Flow Cytometry for In vitro Assessment of Genotoxic Substances

G. Marceau¹, J. Godin-Ethier¹, F. Merah², S. Lavallée¹, F. Leroux¹, and A. Nelson¹,²

(¹)Immunology, ITR Laboratories Canada Inc., Montreal, Canada;
(²)Genetic Toxicology, ITR Laboratories Canada Inc., Montreal, Canada

Rationale:
Flow cytometric analysis offers new possibilities in genotoxicology by providing an easy way to increase both the number of samples and the number of cells per sample, therefore increasing the statistical power of the results. Here we detail the implementation of the micronucleus assay by flow cytometry for the purpose of in vitro genotoxicity assessment.

Micronuclei are created whenever a fragment of DNA, either acentric chromosomes or even complete chromosomes, that won't migrate to the pole during cell division. The method also allows a mechanistic interpretation of DNA damages with the detection of damages caused by anogenus and clastogens. Since micronuclei represent damaged transmitted to daughter cells the demonstration of mitosis is required unless some mitosis blocker is used (hence the population doubling calculation for each experiment).

Anogenus
Substance that interacts with components of the mitotic and meiotic cell division cycle apparatus leading to a deviation from the normal diploid/haploid number of chromosomes.

Clastogens
Substance that causes structural chromosomal aberrations in populations of cells.

Experimental Procedures:
CHO-K1 cells were exposed to either vehicle or known genotoxins (Cytophosphamide, CPA; Clofazime, CMC, and Mitomycin C, MMC) in two modes of exposure, short (approx. 4 hours with additional 24 hours in normal media) and extended (approx. 26 hours). Dead cells were tagged with Ethidium Monoxide (EMA), a fluorescent DNA dye (red) that can be covalently fixed by a simple exposure to visible light wavelengths. Cells were then lyzed and labeled again with a fluorescent DNA dye (green). Fragmented DNA from apoptotic or necrotic cells can be thus excluded from analysis by selecting out-color ed events.

DNA fluorescence intensity was obtained on FacsCanto II flow cytometer for the quantification of haploid/diploid nuclei, hypodiploid nuclei and micronuclei. The relative population doubling of each sample was calculated with the help of fluorescent beads.

Labelling for flow cytometry

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Population</th>
<th>Micronuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1.2 ± 0.3</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>CPA</td>
<td>1.3 ± 0.4</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>MMC</td>
<td>1.4 ± 0.5</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Results:
Exposure with CPA (1, 2, and 5 μg/mL) induced measurable dose-dependent in the short protocol with addition of metabolic activator (55% liver fraction). The MN frequency reached 12.0%. Exposure with MMC (0.05, 0.10, and 0.25 μg/mL) induced measurable dose-dependent increases in micronuclei in the short and extended protocols. The MN frequency reached 9.5% and 12.2% in the short and extended exposure protocols, respectively. Significant toxicity was present in the highest dose at the extended exposure. Exposure with CPA (0.2, 0.3, and 0.5 μg/mL) in the short exposure and 0.05, 0.10, 0.15 μg/mL in the extended exposure protocol) induced measurable dose-dependent increases in both hypodiploids and micronuclei. The MN frequency reached 6.5% and 14.5% in the short and extended exposure protocols, respectively. Significant toxicity was present in the highest dose at both the short and extended exposure. The range of valid genetic effects and cytotoxicity is more limited. The Hypodiploid frequency reached 3.0% and 8.0% in the short and extended exposure protocols respectively.

Discussion:
The assessment of micronucleus formation in cell cultures requires that the cell division machinery be induced. Unlike the microscopic-based method which can rely on visual confirmation of the chromatid duplication after cell division block, the flow cytometry method necessitates the release of the genetic material through cell lysis. Therefore, an indirect method is used to demonstrate active cellular division, through cell population doubling.

Of a total of 27 experiments, 3 had failed the population doubling requirement (including one for an obvious technical error).

Conclusions:
The analysis of in vitro micronuclei by flow cytometry provides an alternative to microscopic slides reading for detecting both anogenus and clastogens, with capacity for high throughput.

Bibliography: