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High Frequency of Double Drug Resistance in the B16 Melanoma Cell Line

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Methotrexate (MTX) and N-(phosphonacetyl)-L-aspartate (PALA) are two agents to which cellular resistance can be conferred by gene amplification, but they do not generally show cross resistance. However, combined treatment with these two agents produced drug resistant cells in the B16 melanoma cell line at a much higher frequency than would be expected if resistance to the two agents was totally independent. An isolated doubly resistant clone, B16-F1 MP, showed a high frequency of resistance to pyrazofurin and ouabain, which are also agents to which resistance can be conferred by gene amplification. Thus MTX combined with PALA selected cells with an 'amplificator' phenotype (an increased ability to amplify parts of the genome). These B16-F1 MP cells had a decreased ability to form experimental lung metastases compared with the parent line but this difference was not found in baby hamster kidney cells with the amplificator phenotype. The mechanism underlying drug resistance may need to be considered when designing combination chemotherapy.
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INTRODUCTION

THE development of drug resistance is a major cause of failure in treating tumours [1]. Resistance is generally attributed to expansion of tumour cell clones that have mutated spontaneously [2] or under the influence of the drugs or treatments used [3, 4]. Combination chemotherapy is one way of attempting to circumvent drug resistance [1] and judicious selection of the combination used has been effective in reducing resistance within heterogeneous cell populations [5]. An important assumption underlying this approach is that the probability of a cell becoming resistant to two drugs simultaneously will be very small, approximately equal to the product of the two independent probabilities. This simple assumption is not valid for several reasons. Multiple drug resistance (MDR), due to overexpression of a membrane protein (P-glycoprotein) that pumps several different classes of chemotherapeutic drugs out of cells, is important in clinical drug resistance [6–8]. In MDR a single mechanism causes simultaneous acquisition of resistance to more than one drug. Another complicating factor is the effect of various therapeutic treatments. Many cytotoxic drugs are potent mutagens and can cause resistance to a second drug to develop more rapidly [9]. Ultraviolet and ionizing radiation, drugs that inhibit DNA replication and even hypoxia can increase the probability of gene amplification [3], a common means of achieving drug resistance. Finally, Giulotto *et al.* [10] reported that a population of baby hamster kidney (BHK) cells contains variants which have a generally increased ability to amplify their DNA, that these variants are selected when two drugs are administered simultaneously and that the cloned variants become resistant to additional agents much more easily than the initial BHK cell populations. The BHK cells have become tumorigenic when passaged *in vitro*, so to assess the possible relevance to tumour cells we have done a double drug selection with B16 murine malignant melanoma cells and found that similar observations can be made with this line.

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MATERIALS AND METHODS

The low lung-colonizing B16-F1 variant [11] or BHK 'amplificator' cells [10] were maintained as monolayer cultures on plastic dishes in Dulbecco's modification of Eagle's medium, with 10% heat-inactivated foetal calf serum (DEM). Cultures were grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

Drugs were administered in DEM containing dialysed foetal calf serum [12]. Methotrexate (MTX), ouabain (Sigma), N-(phosphonacetyl)-L-aspartate (PALA) or pyrazofurin (both from Dr D. Parker Suttle, National Cancer Institute, Bethesda) were added at the time of plating. Selections were done at cell densities of 1.5×10^5–4×10^5 viable cells per 9 cm dish. Three weeks later the cultures were fixed in alcohol and stained with methylene blue. Colonies were counted when they contained more than 50 cells and frequencies were expressed as colonies per number of cells plated.

