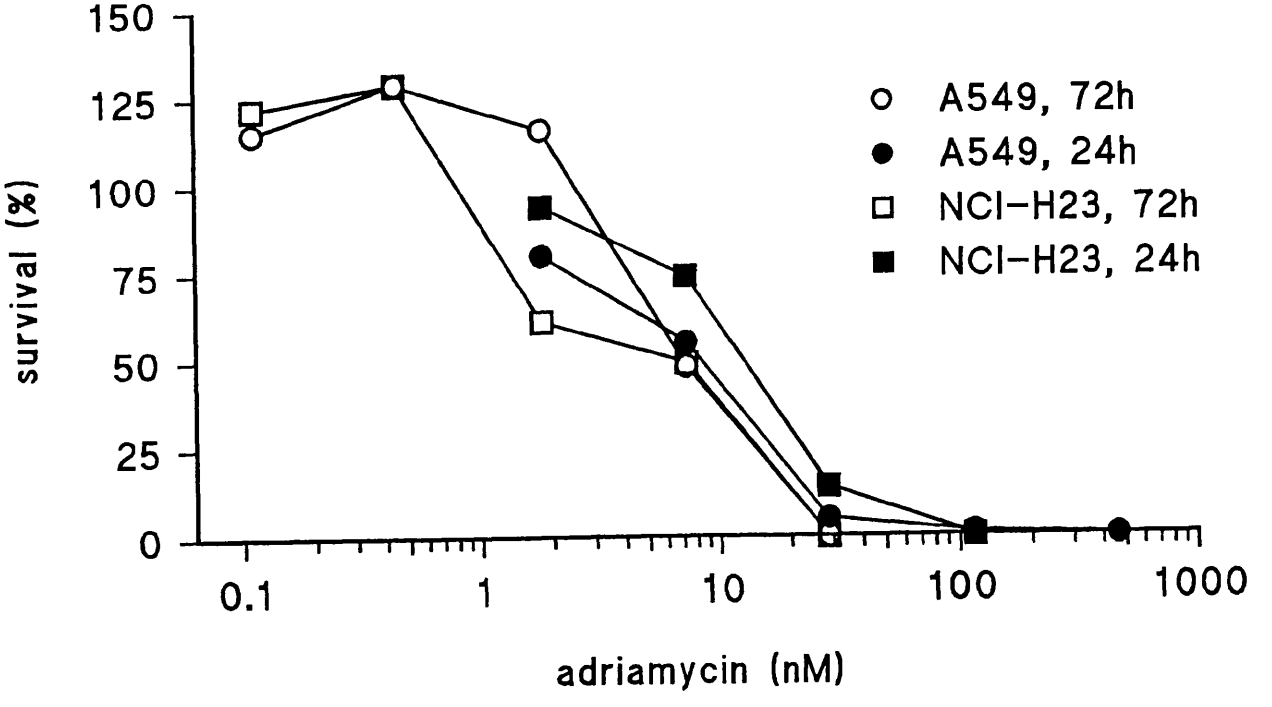
In view of the lack of association between gap junctional communication and chemosensitivity of these cell lines, it was decided to investigate whether, as with radiation response, 3-dimensional contact was a necessary condition for any protective effect of intercellular communication on the cell lines to be manifest.

Two of the cell lines, L-DAN and SK-MES-1, were therefore cultured as multicellular spheroids and a clonogenic assay performed on the spheroid cells by spheroid disaggregation following drug exposure. These two cell lines were selected as representing lines of widely

Figure 5
NSCLC chemosensitivity: clonogenic assay, comparison of 24 and 72 hour drug exposure

24 hour drug exposure was performed as described in Fig 3. The method of 72 hour drug exposure was identical with the exceptions that NCI-H23 were seeded at 2 x 10^3 cells/well and L-DAN at 10^3 cells/well. Triplicate wells were prepared at each drug concentration and medium and drug were replenished at 24 and 48 hours.

FIGURE 5
NSCLC chemosensitivity: comparison of 24 and 72 hour drug exposure



differing junction forming ability, and might therefore be expected to confirm whether junctional communication was associated with increased spheroid resistance to adriamycin.

Survival curves derived from a series of three experiments were very similar for each cell line (fig.6) and produced IC_{50} values of 131 nM for L-DAN and 80nM for SK-MES-1.

Thus, for both cell lines, culture as three-dimensional spheroids produces a 9-fold increase in adriamycin resistance over monolayer (compare table 5). This equal advantage for two cell lines of widely differing communication ability again supports the absence of a role for intercellular communication in adriamycin resistance in this model.

3.4. Discussion

3.4.1. Introduction

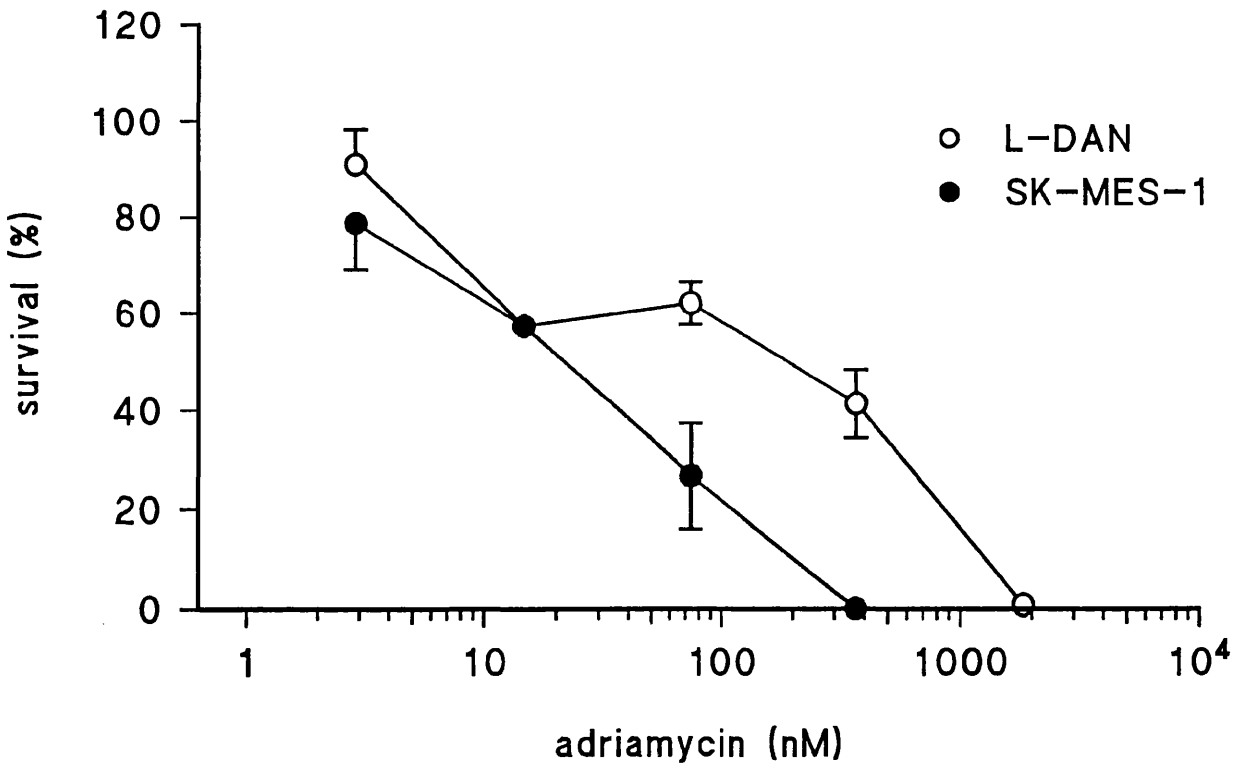
This study confirms the wide variation in gap junction forming ability which has been reported to exist between different tumour cell lines (Morgan et al., 1982, Miller et al., 1983) and demonstrates that the nucleotide transfer technique is able to reproducibly quantify the extent of coupling. If junctional communication plays a significant role in determining the cellular response to

Figure 6
NSCLC chemosensitivity: spheroid clonogenic assay

1.25ml medium containing serial adriamycin dilutions was added to 25cm^2 BCF containing 5ml medium and 5-6 day old L-DAN or 6-8 day old SK-MES-1 spheroids. After 24 hours incubation at 37^{o}C, 10 spheroids from each dish were transferred to one well of a 24 well microtitration dish. 0.5ml of 0.25% trypsin solution was added to each well, the spheroids were disaggregated to a single cell suspension by repeated pipetting and 1.5ml of medium added to each well. 200μl aliquots of this suspension were then added in triplicate to new 25cm^2 flasks and incubated at 37^{o}C. The resulting colonies were fixed in methanol and stained with Giemsa. Colonies > 50 cells were counted and their survival expressed as a percentage relative to control flasks without drug. Each point represents the mean (+/-SEM) of 3 separate experiments. Drug concentrations are calculated assuming even distribution of adriamycin between medium and agar.

FIGURE 6

NSCLC chemosensitivity: spheroid clonogenic assay



cytotoxic drug treatment then this panel of seven human NSCLC cell lines would have been ideally suited to demonstrate a relationship between junctional communication and adriamycin sensitivity.

3.4.2. Cytotoxicity assays

The narrow range of chemosensitivity of the seven cell lines as assessed both by clonogenic and MTT assays was therefore surprising, particularly in view of the previously reported large variation in adriamycin sensitivity as measured by ^{3}H leucine incorporation assay (Merry et al., 1987), although that data is based upon only the results of one or two experiments per cell line.

The results from 72 hour drug exposure in this study suggest that the difference in duration of drug exposure is not a sufficient explanation for this discrepancy. Subsequent clonogenic assay and MTT studies from that laboratory with these lines (R.I. Freshney, personal communication) have also failed to find a significant range of chemosensitivity and thus support the present findings.

Carmichael et al. (1988a) studied a large number of SCLC and NSCLC lines including three of those in this study (NCI-H23, NCI-H25 and A549) and similarly found little difference in their chemosensitivities as measured by MTT

assay, parallelling the consistently adriamycin resistant behaviour of these tumours in the clinical setting.

3.4.3. Role of spheroid geometry

Culture of cell lines as multicellular spheroids increases the resistance of both well-coupled and poorly-coupled cells to an equal extent, therefore in this system at least, there is no evidence to implicate junctional communication in spheroid resistance. The contribution of penetration and kinetic influences to this increased spheroid resistance was not examined in this study although penetration barriers to adriamycin have previously been reported to be present in L-DAN spheroids and to contribute to their drug resistance by Kerr and colleagues (1986), although in their study the duration of adriamycin exposure was only one hour and it is possible that the 24 hour exposure in the current study allows sufficient time to overcome such a barrier.

Therefore, this study using human tumour lines is in agreement with previous reports in rodent lines of increased adriamycin resistance of spheroids over monolayer (Sutherland et al., 1979; Durand, 1981).

Among the mechanisms of spheroid resistance proposed by those earlier studies was that sharing of some intermediary metabolites or messengers between cells in a three dimensional structure might confer increased

resistance on the population in a manner analgous to the metabolic cooperation originally described by Subak-Sharpe et al. in 1966. Cell to cell transfer of glutathione (GSH) would be one plausible explanation.

This model is dependent on communication between the cells and based upon the assumption that such communication might be more extensive in three dimensional spheroid culture. The reports of Dertinger, Hinz and Jacobs (1982) and Sutherland (1986) of reduced rather than increased coupling when cells are cultured as spheroids seems to contradict this explanation and the failure of junctional communication to influence spheroid adriamycin resistance in this study also suggests that some further mechanism may be involved.

3.4.4. Relevance of cytotoxicity assay techniques

It is possible that the assay methods employed in this study have disadvantages which limit their suitability in a study of communication related phenomena. If sharing of GSH or some intermediary metabolite has a role to play in drug resistance, the timing and duration of cell coupling may be critical. Maintainance of communication in the cell population in the period following drug exposure, in addition to communication during the exposure period itself, may be necessary to enable limitation of or recovery from drug induced damage.

In clonogenic assays involving both monolayer and spheroid systems in this study, the cells are trypsinised and separated immediately following drug exposure and plated out at low density, eliminating any junction mediated metabolic cooperation from that time. The ability of the individual cell to survive the period of drug exposure and retain its clonogenic potential is the only property which this assay can detect. For junctional communication to influence this property, it would be essential that communication during drug exposure itself was sufficient to limit drug induced damage.

The introduction of the MTT assay was designed to overcome these reservations regarding clonogenic assays. In the MTT assay cells are allowed a 72 hour period in undisturbed culture to recover from drug exposure and to continue to divide before the reduction of MTT is used as an index of cell number, and, by inference, of the surviving fraction of clonogenic cells. However, although the MTT assay avoids the disruption of junctional contact inherent in clonogenic assays it too has its limitations. In order to allow the surviving cells to continue in exponential growth for 72 hours following drug exposure, it is implicit that at the time of drug exposure itself, they were subconfluent. Therefore although the MTT assay allows maintainance of intercellular communication following drug exposure, this is at the expense of reduced communication during the

early stages of the assay as a result of lower cell density.

Neither assay is therefore capable of allowing optimum communication both during and following drug exposure. As the proposed protective mechanism of intercellular communication is itself unclear, the relevance of these methodological differences between the two assays of chemosensitivity is uncertain, but in conjunction the assays allow assessment of the effects of gap junctional communication at most times of potential importance and might be considered complementary.

An alternative assay method which was not investigated in this study, but which might circumvent these problems, is one based on measurement of spheroid growth delay following drug exposure, where the duration of drug induced delay in spheroid growth relative to untreated control spheroids is used as an index of chemosensitivity. In this system, as in tumours *in vivo*, cell-cell contact is present before, during and after drug exposure, affording optimum opportunity for metabolic cooperation to influence response.

The evidence from this present study suggests that the previously demonstrated relationship between junctional communication and resistance to radiation or to the cytotoxic lymphokines TNF and lymphotoxin may not

represent a generalised phenomenon, applicable to all conventional cytotoxic drugs.

3.4.5. Potential role for GSH in determining cytotoxicity

The mechanism whereby TNF and lymphotoxin exert their cytotoxic effect remains uncertain although there is evidence that free radical induced DNA fragmentation may be involved (Matthews et al., 1987, Rubin et al., 1988 and Yamauchi et al., 1989). It is possible that this common pathway of radiation or lymphokine damage is responsible for the reported association between resistance to these treatment modalities and junctional communication. The role of intercellular GSH transfer in modulation of cytotoxicity in such coupled populations would be of interest.

Carmichael et al. (1988b) measured GSH levels in a large series of human small and non-small cell lung cancer lines. As a group, the NSCLC lines had significantly higher GSH levels than the SCLC lines which may be relevant to their differences in drug sensitivity in the clinical setting. Included in their NSCLC group were three of the lines in this study, NCI-H23, NCI-H125 and A549, and no significant difference in GSH levels was observed between the three. No information is available regarding GSH levels in the other lines used in the present study.

Although intracellular GSH levels may have a role in limiting adriamycin toxicity in some resistant cell lines (Dusre et al., 1989), the importance of this agent in determining the drug response of these NSCLC cell lines is unknown.

Chapter 4 STUDY OF THE INTERACTION OF SENSITIVE AND RESISTANT CELLS IN MIXED CULTURE

4.1. Introduction

The original intention of this project was to employ the human NSCLC cell lines studied in Chapter 3 to investigate whether growth of sensitive and resistant cell lines in mixed culture resulted in interaction between the cell lines leading to modulation of their drug response. The wide spectrum of chemosensitivity of these cell lines reported by Merry et al. (1987) was considered appropriate to allow selection of a relatively resistant and a relatively sensitive pair of lines suitable for combining in mixed culture.

The presence of a pattern of cross resistance to adriamycin, vincristine and etoposide in these cell lines suggested that their drug resistance might result from increased mdr1 expression. This was supported by the ability of the calcium antagonist verapamil to reduce drug accumulation and increase sensitivity in some of the lines (Merry et al., 1987). Therefore as proposed by the model in section 1.7.4. intercellular drug transfer between adjacent sensitive and resistant cells in mixed culture might alter their drug response.

The subsequent contradiction of this chemosensitivity data, at least for adriamycin, by MTT and clonogenic

assays (Chapter 3) rendered these lines clearly unsuitable for use as "sensitive" and "resistant" lines in mixed culture. It was therefore necessary to examine alternative cell lines.

4.2. Studies with sensitive and resistant murine astrocytoma cell lines

4.2.1. Introduction

In view of the unsuitability of the NSCLC lines for mixed culture experiments, it was decided to examine a pair of sensitive and resistant lines where some interaction had already been suggested in mixed culture.

Bradford et al. (1986) had presented a preliminary report on their studies with clonal cell lines derived from the P497 cell line, originally isolated from a spontaneous murine astrocytoma.

Characterisation of the chemosensitivity of these cell lines had shown that two of the lines, C12 and F1, had shown wide differences in sensitivity to the vinca alkaloids vincristine and vindesine, as measured by ^{35}S methionine uptake assay. There was no difference in adriamycin sensitivity between the two lines (table 6). Mixed monolayer culture of the two cell lines had shown that the combination adopted the chemosensitivity of the more resistant line (C12), suggesting possible transfer

	Mean IC_{50} (mg/ml) ± S.E.	
	C12	F1
vincristine	61 ± 2	5 ± 0.8
vindesine	85 ± 1	4.2 ± 0.3
adriamycin	230 ± 10	190 ± 10

Table 6
Chemosensitivity of murine astrocytoma lines:
^{35}S methionine uptake assay

(J. Darling, unpublished data)

of resistance although the individual response of the two cell lines in mixed culture was not examined.

These two cell lines were obtained from Dr. J.L. Darling (London) and it was proposed to perform further mixed culture studies with the lines after genetic marking with ß-galactosidase (ß-gal) to determine whether this reported interaction could be confirmed, and if so to relate this to alterations in the individual chemosensitivity of the two lines.

4.2.2. Genetic marking with ß-galactosidase

Retroviral mediated gene transfer using the ecotropic virus producer line G4-12.2-Y7 (Price, Turner and Cepko, 1987) was performed (section 2.4.2.). A high proportion of cells survived G418 selection and within two weeks there were adequate cell numbers for ß-gal histochemical staining. This was positive for both cell lines. F1 showed approximately 90% positive staining, whereas C12 showed approximately 60% staining.

4.2.3. Assessment of chemosensitivity

Concurrently with the above retroviral infection procedures, a clonogenic assay was performed on the two cell lines in order to confirm that the chemosensitivity differences reported with the ^{35}S radiolabelled

methionine uptake assay were maintained in the assay system necessary for coculture studies.

The survival curves from the first pair of assays using an initial cell density of 10^5 cells/well (figs 7a and 7b), whilst showing some reduction colony survival following drug exposure, have a quite different appearance from those obtained with NSCLC cell lines (see fig.3). The most striking feature of the survival curves is that at higher drug concentrations there is no further reduction in colony survival. It is therefore impossible to accurately calculate the IC_{50} value for either cell line.

It was considered that the most probable explanation for the appearances of these survival curves is the mechanism of action of the two drugs. The cytotoxicity of vinca alkaloids results from their interference with the formation of the mitotic spindle and they therefore only act on cells during division. Although the 24 hour duration of drug exposure employed in the assay should allow all cycling cells to undergo mitosis during this period, it is probable that the number of cells present per well resulted in density limitation of cell growth, particularly at the edges of wells where cells were most confluent on microscopical examination. As a result, a proportion of cells in each well were no longer dividing and were therefore resistant to the drugs, even at high concentration.

Figure 7
Chemosensitivity of murine astrocytoma lines: clonogenic assay

10^5 cells/well were added to a 24 well plate. After 24 hours incubation at 37^0C, the medium was removed and replaced with fresh medium containing serial dilutions of vindesine (a) or vincristine (b). One set of wells contained medium alone and acted as control. Following a further 24 hours incubation, the medium and drug were removed and 200μl 0.25% trypsin solution was added to each well. After 10 minutes incubation 1.8ml/well of medium was added and the cells separated to a single cell suspension by repeated pipetting, then counted on a Coulter counter and 5 x 10^2 cells/well were plated into triplicate 60mm petri dishes, incubated for 8 days, fixed in methanol then stained with 10% Giemsa. Colonies > 50 cells were counted and their survival expressed as a percentage relative to control wells without drug.

FIGURE 7a
chemosensitivity of murine astrocytoma lines
clonogenic assay, vindesine

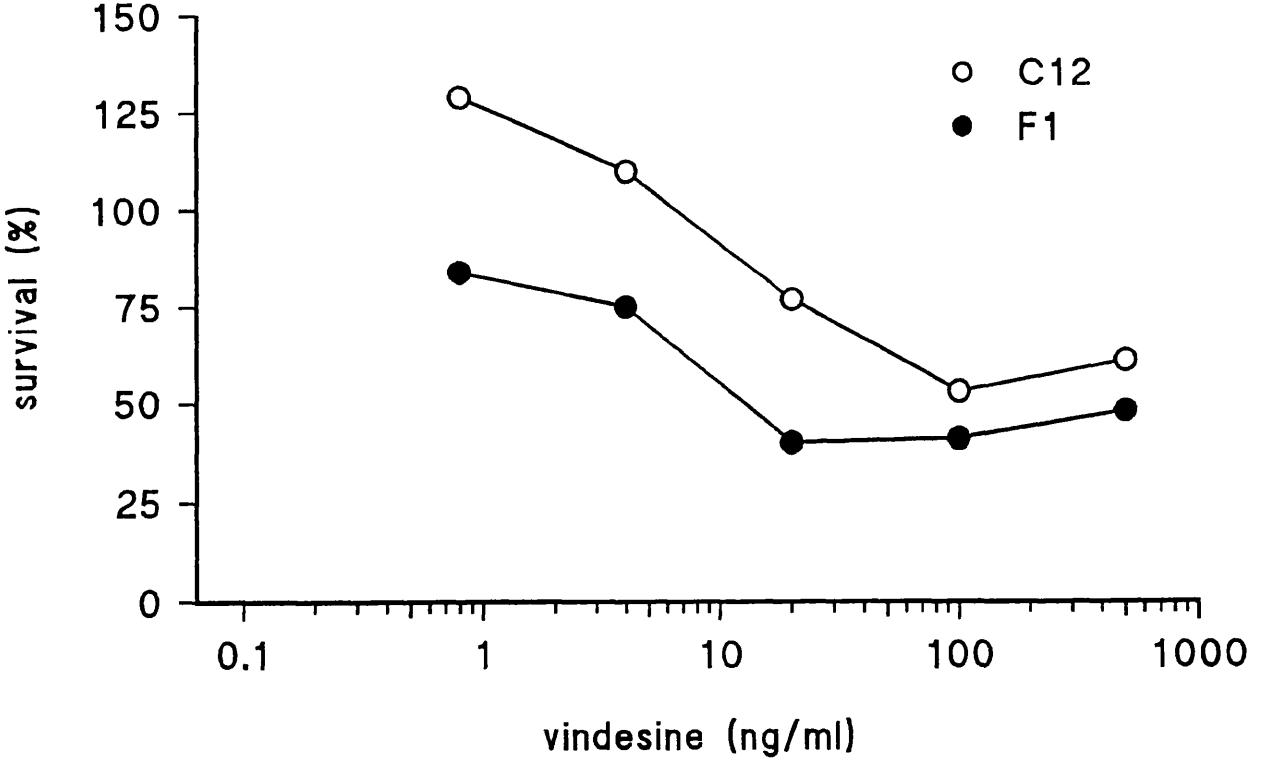
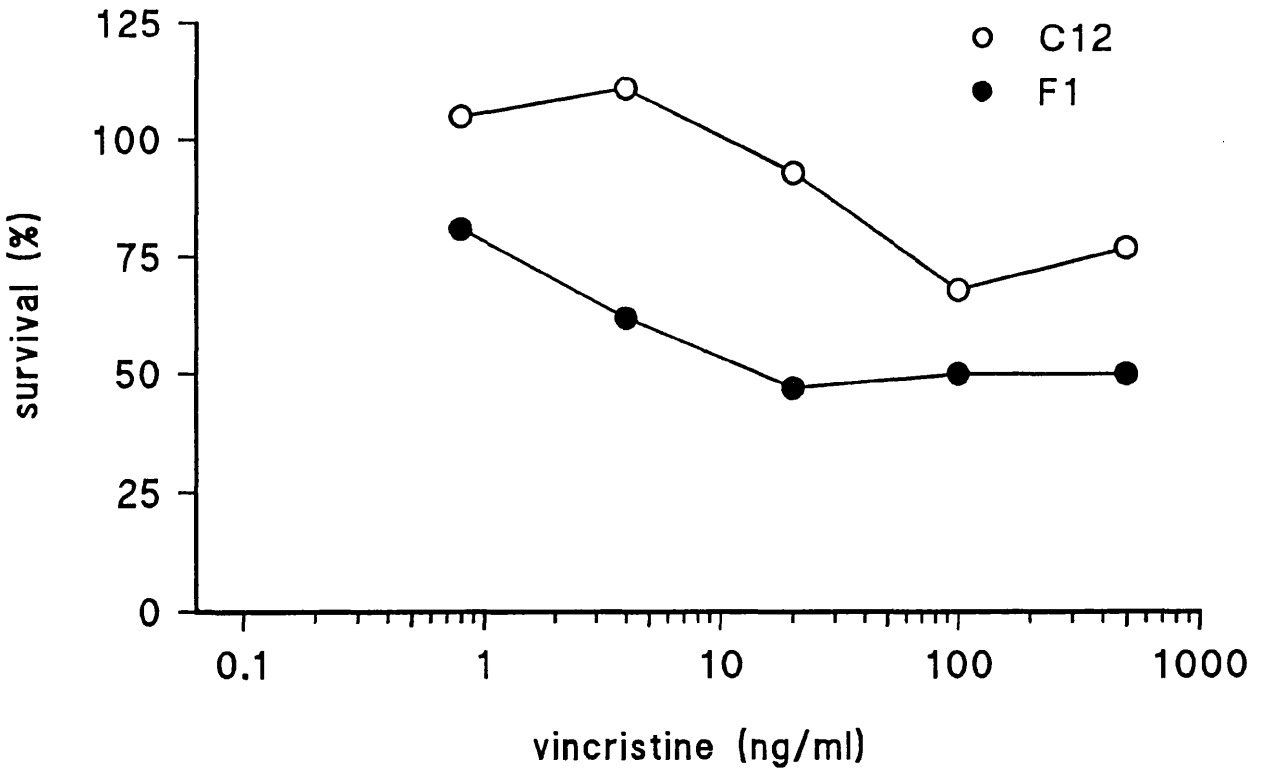


FIGURE 7b
chemosensitivity of murine astrocytoma lines
clonogenic assay, vincristine



Although the separation of the two survival curves suggests some difference in chemosensitivity, this is not marked. In the survival curves following vindesine exposure (fig.7a) in particular there is a striking similarity between the shapes of the two curves. As each point on the curve is calculated relative to the control survival in the absence of drug it is clear that the low survival of the control C12 colonies is the only difference between the two curves. If, as might be expected, the control survival had been approximately equivalent to that of the lowest drug concentration then the two survival curves would have been almost exactly superimposed.

The survival curves following vincristine exposure (fig.7b) appear more clearly separated, perhaps indicative of a genuine difference in chemosensitivity, although it is possible that the faster growth rate of C12 cells allowed them to reach confluence earlier, thus accounting for their apparent increase in resistance.

A second pair of assays was therefore performed at a lower initial cell density of $2x10^4$ cells/well to examine whether this would remove the complicating factor of cell confluence during drug exposure and enable any true chemosensitivity differences to be evident.

The survival curves from these assays (figs 8a and 8b) confirm the reduced survival with increasing drug

concentration which would be expected in the absence of density limitation of growth. It is also clear, however, that with either drug the survival curves of C12 and F1 are closely superimposed suggesting that by clonogenic assay there is no significant difference in vinca alkaloid chemosensitivity.

On the basis of these assays, the IC_{50} value of both cell lines to vindesine is approximately 0.025μg/ml and to vincristine is 0.018μg/ml, both of which are intermediate between the values reported for the two lines by ^{35}S methionine incorporation. The explanation for the discrepancy between clonogenic and ^{35}S methionine incorporation assays is unclear. Previous studies (Morgan et al., 1983) suggest good correlation between the two assays. The influence of cell density on IC_{50} values may be partially responsible.

In the absence of a significant chemosensitivity difference by clonogenic assay, there was no value in proceeding with the proposed mixed culture experiments. Therefore neither the parent nor ß-gal marked lines were studied further.

Figure 8
Effect of reduction in cell density on chemosensitivity of murine astrocytoma lines

Method as described in figure 7 except that initial cell density was 2 x 10^4 cells/well. Colonies > 50 cells were counted and their survival expressed as a percentage relative to control wells without drug.

FIGURE 8a
chemosensitivity of murine astrocytoma lines
effect of reduction in cell density
clonogenic assay, vindesine

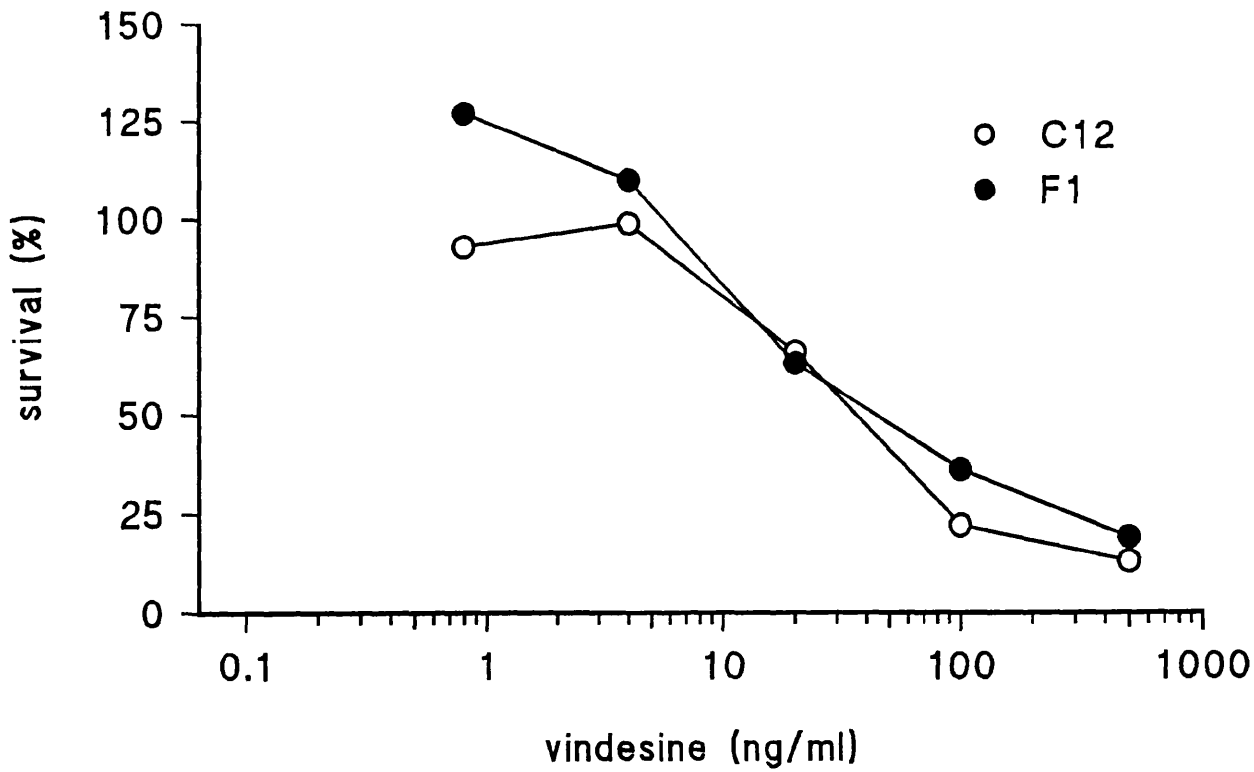
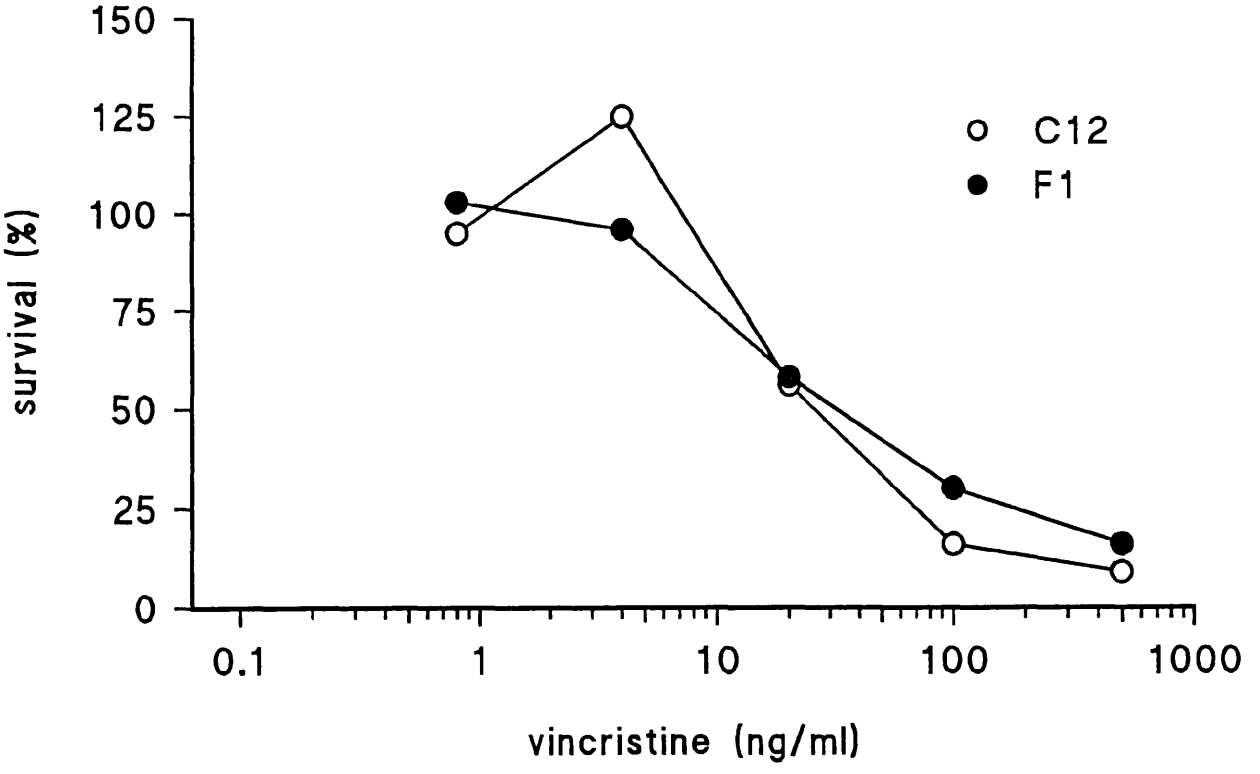


FIGURE 8b
chemosensitivity of murine astrocytoma lines
effect of reduction in cell density
clonogenic assay, vincristine



4.3. Studies with sensitive and resistant Chinese hamster ovary (CHO) cell lines

4.3.1. Introduction

As the murine lines, C_{12} and F_1 were unsuitable for mixed culture studies, an alternative model system was therefore sought. A group of Chinese hamster ovary (CHO) cell lines of varying chemosensitivity was obtained from Dr I Hickson, University of Newcastle on Tyne. These comprised the parental line, CHO-K1, a multidrug resistant cell line, Adr^r and a drug-hypersensitive cell line, ADR-6.

Adr^r was derived from the parental line by culture in adriamycin and exhibits cross resistance to colchicine, mitozantrone, mitomycin C, vicristine and actinomycin D. Amplification of mdr1 sequences together with increased mdr1 mRNA expression and P-glycoprotein have been demonstrated in this line compared to CHO-K1 (Chatterjee and Harris, 1990; I. Hickson, personal communication). The hypersensitive mutant ADR-6 was selected following exposure of the parent line to the mitogen ethyl methanesulphonate, and has therefore never been exposed to adriamycin. It is also cross-sensitive to vincristine and colchicine (I.Hickson, personal communication).

4.3.2. Confirmation of chemosensitivity

Adriamycin chemosensitivity of CHO-K1, ADR-6 and Adr^r was assessed by clonogenic assay. The survival curves generated from this experiment (fig.9) show clear differences in drug sensitivity between the different cell lines. The resultant IC_{50} values were CHO-K1 64nmol, ADR-6 16.6nmol and Adr^r 2390nmol. Thus the MDR cell line Adr^r was 37-fold more resistant to adriamycin than the parental line, whilst the hypersensitive line ADR-6 was almost 4-fold less resistant. These results are in close agreement with the sensitivities reported by the Newcastle group (Chatterjee and Harris, 1990; I. Hickson, personal communication).

4.3.3. Coculture of sensitive and resistant cells: preliminary studies of ^3H thymidine incorporation

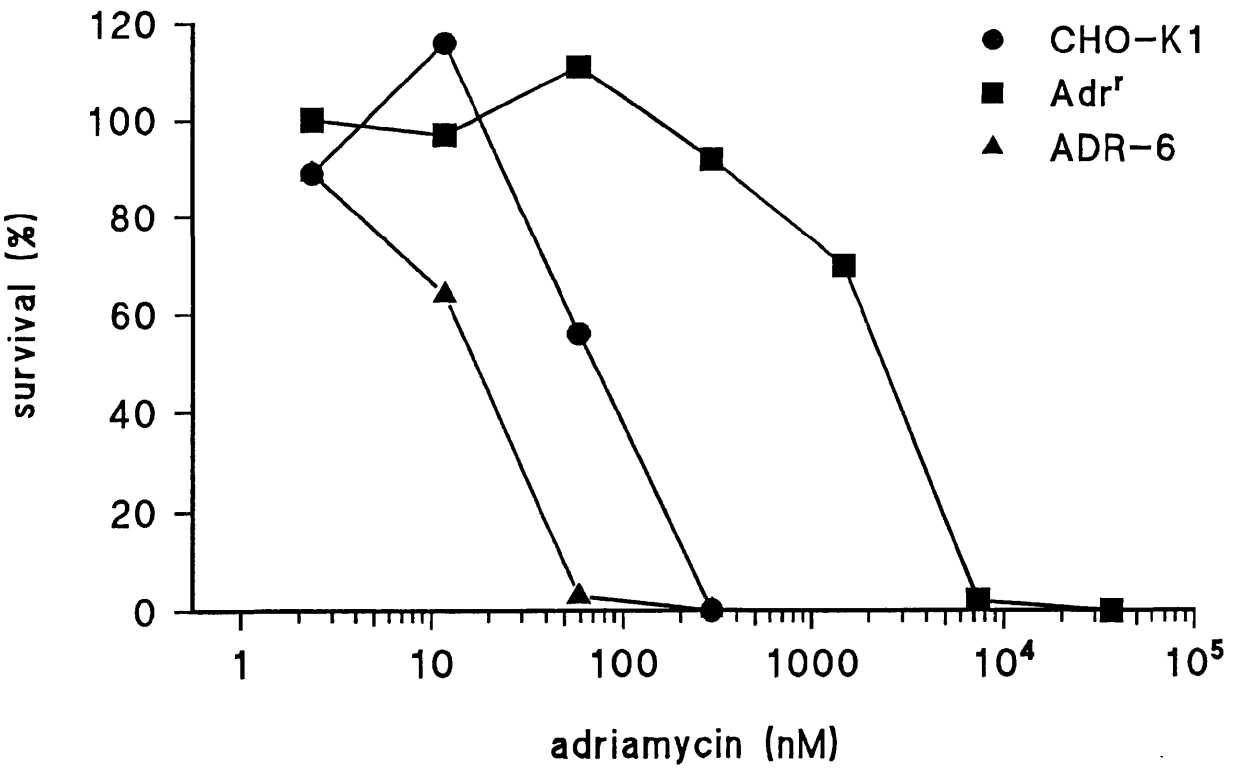
4.3.3.1. Introduction

This initial study employed an assay technique where the cells' ability to incorporate ^3H thymidine into newly synthesised DNA during the S phase of the cell cycle is used as a marker of continued cell division and therefore of survival following drug exposure. Measurement of the total incorporation of label by scintillation counting at a specified time following drug exposure is thus an index both of cell number and reproductive capacity and

Figure 9
CHO chemosensitivity: clonogenic assay

5×10^3 cells in 100μl medium were seeded into each well of a 96 well plate, incubated overnight at 37^{o}C, then the medium was removed and replaced with fresh medium containing serial adriamycin dilutions. After 24 hours incubation the medium and drug were removed and 50μl/well of trypsin added. 150ul medium was then added and the cells were separated to a single cell suspension. 20μl aliquots from each well were then added to 25cm^2 flasks, incubated for 7 days at 37^{o}C, fixed in methanol and stained with Giemsa. Colonies > 50 cells were counted and their survival expressed as a percentage relative to control wells without drug.

FIGURE 9
CHO chemosensitivity: clonogenic assay



therefore, as with the MTT assay (section 2.3.2.), is an indirect measure of the survival of clonogenic cells.

In addition to scintillation counting, the cells were processed for autoradiography. The actively cycling cells could be identified by the presence of autoradiographic grains visible overlying their nuclei, indicative of incorporation of label into their DNA. This allowed some indication of the survival of individual cells, rather than of the entire population, and it was hoped that this would prove useful in studying the individual response of the two cell types in mixed culture.

Based on the doubling time of the cell lines (section 4.3.4.5.), a 24 hour period of labelling was chosen so that all cycling cells would be likely to pass through the S phase of the cell cycle during this period and thus incorporate the label.

The initial experiment was designed to determine the optimum timing of labelling in relation to drug exposure, in order to maximallly differentiate between the response of the two cell lines studied, the parent (sensitive) line CHO-K1, and the resistant line Adr^r. Three labelling periods were studied: day 1, i.e. concurrent with drug exposure, day 2 or day 4 of the assay.

4.3.3.2. Scintillation counting

Survival curves were generated by plotting of total scintillation counts (relative to those of control dishes without drug) versus drug concentration. It was possible to compare the survival curves of CHO-K1 cells alone, Adr^r cells alone and of a 50:50 mixed culture of the two cell types for each of the chosen labelling periods.

Labelling of cells on day 1 showed no differences between the three groups of cells. It was likely that insufficient time had elapsed for the full cytotoxic effects of the drug to occur and allow any differences in drug responses of the lines to be evident. However, labelling on day 2 (fig.10a) and day 4 (fig.10b) showed a clear distinction between the curves of CHO-K1 and Adr^r. In the day 4 label, the survival of the mixed culture was intermediate between that of the two cell types cultured separately, whilst in the day 2 label, the survival of the mixed culture appeared even higher than that of Adr^r alone.

4.3.3.3. Autoradiography and labelling index

Calculation of the labelling index (LI) from coverslips submitted for autoradiography also confirmed a marked difference between CHO-K1 and Adr^r, particularly when labelled on day 4. At this point the LI of Adr^r following exposure to the highest drug concentration was

Figure 10
Coculture of CHO-K1 and Adr^r: ^3H thymidine incorporation assay

(a) day 2 labelling
10^5 cells (CHO-K1 alone, Adr^r alone or a 50:50 mixture of the two cell types (mixed)) in 2ml medium were seeded into 35mm dishes, each containing 3 x 13mm coverslips. Following 2 hours incubation at 37^oC, 1ml medium containing serial dilutions of adriamycin at 3 times the final concentration was added. After a further 24 hours incubation the drug and medium were removed and replaced with fresh medium and ^3H thymidine (200μCi/ml in PBS, 50μl/dish) was added. After 24 hours incubation, the dishes were washed in PBS, fixed in formol-saline and the coverslips washed in ice-cold 5% TCA, H_2O and rinsed in ethanol. The coverslips were then added individually to scintillation vials and counted for one minute on a scintillation counter.

(b) day 4 labelling.
Method as above except that after the period of drug exposure, medium and drug were removed and replaced with fresh medium alone and dishes reincubated at 37^oC for a further 48 hours before addition of ^3H thymidine. The counts for each coverslip are expressed as a percentage relative to control dishes without drug.

FIGURE 10a
coculture of CHO-K1 and Adr^r
^{3}H thymidine incorporation assay
day 2 labelling

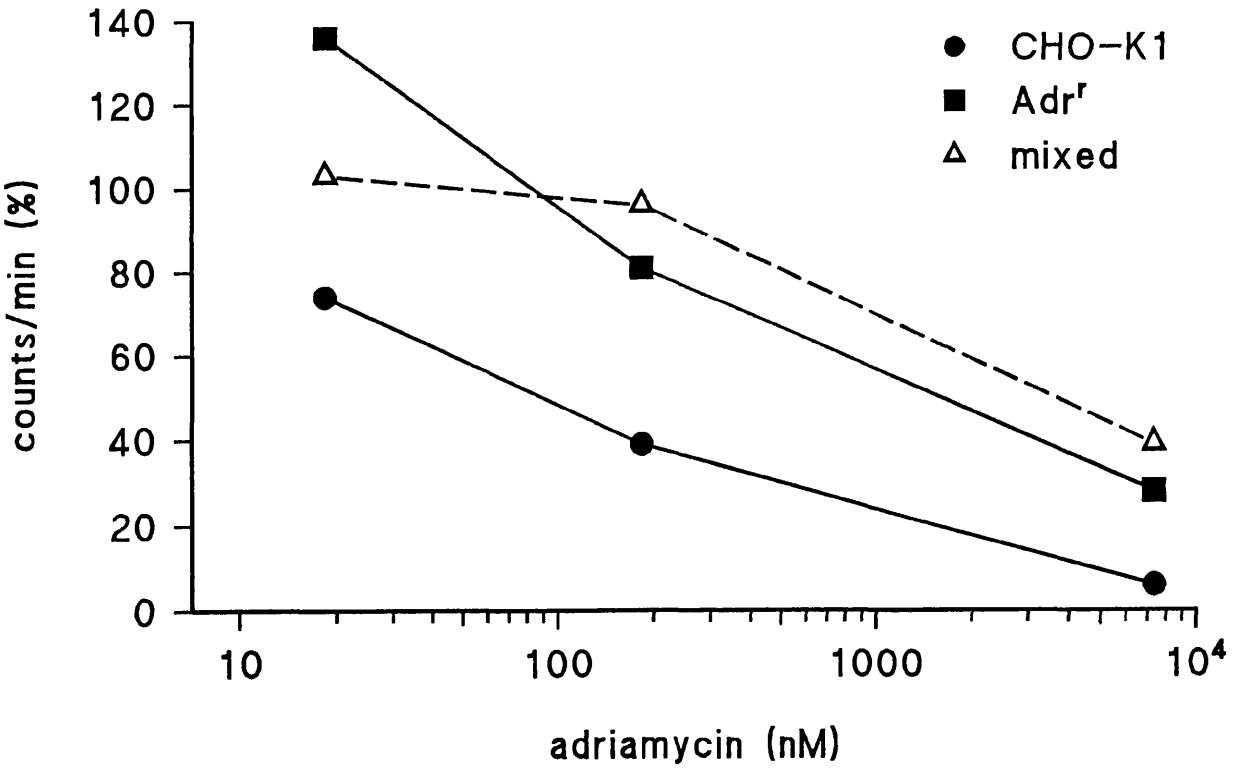
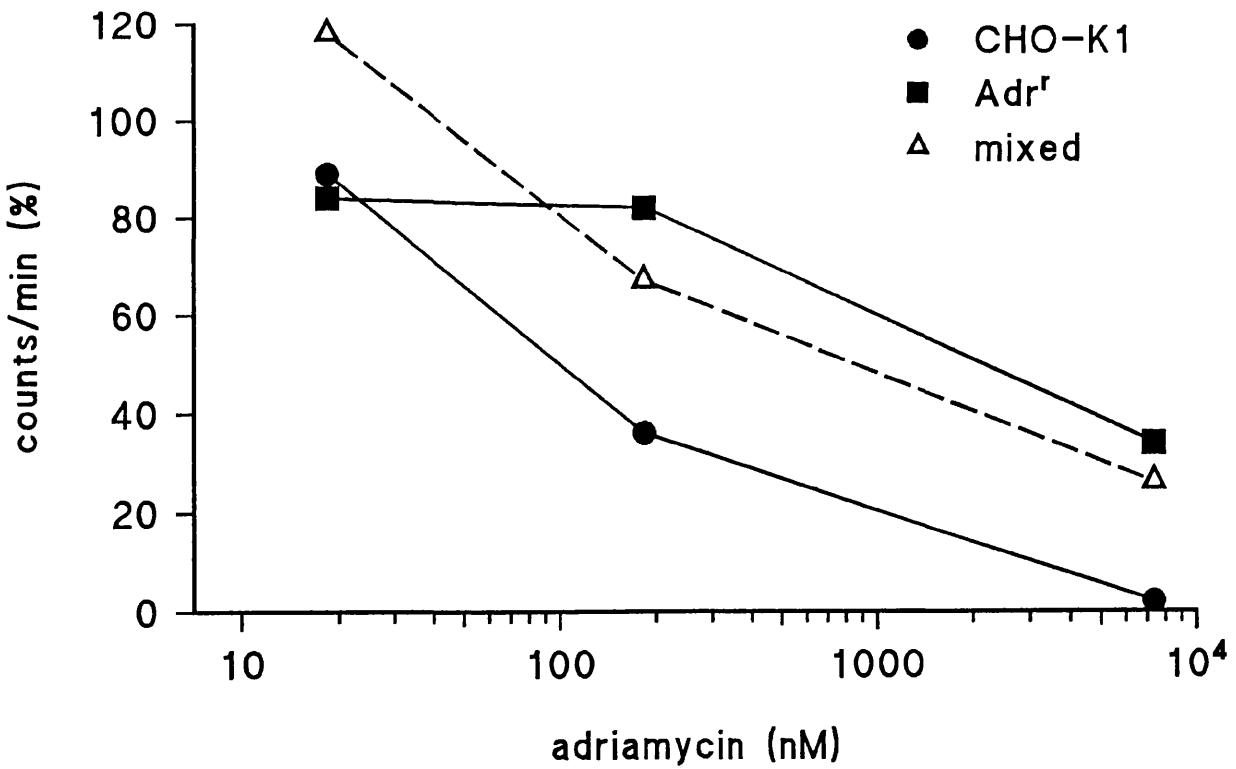


FIGURE 10b
coculture of CHO–K1 and Adr^r
^{3}H thymidine incorporation assay
day 4 labelling



89% whilst for CHO-K1 it was only 12%. The LI of the mixed culture at this adriamycin concentration was 90% and therefore comparable to that of Adr^r alone. Although this result was consistent with resistance transfer, the finding was not confirmed in subsequent experiments. Other considerations discussed below suggest that this result may be spurious.

At the higher drug concentrations where the difference between the sensitive and resistant lines was most apparent it was difficult to identify intact cells in view of considerable cell disruption and debris, and this limited the accuracy of counting. It was clear that in coverslips with CHO-K1 cells alone, the drug toxicity was more severe and cells were more sparse with frequent abnormal forms. Thus, although viable labelled cells could be clearly distinguished, the accuracy of the denominator used for calculation of the LI, i.e. the total number of cells present, was uncertain. The potential error was considered to be sufficiently large to negate the value of the LI in this study and it was not examined further. In a series of subsequent experiments (section 4.3.3.4.), therefore, scintillation counting alone was studied.

4.3.3.4. Scintillation counting: effect of cell density

In selecting the optimum experimental conditions for these subsequent studies, it was important to allow a

sufficient period of time to elapse between drug exposure and labelling for adequate distinction between the responses of Adr^r and CHO-K1. However it was necessary to balance the duration of the experiment with the initial density of cells seeded into the dishes. As previously discussed with regard to the MTT assay, in order to allow for continued growth of cells for several days following drug exposure, the cells must initially be subconfluent, thus limiting the potential for intercellular communication. A long delay before labelling in an attempt to maximise the differential drug responses would inevitably require a correspondingly low initial cell density.

Based on the results of these preliminary experiments further studies were therefore performed with ^3H thymidine labelling on day 3. The effect of different initial densities of 1×10^5 cells/dish and 5×10^5 cells/dish was also studied. The resulting survival curves (figs 11a and 11b) again showed a clear separation between the survival curves of Adr^r and CHO-K1, although on each occasion the curve for the 50:50 mixture lay mid-way between those of the pure cultures i.e. in the position which would have been predicted from the curves of Adr^r and CHO-K1 alone, assuming no interaction.

These survival curves are generated using the mean scintillation counts of two separate coverslips. Although it was attempted to exclude coverslips with a

Figure 11
Coculture of CHO-K1 and Adr^r: ^3H thymidine incorporation assay, day 3 labelling

Method as described in Fig.10a except that initial cell density was 10^5 cells/dish (a) or 5 x 10^5 cells/dish (b). After the period of drug exposure, medium and drug were removed and replaced with fresh medium alone and dishes reincubated at 37^0C for a further 24 hours before addition of ^3H thymidine. The counts for each coverslip are expressed as a percentage relative to control dishes without drug.

FIGURE 11a
coculture of CHO–K1 and Adr^r
^{3}H thymidine incorporation assay
day 3 labelling, 1x10^5 cells/dish

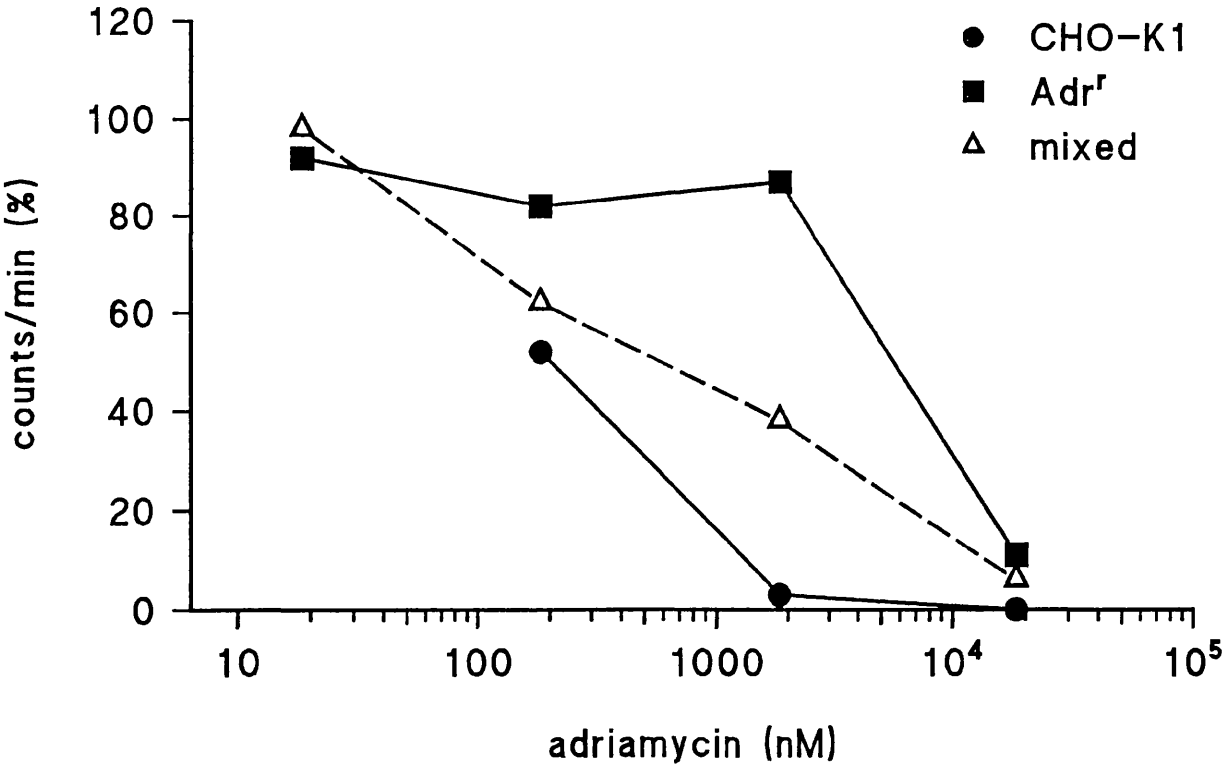
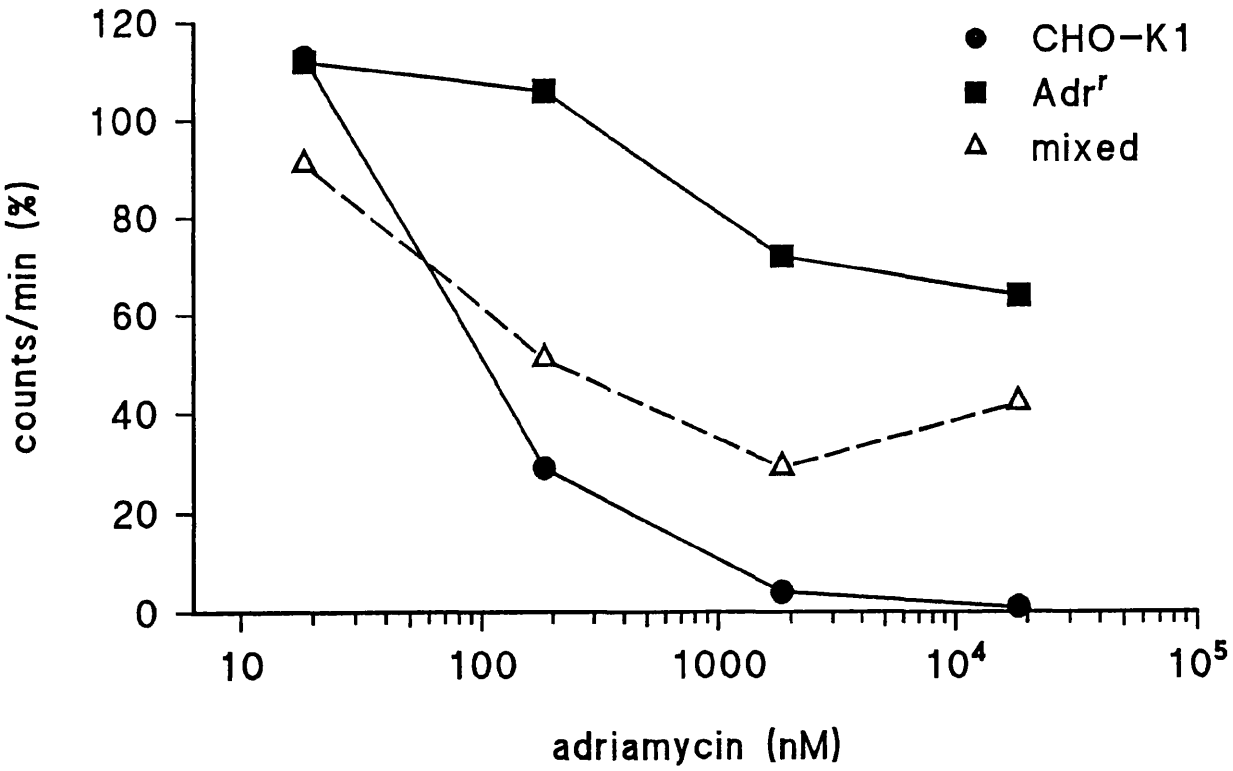


FIGURE 11b
coculture of CHO–K1 and Adr^r
^{3}H thymidine incorporation assay
day 3 labelling, 5x10^5 cells/dish



clearly non-uniform distribution of cells for scintillation counting, some inhomogeneity remained, and therefore limited the accuracy of the survival curves. To exclude the possibility that such inhomogeneity might mask a significant interaction between the two cell types, a further study was performed to compare the results obtained counting intact coverslips as above, with those where the cells were seeded into dishes without coverslips, and the incorporation of label measured following solubilisation of all cells in the dish (see section 2.5.3.2.).

The resulting survival curves (figs 12a and 12b) showed essentially identical results with or without coverslips, suggesting that any inhomogeneity in cell distribution had no substantial effect on these results. To maximise potential interactions a high initial cell density was used in this experiment (1×10^6 cells/dish) and labelling was performed on day 2, before cell division was limited by confluence.

With the exception of one experiment from the preliminary study (fig 10a) these results have therefore consistently shown that ^{3}H-thymidine incorporation, and effectively the survival, of the 50:50 mixture of Adr^r and CHO-K1 to have a value intermediate between that of either cell type cultured alone. This result is that which would have been calculated for such a mixed culture, from the data from the separate cultures, assuming both cell types

Figure 12
Coculture of CHO-K1 and Adr^r: ^3H thymidine incorporation assay, day 2 labelling

(a) method as described in Fig.10a except that initial cell density was 10^6 cells/dish. (b) method as described in Fig.10a except that dishes did not contain coverslips, initial cell density was 10^6 cells/dish and the activity of the label was 40μCi/ml. Following TCA extraction, the cells were solubilised in 2ml 0.1M NaOH, then acidified with 0.3ml 1M HCl and added to scintillation vials for counting. The counts for each dish or coverslip are expressed as a percentage relative to control dishes without drug.

FIGURE 12a
coculture of CHO–K1 and Adr^r
^{3}H thymidine incorporation assay
day 3 labelling, with coverslips

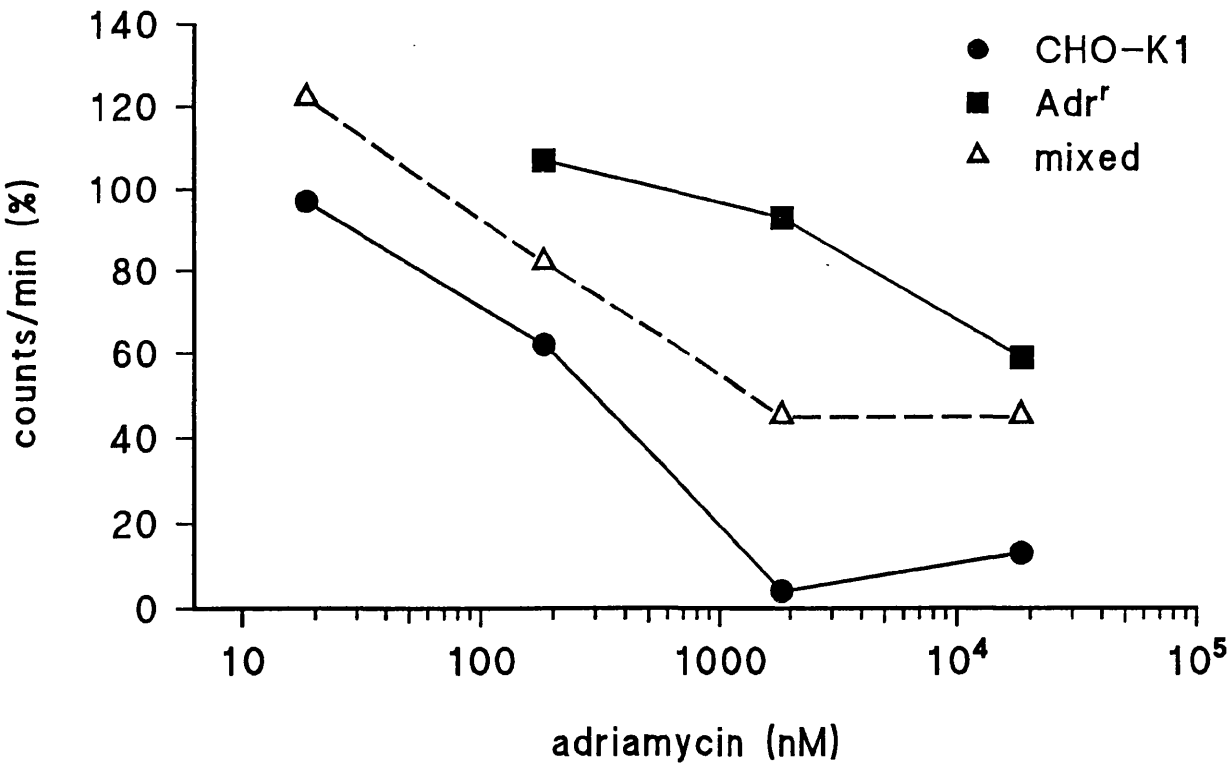
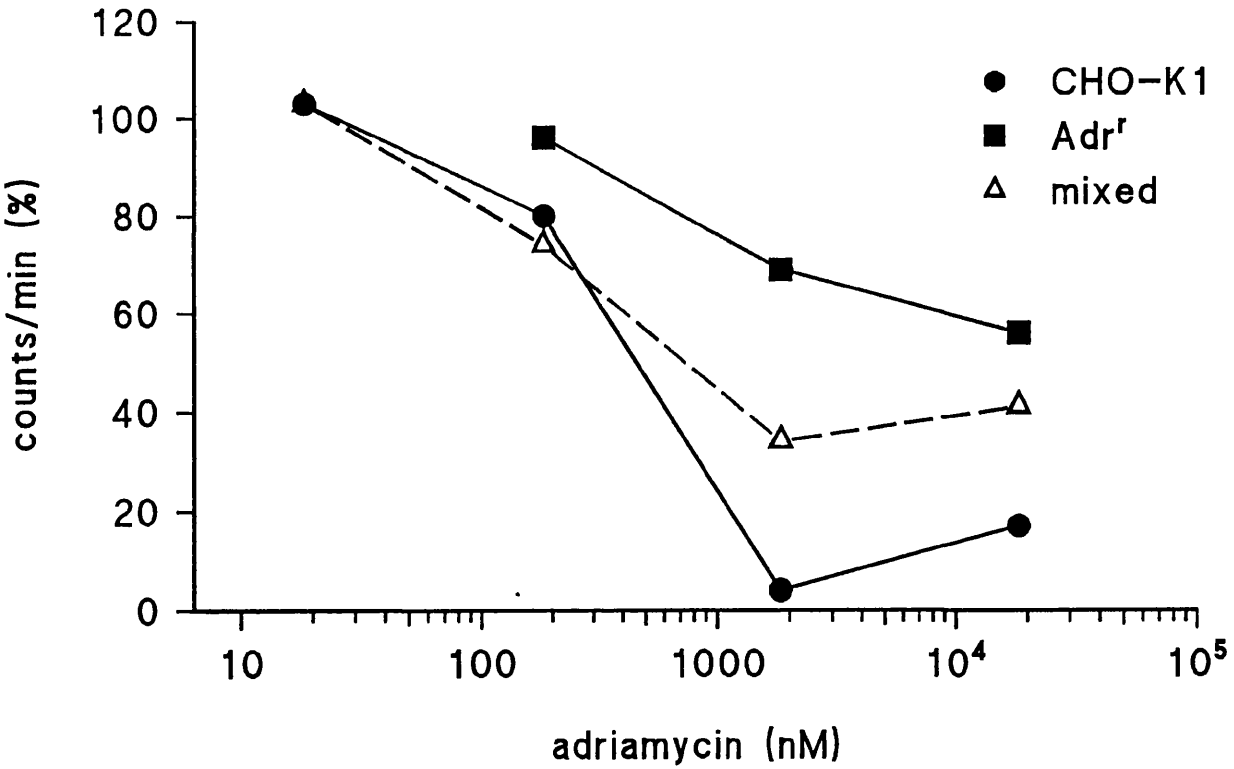


FIGURE 12b
coculture of CHO–K1 and Adr^r
^{3}H thymidine incorporation assay
day 3 labelling, without coverslips



continued to respond individually, and would therefore be consistent with an absence of interaction between the two populations under these conditions.

Although this study would exclude transfer of drug resistance of the magnitude suggested by the work of Tofilon et al. (1987) and Bradford et al. (1986), where the overall response of the mixed population showed a shift towards that of the resistant line, the possibility of some interaction remains.

This study only provides information on the overall response of the mixed population and it is possible that some interaction is occurring which has the effect of reducing the resistance of Adr^r and increasing that of CHO-K1, thus producing an intermediate but uniform response in the mixed population. Such a result might be anticipated if equilibration of adriamycin occurs, but the rate of extrusion of adriamycin required to protect both populations exceeds the capacity of the P-glycoprotein pump in the resistant cells. To identify the individual response of the two cell populations in mixed culture, it is necessary to mark the cells in some way and this is discussed in the following section.

4.3.4. Genetic marking with ß-galactosidase

4.3.4.1. Introduction

In order to assess the individual chemosensitivity of two cell types following a period of mixed culture, some means of marking the cells is required to permit their subsequent identification. Although the work of Bradford et al. (1986) had suggested some interaction between vinca alkaloid sensitive and resistant mouse glioma cells, this was based on the overall population response and their findings could also have been explained by an overgrowth of the resistant line.

Tofilon et al. (1984, 1987) overcame this limitation by their use of the extent of drug induced sister chromatid exchanges (SCE) in rat brain tumour cell lines as a means of identification of individual cell lines as well as of their drug response. They reported that the sensitive and resistant cell populations could be clearly delineated by the reduced induction of SCEs in the resistant compared to the sensitive line.

An ideal cell lineage marker should be stable, should not be diluted by cell division or spread to non-sibling cells and should allow easy identification of marked cells. Genetic markers can fulfill all these criteria and one such marker which has received considerable attention in recent years is the E. coli ß-galactosidase

(ß-gal) gene. The presence of the ß-gal enzyme allows histochemical identification of the marked cell line and has been used to examine cell lineages in developmental studies in vivo (Sanes, Rubinstein and Nicolas, 1986; Price, Turner and Cepko, 1987; Turner and Cepko, 1987). These studies have used a recombinant retroviral vector system for gene transfer which in general has the advantage of greater efficiency of transfer in comparison to tranfection techniques (Gilboa, 1986). The use of this system for marking of the CHO-K1 and Adr^r cell lines was therefore investigated.

4.3.4.2. Retroviral infection

Two retroviral producer cell lines were employed in retroviral infection studies. Virus-containing supernatant from the ecotropic producer line G4-12.2-YZ (Price, Turner and Cepko, 1987) and from the amphotropic line PZ NC1 (Brown and Mee, unpublished) were used for infection of both CHO-K1 and Adr^r cell lines. The inclusion of the neo gene conferring resistance to G418 was used for selection of infected lines.

On transfer of the cell lines to selective medium containing 0.8mg/ml G418 following the infection procedure only CHO-K1 cells from the cultures infected with supernatant from G4-12.2-YZ producer line survived and grew. All CHO-K1 cultures infected with PZ NC1 supernatant and Adr^r cells infected by either vector

failed to survive the selection procedure. Despite growth of the one surviving G4-12.2-YZ infected CHO-K1 line for three weeks in selective medium, subsequent staining of this cell line for ß-gal was negative.

4.3.4.3. G418 titration

As it was possible that the concentration of G418 employed was insufficient for selection and the negative ß-gal staining in the above studies resulted from survival of uninfected cells, the effect of a range of G418 concentrations on plating efficiency of CHO-K1 and Adr^r cells was assessed (table 7). At 0.8 and 1.6mg/ml G418 no colonies > 50 cells were produced, although a few smaller aggregations of cells were visible at the lower concentration. It therefore appeared that although 0.8mg/ml was effective in suppressing clonogenic growth of CHO-K1 and Adr^r cells, the possibility that some normal, uninfected cells might survive G418 selection could not be excluded. In subsequent experiments 1.2mg/ml G418 was accordingly employed in selection.

Once more, however, all cells transferred to selective medium failed to survive and grow. Thus virus-containing supernatant from amphotropic and ecotropic producer cell lines failed to successfully infect either CHO-K1 or Adr^r cell lines, possibly because of a lack of suitable receptor on the CHO lines.

G418 (mg/ml)	Survival	
	CHO-K1	Adriamycin
0.1	98	89
0.2	86	71
0.4	31	62
0.8	0	0
1.6	0	0

Table 7
Effect of G418 concentration on survival of CHO-K1 and Adr^r

2×10^2 cells were seeded into $25cm^2$ flasks containing 5ml medium with serial dilutions of G418. One flask contained medium alone and acted as control. Flasks were incubated at 37^oC for 7-10 days, then medium removed, fixed in methanol, stained with Giemsa and colonies > 50 cells counted. Survival expressed as a percentage of control.

Further attempts to transfer the gene were therefore investigated using the technique of DNA transfection.

4.3.4.4. DNA transfection

Two different recombinant vectors each containing ß-gal and neo were studied, BAG (Price, Turner and Cepko, 1987) and $pLGV_1$ (Debenham and Thacker, unpublished), and used for transfection of CHO-K1 and Adr^r (section 2.5.4.1.). On this occasion, there was survival on G418 selection, although at very low frequency (approximately 1-2 colonies per dish), of both cell lines following transfection with each vector. After 2-3 weeks growth in selective medium the ß-gal activity of the cell lines was assessed by histochemical staining (section 2.5.4.4.).

CHO-K1 cells transfected with either vector showed positive staining, although the extent varied between 10 and almost 100% of cells in each. The proportion of cells staining positively was highest in those cells transfected with $pLGV_1$ vector where staining was consistently > 90%, and this cell line (designated CHO-K1-BG) was used in all subsequent coculture experiments.

CHO-K1-BG was maintained in selective medium for routine culture before transfer to non-selective medium for experiments. This continued selection was necessary for and successful in the maintainance of the ß-gal phenotype. There was some evidence of increased

stability with continued culture (section 4.3.5.5.). The remaining instability of some of the transfected cells in the absence of selection was demonstrated by the loss of ß-gal expression in a proportion of cells within individual colonies examined during the course of clonogenic assays (section 4.3.5.1.).

None of Adr^r cells showed positive staining despite survival of G418 selection and were not studied further.

4.3.4.5. Characteristics of CHO-K1-BG cell line

It was important to establish whether insertion of the $pLGV_1$ genome into the parent cell line CHO-K1 to produce the marked cell line CHO-K1-BG produced any alterations in the growth properties or chemosensitivity of the cells. A clonogenic assay was therefore performed to compare the response of the parent and marked lines to adriamycin.

The resulting survival curves (fig 13) show the two lines have similar responses, although the ID_{50} of CHO-K1-BG at 92nM is slightly less than that of the parent line at 134nM.

Comparison of the growth curves of the two cell lines and of those of Adr^r and ADR-6 (fig.14), show the growth properties CHO-K1 and CHO-K1-BG to again be very similar,

Figure 13
CHO chemosensitivity: comparison of CHO-K1 and CHO-K1-BG

10^5 cells/well were added to a 24 well plate and incubated at 37°C for 24 hours, then the medium was replaced with fresh medium containing serial dilutions of drug. Following a further 24 hours incubation, the medium and drug were removed and 200μl 0.25% trypsin solution was added to each well. After 10 minutes incubation, 1.8ml/well of medium was added and the cells separated to a single cell suspension by repeated pipetting, then counted on a Coulter counter and 5 x 10^2 cells/well were plated into triplicate 60mm petri dishes, incubated for 7 days and then stained with 10% Giemsa. Colonies > 50 cells were counted and their survival expressed as a percentage relative to control wells without drug.

FIGURE 13
CHO chemosensitivity
comparison of CHO-K1 and CHO-K1-BG

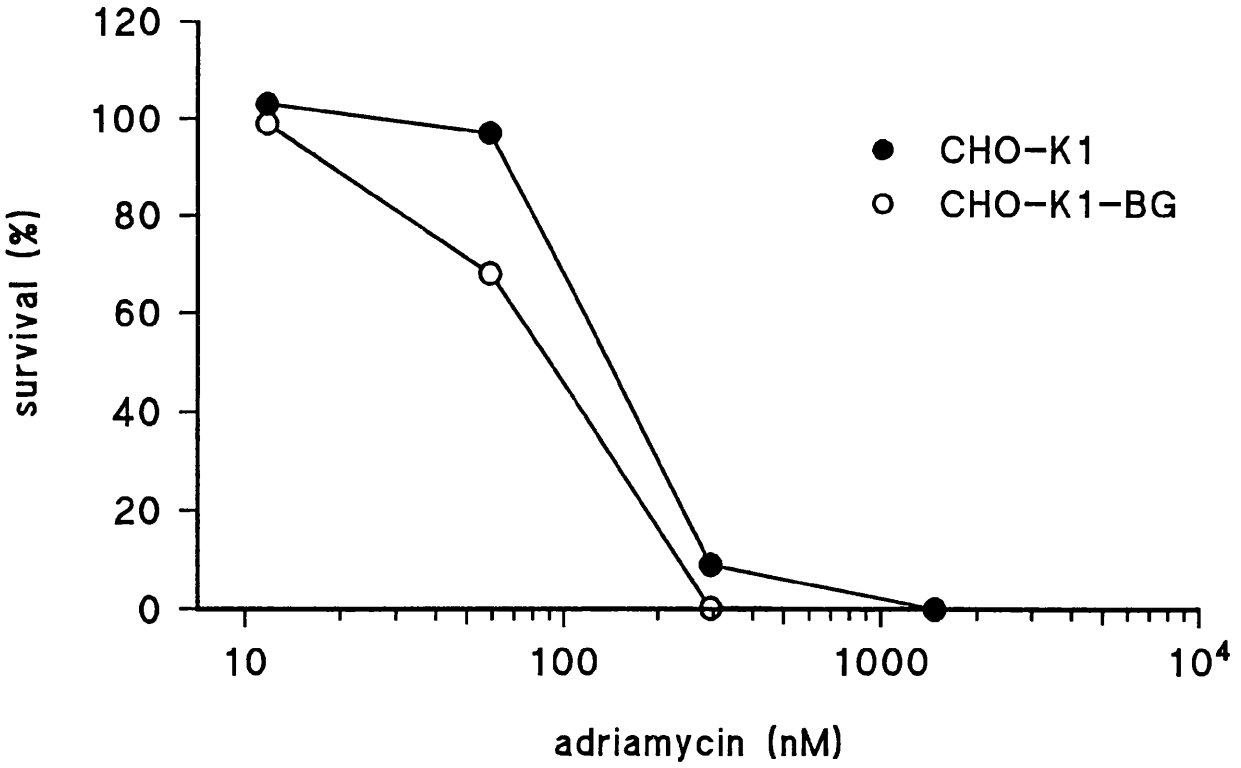
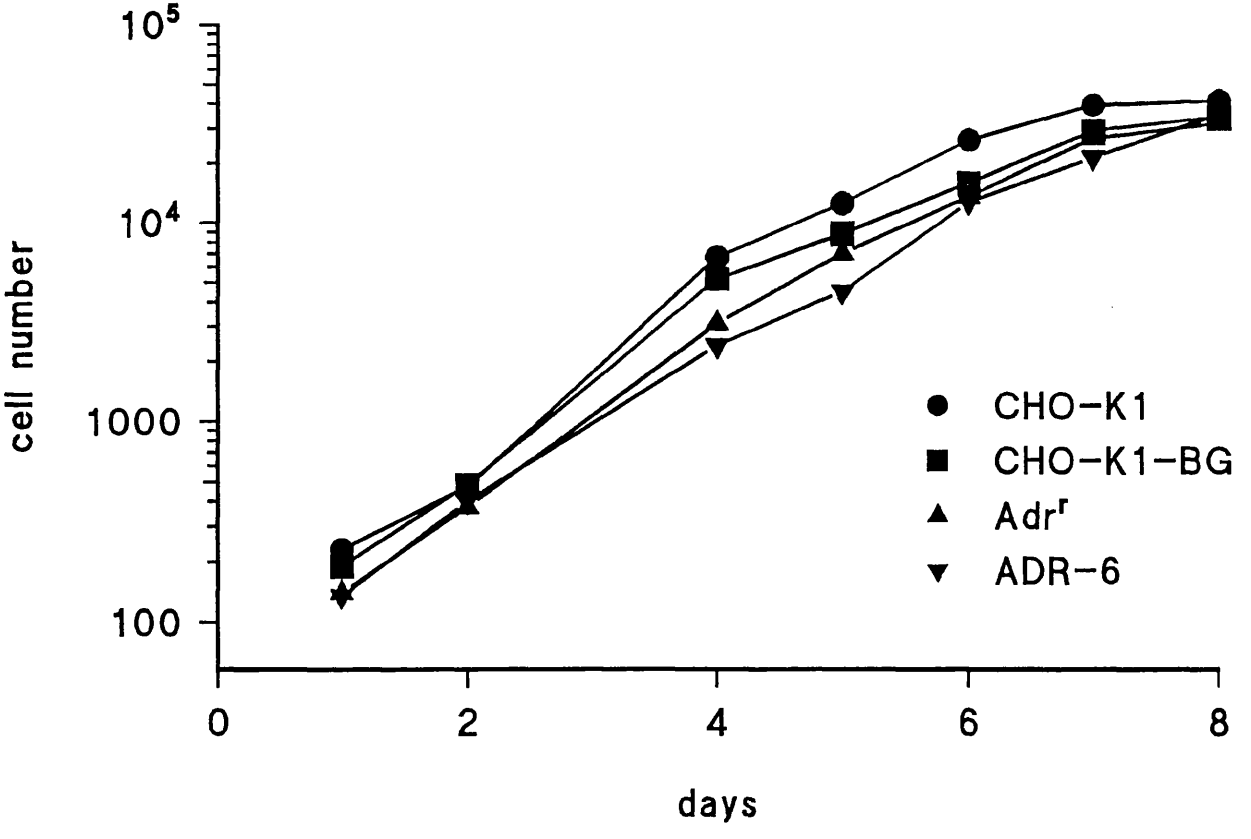


Figure 14
Growth curves of CHO lines

10^4 cells were plated into 35mm dishes and incubated at 37^oC. The total cell number per dish was estimated at regular intervals by trypsinisation of paired dishes at each time point and counting the resulting single cell suspension on a coulter counter. After the third day of growth the medium was replenished every 24 hours.

FIGURE 14
growth curves of CHO lines



with doubling times of 12.6 and 13.8 hours respectively. The MDR line Adr^r had a doubling time of 16.2 hours, whereas that of the hypersensitive line ADR-6 was 18.6 hours. These growth rates of CHO-K1 , Adr^r and ADR-6 are comparable to those reported by the Newcastle group (I.Hickson, personal communication).

It was therefore concluded that transfection of the marker gene had no major effect on either the growth or adriamycin sensitivity of CHO-K1. In particular the chemosensitivity of the two lines was sufficiently close to allow substitution of the marked line CHO-K1-BG for the parental line in planned mixed culture experiments.

4.3.5. Coculture studies with marked lines

4.3.5.1. Rationale of technique

To identify the individual response of the two cell lines in mixed culture, a standard clonogenic assay was performed (section 2.5.5.) using three sets of wells, the first containing CHO-K1-BG, the second containing a more resistant line (Adr^r) or more sensitive line (ADR-6), and a third containing a 50:50 mixture of the two cell lines.

Following drug exposure, plating of cells from the first two sets of wells allowed growth of colonies which could be counted to produce survival curves in standard fashion. After growth of colonies from the mixed wells,

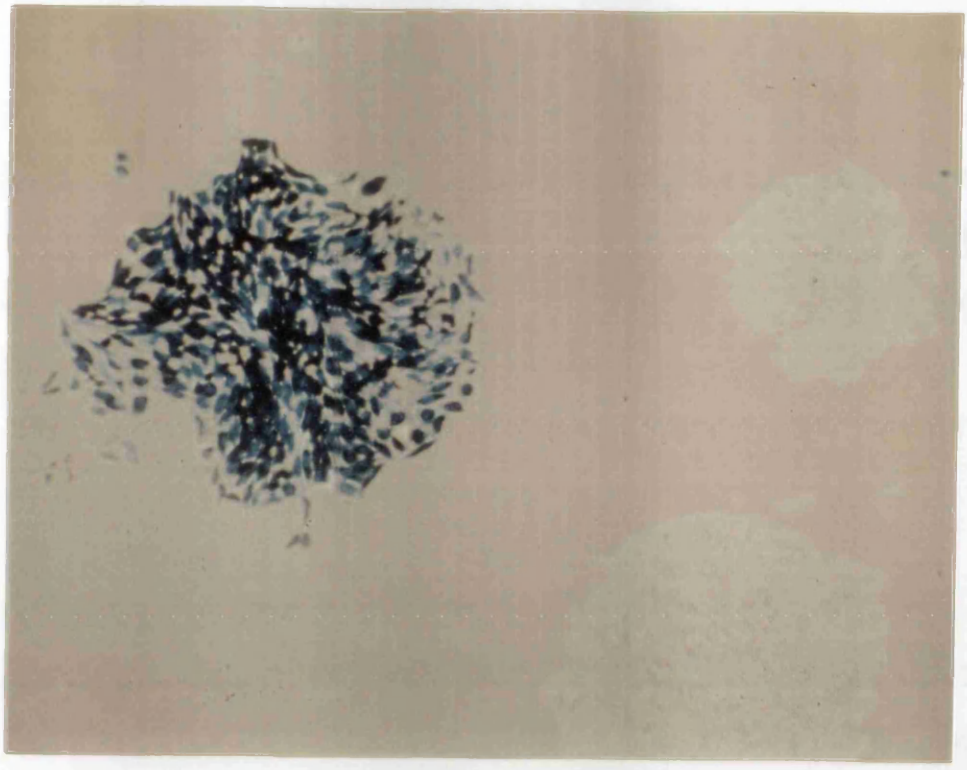
the cells were stained histochemically for ß-gal (section 2.5.4.4.) which produced a blue stain, rendering marked colonies readily identifiable. The intensity of stain varied considerably between colonies. In many colonies every cell within that colony showed clear ß-gal staining, but in others only a proportion of cells within the colony did so, possibly because the nine days growth of the cells in non selective medium over the course of the assay allowed survival of any cells which had lost the marker and selective genes.

