Collateral Sensitivity to Cross-Linking Agents Exhibited by Cultured L1210 Cells Resistant to Oxazaphosphorines

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ABSTRACT

The sensitivity of cultured L1210 and P388 cells, sensitive (L1210/0, P388/0) and resistant (L1210/CPA, P388/CPA) to cyclophosphamide in vivo, to five oxazaphosphorine and eight nonoxazaphosphorine cross-linking agents was determined. Each of the resistant sublines was cross-resistant to all of the oxazaphosphorines tested. The P388/CPA cell line was also cross-resistant to all of the nonoxazaphosphorines but, in most cases, not nearly to the same extent. The L1210/CPA cell line was collaterally sensitive to all but one of the nonoxazaphosphorines, in which case it was equsensitive. Changes in sensitivity could not be accounted for by changes in intracellular pH values, or by changes in intracellular inorganic phosphate or acid-soluble organic phosphate concentrations. Inasmuch as the L1210/CPA cell line was specifically resistant to the oxazaphosphorines, identification of the phenotypic basis for this resistance should serve to identify a potentially important determinant with regard to the basis for the oncotoxic specificity of this group of agents.

INTRODUCTION

The 2-chloroethylamido-oxazaphosphorines, cyclophosphamide and ifosfamide, are nitrogen mustard analogues that exhibit a great deal of clinical utility as antineoplastic and immunosuppressive agents (3, 16). Both of these agents are prodrugs. They are first hydroxylated to 4-hydroxycyclophosphamide and 4-hydroxyifosfamide, respectively. The hydroxylated metabolites are also prodrugs. They give rise to aldehydes which, in turn, give rise to acrolein and the corresponding reactive mustards, namely, phosphoramide and isophosphoramide mustard, respectively. Cytotoxicity is apparently due primarily to cross-linking of DNA by the reactive mustard metabolites. Relative to that exhibited by most cross-linking agents in current clinical use, the oxazaphosphorines exhibit a greater margin of safety in the treatment of a number of neoplasms.

Although the metabolism and mechanism of action of the oxazaphosphorines is fairly well understood, the basis for their relatively favorable margin of safety is not; it does, however, appear to reside with the 4-hydroxy/aldehyde intermediates rather than with the reactive mustard (31, 40). The long-term goal of our efforts is to identify the relevant determinant(s).

Many tumor cell populations become resistant to a cytotoxic agent when repeatedly exposed to it. Identification of the basis for this resistance often serves to identify one of the determinants influencing the sensitivity of a given cell population to the drug. Hence, our initial efforts, the subject of this report, were directed toward identifying cell lines that could be grown in culture (to eliminate host factors) and that had acquired resistance to the oxazaphosphorines but that retained their sensitivity to other cross-linking agents.

L1210 and P388 cells, sensitive (L1210/0, P388/0) and resistant (L1210/CPA, P388/CPA) to cyclophosphamide in vivo and available from the Southern Research Institute, Birmingham, AL, seemed to be good candidates since, in vivo, the resistant sublines were cross-resistant to several oxazaphosphorines but not to other cross-linking agents (32, 33, 35, 43, 45). Moreover, all 4 cell lines had been adapted to growth in culture (32).

MATERIALS AND METHODS

Materials. 2-[Bis(2-chloroethyl)amino]-4-(2-sulfoethylthio)tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide cyclohexylamine salt (ASTA Z 7557)† was supplied by Dr. P. Hilgard, Asta-Werke AG, Bielefeld, Federal Republic of Germany. 4-Hydroperoxycyclophosphamide was supplied by Dr. A. Takamizawa, Shinogi and Co., Fukushima, Japan. 4-Hydroperoxyifosfamide was prepared from ifosfamide by Kathleen Getman, University of Minnesota, according to the protocol described by Peter et al. (29) for the preparation of 4-hydroperoxycyclophosphamide from cyclophosphamide. 4-Hydroxycyclophosphamide and 4-hydroxyifosfamide were prepared from 4-hydroperoxycyclophosphamide and 4-hydroperoxyifosfamide, respectively, as described previously (26, 27, 42). Phosphoramide mustard-cyclohexylamine and 1,3-bis(2-chloroethyl)-1-nitrosourea were supplied by Dr. H. B. Wood, Jr., Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Isophosphoramide mustard was prepared and supplied by Dr. R. F. Borch, Department of Pharmacology, the University of Rochester Medical Center, Rochester, NY. Melphalan-HCl and thiopeta were supplied by Dr. G. M. Lyon, Jr., Burroughs Wellcome Co., Research Triangle Park, NC, and by Dr. E. W. Cantrall, Lederle Laboratories, Pearl River, NY, respectively. Acrolein, mecloethamine-HCl and 2-dimethylaminoethyl chloride-HCl were purchased from Aldrich Chemical Co., Milwaukee, WI. Chlorambucil and cis-platinum(II) diamine dichloride were purchased from the Sigma Chemical Co., St. Louis, MO.

