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Detection of Heterogeneity in the Chemosensitivity of 9L Brain Tumor Cell Lines to 1,3-Bis (2-chloroethyl)-1-nitrosourea by the Sister Chromatid Exchange Assay*

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Abstract—*To investigate the potential use of the sister chromatid exchange assay to analyze the heterogeneity of drug response among tumor cell subpopulations, mixtures of* 1,3-*bis* (2-*chloroethyl*)-1-*nitrosourea-sensitive* (9*L*) *and -resistant* ($9L_2$) *rat brain tumor cells were treated* in vitro *with* 2 μM 1,3-*bis* (2-*chloroethyl*)-1-*nitrosourea. When data were plotted as histograms representing the number of cells vs sister chromatid exchanges/metaphase, two regions corresponding to the* 9*L and* $9L_2$ *populations were obtained. The approximate percentages of* 9*L and* $9L_2$ *in each mixture could be predicted from these histograms. While these results were obtained with a limited model chosen for its simplicity, they suggest that the sister chromatid exchange assay may be useful in the analysis of heterogeneity in drug sensitivity among cell subpopulations in a tumor.*

INTRODUCTION

HUMAN neoplasms consist of discrete cell subpopulations that are heterogeneous with respect to chromosome number [1], metastatic potential [2], DNA content [3], morphology [4] and other biologic characteristics. It has been shown recently that these subpopulations also have different sensitivities to chemotherapeutic drugs [1, 4–6]. Because the success of treatment depends on the sensitivity of the entire tumor cell population to a drug, heterogeneity in drug sensitivity within a tumor can be a major obstacle to successful cancer chemotherapy. At present there is no practical method with which the heterogeneity in drug sensitivity of cells in a tumor can be determined.

The *in vitro* sensitivity of tumor cells to antineoplastic agents is usually determined from survival curves based on CFE assays, which determine the percentage of clonogenic tumor cells that survive drug treatment. The CFE assay has been used to determine the BCNU sensitivity of stem cells cultured from biopsy specimens obtained from patients with brain tumors [7]. In all instances in which the CFE assay predicted resistance, patients failed to respond to therapy; however, in instances in which the CFE assay predicted sensitivity, 60% of patients responded to therapy. This range of accuracy of prediction has been reported for other drugs and other tumor types [8–10]. Unfortunately, unless extensive cloning experiments are performed, the CFE assay cannot measure the heterogeneity of drug response within a tumor.

The SCE assay is a sensitive, simple method for the measurement of damage to DNA. We have investigated the potential use of the SCE assay to determine the *in vitro* sensitivity of neoplastic cells to chemotherapeutic agents by comparing results obtained with SCE and CFE assays in

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Abbreviations: CFE, colony-forming efficiency; BCNU, 1,3-bis (2-chloroethyl)-1-nitrosourea; SCE, sister chromatid exchange.

several studies using rat 9L brain tumor cells [11–13]. The induction of SCEs by several nitrosoureas has been directly correlated to their ability to kill cells, and a linear increase in SCEs has been observed at nitrosourea doses corresponding to the shoulder region of survival curves [12]. 9L cell lines that were resistant to nitrosourea-induced cell kill were also resistant to nitrosourea-induced SCEs [13]. Depletion of intracellular polyamine levels in 9L cells increases BCNU-induced SCEs and decreases *cis*-platinum-induced SCEs; the same relative changes in cell kill, determined with the CFE assay, were found for these agents in polyamine-depleted cells [11]. Thus, in studies conducted on these drugs, the SCE assay provides the same information as the CFE assay. However, because it is more sensitive, the SCE assay may detect small changes in drug-induced cytotoxicity that the CFE assay may not detect. These changes might significantly influence the outcome of clinical treatment [14].

Because the SCE assay is based on the analysis of individual cells, it may be possible to use the assay to evaluate differences in drug sensitivity among tumor cell subpopulations. By combining various proportions of BCNU-sensitive and -resistant 9L cells in monolayer culture, mixtures simulating a tumor composed of sensitive and resistant cells were obtained. The results reported here show that the SCE assay can be used to analyze the relative proportions of these two cell types.

