Immortalization of Carcinogen-treated Syrian Hamster Embryo Cells Occurs Indirectly via an Induced Process

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ABSTRACT

The hypothesis that rodent cells can be immortalized by the direct induction of a single mutation-like event was tested by initiating cultures of benzo(a)pyrene treated Syrian hamster embryo cells with low inocula and expanding these few cells maximally until senescence prevented further culturing or immortalization took place. According to the mutation hypothesis immortalization is hardly to be expected under these conditions. However, immortalization was frequently observed. Therefore the induction of immortalization appears indirect. The progeny of benzo(a)pyrene treated cells immortalized at a rate of 3.9 × 10^-6/cell/generation, which is 64 times higher than the spontaneous rate. The results are in line with the probabilistic theory developed in 1980 by both Fernandez et al. (Proc. Natl. Acad. Sci. USA, 77: 7272-7276, 1980) and Kennedy et al. (Proc. Natl. Acad. Sci. USA, 77: 7262-7266, 1980), which states that treatment of cells with a carcinogen can result in a so-called activated state of the treated cells which is transmitted to the progeny and which results in an enhanced rate of transforming events.

INTRODUCTION

Spontaneous and Induced Frequencies of Immortalization. In a previous study (1) it was observed that induction of immortalization in SHE cells did not occur in a single step but that there are several sequential steps involved. The first step is ELS. The frequency of induction of ELS after treatment with B(a)P, X-rays, or ethylnitrosourea was in the same order of magnitude as induction of mutation at the HPRT gene of rodent cells which suggested that inactivation of one allele of one or at the most of a limited number of loci is sufficient to start the process of immortalization. This is in support of the hypothesis of Shay and Wright (2) which states that immortalization of rodent cells requires one mutational event. However, because no spontaneous immortalization or extension of life span was observed the spontaneous frequency of ELS was estimated to be below 2.4 × 10^-7/cell/generation. Literature data even indicated that it could be as low as 6.1 × 10^-10/cell/generation (1). Because the spontaneous frequencies of mutation at the HPRT gene in rodent cells range from 10^-6 to 10^-5/cell/generation, the question arose whether the induction of ELS should be considered as the induction of a mutation-like event. An alternative explanation could be that the treatment with carcinogen did not directly induce this step but instead only enhanced the rate of occurrence of ELS. There is evidence that carcinogens can cause a single step in immortalization.

Hypothesis of Probabilistic Malignant Transformation. The hypothesis of probabilistic malignant transformation (3, 4) states that carcinogens induce an initial change in a large fraction, perhaps even all, of the treated cells. This high frequency event does not lead directly to transformation of the cells but increases the probability that transformation occurs in the progeny of the treated cells as a secondary low frequency event.

The evidence that a high frequency event is induced by carcinogens both in vitro and in vivo has been reviewed by Kennedy (5). Because it appears from these data that a very small number of carcinogen-treated cells can result in malignant transformation or tumors it was concluded that a directly induced mutation in one of the treated cells could not be responsible for the malignant alteration but that this alteration must have occurred in the progeny of the treated cells. Therefore the initial change in most or all of the treated cells has been called “activation” and this activation is supposed to lead to a probabilistic second step which can be expressed as a rate per cell per generation.

The evidence for a probabilistic second step has been derived from the observations with C3H10T1/2 cells that the number of transformed foci per dish depends on the number of cells at confluence and not on the initial number of treated cells seeded per Petri dish. It was shown that the transforming event was dependent on the number of cell divisions and thus could be expressed as a rate per cell per generation (6). The data have been reviewed by Barrett and Elmore (7). From this review it appears that the transformation rate rises from about 5 × 10^-7/cell/generation in untreated cells to about 5 × 10^-5/cell/generation in X-irradiated cells, i.e., 100-fold enhanced. After treatment with MCA a similar enhancement was observed (1-6 × 10^-7/cell/generation). For the data of Mondal and Heidberger (8) who showed that 100% of treated prostate cells gave rise to transformed foci a rate of 2-7 × 10^-7/cell/generation was calculated for the secondary event.

The probabilistic hypothesis has been challenged several times (9-12) but the theory still stands and could explain the seeming discrepancy observed between induced and spontaneous ELS and subsequent immortalization of SHE cells. Therefore this study was undertaken. Confirmation of a probabilistic process would corroborate the importance of indirect processes inasmuch as it would hold for another type of cell and another type of biological end point. Rejection of the probabilistic theory would lend further support to the hypothesis of Shay and Wright for immortalization which states that immortalization in rodent cells can be induced in a single step.