Except for chlorambucil, 1,3-bis(2-chloroethyl)-1-nitrosourea, and isophosphoramide mustard, all drugs were dissolved in triple-distilled water. The 3 exceptions were dissolved in 85% ethanol, 10% ethanol, and water containing a trace of ethanol, respectively. All drug solutions, except for that containing acrolein, were sterilized by passage through 0.22-µm Millipore filters; all were used within 1 hr of preparation and were kept at approximately 4° prior to their use. Ethanol, at the concen-
tations used, was not cytotoxic to tumor cells.

Drug-exposure media contained (mg/liter) CaCl$_2$, 100; KCl, 200; MgCl$_2$·6H$_2$O, 100; KH$_2$PO$_4$, 42; Na$_2$HPO$_4$·7H$_2$O, 453; and NaCl, 8614 dissolved in triple-distilled water. The pH was adjusted to a value of 7.4. Sterilization was effected by passage through a 0.22-μm Millipore filter.

The media used when intracellular pH and inorganic and acid-soluble organic phosphate concentrations were determined were identical, except for the addition of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (10.412 g/liter) and the reduction of NaCl to 7.894 g/liter to adjust for tonicity. The pH was again adjusted to a value of 7.4.

Cultured mouse L1210 and P388 cells, sensitive (L1210/0, P388/0) and resistant (L1210/CPA, P388/CPA) to cyclophosphamide, were obtained from the Southern Research Institute, Birmingham, AL, through the courtesy of Drs. R. F. Struck and L. J. Wilkoff. The cyclophosphamide-resistant sublines were originally obtained in vivo by means of serial drug treatment (11, 23). In our laboratory, the 4 cell lines were grown in static suppression culture at 37°C. RPMI Medium 1640 supplemented with 10% horse serum and a humidified atmosphere of 5% CO$_2$ in air was used to grow the cells. Mean population-doubling times were approximately 9 hr.

Methods. The back-extrapolation method of Alexander and Mikulski (1) was used, essentially as described previously (4, 28, 37-39), to determine the sensitivity of cultured tumor cells to drugs. Cultured tumor cells in asynchronous exponential growth were harvested and suspended, 1.5 to 2 x 10$^6$ cells/ml, in drug exposure media to which horse serum (final concentration, 10%) had been added. Drug solutions were added to the tumor cell suspension in volumes of 0.4 to 1 ml; the final volume of the tumor cell suspension was 25 ml. Exposure of tumor cells to the drug and/or solvent was for 30 min at 37°C in air. At the end of the exposure period, the cells were harvested by low-speed centrifugation and the drug-containing medium was discarded. The tumor cells were then resuspended in drug-free RPMI Medium 1640 supplemented with 10% horse serum and grown in static suspension culture at 37°C in a humidified atmosphere of 5% CO$_2$ in air. Cell concentrations were determined at zero time and at 1-day intervals thereafter by means of a particle counter. All of the cultures were subcultured when necessary to maintain exponential growth concentrations throughout the experiment. The results of a typical experiment are presented in Chart 1.

The back-extrapolation assay does not distinguish between cell death (inability to divide indefinitely) and delayed cell division. However, it was established, through the use of an in vitro colony-forming assay (28, 37), that several alkylating agents, namely, mechlorethamine, melphalan, chlorambucil, and 4-hydroperoxycyclophosphamide, all cause cell death. Similar experiments have not been conducted with the other agents used in the present investigation. Although unlikely, some of these drugs may have caused a delay in cell division rather than cell death. In that case, the terms “viable cells,” “cell kill,” “99% cell kill concentration,” and “surviving fraction” are inappropriately used.