MATERIALS AND METHODS

Cell lines

The BCNU sensitivity of 9L cell lines used in this study have been determined from survival curves based on CFE data. 9L is most sensitive, $9L_8$ has intermediate sensitivity and $9L_2$ is the least sensitive line [15]. No significant differences in generation time were detected among the cell lines. All cell lines were seeded either as homogeneous populations (2×10^6 cells) or as mixtures (2×10^6 cells total) into 75-cm^2 tissue culture flasks and grown in 15 ml of Earle's minimum essential medium supplemented with non-essential amino acids, 10% newborn calf serum and gentamicin (50 μg/ml). The modal chromosome numbers for 9L, $9L_8$, and $9L_2$ are 55, 56 and 57 respectively. The log cell kill for 9L, $9L_8$ and $9L_2$ cells treated with 30 μM BCNU are approximately 2, 1 and 0.5 respectively [7, unpublished results].

Drug treatment

Stock solutions of BCNU were prepared in absolute ethanol immediately before use. The volume of added vehicle was always less than 1% of the final treatment volume and had no effect on SCE induction. Exponentially growing cells were treated for 3 hr with 2 μM BCNU. Under our culture conditions this is analogous to an 'infinite' exposure; increasing the exposure time beyond 3 hr does not increase cytotoxicity [16].

SCE assay

After treatment cells were rinsed, 15 ml of fresh medium was added and then a concentrated solution of bromodeoxyuridine was added to give a final concentration of 10 μM. Cells were allowed to replicate for 30 hr. Four hours before harvesting, cultures were treated with 0.04 μg/ml of colcemid. Mitotic cells were shaken from the flasks and treated with 0.05 M KCl. Cells were fixed and washed with freshly prepared methanol/acetic acid (3:1). Sister chromatids were differentially stained using the method of Perry and Wolff [17]. SCEs were counted in 50 well-spread metaphase cells by an investigator to whom samples were not identified.

RESULTS

The background levels of SCEs/metaphase were 12.0 ± 2.4 for 9L, 13.2 ± 4.2 for $9L_8$ and 12.6 ± 4.2 for $9L_2$. Histograms obtained by plotting the number of cells vs SCEs/metaphase had well-defined regions characteristic of each cell line (Figs 1A, B). Treatment of 9L cells with 2 μM BCNU produced 58.0 ± 1.9 ($\bar{x}$ ± S.E.) SCEs/metaphase, while treatment of $9L_2$ cells with the same dose produced only 19.4 ± 1.4 ($\bar{x}$ ± S.E.) SCEs/metaphase. Treatment of the intermediately sensitive $9L_8$ cell line with 2 μM BCNU produced 45.3 ± 1.5 ($\bar{x}$ ± S.E.) SCEs/metaphase. A histogram obtained by plotting the number of $9L_8$ cells vs SCEs/metaphase had a single region (Fig. 1C), the mean of which was located between the means of the sensitive (9L) and resistant ($9L_2$) regions.

Figures 2A–E show histograms obtained from SCE data for various mixtures of 9L and $9L_2$ treated with 2 μM BCNU. Histogram regions corresponding to the 9L and $9L_2$ populations did not overlap, and percentages of 9L and $9L_2$ in each flask determined by SCE counts were very similar to the actual mixtures (±10% of the actual percentage).

DISCUSSION

The SCE assay detected two cell populations and predicted the approximate percentages of sensitive (9L) and resistant cells ($9L_2$) in mixtures of BCNU-sensitive and -resistant cells treated with BCNU. The single histogram region obtained after BCNU treatment of $9L_8$, a cell line with intermediate BCNU sensitivity, indicates that $9L_8$

