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Review

Tumour cell resistance to anthracyclines – A review

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Summary. *Resistance to anthracyclines is the major factor limiting their clinical utility. Laboratory studies using cultured experimental and human tumour cells have indicated that reduced intracellular drug accumulation is one important factor underlying resistance. In some systems this results from enhanced active drug efflux, a process which may be circumvented experimentally, for example by calcium antagonists. A specific glycoprotein which is produced in excess and is inherited has been identified in the cell membrane of certain anthracycline-resistant cells, while gene amplification with the appearance of double-minute chromosomes has been noted in others.*

Thus it is possible that anthracycline resistance arises following inherited changes in the cell membrane resulting in failure of drug accumulation. However, other possibilities exist, including differences in drug binding, either to the cell membrane or to nuclei, differences in metabolism to the semiquinone free radical, and differences in drug penetration related to tumour morphology.

For each human tumour type the factor(s) involved may differ, but sufficient clues now exist to suggest that clinical testing of some of the therapeutic possibilities for circumventing anthracycline resistance may soon be appropriate.

Introduction

Adriamycin (doxorubicin) is among the most widely used of cytotoxic agents, being the best known of the anthracycline antibiotics first developed in the early 1960s. Since its initial testing in phase-I and -II studies in 1970 [7], it has become an integral part of the treatment of many tumours, possessing activity against a wide range of solid and haematological malignancies. This has taken place despite well-documented acute toxic effects (alopecia, vomiting, myelosuppression) and longer-term cardiac toxicity [64]. It is becoming increasingly clear that the major limitation to its usefulness in many cases is not toxicity, for the drug has often ceased to be effective before maximum cumulative doses have been reached. The limitation most commonly apparent is the emergence of drug resistance in the tumour being treated, and methods by which such resistance might be circumvented would therefore have major clinical implications.

The purpose of this review is to examine the phenomenon of anthracycline resistance, and to assess those means by which it may be overcome, at least in the laboratory. It will be emphasised that critical clinical testing will soon be necessary.

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1. Experimental models

Much of the data related to anthracycline resistance has been obtained in murine tumour systems, particularly P388 leukaemia [27] and the Ehrlich ascites tumour [15], while other in vitro systems include the Chinese hamster ovary cell [45] and C-46 murine neuroblastoma [1]. More recently a range of cultured human tumour cells have been studied, and the information obtained is of particular interest. These cells include human haematopoietic tumour cell lines established from acute lymphoblastic leukaemia, acute myeloid leukaemia and Burkitt's lymphoma [2, 62], human small cell lung cancer cell lines [49], human glioma cell lines [38] and human breast cancer cell lines [55].

When a drug-resistant tumour cell line is identified it may well be important to distinguish between cells with 'natural' resistance (i.e., previously untreated cells, e.g., those in some human breast cancer lines identified by Smith et al. [54]), and cells with 'acquired' resistance (i.e., cells from previously treated tumours, e.g., those in some human small cell lung cancer lines reported by Shoemaker et al. [49]). The distinction between natural and acquired resistance should be borne in mind, as it is quite likely that different mechanisms underlie the presence of resistance in each case. However, at this time no data are yet available to indicate clearly what these differences might be, and this is certainly an area for further study.

In general, when sensitive and resistant sublines of the same tumour line are compared, it is important at the outset to establish the means by which resistance is derived. Adriamycin-resistant sublines of P388 leukaemia and Ehrlich ascites tumour cells were originally derived in vivo from repeated treatment of tumour-bearing mice, and this is to some extent analogous to the development of drug resistance clinically. On the other hand, adriamycin-resistant sublines of Chinese hamster ovary cells and C-46 murine neuroblastoma cells were derived in vitro by drug exposure and cloning of cultured tumour cells, and the selection pressures in these circumstances are not necessarily the same as apply in vivo. It would clearly be useful to compare data for a given tumour type in which anthracycline resistant sublines are derived both in vitro and in vivo, and such studies are in progress in our laboratory.

