Some Properties of Substrains of L-Cells with a Decreased Sensitivity to Bis(2-chloroethyl)sulfide

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SUMMARY

Three substrains of L-cells with a decreased sensitivity to bis(2-chloroethyl)sulfide were isolated, and some of their properties were compared with those of the parental strain. Although the substrains were isolated after 1, 3, or 4 selective procedures, they all showed the same degree of resistance to bis(2-chloroethyl)sulfide, which was 2.4 times that of the parental strain. The substrains were smaller, grew more slowly, contained on the average fewer chromosomes, and possessed a more diffuse colonial morphology when compared with the parental cells. The substrains excised alkylation products from their DNA two to three times more rapidly than the parental strain. When tested with methylmethanesulfonate, dimethyl myleran, UV, and X-radiation, cross-resistance was noted only for two of the substrains towards dimethyl myleran. The third substrain exhibited an increased sensitivity towards dimethyl myleran and X-radiation.

INTRODUCTION

It is well established that bis(2-chloroethyl)sulfide reacts with the DNA of cells (8), and there is much evidence to suggest that this reaction, particularly the cross-linking component of the reaction, is responsible for subsequent cell death (1). Much remains to be understood, however, about the immediate consequences of this alkylation and about the cellular mechanisms that allow some cells to survive this kind of damage. One approach to these problems is the study of related cell lines, some of which are relatively resistant to alkylating agents. In a previous paper (10), we compared some properties of L-cells and a resistant strain. Both lines contained the same number of chromosomes. There were differences between the lines in acid-soluble sulfhydryl content, which in turn varied characteristically with the age of the culture, but resistance to bis(2-chloroethyl)sulfide was not correlated with this parameter. The resistant cells grew more slowly and were smaller. One hr following drug treatment, the DNA isolated from the resistant cells was alkylated to a lesser extent than that from the parental L-cells. This result was only partially explained by the smaller cell size. Another explanation suggested at the time was that the resistant cells possessed a mechanism for rapidly removing alkylation products.

In the present study, 3 more resistant substrains of L-cells were isolated and some of their properties were compared with those of the parental strain in order to test the generality of the previous findings and to seek a firmer basis for the mechanism of resistance.

MATERIALS AND METHODS

Cells and Cell Culture

The cell strains were grown at 37° in spinner flasks in Medium CRML-1066, lacking TdR³ and supplemented with 6% bovine serum plus the antibiotics penicillin and streptomycin (10). The cultures were kept in exponential growth by daily dilutions.

The parental strain of cells is referred to as L and was obtained from the Ontario Cancer Institute, Toronto, Canada, in 1963. It was designated L 60 T by the Toronto group because it had been adapted to grow in the absence of added TdR and because, when first received in Toronto, it had an average of 60 chromosomes (17). From time to time fresh cultures have been started from frozen stock. The sensitivity of bis(2-chloroethyl)sulfide of these cultures as well as that of several cloned cultures derived from the stock has remained constant.⁴

Resistant substrains were obtained by picking colonies that grew from Petri dish cultures that had been treated with sufficient bis(2-chloroethyl)sulfide (up to 1.5 µg/ml) to reduce the surviving fraction to 10⁻³ to 10⁻⁵. The individual colonies were grown into mass cultures, and the process was repeated. Substrain L/H3 was obtained after 3 separate

³The abbreviations used are; TdR, thymidine; MMS, methylmethanesulfonate; DMM, dimethyl myleran.

⁴The survival data reported by us in 1966 (10) showed the L-cells to be apparently more resistant to bis(2-chloroethyl)sulfide than those used in this study. The difference is due to technique, however. If, as in the earlier work, the Petri dish cultures are not swirled immediately following the addition of bis(2-chloroethyl)sulfide, the cells receive a dose of bis(2-chloroethyl)sulfide smaller than otherwise because of its rapid rate of decomposition, and an apparently higher survival rate is observed. In the present study, care was taken to swirl the dishes immediately after adding the bis(2-chloroethyl)sulfide.
Preparation of Metaphase Chromosomes

A culture was incubated in the presence of $10^{-7}$ M 5-fluorodeoxyuridine for 12 to 14 hr. The 5-fluorodeoxyuridine block was released by adding TdR to a final concentration of $5 \times 10^{-5}$ M. Metaphase cells were accumulated by adding vinblastine, 0.1 $\mu$g/ml, to the culture 4 hr after the addition of the TdR. Four hr later, the cells were collected by centrifuging, and slides containing metaphase cells in which the chromosomes were well spread were prepared and stained by the method of Carr and Walker (2). One hundred cells of each substrain containing well-spread chromosomes were counted.