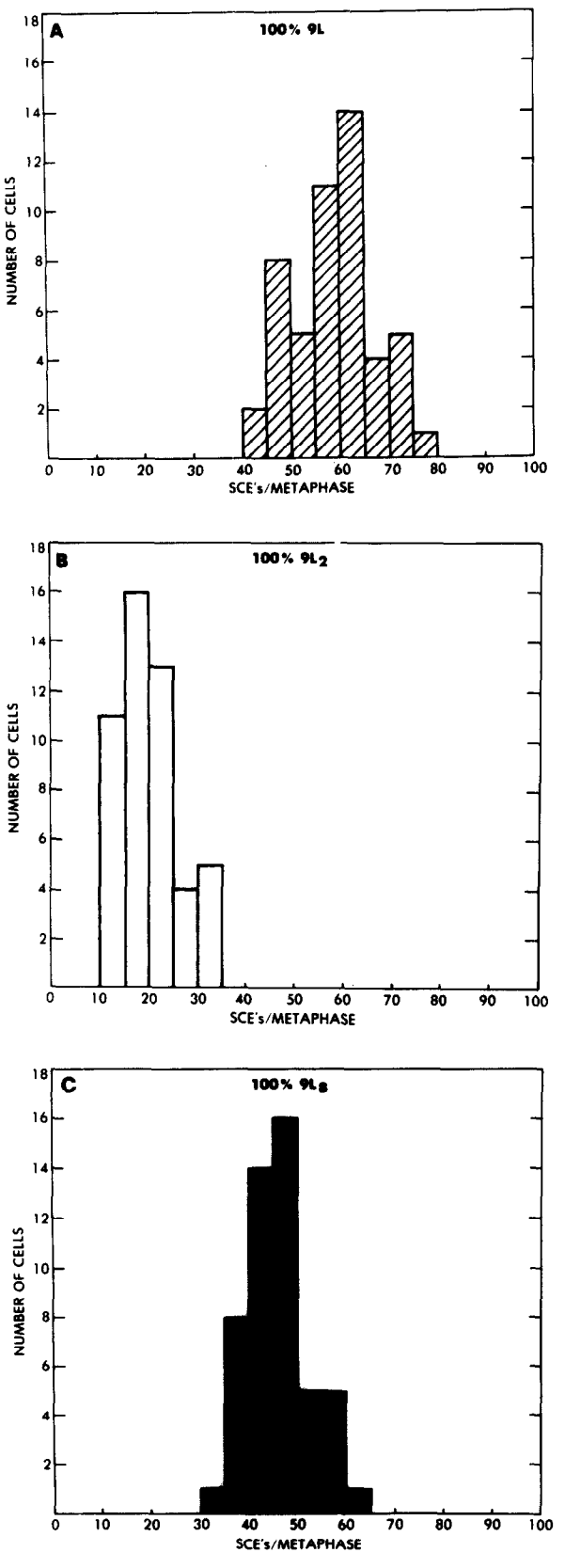

Fig. 1. SCE histograms of BCNU-sensitive (A: 9L), -resistant (B: $9L_2$) and intermediately sensitive (C: $9L_8$) cell lines treated with 2 μM BCNU.

is a relatively homogeneous population and not a mixture of sensitive and resistant cells.

The CFE assay can most accurately analyze the effects of agents against tumor cell populations that are homogeneous with respect to drug sensitivity; results obtained on a heterogeneous population of tumor cells can be misleading. For example, BCNU survival curves generated from CFE data for mixtures of 9L and $9L_2$ and $9L_8$ alone would all fall between the individual survival curves of 9L and $9L_2$; certain mixtures (for example of approximately equal proportions) of 9L and $9L_2$ would then be classified as having the same intermediate BCNU sensitivity as $9L_8$. Thus the CFE assay will not always distinguish between a mixture of sensitive and resistant cells and a population of intermediate sensitivity. The approach to the treatment of a tumor consisting entirely of cells with intermediate drug sensitivity would not be the same as the approach to the treatment of a tumor that contained a subpopulation of resistant cells.

The inability of the CFE assay to detect subpopulations of drug-resistant cells may explain why it is only partially successful as a predictor of drug sensitivity in human tumor cell populations. It is probably the case that human tumors are not homogeneous cell populations but consist of cell populatins with various degrees of drug sensitivity [5, 6]. Resistant cells, which may be only a small percentage of the tumor cell population and may not be detected by the CFE assay, would not be killed and might repopulate the tumor. This inability to detect resistant cells could give a false prediction of sensitivity.

Because of the heterogeneity of a malignant tumor, it is unlikely that a single drug could kill all cells. It has been suggested that combination chemotherapeutic protocols should be designed to take advantage of the susceptibility of individual subpopulations of a tumor rather than the additive or synergistic effects of agents on a homogeneous population [4]. Protocols based on this concept could be designed using data obtained with a method that accurately and rapidly determines the drug sensitivities of tumor cell subpopulations. For example, the BCNU-resistant cell line $9L_2$ is not cross-resistant to *cis*-platinum [Tofilon and Deen, unpublished results]. Thus, in the clinical setting, combination protocols that use BCNU and *cis*-platinum might be efficacious against tumors that contain BCNU-resistant cells.

