Detection of Mutant Diphtheria Toxin-resistant Chinese Hamster Lung Cells in Situ by Autoradiography

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ABSTRACT

An autoradiographic method was developed to detect diphtheria toxin-resistant (DT') Chinese hamster lung cells in situ. With this method, a dose-dependent increase in the number of DT' cells was observed after exposure of the cells to increasing concentrations of ethyl methanesulfonate (EMS). Segregation of EMS-induced DT' cells in a sector of single colonies was clearly demonstrated. It was also found by this method that the number of DT' cells induced by a fixed concentration of EMS was not determined by the number of cell divisions during the expression time but by the period after exposure of the cells to EMS.

INTRODUCTION

Diphtheria toxin enters the DT cells via its receptor and inactivates EF-2 by catalyzing its ADP ribosylation at a unique modified amino acid, diphthamide, leading to inhibition of protein synthesis and death of the cells (1—6). We previously established a mammalian mutation assay system with cultured CHL cells and diphtheria toxin resistance as a marker, in which DT' cells were detected by their ability to proliferate and form colonies in the presence of diphtheria toxin (colony formation method) (7—10). This assay system has been used successfully to detect various mutagens and carcinogens (7—10). The mutant DT' cells obtained in this assay have been characterized. Almost all of the DT' cells induced by mutagens and carcinogens were found to contain EF-2 that could not be ADP ribosylated by diphtheria toxin in vitro with NAD as a substrate; the resistance of more than 90% of the DT' cells tested was not due to a defect in the receptor for the toxin but to alteration of EF-2 itself or deficiency of the enzymes required for synthesis of diphthamide (7, 11, 12). Mutation assays using diphtheria toxin resistance as a marker have proved effective with human fibroblasts (13) and Chinese hamster ovary cells (14).

Since diphtheria toxin is a potent inhibitor of protein synthesis, a single DT' cell could be detected by autoradiography after exposure of the cells to the toxin and then pulse-labeling with L-[¹⁴C]leucine (autoradiographic method). We have reported preliminary results on this method of detecting DT' cells in situ by autoradiography (12). A similar way to detect DT' cells among Chinese hamster ovary cells exposed to mutagens and carcinogens has been reported recently from another laboratory (15).

In the present study, we compared the conventional colony formation method and autoradiographic method for detecting EMS-induced DT' cells. With the latter method, we also analyzed the mutational events occurring at a cellular level during the period of induction of DT' cells after exposure of CHL cells to EMS.

MATERIALS AND METHODS

Materials. EMS and diphtheria toxin were purchased from Aldrich Chemical Co. and Connaught Medical Research Laboratories, Toronto, Ontario, Canada, respectively. Eagle's minimum essential medium containing kanamycin was purchased from Nissui Seiyaku Co., Tokyo, Japan. All other reagents were analytical grade.

Cells and Culture Conditions. CHL cells were originally isolated from the lung tissue of a newborn Chinese hamster (7). Unless otherwise stated, the cells were maintained in Eagle's minimum essential medium containing double the normal concentrations of vitamins and amino acids with 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ in air at 37°C, as described previously (7—10).

Mutation Assay by the Colony Formation Method. Mutation assay by the conventional colony formation method was performed as described previously (7). Briefly, CHL cells in the logarithmic phase of growth were exposed to EMS for 3 h at 37°C, washed, and collected by trypsinization. For determination of cytotoxicity, samples of 200—400 cells were plated in 60-mm dishes with 5 ml of medium and the plating efficiency was measured after culture for 7—8 days. Survival of cells treated with EMS was expressed as their plating efficiency as a percentage of that of untreated cells. For determination of mutation frequency, the remaining portion of the cells was cultured in fresh medium for the indicated expression time. After the expression time, an aliquot was transferred to fresh medium containing diphtheria toxin (0.1 Lf/ml). After culture for 7 to 9 days in the presence of the toxin, the number of DT' colonies was scored. Another aliquot of the cell suspension was transferred to toxin-free medium to determine the number of survivors by measuring the total number of colony developed in the absence of diphtheria toxin, and the number of induced DT' cells was expressed as a frequency per unit survivors.

