Reduced Drug Accumulation in Multiply Drug-resistant Human KB Carcinoma Cell Lines

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ABSTRACT

Human KB cells with increasing resistance to colchicine and other chemotherapeutic agents have been isolated in four sequential steps. This report describes the characterization of drug uptake in the parent and four mutant cell lines. Drug uptake in these cell lines occurred via a nonsaturable process. In general, drug accumulation decreased with increasing drug resistance; this relationship was seen best with colchicine, vincristine, vinblastine, and daunomycin and, to a lesser extent, with actinomycin D. The accumulation of dexamethasone, an agent to which all lines were equally sensitive, was similar for the parent and the four mutants. Drug efflux occurred rapidly, and differences among the various cell lines could be detected within the first minute. In the more resistant lines, a greater percentage of the drug was released more rapidly, although the absolute amount of drug released was less. Verapamil partially reversed the multiple drug-resistance phenotype by increasing the initial rate of uptake and accumulation of drugs in the resistant cell lines without an apparent effect on drug efflux. The results suggest that, in this human epithelial cell, the development of resistance to multiple drugs is complex, with changes in drug uptake, accumulation, and efflux.

INTRODUCTION

The development of resistance to multiple drugs is a common clinical problem in the treatment of various cancers. To analyze this problem, several investigators have developed cell lines which are resistant to multiple drugs, although they were selected with increasing concentration of a single drug. Several rodent lines and one human lymphoblastoid cell line have been characterized and shown to have decreased drug accumulation (1-8). However, no human epithelial cell lines have been studied, despite the fact that, in a clinical setting, these cell types are often resistant to a wide variety of drugs. The studies reported here were undertaken with the aim of characterizing the drug accumulation and efflux from a malignant human epithelial cell line.

We describe elsewhere the isolation and genetic characterization of four mutants of the human KB carcinoma cell lines isolated by successive single-step selections with colchicine (9). These cells were chosen after screening a variety of human epithelial lines because their rapid growth and high cloning efficiency made genetic characterization practical. Cell-cell hybridization studies showed that the colchicine resistance and the multiple drug-resistance phenotypes were incompletely dominant. Segregation analyses suggested that complex genetic loci are involved in the acquisition of multiple drug resistance.

To help in understanding the biochemical basis of drug resistance in these cell lines, detailed studies of drug uptake, accumulation, and efflux have been carried out. They demonstrate a correlation between the degree of drug resistance and changes in these parameters in these human KB carcinoma cell lines.

Verapamil, which decreases the drug resistance of these human cell lines, also increases drug uptake and accumulation, suggesting that the development of drug resistance is related, at least in part, to alterations in cell permeability to multiple drugs.

MATERIALS AND METHODS

Materials. [3H]Colchicine (specific activity, 27.8 Ci/mM) and [3H]daunomycin (specific activity, 2.5 Ci/mM) were obtained from New England Nuclear. [3H]Vincristine (specific activity, 4.4 Ci/mM), [3H]vinblastine (specific activity, 11.4 Ci/mM), and [3H]actinomycin D (specific activity, 13 Ci/mM) were from Amerham/Seear Corp. DMEM* with 1000 mg glucose/liter, without sodium bicarbonate, and 4-(2-hydroxyethyl)-1-piperazineethane sulfoonic acid buffer were Grand Island Biological Co. products. The unlabeled compounds were purchased from Sigma Chemical Co. All other chemicals were at least of reagent grade.

Cells. Human KB carcinoma cells were obtained from the American Type Tissue Culture Collection. The parent cell line was obtained by subcloning the original line; the mutants used were selected with increasing concentrations of colchicine as described by Akiyama et al. (9). The relative drug resistance of the various cell lines is shown in Table 1. To avoid the loss of resistance that occurred upon continuous culture in colchicine-free medium, the cells used for the drug accumulation studies were obtained from recently thawed samples or from cell lines maintained in continuous culture in colchicine-containing medium for a few days before assaying.

