Resistance to Sulfur Mustard: A Comparison of Some Properties of Strain L Cells and a Resistant Subline

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Summary

A line of cells (L/H) that was resistant to sulfur mustard has been isolated and compared in a number of ways to the parent line of L cells. Both strains had an average of 57 chromosomes. The L/H cells were smaller than the L cells, and contained lower concentrations of acid-soluble sulfhydryl compounds. In experiments with sulfur mustard-35S, the whole cell, nucleus, and DNA of the resistant line were found to contain less mustard than the corresponding units of the parent line. For both lines, of the amount of mustard found in the whole cell, an equal proportion was found in the nucleus; of the amount of mustard found in the nucleus, a lesser proportion was associated with the DNA of the resistant cell than with the DNA of the parent cell. The smaller uptake of mustard by L/H cells and their nuclei can be accounted for by their smaller volume; some other factor appears to govern the disproportionately smaller amount found associated with the DNA.

Introduction

As part of our continuing studies on the mechanism of action of biologic alkylating agents, a line of cells (L/H) that was resistant to sulfur mustard was isolated and compared in a number of ways with the parent line of L cells.

The following properties were compared: chromosome number, doubling time, cellular volume, cellular acid-soluble sulfhydryl levels, and the incorporation of sulfur mustard-35S into whole cells, nuclei, and DNA.

Materials and Methods

SULFUR MUSTARD. Unlabeled sulfur mustard was obtained through the courtesy of the Defence Research Station, Suffield, Alberta, Canada. Radioactive sulfur mustard-35S with an original specific activity of approximately 30 mc/mnmole was obtained from the Radiochemical Centre, Amersham, England.

CELL LINES. Spinner flask cultures of a subline of L cells (20) and the derived subline L/H which was resistant to sulfur mustard were maintained at 37°C in medium CMRL 1066, supplemented with 5% normal horse serum, 100 μg/ml of streptomycin, and 60 μg/ml of penicillin, but lacking thymidine and coenzyme A. Both strains had an average of 57 chromosomes. The L/H cells were smaller than the L cells, and contained lower concentrations of acid-soluble sulfhydryl compounds. In experiments with sulfur mustard-35S, the whole cell, nucleus, and DNA of the resistant line were found to contain less mustard than the corresponding units of the parent line.

METHOD FOR OBTAINING SURVIVAL CURVES. To sterile 60-mm Petri dishes containing 4.8 ml of plating medium was added 0.2 ml of an appropriate dilution of cells to be tested. The desired concentration of sulfur mustard in 0.05 ml of methanol was added to the test plates, while the control plates received only methanol. The plates were placed in a humidified incubator at 37°C containing a 5% CO2 atmosphere. After about 1 hr, 5 × 10⁶ "feeder" cells were added along with sufficient plating medium to give a final volume of 10 ml/Petri dish. "Feeder" cells are L cells which have been treated for 1 hr at 37°C with 2 μg/ml of mustard. These cells can no longer form colonies but become very large ("giant" cells (12)) and appear to condition the medium resulting in greater plating efficiency. Four Petri dishes were used for each concentration of mustard and the values averaged.

SELECTION OF RESISTANT SUBLINES. Colonies, picked from Petri dish cultures of cells that had been treated with 1.5 μg/ml of sulfur mustard (and incubated as above), were allowed to form mass cultures in medicine bottles. The same selection process was repeated and from the 2nd selection 1 subline was chosen for the present study and grown thereafter in suspension culture. This subline has been grown continuously for over 2 years and still retains its resistance to sulfur mustard.

MEASUREMENT OF CELLULAR CONSTITUENTS. DNA and RNA concentrations were measured by the methods of Burton (2) and Schneider (15), respectively. Acid-soluble sulfhydryl levels were measured either by the method of Sparkes or Ellman (4).

MEASUREMENT OF RADIOACTIVITY. Radioactive samples were counted in a Nuclear Chicago liquid scintillation counter. Whole cell samples were centrifuged, washed twice with phosphate buffered saline, and were either digested and prepared for counting by the method of Jeffay et al. (9), or were collected on a millipore filter, dried, and counted in a toluene-fluor system (19). Isolated nuclei were counted in a similar manner. DNA after precipitation by cold perchloric acid was dissolved in the toluene-
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**Chart 1.** Survival curves for Strain L (●) and L/H (○) cells as obtained at a population density of 1 × 10^4 cells/ml. Point marked □ not used in computing regression line.

**Isolation of Nuclei.** Nuclei were isolated from 2 × 10^7 cells by the method of Hiatt (7) or that of Baltimore and Franklin (1). It is appreciated that these nuclei would not be completely free of cytoplasmic contamination; however, when these preparations were examined by phase contrast microscopy they appeared to be free of cytoplasmic tags.