Mutation Assay by Autoradiographic Methods. Two methods were used to determine EMS-induced DT' cells by autoradiography: detection of DT' cells in the total population of CHL cells exposed to EMS (method 1); and detection of DT' cells in each colony (method 2). For both methods, the conditions used for culture and exposure of the cells to EMS were the same as in the colony formation method.

For detection of DT' cells in the total population of CHL cells (method 1), the cells were exposed to EMS for 3 h and then 2.5 × 10⁵ cells were transferred to fresh medium and cultured for the indicated expression time. Then the cells were trypsinized, transferred to fresh medium containing diphtheria toxin (1 Lf/ml), and cultured for 16 to 17 h. After two washings with Hanks' solution, containing 10 mM HEPES, pH 7.4, the medium was changed to leucine-free and serum-free medium containing 10 mM HEPES buffer, pH 7.4, and diphtheria toxin at 1 Lf/ml. The cells were labeled with L-[³⁵S]leucine (10 μCi/ml) for 2 h at 37°C. After washing, the cells were fixed and processed for autoradiography.

For detection of DT' cells in individual colonies (method 2) after exposure of the cells to EMS, 300 to 500 cells, instead of 2.5 × 10⁵ cells, were transferred to fresh medium in 90-mm Nunc dishes and incubation was continued for the indicated expression time. After washing, the medium was changed to leucine-free and serum-free medium containing 10 mM HEPES buffer, pH 7.4, and diphtheria toxin at 1 Lf/ml and labeled with L-[³⁵S]leucine as in method 1.

After labeling for methods 1 and 2, the medium was then removed and the cells were washed three times with serum-free medium containing 10 mM L-leucine and once with Hanks' solution containing 10 mM HEPES, pH 7.4. They were then fixed with methanol/acetic acid (3:1, v/v) and washed successively with 5% trichloroacetic acid, 75% ethanol, 90% ethanol, and 100% ethanol. The cells or the colonies were processed for autoradiography by dipping the dishes into Kodak NTB-2.
photographic emulsion. After an exposure time of 7 to 10 days, the cells were counterstained with Giemsa after development and fixation and were examined under a light microscope. In method 1, the plates were code numbered, and numbers of cells with grains and total numbers of cells were counted without previous knowledge of the code number of the sample. Totals of at least $10^4$ cells were counted by two persons. In method 2, numbers of cells with grains and total numbers of cells in each colony were counted. At least a total number of $10^4$ cells were counted, and the minimum number of 200 colonies were analyzed for each experiment.

RESULTS

Detection of DT$^+$ Cells by Autoradiography. When CHL cells were cultured in the presence of diphtheria toxin at 1 $Lf/ml$ for 16 h and then labeled with L-$[3H]$leucine for 3 h, less than 50 cells/$10^5$ cells were diphtheria toxin resistant, revealed by autoradiography (Fig. 1A). Increase in the concentration of the toxin to 10 $Lf/ml$ or increase in the time of exposure to the toxin to up to 24 h did not change the number of DT$^+$ cells. In contrast, CHL cells that were not preincubated with diphtheria toxin and DT$^+$ cells after culture with diphtheria toxin were all heavily labeled with L-$[3H]$leucine (Fig. 1B). When known numbers of DT$^+$ cells were mixed with a fixed number of CHL cells and pulse-labeled with L-$[3H]$leucine, analysis by autoradiography showed that more than 82% of the added DT$^+$ cells were recovered (Fig. 1C; Table 1).

Mutation Assay by the Autoradiographic Method. CHL cells were exposed to EMS at 0.66 mg/ml for 3 h and cultured for the indicated expression time, and then the number of DT$^+$ cells was measured by the conventional colony formation method.