Drug Accumulation Studies. Studies of drug accumulation were performed in a 37°C room. The plates were shielded from light to prevent inactivation of the labeled compounds. Serum-free medium was prepared by dissolving one packet of DMEM containing 1000 mg of glucose without sodium bicarbonate in 1100 ml of distilled water with 50 ml of 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer; the pH was adjusted to 7.3. In preparing the cell monolayers, 5 ml of a cell suspension containing 1 x 10^6 cells plated in 100 mm dishes with 10% FBS were plated in each well the day before use. Blank plates were prepared simultaneously using 5 ml of DMEM with 10% FBS. After an overnight incubation, the medium was decanted, and the wells were washed with 5 ml of the assay medium; then 3 ml of the medium containing the drug to be studied was added. Incubation in the drug-containing medium was carried out for variable time periods, after which the reaction was terminated by decanting the medium, followed by rapid submersion of the plates in 2 beakers in succession, each containing 2.5 liters of PBS. The plates were then turned upside down to drain briefly before the addition of 1.5 ml of 0.1% EDTA and 0.25% trypsin in PBS without calcium or magnesium. Fifteen to 30 min later, the cell suspension was transferred to a scintillation vial containing 13.5 ml of Aquasol and mixed vigorously before counting. Several factors were found to be important in obtaining reproducible results: (a) To obtain a low background, preincubation of the blank plates with serum-containing medium was required. A 50-fold or greater reduction in background radioactivity could be effected by preincubation of the dishes in DMEM with 10% FBS for at least 4 h. Use of 20% FBS or longer incubation times did not further reduce the background. (b) Even with preincubation in serum-containing medium, the background varied when comparing the top 3 wells (Wells 1 to 3) and the bottom 3 wells (Wells 4 to 6). This variation precluded the utilization of individual 35-mm drins for Wells 1, 2, and 3 in a 6-well Costar plate, since the background of Wells 4, 5, and 6 could be as much as 5-fold greater. This difference was less for

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1 The abbreviations used are: DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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RESULTS

Drug Uptake as a Function of Concentration. Reciprocal plots of the rate of drug uptake as a function of drug concentration for the parent line and the most resistant mutant line for colchicine, vincristine, and vinblastine are depicted in Chart 1. The amount of drug taken up in 60 s was directly proportional to drug concentration over a range of 2 to 200 μM, which suggests that uptake is nonsaturable and probably not carrier-mediated. To exclude the possibility that at these high concentrations a saturable process was being obscured by a nonsaturable one, the rate of uptake of vinblastine in the first 30 s was measured over the 1 to 40 nM concentration range. These results demonstrated a concentration-dependent nonsaturating uptake of vinblastine for the 5 cell lines studied (data not shown). The low specific activity of the other labeled compounds and the reduced rates of uptake compared to vinblastine precluded similar studies with the other drugs, although, over a 10-min period, colchicine accumulation was directly proportional to concentration over the 1 to 20 nM range (data not shown).

Drug Accumulation Over Time. Drug accumulation as a function of time is shown in Chart 2. The results with colchicine show slow continued accumulation over the first 8 h, with little additional accumulation observed at 24, 48, and 72 h (data not shown). Vinblastine accumulation occurred more rapidly and, at 20 μM, it reached a plateau after the first 2 h, with the plateau level decreasing with increasing vinblastine resistance (data not shown). Like colchicine, vincristine accumulated slowly, while daunomycin associated rapidly with the cells. Actinomycin D proved most difficult to study, in large part because of the lability of the tritiated material. Although we did not study the effects of temperature and pH in detail, the rate of uptake increased as the temperature increased; for colchicine, vincristine, and vinblastine, uptake rose as the pH increased above 7.3. The dependence of uptake on pH has been described previously (10). Accumulation of dexamethasone is also shown in Chart 2. The rates are similar for the parent and all the mutant cell lines (only the most resistant is shown), results which are consistent with the equivalent sensitivity of these cell lines to dexamethasone.

The uptake data for the various drugs is summarized in Chart 3, with the 1-min accumulation and the maximal accumulation (see Chart 2) plotted relative to the parent value set at 100. For each drug, the panel on the left represents the relative sensitivity as measured with the colony-forming assay used in the isolation of the various mutants; the right panel represents drug accumu-
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Chart 2. Drug accumulation as a function of time. Drug accumulation over 8 h was measured as described in "Materials and Methods." The amount of drug is expressed as pmol accumulated per 5 x 10⁵ cells. The results with dexamethasone were nearly identical for all cell lines and, for clarity, the results with the parent line and the most resistant mutant are shown. Differences at 1 min can be seen best in Chart 3. Parent line, KB 3-1 (•); KB ChB 8 (O); KB Ch" 8-5 (A); KB ChR 8-5-11 (D); KB ChR 8-5-11 -24 (•). Drug concentrations (nM): colchicine, 112.5; vincristine, and the most resistant mutant are shown. Differences at 1 min can be seen best in Chart 3. Parent line, KB 3-1 (•); KB ChB 8 (O); KB Ch" 8-5 (A); KB ChR 8-5-11 (D); KB ChR 8-5-11 -24 (•). Drug concentrations (nM): colchicine, 112.5; vincristine, 25; vinblastine, 25; daunomycin, 50; actinomycin D, 50; dexamethasone, 10.

mulation at 1 min, and the middle panel represents maximum uptake. As can be seen, there is a qualitative correlation between drug resistance and drug accumulation, but there is a quantitative discrepancy between the degree of drug resistance and the extent of drug accumulation which will be discussed below.