Experimental Approach. If a probabilistic process causes the first step in immortalization the rate in the progeny of B(a)P treated cells would be about 5.2 × 10^-8/cell/generation. This figure is based on the 20 immortal cell lines obtained previously (1). The mutation theory using the same data predicted an induced ELS frequency of 1.9 × 10^-4/viable cell (1).

An experimental approach was designed to discriminate between the mutation hypothesis and the activation/ alteration hypothesis. When cultures are initiated with a small inoculum of B(a)P treated cells (e.g., 20 viable cells) there will be hardly any observation possible of induction of ELS according to the mutation hypothesis. However, according to the activation/
ALTERATION HYPOTHESIS. This event can take place in the progeny of these treated cells at the normal rate of \( 5.2 \times 10^{-9} \) cell/generation if the cultures are expanded maximally.

Because induction of ELS is difficult to determine when large numbers of cultures are involved, in this study complete immortalization was used as criterion rather than ELS. This does not influence the problem under study inasmuch as cells with ELS usually undergo complete immortalization if cultured in large numbers (1).

MATERIALS AND METHODS

Cells and Culture Conditions. Syrian hamsters (Cpb-ShGa 51) in their second pregnancy were purchased from CPB-TNO, Zeist, the Netherlands. Preparation and cryopreservation of primary cultures were carried out as described by Pienta et al. (13). All experiments were performed with typical cultures, derived from a single cryopreserved stock. Cells were grown in Dulbecco’s minimal essential medium, supplemented with NaHCO\(_3\) (2.4 g/liter), fetal calf serum, and antibiotics. The cultures were maintained at 37°C in an atmosphere of 10% CO\(_2\) in a humidified incubator. Cells were transferred by a gentle trypsinization with 0.25% trypsin/EDTA solution for 5 min at 37°C. The cultures were refed with fresh medium every 3 or 4 days.

Treatment with Carcinogens. Benzo(a)pyrene was obtained from Sigma Chemical Co. It was dissolved in dimethyl sulfoxide and then diluted to the desired concentration of 20 \( \mu \)g/ml into growth medium. Tertiary passage SHE cells were plated and allowed to attach overnight in normal medium supplemented with 20% serum. The cultures were then rinsed with phosphate buffered saline and treated with B(a)P for 24 h. Control cultures were treated with solvent only (0.1% dimethyl sulfoxide). After treatment the cells were cultured for 7 days in medium with 20% serum for the expression of induced mutations. After the expression period the cells were seeded in medium supplemented with 5% serum in order to create a selective advantage for immortal variants as described before (1).

Determination of the Immortalization Frequency and Immortalization Rate. The determination of the immortalization frequencies and rates is performed as described in a previous study (1). For the estimation of the frequency of induced immortalization per viable cell, the mean number of immortal cells per culture present immediately after treatment is calculated from the \( P_0 \) value. \( P_0 \) is the fraction of flasks in which no immortalization occurred

\[
P_0 = e^{-m}
\]

where \( m \) is the number of immortalising events per culture (14). The immortalization frequency then follows from dividing the number of immortalizing events by the number of viable cells seeded per culture.

For the estimation of the immortalization rates per cell per generation the total amount of cell generations (c.g.) for each mortal culture was calculated as

\[
c.g. = \frac{N_t - N_0}{\ln 2}
\]

and the number of immortalizing events in a particular experiment was divided by the total number of cell generations in that experiment.

RESULTS

Experiment 1. Cells were treated with B(a)P (20 \( \mu \)g/ml). This treatment reduced the relative cloning efficiency to 48%. After an expression time of 7 days, 96 cultures were initiated with 2000 cells each in medium with 5% fetal calf serum. Under these conditions the cloning efficiency is about 1% (3); thus each culture started with about 20 viable cells. The cultures were expanded maximally until senescence prevented further culturing or immortalization took place. The experiment was terminated after 150 days.

The prediction of the expected number of immortal cultures according to the mutation hypothesis is

\[
1.9 \times 10^{-4} \times 96 \times 2000 \times 0.01 = 0.36
\]

in which \( 1.9 \times 10^{-4} \) is the immortalization frequency according to the mutation hypothesis, 96 is the number of parallel cultures, 2000 is the inoculum per culture, and 0.01 is the cloning efficiency. Also the activation/alteration hypothesis can yield a prediction if it is known how many cell generations will be obtained in these cultures if they should have a normal life span. From previous experiments it is assumed that until senescence about \( 10^8 \) cells could be expected in each culture and the prediction for immortalization under this assumption therefore is

\[
96 \times 1.44 \times 10^8 \times 5.2 \times 10^{-9} = 7.2
\]

in which 96 is the number of cultures, \( 1.44 \times 10^8 \) is the number of cell generations per culture, and \( 5.2 \times 10^{-9} \) is the rate of immortalization per cell per generation according to the activation/alteration hypothesis.

