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Review

Resistance to cytostatic drugs at the cellular level

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Introduction

Although the development of new drugs and drug combinations has resulted in more effective treatment of cancer, a major factor limiting the usefulness of cytostatic drugs is the emergence of drug resistance in the tumor being treated. Many data on the causes of resistance have been obtained in murine systems, but information on the mechanisms whereby human tumors develop resistance is scanty and very incomplete.

A distinction between natural resistance (e.g., in previously untreated cells) and acquired resistance (e.g., in cells from previously treated tumors) may be important, as different mechanisms may underlie the development of resistance in each case. However, such a distinction may be artificial, since "acquired" resistance can be obtained by a selection process that results in the outgrowth of cells exhibiting natural resistance. There is also the possibility for cytostatic drugs to induce mutations in cells, leading to resistance. Acquired resistance may also be attributable to spontaneously occurring mutations. Goldie and Coldman [87] developed a mathematical model for this process. If cell mutation is random for any mutation rate, the probability that a tumor may contain a drug-resistant cell increases with the size of the tumor, and this increase will be rapid during a short period of the tumor's early existence.

The purpose of this review is to examine the mechanisms underlying the development of drug resistance at the cellular level. Several mechanisms of resistance can occur, including failure of drug uptake or activation, increased efflux or inactivation, mutation or increase in the level of target enzymes, increased repair of damage to DNA, altered nucleotide pools, and circumvention of the inhibitory effects of some drugs by salvage pathways. A detailed insight into the mechanisms of resistance operating at the cellular level in experimental models and human cancer will eventually provide information of direct clinical value.

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Reduced uptake of drugs into cells

A drop in net drug uptake may be caused by one or more of the following mechanisms: decreased drug influx, decreased intracellular binding, or increased drug efflux. One of the best understood examples of reduced influx is resistance to methotrexate (MTX). Impaired membrane transport of MTX has been well established [78, 99, 150, 164, 217–219]. The transport defect is assumed to be caused by either quantitative (a reduction in the influx V_{max} value) or qualitative (an increase in the influx K_m value) alterations in the transport system for 5-methyltetrahydrofolate, the carrier shared by MTX for cell entry [164, 218, 219]. Cell lines displaying an impaired transport mechanism that exhibit up to 250-fold resistance to MTX have been maintained in vitro [166, 197, 220]; thus, it has been suggested that the folate requirements for the growth of these cells are provided by a second carrier system, preferentially transporting folic acid [99, 119, 174, 220]. Such a carrier system has been described in L1210 cells and in an MTX-transport-defective subline [221].

Recently, Jansen et al. [113] have reported data suggesting that in an MTX-transport-defective subline of CCRF-CEM cells grown at folate levels in the physiological range, folate uptake is mediated by a highly specific membrane-associated folate-binding protein/folate receptor. This folate-binding protein was down-regulated when the cells were grown in a standard medium with a high folate content. The resistance of the cells appeared to be related to the relatively poor affinity of this folate-binding protein for MTX. At high extracellular concentrations of MTX, the transport barrier existing in resistant L5178Y cells is overcome and other properties such as the amount and type of folate reductase involved may be more important in determinations of drug sensitivity [100].

The transport of nitrogen mustard has been found to be an active carrier-mediated process in L5178Y lymphoblasts [85]. This mechanism appeared to be the carrier for the natural substrate choline [86]; kinetic analysis provided evidence of a decrease in the affinity of the transport carrier for nitrogen mustard in a resistant subline of L5178Y

cells. Studies on L1210 cells have indicated that the uptake of melphalan also appears to be an active carrier-mediated process [178]; inhibition of transport is provided by physiological concentrations of L-leucine and L-glutamine. In these melphalan-resistant L1210 cells, an alteration in drug transport may be caused by a specific mutation in the low-affinity leucine transport system.

The role of transport in resistance to cytoxic purine and pyrimidine nucleosides is difficult to evaluate because of the extremely rapid uptake of these drugs [88, 134, 170]. Of course, reduced transport is relevant only when it limits the rate of phosphorylation.

A number of *cis*-diamminedichloroplatinum(II) (CDDP)-resistant murine and human tumor cell lines exhibit a reduced uptake of CDDP [2, 105, 183, 236, 253], whereas the efflux of the drug is similar, if not higher, in the resistant lines [104, 236]. Decreased accumulation of CDDP that is closely related to a decrease in the formation of DNA interstrand cross-links has been described in CDDP-resistant human non-small-cell lung cancer lines; as no enhancement of repair was noted, it was concluded that this decrease in accumulation was the most important mechanism of resistance in this model [26]. However, other investigators have suggested that reduced accumulation could also be the result of an increased efficiency of repair mechanisms in resistant cells [63].

P-glycoprotein

A number of highly resistant cell lines have been isolated in vitro, and they appear to be resistant to several structurally unrelated cytostatic agents such as vinca alkaloids, colchicine, actinomycin D, and anthracyclines. These drugs display diverse structures and mechanisms of action. This phenomenon is known as pleiotropic drug resistance, or multidrug resistance (MDR). As it has been demonstrated both in vivo [127] and in vitro, MDR is not simply a phenomenon confined to the conditions of cell culture.

Cellular resistance to these compounds has been attributed to the augmentation of an energy-dependent efflux mechanism. Inhibition of energy metabolism in resistant cells can increase the net accumulation of many of these agents, whereas the accumulation can be markedly reduced by glucose [45, 108, 110, 222, 223]. Several studies indicate that the uptake of drugs is reduced in these MDR cell lines and that the extent of this reduction in uptake correlates with the increased resistance of the cells [6, 120, 140, 143]. Therefore, it seems likely that the molecular alterations responsible for MDR are localized at the cell membrane.

Comparison of cell membranes prepared from drug-sensitive and -resistant cells indicated that the resistant cells contained an increased amount of a 170,000-Da glycoprotein called the P-glycoprotein [118, 121, 142, 185]. The use of an antiserum raised against membranes from drug-resistant cells and absorbed with membrane proteins from drug-sensitive cells to examine membranes from a variety of cell lines expressing the MDR phenotype revealed that in each case, an increase in the staining of a component in the 170,000-Da region occurred [121]. Using a monoclonal antibody against P-glycoprotein, Bell et al. [17] found evidence of P-glycoprotein overexpression in tumor cells derived directly from patients presenting with ovarian cancer. In one case, an increase in P-glycoprotein expression was observed when the tumor became resistant to the cytostatic drug used, supporting the hypothesis that the expression of P-glycoprotein correlates with a growth advantage during chemotherapeutic treatment. Many other cancers have also been shown to express P-glycoprotein either before or after chemotherapy, including acute lymphocytic and nonlymphocytic leukemia, neuroblastoma, breast cancer, sarcoma, non-Hodgkin's lymphoma, and multiple myeloma [43, 81, 89, 147]. For instance, Kuwazuru et al. [135] studied P-glycoprotein expression in leukemia cells from patients presenting with acute myelogenous and lymphoblastic leukemia. Clinical refractoriness to chemotherapy appeared to be highly correlated with the expression of P-glycoprotein. However, as some patients who failed to express P-glycoprotein were resistant to treatment, other mechanisms should be considered.

