A Practical Cytogenetic Protocol for In Vitro Cytotoxicity and Genotoxicity Testing*

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ABSTRACT

In vitro cytogenetics has been established as a valid method for evaluating the genotoxic potential of chemical agents. Armstrong et al. have described a simple, quantitative approach to in vitro cytotoxicity and genotoxicity testing by using Chinese hamster ovary (CHO) cells. This approach can also be sensitive and repeatable in an inter-laboratory setting, a prerequisite for routine testing of compounds suspected of having genotoxic properties. In the present study, cytotoxicity was evaluated by the parameter of mitotic index (MI). Genotoxicity is measured by the chromosome aberration (Abs) assay as described by Armstrong et al. using CHO cells. The basic analytic principles proposed were extended to include human lymphocytes. Sister chromatid exchange (SCE) analysis was used to establish an additional endpoint. Mito­mycin C (MMC), an established clastogen, was used as the model compound for protocol validation. Dose response curves for MI and Abs in CHO cells were found to be consistent with those reported by Armstrong et al. Results from our extended study on lymphocytes and using SCE analysis were analogous. Our experience is that this standardized approach is indeed sensitive and reliable and can serve as a basis for an inter-laboratory testing program.

Introduction

Chemical agents, known to be genotoxic, have been shown to induce sister chromatid exchanges and chromosome aberrations. The degree of damage has been found to be proportional to the length of exposure and the concentration of the genotoxicant. Therefore, as Tsongas pointed out, chromosome aberrations (Abs) and sister chromatid exchange (SCE) analyses are valid screening tools...
for reliable genotoxicity testing, recognizing that they are a component of a test battery. The chromosome aberrations assay in Chinese hamster ovary (CHO) cells is often applied as an indicator of in vitro genotoxicity, despite the recognition that the human and hamster genomes may differ in their responses to genotoxicants. The advantages of using CHO cells include low chromosome number, large chromosome size, and the fact that they can be cloned so that variability in response is minimized. Therefore, in this study, genotoxicity studies were carried out using CHO cells.

Human peripheral blood lymphocyte (PBL) studies, on the other hand, are difficult to interpret because PBL sources are from different individual sources as opposed to being drawn from a single standardized cell line. The advantages of using peripheral blood lymphocytes (PBLs) in a cytogenetic assay system for environmental toxicants include the fact that 1 ml of human blood can have 1 to 3 million lymphocytes. Circulating throughout the body, some PBLs are long-lived. The PBLs are a synchronized population in Go and can be stimulated to undergo mitosis producing many dividing cells for use in chromosome analysis.8,9

By using both CHO cells and PBLs and by also using more than one endpoint (through chromosome aberration assay and SCE analysis), it is felt that a more accurate assessment of genotoxicity can be achieved.

As regulatory guidelines suggest testing chemicals up to cytotoxic doses in chromosome aberration assays, dose selection depends on the particular measure of the cytotoxicity that is chosen. Armstrong et al3 conducted an extensive quantitative analysis of cytotoxicity associated with chromosome aberrations in CHO cells. The parameter of mitotic index has been chosen as a sensitive measure of cytotoxicity based on the findings of Armstrong et al3 as well as our own experience. Cytotoxicity data obtained in this and other studies are important when used in the risk assessment of compounds to which humans are exposed.

Materials and Methods

CHINESE HAMSTER OVARY CELLS

The CHO-WBL (Wolff, Bloom, and Litton) cloned cells were obtained* and were sub-cultured and maintained in McCoys 5A medium supplemented with 10 percent fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin.† Cells were kept in a 5 percent CO2 incubator at 37°C and were plated at 1.3 × 10^6 cells/10 ml of culture media in 75 cm² flask a day before they were treated with Mitomycin C (MMC, CAS#50-07-7).‡