As any colony which contained even a small proportion of positively staining cells was derived, in whole or in part, from the CHO-K1-BG cell line, all colonies containing positively staining cells were scored as "blue" colonies. The number of "blue" and "white" (unstained) colonies arising from the mixed wells could therefore be counted separately (fig 15) and individual survival curves for the two cell populations derived. The counts of blue and white colonies could also be summed to produce an overall survival curve for the mixed wells.

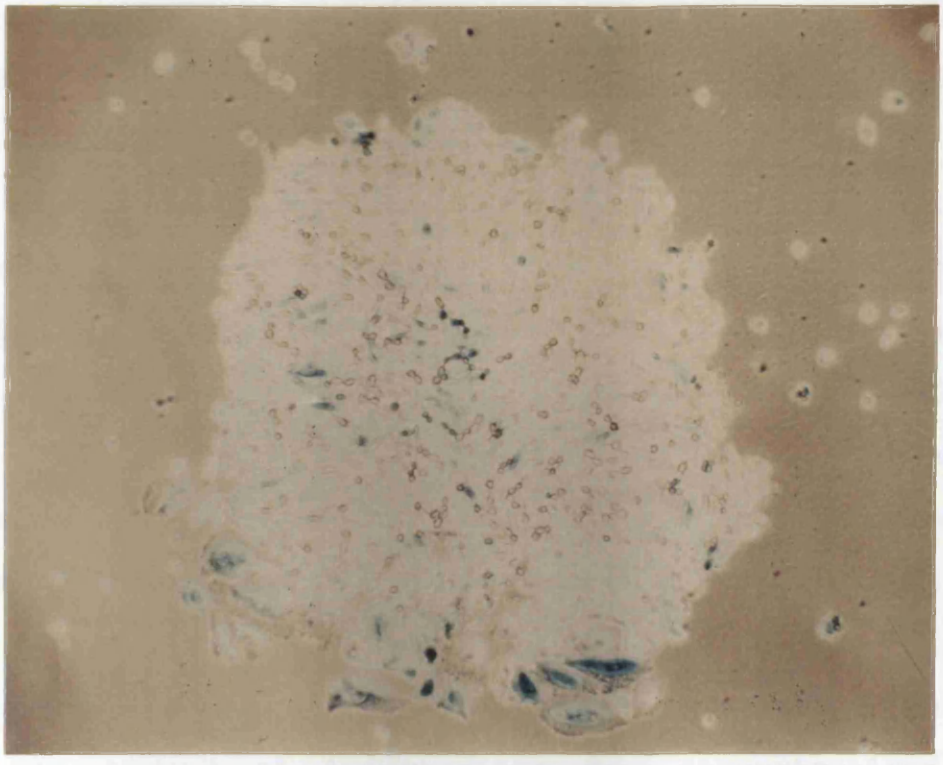
As a control, the colonies arising from wells containg CHO-K1-BG alone were also stained for ß-gal. These showed a similar range of staining intensity and frequency within the individual colonies. In addition it was established that a small proportion of these colonies failed to show any ß-gal staining and in mixed culture

Figure 15
ß-galactosidase as a lineage marker in coculture studies

(a) In dishes derived from mixed wells, colonies staining positively (blue) and negatively (white) for ß-gal can be clearly distinguished; (b) variability of ß-gal expression within individual colony.



(a)



(b)

would therefore be counted as a white colony and would be erroneously attributed to the unmarked lineage. In a series of six experiments the percentage of positive staining of CHO-K1-BG colonies varied from 97.9 to 99.3% suggesting that the error was small enough not to significantly to reduce the validity of this technique.

4.3.5.2. Monolayer coculture: adriamycin

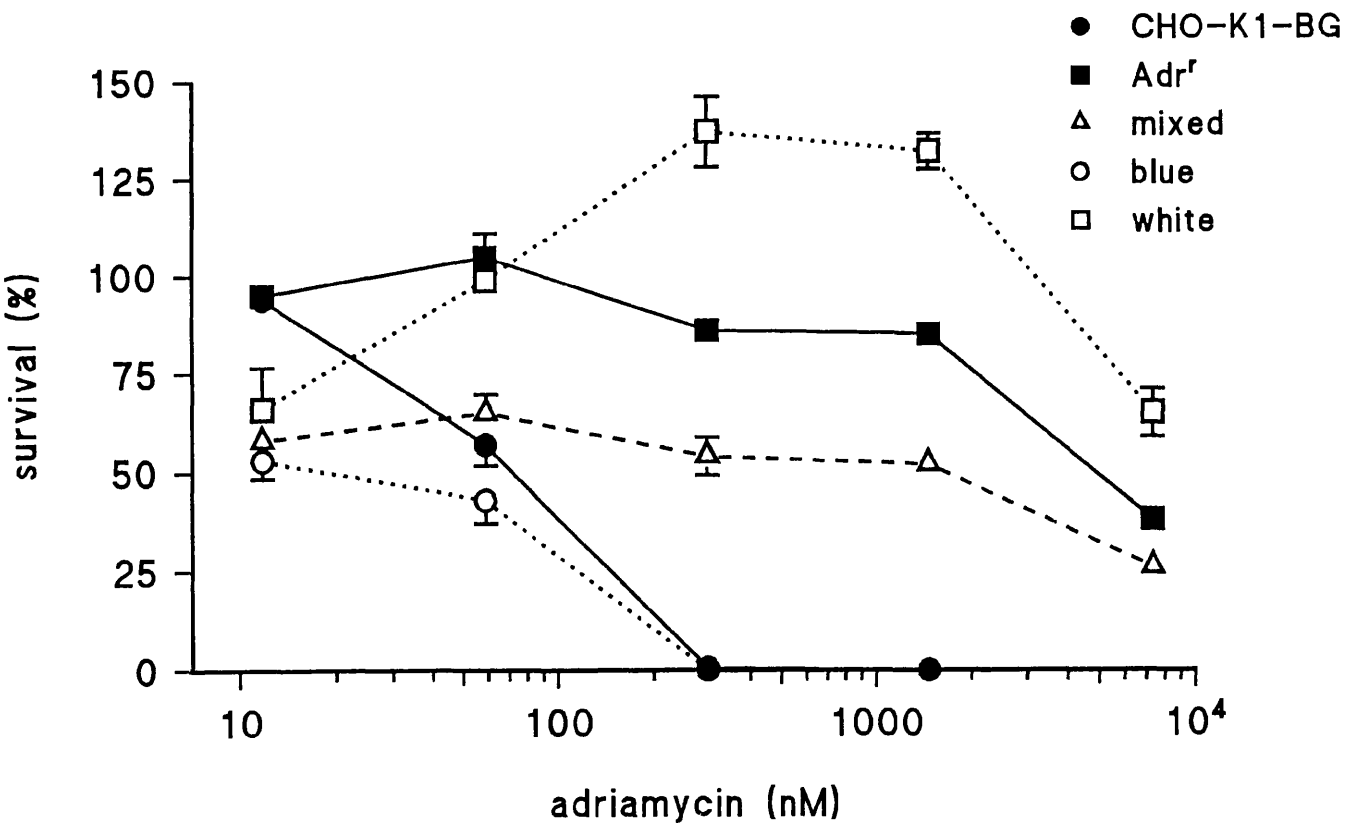
Survival curves following adriamycin exposure of CHO-K1-BG alone, Adr^r alone and a 50:50 mixture of the two cell types were generated (fig.16). The curves for CHO-K1-BG and Adr^r are clearly separated, confirming their differing chemosensitivity. Following exposure to 295 and 1472nM adriamycin the survival of Adr^r is almost unaffected, whereas that of CHO-K1-BG is reduced to less than 1% of control at the lower drug concentration, with no survival at the higher concentration.

The overall survival of the 50:50 mixture shows intermediate values, but by separately counting blue and white colonies following ß-gal staining the survival curves for the individual cell lines within this mixed culture can be obtained. The survival of blue colonies from the mixed culture is similar to that of CHO-K1-BG, with no survival at 295nM adriamycin and above. In contrast the curve of white colonies shows apparent increased survival at intermediate drug concentrations,

Figure 16
Coculture of CHO-K1-BG and Adr^r: monolayer clonogenic assay, adriamycin

10^5 cells/well (CHO-K1-BG alone, Adr^r alone or a 50:50 mixture of the two cell types (mixed)) were added to a 24 well plate and incubated at 37^oC for 24 hours, then the medium was replaced with fresh medium containing serial dilutions of drug. Following a further 24 hours incubation, the medium and drug were removed and 200µl 0.25% trypsin solution was added to each well. After 10 minutes incubation 1.8ml/well of medium was added and the cells separated to a single cell suspension by repeated pipetting, then counted on a Coulter counter and 5×10^2 cells/well were plated into triplicate 60mm petri dishes, incubated for 7 days and then stained for ß-galactosidase (section 2.5.4.4.). Colonies > 50 cells were counted and their survival expressed as a percentage relative to control wells without drug. In dishes derived from mixed wells, colonies staining positively (blue) and negatively (white) for ß-gal were also counted separately. Points represent the mean (+/- SEM) of 6 dishes from 2 separate experiments. Error bars omitted if dimensions less than that of symbols.

FIGURE 16
coculture of CHO-K1-BG and Adr^r
monolayer clonogenic assay
adriamycin



with survival falling again only at the highest concentration.

The shape of this latter curve can be explained by consideration of the methodology involved. Following 24 hour drug exposure the number of surviving cells in each well are counted and 5×10^2 cells from each well plated into dishes for colony formation. At these drug concentrations the survival and growth of CHO-K1-BG is reduced in comparison to Adr^r (fig.17) as shown by the lower cell count following drug exposure. Thus in mixed wells, in the presence of drug, the proportion (and absolute number) of Adr^r in cells plated out is increased compared to control wells without drug, resulting in the apparent increase in Adr^r survival at these concentrations.

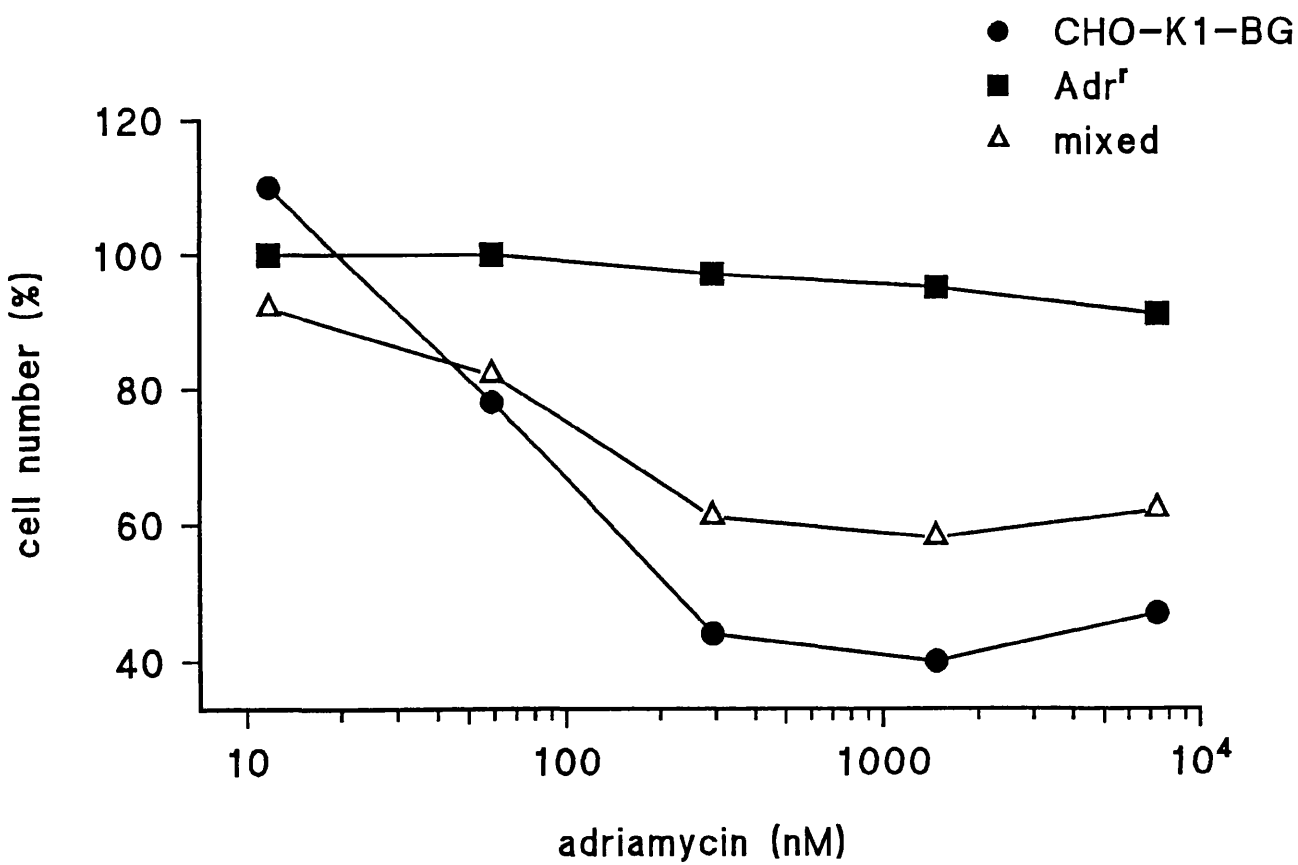
The corollary of this is that a corresponding apparent reduction in survival of CHO-K1-BG cells at these drug concentrations would be expected. As their clonogenic survival was virtually zero at this point, the influence of this factor on the survival curve is minimal.

Therefore, in summary, the use of a 24 hour period of drug exposure allows differential growth of the two cell types in the presence of drug which limits the accuracy of the derived survival curve of the more resistant cell line in mixed culture. However, the survival curve of the more sensitive line is relatively unaffected and its

Figure 17
Coculture of CHO-K1-BG and Adr^r: cell counts after 24 hour drug exposure

10^5 cells/well (CHO-K1-BG alone, Adr^r alone or a 50:50 mixture of the two cell types (mixed)) were added to a 24 well plate and incubated at 37°C for 24 hours, then the medium was replaced with fresh medium containing serial dilutions of drug. Following a further 24 hours incubation, the medium and drug were removed and 200µl 0.25% trypsin solution was added to each well. After 10 minutes incubation 1.8ml/well of medium was added and the cells separated to a single cell suspension by repeated pipetting, then counted on a Coulter counter. Cell number expressed as a percentage relative to control wells without drug.

FIGURE 17
coculture of CHO-K1-BG and Adr^r
cell counts after 24h drug exposure



close similarity to that of CHO-K1-BG alone is indicative of the absence of interaction between the two lines with respect to chemosensitivity.

4.3.5.3. Monolayer coculture: mitozantrone

Further experiments were performed with the anthracenedione, mitozantrone, to which Adr^r is cross-resistant, to determine whether there was any evidence of interaction between CHO-K1-BG and Adr^r with respect to mitozantrone chemosensitivity.

