Increased Persistence of Induced Mutants of Chinese Hamster Cells by 12-O-Tetradecanoylphorbol-13-acetate\(^1\)

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ABSTRACT

Hypoxanthine phosphoribosyltransferase deficient mutants of Chinese hamster ovary cells induced by ethyl methanesulfonate usually do not maintain their phenotype during growth in non-selective medium immediately following the induction. This phenomenon, called poor “persistence” of the induced mutation, is in most cases unrelated to growth rate but results from establishment of contact with wild type cells (Bradley, W. E. C. Exp. Cell Res. 129: 251, 1980). We report here that 12-O-tetradecanoylphorbol-13-acetate, a strong tumor promoter, increases the persistence of these mutants.

INTRODUCTION

Mutants of CHO\(^2\) cells induced by a mutagen must be allowed to grow for several generations before the population is subjected to selective pressure (1). At the end of this period of phenotypic expression most of the mutants yield fewer colonies in the selective medium than the number expected if the mutations were “fixed” immediately and behaved in a stable fashion. This effect, which we have called poor persistence (2), may be the result of delayed fixation of the mutation (3), or, in a limited number of cases, it could be due to slow growth or instability of the phenotype. We have reported (2) that in many cases hypoxanthine phosphoribosyltransferase (hprt\(^-\)) mutants exhibit poor persistence because they are unable to maintain the mutant phenotype in the presence of WT cells, although otherwise they are stable. In this communication we describe the effect of the TPA, a strong tumor promoter, on persistence of mutants induced by EMS, a commonly used mutagen. The rationale for these experiments stems from the fact that TPA increases the yield of hprt\(^-\) mutants of Chinese hamster cells after mutagenesis (4). It would be useful to know whether TPA affected persistence, since this knowledge may help elucidate the mechanism of TPA-mediated tumor promotion.

MATERIALS AND METHODS

CHO cells, obtained originally from C. Stanners, or cells of the CHO-derived line D422 (5) were propagated in α-medium (6) in Lux or Corning plastic Petri dishes. Mutagenesis was carried out by incubating cells in α-medium with EMS (150 μg/ml) and subsequently plating in small Linbro wells (0.3 ml, 96 wells/tray capacity) in α-medium with or without TPA (1 μg/ml) or an equimolar concentration of the related, but non-promoting chemical, phorbol (0.59 μg/ml), at a density of 350 viable colony-forming cells/well. These mutagenesis conditions result in 50% killing and, when the culture is propagated en masse in non-selective medium for 4–10 days (2) before selection, an average mutation frequency of 1.0 x 10\(^{-5}\) per cell. Neither TPA nor phorbol at these concentrations affects cell viability or mutation frequency of non-mutagenized cells (4).\(^2\) After 5 days, the entire contents (or, where indicated, a known proportion) of each well were transferred to a 35-mm Petri dish containing α-medium and 6-TG (3 μg/ml) and resistant colonies, which are hprt\(^-\), were counted about a week later. TPA and phorbol were obtained from CCR, Inc., Eden Prairie, MN or Sigma Chemical Co., St. Louis, MO. 6-TG was obtained from Sigma.

RESULTS AND DISCUSSION

Persistence of mutants is measured by dividing a culture of cells immediately after mutagenesis into many small populations of size such that almost all contain either 0 or 1 mutant. Cells are then propagated in non-selective medium and are plated in selective medium (6-TG) after a known number of generations. A portion of each population can also be retained for further propagation in non-selective medium and tested later if persistence is to be followed over an extended period. The number of colonies appearing in 6-TG can be expressed as RCE which is the ratio of the number of resistant colonies observed to the number of colonies expected assuming a single stable mutant had been present in the population at the end of the mutagenesis period.

If all mutants had complete persistence, the number of mutant colonies per dish would be relatively uniform and would reflect the number of cell divisions (which was determined to be between 6 and 7) during the 5 days of growth in non-selective medium. Since the initial event in EMS-induced mutagenesis is thought to involve modification of only one of the DNA strands (7), a reasonable expectation of 6-TG-resistant colony yield is therefore probably 32–64 in the case of mutants with complete persistence.

The results of several experiments are presented in Fig. 1, as a distribution histogram, with intervals of powers of two. By simple visual inspection, it is clear that TPA-treated mutants (represented by black bars in Fig. 1) had a higher persistence as a group than did the control or phorbol-treated mutants. This effect is emphasized in the inset, which portrays the ratios between the heights of the bars (TPA:control, the latter being the average of α-medium only and phorbol-treated populations). To quantitatively assess the difference between TPA-treated and control populations we first used the Poisson equations to estimate both the total number of mutants distributed among the populations with two or three independent mutants (called “doubles” and “triples,” respectively; Table 1). These calculations showed that between 6 and 9 doubles were expected in each of the three experimental groups, and about one triple in each group. If 33–64 colonies (“25” on the abscissa of Fig. 1) were taken as an indication of a single high-persistence mutant, the results of Fig. 1 fit quite well with Poisson predictions for the TPA-treated cultures, with four apparent doubles and one triple (“26” and “27,” respectively). This gives us confidence that our initial prediction of expected yield was accurate. (The fit would not be expected to be as good as for the other cultures, since they clearly have many low-persistence mutants, and a double or triple population would probably not yield enough resistant colonies to be scored as such.) These calculations lead to the estimate that 42% (21 of 50) of the TPA-treated mutants had high persistence, compared with 16%.