**Isolation of DNA.** DNA was isolated from 2 × 10^7 cells by the method of Djordjevic and Szybalski (3). Consistant yields of 84% or better were obtained by this method. Such preparations were free of measurable amounts of RNA and protein. The DNA isolated by this procedure was considered to have a high molecular weight since it could be “spooled” when precipitated with ethanol. Chain scission due to alkylation of the N7 position of guanine has a half-life of 50 days, while splitting off of alkyl-guanine at neutral pH has a half-life of 50 hr (10). The elapsed time from the initiation of the experiment to the measurement of radioactivity was approximately 30 hr. Further, the number of alkylations for the concentration of mustard used is approximately 1/30,000 nucleotides for L cell DNA and about 1/65,000 nucleotides for L/H cell DNA. These values have been calculated from the data given in reference 19. If scission occurred with this frequency, (which is most unlikely), the resulting DNA would still have a high molecular weight since such scissions would only be apparent after the DNA had been denatured.

*The Dv value is that dose of mustard, measured in the exponential portion of the survival curve, which will reduce the survival to 37%.*

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**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Population density (× 10^4 cells/ml)</th>
<th>Mean particle size (threshold units)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>1.4</td>
<td>40.8</td>
</tr>
<tr>
<td></td>
<td>2.1</td>
<td>39.0</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>35.6</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>35.5</td>
</tr>
<tr>
<td></td>
<td>5.1</td>
<td>33.2</td>
</tr>
<tr>
<td>L/H</td>
<td>1.0</td>
<td>27.7</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>33.7</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>29.3</td>
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<tr>
<td></td>
<td>2.4</td>
<td>28.1</td>
</tr>
<tr>
<td></td>
<td>6.1</td>
<td>21.9</td>
</tr>
</tbody>
</table>

* One threshold unit is equivalent to 68 cu μ.

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**Chart 2.** Cellular volume distribution curves for strain L and L/H cells at various population densities, using the Coulter electronic counter. Each curve was obtained from a sample containing 5 × 10^4 cells/ml. One threshold unit = 68 cu μ. ●——●, L/H 2.0 × 10^4 cells/ml; ●——●, L/H 6.1 × 10^4 cells/ml; ○—○, L 1.1 × 10^4 cells/ml; ○—○, L 5.1 × 10^4 cells/ml.
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TABLE 2
THE EFFECT OF POPULATION DENSITY AND CELLULAR VOLUME ON THE INCORPORATION OF SULFUR MUSTARD IN L CELLS

<table>
<thead>
<tr>
<th>Population density (X 10^3 cells/ml)</th>
<th>Volume (cu mm) occupied by 10^9 cells</th>
<th>Sulfur mustard incorporation (cpm/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3</td>
<td>0.126</td>
<td>505</td>
</tr>
<tr>
<td>5.0</td>
<td>0.085</td>
<td>368</td>
</tr>
</tbody>
</table>

* Calculated by summing up the threshold-frequency data obtained with the Coulter counter.

b Concentration of mustard, 0.5 μg/ml of cell suspension.

Results

The survival curves obtained for L/H and L cells shown in Chart 1 demonstrate the relative resistance of L/H cells to sulfur mustard. The curves are computed regression lines and each point is the average of 4 trials. For purposes of comparison the D_0 values are 0.24 μg/ml and 0.18 μg/ml for L/H and L cells, respectively. While obtaining data for the survival curves we observed, as did Levis and Colussi (11), that the cells in colonies surviving mustard treatment had a tendency to migrate. The cells in our isolated resistant sublines continue to show this characteristic after many generations in the absence of mustard. In 1 of the sublines isolated, the migrating tendency was so great as to make colony counting virtually impossible.

Having shown L/H cells to be less sensitive to sulfur mustard than the parent line, some properties of the 2 lines were compared in an attempt to learn the basis of the sensitivity difference.

Since changes in sensitivity to sulfur mustard could be associated with a change in chromosome number (11, 14), this value was obtained for both cell lines. Twenty mitotic figures were counted for each line. L cells were found to contain 57.35 ± 0.56 chromosomes and L/H cells 57.10 ± 0.57. Therefore both sublines of cells contained an average of 57 chromosomes.

While counting the cells with the Coulter instrument it was noted that L/H cells produced smaller pulses on the oscilloscope than did the L cells. Therefore, the mean cellular volumes of the 2 lines were measured and are compared in Table 1. When cells at a high population density are diluted to 0.5 × 10^4 cells/ml then, for a short time, the cellular volume will be unchanged. As the cell number begins to increase, the cellular volume (initially low) will also increase until a maximum value is reached, after which the cellular volume will begin to decrease (Table 1). The maximum volumes as shown in Table 1 occur at different population densities (i.e., at population densities of 1 and 2 × 10^4 cells/ml for L and L/H cells, respectively); furthermore, the resistant cell is smaller than the sensitive cell when the comparison is made at similar population densities. Goldenberg and Alexander (6) have reported that a radiation and mustard "resistant" subline of L5178Y lymphoma cells was smaller than a "sensitive" subline, which is similar to our finding. Chart 2 illustrates the size distribution for the 2 cell lines at their maximum and minimum volumes.