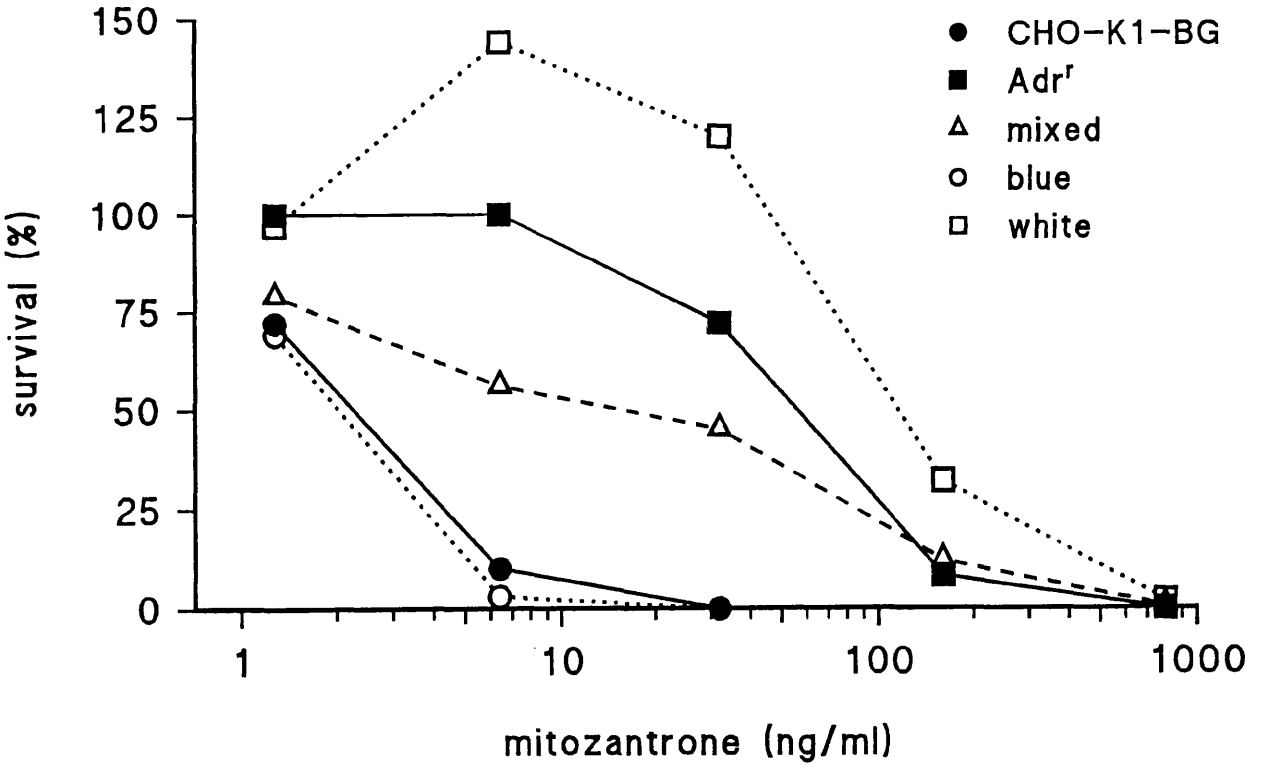
The pattern of survival curves obtained following 24 hour drug exposure (fig.18) shows a close similarity to that of adriamycin (fig.16). The curves for CHO-K1-BG and Adr^r are widely separated showing a marked difference in chemosensitivity, whilst the overall survival of the 50:50 mixture had intermediate values.

When counted individually, the survival of blue colonies from the mixed culture closely parallels that of CHO-K1-BG alone, and there is again an apparent increase in survival of white colonies at intermediate drug concentrations. There was therefore no evidence of transfer of mitozantrone resistance between the two cell lines.

Figure 18
Coculture of CHO-K1-BG and Adr^r: monolayer clonogenic assay, mitozantrone

Method identical to that described for figure 16. Survival expressed as a percentage relative to control wells without drug. In dishes derived from mixed wells colonies staining positively (blue) and negatively (white) for ß-gal were also counted separately. Points represent the mean of 6 dishes from 2 separate experiments. Error bars omitted for clarity as dimensions of majority less than those of symbols.

FIGURE 18
coculture of CHO-K1-BG and Adr^r
monolayer clonogenic assay
mitozantrone



4.3.5.4. Monolayer coculture: vincristine

As the above studies with adriamycin and mitozantrone had shown no evidence of resistance transfer between Adr^r and CHO-K1-BG, it was decided to investigate whether such an interaction was present when CHO-K1-BG and the drug-hypersensitive cell line ADR-6 were combined in mixed culture. For these studies it was necessary to use a third drug from the MDR group, vincristine.

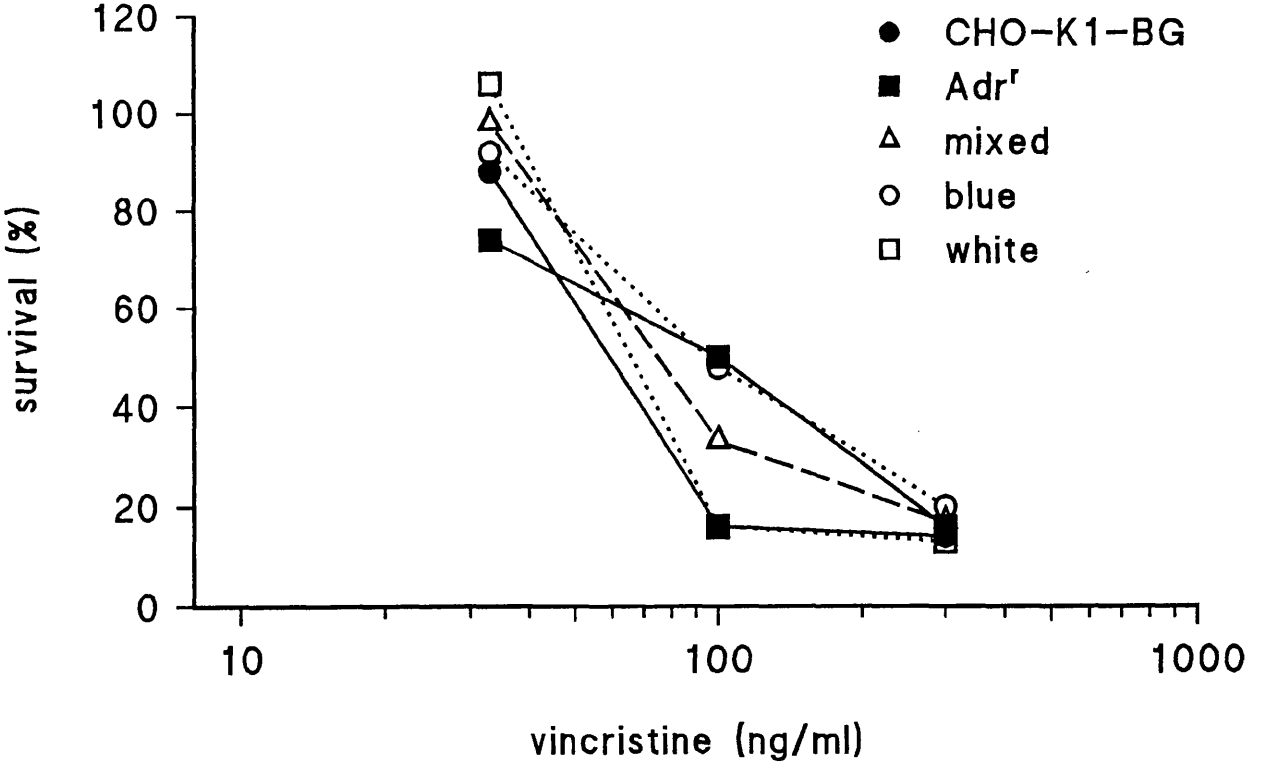
ADR-6 had previously been found to be only 5-fold more sensitive to adriamycin than CHO-K1, but to be approximately 12-fold more sensitive to vincristine (I.Hickman, unpublished data). In order to clearly detect any interaction between the cell lines it is desirable that the magnitude of the chemosensitivity difference and therefore the separation of survival curves is as great as possible. For these experiments with vincristine, ADR-6 is therefore the "sensitive" cell line and CHO-K1-BG is now the more resistant of the two cell lines in mixed culture.

The resulting survival curves show the difference in chemosensitivity between the two lines when cultured individually to be smaller than that predicted, with the IC_{50} of CHO-K1-BG being less than twice that of ADR-6 (fig.19). At a vincristine concentration of 100ng/ml however there is a clear separation between the two survival curves, with a 50% survival of CHO-K1-BG

Figure 19
Coculture of CHO-K1-BG and ADR-6: monolayer clonogenic assay, vincristine

Method identical to that described for figure 15 except that wells contained either CHO-K1-BG alone, ADR-6 alone or a 50:50 mixture of the two cell types (mixed). Survival expressed as a percentage relative to control wells without drug. In dishes derived from mixed wells colonies staining positively (blue) and negatively (white) for ß-gal were also counted separately. Points represent mean of 6 dishes from 2 separate experiments. Error bars omitted for clarity.

FIGURE 19
coculture of CHO-K1-BG and ADR-6
monolayer clonogenic assay
vincristine



compared to a 17% survival of ADR-6. The overall survival of the mixed population has an intermediate value (33%). When the number of blue and white colonies are counted separately, however, their respective survival is virtually identical to that of CHO-K1-BG and ADR-6 alone. There is again, therefore, no evidence of interaction between the two cell types under these conditions.

4.3.5.5. Spheroid coculture

4.3.5.5.1. Introduction

In view of the absence of interaction between the cell lines in monolayer culture, and in order to ascertain whether the increased intercellular contact provided by three dimensional culture is a necessary condition for interaction to be manifest, culture of CHO-K1-BG and Adr^r as multicellular spheroids was established (section 2.5.6.).

In comparison with monolayer, spheroid culture was associated with additional complications which had to be considered in the design of coculture studies. As culture as spheroids entailed growth of cells for six days between spheroid initiation and disaggregation for clonogenic assay, the differential growth rates of the two cell lines would be expected to lead to overgrowth of CHO-K1-BG relative to Adr^r in mixed spheroid culture. A

further complication was the difference in the ease with which the two cell lines will aggregate and form spheroids.

Adr^r readily formed relatively compact spheroids, whilst CHO-K1-BG tended to produce more irregular aggregations with numerous smaller clumps and individual cells. Moreover, in initial experiments at least, this longer period of growth of CHO-K1-BG cells in non selective medium compared to earlier monolayer assays led to loss of ß-gal activity, so that the percentage of CHO-K1-BG colonies which failed to show positive ß-gal staining increased markedly compared to that seen in the earlier monolayer experiments.

In a preliminary experiment, only 88.6% of colonies derived from CHO-K1-BG spheroids showed positive ß-gal staining. Continued passage of the CHO-K1-BG cell line, however, appeared to produce increased stability of the ß-gal phenotype. The experiments described in section (4.3.5.5.4.) were performed over the following two months and the percentage of positively staining colonies from the pure CHO-K1-BG spheroids increased to 99.3%, i.e. to a higher value than that seen in earlier monolayer studies.

4.3.5.5.2. Initiation ratio of mixed spheroids

It was thererfore necessary to establish the most appropriate ratio of CHO-K1-BG : Adr^r to be used in initiation of mixed spheroids, in order that at the time of drug exposure an approximately 50:50 ratio would exist. Six day old spheroids initiated from CHO-K1-BG cells alone and from varying ratios of CHO-K1-BG : Adr^r were therefore disaggregated and plated out to allow colony growth, and the ratio of blue to white colonies calculated following ß-gal staining. The results (table 8) suggest that an initiation ratio of CHO-K1-BG : Adr^r of between 1:4 and 1:8 would produce approximately equal numbers of the two cell types in the resulting mixed spheroids, although the results of this assay were again complicated at this stage in the work by the low percentage of positive ß-gal staining in the colonies derived from pure CHO-K1-BG spheroids.

On the basis of their growth rate in monolayer culture (section 4.3.4.5.) CHO-K1-BG would be expected to undergo 10.4 divisions in the six days of spheroid growth, compared to 8.9 divisions for Adr^r, and therefore the calculated optimum ratio of CHO-K1-BG : Adr^r for spheroid initiation would be approximately 1:3. As both the calculations based on growth rate and the ratio suggested by the above study entail a considerable margin of error, a compromise ratio of 1:5 was selected for the subsequent mixed spheroid studies.

Initiation ratio CHO-K1-BG:Adr^r	Number of colonies blue	white	ratio blue:white
1:1	285	67	4.25
1:4	527	329	1.6
1:8	335	347	0.97
1:12	83	311	0.27

Table 8
Effect of initiation ratio of CHO-K1-BG:Adr^r on composition of mixed spheroids

Six day old spheroids were prepared from CHO-K1-BG cells alone and from ratios of CHO-K1-BG : Adr^r of 1:1, 1:4, 1:8 and 1:12. Ten spheroids from each group were transferred to one well of a 24 well microtitration plate. 0.5ml of 0.25% trypsin solution was then added to each well and the plate was incubated at 37^{o}C for 10 minutes. The spheroids were disaggregated to a single cell suspension by repeated pipetting and 1.5ml/well of medium added. 50μl aliquots of this suspension were then added in triplicate to new 60mm dishes, incubated for 7 days then fixed and stained for ß-gal (section 2.5.4.4.) and the number of colonies > 50 cells staining positively (blue) and negatively (white) for ß-gal were counted separately. Figures represent the mean of 3 dishes.

4.3.5.5.3. Confirmation of heterogeneity within individual spheroids

In addition to compensating for the differential growth rate of CHO-K1-BG and Adr^r in mixed spheroids, it was also necessary to confirm that each individual spheroid consisted of an approximately equal mixture of the two cell types, and that following spheroid initiation the cells did not preferentially form relatively homologous spheroids consisting exclusively or predominantly of a single cell type. If the "mixed" spheroid flask contained two separate populations of relatively pure CHO-K1-BG or pure Adr^r spheroids then the potential for interaction would be severely limited.

To investigate this possibility "mixed" spheroids initiated from a 1:5 ratio of CHO-K1-BG : Adr^r were disaggregated individually and plated out to allow colony growth. Following ß-gal staining the number of blue and white colonies were counted separately. In a series of 10 spheroids of differing sizes (table 9), the percentage of blue colonies varied from 31-64% with a mean value of 48.4%, confiming that the mixed spheroids examined contained approximately equivalent numbers of the two cell types. The mean value also confirms that selection of the 1:5 ratio of CHO-K1-BG : Adr^r used in spheroid initiation was appropriate and succeeded in compensating for the differential growth rate of the two cell lines.

Spheroid	Number of colonies blue	white	% blue
1	58	45	56.3
2	109	96	53.2
3	16	9	64.0
4	37	49	43.0
5	68	78	46.6
6	35	32	52.2
7	39	40	49.4
8	38	59	39.2
9	20	44	31.2
10	86	88	49.4

Table 9
Confirmation of heterogeneity within individual spheroids

Ten 6 day old spheroids initiated from a 1:5 ratio of CHO-K1-BG:Adr^r were each transferred to a single well of a 24 well dish. 0.5ml of 0.25% trypsin solution was then added to each well and the dish was incubated at 37^{o}C for 10 minutes. The spheroids were disaggregated to a single cell suspension by repeated pipetting and 1.5ml/well of medium added. 200μl aliquots from each well were then added to new 60mm dishes, incubated for 7 days, then fixed and stained for ß-gal (section 2.5.4.4.) and the number of colonies > 50 cells staining positively (blue) and negatively (white) for ß-gal were counted separately.

4.3.5.5.4. Mixed spheroid clonogenic assay: adriamycin

A standard clonogenic assay was performed following adriamycin exposure of three sets of spheroids: one initiated from CHO-K1-BG cells alone, one from Adr^r cells alone and one from a 1:5 mixture of CHO-K1-BG and Adr^r. As before, ß-gal staining of the resulting colonies allowed identification of the individual survival of the two cell types from mixed spheroids. As in monolayer culture, the survival of Adr^r was substantially greater than that of CHO-K1-BG, whereas mixed spheroids showed intermediate survival (table 10).

When the survival of blue colonies from the mixed spheroids was assessed separately, however, this parallelled that of CHO-K1-BG alone. Similarly the survival of white colonies was comparable to that of Adr^r alone. In summary, therefore, there was no evidence from these studies that three dimensional culture of CHO-K1-BG and Adr^r in mixed spheroids produces any interaction leading to an alteration in the distinct adriamycin sensitivity of the two lines.

4.3.6. Gap junctional communication between CHO cells

4.3.6.1. Introduction

Intercellular communication via gap junctions has been reported to occur between CHO cells (Weiner and

	Survival (%)	
	2μM adriamycin	10μM adriamycin
CHO-K1-BG	11	0
Adr^r	89	55
mixed	38	32
blue	1	0
white	66	56

Table 10
Coculture of CHO-K1-BG and Adr^r: Spheroid clonogenic assay

5×10^5 cells were seeded into base coated flasks: CHO-K1-BG alone, Adr^r alone or a 1:5 mixture of the two cell types. After 5 days incubation at 37°C, 1.25ml medium containing serial adriamycin dilutions was added to each flask, and after a further 24 hours incubation the medium and spheroids from each flask were transferred to 60mm dishes and 10 spheroids from each dish were then transferred to one well of a 24 well plate. 0.5ml of 0.25% trypsin solution was then added to each well and the dish was incubated at 37°C for 10 minutes. The spheroids were disaggregated to a single cell suspension by repeated pipetting and 1.5ml of medium added. 20μl aliquots of this suspension were then added in triplicate to new 60mm dishes and incubated for 7 days, then fixed and stained for ß-gal (section 2.5.4.4.) and the number of colonies > 50 cells were counted and expressed as a percentage of control in the absence of drug. In those dishes derived from mixed spheroids, colonies staining positively (blue) and negatively (white) for ß-gal were counted separately.

Lowenstein, 1983). For the purposes of the present study it was however necessary to confirm that communication could be demonstrated in both the parent line, CHO-K1, and in its variants Adr^r, ADR-6 and CHO-K1-BG.

4.3.6.2. Microinjection

Communication was initially assessed in CHO-K1 and Adr^r by iontophoretic microinjection of Lucifer Yellow into individual cells and observation of the extent of dye spread to neighbouring cells (section 2.6.).

The degree of spread observed varied widely between individual injections. Following injection of some cells, no spread was detected over a five minute period of observation, whilst with others there was a rapid passage of dye to all surrounding cells. Of 22 separate injections of CHO-K1 cells performed, 15 (68%) produced spread of dye to two or more contiguous cells indicative of functional coupling (gap junctional communication could not be inferred from spread to one cell alone, as this could arise as a result of incomplete mitotic separation). Of 13 separate injections of Adr^r cells, dye spread to two or more cells was seen on 7 occasions (54%).

Comparison of these results with microinjection of L-DAN cells, which was one of the best coupled of the human NSCLC cell lines on the basis of the nucleotide transfer

method, is of interest. Dye spread to two or more cells was seen following only one of six injections of L-DAN. This contrast in percentage coupling values of L-DAN cells obtained by nucleotide transfer (table 4) and microinjection techniques suggests that nucleotide transfer may be the more sensitive of the two methods of assessing coupling.

4.3.6.3. Nucleotide transfer

Both homologous and heterologous communication between the CHO lines was confirmed by the use of nucleotide transfer. Using CHO-K1-BG as donor cells and ADR-6, Adr^r or CHO-K1-BG itself as recipient cells, examination of the resulting autoradiographs showed extensive transfer of ^3H nucleotides from donor to adjacent recipient cells. The extent of nucleotide transfer was not quantified in the manner described for NSCLC lines in Chapter 3, although the earlier microinjection studies indicated that communication in CHO lines was at least as good as one of the best coupled NSCLC lines, L-DAN.

In summary, therefore, these studies have confirmed that the previously reported gap junctional communication between CHO cells (Weiner and Lowenstein, 1983) is demonstrable by both microinjection and nucleotide transfer techniques in the cell lines used in the present study. Gap junctional passage of free small molecules would therefore be expected between the cells in mixed

monolayer and spheroid culture. Consequently poor intercellular communication between sensitive and resistant cells would not seem to explain the absence of interaction observed in mixed culture experiments.

4.4. Passage of adriamycin through gap junctions

4.4.1. Introduction

The model proposed in section 1.7.4. as a mechanism of resistance transfer is dependent on the free passage of drug from cell to cell through gap junctions along diffusion gradients. Thus the P-glycoprotein efflux pump in the membrane of an MDR cell could act to regulate the cytoplasmic drug concentration both in the MDR cell itself and in a sensitive cell to which it is linked by gap junctions and thus protect both cells from drug induced damage.

The molecular weight exclusion limit for passage through mammalian junctions has been calculated to be approximately 900 (Flagg-Newton, Simpson and Loewenstein, 1979) and therefore adriamycin (M.W. 543) and mitozantrone (M.W. 517.4) should pass without difficulty. Vincristine (M.W. 824.9) is close to the exclusion limit, where the ease of passage is uncertain and may depend on the molecular configuration of the drug. The estimation of the exclusion limit is based on the passage of fluorescent probes through junctions, and as adriamycin,

in common with other anthracyclines, displays fluorescence it seemed appropriate to exploit this property to confirm that drug passage through junctions does occur.

4.4.2. Microinjection

Microinjection of adriamycin into individual BRL cells was performed in order to monitor the spread of drug associated fluorescence to adjacent cells as described with Lucifer Yellow in the preceding section.

As adriamycin is positively charged at physiological pH and would therefore require a positive current pulse for iontophoresis with consequent depolarisation of the injected cell, a positive pressure injection system was investigated for adriamycin microinjection although this had the disadvantage of entailing a substantial increase of perhaps 10-20% in cell volume during the injection procedure.

Unfortunately, microinjection of the drug produced additional technical and interpretative problems. In a series of injections attempting to demonstrate junctional spread of adriamycin from the injected cell, it was never possible to unequivocally confirm such drug transfer.

This difficulty was attributable to a number of disadvantageous features of adriamycin with regard to

microinjection studies. A large number of dyes have been investigated for this purpose. Very few are suitable, but several characteristics of Lucifer Yellow have led to its selection as an agent of choice in the study of gap junction mediated dye transfer. In addition to its molecular weight these include its intense fluorescence, negative charge at physiological pH and inability to traverse intact cell membranes. (El Fouly, Trosko and Chang, 1987).

Adriamycin is a less satisfactory agent in several respects. Its fluorescence is much less intense which complicates adequate monitoring of injections. The ability of adriamycin to enter cells by passive diffusion across the cell membrane is a further adverse characteristic of the drug. During microinjection, particularly with the positive pressure system necessary for adriamycin, there is a variable amount of leakage of dye or drug into the medium bathing the cells. With Lucifer Yellow this is not problematic as the dye is unable to enter the cells by this route. In the case of adriamycin however there is some direct uptake of drug across the cell membrane from the bathing medium. This leads to a background level of cellular fluorescence which is particularly marked in those cells nearest to the injected cell, and which therefore masks the detection of any junction mediated spread of drug from the injected cell. On the few occasions when fluorescence was clearly observed in one or more

neighbouring cells after adriamycin microinjection, direct uptake of drug from the medium could not be excluded. In view of these problems, it was not possible to draw any conclusions about cell-cell transfer of adriamycin through gap junctions on the basis of these studies.

4.4.3. Scrape loading.

In view of the problems associated with microinjection of adriamycin an alternative technique was investigated in an attempt to demonstrate junctional transfer of the drug.

Scrape loading is a method of promoting cellular uptake of dye or other substances by producing a transient tear in the plasma membrane, through which the dye can gain entry to the cell (McNeil et al., 1984). If the tear is sufficiently small the membrane defect is rapidly repaired and there is no loss of cell viability. El Fouly, Trosko and Chang (1987) adopted this technique as a means of demonstrating gap junctional transfer of Lucifer Yellow from scrape loaded cells into contiguous cells. In the present experiments, as in their original study, the concurrent introduction of a high molecular weight dye, rhodamine dextran (M.W. 10,000), which cannot traverse gap junctions was also employed. This dye remains localised in those membrane-damaged cells at the edge of the scrape and permits their identification.

Following scrape loading of BRL cells with 0.5% Lucifer Yellow and 0.1% rhodamine dextran (section 2.7.), uptake of both dyes was clearly visible in those cells at the edge of the scrape. Spread of lucifer yellow was also observed across several cell widths distance from the line of scrape, confirming coupling of the cells. When scrape loading was attempted with 0.05% adriamycin, fluorescence was also most marked in cells at the edge of the scrape, but uptake of adriamycin from the medium produced background fluorescence in all other cells on the coverslip, thus masking any evidence of junctional transfer.

Modification of the experimental conditions in an attempt to overcome this problem was unsucessful: reduction in the duration of exposure of the cells to drug containing medium did not substantially reduce the background fluorescence, and execution of the procedure using ice cold medium to increase drug uptake by reducing the rate of membrane repair and therefore increase the fluorescence intensity of scrape loaded relative to background cells also produced no improvement.

In summary, therefore, it was not possible with either microinjection or scrape loading techniques to confirm whether or not adriamycin can pass through gap junctions. A major limitation of both methods was background fluorescence secondary to direct diffusion of drug into cells from the bathing medium.

4.5. Discussion

4.5.1. Introduction

Numerous examples have been reported in support of the occurence of interaction between phenotypically distinct cellular subpopulations (Heppner, Miller and Miller, 1983). In some cases where the interaction is between drug sensitive and resistant subpopulations the mechanism underlying this phenomenon is clear, as in the cases of transfer of thioguanine sensitivity (Fujimoto et al., 1971) or of ouabain resistance (Corsaro and Migeon, 1977). In other cases the mechanism of interaction remains obscure or poorly defined (Miller, Miller and Heppner, 1981; Tofilon, Buckley and Deen, 1984; Tofilon, Arundel and Dean, 1987).

The rationale for the present study is based upon presently available knowledge of the role of the mdr1 gene and P-glycoprotein as mediators of the MDR phenotype. If cellular resistance is dependent on active drug efflux from the cell by the P-glycoprotein membrane pump then in a pair of sensitive and resistant cells coupled by gap junctions, free junctional passage of drug between the cells should produce a near-uniform drug concentration in each cell. The P-glycoprotein pump in the resistant cell would therefore contribute to the reduction of intracellular drug concentration, and therefore to the resistance, of both cells.

This study has examined the functional consequences in a number of in vitro systems of mixed culture of sensitive and MDR CHO cells. For each assay system and each member of the MDR group of drugs studied there has been no evidence of interaction in the series of experiments reported here. The clonogenic assays following genetic marking of the parent line to produce CHO-K1-BG would have been expected to be particularly sensitive to any alterations in chemosensitivity resulting from the coculture. The ease of identification of marked colonies of the sensitive line CHO-K1-BG and the wide separation of the survival curves of CHO-K1-BG and Adr^r allowed drug concentrations to be selected where there was little or no survival of CHO-K1-BG but where the survival of Adr^r was virtually unaffected. In this situation an increase in resistance of a single CHO-K1-BG cell towards of Adr^r would have been detected by its survival and growth to form a blue colony.

4.5.2. Alternative explanations for absence of interaction

4.5.2.1. Inhomogeneity of cell distribution

One factor which could diminish the potential for interaction in these experiments would be preferential clustering of the two cell types. If this resulted in a non-random distibution of cells and produced discrete areas consisting predominantly of a single cell type

either in monolayer or spheroid, the opportunity for heterologous junction formation would clearly be restricted.

Such zonal distribution of cellular subpopulations has frequently been observed in vitro (Heppner, Miller and Miller, 1983), but the extent of this in the present study has not been fully explored. Extensive clustering seems unlikely in monolayer in view of the brief period of co-culture before drug exposure. In spheroid culture, although the precise spatial distribution of cells in the intact spheroid was not assessed, the heterogeneous composition of individual spheroids was clearly confirmed. It was not considered necessary to undertake sectioning of spheroids to examine the distribution further.

Although mixing of the two cell types may not be optimum at all sites, at some locations at least CHO-K1-BG cells must be adjacent to and in communication with a number of Adr^r cells. As discussed above, the assay method should have been sufficiently sensitive to demonstrate any increase in survival, even if only a small proportion of CHO-K1-BG cells came into this category.

4.5.2.2. Duration of cell-cell contact

One further limitation of the clonogenic assay is that the two cell types are only in contact before and during

the period of drug exposure. Following this they are separated into a single cell suspension and plated out at low density to allow colony growth. For the proposed model of resistance transfer based on the P-glycoprotein efflux mechanism, this arrangement might be expected to be adequate for interaction as separation of the two populations following drug removal would not interfere with this process. If, however, some further mechanism such as mutual support of cells with drug induced damage by sharing of intermediary metabolites between sensitive and resistant cells was necessary for increased survival, then continued contact following drug exposure might be necessary for optimum limitation of or repair of the damage.

As discussed in section 3.4.4. with regard to the NSCLC study, a spheroid growth delay assay would allow two populations to continue to grow in contact and might be considered to offer advantages over the present assay. The major limitation of a spheroid growth delay assay, however, is that it would only be able to assess the overall response of the mixed population and its use would forfeit the sensitivity conferred by genetic marking with ß-gal for identification of the individual response of the two populations.

4.5.2.3. Adequacy of communication

Inadequate gap junctional communication in either the two or three dimensional systems studied could also provide an explanation for the absence of interaction.

Communication between CHO cells was confirmed in monolayer culture by both dye injection and nucleotide transfer techniques. There is ample evidence that the communication which exists between coupled cells in monolayer culture is sufficiently extensive to allow functional consequences such as transfer of 6-thioguanine or ouabain resistance to be clearly evident (Fujimoto et al., 1971; Corsaro and Migeon, 1977). Some reduction in junctional communication in spheroids compared to monolayer has been demonstrated in other cell types (Dertinger, Hinz and Jacobs, 1982; Sutherland, 1986) but the effect of culture of CHO cells in multicellular spheroids on their junctional communication relative to monolayer was not assessed in the present study.

It seems unlikely, however, that this three dimensional arrangement would substantially diminish interaction. Miller and colleagues (1990) have recently described, using ouabain sensitive and resistant cell lines from a mouse mammary tumour growing in three dimensional array in collagen gel culture, that a resistant subpopulation comprising as little as 5% of the total cell number is

sufficient for metabolic cooperation and transfer of ouabain resistance.

This finding shows that extensive junctional communication can exist in three dimensional culture. If these results can be extrapolated to spheroid culture, they suggest that the potential for metabolic cooperation between two populations is sufficiently great so as to be unlikely to be affected by zonal distribution or limited heterogeneity within individual spheroids. A further implication is that if transfer of cytotoxic drug resistance within a coupled tumour can occur, then a relatively small resistant population within a predominately sensitive tumour may have a pronounced effect on its drug response.

4.5.2.4. Drug passage between cells

Although the ease of passsage of vincristine through gap junctions may be uncertain on the basis of its molecular weight, adriamycin and mitozantrone would be expected to pass freely, although as it has not been possible to confirm this experimentally, some doubt must remain regarding these drugs also.

In addition, before the drugs can pass from sensitive to resistant cells they must gain free access to the cytoplasm of the sensitive cell. If such access is associated with either irreversible binding or

irreparable damage to key intracellular sites of action, either directly or through free radical generation, subsequent passage of drug to the resistant cell would have no effect on the extent of damage sustained by the sensitive cell.

It is possible that compartmentalisation of intracellular adriamycin could reduce the availability of freely diffusible drug for junctional passage between cells. In addition, such compartmentalisation would restrict the capacity of MDR cells to rescue coupled sensitive cells.

Differences have been reported in cellular distribution of drug in MDR compared to sensitive cells. In adriamycin cytofluorescence studies in colon cancer lines, Chauffert et al. (1984) have shown predominantly nuclear localisation of adriamycin in sensitive cells, whereas MDR cells exhibited increased, possibly lysosomal, cytoplasmic fluorescence but reduced nuclear fluorescence. These observations are consistant with increased lysosomal/endosomal trapping of drug in MDR cells (Sehested et al., 1987a) reducing drug access to the nucleus.

If P-glycoprotein-associated drug efflux in MDR cells is mediated by drug accumulation in the endosomal compartment and subsequent exocytosis (Sehested et al., 1987a), it seems likely that this drug efflux mechanism would act principally on drugs presenting via the

extracellular route, and that this mechanism would be less suited to dealing with drug delivery into the cytoplasm via the junctional route. In mixed culture such a mechanism would be expected to allow transfer of sensitivity rather than of resistance between coupled cells, as the drug would gain indirect access to the resistant cell cytoplasm from an adjacent sensitive cell via the gap junctional route.

No evidence of such transfer was observed in this study although, in view of the overshoot in the survival curve of the more resistant cells inherent in the assay technique, only a relatively large effect would be evident, and this possibility cannot, therefore, be totally discounted.

4.5.2.5. Multiplicity of resistance mechanisms

The CHO cells examined here were selected for study in view of the known increased expression of mdr1 gene and P-glycoprotein in the resistant line Adr^r. Further characterisation of Adr^r has since revealed that although there is a 5-fold increase in mdr1 expression, 2-fold increase in drug efflux and 5-fold reduction in drug accumulation (assessed with ^3H daunomycin), it also exhibits a 4-fold reduction in topoisomerase II (topo II) activity compared to the parent line CHO-K1 (I. Hickson, personal communication). There is no significant difference in GST activity between the two lines.

Thus the chemosensitivity differences of the cell lines may not be wholly attributable to their documented differences in intracellular drug concentration. If the reduction in topo II activity of Adr^r has a major influence on its chemosensitivity, this form of drug resistance seems unlikely to be amenable to transfer to adjacent sensitive cells. Those chemosensitivity differences attributable to topo II should be unaffected by intercellular passage of drug between coupled cells. Reduction of either levels or activity of the enzyme affords fewer opportunities for drug binding to stabilise the cleavable complex and produce double strand breaks. Thus less damage is sustained by the cell.

It is possible that drug transfer through gap junctions and changes in intracellular drug distribution do occur as a consequence of mixed culture of CHO-K1-BG and Adr^r but that such changes have a relatively small role to play in their relative drug resistance. If this were the case, it could account for the apparent absence of interaction in the present study.

Further studies (I. Hickson, personal communication) with the hypersensitive line ADR-6 have demonstrated a reduction in active drug efflux, even compared to the relatively modest levels observed in CHO-K1. The contribution of this reduction to the chemosensitivity of ADR-6 is not clear. No information is available on the mdr1 expression of this line.

The coincidence of multiple and distinct mechanisms of resistance in MDR as well as other forms of cytotoxic drug resistance is increasingly recognised (Kaye, 1988). The presence of several potential mechanisms in Adr^r complicates its use in the present study. Resistance resulting purely from mdr1 overexpression (or perhaps from alteration in GST activity, where glutathione transfer through junctions may play some role) might potentially be transferred to a sensitive cell, but this would not seem possible in the case of topo II mediated resistance, as there seems no clear route through which the topo II enzyme activity of one cell can influence the activity of another.

Perhaps the ideal model for investigation in the present study would have been a cell line whose resistance was conferred by transfection of the mdr1 gene and where drug extrusion by the P-glycoprotein pump was the sole mechanism of resistance. It now seems likely, however, that tumour resistance <u>in vivo</u> is frequently more complex.

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