Alkylating agent resistance: *In vitro* studies with human cell lines
(resistant cells)

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ABSTRACT Development of *in vitro* resistance to HN2 (also called mustargen or mechloethamine hydrochloride), \(N,N',N'-\text{bis}(2\text{-chloroethyl}) N'\text{-nitrosourea (BCNU)}, \) and cisplatin [cis-diamminedichloroplatinum(II)] was achieved in two human cell lines, the Raji/Burkitt lymphoma and a squamous cell carcinoma of the tongue. A 10- to 20-fold increase in resistance relative to the parental line was achieved in 3- to 4-months of continuous selection pressure. At this time, further increase in selection pressure resulted in cell death, while removal of drug led to rapid loss of resistance. However, by holding selection pressure constant over 8- to 12-months, semistable clones ranging in resistance up to 8- to 12-fold were obtained. The half-life for resistance loss upon removal of drug was 2- to 3-months. In the presence of intermittent low concentrations of the alkylating agent, resistance has been maintained in excess of 9 months. With one exception, the growth kinetics of the resistant clones were slightly slower than those of the parental lines. Cross-resistance studies were performed against HN2, BCNU, cisplatin, phenylalanine mustard, and hydroperoxycyclophosphamide. There was, in general, a lack of cross-resistance. We conclude that stable resistance to alkylating agents is produced with difficulty. We propose that these semistable cloned human tumor lines represent clinically relevant models for the study of alkylating agent resistance and that the cross-resistance patterns among these cells have important therapeutic and mechanistic implications.

The alkylating agents represent one of the most important classes of antitumor drugs. Recently introduced alkylating agents such as cisplatin [cis-diamminedichloroplatinum(II); cis-Pt] have produced significant advances in the treatment of testicular, ovarian, head and neck, and lung cancer, and of selected other tumors, particularly squamous cell origin. Not all patients with a given tumor respond to alkylating agent therapy initially, and most patients who do respond eventually become refractory. Thus, natural and drug-induced resistance are major obstacles to clinical treatment as well as major challenges for therapeutic research.

It has been generally assumed that there is cross-resistance among the alkylating agents; that is, if a patient fails to respond to one alkylating agent, he is unlikely to respond to another. However, the data on clinical cross-resistance are conflicting and there have been no quantitative comparative studies addressed to this issue. In 1978, Schabel et al. reported the development of alkylating agent-resistant L1210 and P388 murine leukemia cell lines *in vivo* and observed that, in general, cross-resistance among the alkylating agents did not occur. Experimental studies of alkylating agent-resistant murine lines have indicated that resistance may occur by a variety of mechanisms. However, cross-resistance among alkylating agents in experimental tumors has received only limited study.

The presence or absence of cross-resistance among alkylating agents has major therapeutic implications. If mechanisms for selective cytotoxicity and resistance vary among these drugs, then their use in combination, either concurrently or in sequence, represents a rational therapeutic approach. Indeed, it has been demonstrated experimentally that substantial synergism among alkylating agents can occur.

The above considerations prompted the present study of acquired resistance, including cross-resistance patterns, to three clinically important alkylating agents, HN2 (also called mustargen and mechloethamine hydrochloride), \(N,N',N'-\text{bis}(2\text{-chloroethyl}) N'\text{-nitrosourea (BCNU)}, \) and cis-Pt, in two human cell lines, the Burkitt (Raji) lymphoma and a squamous cell carcinoma (SCC25) of the head and neck. (The term "alkylating agent," as used in this paper, includes cis-Pt and other compounds with metallation activity).

MATERIALS AND METHODS

Drugs. HN2 was obtained in pure powder form from the Dana-Farber Cancer Institute pharmacy as the hydrochloride salt. When resuspended in 0.1 M HCl, it remains stable for up to 1 year at \(-20^\circ C\) (L. C. Erickson, personal communication). Aliquots were thawed and used immediately. cis-Pt was obtained in powder form from Matthey-Bishop, Malvern, PA. BCNU was obtained from the Dana-Farber Cancer Institute pharmacy. The lyophilized powder was resuspended in 95% ethanol and stored, protected from light, at \(4^\circ C\). This preparation results in 10% degradation in 78 days. Hydroperoxycyclophosphamide (HO2-CPA), kindly provided in powder form by M. Colvin of Johns Hopkins University, was prepared in serum-free Dulbecco's modified Eagle's medium (DME medium) just before use. Phenylalanine mustard was obtained from this institute's pharmacy and was dissolved in HCl-acidified ethanol and diluted in DME medium just before use.

