Int. J. Cancer: **71,** 907–915 (1997)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

ISOLATION FROM A HUMAN MDR LUNG CELL LINE OF MULTIPLE CLONAL SUBPOPULATIONS WHICH EXHIBIT SIGNIFICANTLY DIFFERENT DRUG RESISTANCE

Mary HEENAN*, Lorraine O'DRISCOLL, Irene CLEARY, Lisa CONNOLLY and Martin CLYNES

National Cell and Tissue Culture Centre/BioResearch Ireland, Dublin City University, Glasnevin, Dublin, Ireland

The heterogeneous nature of an adriamycin-selected human MDR squamous lung cell line, DLKP-A, was investigated by isolating and characterising 9 of its clonal subpopulations. The DLKP-A cell line exhibits resistance to the classical MDR drugs, overexpresses P-glycoprotein and displays reduced topoisomerase II amounts. The clonal cell lines exhibit a wide range of resistance extents, with the most resistant clone displaying 9 times the extent of adriamycin resistance observed in the least resistant clone. A number of clones exhibit sensitivity to the concentration of adriamycin in which the parental cell line was selected, possibly indicating cooperation between the more and less resistant cells. Detailed analysis of 4 of the clonal subpopulations revealed broadly similar drug resistance mechanisms. Alterations in expression of the MDR-associated genes *MDR1* and *Topo IIα* were observed, with no detectable changes in the expression of *MDR3, MRP, GSTπ*, *Topo IIβ*, *Topo I* and *CYP1A1* noted. However, each clonal cell line displayed a distinct extent of expression of *MDR1* and *Topo IIα* and further characterisation of the clones indicated that other modes of drug resistance may exist in at least one of the cell lines. In particular, 2 of the clones (DLKPA6B and DLKPA11B) which have almost identical drug resistance profiles appear to have quite different mechanisms of resistance. The clonal subpopulations possess individual growth rates, amounts of adriamycin accumulation and susceptibility to toxicity-enhancement by MDR-modulating agents. It was possible to generate a cell line with a drug toxicity profile similar to DLKP-A by mixing some of the clonal subpopulations. Our results provide evidence of heterogeneity within an MDR human cell population with respect to resistance and expression of MDR-associated genes. *Int. J. Cancer* 71:907–915, 1997.
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The resistance of tumours to cytotoxic drugs is a major cause of chemotherapy failure. In multidrug resistance (MDR), cells display resistance to a variety of structurally and functionally unrelated chemotherapeutic drugs. The MDR phenotype may be an inherent property of the cells or acquired following exposure to one or more cytotoxic agents. The chemotherapeutic drugs to which the cells are resistant generally include the anthracyclines, Vinca alkaloids, podophyllotoxins, peptide antibiotics and taxol, but usually exclude nucleotide analogues, metabolic inhibitors, alkylating agents and platinum analogues. The acquisition of an MDR phenotype is associated with a range of biochemical and biophysical alterations. The most frequently identified change is reduced drug accumulation and the associated overexpression of a 170kDa membrane-associated energy-dependent drug-efflux pump, termed P-glycoprotein, which is coded for by the *MDR1* gene (Roninson *et al.*, 1986). The overexpression of a second 190kDa drug-efflux pump, the multidrug resistance-related protein (MRP) (Cole *et al.*, 1992), is also capable of conferring an MDR profile on cells. Reduced concentration and activity of a nuclear enzyme involved in unwinding DNA, topoisomerase II (Beck *et al.*, 1987), and overexpression of the human major vault protein, the lung resistance-related protein (LRP) (Scheper *et al.*, 1993) are also correlated with the acquisition of MDR phenotypes in various cell lines. Altered levels of glutathione and detoxifying glutathione-S-transferase enzymes, especially the overexpression of the π isoform, are also associated with increased drug resistance (Moscow and Dixon, 1993). In general, the acquisition of resistance by cells to a range of chemotherapeutic drugs is mediated by the altered expression of a number of the above biochemical characteristics, and is seldom due to a single modification.

The heterogeneous nature of tumours and of their subsequently established cell lines has long been recognised and characterised (Heppner and Miller, 1983). Heterogeneity in tumours is manifested by a variety of phenotypic differences, but from the point of clinical treatment, the variation in the sensitivity of cells to chemotherapeutic drugs and radiation is of paramount importance. Tumour heterogeneity suggests the possibility that an inherently drug resistant population may exist in some tumours. Following initial chemotherapy, this subpopulation may be selected for, or a combination of mutation and selection processes may result in the selection of specific subpopulations. The resulting MDR population may, in turn, be either homogeneous or heterogeneous in terms of resistance extent and mechanisms. Yang *et al.* (1993) have described the *in vitro* establishment and analysis of a heterogeneous MDR population.

In this study, we describe the isolation and characterisation of clonal subpopulations from an MDR human lung cell line. The clonal subpopulations were analysed to determine whether their resistance extent was associated with biochemical and genetic mechanisms different to those identified in the parental population. Possible methods by which the subpopulations survived in the ''mixed'' parental cell line, in the presence of drug, are suggested.

MATERIAL AND METHODS

Chemicals

Adriamycin and 5-fluorouracil were obtained from Farmitalia Carlo Erba (Milton Keynes, UK), VP-16 from Bristol Myers Squibb, (Dublin, Ireland) and vincristine and cisplatin from Lederle (Dublin, Ireland). All media used in the maintenance of the cell lines was obtained from GIBCO BRL (Paisley, UK) and the FCS (batch 101024) from Sera-Lab (Crawley Down, UK). Cyclosporin A was purchased from Sandoz (Basle, Switzerland). Reagents used in RT-PCR analysis was purchased from Promega (Southampton, UK). All other chemicals were obtained from Sigma (Poole, UK).

The anti-P-glycoprotein monoclonal antibodies were obtained from BioResearch Ireland (Dublin, Ireland) (BRI MAB MDR-1 [Clone 6/1C]) and Centocor (Malvern, PA) (C219). The topoisomerase II polyclonal antibody was a gift from Dr. W. Beck (Memphis, TN) and the topoisomerase IIα and topoisomerase IIβ monoclonal antibodies (MAbs) were gifts from Dr. G.C. Astaldi-Ricotti (Pavia, Italy).

Cell lines

The poorly differentiated human lung squamous cell line, DLKP (Law *et al.*, 1992) and its adriamycin-selected MDR variant, DLKP-A (Clynes *et al.*, 1992) were cultured in DMEM/Ham's F12 (1:1) medium, supplemented with 5% FCS and 1% L-glutamine.

Contract grant sponsor: BioResearch Ireland.

*Correspondence to: National Cell and Tissue Culture Centre/BioResearch Ireland, Dublin City University, Glasnevin, Dublin 9, Ireland. Fax: 353 1 7045484; E-mail: heenanm@ccmail.dcu.ie

Received 3 September 1996; revised 8 January 1997

The cells grew as monolayers in tissue culture flasks, in the presence of 5% CO_2 and at 37°C.