Drug Efflux. To characterize drug disposition further, we examined the release of drug from cells. Three factors were found to be important in these studies: the accumulation time, the extracellular concentration during this period, and the time allowed for drug release.

Release of colchicine and vinblastine as a function of time following a 2-h period of accumulation is shown in Chart 4. With both agents, the more drug-resistant mutants release a higher percentage of drug. The percentage and not the absolute amount of drug released is increased, since the parent and the less resistant lines have a larger intracellular pool of drugs. Similar results were obtained with daunomycin (data not shown).

The results depicted were obtained after incubation in 112 nM colchicine and 25 nM vinblastine for 2 h. At higher concentrations of colchicine (20 μM), the levels reached by 1 h declined slowly on incubation in drug-free medium, suggesting that drug was being released from sites which were only slowly reversible (data not shown).

Studies with Verapamil. The effect of verapamil on cloning efficiency and drug uptake is shown in Charts 5 to 7 and Table 2. Chart 5 shows the effects of verapamil (10 μg/ml) on the parent and the two most resistant clones when evaluated in a colony-forming assay. As can be seen in the experiment shown here, the parent line is unaffected, while the resistant clones demonstrate increased sensitivity to colchicine. In other experiments, we have observed some increased sensitivity of the parent cells to colchicine and other drugs in the presence of verapamil (10 μg/ml). Table 2 summarizes the data for a variety of drugs, showing that, in every case, verapamil raises the sensitivity of the resistant cells close to that of the parent cell line. The concentration of verapamil which is optimal for increasing sensitivity of the mutants to drugs (10 μg/ml) was also found to be optimal in the studies of drug uptake. As shown in Chart 6, the uptake of vinblastine increases with increasing verapamil concentration and reaches a maximum at 10 μg/ml. A summary of various studies of drug accumulation is depicted in Chart 7; drug accumulation without verapamil is shown by open bars, and accumulation with verapamil is shown by hatched bars. Verapamil at 10 μg/ml increased the 2-h accumulation values for the various drugs. Because vinblastine was taken up rapidly, we could also measure the effect of verapamil at an early time, and we readily detected a verapamil effect at 1 min. In most cases, the increases produced by verapamil represented only partial restoration of the accumulation when compared to the parent cell line.

DISCUSSION

This paper describes the characterization of a human KB carcinoma cell line and four mutants selected for increasing colchicine resistance and demonstrated to have cross-resistance to a wide variety of chemotherapeutic agents (9). These lines were selected as a model for drug resistance in a human epithelial cell line because these cell types are usually refractory to multiple drugs. Several authors have previously isolated similar rodent and a human lymphoblastoid line, and reports concerning drug accumulation in these cells have appeared (1–8).

We have measured the accumulation of 6 drugs in our cell lines, documenting the multiple drug-resistance phenotype by these assays, and we have found an inverse correlation between the degree of drug resistance determined by the colony-forming assay and drug accumulation. These results argue that the sequential development of drug resistance in our KB cell lines is related, in every step, to the extent of drug accumulation. Our results show differences in the initial rates of uptake, drug accumulation with time, and efflux and suggest that several mechanisms may mediate the multiple drug-resistance phenotype. It is not clear at this time precisely how these observations...
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Chart 3. Comparison of drug sensitivity and drug accumulation. The sensitivity of the various cell lines as determined with the colony-forming assay is compared with the results of drug uptake at 1 min and accumulation at 8 h. For the 6 drugs studied, the LD10 for each drug was determined from killing curves obtained with the colony-forming assay as described in “Materials and Methods.” The sensitivity of the parent line is arbitrarily defined as 100, while the sensitivity of the 4 mutants relative to the parent line is calculated from the respective LDx0 values. The amount of drug accumulated in 8 h and that taken up in the first minute is expressed relative to the values for the parent line, which are arbitrarily represented as 100 for each drug. For each drug, the left panel shows the results of drug sensitivity determined with the colony-forming assay, while the middle and right panels are the values for 8-h accumulation and 1-min uptake, respectively.

Chart 4. Drug efflux with time. Drug efflux over time is shown following a 2-h period of drug accumulation with colchicine (112.5 nm) and vinblastine (25 nm). For each drug, the upper panel shows the amount of drug remaining cell-associated, and the lower panel shows that being released into the medium. Parent line, KB 3-1 (■); KB ChB 8 (○); KB ChR 8-5 (▲); KB Chn 8-5-11 (□); KB Chn 8-5-11-24 (●).