Adriamycin-resistant sublines in culture have been studied either in suspension or as monolayer cultures, while most of the in vivo studies have examined ascites-tumour-bearing mice. Two groups have studied anthracycline resistance in drug-resistant solid murine tumours, namely mammary adenocarcinoma 16/C [48] and Ridgway osteogenic sarcoma [32]; further studies with solid tumours, including human tumour xenografts, are in progress and their results are awaited with interest.

2. Cross-resistance

An early feature of studies of anthracycline resistance was the recognition of the phenomenon of cross-resistance. As early as 1968, it was noted that vinblastine-resistant P815 cells (a transplantable murine mast cell tumour) were cross-resistant to daunorubicin [35], and since then cross-resistance between anthracyclines, vinca alkaloids, actinomycin D and also epidopodophyllotoxins has been demonstrated for several experimental tumour cell lines, which remained generally sensitive to treatment with other classes of cytotoxic drugs (Table 1). This phenomenon, known as pleiotropic drug resistance (PDR), is particularly intriguing because of the structural and biological dissimilarity of the drugs in question. Clearly it is conceivable that a common mechanism underlies the emergence of resistance to these compounds, and this will be considered in detail in this review. Although PDR has been demonstrated to some extent in a few human tumour cell lines in vitro [2, 38, 49], it remains unclear whether this phenomenon is a common finding in human cancer, and further in vivo studies using human tumour xenografts may help to clarify the issue.

There are few clinical data regarding the emergence of PDR. Nevertheless, it is a general impression that when resistance to cytotoxic drugs develops clinically, although it is sometimes possible to identify other agents which have short-lived activity (at least in solid tumours), universal drug resistance usually ensues. However, the clinical situation is a complex one, in which the factors underlying the development of resistance are likely to be multiple, and since cytotoxic drugs are usually given in combination it would be indeed surprising if any semblance of PDR were recognisable clinically. Nevertheless, PDR has been demonstrated to occur both in vivo [32] and in vitro, and it thus seems that it is not simply a phenomenon confined to the conditions of cell culture. Thus, although its relevance to clinical practice at present is quite uncertain, the phenomenon of PDR is still an important observation, since clear elucidation of the underlying mechanisms might lead to the rational development of therapy aimed at circumventing resistance to specific cytotoxic drugs.

3. Transport studies

The precise mechanisms by which anthracyclines are transported in and out of cells are not clear,but it has generally been thought that uptake occurred via a carrier-mediated transport mechanism. However, recent evidence indicates that transport might in fact occur by simple (Fickian) diffusion across the cell membrane and that saturation kinetics previously noted resulted from self-association of anthracycline molecules [14]. The implication is that if changes in the physicochemical composition of the cell membrane were to occur, this might adversely affect drug uptake (and efflux) and that if these changes could be identified it might be possible to design means of overcoming such effects. This will be considered in more detail in the next section.

It has in fact been recognized since 1968 that anthracycline-resistant cultured tumour cells accumulate lower quantities of drug than sensitive cells [35]. The primary event leading to a drop in net drug uptake may be one or more of the following; decreased intracellular binding capacity, decreased drug influx and increased drug efflux. Many of the recent data indicate that increased drug efflux is present in most cases, and some workers have suggested (vide infra) that this is the primary cause of anthracycline resistance. However, this remains to be proven, and it is quite conceivable that different mechanisms will be found to operate in different systems.

Decreased retention of drug in daunomycin-resistant tumours cells was first reported by Kessel et al. in 1968 for the P3815 tumour model [35], but Dano was the first to emphasise that this process of outward transport is an active one requiring energy, at least in studies with resistant Ehrlich ascites tumour cells in 1973 [17]. These findings were subsequently confirmed by Skovsgaard in the same cell line [51], and he demonstrated that drug efflux was energy-dependent, using metabolic inhibitors and glucose deprivation. He also showed that a reduction both in drug influx and in affinity for intracellular binding occurred in resistant cells, at least at high drug concentrations.