Selection of MDR clones

The clonal subpopulations of DLKP-A were obtained by isolating individual colonies of cells, that were established from a single cell. Briefly, DLKP-A cells were plated at 50 cells per 35 mm tissue culture Petri-dish in 5 ml of culture medium. The cells were grown for 24 hr in 5% CO_2 at 37°C to allow cell attachment and were microscopically examined to ensure that the cells adhered singularly and in a dispersed manner. The cells were grown, with constant monitoring and feeding with cloning medium (a 1:1 mixture of culture medium to conditioned medium (medium exposed to DLKP-A for 24 hr, filtered through a 0.2 μm low protein-binding filter and stored at 4°C), supplemented with 10% FCS and 1% L-glutamine), until individual cells had formed single colonies of approximately 50 cells. The colonies were separately sub-cultured using cloning rings, which formed individual trypsination chambers around the colonies. The cells from each colony were transferred to a single well of a 96-well plate in 100 μl of medium. The cells were allowed to attach overnight at 37°C in 5% CO_2 and then were refed with fresh medium. When the cells reached approximately 90% confluency, they were sub-cultured into a single well of a 48-well plate. This procedure was repeated into a 24-well plate, 6-well plate, 25 cm^2 flask and 75 cm^2 flask. Stocks of the randomly selected clones were maintained frozen and all clones were routinely maintained in the absence of adriamycin, when in culture. The clonal subpopulations were cultured in DMEM/Ham's F12 (1:1) medium, supplemented with 5% FCS and 1% L-glutamine. Characterisation of the clones was performed within a 10 passage span following revival of the cells from cryo-preservation.

Cytotoxicity assays

In all assays, cells were pre-treated in a standard manner to ensure that the cells were healthy and in a logarithmic phase of growth prior to analysis. This involved sub-culturing the cells into 75 cm^2 flasks at a density of 2×10^5 cells/flask, 2 days prior to the assay and feeding the cells the following day. The assay involved plating the cells, from a single cell suspension, into 96-well plates at a density of 10^3 cells/well in 100 μl of medium. Cells were allowed to attach for 24 hr, at 37°C in 5% CO_2.

In the case of standard cytotoxicity assays, 100 μl of the required drug dilution were added to each well, with 8 replica wells set up for each drug dilution. The plates were then incubated for a further 6 days at 37°C in 5% CO_2, or until control wells reached approximately 80% confluency.

Drug toxicity was determined by intracellular acid phosphatase analysis (Martin and Clynes, 1993). In brief, the medium was removed, the cells washed twice with PBS and 100 μl of assay substrate (10 mM ρ-nitrophenyl phosphate in 0.1 M sodium acetate, pH 5.5; 0.1% Triton-X-100) were added to each well. The plate was incubated for 2 hr at 37°C in darkness and the reaction then stopped by the addition of 50 μl of 1M NaOH. The optical density of each well was read at a wavelength of 405 nm, in a dual beam plate reader, against a reference wavelength of 620 nm.

In the MDR modulating assays, 50 μl of a 4× concentration of the required drug and/or circumventing agents (verapamil and cyclosporin A) were added to the appropriate wells, with 8 replica wells per assay plate. The volume in each well was adjusted to 200 μl with medium and the plates were incubated and analysed as outlined above.

Cell growth rate determination

The growth rate of the cells was determined by monitoring their extent of growth over consecutive 24 hr time periods. The cells were plated in 25 cm^2 flasks at a cell density of 10^4 cells per flask, in 10 ml of growth medium. At successive 24 hr time periods, the number of cells present in duplicate flasks was determined, by performing cell counts of viable cells. The cell doubling time was calculated from a graph of cell number against time.

RNA analysis

Gene transcript levels, for a range of genes associated with drug resistance, were analysed using a semi-quantitative RT-PCR method. The total RNA extraction protocol, a detailed description of all primers used in the study and the RT-PCR reaction procedure is as outlined by O'Driscoll *et al.* (1993). The genes investigated included those coding for P-glycoprotein (*MDR1*), MRP (*MRP*), glutathione-S-transferase π (*GST*π), cytochrome P450 1A1 enzyme (*CYP1A1*), topoisomerase I (*Topo I*), topoisomerase II (*Topo II*), its α and β isoforms (*Topo II*α and *Topo II*β, respectively) and the gene homologous to *MDR1, MDR3*. The number of PCR cycles used in the detection of the gene transcripts of interest were the maximum number of cycles which still gave a linear amplification of product, as determined by densitometrical analysis. This was 20 cycles for *MDR1* and *GST*π analysis; 25 for *MRP, Topo II, Topo II*α and *Topo I;* 27 for *MDR3* and *Topo II*β and 30 for *CYP1A1*. β-actin was used as an internal control and was co-amplified with the gene of interest, in the same reaction mixture, in all PCR reactions. The resulting PCR products were analysed by agarose gel electrophoresis and their size determined by comparison with the Phi-X Hae III digested m.w. marker (Promega). The marker consisted of 22 fragments with the following number of base pairs: 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11 and 8. RT-PCR analysis of the MDR-associated gene transcripts, using the primers as described in O'Driscoll *et al.* (1993), resulted in the amplification of a 157 bp DNA fragment for *MDR1,* a 321 bp fragment for *MDR3,* a 203 bp fragment for *MRP,* a 270 bp fragment for *GST*π, a 327 bp fragment for *CYP1A1,* a 180 bp fragment for *Topo I,* a 216 bp fragment for *Topo II* and 139 and 118 bp fragments for *Topo II*α and *Topo II*β, respectively. Densitometrical analysis of band intensities in ethidium bromide-stained agarose gels was performed on photographic negatives of the gels, using a GS670 densitometer with Molecular Analyst software (Bio-Rad, Hercules, CA). The intensities of the bands of interest, and their corresponding β-actin band, were determined, using local background subtraction, which accounted for the optical density reading of the negative and gel background. The RT-PCR protocol used resulted in linear amplification of β-actin for 27 PCR cycles and so, it was also linearly amplified in the reactions where differential expression of gene-transcripts were noted.

P-glycoprotein detection

Membrane-enriched protein fractions were prepared from the relevant cell lines, the protein concentration determined and 20 mg of protein was applied per lane of a 7.5% SDS-PAGE gel. Membrane preparation, gel electrophoresis and protein transfer to nitrocellulose membrane was performed as described by Clynes *et al.* (1992). Following transfer of the protein, non-specific binding was blocked by incubating the nitrocellulose for 2 hr at room temperature in TBS (500 mM NaCl, 20 mM Tris; pH 7.5) containing 5% non-fat dried milk. The blot was then exposed to the anti-P-glycoprotein monoclonal antibody, BRI MAB MDR-1 [Clone 6/1C], overnight at 4°C. The blot was washed 3 times with TBS/0.5% (v/v) Tween 20 and incubated with the secondary antibody (horseradish peroxidase labelled rabbit anti-mouse IgG) for 90 min at room temperature. The blot was again washed and bound antibody detection was by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham, Aylesbury, UK).