Cells. The Raji cell, derived from a human Burkitt lymphoma, was obtained from H. Lazarus of the Miami Comprehensive Cancer Center. Cells were grown in suspension in DME medium supplemented with 10% fetal bovine serum, 1-glutamine (292 \(\mu g/ml\)), penicillin (10 \(\mu g/ml\)), and streptomycin (100 \(\mu g/ml\)) in 8% CO2/92% air at 37°C. The SCC25 cell line was derived from biopsy of a human squamous cell carcinoma of the tongue and was established and characterized initially by J. G. Rheinwald at this institute (4). Monolayers were maintained in DME medium supplemented with 5% fe-

Abbreviations: BCNU, \(N,N',N'-\text{bis}(2\text{-chloroethyl}) N'\text{-nitrosourea; HO2-CPA hydroperoxycyclophosphamide; cis-Pt, cisplatin [cis-diamminedichloroplatinum(II)].\)
tural bovine serum and antibiotics. For the SCC25 line, hydrocortisone (0.4 μg/ml) was included in the medium.

Cytotoxicity. The drug concentration that reduced the number of viable cells to 50% of control (IC50) was determined as follows. For the SCC25 line, 1 × 10^5 cells were plated on a 60-mm dish and allowed to attach overnight; 24 hr later the medium was replaced with serum-free DME medium containing drug. Treatment was terminated after 30 min by washing with DME medium and replacing with fresh medium. After 2 weeks, the plates were fixed with phosphate-buffered 10% formalin, and the colonies were stained with 0.2% methylene blue. An appropriate concentration range was used. Raji cells were diluted to 5 × 10^3 cells per ml in serum-free DME medium. Drugs were added, and the cells were incubated (30 min at 37°C), pelleted, washed, and resuspended in fresh medium. The number of viable cells was determined on day 3, initially by trypan blue exclusion. In the majority of experiments, the flow cytometric assay with rhodamine, which has been shown to correlate with clonogenic assays (5) generally and for Raji cells specifically, was used. In addition, confirmatory studies were performed with a limiting dilution technique as a clonogenic assay for cytotoxicity (6).

Production of Resistant. Two drug schedules were used to develop resistant cell lines: intermittent and continuous (daily). For the intermittent schedule, the SCC25 cells (2 × 10^5 cells per 100-mm dish) and Raji cells (5 × 10^5 cells per ml) were treated for 30 min with the IC50 concentration of drug. The cultures were observed daily and allowed to grow until they reached the initial density or greater as determined by trypan blue exclusion (Raji) or microscopic observation (SCC25). For the Raji cells, the concentration was increased primarily on the basis of recovery time. For short times (1–2 weeks), a 1.5- to 2-fold increase in concentration was made. For recoveries of 2–4 weeks, the increase was smaller (10–50%); and for recoveries exceeding 4 weeks, the concentration was either kept the same or decreased. For the SCC25 cells, concentration changes were based on the reduction and recovery of colony number and size. For continuous (daily) exposure of Raji and SCC25 cells, daily “pulsing” for 30 min with selected alkylating agents was used.

Cloning. The cloning of resistant populations of Raji cells was accomplished by the limiting dilution technique (6) and was performed at least twice to ensure clonal derivation. Daily microscopic observation confirmed that the clone was derived from a single cell. Cloning of SCC25 monolayers was accomplished by isolation of a single large colony in a cloning chamber, followed by removal and trypsinization. Measurement of the IC50 was made periodically with each clone to determine the degree of resistance.

Resistance and Cross-Resistance. These were obtained by cytotoxicity (growth curve) assay, limiting dilution (cloning) assay, or both.

Growth Kinetics. The cytokinetics of the sensitive and resistant Raji cell lines were determined by cell-counting techniques through a 13-fold increase in cell number. The cytokinetics of the SCC25 line was determined as described (7). DNA histograms for the Raji cells were obtained and analyzed by a method (8) using propidium iodide.

Biostatistics. Since the procedures for obtaining IC50s for Raji and SCC25 cells differ, the estimates of variance were calculated separately for the two groups of experiments but were pooled within cell types. The pooled data were used to determine P values according to the BonFerani i test (9).

RESULTS

Production of Resistant Cell Lines. Attempts were made to produce six resistant cell lines with three alkylating agents and two parental cells. We succeeded in producing and finally cloning three resistant lines: a Raji line resistant to HN2 (mustargen), Raji/HN2; a Raji line resistant to BCNU, Raji/BCNU; and an SCC25 line resistant to cis-Pt, SCC25/cis-Pt (Fig. 1). Failure in the other circumstances was characterized by a lack of increase in drug resistance during in vitro selection pressure over a minimum of 3–4 months.