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Fig. 1. A to C, autoradiographic demonstration of DT$^+$ cells and DT$^-$ cells. DT$^+$ cells (A), DT$^-$ cells (B), and a mixture of DT$^+$ cells and DT$^-$ cells (C) were incubated with diphtheria toxin and pulse-labeled with L-$[3H]$leucine. Autoradiographs were prepared as described in the text. D to F, types of colonies seen after exposure of the cells to EMS. The cells were exposed to EMS at 1.32 mg/ml for 3 h and then 300 to 500 cells were transferred to 90-mm dishes. After an expression time of 7 days, the cells were labeled with L-$[3H]$leucine after incubation with diphtheria toxin as described in the text (method 2). D, type 1 colony; E, type 2 colony; F, type 3 colony. x 61.
Cells were cultured in the presence of EMS, 0.66 mg/ml, or in the absence (method 1) as described in the text. After the indicated expression times, the cells were trypsinized and the numbers of DV cells were measured by the colony formation method (method 1) and the autoradiographic method (method 1). With both methods, the number of cell divisions was not important for induction of the maximum number of DT cells. The relationship between the number of induced DT cells and the number of cell divisions during the expression period was examined further by the autoradiographic method (method 2) by counting numbers of EMS-induced DT cells after culture in medium with low and high serum contents and by counting the numbers of cells in each colony. In this way it was possible to measure simultaneously the number of induced DT cells and the number of cell divisions in the same cell population, since the number of cell divisions could be calculated from the number of cells in each colony. Reduction of the serum concentration of the medium from 10% to 1.5% resulted in a 50% decrease in the number of cell divisions, but the numbers of DT cells obtained in medium with serum at 10 and 1.5% were the same (Table 3). Furthermore, the number of divisions of cells cultured for 3 days in medium with 10% fetal calf serum was approximately the same as that of cells cultured for 7 days in medium with 1.5% serum, whereas the number of DT cells was much higher after culture for 7 days than after culture for 3 days.

**DISCUSSION**

We previously established a method for detecting the mutagenic activity of various compounds on CHL cells using diphtheria toxin resistance as a selection marker and DT cells obtained by colony formation assay have been characterized (7–10).

Any change of DNA sequences leading to a defect in the receptor for the toxin, alteration of EF-2 itself, or deficiency in enzymes required for synthesis of diphthamide (5, 11) gives rise to DT cells. The changes might include not only point mutation

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**Table 1 Recovery of diphtheria toxin-resistant cells assayed by autoradiography**

<table>
<thead>
<tr>
<th>No. of DT cells plated/10^9 CHL wild-type cells</th>
<th>No. of DT cells/10^9 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Expected</td>
</tr>
<tr>
<td>5 x 10^3</td>
<td>100</td>
</tr>
<tr>
<td>1 x 10^4</td>
<td>2,000</td>
</tr>
<tr>
<td>5 x 10^4</td>
<td>10,000</td>
</tr>
</tbody>
</table>

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**Table 2 Percentages of different types of colonies after exposure of CHL cells to ethyl methanesulfonate**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of types of colonies</th>
<th>DT cells/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>88.9</td>
<td>34</td>
</tr>
<tr>
<td>EMS (0.66 mg/ml)</td>
<td>81.7</td>
<td>714</td>
</tr>
<tr>
<td>EMS (1.32 mg/ml)</td>
<td>84.0</td>
<td>1887</td>
</tr>
</tbody>
</table>

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**Table 3 Relation of mutation frequency and number of cell divisions**

<table>
<thead>
<tr>
<th>EMS (mg/ml)</th>
<th>Serum (%)</th>
<th>No. of cell divisions</th>
<th>DT cells/10^6 cells</th>
<th>No. of cell divisions</th>
<th>DT cells/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>10</td>
<td>5.0</td>
<td>290</td>
<td>12.3</td>
<td>722</td>
</tr>
<tr>
<td>1.32</td>
<td>10</td>
<td>3.5</td>
<td>462</td>
<td>8.6</td>
<td>1050</td>
</tr>
</tbody>
</table>

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*Calculated from average number of cells per colony.
*Expression time was 7 days.
*Numbers of DT cells were detected by method 2.
but also rearrangement of structural and regulatory genes. These multiple possible target sites in the cellular genome might account for high spontaneous background frequencies. It is also possible that there might be yet unexplained nongenetic mechanisms to cause stable DT resistance in these cells.