Topoisomerase II detection

Nuclear protein was extracted by the method of Danks *et al.* (1988). Equal amounts of nuclear protein (35 mg) were loaded per lane of a 7.5% SDS-PAGE gel and protein separation and transfer were carried out as for detection of P-glycoprotein. Following transfer of the protein to the nitrocellulose, the nitrocellulose was blocked at 4°C overnight in blocking solution. In the case of

topoisomerase II analysis, the blocking solution consisted of 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5% non-fat dried milk, 3% BSA and 0.2% Tween-20. The nitrocellulose was then incubated with the primary antibodies (polyclonal rabbit serum that recognises topoisomerase II, using the pre-immune rabbit serum as the negative control (Friche *et al.*, 1991)) for 24 hr at 4°C. The filters were then washed with 10 mM Tris, pH 7.4, 140 mM NaCl (3 washes/20 min) and incubated with the secondary antibody (alkaline phosphatase labelled goat anti-rabbit IgG) at room temperature for 2 hr. The filters were then re-washed and the bound antibody was detected by the addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate substrate, according to the manufacturer's instructions (Boehringer Mannheim, Germany). In relation to the detection of topoisomerase IIα and topoisomerase IIβ, the blocking solution consisted of PBS/3% BSA, the primary antibodies were the MAbs 6G2 and 8F8, which recognise topoisomerase IIα and topoisomerase IIβ, respectively (Negri *et al.*, 1992), the secondary antibody was an alkaline phosphatase labelled rabbit anti-mouse IgG and bound antibody detection was as outlined above.

Adriamycin accumulation studies

The cellular accumulation of adriamycin was measured by a modification of the method of Ganapathi and Grabowski (1983). In brief, the cells were plated at a cell density of 5×10^5 cells/well in 5 ml of growth medium, in a 6-well plate. The cells were incubated at 37°C in 5% CO_2 for 48 hr, after which the medium was replaced by 4 ml of fresh medium containing 10 μM adriamycin. Control wells were set up containing adriamycin-free medium. The cells were re-incubated at 37°C in 5% CO_2. At specific time intervals (0, 2 and 4 hr time points) the adriamycin-containing medium was removed from duplicate wells and the cells washed twice with ice-cold PBS. Ice cold water (2 ml) was then added to each well until cell lysis was observed (approximately 5 min), 2 ml of an ice-cold 0.6 M HCl solution in methanol was then added and the plates incubated for a further 3 min. The solution was then centrifuged at 4000 g for 10 min and the fluorescence of the supernatant was spectrofluorometrically measured, against the adriamycin-free control, at an excitation wavelength of 470 nm and an emission of 585 nm. The concentration of adriamycin in the solution was determined from a standard curve, prepared from the fluorescence of known concentrations of adriamycin.

Statistical analysis

Differences between cell lines was assessed using a 2-tailed Student's *t*-test.

RESULTS

Establishment of clonal subpopulations

Nine clonal subpopulations of the MDR human lung cell line, DLKP-A, were isolated in order to study the heterogenous nature of the DLKP-A cell line and to determine if different subpopulations within the cell line were responsible for specific characteristics of the overall population. The clones of DLKP-A were maintained as individual cell lines without selective pressure and randomly designated DLKPA2B, DLKPA6B, DLKPA9B, DLKPA11B, DLKPA3C, DLKPA10C, DLKPA2D, DLKPA5D and DLKPA5F. DNA fingerprint analysis (Cellmark, Abingdon, UK; data not shown) confirmed the genetic identity of the clones with DLKP-A. The adriamycin toxicity profiles of DLKP, DLKP-A and DLKPA5F (as a representative of a clonal subpopulation of DLKP-A) were stable when the cells were cultured for at least 3 months in the absence of adriamycin and following recovery of frozen stocks (data not shown).

Resistance profiles of DLKP-A clonal subpopulations

The sensitivities of the clonal populations to a variety of chemotherapeutic compounds (Table I) were established to determine if the subpopulations present in the DLKP-A cell line were multidrug resistant, or whether specific clones were responsible for resistance to different drugs.

DLKP-A, as previously characterised by Clynes *et al.* (1992), exhibits an MDR profile and is resistant not only to its selective agent, adriamycin, but is highly cross resistant to vincristine and resistant to a lesser extent to VP-16. It showed neither significant cross resistance nor sensitivity to cisplatin or 5-fluorouracil, when compared with the parental cell line.

All clonal subpopulations of DLKP-A also exhibited a classical MDR profile, in that they were highly resistant to adriamycin, vincristine and VP-16 and all the clones exhibited the same rank order of resistance as DLKP-A *i.e.*, significantly more resistant to vincristine than to adriamycin and least resistant to VP-16. Very low extent of either resistance or sensitivity to cisplatin and 5-fluorouracil was present in some clones. Within the clonal subpopulations there was a 9-fold span in resistance to adriamycin, an 8-fold variation in resistance extent to vincristine and a 5-fold variation in their resistance to VP-16. The clone most resistant to one specific MDR-associated drug was not most resistant to all MDR drugs, but DLKPA2B was the least resistant clone with respect to resistance to all the typical MDR drugs tested. The clonal populations differ significantly, not only from DLKP-A, but also from each other.

TABLE I – THE RELATIVE RESISTANCE OF DLKP-A AND ITS CLONAL SUBPOPULATIONS TO A NUMBER OF CHEMOTHERAPEUTIC DRUGS, WITH RESPECT TO THE DRUG SENSITIVITY OF DLKP CELLS

	Adriamycin	Cisplatin	Vincristine	VP-16	5-Fluorouracil
DLKP	1	1	1	1	1
DLKP-A	254.14[4,8,10]	1.46	1504.26[4,7,9]	60.67[4,7,10]	1.75
DLKPA2B	36.80[1,12]	0.57	228.32[1,13]	18.17[1,13]	1.28
DLKPA11B	83.88[2,12]	0.62	328.61[1,14]	25.19[2]	0.54
DLKPA10C	88.88[2,12]	0.95	974.18	23.09[1,14]	0.98
DLKPA6B	94.59[3,12]	0.71	367.11[2,14]	29.77[2]	0.77
DLKPA2D	165.50[13]	1.62	1780.93[4,6,9]	42.49	1.46
DLKPA5D	213.21[5]	1.95	750.34	96.18[2,4,6,9,12]	1.01
DLKPA9B	245.16[4,8,11]	0.91	1794.81[4,6,9]	61.19[4,7,10]	1.11
DLKPA3C	327.68[4,6,9]	1.09	1985.08[4,6,9]	35.35	0.90
DLKPA5F	330.59[4,6,9]	1.39	1275.04[5,8,11]	50.60[5]	0.98