TREATMENT, HARVEST AND STAINING FOR SISTER CHROMATID EXCHANGE AND CHROMOSOME ABERRATION ANALYSES

Based on the results of cytotoxicity reported by Armstrong et al3, seven different concentrations of Mitomycin C (MMC) in addition to a control were chosen. Cultures were harvested at 24 hours after the beginning of the MMC treatment. The selected doses were: 0.0 µM, 0.1 µM, 0.5 µM, 0.8 µM, 1.0 µM, 2.0 µM, 4.0 µM, and 8.0 µM. Duplicate cultures were established for each dose, with one set of cultures receiving 5-bromodeoxyuridine (BrdU) at 10 µM for sister chroma-

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† Gibco, Life technologies Inc., P.O. Box 68, Grand Island, NY 14072.
‡ Sigma Chemical Company, St. Louis, MO 63178.
tid exchange induction. All cultures were harvested 24 hours after the beginning of the treatment. Galloway et al\textsuperscript{10} and Bean and Galloway\textsuperscript{11} using various parameters, such as average generation time\textsuperscript{12} and mitotic index, to evaluate cell cycle delay and optimal time and harvest, concluded that harvest time is one of the most important variables that determines the success or failure of the protocol.

Harvesting was performed according to a modification of the method of Moorhead et al.\textsuperscript{13} Three hours prior to harvest, 32 ìl of stock Colcemid (deacetyl methylocolchicine) at 10 ìg/ml were added to all the cultures. Cells were collected by a combination of the trypsinization and the mitotic shake-off method into centrifuge tubes. Hypotonic treatment was carried out using 75 mM KCl for 5 to 10 minutes. Cells were fixed with Carnoy’s fixative (3 parts methanol to 1 part acetic acid) and rinsed three times until the appearance of the pellet became whitish. Air-dried slides were prepared and stained according to the methods of Perry and Wolff.\textsuperscript{14} Cell cultures which did not undergo BrdU treatment were stained in 4 percent Giemsa§ in a pH 6.8 buffer. Sister chromatid exchange (SCE) analysis slides were differentially stained by the fluorescence plus Giemsa (FPG) method of Perry and Wolff.\textsuperscript{14}

**HUMAN PERIPHERAL BLOOD LYMPHOCYTE PROCEDURES**

Blood was obtained from a healthy male donor. Previous research had reported SCE levels in human PBLs to be independent of age and sex.\textsuperscript{6,7,15} Whole blood lymphocyte cell cultures were set up in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented by fetal bovine serum (0.15 ml/ml of medium), penicillin (100 units/ml of medium), streptomycin (100 ìg/ml of medium), L-glutamine (2 mM/ml of medium) and 2 percent v/v PHA-M (all from Gibco\textsuperscript{8}). Duplicate cultures were maintained, and BrdU was added to one set of cultures to enable visualization of sister chromatid exchanges. For chromosome aberration scoring, 0.4 ml of blood in 4.6 ml of medium was used. For sister chromatid exchange analysis, 0.5 ml of blood in 9.5 ml of medium was used.

Cells were treated with eight different concentrations of MMC (0.0 ìM, 0.1 ìM, 0.5 ìM, 0.8 ìM, 1.0 ìM, 2.0 ìM, 4.0 ìM, and 8.0 ìM) at 48 hours after culture initiation. All cultures were harvested 24 hours after MMC treatment. Colcemid (10 ìg/ml) was added three hours before the harvest. Although mitotic index was recorded for all concentrations in both (CHO and human peripheral lymphocytes) systems, chromosome aberrations were examined in the five lower doses (0.1 ìM to 2.0 ìM) only. The chromosome aberrations in the two highest doses (4.0 ìM and 8.0 ìM) were found to be too numerous for accurate enumeration and analysis. For sister chromatid exchange analysis only, two dose groups (0.1 ìM and 0.5 ìM) were scored, as there are predominantly first-cycle cells in the higher dose groups.

One hundred metaphases from CHO cells and 50 metaphases from human peripheral lymphocytes were analyzed for chromosome aberrations. Between 25 and 50 metaphases were analyzed for sister chromatid exchanges.

Metaphase cells with either few or no overlapping chromosomes were selected for scoring. The coordinates (identified

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\textsuperscript{§} Gurr’s improved R66, Bio-medical Specialties, P.O. Box 1687, Santa Monica, CA 90406.