The strong association of P-glycoprotein with MDR is also demonstrated by the correlation of the level of this protein with the degree of drug resistance [118, 120, 121, 141, 145]. P-glycoprotein is greatly reduced in revertant cells [121, 185] and is expressed in cells that have been transfected with DNA from MDR cells [45]. Increased expression of P-glycoprotein also correlates with an increased number of small extrachromosomal elements (double minute chromosomes) and increased MDR in a mouse cell line [188]. More direct evidence of gene amplification in MDR has been provided by Roninson et al. [192]. Using the technique of in-gel renaturation, these authors demonstrated the presence of amplified DNA sequences in two MDR sublines of Chinese hamster cells, C_5 and LZ. A subset of amplified LZ DNA sequences was amplified in common between the two lines. One of the commonly amplified DNA fragments was cloned, and the authors showed that the degree of drug resistance correlated with the number of copies of this segment in the DNA of resistant cells. This amplified common region was found to contain a transcription unit that encodes an mRNA of about 5 kb. Expression of this mRNA correlated with MDR in hamster cells [91].

Van der Bliek et al. [248] from the Netherlands Cancer Institute isolated complementary DNAs derived from five gene classes that were amplified and overexpressed in the MDR Chinese hamster ovary cell line CH^RC5; class 2 appeared to code for P-glycoprotein and class 4, for the cytoplasmic calcium-binding protein sorcin [186]. The products of the other genes are unknown. After it had been shown that the five genes were linked in two groups [247], a subsequent paper provided evidence that all five genes were linked in one large amplicon [117]. Other findings indicate the differential expression of genes within the large domain [48, 64]. Moreover, multiple transcripts may be generated from a single P-glycoprotein gene [64, 249]. Direct proof of the causative role of P-glycoprotein in the MDR phenotype has been provided by Gros et al. [93]. These investigators isolated a full-length cDNA encoding P-glycoprotein from drug-sensitive mouse cells and, after

its transfection into drug-sensitive hamster cells, demonstrated that the progeny of the transfected cells showed MDR. The MDR phenotype was consistently associated with overexpression of the transfected P-glycoprotein, whereas endogenous P-glycoprotein was not overexpressed.

To analyze the mechanisms of MDR in tumor cells, MDR sublines of the human KB carcinoma cell line were isolated [212]. These cell lines contain an amplification of two related DNA sequences that are homologous to the Chinese hamster mdr gene [193]. Segments of the human mdr sequences, designated mdr-1 and mdr-2, were cloned; mdr 1 sequences were amplified in all of the MDR sublines and were expressed as an RNA species of 4.5 kb in the resistant cells but not in the parent line. Expression of the mdr-2 sequences was not detected, but this gene was linked to mdr-1 and coamplified in some cell lines. The authors suggest that the mdr-1 gene is involved in MDR in human cells. Van der Bliek et al. [249] have recently described a human mdr-3 gene that is expressed in human liver and encodes a novel P-glycoprotein. In summary, characteristic of highly resistant cell lines is the amplification of a small group of closely related genes termed mdr-1 for multiple drug resistance.

In human MDR cell lines the increase in mRNA expression can be greater than the extent of the mdr gene amplification, suggesting the possibility of increased expression without gene amplification [213]. The characteristic variability of the cross-resistance profiles among MDR cell lines (reviewed in [22]) may result from differential expression of P-glycoprotein family members modulating the MDR phenotype or from the generation of P-glycoprotein molecules exhibiting different structures and functions. In addition, alternative splicing can vary the P-glycoprotein structure [249].

The nucleotide sequence of mdr cDNA that is capable of conferring a complete MDR phenotype has been determined [92]. The deduced aminoacid sequence shows structural similarities to bacterial membrane transport proteins. The glycoprotein includes 12 hydrophobic regions that may represent transmembrane loops. On the outside of the cell is a cluster of potentially N-linked glycosylation sites, and on the inside are nucleotide-binding domains that may bind adenosine triphosphate (ATP). It is likely that the predicted MDR polypeptide represents the P-glycoprotein, as both peptides share at least four structural features [32, 80, 92].

Apart from this P170 glycoprotein, which was first described by Ling and co-workers, other marker proteins for MDR have been reported [12, 31, 47, 200]. For instance, Fine et al. observed a 20,000-Da phosphoprotein in MDR human breast-cancer cell lines [70] and in a human small-cell lung cancer line [71]. A 150,000-Da glycoprotein was described by Peterson et al. [169] in Chinese hamster cells resistant to daunomycin and vincristine. It is not clear whether MDR cells contain one or several of these marker proteins, and their role in drug transport and sensitivity is not clear. It seems unlikely that the carbohydrate moiety of the glycoprotein is a necessary factor for the expression of resistance [11, 33].

MDR cells exhibit increased drug efflux [74, 109, 256]. Membrane vesicles prepared from MDR KB cells possess large amounts of P-glycoprotein; they also bind increased amounts of [^{3}H]-vinblastine [36]. Using photoaffinity labeling, the drug-binding component has been identified as P-glycoprotein; the labeling can be inhibited by an excess of cold vinblastine or vincristine and, to a lesser extent, by daunomycin [37]. These results suggest that P-glycoprotein can bind these drugs. It likely functions as an energy-dependent efflux pump. However, binding studies using colchicine and actinomycin D have raised questions about the precise role of P-glycoprotein; these drugs, both of which are involved in the MDR phenotype, did not compete with vinblastine binding. The possibility exists that there may be more than one binding site on the P-glycoprotein [36].

The disappearance of a lower-molecular-weight glycoprotein from the cell membrane has also been noted in resistant cells [79, 182], and it cannot be excluded that this loss is in fact responsible for the diminished drug accumulation in these cells.

P-glycoprotein is also overproduced in the endometrium of the mouse gravid uterus [3]. Huang Yang et al. [106] studied the interaction of progesterone, which is produced in large quantities during gestation, with P-glycoprotein. Their results indicate that these steroids can inhibit the efflux of antitumor drugs from MDR cells and may be helpful in maintaining drug sensitivity.

P-glycoprotein is not limited to MDR tumor cells but can also be found in normal human tissues [76, 101, 229]. Using a monoclonal antibody, relatively high levels of P-glycoprotein could be detected in samples of liver membranes, whereas samples of small-bowel membranes displayed lower levels. In other intraabdominal organs (spleen, stomach, pancreas, kidney, large bowel, urinary bladder, prostate, uterus, ovary), P-glycoprotein could not be detected by this method; it is possible that the low levels of P-glycoprotein found in these organs were below the limits of detection of the immunoblot assay used [101]. The expression of P-glycoprotein in normal human tissues has also been studied by immunohistochemical techniques using a monoclonal antibody. High levels could be detected in the liver, pancreas, kidney, colon, jejunum, and adrenals. Except in the adrenals, P-glycoprotein was always localized at the luminal face of the cells [238]. These data suggest that P-glycoprotein may play a physiological role in secretory (detoxification?) processes.