Measurement of Cellular Volumes

Cellular volume measurements were made with the Coulter electronic particle counter that had been calibrated with ragweed pollen. The frequency-volume data obtained were summed and divided by the number of cells in the sample to give the average cellular volume.

Cell Cycle Parameters

Doubling Time. Cell counts on spinner flask cultures were made twice daily, and the overnight growth was used to calculate the doubling time.

Percentage of Cell Population Engaged in DNA Synthesis. A 5-ml cell sample was exposed for 20 min at 37° to TdR-3H, 0.5 $\mu$Ci/ml (specific activity, 21.9 Ci/mmole). The label was diluted by the addition of 0.1 ml of 0.005% TdR, and the cells were pelleted by centrifugation. The cells were swelled in hypotonic salt solution, fixed, and spread on slides for autoradiography. Slides were developed at 6 to 8 days, and the proportion of labeled cells was enumerated.

Duration of $G_2$ Phase. Petri dish cultures containing TdR-3H, 0.5 $\mu$Ci/ml, were prepared for autoradiography at 1-hr intervals following addition of the TdR-3H. Labeled metaphases were enumerated, and the time taken for 50% of the figures to become labeled was taken as the $G_2$ period.

Dose-Survival Curves

Incubations were done at 37° in a humidified CO$_2$ incubator. For all treatments, plastic Petri dishes, 5 cm in diameter, 3/point, were seeded with 5 ml of a diluted cell suspension taken from an exponentially growing spinner culture. The dishes were incubated for about 3 hr to allow the cells to adhere to the dishes.

With Drugs. The drug dissolved in methanol was added (0.05 ml of methanolic solution/dish), and after a further 60-min incubation the medium was removed and replaced with medium containing twice the usual amount of bovine serum and referred to as “plating medium.”

With X-Rays. Cells were seeded initially in plating medium. The dishes were placed in a Gamma Cell 20 radiation unit (Atomic Energy of Canada Ltd., Ottawa, Canada), which delivered 120 rads/min, and were radiated for various lengths of time.

With UV Light. The medium was removed from the dishes, and after being rinsed with phosphate-buffered saline the cells were radiated for various lengths of time; then plating medium was added. A 15-watt General Electric germicidal lamp mounted 54 cm above the dishes delivered 20 ergs/sec/sq mm to the surface of the dishes, as measured by a Blak-Ray UV intensity meter (Fisher Scientific Co., Pittsburgh, Pa.).

After the treatments, L-cells that had been exposed to bis(2-chloroethyl)sulfide, 2 $\mu$g/ml, for 1 hr were added to bring the total number of cells in each dish to $10^5$. The dishes were incubated for 7 to 12 days, after which the colonies were stained with methylene blue and enumerated with the aid of a low-power microscope. A colony was scored if it contained more than 25 cells. The vast majority of the colonies contained many times that number. Plating efficiency is defined as the percentage of control cells plated (manipulated but not treated and determined from a cell count and dilution) that form colonies.

Other Techniques and Materials

The techniques for isolation of DNA, denaturation of DNA with alkali and subsequent neutralization, centrifugation in cesium chloride solution, measurement of alkylation products by hydrolysis of DNA and chromatography, and the measurement of radioactivity have been described (11), as were the sources of the radioactive and other chemicals. 1,4-DMM was obtained through the kindness of Dr. T. A. Connors, Chester Beatty Institute for Cancer Research, London, England. The bis(2-chloroethyl)sulfide-3$^3$S obtained from Amersham/Searle Corp., Arlington Heights, Ill., was used while its specific activity was between 0.2 and 1 Ci/mmole.

RESULTS

Sensitivity of the Cell Strains to Bis(2-chloroethyl)sulfide. The survival curves for for L-cells and 3 resistant substrains are shown in Chart 1. The lines drawn are computed regression lines. The resistant substrains all have the same degree of resistance within 1 S.E. of the estimate of the line drawn for L/H3 cells, although they represent substrains isolated after 1, 3, and 4 selection procedures. Relative to L-cells, the substrains are 2.4 times less sensitive to bis(2-chloroethyl)sulfide.