The other model extensively studied in this context is the P388 leukaemia cell line, and Inaba et al. showed in 1978 and

Table 1. Cross-resistance detected in experimental tumour cell lines with various anticancer agents

Tumour type	Drug					Reference
	ADR[a]	ACT	VCR	VBL	VP16	
P815	CR	–	–	CR	–	[35]
Ehrlich ascites	CR	CR	CR	CR	–	[16]
P388	CR	CR	CR	CR	CR	[28]
Chinese hamster (transformed)	CR	CR	CR	CR	–	[45]
C-46 murine neuroblastoma	CR	–	CR	–	–	[1]
Murine L51787 lymphoblastoma cells	CR	–	–	CR	–	[41]
Murine L1210	CR	–	CR	–	–	[10]
CCRF-CEM	CR	CR	CR	CR	CR	[2]

CR, cross-resistance

[a] Adriamycin or related anthracycline

1979 that active efflux was the major factor underlying development of resistance and that in this particular cell line, differences in drug influx and intracellular binding were not apparent between sensitive and resistant sublines [24, 25]. It may be that the differences in drug binding seen in the studies using Ehrlich ascites tumour cells and those using P388 leukaemia cells are related to the level of drug used. More recently, impaired net uptake has been noted for other resistant tumour cell sublines, including C-46 murine neuroblastoma [1] and the Chinese hamster ovary cells [30], and studies with cultured human tumour cells are in progress.

The recognition of active drug efflux as one factor in the development of resistance to anthracyclines has led to the examination of a range of compounds which act by inhibiting their outward transport. In resistant P388 leukaemia cells, Tsuruo et al. have shown that some calcium antagonists (such as verapamil) and some calmodulin inhibitors (such as prenylamine, trifluoperazine, and clomipramine) are capable of increasing cellular accumulation of adriamycin by inhibiting its efflux [60], and at nontoxic doses of these compounds the activity of adriamycin is potentiated in resistant cells [62]. Inaba et al. have reported similar promising results with reserpine and resistant P388 leukaemia cells [26]. Similar (and even more striking) results have been obtained for P388 cells resistant to vincristine [26, 62].

The actual mechanisms involved are unclear. Calcium antagonists lower the intracellular calcium content, and this might interfere with membrane function. Calmodulin is certainly involved in cellular calcium transport, and inhibitors may directly inhibit the calcium-calmodulin complex formation with an adverse effect on membrane efflux mechanisms. However, in separate experiments, Ramu et al. [44] showed that changes in the calcium concentration in the medium over a wide range had no effect on the sensitivity of P388 cells to adriamycin, suggesting that a change in calcium flux per se might not be the central event involved. Clearly further information on the precise mechanism of adriamycin efflux is required.

The Danish group, using Ehrlich ascites cells resistant to anthracyclines, also examined ways of circumventing resistance, specifically aimed at blocking drug efflux. They confirmed that verapamil circumvents resistance both in vitro and in vivo in these cells and have also shown that quinidine has a similar effect [53]. In addition, they have examined the use of nontoxic analogues of anthracyclines, which it was hoped would competitively inhibit the outward transport of the drug under study, e.g., daunorubicin, when resistant cells were exposed to the two compounds simultaneously. They have succeeded in showing that the nontoxic analogue *N*-acetyl daunorubicin is capable of circumventing daunorubicin resistance both in vitro and in vivo [52], and this group is now concentrating on a search for more effective noncytotoxic anthracycline analogues for this purpose. In addition, they have shown that certain cytotoxic anthracycline analogues, such as aclacinomycin A, are capable in their own right of reaching higher intracellular levels than daunorubicin itself in resistant cells [20], and these data certainly encourage clinical studies in the near future.