The fold-resistance was determined by dividing the average 50% inhibitory concentration (IC_{50}) value for the cell line of interest by the corresponding average IC_{50} value for DLKP. The IC_{50} values are the mean of a minimum of 3 repeat experiments, with 8 intra-assay repeats in each experiment. In all cases the intra-assay variation was less than 10%. The IC_{50} values of the drugs to DLKP cells are 24.83 nM for adriamycin, 873.00 nM for cisplatin, 1.21 nM for vincristine, 146.45 nM for VP-16 and 9551.11 nM for 5-fluorouracil. Data are significantly different from DLKP-A ($^1p < 0.001$, $^2p < 0.01$ and $^3p < 0.05$). Data are significantly different from DLKPA2B ($^4p < 0.001$, $^5p < 0.01$). Data are significantly different from DLKPA6B ($^6p < 0.001$, $^7p < 0.01$ and $^8p < 0.05$). Data are significantly different from DLKPA11B ($^9p < 0.001$, $^{10}p < 0.01$ and $^{11}p < 0.05$). Data are significantly different from DLKPA5F ($^{12}p < 0.001$, $^{13}p < 0.01$ and $^{14}p < 0.05$).

The relative extent of resistance to the chemotherapeutic drugs varied in the clonal subpopulations (Table II). When the resistance of the DLKP-A cell line to adriamycin was taken as 1 and the cells' resistance to vincristine and VP-16 compared to this, DLKP-A possessed a fold-resistance profile of 1:5.92:0.24 in relation to adriamycin, vincristine and VP-16, respectively. Although all clones possess fold-resistance ratios similar to DLKP-A, they were not identical. DLKP-A and DLKPA3C possessed similar fold-resistance ratios in terms of adriamycin to vincristine resistance but for adriamycin for VP-16 the ratios were different. The cell clones DLKPA10C and DLKPA2D exhibited similar fold-resistance ratios, but DLKPA2D cells were twice as resistant to the drugs as the DLKPA10C cells (Table I). Although both cell lines exhibited a ratio of adriamycin to VP-16 resistance similar to DLKP-A cells, the relative ratio of adriamycin to vincristine resistance was twice that noted for DLKP-A. The cell lines DLKPA6B and DLKPA11B possessed identical fold-resistance ratios and both cell lines also exhibited similar extent of drug resistance. Although their relative fold-resistance ratio of adriamycin resistance to vincristine resistance was similar to that noted for DLKPA5F, the fold-resistance ratio of adriamycin to VP-16 in DLKPA5F was half that observed for DLKPA6B and DLKPA11B. DLKPA5F cells were also 4 times more resistant to adriamycin and vincristine than DLKPA6B and DLKPA11B cells.

Growth rates of cell lines

Cell doubling times were determined for all variants of DLKP (Table III). Although DLKP-A did not possess a significantly faster growth rate than that observed for the drug-sensitive DLKP cells, significant variation existed within the growth rate of the clones of DLKP-A both in comparison with each other and with DLKP-A (*e.g.*, DLKPA5D vs DLKPA2D, $p < 0.001$). However, growth rates did not correlate directly with drug resistance in the cell lines.

TABLE II – THE RATIO OF FOLD-RESISTANCE OF ADRIAMYCIN TO VINCRISTINE AND VP-16, FOR EACH MDR CELL LINE[1]

	Adriamycin	Vincristine	VP-16
DLKP-A	1	5.92	0.24
DLKPA2B	1	6.20	0.49
DLKPA11B	1	3.92	0.30
DLKPA10C	1	10.96	0.26
DLKPA6B	1	3.88	0.31
DLKPA2D	1	10.61	0.26
DLKPA5D	1	3.52	0.45
DLKPA9B	1	7.32	0.25
DLKPA3C	1	6.06	0.11
DLKPA5F	1	3.86	0.15

[1]The fold-resistance to adriamycin in each cell line (as shown in Table I), was taken as 1 and the fold-resistance to vincristine and VP-16 was normalised to this value.

TABLE III – DOUBLING TIMES OF VARIANTS OF THE DLKP CELL LINE, DETERMINED DURING THE EXPONENTIAL PHASE OF CELL GROWTH

	Doubling time (hr)
DLKP	20.3 ± 3.6
DLKP-A	18.5 ± 1.1
DLKPA2B	23.7 ± 1.9[3]
DLKPA11B	24.9 ± 1.9[1,2]
DLKPA10C	20.3 ± 0.8[4]
DLKPA6B	22.2 ± 0.6[3]
DLKPA2D	16.0 ± 0.6
DLKPA5D	26.2 ± 2.4[1,2]
DLKPA9B	19.8 ± 4.0[4]
DLKPA3C	16.9 ± 2.3
DLKPA5F	16.4 ± 0.3

The doubling time of the cell lines was determined from 3 repeat experiments and the data above represent the mean growth rate ± the standard deviation on the mean. Statistical analysis revealed that cell growth rates were significantly different from [1]DLKP-A ($p < 0.001$), [2]DLKPA2D ($p < 0.001$), [3]DLKPA2D ($p < 0.05$) and [4]DLKPA5D ($p < 0.05$).

Alterations in MDR-associated mRNA and protein levels

A detailed analysis of the mechanisms supporting the MDR profiles in 4 of the clones of DLKP-A was performed. The most, DLKPA5F, and least, DLKPA2B, resistant subpopulations with respect to adriamycin sensitivity and 2 clones of intermediate resistance and similar cross resistance profiles and growth rates, DLKPA11B and DLKPA6B, were analysed and compared with both the drug-sensitive DLKP cells and the MDR parental population, DLKP-A.

Genes associated with drug resistance were analysed by a semi-quantitative RT-PCR method. The concentrations of gene transcripts of *MDR1, MDR3, MRP, GST*π, *Topo I, Topo II, Topo II*α, *Topo II*β and *CYP1A1* in the drug resistant and sensitive cell lines were studied. RT-PCR analysis suggests that *MDR1* overexpression may be a major mechanism of resistance associated with DLKP-A and its clonal subpopulations (Fig. 1*a*). *MDR1* expression was detected in the sensitive cell line DLKP, at a low extent, and in general, the rank order in the amount of *MDR1* mRNA detected in the resistant cell lines corresponded to their resistance to MDR-associated chemotherapeutic drugs. However, although DLKPA6B and DLKPA11B exhibited similar extent of resistance to all the MDR drugs, DLKPA6B had substantially lower amounts of *MDR1* mRNA than DLKPA11B. Detectable amounts of gene transcripts of *MRP, GST*π, *Topo I, Topo II, Topo II*α and *Topo II*β were present in all cell lines (Fig. 1*c-h*, respectively), but no alterations in the