For production of the Raji/HN2 line, intermittent exposure to increasing HN2 concentrations resulted at 2 months in cells growing in concentrations 20-fold higher than the initial IC50. At this point (data not shown), multiple attempts to further increase drug concentration resulted in total cell death rather than increased resistance. At 3 months when drug was removed, reversion to sensitivity occurred within 4 weeks (Fig. 1). Accordingly, an attempt was made to stabilize resistance by extending the duration of treatment, with intervals between dose escalations being adjusted depending on the rate of growth recovery. Then, at 20 months, the cell line was cloned.

Resistance development in the SCC25/cis-Pt line followed a similar course (Fig. 1). After 3 months of intermittent exposure, there was a 20-fold increase in the IC50. As with the Raji/HN2 cells, a further increase in cis-Pt concentration resulted in cell death, whereas cells cultured in the absence of drug rapidly lost the resistance phenotype. Accordingly, the resistant line was exposed to a constant cis-Pt concentration with the intent of stabilizing resistance. As the treatments progressed, shorter intervals were tolerated between successive drug treatment (Fig. 1). Fifteen months after the initial selection pressure was applied, the concentration of drug was increased. In contrast to the lack of success in increasing resistance at earlier times, a further 10-fold increase in resistance was achieved. Clones were established from the culture at this time.

Exposure of the Raji cells intermittently to BCNU failed to elicit stable resistance. Accordingly, continuous daily exposure was used. In contrast to intermittent treatment, this allowed for a progressive increase in resistance to 20 times the original IC50. The line was cloned at 5 months.

Killing curves for the most resistant of each of the clones of the Raji/HN2, Raji/BCNU, and SCC25/cis-Pt cells are given in Fig. 2. In addition to these resistant clones, the following were obtained and cryopreserved: Raji/HN2, five clones with 3- to 7-fold resistance; Raji/BCNU, six clones

![Fig. 1. Time course for the development of alkylating agent-resistant cell lines. Arrows indicate the time at which the lines were cloned. Individual points corresponding to a change (or lack of change) in drug concentration are shown for the SCC25/cis-Pt cells, but are omitted for other cells for the sake of clarity. The interrupted line for the Raji/HN2 cells represents loss of resistance when drug selection pressure was withheld.](image-url)
with 5- to 8-fold resistance; SCC25/CP, two clones with 5- and 8-fold resistance. Further studies were carried out on the clones described in Fig. 2.

Stability. The stability of the three cloned resistant lines is presented in Fig. 3. In the absence of drug, resistance was stable for 1 month, after which there was a gradual loss so that by 3 months resistance was one-third the original level. Thus, these cloned lines were more stable in terms of resistance than the uncloned lines were after selection pressure (compare Figs. 1 and 3). Resistance has been maintained by low-concentration intermittent treatment with the appropriate alkylating agent for over 9 months.

Growth Kinetics. The growth curves for the sensitive and resistant Raji lines are presented in Fig. 4. The parent line and Raji/BCNU clone had a doubling time of 20 and 27 hr, respectively, whereas that of the Raji/HN2 clone was 38 hr. The doubling time for the parental SCC25 line was 48 hr and that of the SCC25/cis-Pt clone was 50 hr. DNA distribution analyses by flow cytometry (Table 1) confirmed the slower growth kinetics of the resistant Raji clones.

Cytogenetic Analysis. Two of our resistant lines, the Raji/HN2 and the SCC25/cis-Pt, were examined cytogenetically at the time of early resistance and later when cloned. Double-minute chromosomes were not observed.

Cross-Resistance. Patterns of cross-resistance are presented in Table 2. The Raji/HN2 clone remained sensitive to BCNU and cis-Pt but was partially cross-resistant to HO2-CPA and phenylalanine mustard, the other bis(2-chloroethyl)amines tested. The Raji/HN2 clone grown at low HN2 levels to maintain resistance was retested after 4 months and produced results comparable to the original resistant clone except for loss of cross-resistance to phenylalanine mustard. The Raji/BCNU clone had low cross-resistance to HN2 and HO2-CPA and partial cross-resistance to cis-Pt and phenylalanine mustard. The SCC25/cis-Pt clone had low levels of resistance to BCNU, HN2, and HO2-CPA and was partially cross-resistant to phenylalanine mustard.

DISCUSSION

We have produced resistance to three alkylating agents in two human cell lines, a lymphoma and a squamous cell carcinoma. These lines are ca. 10-fold resistant and semistable and, thus, can be used for biological studies of cross-resistance and for biochemical studies of the mechanism of drug resistance. In view of the steep dose-response for alkylating agents in the clinic, it is highly probable that 10-fold resistance in vitro would correspond to refractoriness in the patient (10). This is supported by experimental studies of Schabel et al. wherein resistance was induced in L1210 and P388 murine leukemia by serial transplantation and progressive dose escalation of the alkylating agent (3). After 3–5 transplant generations, resistance was “clinically complete” even though the same tumors were only 5-fold resistant when grown in vitro. Therefore, by analogy, the 10-fold resistant human cell lines we have developed in vitro should likewise be clinically relevant models.