It should be stated at this stage that although enhanced drug efflux does seem to be a major factor underlying resistance in some experimental tumours, it is not possible to infer that this factor is important (or indeed present at all) in adriamycin resistance in human cancer. Nevertheless, it is a valid hypothesis, which should be examined critically in human tumour models. In this respect the data of Tsuruo et al. are encouraging insofar as they indicate that adriamycin (and vincristine) activity in a variety of human haematopoietic tumour cell lines may be enhanced in vitro by the use of calcium antagonists and calmodulin inhibitors [61], and they propose the initiation of clinical studies. Similarly, preliminary data from our laboratory indicate that the cytotoxicity of adriamycin in a resistant human glioma cell line can be enhanced by means of verapamil, in association with an increase in intracellular drug level [39].

4. Cell membrane studies

The evidence previously presented, which points towards changes in drug transport as the underlying factor in the development of anthracycline resistance, has focused attention on the tumour cell membrane. Earlier studies concentrated on drug-resistant Chinese hamster cells, and altered cell membrane properties were soon apparent [5]. In comparison with parent drug-sensitive cells, resistant cells showed marked differences in cell morphology and patterns of growth, resembling the characteristics of normal nonmalignant cells in culture. These included a compact cell arrangement indicative of increased cell-substrate and cell-cell adhesiveness. In addition, resistant cells were either weakly tumorigenic or nontumorigenic in vivo, compared with sensitive cells. The data indicated a phenotypic reversion to normal growth behaviour, and these changes in Chinese hamster cells have recently been correlated with specific alterations in membrane glycopeptides and gangliosides.

Thus, resistant cells have been shown to synthesise a major glycoprotein species (molecular weight 150,000) not present in sensitive cells, and comprising a family of glycopeptides [42]. Furthermore, analysis of membrane gangliosides revealed a block in synthesis at the level of haematosides, which are present in excess in comparison with sensitive cells. How gangliosides and glycoproteins interact to influence drug sensitivity as well as tumorigenic capacity is not clear, but it is possible that the glycopeptide is a product of amplified genes. This will be discussed further in the next section.

These data were confirmed and extended, again in anthracycline-resistant Chinese hamster ovary cells, by Ling et al. in Toronto [30]. This time a prominent cell surface glycoprotein with a molecular weight of about 170,000 was identified by gel electrophoresis in daunorubicin-resistant clones, which were characterised by reduced drug accumulation compared with sensitive cells. Previous studies on colchicine-resistant sublines of Chinese hamster ovary cells had identified a cell surface P-glycoprotein present in large quantities, and this was immunologically cross-reactive with the glycoprotein present in the anthracycline-resistant cells.

More recently, the Toronto group extended this observation to a human tumour cell line, namely the vinblastine-resistant CCRF-CEM leukaemic lymphoblast line [29]. They again identified larger amounts of P-glycoprotein in resistant than in sensitive cells, thus confirming an earlier observation on the same cell line made by Beck et al. in 1979 [4]. Clearly further studies are indicated to establish whether expression of the P-glycoprotein-like molecule is a common feature of human tumour cells in which anthracycline resistance has developed.

The mechanism by which this cell surface glycoprotein influences drug transport and sensitivity is not known, but currently it seems unlikely that the carbohydrate moiety is a

necessary factor for expression of resistance. This conclusion is reached from studies with resistant P388 cells and resistant CCRF-CEM cells, in which tunicamycin and/or pronase have been used to inhibit glycoprotein synthesis [3, 12]. In both studies, cells remained viable and resistance (to anthracyclines and vinblastine respectively) was maintained. However, such treatment does not apparently affect protein synthesis in resistant cells and it thus remains conceivable that it is the noncarbohydrate component of the glycoproteins which exerts the major effect of the cell surface. Studies involving the direct isolation and insertion of the glycoprotein into membranes of sensitive cells, together with the use of recombinant DNA techniques for insertion of genetic material from resistant into sensitive cells, should answer the remaining questions concerning the relationship between the P-glycoprotein and anthracycline resistance.