Apart from MDR due to the expression of P-glycoprotein, another mechanism of MDR relates to intracellular levels of glutathione (GSH). Resistance and cross-resistance between alkylating agents, radiation therapy, and CDDP have been associated with elevations in cellular GSH levels [98, 146]. MDR can also be caused by alterations in the activity or the amount of DNA topoisomerase II, and a number of cells displaying this characteristic have been isolated [44, 52, 67, 172].

A non-P-glycoprotein-mediated MDR human leukemia cell line that does not overexpress mdr-1 mRNA has been described by Takeda et al. [232]. These authors showed that the mechanism of resistance to Adriamycin in this cell

line might be explained by alterations in the intracellular distribution of this drug [232].

Enhanced drug efflux can also be caused by the conversion of hydroxylated molecules such as anthracyclines and vinca alkaloids to glucuronide conjugates. Glucuronides are readily eliminated from cells. Gessner et al. [82] have provided evidence that daunorubicin-sensitive and -resistant P388 cells can glucuronidate daunorubicinol, a known active metabolite of daunorubicin, and eliminate it into the surrounding medium. The activity of the enzyme glucuronyltransferase, which can glucuronidate a variety of substrates, was found to be 4 times higher in the resistant cells as compared with the sensitive cells. These authors did not mention P-glycoprotein measurement in their paper [82].

Calcium-channel-blocking agents and calmodulin inhibitors

Since P-glycoprotein plays a central role in MDR, efforts have been made to identify drugs that interact with P-glycoprotein and can reverse MDR. Reversal of the activity of the efflux pump can be brought about by a range of membrane-active noncytotoxic agents, most of which act by directly binding to P-glycoprotein, thereby preventing the efflux of cytotoxic drugs by competitive inhibition [260]. Modulators of MDR can act as a substrate for the efflux pump mediated by P-glycoprotein, competitively inhibiting the efflux of cytotoxic drugs that bind to P-glycoprotein.

P-glycoprotein from a Chinese hamster lung cell line can bind to calcium-channel-blocking agents, Adriamycin, vincristine, and dactinomycin, and it has been suggested that calcium-channel blockers reduce MDR by acting as a substrate for the P-glycoprotein-mediated efflux pump, thereby inhibiting the efflux of cytostatic drugs [202]. In other MDR cell lines, resistance to anthracyclines and vinca alkaloids can be reversed by calcium-channel-blocking agents and calmodulin inhibitors [190, 225, 236, 244, 245]. Calmodulin is a calcium-binding protein that occurs at high levels in cells and is thought to be responsible for the intracellular actions of calcium. Results similar to those obtained in Adriamycin- and vincristine-resistant P388 leukemia cells have been reported for reserpine by Inaba et al. [111]. Skovsgaard and Friche [224] have shown that quinidine produces the same effect as verapamil in circumventing resistance in Ehrlich ascites cells.

It is not clear how calcium-channel blockers affect the drug-resistant cell, since these cells do not exhibit the high-affinity binding site for nitrendipine that has been found in cardiac and brain tissue [69]. Calcium antagonists lower the intracellular calcium content, and this might interfere with membrane function. As calmodulin is involved in cellular calcium transport, its inhibitors may directly suppress the formation of the calcium-calmodulin complex, resulting in an adverse effect on membrane efflux mechanisms. However, changes in the calcium concentration in the medium had no effect on the sensitivity of P388 cells to Adriamycin [175]. Also, Kessel and Wilberding [131] reported that no relationship could be found between calcium flux and anthracycline transport in P388 cells. A change in calcium flux per se might not be the only event involved.

A number of investigators have suggested that verapamil enhances the cellular accumulation of anthracyclines and vinca alkaloids by inhibiting a putative efflux pump [91, 244–247]. Kessel and Wilberding [130] have suggested that verapamil itself may be handled by this pump, thus competing with anthracyclines for release from the cells. Beck et al. [13] proposed another mechanism for verapamil's enhancement of vinca-alkaloid cytotoxicity. These investigators reported that the rapid lytic effect of the alkaloid-verapamil combination on resistant human leukemic cells was associated with cellular vacuolization. They propose that verapamil might perturb cell membranes and expose ordinarily "cryptic" targets that may be associated with these vacuoles to cytotoxic drug binding [13].

Other mechanisms of action have also been proposed for these drugs. Stimulation of the phospholipid-sensitive and Ca^{2+}-dependent phosphorylating enzyme protein kinase C (PKC) is known to reduce intracellular drug concentrations. Fine and co-workers [70] have proposed that stimulation of the enzyme could lead to an increase in the phosphorylation of a 20,000-Da protein in human breast-cancer cell lines exhibiting MDR. This enzyme is inhibited by verapamil and by the calmodulin inhibitor trifluoperazine.

Fine et al. [72] have demonstrated that the activation of PKC by phorbol esters can induce MDR in drug-sensitive human breast-cancer cells and can further increase the resistance in drug-resistant mutants of the same cell line. The decreased intracellular accumulation of drugs after exposure to phorbol esters was associated with an increase in the phosphorylation of proteins. The enhanced resistance could be partially or fully reversed by the application of verapamil and trifluoperazine. These investigators suggest that a decrease in the accumulation of drugs may be caused by the phosphorylation of an efflux pump or a carrier protein. Perhaps a combination of anticancer drugs with inhibitors of PKC has potential for the development of new approaches to cancer treatment.

In the above-mentioned studies using membrane vesicles of human KB carcinoma cells, verapamil and quinidine appeared to be excellent inhibitors of the binding of the iodinated photoaffinity analog of vinblastine to P-glycoprotein, resulting in the decreased efflux of this drug [38]. These data suggest that verapamil may bind to P-glycoprotein, thus resulting in a loss of drug resistance. Verapamil and other Ca^{2+} antagonists have also been found to potentiate DNA damage by etoposide (VP-16) in L1210 cells in vitro [257]. The authors believe that the most probable mechanism for this effect involves an increase in the available intracellular drug brought about by altered transport.

Enhancement of the antitumor effect of CDDP by verapamil has been described in human neuroblastoma grown in athymic nude mice, but the authors offered no explanation for their finding [107]. The antineoplastic effects of CDDP can also be enhanced by calmodulin antagonists in athymic nude mice bearing ovarian carcinoma [133]. The

authors suggest that these drugs could either disturb the repair process in DNA damaged by CDDP or limit CDDP-induced damage to cytotoxic spleen cells. In addition to using the plasma membrane as a site of action, verapamil can also act by binding to α_1-acid glycoprotein, an acute-phase plasma protein. The effect of verapamil on MDR can indeed be modulated by this protein [29]. Verapamil is the drug that is most often used for reversing MDR. Unfortunately, the levels achievable in humans are limited to approximately 2 μM because of the drug's adverse effects on heart rhythm and blood pressure, and these levels are not adequate to reverse the resistance of many MDR tumors in culture.