The schedule of administration of an alkylating agent can markedly influence the therapeutic index and duration of remission in vivo, and we considered it likely that a form of “dose scheduling” could also influence the time needed to achieve resistance in vitro. Accordingly, we varied the schedule in an effort to produce and maximize alkylating agent resistance in culture. Continuous drug exposure is considered more effective in producing methotrexate resistance than is intermittent treatment (11). We observed simi-
Resistance to phenylalanine mustard has been shown by Vistica et al. (17) and by Redwood and Colvin (16) to be due to a transport defect, probably involving the leucine carrier. The same carrier also is thought to mediate cis-Pt uptake, since leucine provides protection from both phenylalanine mustard and cis-Pt cytotoxicity (26). Thus, one might expect some cross-resistance between phenylalanine mustard and cis-Pt but not between BCNU and cis-Pt, since BCNU probably enters cells by passive diffusion. Our results with SCC25/cis-Pt cells (Table 2) appear to support this view. It should be noted, on the other hand, that phenylalanine mustard resistance may also be associated with an elevation in intracellular glutathione (19, 27). This strong nucleophile reacts rapidly with alkylating agents and, therefore, can inactivate them before they reach critical targets such as DNA. An ovarian cell line 4-fold resistant to phenylalanine mustard was found by Vistica and co-workers to contain a 2- to 3-fold increase in glutathione over control values (19), and reduction of the glutathione level by use of cysteine-free medium or inhibition of glutathione synthetase restored sensitivity (27). These cells, like ours, exhibited cross-resistance to cis-Pt but not to BCNU. This suggests that a small increase in glutathione level may suffice to inactivate agents such as phenylalanine mustard and cis-Pt that enter cells via carrier-mediated transport but not those (e.g., BCNU) that enter cells by passive diffusion.

For CPA, a distinct type of biotransformation has been observed, which sets this drug apart from the other alkylating agents in our study. Hilton and Colvin (18) have reported CPA-resistant tumors with increased levels of aldehyde oxidase, which converts aldophosphamide to the inactive product carboxyphosphamide. This mechanism would not affect BCNU, HN2, or cis-Pt sensitivity; therefore, it was not surprising to find no cross-resistance between these drugs and HO2-CPA (Table 2).

An important mechanism for resistance to alkylating agents involves increased DNA repair. Alkylating agents differ considerably in terms of the site of DNA attack, the rate of reaction, and the nature of the covalent link. Interstrand links are formed within minutes with HN2, whereas at the other extreme, nitrosoureas form monoadducts rapidly but give crosslinks only after many hours. Monoadduct repair may occur via the action of O6-methyltransferase (22, 28); indeed, increased transferase activity has been found in a P3U1-resistant human line (29). The importance of this process is supported by the fact that repair-deficient mutants show increased alkylating agent sensitivity (30).

Ling and co-workers (31) have reported for antitumor antibotics and plant products that pleiotropic resistance occurs; that is, the production of resistance to one agent is associated with cross-resistance to others. There is evidence that this resistance is due to a decrease in net drug accumulation and is related to the presence of a glycoprotein on the cell surface (31). Although this pleiotropic resistance as described for these plant products, is not in our results, where we have observed resistance to a single agent associated with resistance to all other agents tested. Our results do suggest, however, that the biological properties of the different alkylating agents may be different in the same cell line, and that this difference may be related to the nature of the drug-induced alterations in the cell. Further investigation is needed to determine the biological basis of these differences.
scribed by Ling and co-workers has not yet been demonstrated for alkylating agents, it represents another possible mechanism of cross-resistance.

In summary, the results reported in this paper have relevance to clinical and basic science and deserve to be explored further, particularly from a mechanistic standpoint. To the extent that alkylating agents may not be uniformly cross-resistant, they represent clinically "different" compounds and are candidates for combination therapy. Alkylating agent synergism has been demonstrated in a number of experimental transplanted tumors (3). Alkylating agent-resistant human cell lines provide an opportunity to test new congeners, e.g., platinum analogs, for cross-resistance in a clinically relevant setting. Studies of the mechanism of resistance and cross-resistance among alkylating agents pose a major challenge for molecular biology, especially at the level of DNA damage and repair. In addition to furthering our knowledge of these fundamental processes, such studies should yield guidelines for more rational structure-activity-based synthesis of alkylating agent congeners and for related approaches involving alkylating agent modulation and combination chemotherapy.

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