It should also be noted that as well as increased amounts of the P-glycoprotein, disappearance of a lower-molecular-weight glycoprotein from the cell membrane has also been noted in anthracycline-resistant Chinese hamster lung cells [21], and it remains possible that it is in fact the loss of such a glycoprotein that is responsible for reduced drug accumulation.

Another aspect of the cell membrane of anthracycline-resistant cells which has receivd attention is its lipid structural order. This has been examined in detail in P388 leukaemia cells by electron spin resonance spectroscopy and fluorescence depolarisation measurement. In essence these studies have shown a higher degree of structural order in the lipid phase of the cell membrane of anthracycline-resistant P338 leukaemia cells than in sensitive cells (as well as increased amounts of cytoplasmic lipid in the resistant cells [43]. The authors speculated that the reduced rate of accumulation of drug in resistant cells results from these differences observed in the cell membrane, and more recently they have extended these studies to examine the effects of perhexiline maleate, a drug which affects cellular phospholipids [44]. Although they have not examined the changes which might occur in membrane lipid composition in detail, they have shown that this drug enhances the accumulation and the activity of adriamycin in resistant P388 leukaemia cells. The drug is also a calcium antagonist, but the authors suggest that its ability to increase drug accumulation rests in its ability to affect the cell membrane lipid domain rather than through any effect on calcium transport.

The relationship between cell membrane lipid composition, particularly lipid fluidity, and anthracycline resistance has also been studied in two other cell lines. Siegfried et al. [50] studied resistant sarcoma 180 cells using a similar technique of electron spin resonance spectroscopy, and concluded that increasing anthracycline resistance correlated with a progressive increase in membrane fluidity. Wheeler et al. [63] studied a resistant murine tumour, MDAY-K2, using fluorescence polarisation with diphenylhexatriene as a marker, and reached a similar conclusion; in addition they showed that the increase in fluidity was associated with increasing drug efflux as well as resistance. As discussed in section 6 (b), anthracyclines themselves do affect membrane fluidity. It is thus uncertain whether the changes in membrane fluidity seen in resistant cells reflect a primary difference in drug activity or whether they are a major factor in actually causing resistance to develop. Further detailed studies, preferably with human cancer cell lines, are clearly indicated.

Finally, another group of noncytotoxic drugs which might influence membrane transport of adriamycin are local anaesthetics, and these have in fact been tested in a human tumour line, namely a human melanoma cell line (SHG). It was apparent that the cytotoxicity of adriamycin was significantly enhanced by combined incubation with procaine or lidocaine at concentrations which are achievable clinically [11]. Although no drug uptake studies were performed, it is known that these compounds do alter surface membrane characteristics and also increase permeability, and this interaction thus seems to justify further study.

5. Chromosome studies

It is becoming widely accepted that most drug-resistant cancer cells have a genetic basis, and that the resistant phenotypes generally arise spontaneously from mutations [36]. They are thus inherited and propagated, and evidence for this being the case in anthracycline resistance is presented in the studies of Ling et al., who have demonstrated DNA-mediated transfer of resistance in Chinese hamster ovary sublines [18]. In addition, they have shown, by means of cell : cell hybrid formation, that in this particular case the drug-resistant phenotype is dominant. Thus in theory it may be expressed in polyploid cells as a result of only a single mutation.

Since, as previously described, these resistant cells appear to overproduce a specific P-glycoprotein located in the cell membrane, it seems clear that one result of the inheritance of the drug-resistant phenotype would be the production of this glycoprotein, and this was indeed seen [18]. However, these transfection studies suggested that genes coding for other proteins were transferred as well, and the case for the primary involvement of the P-glycoprotein is thus still not proven. Studies aimed at isolating the P-glycoprotein gene are now under way, and these should clarify the issue.