In primary human tumors such as MDR renal-cell carcinomas, an evident correlation also exists between P-glycoprotein expression and sensitivity to vinblastine. Furthermore, the addition of a number of calcium antagonists (verapamil, diltiazem, nifedipine, nitrendipine, nimodipine) to vinblastine therapy has caused a significant decrease in the number of viable tumor cells as compared with treatment with vinblastine alone [155]. In a clinical study, Dalton et al. [43] used verapamil in an attempt to improve the efficacy of a chemotherapy regimen consisting of vincristine, Adriamycin and dexamethasone (VAD) in patients suffering from multiple myeloma and non-Hodgkin's lymphoma. These investigators showed that cells from six of eight patients exhibited elevated levels of P-glycoprotein after they had failed VAD therapy and that three of six positive patients appeared to show a transient response to VAD plus verapamil. Maximal doses of verapamil were used by Miller et al. [157], who obtained a high degree of response in some patients who had been selected for clinical drug resistance. Ozols et al. [167] could not demonstrate a potentiating effect of verapamil on Adriamycin in eight drug-resistant ovarian cancer patients; however, expression of P-glycoprotein was not documented in these subjects.

Alterations in membrane lipid fluidity, which lead to an overall increase in drug efflux or to an overall decrease in drug influx, must also be considered as a mechanism of resistance. A higher degree of plasma-membrane-lipid structural order corresponding to a decrease in the fluidity of membrane lipids has been found in anthracycline-resistant P388 cells as compared with the sensitive cells [176]. Moreover, in studies on resistant sarcoma 180 and MDAY-Kr cells, it was concluded that increasing anthracycline resistance correlated with a progressive increase in membrane fluidity [215, 255]. The increase in fluidity appeared to be associated with increasing drug efflux [255].

Defective metabolism of drugs to active compounds

Membrane transport of cytotoxic purines and pyrimidines is a very fast process [170]. To exert antitumor activity, these drugs must first undergo intracellular phosphorylation. A decrease in the activity of enzymes required for nucleotide formation is associated with the development of drug resistance in a number of murine tumors.

The toxicity exerted by 5-fluorouracil after its incorporation into RNA requires either conversion of the drug to fluorouridine monophosphate by orotic acid phosphoribosyl transferase or its metabolism to fluorouridine by uridine phosphorylase followed by the phosphorylation of fluorouridine by uridine kinase. Decreased activity for orotic acid phosphoribosyl transferase [123, 160], uridine phosphorylase [179], and uridine kinase [179, 180] has been found in 5-fluorouracil-resistant tumor cells.

Arabinoside cytosine (ara-C) is intracellularly converted to the active metabolite ara-CTP by the enzyme deoxycytidine kinase. The metabolite directly inhibits the DNA polymerases and can be incorporated into DNA, causing lethal toxicity. The initial step to the monophosphate ara-CMP is mediated by the enzyme deoxycytidine kinase. This phosphorylation is believed to be the rate-determining step in drug activation. Decreased activity of this enzyme in ara-C-resistant murine and human leukemia cell lines has been reported [54, 60]. In addition, the existence of two forms of deoxycytidine kinase activity exhibiting different biochemical properties has been demonstrated in Chinese hamster fibroblasts; one enzyme was localized in the mitochondrial cell fraction and one, in the cytosol [54]. Only the latter enzyme was capable of phosphorylating ara-C, and resistant lines showed a vast reduction in the level of activity of this enzyme. Most experiments have used cells made resistant to ara-C in vitro. However, Kees et al. [129] tested leukemia cell lines derived from a patient before treatment and at the moment of relapse. The latter cell line appeared to be highly resistant to ara-C and showed a marked reduction in deoxycytidine kinase activity.

Decreased levels of hypoxanthine guanine phosphoribosyl transferase, which is required for the conversion of 6-mercaptopurine and 6-thioguanine to their active metabolites, are held responsible for the resistance of some murine tumors to these thiopurines [23], but enzyme studies to test this possibility in man have indicated that this mechanism is not operative in most human leukemias [46, 195].

Exposure of cells to methotrexate (MTX) can result in the intracellular accumulation of large quantities of polyglutamate derivatives. These compounds show a similar, if not greater, ability to interact with dihydrofolate reductase and may exhibit a lower capacity to permeate the cell membrane, thus increasing the duration of inhibition of the target enzyme [116, 258]. Defective metabolism of MTX to its polyglutamate forms has been purported to cause resistance to this drug in a human breast-cancer cell line [116]. The same mechanism has recently been reported by Frei et al. [77] and Rosowsky et al. [198] for human epidermoid tumor cell lines.

Intracellularly, doxorubicin can be reduced by P450 reductase to a free radical semiquinone that can react with oxygen to form cytotoxic oxygen radicals. A decrease in the activity of the enzymes that bring about this reduction may then result in anthracycline resistance [161].

Increased intracellular degradation of cytostatic drugs

Apart from defective metabolism of cytostatic agents to active species, increased enzymatic degradation of antimetabolites also results in resistance to these drugs. Ara-C is degraded by the enzyme cytidine deaminase to arabinosyluracil. High levels of this enzyme have been found by Steward and Burke [228] in patients with leukemia exhibiting de novo or acquired resistance to the drug. However, other investigators have disputed this mechanism of resistance, as subsequent work has emphasized the heterogeneity of enzyme activities in samples from patients [226, 233].

An increase in the activity of alkaline phosphatase in human leukemic cells displaying resistance to the thiopurine 6-mercaptopurine has been reported [196, 209]. This membrane-associated enzyme degrades 6-mercaptopurine nucleotides to inactive nucleosides. Lee et al. [138, 139] have demonstrated a 100-fold difference in alkaline phosphatase activity in sensitive and resistant variants of a murine sarcoma cell line. Their results also suggested a difference in the physicochemical and immunological properties of this enzyme in sensitive vs resistant lines, possibly due to the expression of a new gene product.

Increased drug inactivation by enzymatic degradation has also been demonstrated in bleomycin-resistant cells exhibiting an increase in the activity of bleomycin hydrolase [1]. This enzyme is a cysteine proteinase that displays cathepsin H-like activity [210] and can be inhibited by L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane (E-64). Using E-64, Sebti et al. [211] have demonstrated that inhibition of bleomycin metabolism in vivo can enhance the antitumor activity of this drug and hence overcome resistance.

The principal nonprotein thiol of the cell, glutathione (GSH), is capable of inactivating alkylating agents. Suzukake et al. [229, 231] have recently described an L-phenylalanine-resistant L1210 cell line showing increased intracellular levels of GSH and GSH disulfide (GSSG), which are the critical determinants in the dechlorination of L-phenylalanine mustard to its noncytotoxic derivative dihydroxy-L-phenylalanine mustard. Resistant cells could again be sensitized to the drug by reduction of the intracellular concentration of GSH via nutritional restriction of L-cysteine. By scavaging and detoxifying free-radical species, GSH may also be responsible for anthracycline resistance [4, 10, 59]. Increases in the activity of a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-GSH-reductase [4] and of a GSH-S-transferase in cells exhibiting altered physicochemical properties as compared with those of the parent line [10] may also be associated with the emergence of Adriamycin resistance.