To assay malignancy, cells were harvested by brief exposure to 0.25% trypsin, 1% EDTA in medium, washed once, resuspended in serum-free medium, counted in a haemocytometer and adjusted to the appropriate concentration. Only cell suspensions more than 90% viable (trypan blue exclusion) were injected into animals. Specific pathogen-free C57 BL/6 mice and athymic nu/nu mice of BALB/c background were from the ICRF Animal Breeding Unit. Eight mice per group were injected with 5×10^4 cells in 0.2 ml, via the lateral tail vein. Three weeks later the

Table 1. *Colony formation by B16-F1 cells in MTX and PALA**

Experiment	Colonies per 10^6 cells: MTX Observed	PALA Observed	MTX + PALA Expected	MTX + PALA Observed	Fold increase (O/E)
1	6	108	0.00065	0.15	231
2	36	106	0.0038	1.35	355

*5×10^5 cells were plated in each single drug and 1.35×10^7 cells in combination. MTX was used at 125 nmol/l and PALA at 20 μmol/l.

mice were killed and the lungs were removed and fixed in formol-saline. Pulmonary colonies were counted with the aid of a dissecting microscope.

RESULTS

Results from two independent experiments in which B16-F1 cells were grown in the presence of 125 nmol/l MTX and 20 μmol/l PALA, either as single agents or in combination, are shown in Table 1. The observed frequency of double resistant colonies was more than 200 times greater than that expected from the product of the frequencies with each drug alone. Of the colonies that grew in the double selection, B16-F1 MP was isolated and grown continuously in MTX and PALA. Resistance to MTX and PALA was confirmed with growth curves and it was tested for its relative resistance to two other drugs which select for gene amplification [10]. B16-F1 and B16-F1 MP cells were plated in 750 nmol/l pyrazofurin or 1.5 mmol/l ouabain at 1×10^5 cells per dish. No colonies arose from B16-F1 cells in either drug at this dose whereas B16-F1 MP cells gave rise to 213 and 100 colonies per 10^5 cells, respectively. Thus, selection for simultaneous resistance to two drugs gave a variant that was more resistant to drugs not yet encountered.

Two similar experiments were done to assess the metastatic capacity of the two lines. Parental B16-F1 cells produced a median of 12 lung nodules (range 5–28) after the injection of 5×10^4 viable cells, whereas B16-F1 MP cells produced 4 (range 113, $P \leq 0.01$, U test). In the second experiment the median colony number for B16-F1 was 19 compared with 2 for B16-F1 MP. In similar experiments, MP1 and MP5, two independent clones of BHK amplificator cells selected with MTX and PALA simultaneously [10], were injected intravenously into athymic nude mice. There was no significant difference between MP1, MP5 and parental BHK cells in this assay. All gave rise to more than 200 lung nodules per 10^5 cells injected.

DISCUSSION

Simultaneous exposure to PALA and MTX resulted in many more doubly resistant colonies than expected from the independent frequencies. In a related study concurrent selection of Chinese hamster ovary cells with MTX and doxorubicin gave similar results [13]. It is interesting that doxorubicin is one of the set of drugs that selects for MDR gene amplification. Thus, the phenomenon of 'greater-than-expected' double resistance is not uncommon and it is appropriate therefore to consider its importance in cancer chemotherapy. It is possible that combination chemotherapy regimens select in a tumour for 'amplificator' cells that have an enhanced ability to amplify any gene and that such cells, even if selected with drugs which are not a part of the MDR set, would have a higher probability of amplifying the MDR or any other gene that can mediate resistance.

Giulotto *et al.* [10] found that amplificator BHK cells selected with MTX and PALA were able to amplify other genes, conferring resistance to pyrazofurin, ouabain, or coformycin at rates 10–20 times greater than the parental cells. They suggested that such amplificator cells could amplify virtually any part of the genome at a higher rate. Our results are consistent with these findings, since B16-F1 MP cells had a greatly increased ability to form colonies in ouabain and pyrazofurin.

Gene amplification often occurs in tumours [4], as suggested first by the observation of homogeneous staining regions and double minute chromosomes [14–16]. Later studies showed that many cellular oncogenes are amplified in primary tumours and that the degree of amplification correlates with increased malignancy [17, 18]. Therefore, we wanted to establish whether amplificator cells were more metastatic than control cells, particularly since it had already been suggested that an increased ability to carry out amplification was correlated with an increased probability of metastasis in B16 melanoma [19]. Also, MDR cells in untreated solid tumours are associated with areas of increased growth [20]. However, our experiments revealed no enhancement of metastatic behaviour for the three cell lines tested. Since the sample size was small, we would not want to generalize this conclusion.