\(^1\) W. E. C. Bradley and F. Laviolette, unpublished results.


\(^1\) The abbreviations used are: CHO, Chinese hamster ovary; WT, wild type; TPA, 12-O-tetradecanoylphorbol-13-acetate; EMS, ethyl methanesulfonate; RCE, relative cloning efficiency; 6-TG, 6-thioguanine.
INCREASED PERSISTENCE OF MUTANTS BY TPA

Fig. 1. Histogram of the distribution of numbers of 6-TG-resistant colonies obtained in 139 populations, of which 50 were grown in α-medium only (open bars) 45 in α + phorbol (stippled), and 44 in α + TPA (solid bars). Experimental details are as described in Table 1. Intervals labeled as powers of 2 (abscissa) indicate colony numbers up to and including the indicated power of 2 but higher than the preceding power of 2. Inset, the ratio of the heights of the bars representing TPA-treated populations to the average of those representing untreated and phorbol-treated populations.

Table 1 Persistence of EMS-induced mutants in the presence or absence of TPA and phorbol

<table>
<thead>
<tr>
<th></th>
<th>α-Medium</th>
<th>+TPA</th>
<th>+Phorbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of populations</td>
<td>192</td>
<td>185</td>
<td>141</td>
</tr>
<tr>
<td>Population size (number of viable cells)</td>
<td>350</td>
<td>350</td>
<td>350</td>
</tr>
<tr>
<td>Number of populations yielding TG' colonies</td>
<td>50</td>
<td>44</td>
<td>45</td>
</tr>
<tr>
<td>Number of mutants</td>
<td>7.1/1</td>
<td>6/4</td>
<td>9.1/1</td>
</tr>
<tr>
<td>Number of populations with two mutants expected/obseved</td>
<td>0.8/0</td>
<td>0.75/1</td>
<td>1.6/0</td>
</tr>
<tr>
<td>Number of populations with three mutants expected/observed</td>
<td>6</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>Number of mutants with high persistence</td>
<td>33</td>
<td>15</td>
<td>30</td>
</tr>
</tbody>
</table>

* Calculated from the Poisson distribution based on the proportion of populations with no mutant colonies.
* Populations yielding between 65 and 128 TG' colonies were deemed to have had two independent mutants initially; those yielding 128 colonies, three.
* A yield of 33–64 resistant colonies was deemed to reflect high persistence.
* Thus the two or three mutants assumed to be present in the high-yield populations (Footnote b) were scored as, respectively, two or three high persistence mutants. A yield of 16 resistant colonies or fewer was deemed to reflect low persistence.

for the controls (Table 1). Low persistence mutants, which we arbitrarily define as those yielding 16 or fewer resistant colonies, made up 30 and more than 65% of the α-Medium and control populations, respectively. These differences are significant at 95% by statistical analysis of the proportions. It should be noted that these estimates of the TPA effect are conservative, since among control populations several expected doubles and triples were not observed and were presumably scored as singles with higher persistence than they actually exhibited.

In order to test the effect of TPA on induced mutants more directly, the protocol was modified so that each mutagenized population was grown first in α-medium without TPA, then split after 3 days, one-half of each being seeded in medium containing TPA and the other in α-medium only. Two experiments were performed where the protocol differed slightly (Table 2), but both gave similar results. After several days of further growth, known numbers of cells from each population were plated in 6-TG. Again, a difference was seen between TPA-treated and control cells, with 19 of 27 mutants yielding at least twice as many resistant colonies after TPA treatment than without treatment (Table 2) and only five yielding fewer colonies after TPA treatment compared to the non-treated control. It should be emphasized that the small numbers of cells involved introduce the probability of uneven distribution at the point of splitting the cultures at 3 days and may to some extent explain why some mutants yielded fewer colonies after TPA treatment than after growth in α-medium alone. Nevertheless, the difference between the TPA and control portions was significant at 95% by statistical analysis of the proportions.