In the studies reported here it was possible to clearly distinguish between BCNU-sensitive and -resistant cells by counting only 50 metaphases. If more cells per sample are counted, the sensitivity of the assay should increase because the larger the sample, the higher the probability of detecting cells that are infrequent in the population. Moreover, by counting more metaphases, it may

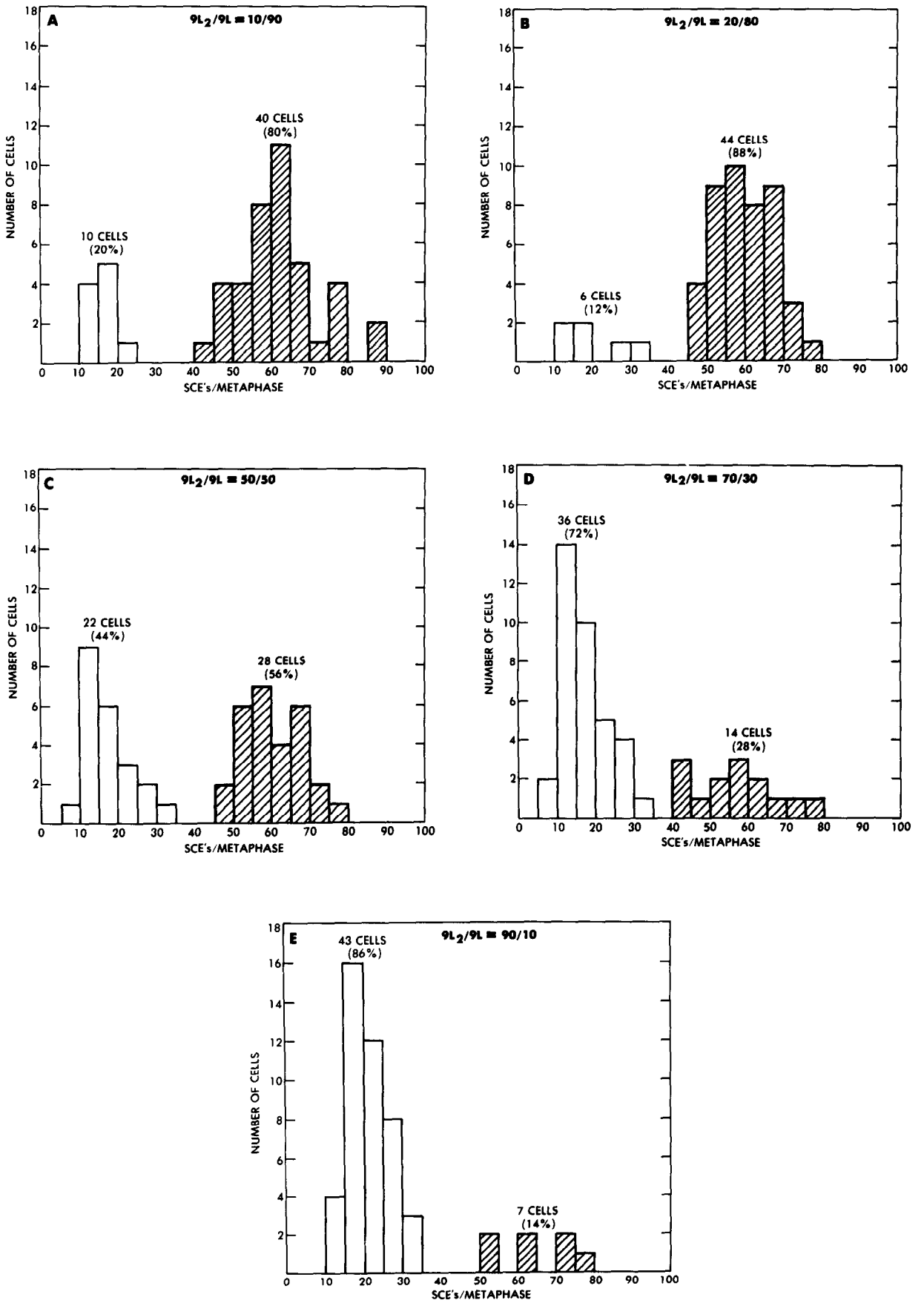

Fig 2. SCE histograms of mixtures of 9L and $9L_2$ cell lines treated with 2 μM BCNU. Actual percentages of mixtures are listed above each histogram.

be possible to detect additional subpopulations of tumor cells that may exist. Obviously, either approach may be possible only for drugs for which cell survival and induction of SCEs can be correlated. We have used the least complicated model for these studies: a mixture of one sensitive and one resistant cell line. If the results obtained with the SCE assay in the 9L model can be duplicated with cultures of human tumor cell lines that contain several cell subpopulations, the SCE assay may be a valuable method for the evaluation of the heterogeneity in drug sensitivity of subpopulations of cells in human tumors.

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