Our initial studies were aimed at defining the mechanism of drug uptake in the human KB carcinoma cell line. We conclude that uptake of drugs over a range of concentrations which are clinically relevant occurs via a nonsaturable process, most likely...
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Chart 5. Effect of verapamil on drug resistance. The effect of verapamil (10 μg/ml) on the parent cell line (KB 3-1) and the two most resistant clones (KB Ch'8-5-11 and KB Ch'8-5-11-24) as evaluated by the colony-forming assay. The assay was performed as described in "Materials and Methods."

Chart 6. Vinblastine accumulation as a function of added verapamil. Accumulation of vinblastine after a 1-h incubation in media containing 15 nM vinblastine and various amounts of verapamil. Results are shown for the parent line (○), the least resistant mutant KB Ch'8 (□), and the most resistant mutant KB Ch'8-5-11-24 (△).

Diffusion. Over the large concentration range studied, the uptakes of colchicine, vincristine, and vinblastine were proportional to concentration in both the parent and the most resistant mutant, in agreement with published results for colchicine in a Chinese hamster ovary cell line (4). A previous study in murine leukemia cells reported that uptake of vincristine was carrier-mediated (10); however, the assay conditions and the limited concentration range used in the previous study may be responsible for the difference in results. We looked for a carrier-mediated system at lower drug concentrations to exclude the possibility that, at high concentrations (2 to 200 μM), such a system was being masked by a nonsaturable process, but with both vinblastine and colchicine, we were unable to document the existence of a saturable uptake system. The finding of such a system would be of questionable significance for clinically significant drug resistance, since serum drug concentrations higher than 40 nM are achieved during therapy.

Drug accumulation varied for the different agents that were studied, probably as a result of differences in membrane permeability and intracellular distribution and storage. It is likely that the differences in uptake observed at 1 min are primarily a reflection of differences in membrane binding and permeability, while the accumulation over time is influenced by additional factors, including uptake, efflux, and binding to cellular storage sites. For all of the drugs studied, the differences in the amount of drug accumulated over time were larger than the variations in initial uptake rates, suggesting that resistance involves more than membrane permeability changes. Differences in efflux were noted, and these are discussed below. Changes in target sites such as binding to tubulin are also possible, although, in the first
3 steps, a mutation affecting tubulin seems unlikely, since resistance to drugs which do not interact with tubulin developed at each step along with a decrease in drug accumulation, which is largely reversed by verapamil (12).

For all drugs studied, the percentage of reduction in drug accumulation was not the same as the degree of resistance, as determined by colony-forming assays. This discrepancy was not surprising, since the assay conditions (5 × 10⁶ cells per dish versus 300 cells per dish), the duration of the studies (hours versus 10 days), and what is measured (drug accumulated versus survival and colony formation) are very different in the 2 assays. Similar discrepancies have been described by other investigators (1, 7, 8).

The results of the efflux experiments illustrate some practical points to be considered in studies of drug accumulation and release. Since a large fraction of drug escapes in the first few minutes, studies which involve extensive washing before measurement of drug uptake or release are likely to miss this rapid efflux. In addition, although the amount of readily exchangeable drug increases with time, the percentage decreases with longer incubations, since a greater fraction becomes part of a less accessible pool (data not shown). The data obtained with our cell lines demonstrate differences in the rates and absolute amounts of drug efflux. Although the absolute amount of drug released was decreased in the more resistant mutants, the percentage was increased, suggesting that efflux occurred more rapidly in these cell lines. Since efflux reflects the size of the freely exchangeable pool, the differences could be a result of variations in this compartment among the various cell lines. The possibility that this pool is larger in the more resistant mutants cannot be excluded.

The results with verapamil clearly demonstrate the partial reversibility of the resistant phenotype, both in the colony-forming assay and in the studies of drug accumulation. Other authors have reported recently that verapamil can sensitize resistant cells to certain drugs (13, 14). It is interesting that, in the system we have studied, verapamil affects both the initial uptake, suggesting a possible effect on drug entry, and the accumulation that occurs with time. Whether verapamil acts at more than one site cannot be determined from our data. In support of the idea that verapamil has multiple modes of action is the finding that it also slows degradation of [125i]-epidermal growth factor in the lysosomes in KB cells (15). Effects of verapamil on drug uptake and resistance are observed to some extent at concentrations that have been attained with continuous verapamil infusions (13), so that it may be possible to test its efficacy in a clinical setting.

In summary, we have examined the accumulation and release of various labeled compounds in a human KB carcinoma cell line and four mutants with cross-resistance to a wide range of chemotherapeutic agents. Drug accumulation correlated qualitatively with the degree of drug resistance determined by colony-forming assays, but the degree of this correlation varied for different drugs. Differences in drug uptake, efflux, and accumulation were apparent, but how these effects are mediated cannot be determined from our data. The partial reversibility of the drug resistance by verapamil and previous genetic analyses (9) suggests that more than one mechanism may be responsible for the phenotype.

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