The effect of TPA on the long-term persistence of some of the mutants generated in Experiment 1 of Table 2 was assessed by maintaining a portion of each population in non-selective medium. At various times up to 24 days after EMS treatment, known numbers of cells were seeded in 6-TG, and the resistant colonies were counted a week later. The results were calculated as the RCE, defined above. As shown in Fig. 2 for four of these mutants, the TPA treatment conferred higher persistence on several mutants which, in its absence, yielded progressively fewer 6-TG-resistant colonies. Mutants in a total of 12 populations were followed in this manner, and in the TPA-treated portions, 11 maintained at least 50% of the initial RCE, whereas non-TPA-treated mutants all yielded fewer resistant colonies in a manner similar to that exhibited by the non-treated mutants in Fig. 2. These experiments therefore support the conclusion drawn above that TPA enhances the persistence of EMS-induced mutants, and further show that the enhancement can occur when treatment is as much as 3 days after the mutagenesis.

How does TPA increase the persistence of these mutants? As mentioned above, low persistence could be due to relatively slow growth, delayed “fixation” of the mutation, or actual loss of the mutant phenotype in the presence of WT cells. TPA could conceivably act on any of these processes, but we consider the first to be unlikely, since both we (2) and others (3) have eliminated slow growth of mutants as a significant factor in low persistence.

The results of the second set of experiments described here (Fig. 2, Table 2) suggest that TPA action is not involved in accelerating the fixation process. Nearly all mutations are fixed within three generations, as has been shown at the glucose-6-phosphate dehydrogenase locus (3). Since TPA was only introduced into the culture after 3 days or about 4–5 generations, fixation processes should have already been completed, and any effect of TPA should not have increased the persistence. This argument also applies to repair mechanisms, so increased persistence conferred by TPA probably did not result from inhibition of repair.

The alternative which we favor is that TPA interferes with the process resulting in loss of the hprt* mutant phenotype induced by EMS. The results of this phenomenon are not yet understood, but high cell density and thus, presumably, establishment of contact with a WT cell is necessary for phenotype reversal (2). TPA has been shown to affect cell surface phenomena by interrupting metabolic co-operation (8, 9) altering phospholipid metabolism (10), and phosphorylating growth factor.
INCREASED PERSISTENCE OF MUTANTS BY TPA

Table 2  Effect of TPA on persistence of individual EMS-induced mutants

EMS-treated CHO cells were distributed into 24, 2-ml capacity Linbro wells, 400 viable cells/well (Experiment 1) or 48, 0.3-ml Linbro wells, 350 viable cells/well (Experiment 2), in α-medium. Three days later each population was divided into two fresh wells of the same size as the initial well, one with α-medium and one with α-medium containing TPA (1.0 μg/ml). In experiment 1, TPA was removed 24 h later, and known numbers of cells were plated in 6-TG 8 days after that; cells were passaged as necessary until then. Most populations were maintained in α-medium for a further 12 days with passaging as necessary, and known numbers were seeded in 6-TG at various times (data presented in Fig. 2). In experiment 2, TPA was not removed until 4 days later, when all cells were plated in 35-mm dishes containing 6-TG. Results are shown for populations yielding a relative cloning efficiency of at least 0.05 in at least one of the two portions.

<table>
<thead>
<tr>
<th>Number of populations with mutants</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Populations yielding 6-TG' colonies in both TPA-treated and control</td>
<td>17</td>
<td>10</td>
<td>27</td>
</tr>
<tr>
<td>Number of 6-TG' colonies from TPA-treated &gt; number from control</td>
<td>7</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Number of 6-TG' colonies from control &gt; number from TPA-treated</td>
<td>5</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>α-Colonies = TPA colonies</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Populations with 6-TG' colonies in one portion, not other</td>
<td>10</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>6-TG' colonies obtained from TPA-treated, not from control</td>
<td>9</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>6-TG' colonies obtained from control, not from TPA-treated</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of TPA on the persistence of four mutants induced by EMS. Experimental details are as described in the description of Table 2, experiment 1. Results from four independent mutants are presented, each represented by different symbols: (□, ○, △, □, △, ○, and △). Filled symbols, portion of population treated with TPA; open symbols, untreated.

receptors (11). It can be hypothesized that TPA in our experiments affects persistence by interfering with cell contact-mediated phenotype reversal. Many groups, in attempting to elucidate how TPA and other promoters exert their biological effect, have identified various cell changes induced by these substances (12), but no general mechanism for cancer promotion has yet been proven. The data presented here suggest the hypothesis that in addition to inducing a secondary effect increasing the initiated cell’s neoplastic potential, TPA increases its persistence. Thus, if a cell initiated in vivo behaves similarly to the hprt CHO cells induced by EMS in our experiments, TPA treatment would increase the time that the initiated phenotype would be maintained, thus increasing the risk of further neoplastic development. Clearly further investigation is necessary before such a hypothesis can be assessed. Once the cellular and/or molecular mechanism of poor persistence is understood, the effect of TPA can be directly tested to determine whether this hypothesis may be valid.

REFERENCES


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