\textsuperscript{8} Gibco, Life technologies Inc., P.O. Box 68, Grand Island, NY 14072.
by England Finder ID slide), slide identification number, and chromosome counts as well as other pertinent details, were recorded for each of the selected cells.

**Criteria and Methods for Scoring Chromosome Aberrations**

Metaphase cells with few or no overlapping chromosomes were selected. Chromosome aberrations (Abs) were scored analogous to Armstrong et al.\(^3\) Both the total number of chromosome abnormalities and the number of cells with abnormalities were recorded. For example, a cell with 10 or more Abs was scored as 1 aberrant cell, but as 10 Abs when calculating the total number of Abs. Results were summarized as the percentage of cells with Abs.

The following chromosome aberrations were recorded: chromatid gaps, chromosome gaps, chromatid breaks, chromosome breaks, triradials, quadriradials, complex rearrangements, dicentrics, rings, polyploid cells, and pulverization. A chromatid gap is defined as a gap present on only one of the chromatids. A chromosome gap is defined as a gap that is present and aligned on both chromatids. The aberration count per metaphase was capped at 10. Thus, cells having 10 or more aberrations were counted as having 10 aberrations. All details were recorded. A representative metaphase showing a chromosome aberration is given in figure 1b. Figure 1a shows a normal CHO metaphase.

**Criteria for Sister Chromatid Exchange Scoring**

Exchanges were enumerated according to the scheme described in figure 2.

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\(^3\) Graticules Limited, Sovereign Way, Tonbridge, Kent, England.
Metaphases with few or no overlaps were selected from slides coded for blinded scoring. The locations of these metaphases were identified by using coordinates and England finder ID slides. The number of chromosomes were counted and the exchanges (including centrometric exchanges) were identified according to the scheme depicted in figure 2. The categories scored are 0 exchange, 1 exchange, 2 exchanges and 3 exchanges.

**Statistical Analysis**

Statistical analysis was carried out as described in Armstrong et al. A Chi-squared test based on a standard normal approximation was utilized to compare the frequencies of cells with aberrations in the treated samples versus those in the control samples.

**Results and Discussion**

Data on cytotoxicity (table I) as measured by mitotic index (MI) for CHO-WBL cells and human peripheral lymphocytes are presented graphically in figure 3. As expected, inverse proportionality was observed. With increasing doses of MMC, the MI decreases in a
Mitotic Index in Chinese Hamster Ovary–WBL Cells and Human Lymphocytes Treated with Different Doses of Mitomycin C for 24 Hours

<table>
<thead>
<tr>
<th>MMC Dose (μM)</th>
<th>CHO–WBL MI (%)</th>
<th>Human Lymphocytes MI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>19.50</td>
<td>7.7</td>
</tr>
<tr>
<td>0.1</td>
<td>15.50</td>
<td>5.8</td>
</tr>
<tr>
<td>0.5</td>
<td>12.60*</td>
<td>3.8*</td>
</tr>
<tr>
<td>0.8</td>
<td>10.70*</td>
<td>3.3*</td>
</tr>
<tr>
<td>1.0</td>
<td>13.70*</td>
<td>2.3*</td>
</tr>
<tr>
<td>2.0</td>
<td>11.60*</td>
<td>1.6*</td>
</tr>
<tr>
<td>4.0</td>
<td>7.30*</td>
<td>0.6*</td>
</tr>
<tr>
<td>8.0</td>
<td>3.80*</td>
<td>0.3*</td>
</tr>
</tbody>
</table>

*Statistical analysis shows that the difference between test and control doses is significant (p < 0.05).

WBL = Wolff, Bloom, and Litton.

There was an increase in the frequency of aberrations and also the number of cells with aberrations in the MMC-treated groups as compared to the control group. Chromosome aberrations in CHO-WBL cells and human peripheral lymphocytes, as summarized in Table II, can be seen to increase proportionately with increasing dosage and is in agreement with the results of Armstrong et al.3 The two sets of data, derived from two different systems, agree remarkably well with each other, as can be seen in figure 4. Note that 4.0 μM and 8.0 μM of MMC were not scored for chromosome aberrations as the aberrations were too numerous for reliable scoring. At 2.0 μM, 76 percent of the cells had aberrations.