If selection *in vivo* for the ability to amplify oncogenes is an important aspect of tumour progression, an advanced tumour may already contain a high proportion of amplificator cells with an increased ability to generate drug resistant variants. Conversely, resistant cells selected from early stage tumours by exposure to several drugs simultaneously may have higher rates of amplification (and perhaps higher rates of generating other chromosomal abnormalities) and thus may be more likely to amplify oncogenes and generate more malignant variants.

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Syngeneic Anti-idiotypic Antibody Prevents Localization of a Murine Monoclonal Antibody in Human Tumour Xenografts

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BALB/c mice were immunized against syngeneic mouse monoclonal antibody (Mab) 791T/36 to produce anti-idiotypic antibody. To examine the effect of this antibody on tumour localization of the Mab, serum from these mice was transferred to nude mice with human tumour xenografts and distribution was studied with I-125 labelled Mab. Serum containing anti-idiotypic antibody prevented tumour localization of the Mab. This finding has implications for the clinical use of human or humanized Mab since, if these evoke anti-idiotypic antibody, this alone may be sufficient to prevent tumour targeting.
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INTRODUCTION

PATIENTS given mouse monoclonal antibodies (Mabs) to tumour associated antigens for immunoscintigraphy or therapy frequently produce anti-mouse antibody (AMA) [1]. AMAs can be a limitation to repeated administration of Mabs because they form immune complexes with the further doses of Mab, and these complexes are cleared to the liver or spleen [2, 3].

AMA may contain anti-species, anti-isotype and anti-idiotypic antibody [4–7], but the relative contributions of these to the perturbation of distribution is unclear. Animal models are needed to investigate this question [8, 9]. Anti-idiotypic AMA against 791T/36 (directed against a human tumour associated gp72 antigen) was generated in syngeneic (BALB/c) mice by injection of 791T/36–ricin toxin conjugate. In the mice the distribution of radiolabelled 791T/36 was altered, with hepatic clearance of the label [9]. Thus it would appear that anti-idiotypic antibodies alone can affect the distribution of Mab. It is unclear whether this would also affect localization of the Mab in tumour, or whether the affinity of Mab for the tumour target antigen would be sufficient to overcome the formation and eventual clearance of immune complexes with the anti-idiotypic antibody in the circulation. An analogous situation is the formation of immune complexes between Mab and circulating carcinoembryonic antigen (CEA), where tumour localization is not affected because of different affinities of the Mab for tumour-associated CEA compared with circulating CEA [10].

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It would be difficult to devise a model to determine directly the effect of anti-idiotypic AMA on tumour localization of Mabs to human tumours, because immunologically competent animals are needed to generate AMA, and these cannot support the growth of human tumour xenografts. Consequently, we transferred serum from mice with AMA into nude mice with human tumour xenografts and examined the localization of radiolabelled 791T/36 Mab in the tumours.

MATERIALS AND METHODS

Anti-idiotypic AMA was generated in BALB/c mice by two intraperitoneal injections of 1 mg/kg 791T/36-ricin toxin A chain immunotoxin [9] 10 days apart. Mice were bled out after a further 10 days and serum stored at −20°C. The presence in the serum of antibody capable of preventing binding of fluorescein isothiocyanate (FITC) labelled 791T/36 Mab against 791T tumour target cells was confirmed by a flow cytometric blocking assay [9]. Thus 0.1 ml test serum reduced the mean linear fluorescence (MLF) obtained by treating 2×10^5 791T cells with 100 ng FITC-791T/36 from 64 to14, while control mouse serum had no effect (MLF 77).

Groups of three athymic nude mice with subcutaneous xenografts of osteosarcoma 788T [11] were injected twice 2 h apart with 1 ml test or control mouse serum. Six hours after the second injection these mice and an additional group of untreated mice were injected intravenously with 10 μg I-125 labelled 791T/36 (specific activity 10 MBq/mg) [11]. All mice were killed after a further 4 days, dissected and radioiodine was measured in weighed samples of blood, tumour and organs. This time was