Buthionine sulfoximine (BSO) is a specific inhibitor of the enzyme gamma-glutamyl cysteine synthetase, which is a key enzyme in the synthesis of GSH [90]. Hamilton et al. [98] demonstrated that BSO produced a 6-fold enhancement of carboplatin cytotoxicity in a human ovarian-cancer cell line in which resistance to CDDP had been induced. The effect of BSO on increasing platinum cytotoxicity may also be attributable in part to a decrease in the repair of platinum damage, which is associated with GSH depletion [136].

Reductions in GSH levels by BSO can also result in the reversal of resistance to L-phenylalanine mustard (L-PAM) in tumor cell lines [75, 97, 168]. The effects of BSO on the cytotoxicity of L-PAM in vivo has also been examined. In tumor-bearing athymic nude mice, L-PAM treatment following GSH depletion was markedly superior to treatment with L-PAM alone [168]. Rosenberg et al. [194] established a melphalan-resistant rhabdomyosarcoma xenograft that exhibited cross-resistance with vincristine but showed no elevation in the expression of mdr1 mRNA. The GSH level was 2-fold that found in the parent line. In vivo treatment of the resistant xenograft-bearing animals with BSO increased their sensitivity to melphalan but not to vincristine, suggesting that cellular GSH levels are not an important modulator of vincristine cytotoxicity in this tumor and that an increase in the GSH content is probably not the only difference between the resistant and the parent strain.

Glutathione-S-transferase (GST) isoenzymes conjugate electrophilic cellular poisons with GSH to produce a less toxic and more easily excreted metabolite. Wang and Tew [251] isolated a cell line that was resistant to bifunctional nitrogen mustards and displayed an approximately 2-fold increase in the bulk GST activity as compared with the parent line. An increase in GST has recently been described in a CDDP-resistant human squamous-carcinoma cell line [235].

Some L-PAM-resistant cell lines have been shown to be cross-resistant to CDDP, and recent evidence suggests that this resistance is due to increased levels of GSH [105, 205, 230, 235]. GSH levels in the cell could mediate platinum toxicity, as CDDP could directly bind to the sulfhydryl residues of GSH; this would inactivate the drug before it could reach the DNA target. GSH may also reduce CDDP toxicity by quenching DNA-Pt monofunctional adducts [61, 154]. Exposure to BSO reduced the GSH content of resistant L1210 cells and abrogated their resistance to CDDP in the experiments of Hromas et al. [105]. However, Richon et al. [183] concluded that a reduction in GSH levels did not sensitize their CDDP-sensitive or -resistant L1210 cells.

In studies on human tumor cell lines, CDDP-resistant human ovarian-carcinoma cell lines exhibiting marked elevations of GSH levels were developed; resistance was partially reversible via the depletion of GSH by BSO [59, 168, 181]. However, acquired CDDP resistance appears to be a complex phenomenon, as some resistant phenotypes also show decreased uptake rates [2]. GSH depletion failed to reverse CDDP resistance in a human head and neck squamous-carcinoma cell line [236]. Since the depletion of GSH reverses CDDP resistance in some cases but not in others, this phenomenon does not appear to be a general mechanism of CDDP resistance.

GSH peroxidase can detoxify hydrogen peroxide and organic peroxides; increases in the activity of detoxifying enzymes can render tumor cells less susceptible to damage by Adriamycin [216]. Samuels et al. [203] have indicated that GSH peroxidase-dependent detoxification of doxorubicin-induced oxygen radicals contributes to clinical resis-

tance to the drug. The resistance of MDR human breast-tumor cells to Adriamycin appears to be associated with tolerance of superoxide and hydrogen peroxide, likely caused by an elevation in the activity of superoxide dismutase and GSH peroxidase [158].

GST isoenzymes also play a role in the inactivation of anticancer drugs. An elevation in GST activity has been found in a nitrogen-mustard-resistant tumor cell line; inhibition of GST can restore sensitivity to alkylating agents [251]. A particular GST isoenzyme, GST-π, appears to occur in some resistant carcinoma cell lines as well as in a variety of human tumors. Wang et al. [252] described increased GST-π activity in four human melanoma drug-resistant sublines, each of which was resistant to a different alkylating agent. However, as these cell lines demonstrated no cross-resistance, the observed increase in the level of GST-π could not have been the predominant mechanism of resistance to the drugs tested in these cell lines. Saburi et al. [201] also found higher GST activity and an increase in GST-π mRNA in CDDP-resistant chinese hamster ovary (CHO) cells but not in a CDDP-resistant prostate-carcinoma cell line.

Another factor in the protection against intracellular drugs may involve an elevated pentose phosphate cycle (hexose monophosphate shunt), leading to the generation of more NADPH for the continual regeneration of GSH. Gessner et al. [82] examined the level of the pentose phosphate cycle in daunorubicin-sensitive and -resistant P388 cells and found a 2-fold higher capacity for this cycle in resistant cells.

In some resistant cell lines, high levels of metallothioneins are found [5, 65]. Metallothionein is a small (6,000 Da), soluble, cytoplasmic, cysteine-rich protein that is essential for the detoxification of heavy metals. Its synthesis can be induced by the presence of cadmium, zinc, and copper [254]. By measuring the metallothionein content of and metallothionein mRNA levels in human small-cell carcinoma cell lines, Kasahara et al. [122] demonstrated the importance of metallothionein in the development of resistance to CDDP [122]. A high degree of resistance to CDDP has recently been found in metallothionein-containing cells [5, 66, 162]. As virtually no metallothionein was induced during the treatment of these cells, it was concluded that the CDDP had probably bound to the preexisting metallothionein [66]. In contrast, Schilder et al. [206], who studied metallothionein gene expression in human ovarian-cancer cell lines, concluded that no causal relationship exists between metallothionein expression and CDDP resistance. Clearly, a role for metallothionein in the resistance to CDDP has not been established [206]. Other cytotoxic drugs can also bind to thiol-containing compounds [35]. Endresen et al. [66] developed two cell lines displaying a high content of metallothioneins that exhibited resistance to the nonmetal-containing alkylating drug chlorambucil.

Gene amplification and increased production of target enzymes

Since 1978, Schimke and co-workers [207] have demonstrated that the amplification of genes coding for target proteins can be associated with the development of drug resistance. Gene amplification can occur spontaneously in the absence of selection pressure but is facilitated by the treatment of cells with agents that alter DNA replication or DNA structure. Gene amplification has been observed not only for genes coding for enzymes such as dihydrofolate reductase, hypoxanthine guanine phosphoribosyl transferase, thymidylate synthetase, hydroxymethylglutaryl coenzyme A reductase, adenosine deaminase, glutamine synthetase, ornithine decarboxylase, and ribonucleotide reductase but also for those coding for metallothioneins and for the emergence of MDR.