In other systems, as increased expression of gene products associated with drug resistance occurs, gene amplification, with the appearance of double-minute chromosomes or homologously staining regions in metaphase chromosomes, has been observed. Such phenomena have been observed, for example, in methotrexate-resistant murine sarcoma cells, in which increased production of dihydrofolate reductase (DHFR) is associated with amplification of the relevant DHFR genes [31]. Recently, DHFR gene amplification has also been demonstrated in human tumour cells taken from patients with small cell lung cancer, leukaemia, and ovarian cancer [9, 13, 58].

Similar observations have now been made for certain experimental tumour cells resistant to adriamycin (and other drugs). These cells are resistant sublines of C-46 murine neuroblastoma, derived in vitro by cloning techniques [1]. They failed to accumulate drug as the mechanism apparently underlying resistance (at least for radiolabelled vincristine), and were found to contain numerous double-minute chromosomes, which were absent from the drug-sensitive parental clones. The number of double-minute chromosomes progressively diminished as the initially unstable drug resistant clones were propagated and as stable resistant progeny of these clones emerged. This is consistent with the theory, proposed on the basis of data accumulated in methotrexate-resistant cells, that amplified gene-containing double-minute chromosomal spheres are responsible for the initial (epigenetic) phase of drug resistance. Similar data indicating the presence of amplified genes have been presented by Biedler et al. [6] in studies of vincristine-resistant Chinese hamster lung cells, although neither double-minute chromosomes nor homo-

geneously staining regions were detected in adriamycin- or actinomycin D-resistant cells. Nevertheless all the resistant cells clearly showed increased expression of the membrane glycoprotein gp150, and this can be considered as at least one product of amplified genes.

Although no data have yet been reported on the existence of gene amplification in anthracycline-resistant human tumour cells, it seems likely that such a mechanism does exist. Indeed it may be shown that gene amplification coding for several different drug resistance mechanisms might occur in human tumour cells which develop resistance following multiple-drug therapy.

6. Other possibilities

The data presented above appear to form a cohesive and logical explanation for the emergence of anthracycline resistance in some experimental tumours, linking as they do cross-resistance, impaired drug accumulation (possibly enhanced drug efflux), expression of a specific cell surface glycoprotein and the appearance of specific and inheritable chromosomal abnormalities. However, in other tumour systems, and human cancer in particular, other mechanisms are possible and indeed probable.

At this stage it is pertinent to list the proposed mechanisms of action of this class of drugs [64] as the basis for an examination of other potential means by which anthracycline resistance may develop. These include:

a) DNA intercalation,
b) Membrane binding,
c) Free-radical formation,

and these will be considered in turn in the context of drug resistance.

a) The best documented mechanism of action is the interaction with DNA, whereby the amino sugar portion of the anthracycline binds strongly to the sugar-phosphate backbone of DNA, blocking synthesis of DNA, RNA, and proteins. There are few published data on intracellular binding of anthracyclines in relation to drug sensitivity. As mentioned previously, no differences were seen in binding between isolated nuclei from sensitive and resistant P388 cells at low doses (of daunorubicin [24]), but at higher doses of daunorubicin (> 7 μg/ml) intracellular binding was lower in resistant than in sensitive Ehrlich ascites cells [50]. The importance of any difference in binding is thus unclear and further data, particularly data obtained with human tumour cells, are needed. It remains possible that the change in drug efflux which occurs following manipulation of cellular metabolism might have resulted from an alteration in intracellular binding sites, possibly by phosphorylation and a change in configuration, thus causing a change in the 'releasable' fraction of drug. Beck has speculated along these lines in terms of vinblastine resistance [2], calling into question the whole concept of an 'active efflux pump'.

b) It is known that anthracyclines bind to cell membranes, altering a variety of membrane functions at concentrations certainly no higher than those which affect DNA function. These membrane changes include changes in fluidity, phospholipid structure and also glycoprotein synthesis. In the last case it has been shown for drug-sensitive P388 cells that increases in membrane glycoproteins occurred within 30 min of exposure to adriamycin, but this did not occur in the case of adriamycin-resistant P388 cells [34]. It would be interesting to speculate that part of the basis of adriamycin resistance is an inherent difference in the membrane structure of certain cells, rendering them resistant to at least one of the drug's modes of action. The link, if any exists, between this suggestion and the previous findings in resistant cells of enhanced membrane glycoprotein production and enhanced drug efflux is unclear, but it is clearly possible that a number of changes in the tumour cell membrane will occur in drug-resistant cells, perhaps as a result of gene amplification.