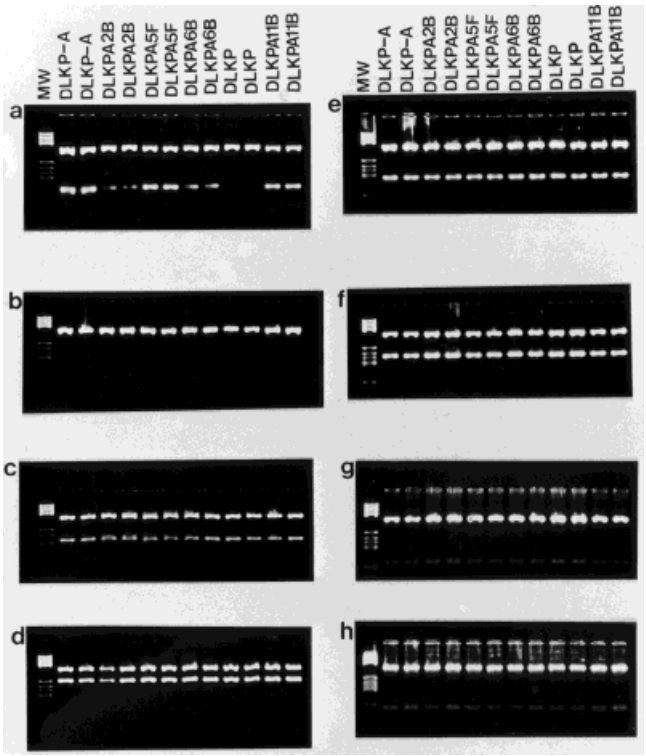

FIGURE 1 – RT-PCR analysis of (*a*) MDR1, (*b*) MDR3, (*c*) MRP, (*d*) GSTπ, (*e*) Topo I, (*f*) Topo II, (*g*) Topo IIα and (*h*) Topo IIβ in DLKP-A, DLKPA2B, DLKPA5F, DLKPA6B, DLKP and DLKPA11B cells. The left-hand column contained the molecular weight marker Phi-X, Hae III digested (Promega) and RT-PCR analysis of each gene transcript was performed in duplicate for each cell line. The upper band (383bp) relates to β-actin, which acted as an internal control and was used to correlate the intensities of the bands related to the MDR-associated genes (lower bands). No mRNA band was detectable for *MDR3* (*b*) or *CYP1A1* (not shown). RT-PCR analysis of the MDR-associated gene transcripts resulted in the amplification of a 157bp DNA fragment for *MDR1*, a 321bp fragment for *MDR3*, a 203bp fragment for *MRP*, a 270bp fragment for GSTπ, a 180bp fragment for *Topo I*, a 216bp fragment for *Topo II*, 139 and 118bp fragments for *Topo II*α and *Topo II*β, respectively and a 327bp fragment for *CYP1A1*.

TABLE IV – INTRACELLULAR ADRIAMYCIN ACCUMULATION BY VARIANTS OF THE DLKP CELL LINE

	ρmoles adriamycin accumulated/10^6cells	
	2 hr	4 hr
DLKP	501.0 ± 21.0	618.0 ± 39.0
DLKP-A	95.0 ± 8.1	138.0 ± 12.1
DLKPA2B	383.1 ± 16.0	424.5 ± 31.0
DLKPA6B	177.9 ± 13.0[1]	232.2 ± 33.0[1]
DLKPA11B	126.0 ± 3.4[3]	204.0 ± 12.0
DLKPA5F	99.9 ± 4.5[2]	160.5 ± 12.0[4]

The data presented are the mean of 4 independent repeat experiments ± standard deviation. All cell lines have significantly different accumulation extent than DLKPO and DLKPA2B ($p < 0.001$), at both the 2 and 4 hr time points. In addition, some cell lines also exhibit significantly different adriamycin accumulation amounts than [1]DLKP-A ($p < 0.01$), and DLKPA6B ([2]$p < 0.001$, [3]$p < 0.01$ and [4]$p < 0.05$).

extent of expression of these genes was noted between drug-sensitive and resistant cells. No *MDR3* (Fig. 1*b*) and *CYP1A1* (not shown) gene transcripts were detectable in the cell lines.

The expression of the MDR-associated proteins, P-glycoprotein and topoisomerase II (and its α and β isoform) were studied by Western blot analysis (Fig. 2). Detectable amounts of P-glycoprotein, as determined by Western blot analysis with the anti-P-glycoprotein MAb, BRI MAB MDR-1 (Clone 6/1C), were noted in membrane extracts from all cell lines (Fig. 2*a*). P-glycoprotein was noted in the drug-sensitive DLKP cell line, but the protein levels were elevated in the drug-resistant DLKP-A cells. Concentration of P-glycoprotein in the clonal subpopulations increased with increasing drug resistance, with the exception of DLKPA6B, which revealed low P-glycoprotein expression, comparable to that noted in the drug-sensitive DLKP cells. Immunocytochemical analysis of P-glycoprotein, undertaken using the C219 MAb, confirmed the presence of P-glycoprotein in the cell lines. Therefore, P-glycoprotein expression in the cells was reflective of their drug-resistance profile and *MDR1* mRNA concentrations, with the exception of DLKPA6B cells, which expressed lower amounts of immunodetectable P-glycoprotein than anticipated from its drug resistance extent.

Concentrations of the nuclear enzyme topoisomerase II and its α isoform were decreased in the resistant cell line DLKP-A, in comparison with the drug-sensitive DLKP cells (Fig. 2*b,c*). In the clonal subpopulations, the concentrations of both proteins were also lower than noted in DLKP. Low levels of both topoisomerase II and its α isoform were present in the least resistant clonal cell line, DLKPA2B and concentrations of topoisomerase IIα were also drastically reduced in DLKPA11B cells, as compared to DLKP cells (Fig. 2*c*). Concentrations of both topoisomerase II and topoisomerase IIα were only slightly reduced in DLKPA6B cells, in comparison with DLKP, implying that altered topoisomerase II is not a significant mechanism of resistance in DLKPA6B cells. In comparison with DLKP cells, immunodetectable topoisomerase II levels in DLKPA5F cells appeared to be substantially decreased, when analysed using the polyclonal antibody, but levels of the α isoform were only slightly reduced, as determined by Western blot analysis with the MAb directed against topoisomerase IIα. Immunodetectable amounts of topoisomerase IIβ were similar for all cell lines (not shown).