A representative cell with a chromosome aberration is given in figure 1b; figure 1a is a normal CHO-WBL cell.

The Chi-square test was used to test the null hypothesis of no difference between the experimental and control groups. Results of our analysis indicated that the number of aberrations in the treated group was significantly different from the control group at the 0.05 proba-

![Figure 3. Mitotic index in CHO-WBL and human peripheral lymphocytes treated with MMC.](image-url)
**TABLE II**

<table>
<thead>
<tr>
<th>MMC Dose (μM)</th>
<th>Chinese Hamster Ovary-WBL Cells</th>
<th>Human Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># of Cells</td>
<td># of Abs</td>
</tr>
<tr>
<td>0.0</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>0.1</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>32</td>
</tr>
<tr>
<td>0.8</td>
<td>100</td>
<td>32</td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>42**</td>
</tr>
<tr>
<td>2.0</td>
<td>100</td>
<td>61**</td>
</tr>
</tbody>
</table>

*Statistical analysis shows that the difference in chromosome aberrations (Abs) between test and control doses is significant (p < 0.05).

**Cells with more than 10 Abs are also included.

Note: Dosages of 0.4 μM and 0.8μM were not tested for chromosome aberrations because the aberrations were too numerous for reliable scoring. At 2.0 μM, 76 percent of the cells had chromosome aberrations.

WBL = Wolff, Bloom, and Litton.

**Figure 4.** Chromosome aberrations in CHO-WBL Cells and human peripheral lymphocytes treated with MMC.
TABLE III
Sister Chromatid Exchanges in Chinese Hamster Ovary-WBL Cells and Human Lymphocytes Treated with Mitomycin C

<table>
<thead>
<tr>
<th>MMC (µM)</th>
<th># of Cells Scored</th>
<th># of Exchanges Per Cell</th>
<th># of Cells Scored</th>
<th>Exchanges Per Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>50</td>
<td>6.08</td>
<td>50</td>
<td>6.24</td>
</tr>
<tr>
<td>0.1</td>
<td>50</td>
<td>10.72</td>
<td>50</td>
<td>10.84</td>
</tr>
<tr>
<td>0.5</td>
<td>25</td>
<td>15.20*</td>
<td>25</td>
<td>14.60*</td>
</tr>
</tbody>
</table>

*Statistical analysis shows that the difference in sister chromatid exchanges between test and control doses is significant (p < 0.05).

Note: Higher doses could not be scored owing to too many cells with multiple chromosome aberrations and too few second division (M2) metaphases.

WBL = Wolff, Bloom, and Litton.

The direct proportionality observed by us is consistent with the findings of Armstrong et al\(^3\) and also of others. Sister chromatid exchange analysis is a sensitive method for assaying DNA damage and repair processes and can be used reliably as a cytogenetic tool for the testing of potential genotoxicants (Bloom,\(^7\) Kato and Shimada,\(^5\) Perry and Evans\(^6\)).

In conclusion, the present study validates the approach of Armstrong et al\(^3\) using mitotic index and chromosome aberration as measurable endpoints for evaluating cytotoxicity and the genotoxic potential of chemical agents. An additional endpoint of sister chromatid exchange analysis was employed in the present study. Armstrong et al\(^3\) described their simple quantitative approach to cytotoxicity and genotoxicity testing in Chinese hamster ovary (CHO) cells. Using mitomycin C (MMC), an established clastogen, as the model compound, their protocol has been extended by us to testing using human peripheral lymphocytes in vitro as well. This approach has been shown by us to be repeatable in an inter-laboratory setting. This effort is a proper prerequisite for routine testing of compounds suspected of having genotoxic properties. Our experience is that this standardized protocol is indeed sensitive and reliable and can serve as a basis for an inter-laboratory testing program. Application of this approach in the testing of potentially genotoxic compounds produced during the drying of lumber will be reported elsewhere.

Acknowledgments

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References