Methotrexate (MTX) has become a model compound in the study of gene amplification as a cause of resistance. MTX is a potent inhibitor of dihydrofolate reductase, the enzyme that converts dihydrofolate to tetrahydrofolate and thus replenishes the intracellular pool of reduced folates that are required for thymidylate and purine synthesis, for certain amino-acid interconversions, and possibly for the synthesis of neurotransmitters [7, 18, 115]. When the block is released after removal of the drug, chromosomal segments that have undergone partial replication again replicate, producing an extra copy of that segment, which may either be reinserted into chromosomes or remain as extrachromosomal elements. Amplified dihydrofolate reductase genes can be stable in some cell lines that have been grown in the absence of selective pressure, whereas in other cell lines, 50% of the genes can be lost in 20 cell doublings [25]. Stable amplified genes are present on one of two homologous chromosomes at the site of the resident nonamplified gene [165, 208], and cytogenetic analysis reveals elongated marker chromosomes displaying a homogeneously staining region [19, 39, 57, 156, 165, 166]. In situ hybridization studies have shown that the amplified genes are specifically localized in the homogeneously staining region [57, 165]. Unstably amplified dihydrofolate reductase genes reside on double minute chromosomes [25, 125, 126]; as the latter contain no centromers, they can be proportioned unequally into daughter cells and can be rapidly lost.

In cells that overproduce dihydrofolate reductase, the number of dihydrofolate reductase gene copies and the amount of messenger RNA coding for the enzyme may not be quantitatively related to the amount of gene product [21, 77]. Domin et al. [58] developed a series of human KB cell lines showing increased methotrexate resistance; a high level of dihydrofolate reductase activity was found in relatively few gene copies. In some subclones, the level of dihydrofolate reductase did not correlate with the expression of dihydrofolate reductase mRNA, suggesting the existence of differences in transcription and translation. Frei et al. [77] and Rosowsky et al. [198] recently reported on MTX-resistant cultured human squamous-cell carcinoma lines exhibiting dihydrofolate reductase gene amplification. Again, the increase in gene-copy number proved to be lower than that in the gene product.

It appears that gene amplification leading to resistance also occurs in the clinical setting. In an analysis of dihydrofolate reductase gene-copy numbers prior to treatment and after the development of resistance to MTX, Horns et al. [102] and Carman et al. [28] found a 3- to 6-fold amplification of dihydrofolate reductase genes in two leukemia patients. Curt et al. [41] reported on a tumor cell line derived from a patient presenting with small-cell lung cancer who was treated with MTX. This cell line contained double minute chromosomes and approximately 5 times as many dihydrofolate reductase genes as normal cells. Trent et al. [242] studied cells from a patient suffering from ovarian carcinoma who had previously been treated with MTX for psoriasis. Using karyological analysis, these authors observed a homogeneously staining region containing large numbers of dihydrofolate reductase genes.

These cases suggest that amplification of the gene coding for dihydrofolate reductase is associated with clinical drug resistance. It is not clear whether an increase in the production of the target enzyme thymidylate synthetase in some fluorodeoxyuridine-resistant cell lines [9, 173, 199] is also associated with gene amplification or with other mechanisms such as increased mRNA stability, transcription efficiency, or enzyme half-life [42].

Alteration of target proteins

Acquired MTX resistance can also result from the production of altered dihydrofolate reductase displaying lower affinity for the drug or altered dihydrofolate reductase exhibiting higher activity. A number of altered dihydrofolate reductases demonstrating different kinetic and physical properties have been described [73, 95, 96, 112], probably reflecting mutations in the various amino acids involved in the binding of MTX with the catalytic pocket of the enzyme molecule. For instance, Haber et al. [95] studied a line of mouse fibroblasts grown in progressively increasing concentrations of MTX. Initially, resistance resulted from gene amplification leading to increased concentrations of normal dihydrofolate reductase, but the growth of these resistant cells at higher drug concentrations caused the emergence of cells containing high levels of dihydrofolate reductase exhibiting a 270-fold reduction in binding affinity for MTX. As the variant gene was not detected in the population of cells that were resistant to the lower dose of MTX, these authors postulated that a mutational event occurred after the initial gene amplification.

Dedhar and Goldie [49] described a highly MTX-resistant L5178Y mouse-leukemia cell line displaying overproduction of two antigenically distinct forms of dihydrofolate reductase. This overproduction was associated with abundant mRNA enzyme activity, and chromosome analysis revealed a homogeneously staining region in one of the chromosomes of the resistant cells. Obviously, in this case the gene amplification resulted in the overproduction of two distinct enzymes [49]. Crouse et al. [40] studied amplified dihydrofolate reductase genes in mouse sarcoma S180 cells. In addition to the normal gene, they found two classes of variant dihydrofolate reductase genes in the amplified cell line.

Dedhar et al. [50, 51] described an MTX-resistant human leukemia cell line that showed higher specific affinity for dihydrofolate reductase as compared with the sensitive cells, the enzyme from the resistant cells being markedly sensitive to heat and exhibiting molecular-weight and kinetic properties that differed from those of the dihydrofolate reductase from the sensitive cells. As there were no differences between the two cell lines in terms of the amount of dihydrofolate reductase protein and the rate of dihydrofolate reductase synthesis, these results suggested that the observed increase in dihydrofolate reductase activity was not attributable to the expression of a more active form of the enzyme. The appearance of variant dihydrofolate reductases is not necessarily caused by mutation(s). Melera et al. [151, 152] reported on the overproduction of two molecular-weight classes of the enzyme in MTX-resistant Chinese hamster cells and presented evidence that both weight classes had previously been present in drug-sensitive cells.

A number of investigators have provided evidence that the cell surface may be a possible site of action for Adriamycin. Adriamycin coupled to polyglutaraldehyde microspheres or to an insoluble agarose support was actively cytotoxic to a number of cell lines [191, 240, 243]. Interaction with cell-surface components may be a factor leading to cytotoxicity. Taylor et al. [234] have reported the isolation of macromolecular lipids from sensitive and resistant L1210 cells that specifically bind Adriamycin. In the resistant cells, Adriamycin binding of the lipids was altered such that 2–10 times more drug was required for saturation and the K_m value was 3-fold that of the lipid from sensitive cells. These data suggest that resistance may be the result of decreased binding to altered membrane components. Perhexiline maleate, a drug known to raise the phospholipid content of fibroblasts, markedly increased the sensitivity of an Adriamycin-resistant P388 cell line in the experiments of Ramu et al. [177] who suggested that this effect might have been associated with perhexiline maleate-induced alterations in the lipid metabolism of the cells.