Cellular accumulation of adriamycin

One of the most frequently MDR-associated cellular changes is the reduced intracellular accumulation of drug. The extent of

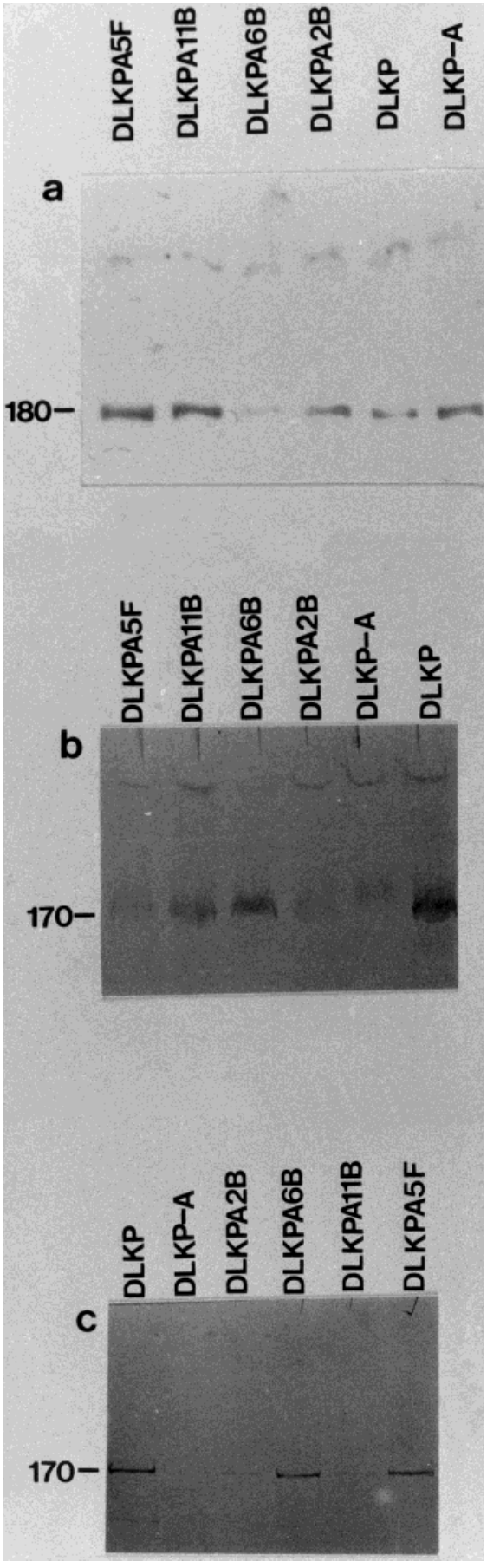

FIGURE 2 – Western blot detection of (*a*) P-glycoprotein, (*b*) Topoisomerase II and (*c*) Topoisomerase IIα in DLKP, DLKP-A, DLKPA2B, DLKPA6B, DLKPA11B and DLKPA5F cell lines.

TABLE V – TOXICITY OF CYCLOSPORIN A AND VERAPAMIL, AT VARYING CONCENTRATIONS, ON THE DLKP VARIANTS

	% cell survival[1]					
	Cyclosporin A (μg/ml)			Verapamil (μg/ml)		
	0.2	1.0	2.0	0.2	1.0	3.0
DLKP	95.23 (4.42)	93.38 (8.05)	92.61 (8.01)	101.34 (3.66)	101.23 (5.71)	102.69 (3.23)
DLKP-A	103.18 (7.27)	98.45 (8.95)	70.15 (13.81)	92.58 (13.31)	73.37 (16.35)	50.34 (19.11)
DLKPA2B	93.93 (3.34)	83.88 (7.95)	66.41 (8.02)	89.22 (10.16)	79.36 (12.96)	63.90 (16.09)
DLKPA6B	93.63 (3.73)	81.82 (5.04)	61.40 (9.23)	99.20 (11.38)	91.89 (11.13)	86.30 (11.24)
DLKPA11B	88.23 (6.50)	76.37 (8.45)	77.83 (12.60)	99.94 (5.99)	97.87 (8.46)	88.56 (3.45)
DLKPA5F	97.14 (5.85)	85.75 (13.12)	68.60 (5.02)	97.98 (15.36)	81.41 (20.15)	65.98 (19.44)

[1]Cell growth in the absence of either cyclosporin A or verapamil is taken as 100% survival and growth in the presence of varying concentrations of the circumvention agents is presented as percentage growth relative to that observed in the 100% controls. The values in parenthesis are the standard deviations of the percentage survival values.

TABLE VI – THE ENHANCEMENT OF ADRIAMYCIN, VINCRISTINE AND VP-16 TOXICITY TO VARIANTS OF DLKP, BY THE PRESENCE OF CYCLOSPORIN A AND VERAPAMIL

	% cell survival[1]					
	Cyclosporin A (μg/ml)			Verapamil (μg/ml)		
	0.2	1.0	2.0	0.2	1.0	3.0
Adriamycin						
DLKP	61.07	32.25	28.92	89.09	84.66	59.40
DLKPA	101.85	89.86	50.19	74.61	48.78	29.62
DLKPA2B	77.42	29.09	19.66	85.46	60.73	36.22
DLKPA6B	68.02	27.70	16.45	95.85	62.56	42.26
DLKPA11B	95.85	57.74	48.70	94.92	81.44	59.45
DLKPA5F	80.94	40.53	16.52	86.90	56.74	23.07
Vincristine						
DLKP	87.89	21.86	3.69	100.71	86.62	54.38
DLKPA	89.14	75.86	33.94	89.20	66.45	27.17
DLKPA2B	73.19	25.43	15.19	85.61	44.33	23.26
DLKPA6B	71.86	19.24	4.45	87.45	43.75	19.44
DLKPA11B	88.86	76.26	41.20	100.76	82.17	50.06
DLKPA5F	83.26	44.26	16.86	76.00	41.81	7.35
VP-16						
DLKP	93.90	71.27	54.58	100.20	97.62	91.90
DLKPA	98.19	66.16	19.49	102.01	93.27	39.40
DLKPA2B	38.70	12.67	7.02	118.70	98.90	63.12
DLKPA6B	102.12	71.56	67.89	108.40	125.21	114.34
DLKPA11B	94.78	79.09	60.33	102.61	106.36	76.77
DLKPA5F	104.52	70.59	52.94	104.14	105.53	85.53

[1]The chemotherapeutic drug was used at a concentration that resulted in 50–70% cell survival. The respective concentrations of adriamycin, vincristine and VP-16 used in this assay (all in μg/ml) were: DLKP: 0.02, 0.01, 0.075; DLKP-A: 1.0, 0.5, 2.0; DLKPA2B: 1.0, 0.5, 1.0; DLKPA6B: 1.0, 0.5, 2.0; DLKPA11B: 1.0, 0.5, 2.0; DLKPA5F: 1.0, 0.5, 4.0. In relation to the data presented here, the survival in the presence of the chemotherapeutic drug alone was taken as 100% survival and the survival in the combined presence of chemotherapeutic drug and circumvention agent is given as a percentage of this 100% value (the toxicity of the circumventing agent alone is given in Table V). The standard deviation on all data was less than 10%.

adriamycin accumulation was determined, to establish if this property was also observed in the clonal subpopulations of DLKP-A. All 4 clonal sublines have reduced intracellular concentrations of adriamycin when compared with the drug sensitive DLKP cell line (Table IV). The concentration of intracellular adriamycin detected at 2 and 4 hr reflected both *MDR1* mRNA and protein expression in the cells and their drug resistance extent, with the exception of resistance in DLKPA6B cells.