Intracellular pH determinations were based on the distribution of a weak acid, 5,5-dimethyl-1,3-cyclohexadiene-2,4-dione, between the intracellular and extracellular space (30, 44), and were made essentially as described previously (4). Intracellular volume determinations were based on the differential distribution of $^3$H$_2$O and $^{14}$C-inulin (30, 44) and were also made essentially as described previously (4). Detailed descriptions of the methods used are available (25).

Inorganic and acid-soluble organic phosphate concentrations were determined essentially as described by others (2, 19, 24). A detailed description of the method used is available (25).

RESULTS

The L1210/CPA and P388/CPA lines were each cross-resistant to all of the oxazaphosphorines tested, namely, 4-hydroxycyclophosphamide, 4-hydroxyifosfamide, ASTA Z 7557, 4-hydroperoxycyclophosphamide, and 4-hydroperoxyifosfamide (Table 1). The magnitude of the resistance to each of these agents was approximately the same within each cell line. These findings mirror those observed in vivo with the exception that, in vivo, the results of a single experiment indicated that the P388/CPA cell line was only minimally cross-resistant to 4-hydroperoxyifosfamide (32).

The L1210/CPA cell line was not cross-resistant to other cross-linking agents, namely, several nitrogen mustards, 1,3-bis(2-chloroethyl)-1-nitrosourea, thiopeta, and cis-platinum (Table 1), thus demonstrating the specificity of the resistance by this cell line to oxazaphosphorines. Indeed, the L1210/CPA cells exhibited a collateral sensitivity4 to all of the cross-linking nonoxazaphosphorines except isophosphoramide mustard, in which case they exhibited equisensitivity. Our findings in culture are somewhat different from those made in vivo. Several laboratories found their L1210/CPA cells to be, in most cases fully, but never collaterally, sensitive to nonoxazaphosphorine cross-linking agents (32, 33, 43, 45). One laboratory reported data suggestive of collateral sensitivity, although it was not identified as such (34).

The P388/CPA cell line was resistant not only to oxazaphosphorines but also to all of the nonoxazaphosphorine cross-linking agents (Table 1). However, the magnitude of the resistance to the nonoxazaphosphorines was generally smaller, the major exceptions being full cross-resistance to mechlorethamine and

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3 The term “collateral sensitivity” is used to describe the phenomenon whereby a cell population, while acquiring resistance to one drug or group of drugs, becomes more sensitive to another drug or group of drugs (22).

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cis-platinum. Again, our findings in culture differ somewhat from those made in vivo, where even partial resistance to nonoxazaphosphorines was not detected (32, 43).

Identified determinants potentially influencing the sensitivity of a cell population to the oxazaphosphorines include intracellular pH and phosphate concentration (26, 27, 41). Differences in intracellular pH and phosphate concentrations did not account for the resistance to the oxazaphosphorines that we observed (Table 2).

**DISCUSSION**

Results of the present investigation demonstrate that the cultured L1210/CPA cell line is resistant specifically to the oxazaphosphorines; hence, the cultured L1210/0-L1210/CPA cell line pair should be useful with regard to identifying at least one of the determinants (specific to oxazaphosphorine action) influencing the sensitivity of a given cell population to these agents. The P388/CPA cell line was also resistant to oxazaphosphorines but, in contrast to the L1210/CPA cell line, it exhibited partial or, in some cases, even full cross-resistance to nonoxazaphosphorine cross-linking agents. Hence, the P388/0-P388/CPA cell line pair may yet prove to be useful with regard to identifying the determinants in question.

Any one of several changes in phenotype could account for resistance specific to the oxazaphosphorines. For example, such resistance would develop if the tumor cell population became less able to convert the nontoxic 4-hydroxy intermediates to the cytotoxic reactive mustards (26, 27, 41). General bases such as dianionic phosphate are known to catalyze this reaction (26, 27).

Thus, the intracellular concentration of dianionic phosphate or some other general base catalyst, e.g., carbonate, could serve as an important determinant with regard to the oncotoxic potential of the oxazaphosphorines. Intracellular pH could also be an important determinant, since it influences the concentration of these catalysts (26, 27). However, the resistance to the oxazaphosphorines observed in the present experiments could not be accounted for by differences in intracellular pH or phosphate concentrations. This possibility should not be entirely abandoned, since the average intracellular values that we obtained do not take into account the possibility of differences in intracellular pH or phosphate domains due to compartmentalization of the intracellular environment.