Several reports in the literature implicate a reduction in topoisomerase II activity in drug-resistant cells. Type II topoisomerases are adenosine triphosphate (ATP)-dependent enzymes that function in a variety of genetic processes and induce topological changes in DNA. They create (and then reseal) DNA double-strand breaks, presumably via a transient intermediate complex of the enzyme covalently linked to the 5′-termini of the DNA breaks. Stabilization of this cleavable complex results in increased DNA scission and inhibition of the rejoining reaction. The cytotoxic effect of the intercalators or epipodophyllotoxins is thought to be due to stabilization or trapping of these complexes by the drugs, resulting in the stimulation of DNA cleavage [159, 163, 171, 259]. Danks et al. [44] reported on teniposide (VM-26)-resistant human leukemic cells that showed cross-resistance but displayed no defect in drug accumulation and failed to express P-glycoprotein but exhibited altered topoisomerase II activity. Ferguson et al. [67] found decreased levels of topoisomerase II in etoposide (VP-16)-resistant human nasopharyngeal carcinoma KB cells; however, in this model a reduction in

drug accumulation also contributed to the observed resistance [67].

Topoisomerase II-mediated DNA cleavage is stimulated by Adriamycin at low concentrations; anthracyclines do not produce DNA double-strand breaks in the absence of the enzyme [237]. Also, the work of Glisson et al. [84] suggests that the cytotoxicity of Adriamycin may be related to interference with the type II topoisomerase-mediated cleavage of DNA. A reduction in topoisomerase II activity in Adriamycin-resistant P388 leukemia cells has also been described [52]. In studies on resistant cell lines, Pommier et al. [172] and Glisson et al. [83] put forward the hypothesis that a qualitative change in type II topoisomerase by mutation alters the interaction of VP-16 with the enzyme-DNA complex. Their data also suggest that a qualitative change in the enzyme may lead to MDR [84, 172]. However, the occurrence of MDR due to altered topoisomerase in drug-resistant patients remains to be determined.

Altered expression of tubulin may be important to vincristine resistance. Several workers have isolated mutant clones of CHO cells that proved to be resistant to a variety of antimitotic drugs [27, 94, 144]. The emergence of resistance was associated with reductions in the binding affinity of tubulin for these drugs [128, 144], with alterations in the electrophoretic patterns of tubulin subcomponents [27, 128], and with changes in microtubule-associated proteins [94]. More recently, a human rhabdomyosarcoma grown as a xenograft in immune-deprived mice has been selected for resistance to vincristine [103]; electrophoretic analysis of β-tubulins from resistant vs sensitive cells revealed differences in migration patterns.

Increased efficiency of DNA repair

Cell sensitivity to DNA-damaging agents depends on many factors, including those related to the intracellular presence or absence of enzymes that survey the DNA for damage and repair it by restoring its normal nucleotide sequence and stereochemistry. The most general mechanism is excision repair, which is responsible for the removal of many types of lesions. The sequential steps include recognition of the damage, incision of the damaged strand at or near the site of the defect, excision of the defective site, repair replication to replace the excised region, and ligation to join the repair patch to the contiguous parental DNA strand.

Repair can be followed by the appearance and subsequent ligation of DNA strand breaks, e.g., by alkaline elution or by other methods that assess the unwinding of DNA-containing strand breaks. Repair replication may be measured by the determination of unscheduled DNA synthesis. Antibodies to bromodeoxyuridine have been used to identify DNA molecules containing bromodeoxyuridine-labeled repair patches. Bromodeoxyuridine labeling of newly synthesized DNA is also used to facilitate the resolution of parental DNA from replicated DNA via equilibrium sedimentation. Repair of essential genomic regions to facilitate transcription may be more essential than repair to enable the cells to complete the replication of their genomes [20].

A number of repair-mutant cell lines have been isolated on the basis of their increased sensitivity to DNA-damaging drugs such as alkylating agents [66, 149, 204, 214, 239], mitomycin C [153, 189], and bleomycin [189]. Moreover, cell lines deficient in DNA repair were found to be markedly sensitive to CDDP [153, 189]. Repair of sublethal DNA damage produced by alkylation in mammalian cells was first suggested by Crathorn and Roberts [30]. These investigators observed that HeLa cells treated with varying doses of mustard gas exhibited a dose-survival curve with a shoulder, suggesting that repair of alkylated DNA had occurred. It has also been shown that exposure of various mammalian cells to nitrogen mustard stimulates unscheduled DNA synthesis [187].

In many resistant cell lines, an increase in the efficiency of DNA repair mechanisms has been described. Chou and Yost [34] examined the DNA repair status of Adriamycin-sensitive and -resistant P388 cells. Following alkaline elution, the resistant cells showed immediate and continued repair after removal of the drug. Zuckerman et al. [261] developed a subline of B16 melanoma that exhibited 10-fold resistance to bleomycin as compared with the parent line; slight cross-resistance with etoposide was noted. The overall number of single- and double-strand DNA breaks produced by bleomycin treatment did not differ in sensitive vs resistant cells. However, the cross-resistance with etoposide suggests an enhanced capability of the resistant cells to withstand or repair single-strand breaks [261]. Bedford and Fox [14] used alkaline elution to study the removal of DNA interstrand cross-links after the interaction of busulphan with DNA in Yoshida sarcoma cells. Cross-links were removed from the resistant cells after 6 h but were not removed from the sensitive parent cells, even after 24 h [14]. Masuda et al. [148] developed ovarian-carcinoma cell lines in which primary resistance to Adriamycin, melphalan and CDDP was induced (overexpression of P-170 glycoprotein could not be detected). Using [^{3}H]-thymidine incorporation, these authors found a clear increase in the ability of resistant cells to repair DNA damage; moreover, their studies with aphidicolin, an antibiotic isolated from *Cephalosporium aphidicola* that inhibits polymerase α, responsible for the movement of the replication fork, indicated a causal relationship between resistance and repair activity [148]. Alkylating agents and nitrosoureas induce DNA cross-links by methylation of the N^7 position of guanine; enhanced repair of these lesions is associated with drug resistance in vitro [114] and in vivo [241]. The DNA repair enzyme *O*^{6}-alkylguanine-DNA alkyltransferase, which repairs alkylation at the O^6 position of guanine, plays a role in mediating resistance to alkylating agents. Increased levels of this enzyme have been correlated with a reduction in alkylation-induced cytotoxicity [53]. However, the observed resistance is not necessarily due to the repair of the O^6-guanine adducts. The increased alkyltransferase activity may be closely linked to the activity of other DNA repair mechanisms [8].

Eastman et al. have developed resistant L1210 cell lines by stepwise exposure of the cells to increasing CDDP concentrations; these lines show a very high level (up to 100-fold that of the parent line) of resistance to CDDP [62, 184]. Using exposure to a radiolabeled analog of

CDDP, all of the resistant cells exhibited a decrease of about 40% in drug accumulation [184]. This reduction in accumulation was not proportional to the degree of resistance in these cells. However, decreasing the intracellular concentration of drug in the resistant cells to a level equal to that in the sensitive cells did not sensitize the former to CDDP [184]. After exposure of the various cell lines to cadmium chloride, the resistant lines appeared to be very sensitive. This finding obviates a role for metallothionein in CDDP resistance in these lines, and Eastman and coworkers [63] concluded that the above-mentioned mechanisms could account only for a minor degree of the resistance shown by these L1210 cells. Subsequently, they analyzed the individual nucleoside-bound adducts and found that the rate of repair of adducts at GG sequences was markedly enhanced in the resistant cells. The reduced rate of accumulation discussed above may in fact result from these differences in the DNA repair rate.