Circumvention of drug resistance

The ability of the MDR resistance-modulators, verapamil and cyclosporin A to chemosensitise the parental cell line, DLKP, its MDR variant DLKP-A and the 4 representative subpopulations, DLKPA2B, DLKPA6B, DLKPA11B and DLKPA5F, was investigated. The MDR cell lines were more sensitive to both cyclosporin A and verapamil toxicity than the drug-sensitive DLKP cells (Table V). Cyclosporin A and verapamil were effective chemosensitisers of both MDR and drug-sensitive cells to MDR-associated drugs (Table VI). Cyclosporin A was a more potent enhancer of drug toxicity to DLKP cells than verapamil. This is especially notable in its ability to sensitise the cells to adriamycin and vincristine and less so to VP-16, where the presence of verapamil exhibited no major enhancing effect. Both cyclosporin A and verapamil were potent enhancers of drug toxicity in the MDR cell line, DLKP-A, and its clonal subpopulations. However, in general, enhancement

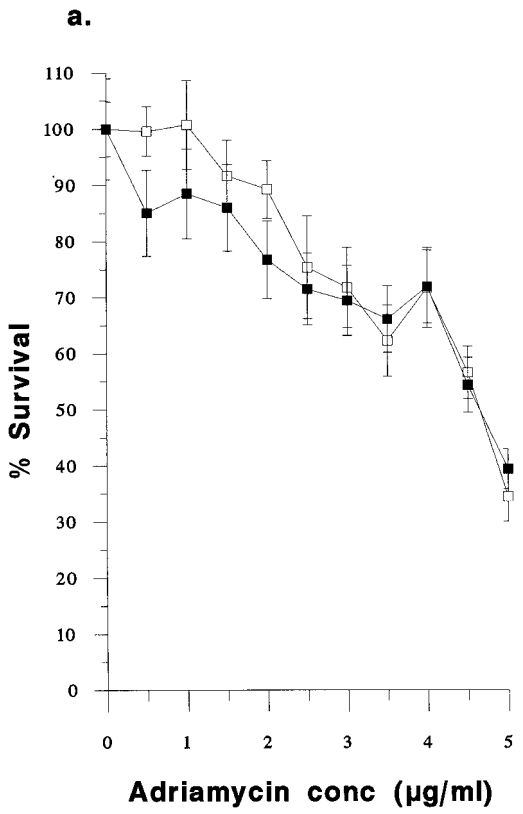

FIGURE 3 – The toxicity of (*a*) adriamycin, (*b*) vincristine and (*c*) VP-16 to DLKP-A cells (□) and the cell line generated by mixing DLKPA5F, DLKPA2B, DLKPA6B and DLKPA11B in the ratio 2:1:1:1 (■).

FIGURE 3

of toxicity in the more resistant MDR cell lines was only partially accomplished when using non-toxic concentrations of the circumventing agents and sensitivity to VP-16 was not as effectively enhanced as that of adriamycin and vincristine, particularly when verapamil was used as the modulating agent.

Although DLKPA6B and DLKPA11B possess similar MDR resistance extent and profiles, the cell lines respond quite differently to the MDR modulating agents. Both cyclosporin A and verapamil were much more effective at enhancing adriamycin and vincristine toxicity to DLKPA6B than to DLKPA11B, while both circumventing agents were of comparable potency in enhancing VP-16 toxicity to the DLKPA11B and DLKPA6B cell lines.

Generation of DLKP-A-like population

No clonal subpopulation of DLKP-A had an identical toxicity profile to that observed in the parental cell population, therefore the 4 most characterised clones (DLKPA2B, DLKPA6B, DLKPA11B and DLKPA5F) were mixed at varying ratios, to determine if a DLKP-A-like population could be generated, as determined by its toxicity to adriamycin. A mixture of DLKPA5F, DLKPA2B, DLKPA6B and DLKPA11B in a ratio of 2:1:1:1, respectively, generated a cell line with a toxicity profile most like that of DLKP-A (Fig. 3*a*). The highest amount of deviation between DLKP-A and the mixed populations occurred at low drug concentrations. Therefore, one or more clonal sub-populations, present in the DLKP-A cell line and which contributed to its adriamycin-resistance profile, were assumed to be absent from the mixed clones. The cell mixture with an adriamycin toxicity profile most like DLKP-A also possessed resistance profiles to vincristine and VP-16 which were similar to those observed for DLKP-A (Fig. 3 *b,c*).

DISCUSSION

DLKP-A is an adriamycin-selected MDR cell line (Clynes *et al.*, 1992) derived from a poorly differentiated squamous lung cell line,

which was established from a lymph node metastasis biopsy of a human lung tumour (Law *et al.,* 1992). Primary tumours are well documented as heterogeneous in nature, in relation to a range of biophysical and biochemical properties, including morphology, chromosomal number and chemotherapeutic drug sensitivity (Heppner and Miller, 1983). Therefore, it is not surprising that cell lines derived from tumours maintain a degree of heterogeneity. When a cell line is selected for the MDR phenotype by exposure to stepwise increasing concentrations of chemotherapeutic drugs over a prolonged time period, it undergoes both mutagenic and selective processes. Both from its origin as a heterogenous cell line (several clonal subpopulations have been isolated from DLKP, (data not shown), and because of its selection by a mutagenic drug, the heterogenous nature of DLKP-A is not entirely unexpected and is demonstrated by the presence of at least 9 clonal subpopulations with varying drug resistance extent and profiles, and with individual cell growth rates.