As controls 20 cultures were initiated. The results from this experiment (Table 1) show that according to the mutation hypothesis an induction of \( 4.9 \times 10^{-9} \) has been observed which is 25-fold higher than the \( 1.9 \times 10^{-4} \) previously found. The immortalization rate per cell per generation according to the activation/alteration hypothesis is

\[
\frac{9}{2.2 \times 10^3 \times 87} = 3.7 \times 10^{-8}
\]

which agrees rather well with the previously observed \( 5.2 \times 10^{-8} \).

Because spontaneous immortalization was not observed the spontaneous rate of immortalization must be below \( 1.0 \times 10^{-4} \)/cell/generation.

Experiment 2. Cells were treated with B(a)P and seeded in normal growth medium supplemented with 20% serum for clone formation and expression of mutations; cultures were initiated via the isolation of clones. All cultures were expanded until senescence prevented further culturing. The experiment was terminated after 106 days. For the control 62 clones were isolated, whereas 96 clones were isolated from the B(a)P treated cells; 48 of these 96 clones had a normal morphology and 48

| Table 1 Immortalization in cultures initiated with 2000 B(a)P treated cells |
|--------------------------------|---|---|
| Control | B(a)P |
| No. of cultures | 20 | 96 |
| No. of cells seeded | 2000 | 2000 |
| No. of PD* of mortal cultures | 11.4 ± 5.0 | 9.9 ± 5.4 |
| Days until last passage of mortal cultures | 19.8 ± 10.3 | 27.5 ± 12.8 |
| PDT of mortal cultures | 41.5 ± 8.4 | 57.0 ± 21.0 |
| PDT of immortal cultures | 34.8 ± 14.6 |
| Total no. of cell generations of mortal cultures | 2.2 \times 10^9 | 9.5 \times 10^9 |
| No. of immortal cultures expected | 0 | 0.4 or 7.2 |
| No. of immortal cultures found | 0 | 9 |
| Induced immortalization frequency/ viable cell according to mutation hypothesis | Observed: 4.9 \times 10^{-9} | Expected: 1.9 \times 10^{-4} |
| Induced immortalization rate/cell/ generation according to activation/alteration hypothesis | Observed: 3.7 \times 10^{-8} | Expected: 5.2 \times 10^{-8} |

* PD, population doublings; PDT, population doubling time (h). The experiment was terminated after 150 days.

** Mean ± SD.
had a transformed morphology. The prediction of the expected number of immortal cultures according to the mutation hypothesis is

\[ 1.9 \times 10^{-4} \times 96 = 0.02 \]

in which \( 1.9 \times 10^{-4} \) is the induced immortalization frequency and 96 is the number of parallel cultures. For the activation/alteration hypothesis the expected rate per cell per generation is again

\[ 96 \times 1.44 \times 10^{6} 	imes 5.2 \times 10^{-8} = 7.2 \]

The results from this experiment are shown in Table 2. According to the mutation hypothesis the induced immortalization frequency was \( 3.1 \times 10^{-2} \) which is 160-fold higher than the predicted \( 1.9 \times 10^{-4} \). According to the activation/alteration hypothesis the observed immortalization rate per cell per generation is

\[ \frac{3}{60.1 \times 10^{6} \times 96} = 4.8 \times 10^{-8} \]

which agrees with the previously observed rate of \( 5.2 \times 10^{-8} \).

No spontaneous immortalization was observed which indicates that the spontaneous immortalization rate must be below \( 1.9 \times 10^{-8}/\text{cell/generation} \); experiment 1 and experiment 2 taken together indicate that the spontaneous rate must be below \( 6.8 \times 10^{-9}/\text{cell/generation} \). The induced rate from the combined data of experiment 1 and 2 amounts to \( 3.9 \times 10^{-8}/\text{cell/generation} \) which is 64-fold higher than the spontaneous rate.