Studies on cultured human fibroblasts have clearly shown the great importance of the DNA repair capacity of cells to their survival after CDDP treatment [56]. Comparisons of adduct formation and repair in human testicular and bladder cancer have suggested that differences in the levels of adducts formed, in combination with a more or less efficient repair of DNA, can explain the discrepancies observed in the effect of CDDP on cell survival [15]. In a study on platinum concentrations and DNA-adduct levels in tumors of CDDP-treated LOU/M rats that were inoculated with CDDP-sensitive or -resistant IgM immunocytoma, Fichtinger-Schepman et al. [68] could find no difference in the platinum levels achieved shortly (1 h) after the administration of a high dose of CDDP (10 mg/kg) or in the formation of CDDP-DNA adducts in the CDDP-sensitive vs resistant tumors, indicating that the acquired CDDP resistance was not attributable to a decrease in the formation of CDDP-DNA adducts, at least in this tumor model. However, the large variations in the values found for the adduct levels in the tumors make it difficult to reach a conclusion with regard to the existence of differences in repair capability [68]. Behrens et al. [16] have reported that induced resistance to CDDP in a human ovarian-cancer cell line is accompanied by cross-resistance to other DNA-damaging agents such as melphalan and irradiation, and they suggest that a common repair mechanism may be responsible. Their experiments indicated the occurrence of a CDDP- and melphalan-dose-dependent increase in unscheduled DNA synthesis in resistant cells [16].

In vivo studies on the effect of platinum derivatives on the above-mentioned IgM immunocytoma in the LOU/M Wsl rat have shown that cross-resistance with other platinum-containing drugs such as *cis*-diammine(1,1-cyclobutanedicarboxylato)-platinum(II) (CBDCA, carboplatin), *cis*-dichloro-*trans*-dihydoxybis-(isopropylamine)-platinum(IV) (CHIP, iproplatin), and (*trans-d*,1)1,2-diamminocyclohexanetetrachloroplatinum(IV) (tetraplatin) can be induced [227]. Also, the induction of resistance to CDDP in vitro is accompanied by cross-resistance with these platinum analogs. In these experiments, cross-resistance was also observed with other drugs that interact with DNA, including Adriamycin, mitomycin C, and melphalan. Preliminary results obtained using ara-C as the cytostatic drug also indicate the occurrence of cross-resistance, at least after exposure to the drug for b48 h. No difference in sensitivity could be detected following exposure of the two cell lines to vincristine or VP-16, cytostatic drugs that do not directly interact with DNA [250]. These data also suggest the possibility of a common (enhanced) repair mechanism for drugs that interact with DNA.

Altered nucleotide pools

Increases in intracellular pools of deoxycytidine triphosphate (due to decreases in the activity of deoxycytidine monophosphate deaminase and/or to increases in the activity of cytidine triphosphate synthetase) can result in resistance to ara-C by increasing the feedback inhibition of deoxycytidine kinase (necessary for metabolism of ara-C to ara-CMP) and by competing directly with ara-CTP for incorporation into DNA. Expanded intracellular pools of this nucleotide have been described in ara-C-resistant Chinese hamster fibroblasts [55]. The importance of this mechanism in clinical drug resistance remains uncertain.

Increases in cytidine triphosphate levels associated with resistance to 5-fluorouracil have been found by Kaufman [124] in V 79 Chinese hamster cells. The primary lesion in these mutants appears to involve an alteration in cytidine triphosphate synthetase activity, which is no longer sensitive to negative regulation by cytidine triphosphate. The resulting high levels of cytidine triphosphate and deoxycytidine triphosphate subsequently induce negative feedback of enzymes required for the activation of 5-fluorouracil.

Salvage pathways

Salvage pathways can circumvent the inhibitory effects of some antimetabolites on the de novo synthesis of nucleotides. Thymidine kinase transforms the drug fluorodeoxyuridine to deoxyfluorouridine monophosphate but also provides an alternative pathway for circumventing the deoxyfluoromonophosphate blockade of thymidylate synthesis. Kessel and Wodinsky [132] described an inverse correlation between the responsiveness of different transplantable mouse leukemias in vivo and the levels of thymidine kinase in the tumor cells. Laskin et al. [137] found higher thymidine kinase activity in human and mouse cell lines exhibiting less sensitivity to 5-fluorouracil.

The availability of salvage pathways may also be an important factor for resistance to MTX as this drug inhibits both de novo thymidine and purine synthesis. In L1210 cells selected for impaired purine salvage, Browman and Csullog [24] demonstrated increased sensitivity to MTX. The reason for this increase in sensitivity may be the relative inability of tumor cells to overcome the antipurine effects of MTX by utilizing exogenous purines.

Conclusion

Foremost among the obstacles encountered in the treatment of cancer is de novo (colorectal carcinoma, malignant melanoma) or acquired (breast cancer, small-cell lung

cancer) drug resistance. Comparative investigation of parental cells vs resistant sublines for biochemical and genetic differences is a powerful research tool. Genetic changes in individual tumor cells can induce changes in the biochemical properties of the cells, rendering them resistant. The emergence of drug resistance appears to be mediated at the level of the cell membrane for some agents and at the cytoplasmic or nuclear level for others. The biochemical basis of resistance may involve an alteration in drug entry into the cell, binding to a target, intracellular activation or inactivation, or efflux. Amplification or altered expression of genes may be responsible for the synthesis of an increased amount of target or of mutant targets exhibiting lower affinity for the drug.

Alterations in the cell membrane appear to induce resistance to several different types of drugs – so-called pleiotropic or multidrug resistance. Increased expression of P-glycoprotein, which is strongly associated with the phenomenon of pleiotropic drug resistance, and enhancement of DNA repair mechanisms may also result from gene alteration. Multidrug resistance poses a special problem in the treatment of cancer patients; theoretically, it inhibits successful cytostatic therapy.

Basic information about a drug-resistant tumor and sufficient understanding of the intracellular and membrane changes that occur in resistant cells should provide us with a rationale for the development of strategies for the identification and prevention of drug resistance, leading to more effective therapy. Research should also be aimed at modulating or circumventing resistance to clinically useful drugs, thus providing more effective treatment modalities using agents with known toxicities. Increasingly more research must be aimed at the use of a nontoxic "modulator" in combination with a standard dose of antitumor agents so as to enhance the effect of the cytostatic drugs without producing increased damage to normal tissues. These modulators should interact with the drug target or interfere with specific resistance mechanisms.

However, it should be borne in mind that resistance in individual patients does not always imply the presence of drug-resistant tumor cells. Other factors such as the patient's tolerance of drugs, the tumor's size, the vascularization and localization of the tumor, individual pharmacokinetics, the inactivation of drugs, and the unfavorable kinetics of solid tumors, particularly that of a large fraction of tumor cells that do not actively progress through the cell cycle, can play a significant role. The observation that most normal cells do not become resistant is fascinating; one could speculate that the greater genetic stability of normal cells might be a reason for this phenomenon.

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