The 4 clonal subpopulations analysed in detail exhibited reduced drug accumulation and levels of adriamycin accumulation inversely reflected their extent of drug resistance. Overexpression of the drug-efflux pump, P-glycoprotein, and alterations in topoisomerase II, either individually, in combination or in association with other genetic alterations, are widely correlated with the acquisition of an MDR profile by cell lines (Beck and Danks, 1991). The DLKP-A population exhibited increased P-glycoprotein expression and reduced levels of topoisomerase II, reflected by a decrease in topoisomerase IIα. It should be kept in mind that while we examined mRNA and protein levels for topoisomerase II, no functional assessment has been undertaken. The 4 clonal cell lines also exhibited altered concentrations of these MDR-associated proteins. The MDR profile of DLKPA5F, the most resistant clone, appears to be maintained primarily by P-glycoprotein overexpression, although alterations in topoisomerase II content were also noted. In the least resistant cell line, DLKPA2B, the resistance profile is associated with a substantial decrease in topoisomerase content and a slight increase in P-glycoprotein. DLKPA6B exhibited low amounts of immunodetectable P-glycoprotein and unaltered amounts of topoisomerase II, in comparison with the drug sensitive DLKP cells, while DLKPA11B, a cell line of comparable resistance extent and profile as DLKPA6B, exhibited a significant increase in P-glycoprotein and a decrease in topoisomerase IIα. The amounts of P-glycoprotein present in the cell lines is not fully reflected by the concentration of *MDR1* mRNA, implying that control of expression at the translational level may be important in generating the MDR profile of the cell lines. Previous studies have revealed the overexpression of P-glycoprotein, but not its mRNA, in MDR cells (Zhao *et al.,* 1994) and the generation of P-glycoproteins with various half-lives, depending on the cell line or the presence of extrinsic modulating factors (Mickley *et al.,* 1989). The presence of truncated or partially degraded mRNAs, which may be detected by the PCR primers but which are not translatable or code for a non-immunodetectable protein, could also play a role in the lack of correlation between RNA and protein concentrations.

The resistance profile of DLKPA6B cannot be fully explained by the cellular amounts of P-glycoprotein and topoisomerase II, as these would suggest it to be more sensitive than DLKPA11B, rather than exhibiting comparable resistance. The effect of the MDR modulating agents in enhancing drug sensitivity also indicates differing mechanisms of resistance in the 2 cell lines, as both verapamil and cyclosporin A are more potent enhancers of adriamycin and vincristine toxicity to DLKPA6B than to DLKPA11B. The amount of P-glycoprotein expression is not necessarily reflective of its function (Bailly *et al.,* 1995) as phosphorylation may enhance its activity (Hamada *et al.,* 1987). However, P-glycoprotein concentration in DLKPA6B is lower and intracellular adriamycin accumulation higher than that observed in DLKPA11B. Additional mechanisms of resistance, or factors which enhance P-glycoprotein activity and response to modulators, may be present in DLKPA6B. Nevertheless, RT-PCR analysis of a range of drug-resistance associated genes failed to indicate a definitive alternative resistance mechanism.

The wide band of drug resistance extent displayed by the clones is surprising, given the fact that the DLKP-A cell line was selected and maintained at an adriamycin concentration of 3.5 μM and stably sustained its resistance profile in the absence of adriamycin. Therefore, within the DLKP-A population, distinct clonal populations are surviving during prolonged exposure to concentrations of adriamycin which is toxic to the same cells when they are analysed in isolation. DLKPA2B, which has a stable IC_{50} value of less than 1 μM, in terms of adriamycin sensitivity, was isolated from a population maintained in a concentration of adriamycin which was nearly 4 times the dose that would kill 50% of its population, when assessed in a standard 96-well plate assay. DLKPA6B and DLKPA11B are also isolated from a population maintained at a concentration of drug which would result in 50% cell kill of each of the individual cell lines. Although it is plausible to find cells of a higher resistance, *e.g.,* DLKPA5F, surviving in a population of cells maintained at a drug concentration less than their IC_{50} value, it is unexpected to find cells with a lower resistance extent present. This raises the questions as to why and how the cells are maintained in the mixed population, in a situation where, in terms of adriamycin selective pressure alone, they should be lost. The selection of an MDR population from a sensitive human colon cell line by continuous drug exposure and the emergence of a heterogeneous MDR population has previously been reported by Yang *et al.* (1993). Although the 2 characterised clonal populations exhibited different drug resistance profiles and mechanisms, the extent of resistance in both was greater than the maximum concentration of the selective drug. In order for the less resistant subpopulations of DLKP-A to survive, the sensitive cells may have developed mechanisms for escaping the toxic effects of adriamycin, or co-operation between the more and less resistant cells may have occurred, resulting in a cell protective effect.

Modification of the sensitivity of cells to toxins by both contact-dependent and independent mechanisms have been observed both *in vitro* and *in vivo* (Heppner and Miller, 1983). Cell contact-dependent modification of toxicity would involve either, or a combination of, the transfer of toxin from sensitive to resistant cells or the transfer of protective agents from the resistant cells back to the sensitive. Modification of the cells' environment may diminish the apparent toxicity of the chemotherapeutic drug. A decrease in extracellular pH has been shown to protect cells from cytotoxicity (Jahde *et al.,* 1990). Confluency-dependent resistance to a range of chemotherapeutic drugs has been observed and is generally reflected by a decrease in drug accumulation (Pelletier *et al.,* 1990). Both the cytotoxicity of the drug and its intracellular concentration progressively decreased with increasing confluency. Intracellular localisation of drug is also density-dependent (Pelletier *et al.,* 1990). The DLKP-A cell line has been observed to exhibit density-dependent adriamycin resistance (data not shown) and so the more adriamycin-sensitive subpopulations may be surviving in the parental population by a masking of toxicity, due to a density-dependent phenomenon.

The DLKP-A cell line has been observed to possess functional gap junctions when the cells are grown to areas of confluency (data not shown). Gap junctions allow for the transfer of small hydrophilic molecules and ions between cells (Loewenstein, 1981). Gap junctional transfer of H^+ has also been widely documented and alterations in intracellular pH has also been associated with modified cell kill by drugs (Barry *et al.,* 1993). Some MDR cells exhibit an increase in intracellular pH (Boscoboinik *et al.,* 1990) and circumventing agents have been shown to decrease the

cytosolic pH (Hamilton *et al.,* 1993) resulting in increased drug accumulation (Simon *et al.,* 1994). Resistance modifying molecules may be transferred intercellularly in the DLKP-A population, resulting in a cell population with an apparent uniform and intermediate extent of drug resistance, while also allowing drug sensitive cells to escape cell kill. Frankfurt *et al.* (1991) have observed a decrease in drug-induced DNA damage in sensitive cells when grown both in direct contact with resistant cells and in the absence of gap junction inhibitors.

In conclusion, the MDR squamous lung carcinoma cell line DLKP-A, is a heterogeneous population consisting of a number of MDR subpopulations, of which 9 have been isolated and characterised. The resistance levels of the subpopulations vary substantially and a number of the clonal cell lines are less resistant than the population from which they were isolated. The MDR profiles of the subpopulations are supported by a number of mechanisms, including overexpression of P-glycoprotein and altered amounts of topoisomerase II. Characterisation of the clonal populations indicate that other modes of resistance may also exist in at least one of the cell lines. When a number of the clonal cell lines were mixed at a specific ratio, it was possible to generate a population similar, but not identical to the parental cell line, in terms of drug resistance. The sensitive subpopulations of DLKP-A may have survived in the presence of toxic concentrations of adriamycin by a modification of toxicity, as a result of cell density and/or intercellular metabolic co-operation.

ACKNOWLEDGEMENTS

Financial support from BioResearch Ireland is gratefully acknowledged.

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