Colony Morphology, Plating Efficiency, and Chromosome Number. Fig. 1 illustrates the different morphologies of L-cell and L/H3 cell colonies after 5 days of growth from single cells in control plates. Whereas the L-cell colony is compact and well delineated, the L/H3 cell colony is very diffuse. The colonial morphologies of L/H4 and L/H17 cells were similarly...
diffuse. Levis and Colussi (9) reported similar observations for a cell line that was resistant to nitrogen mustard. The tendency towards diffuse colonies becomes more marked after the cells have been treated with alkylating agents, X-rays, or UV, particularly at higher doses, and colony counting becomes more difficult. The diffuse colonial morphology of the substrains probably contributes to their more variable and lower plating efficiency (Table 1). The 3 resistant substrains have chromosome numbers that statistically are significantly lower than that of the L-strain ($p < 0.05$ to $p < 0.001$).

**Cell Cycle Parameters.** The population doubling times of the resistant cells were considerably longer than that of the L-cells (Table 2). This effect appears to result from a general increase in all phases of the cell cycle.

**Cellular Volume Changes with Increasing Population Density.** When substrains of L-cells were grown in suspension culture, they attained a maximum cellular volume at a population density that was peculiar to the substrate, and then the volume decreased as the population density increased (10). These changes were actually a reflection of the age of the culture, a nutritional phenomenon, and were unrelated to population density per se (15). Thus comparisons of cellular volume are meaningful only when the population density or culture age is stated. At all population densities, the L/H3 and L/H4 cells were smaller than the L-cells (Table 3). The cellular volume pattern of L/H17 cells was similar to that of L-cells, although the former did not become quite as large.

**Alkylation of DNA and Subsequent Loss of Alkylation Products in L-Cells and Resistant Substrains.** Suspension
cultures were treated with bis(2-chloroethyl)sulfide. $^{35}$S, 1 µg/ml; at intervals, samples of cells were removed; DNA was isolated, and its radioactivity was measured. Ten min after treatment with bis(2-chloroethyl)sulfide, the DNA of all cell strains was alkylated to approximately the same extent. In L-cells, the extent of alkylation of DNA had increased at 1 hr and then fallen with time. In contrast, the extent of alkylation of DNA in the substrains fell by 1 hr. A semilogarithmic plot of these data showed the half-life for the loss of alkylation products in L-cells to be about 16 hr. For the resistant cells, the half-life of the process was 5 to 7 hr (Table 4).

At the same time that the above measurements were made, portions of the alkylated DNA were hydrolyzed and analyzed chromatographically to obtain the relative proportion of monoguaninyl (7-hydroxyethylthioethylguanine) and diguaninyl (di-(guanin-V-y1)ethylsulfide) mustard derivatives. In L-cells, the proportion of monoguaninyl to diguaninyl derivative did not change significantly with time, and thus there was no preferential loss of either product (Table 5). For the substrains, the proportion of monoguaninyl derivative increased with time, suggesting a preferential loss of diguaninyl derivative.

The formation of cross-links in the DNA of bis(2-chloroethyl)sulfide-treated substrain cells is demonstrated by the upper set of tracings in Chart 2. These were obtained with DNA isolated 10 min after treatment. The DNA was denatured in alkali, neutralized, and isopycnically banded by centrifugation in a cesium chloride solution. The peak next to the marker has a buoyant density of 1.718 and corresponds with that of normal L-cell DNA that was denatured and neutralized. The peak farthest from the marker has a buoyant density of 1.703 and corresponds with that of native L-cell DNA; it signifies a renaturable (cross-linked) fraction of DNA molecules. The lower set of tracings in Chart 2 was obtained with DNA isolated 6 hr after the cells had been treated with bis(2-chloroethyl)sulfide. It demonstrates the same rapid loss of renaturable DNA that was observed with parental L-cells (11).

**Sensitivity of Substrains to Other Agents.** The sensitivity of the parental strain and substrains to X-rays, UV, MMS, and DMM were surveyed (Chart 3), and the results are summarized in Table 6.
Table 5
Molar ratio of 7-hydroxyethylthioethylguanine to di(guanin-7-yl)ethylsulfide in DNA at various times following treatment of cells with 1 μg/ml of $^{35}$S-labeled bis(2-chloroethyl)sulfide

DNA was isolated from the cells at the times indicated. The DNA was hydrolyzed in acid and subjected to paper chromatography to separate the radioactive alkylation products. The L-cell data were taken from a previous study (11). S.D. is given, with no. of trials in parentheses.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>L/H3</th>
<th>L/H4</th>
<th>L/H17</th>
</tr>
</thead>
<tbody>
<tr>
<td>~0.2</td>
<td>2.20 ± 0.46 (4)</td>
<td>2.87 ± 0.02 (4)</td>
<td>2.16 (2)</td>
</tr>
<tr>
<td>1.0</td>
<td>3.15 ± 0.40 (6)</td>
<td>3.57 ± 0.24 (6)</td>
<td>2.15 (2)</td>
</tr>
<tr>
<td>2.5</td>
<td>2.24 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>2.67 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>2.35 ± 0.46 (6)</td>
<td>4.15 (2)</td>
<td>3.49 (1)</td>
</tr>
<tr>
<td>24.0</td>
<td>2.55 ± 0.55 (5)</td>
<td>4.95 (2)</td>
<td>3.89 (1)</td>
</tr>
</tbody>
</table>

Table 6
Sensitivity of L-cell substrains to X-rays, UV, MMS, and DMM relative to that of the parental strain

<table>
<thead>
<tr>
<th>Substrain</th>
<th>X-rays</th>
<th>UV</th>
<th>MMS</th>
<th>DMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>L/H3</td>
<td>0°</td>
<td>0</td>
<td>sR</td>
<td>R</td>
</tr>
<tr>
<td>L/H4</td>
<td>0°</td>
<td>0</td>
<td>0</td>
<td>R</td>
</tr>
<tr>
<td>L/H17</td>
<td>S</td>
<td>0</td>
<td>0</td>
<td>S</td>
</tr>
</tbody>
</table>

a 0, not appreciably different; R, more resistant; S, more sensitive; sR, slightly resistant.

DISCUSSION

A number of changes in the properties of a cell can be conceived of that would confer resistance against the lethal effects of an alkylating agent. An increase in chromosome number is an obvious situation because this would increase the target size. There are several reports of resistance to alkylating agents or ionizing radiation associated with an increased ploidy in tumor cells (3, 6, 12), but in this study the chromosome number of the resistant substrains was decreased slightly. The meaning of this finding is not certain and may be simply fortuitous because the chromosome number of the L-cell is unstable.

The resistant substrains of cells were generally smaller than the parent. Previous work showed that the larger the cell was, the more bis(2-chloroethyl)sulfide was bound at a given dose.

Chart 2. Cross-linked (renaturable) DNA in the resistant substrains L/H3, L/H4, and L/H17 10 min or 6 hr after the cells were treated with bis(2-chloroethyl)sulfide.
In the present study, the presumed target, DNA, was alkylated to a similar extent in all cell strains immediately after the treatment. Cell size does not appear therefore to be a significant factor in explaining the relative resistance of these cells. The smaller average size of the resistant cells in this and the previous study (10) may be a result of their slower growth rate, if it is accepted that all of these cells attain a similar maximum size just prior to cell division. Our results also exclude a decreased permeability of the cells for bis(2-chloroethyl)sulfide as a cause of their resistance. In contrast, the nitrogen mustard-resistant cell lines derived by Rutman et al. (13) and Goldenberg (5) clearly owed their resistance to a lessened permeability to the drug. [See also Refs. 7 and 19, in which the authors utilized the cells derived by Rutman et al. (13).]

It was anticipated that our substrains would exhibit cross-resistance to the alkylating agents MMS and DMM as well as to UV and X-radiation because all of these agents are thought to exert their toxic effect by damaging DNA. Cross-resistance was seen clearly only for substrains L/H3 and L/H4 towards DMM. The increased sensitivity of L/H17 cells towards DMM and X-rays was unexpected. Sinclair (14) and Suit (16) have described, respectively, the properties of Chinese hamster and mouse mammary tumor cells that survived a large dose of ionizing radiation. The Chinese hamster cells, in common with our resistant substrain cells, were smaller and had a longer doubling time than the parental strain, but their radiosensitivity was increased. A similar finding was reported for the mammary tumor. All of these divergent results point up the complex nature of acquired resistance to alkylating agents and radiation and suggest that there are numerous mechanisms in mammalian cells that can lead to the resistant state, not all of which are common when the comparison is made between different alkylating agents, ionizing radiation, and UV radiation.

The most promising difference between L-cells and the resistant substrains that can be used to provide an explanation of the resistance to bis(2-chloroethyl)sulfide was the ability of the latter to excise alkylation products at a rate 2 to 3 times faster than the former. This finding is undoubtedly insufficient to account completely for the resistance, but it may reflect an increased overall ability of the resistant cells to “repair” damaged DNA. Rutman et al. (13) and Connors and Double (4) have described tumor cells that remained viable after degrees of DNA alkylation that were lethal to the parental cells from which they were derived, and although the mechanism was not clear it appeared likely that the resistant cells had an enhanced mechanism for repairing alkylated DNA.

Finally this study confirms the previous findings with L-cells (11) that bis(2-chloroethyl)sulfide cross-links the DNA molecule in mammalian cells and that the cross-links can be removed rapidly.

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REFERENCES


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