Specific resistance to the oxazaphosphorines would also develop if the tumor cell population became more able to convert the 4-hydroxy/aldehyde intermediates to the corresponding acids, since the latter are not particularly cytotoxic themselves and are not known to give rise to cytotoxic metabolites. One way that this could occur would be if aldehyde dehydrogenase activity was increased in the resistant cell population (6-9, 13-15, 36). Hilton et al. (20, 21) have obtained evidence indicating...
this to be the case in the L1210/CPA cells that they utilize.

Relative to their sensitivity to phosphoramide and isophosphoramide mustard, L1210/0, P388/0, and, in all but one case, P388/CPA, cells were substantially more, and L1210/CPA cells were approximately equi- or somewhat less, sensitive to the oxazaphosphorines. These observations and the observed absence of any (L1210/CPA) or full (P388/CPA) cross-resistance to phosphoramide mustard or isophosphoramide mustard are consistent with the conclusion that 4-hydroxycyclophosphamide and 4-hydroxyfosfamide, rather than phosphoramide mustard and isophosphoramide mustard, are the circulating metabolites of therapeutic significance when cyclophosphamide and ifosfamide are used clinically (31, 40) except perhaps in those cases where the tumor cells are relatively insensitive to these agents. However, the exception is probably not clinically relevant, since they are not likely to be used in such a situation. It should also be noted that acrolein is a very potent cytotoxic agent, more potent against L1210 cells than the oxazaphosphorines, from whence it can derive. Inasmuch as the oxazaphosphorines can very quickly give rise to acrolein at approximately physiological conditions (26, 27), appropriate experimental conditions are essential if complications with regard to interpretation are to be avoided when the desire is to quantify the cytotoxic action of oxazaphosphorines in culture.

Acquired resistance to cyclophosphamide has been induced in several experimental tumors growing in vivo. Usually, such tumors are cross-resistant to other oxazaphosphorines, and at least partially cross-resistant to nonoxazaphosphorine cross-linking agents; occasionally, their sensitivity to nonoxazaphosphorines is not altered (12, 17, 18, 32–35, 43, 45). Our findings demonstrated that acquired resistance to the oxazaphosphorines can also be accompanied by a collateral sensitivity to nonoxazaphosphorine cross-linking agents. The phenomenon of collateral sensitivity has been reviewed (22, 32). Examples in experimental tumor systems abound, although we know of only one other instance (34) where resistance/collateral sensitivity was observed within the cross-linking agent group. Important from the viewpoint of therapeutic strategy is what happens in humans, where acquired resistance to cyclophosphamide can also be accompanied by a collateral sensitivity to nonoxazaphosphorine cross-linking agents. The phenomenon of collateral sensitivity has been reviewed (22, 32). Examples in experimental tumor systems abound, although we know of only one other instance (34) where resistance/collateral sensitivity was observed within the cross-linking agent group. Important from the viewpoint of therapeutic strategy is what happens in humans, where acquired resistance to cyclophosphamide is often observed. Chemosensitivity testing of the type described by Daniels et al. (10) could answer the question. In such experiments, the sensitivity of neoplastic biopsy material, taken prior to the initiation of cyclophosphamide therapy and following relapse, to oxazaphosphorines must be assessed.

The phenotypic basis for the collateral sensitivity that we observed was not pursued in the present investigation, but we suggest that 2 changes in L1210 cell phenotype, one causing only the oxazaphosphorines to be less potent, and a second one causing all cross-linking agents to be more potent, may be operative. According to this scenario, the magnitude of the decrease in sensitivity to the oxazaphosphorines effected by one change in phenotype would have had to have been greater than the magnitude of the increase in sensitivity to these agents effected by the other change in phenotype, so that the net effect was a decreased sensitivity to the oxazaphosphorines, as observed. General possibilities with regard to changes in phenotype that might account for the phenomenon have been presented (22); for the most part, these possibilities are simple reversions of the postulated mechanisms for resistance (5).

REFERENCES

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