Two groups have recently confirmed that adriamycin can be cytotoxic without entering tumour cells, using adriamycin coupled to an insoluble agarose support [59] or to glutaraldehyde microspheres [57]. In the latter case, Tokes et al. succeeded in demonstrating that adriamycin-resistant L1210 cells, when exposed to adriamycin presented in this way in vitro, were rendered sensitive [57]. The suggestion was made that the glutaraldehyde microspheres facilitated increased exposure of the cell membranes of resistant cells to adriamycin, and this points to yet another possible means by which anthracycline resistance may be circumvented. However, it is clearly important to establish the relevance of anthracycline cytotoxicity at the level of the cell membrane for human tumour cells, before further speculation can be made.

c) It is becoming increasingly apparent that one of the major routes by which anthracyclines are metabolised intracellularly is reduction by microsomal P_{450} reductase to a semiquinone-free radical [47]. This in turn rapidly reduces molecular oxygen to the superoxide ion, which is highly reactive and may well be responsible for DNA strand scission as well as damage to cytoplasmic constituents such as thiols, lipid membranes and susceptible proteins. It is of interest that among those intracellular enzyme defence mechanisms, which exist naturally to protect against superoxide and hydrogen peroxide production, one of the most important is catalase, which is present in very low levels in cardiac tissue. This may well underlie the potential for anthracyclines to cause cumulative cardiac toxicity [19].

The role of free radical formation in tumour cell cytotoxicity is unclear, although it has been demonstrated to occur in at least one experimental tumour model, namely Ehrlich ascites tumour cells [47]. Cells certainly differ in their ability to reduce anthracyclines, and it would be tempting to speculate that anthracycline-resistant cells may have defective reductase enzyme systems, whereby the drug is inadequately activated. Some support for this suggestion is forthcoming from a study of adriamycin-sensitive and -resistant P388 leukaemia cells, in which decreased levels of cytochrome P450 enzymes were noted in resistant compared with sensitive cells [40]. In addition, Bozzi et al. [8], noted that sensitive Ehrlich ascites cells and resistant Yoshida ascites cells differed in their ability to protect enzymatically against hydrogen peroxide production. Further studies of the relevance of free radical formation to anthracycline resistance in human tumour systems are clearly appropriate and are in progress in our laboratory. Markland et al. [37] have in fact already shown differences in the activity of the enzymes protecting against free radical formation among a range of human tumour cells, and it may prove to be the case that the balance of the factors underlying anthracycline resistance will vary from one tumour type to another.

Finally, another aspect of the development of anthracycline resistance in vivo is the degree of penetration of drug to the target tumour cells within a solid tumour mass. Poor penetration, due to morphological factors such as poor vascularity, is likely to be related to drug resistance, and in that

particular regard efforts to overcome resistance clearly should address the problem of improving penetration. An important laboratory model for examining these phenomena is the multicellular tumour spheroid, which provides a three-dimensional system in vitro with a microenvironment and with cell characteristics similar to tumours in vivo.

Studies with EMT-6 mammary tumour spheroids showed that adriamycin resistance was most pronounced in inner spheroid cells [56]. This was accounted for only partly by poor drug penetration, and the authors speculated that changes in the microenvironment of inner spheroid cells may have been crucial. They showed that these cells were relatively hypoxic, and indeed succeeded in reducing the adriamycin-resistant population of cells by pretreatment of spheroids with the radiation sensitiser misonidazole. Smith et al. [54] have also reported that adriamycin resistance in vitro (of Chinese hamster V79 cells) is correlated with the degree of hypoxia present, but studies with other cell lines have reported contradictory effects of the state of oxygenation of tumour cells on the activity of anthracyclines, and further clarification is needed [33]. It is likely that for a given cell line, the effect of differing states of oxygenation on anthracycline cytotoxicity will be determined by the extent to which free radical formation occurs in those cells. In this respect human tumour spheroids may provide an important means of elucidating some of the mechanisms underlying anthracycline resistance.

7. Clinical relevance, summary and conclusions

In general the development of clinical drug resistance can be ascribed to one or more of the following factors: pharmacological, immunological, kinetic, and biochemical. With respect to anthracyclines there is little firm evidence of a major role for alteration in host immunity. Cell kinetic characteristics may be relevant, and one study on Chinese hamster V79 cells indicated reduced uptake and sensitivity to adriamycin for plateau phase cells compared to cells in exponential growth [54]. However there are few other studies in vitro or in vivo on the importance of cell kinetics, and thus pharmacological and biochemical factors assume major importance.

In pharmacological terms the chief parameters of interest are the concentration of the drug at the tumour site and the duration of time that the concentration is maintained there ($c \times t$). This will of course vary with the dosage and the schedule of the drug, as well as the tumour location, tumour bulk, and the vasculature (as outlined in the previous section). The pharmacokinetics of the drug may also be relevant, and for adriamycin wide interpatient variations in pharmacokinetics and metabolism have been noted.

An attempt has been made to correlate directly the pharmacokinetics of adriamycin clearance and the clinical response in patients with breast cancer [46], but it would be necessary to confirm this suggested correlation in other studies before a firm role for pharmacokinetic analysis in determining sensitivity and resistance can be asserted.

Thus it would seem that the previous biochemical factors outlined in the above review are indeed of major importance. As Goldie and Coldman have suggested [22], clinical drug resistance arises from a wide genetic variability, with a drug-resistant phenotype emerging as a result of spontaneous mutation. As pointed out earlier, gene transfer studies have been carried out using resistant mutants of Chinese hamster ovary cells [17], and further studies are in progress. When the genes responsible for human tumour drug resistance have been identified these techniques might eventually be exploited clinically, but in the shorter term other possibilities for clinical application exist.

For instance, if the P-glycoprotein does indeed prove to be commonly present in resistant human tumour cell membranes, immunochemical screening for resistant cells might be possible. In addition the P-glycoprotein might become a target for monoclonal antibodies conjugated with toxins, with the aim of directly preventing the expression of the drug-resistant phenotypes.

This speculation, of course, begs the question of the relevance of some of the in vitro models used, for example the Chinese hamster ovary cell. Although such models may have given important clues as to the mechanisms by which anthracycline resistance may develop, studies in human tumour models are now clearly needed. These should include the use of human tumour xenografts, in which it should be possible to develop resistance by in vivo treatment in a manner somewhat analogous to the clinical situation [23]. In addition, it is now reasonable to consider clinical studies of some of the possible methods outlined above by which anthracycline resistance may be circumvented. These include the use of calcium antagonists such as verapamil, and data from such clinical studies are awaited with intense interest.

The other possibilities outlined above, whereby anthracycline resistance might arise, should also be emphasised at this stage. If, for instance, differences in free radical formation and protection were clearly seen to be important, the therapeutic implications would be considerable. Indeed for any given tumour type more than one factor might apply or the balance might change with time.

Current results with conventional cytotoxic chemotherapy have in general reached a plateau, and new approaches using alternative forms of drug therapy are needed. Meanwhile there clearly remains considerable scope for improving results with currently available drugs such as anthracyclines. It is hoped and expected that, despite the complexity of the situation, some of the potential for therapeutic gain discussed here might be realised within the next few years.

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