From the following results we concluded that the autoradiographic method described here can detect a single DT' cell in situ and can be used to measure the mutagenic activity of a compound on CHL cells, like the colony formation method. (a) When a known number of DT' cells was mixed with a fixed number of parent CHL cells, the expected number of DT' cells was scored with this method. This finding also showed that there was no metabolic cooperation in the assay system. (b) The number of DT' cells scored did not change on increasing the toxin concentration or prolonging the time for exposure of the cells to toxin up to 24 h. (c) With an expression time of 7 days after exposure of CHL cells to EMS, the number of DT' cells detected by the autoradiographic method described here as well as by the colony formation method increased with increasing concentrations of EMS. The number of DT' cells scored by the autoradiographic method was always higher than that scored by the colony formation method, because with the autoradiographic method all the DT' cells were scored, while with the conventional colony formation method only DT' cells with sufficient proliferative activity to form colonies of detectable size were measured; some DT' cells were at a selective disadvantage and might grow more slowly than wild-type cells. It is also possible that the autoradiographic method might score DT' cells other than those with alteration in EF-2 or in its modification; this method might also score DT' cells with a defect in the receptor for the toxin.

This autoradiographic method (method 2) allowed analysis of the induction of DT' cells at a cellular level. After exposure of the cells to EMS, DT' cells appeared in segregated sectors within a single colony.

Three types of colonies were observed: type 1, composed entirely of DT' cells; type 2, composed of both DT' and DT cells; and type 3, composed entirely of DT' cells. In the control preparation, most of the colonies were type 1; but after exposure of the cells to EMS, the number of type 2 colonies increased; and with the higher concentration of EMS, type 3 colonies were observed. At higher concentrations of the mutagen, cells would contain other genetic and nongenetic defects; as a result, when the target cell divided, some of the daughter cells not carrying diphtheria toxin resistance, but other defects, might die, leaving only the DT' daughter cells to form colonies. Alternatively, type 3 colonies might represent the cellular events in which at the first cell division after exposure to EMS both daughter cells from a DT' cell were converted to DT' cells. It is very unlikely that type 3 colonies were observed as a result of spontaneous mutations, since they were seen only in treated plates.

The relationship between the number of cell divisions during the expression period and the number of DT' cells induced was investigated by this autoradiographic method (method 2). Results showed that the number of EMS-induced DT' cells did not change when the number of cell divisions was decreased by lowering the serum concentration of the medium but was dependent on the time after exposure of the cells to EMS. This finding clearly demonstrated that time rather than the number of cell divisions after exposure of the cells to EMS determined the number of DT' cells scored. This observation is compatible with the idea that 6 to 9 days are necessary for accumulation of sufficient altered EF-2 for change from the DT' to the DT phenotype. A similar conclusion was reached in studies by the colony formation method on induction of 6-thioguanine-resistant Chinese hamster ovary cells (16). It was reported previously that the optimum expression time was not the same for all mutagens (15). Autoradiographic method 2 may be useful in the study of the segregation and time of induction of DT' cells by different mutagens.

Demonstration of DT' cells in situ by the autoradiographic method described here has wide applications. The method can be used to analyze the events occurring during mutagenesis of a single cell. It can also be used to measure the mutation of cells with no ability to form colonies. Furthermore, it may be used to measure somatic mutation of human cells in vivo.

REFERENCES

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