Experiments by Révész et al. (13) and Hirono (8) have shown increased acid-soluble sulfydryl (ASSH) levels to be associated with increased resistance to radiation and nitrogen mustard N-...
oxygen, respectively. Measurement of the ASSH levels in L and L/H cells (Chart 3) showed that the levels fluctuated with population density in the same manner as the cellular volume; however, the cellular volume changes were much less than the changes in ASSH. When the 2 cell lines are compared it should be noted that the ASSH level in the resistant cell is generally lower.

Studies with sulfur mustard-35S yielded the following results. First, the incorporation of radioactivity into whole cells was proportional to the dose, e.g., when the concentration of sulfur mustard-35S was 0.5 µg/ml of culture, the incorporation was found to be 570 cpm/10⁶ cells; at a concentration of 1 µg/ml the incorporation was 1140 cpm/10⁶ cells. This experiment was repeated using 0.3, 0.6, and 0.9 µg/ml of mustard and the incorporation again was found to be proportional to the dose. Second, the incorporation of mustard into whole cells depended on the cellular volume. As shown in Table 2, as the population density of the L cells increased from 1.3 × 10⁶ to 5.0 × 10⁶ cells/ml, the cellular volume decreased by 30%. Similarly the incorporation per cell of mustard at the higher population density was 30% lower than that at the lower population density. In Table 3, the incorporation of mustard (at a concentration of 1 µg/ml) into whole cells, nuclei, and DNA is compared for L and L/H cells, both at a population density of 2 × 10⁶ cells/ml. It will be noted that incorporation into all 3 units is lower for the L/H cells. If one compares the ratio of radioactivity in the nucleus to that in the whole cells (Ratio 1 of Table 3), it can be seen that the values are identical for both lines. This means that for both lines, of the total amount of sulfur mustard in the whole cells an equal proportion is found in the nucleus. When one compares either the ratio of counts in the DNA to those in the nucleus (Ratio 3) or to those in the whole cell (Ratio 2), it is seen that a smaller proportion is associated with the DNA of the resistant cell.

Discussion

Révész et al. (14) found that in a cell line with a doubled chromosome number there was an increased resistance to radiation. In the present study, both L and L/H cells were found to have an average of 57 chromosomes. Therefore, a change in chromosome number cannot explain the observed resistance to sulfur mustard.

The L cell in the present study had an average doubling time of 16 hr, while that of the L/H cell was 21 hr. It has been shown that the sensitivity of cells to radiation (17, 18) and sulfur and nitrogen mustards (20), varies with the position of the cell in the cell cycle. The duration of the various phases of the cycle has not been determined for our resistant subline. Even if this information were available it could only be considered in a speculative manner.

Hirono (8) and Révész et al. (13) have shown increased ASSH levels to be associated with an increased resistance to nitrogen mustard N-oxide and radiation, respectively. In the present study, although the intracellular ASSH level varies with the population density, the resistant line was found generally to have a lower sulfhydryl content than the parent line (Chart 3). Both survival curves shown in Chart 1 were obtained at a population density of 1 × 10⁵ cells/ml. At this density the parent line has almost twice the ASSH content of the resistant line. It would appear, then, that the acquisition of resistance by the L/H line was not associated with an increase in ASSH. It is not be implied however, that ASSH plays no protective role.

Since a reduction in cellular volume results in a reduced incorporation of labeled mustard into the whole cell (Table 2), it might be supposed that the smaller volume of the L/H cells was responsible for their resistance. However, this factor affords only a partial explanation because Ratios 2 and 3 of Table 3 show that a disproportionately smaller amount of mustard is associated with the DNA of the resistant cell. Since for both cell lines Ratio 1 is identical, the disproportionately smaller amount of sulfur mustard associated with the DNA of the L/H cells cannot be the result of a nuclear barrier in these cells. The explanation of this phenomenon is not known, but one could speculate that the DNA of the resistant cell is either truly less reactive or that a mechanism exists in the resistant cell for the very rapid removal of alkylated DNA. Further studies to test the generality of the association between resistance to sulfur mustard and (a) decreased cellular volume, and (b) decreased ASSH levels are planned using other sublines. Levis and Colussi (11) noted that the cells in colonies surviving nitrogen mustard treatment had a great tendency to migrate. Our experience has been similar. Further, the cells in our isolated sublines continued to display this behavior even without further mustard treatment. This finding suggests that the cells have acquired this characteristic through mutation. The relationship, if any, between this migratory tendency and the acquisition of resistance to mustard is interesting but unexplained.

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References


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