**TABLE 2 Immortalization in cultures initiated with one B(a)P treated cell**

The experiment was terminated after 106 days.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>B(a)P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cultures</td>
<td>62</td>
<td>96</td>
</tr>
<tr>
<td>No. of cells seeded</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>No. of PD of mortal cultures</td>
<td>8.5 ± 2.1</td>
<td>6.2 ± 1.5</td>
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<tr>
<td>Days until last passage of mortal cultures</td>
<td>10.8 ± 4.3</td>
<td>9.8 ± 5.8</td>
</tr>
<tr>
<td>PDT of immortal cultures</td>
<td>30.6 ± 14.5</td>
<td>32.3 ± 23.8</td>
</tr>
<tr>
<td>PDT of immortal cultures</td>
<td>34.0 ± 7.3</td>
<td>35.2 ± 29.0</td>
</tr>
<tr>
<td>No. of cell generations of mortal cultures</td>
<td>5.3 x 10^7</td>
<td>6.1 x 10^7</td>
</tr>
<tr>
<td>No. of immortal cultures expected</td>
<td>0</td>
<td>0.02 or 7.2</td>
</tr>
<tr>
<td>No. of immortal cultures observed</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Induced immortalization frequency/viable cell according to mutation hypothesis</td>
<td>Observed: 3.1 x 10^{-2}</td>
<td>Expected: 1.9 x 10^{-4}</td>
</tr>
<tr>
<td>Induced immortalization rate/cell/generation according to activation/alteration hypothesis</td>
<td>Observed: 4.8 x 10^{-8}</td>
<td>Expected: 5.2 x 10^{-8}</td>
</tr>
</tbody>
</table>

* MT, morphologically transformed clones; N, clones with normal morphology; PD, population doubling; PDT, population doubling time (h).

**DISCUSSION**

Indirect Induction of Immortalization. In the present study it was tested whether or not the discrepancy between the very low spontaneous rate of immortalization in SHE cells (6.1 x 10^{-10}/cell/generation), which does not point to mutation as cause of immortalization, and the frequencies of induction of ELS in these cells, which do point to mutation, are compatible by assuming that in immortalization a probabilistic process is generated in carcinogen treated cells which leads to a higher rate of ELS in the progeny of the treated cells. Therefore experiments were initiated with a very low amount of cells per culture, which were expanded maximally and grown until senescence prevented further culturing or immortalization took place. Although only two of these laborious experiments have been performed the data of the two experiments agree closely with each other and indicate unequivocally that the induced immortalization frequencies (4.9 x 10^{-3} and 3.1 x 10^{-2}) do not correspond with the frequency of 1.9 x 10^{-4} which should have been found if direct induction of a mutation-like event should have taken place. In contrast the activation/alteration hypothesis is in agreement with the data as immortalization rates per cell per generation of 3.7 x 10^{-8} and 4.8 x 10^{-8} have been observed which correspond with the predicted value of 5.2 x 10^{-8}. Therefore it is indicated that the immortalization takes place via the induction of a probabilistic process and that due to the treatment with B(a)P the immortalization rate per cell per generation is enhanced from 6.1 x 10^{-10} to about 3.9 x 10^{-8} (64-fold) or to 4.7 x 10^{-8} (77-fold) if our previously obtained data (1) are also taken into consideration.

Heidelberg's Probabilistic Theory. Heidelberg's probabilistic view has been challenged several times. Apart from the observation that the original data obtained with MCA (3) could be influenced by residual MCA (15), the criticism relates to absence of the activation/alteration phenomenon after treatment with N-methyl-N-nitro-N-nitrosoguanidine (11, 15) or to absence of correction for influence of spontaneous transformation (10, 11) and to neglect of a possible inhibitory effect of normal cells on focus formation of transformed cells (5, 9, 10, 12, 16). In some cases an alternative explanation of observed effects appears possible (17).

However, the probabilistic second event hypothesis is the only hypothesis which can explain the occurrence of malignant transformation in the progeny of a small number of carcinogen treated cells as observed for mouse prostate cells (8), C3H10T'/2 cells (3, 4, 16, 18-20), rat tracheal cells (21), rat thyroid clonogens (22), and rat mammary clonogens (23). To this list immortalization of SHE cells has now been added.

Nature of Probabilistic Event. The nature of the secondary low frequency event which induces the process of immortalization is a matter of speculation. It still could be a mutation if this mutation is very rare. For instance if the mutation must be one specific base pair change in an oncogene, such as has been found for activation of ras and neu oncogenes (24, 25). Our data base on mutation spectra in the HPRT gene shows that mutations in 151 of the 218 codons can impair the gene (26). This means that the target will be at least 300 base pairs. Because the spontaneous mutation frequencies for a whole HPRT gene are around 2 x 10^{-7}/cell/generation (27), the spontaneous frequency of a specific base pair change could be in the order of 3 x 10^{-10}. Direct induction of such a mutation would be possible but the frequency after mutagenic treatment would be too low to be detected. The situation appears somewhat different in C3H10T1/2 cells because in that case the transformation rates per cell per generation for carcinogen treated cells are much higher, at 10^{-6} to 10^{-5} (4, 5, 18, 20). Therefore the target for transformation of C3H10T1/2 cells and for immortalization of SHE cells appears different in size. Therefore as could be expected for a larger target in C3H10T1/2 cells, observation of direct induction of transformation appeared experimentally possible (28).

Another explanation might be that the immortalizing event itself cannot be induced directly in normal cells. This would for example be the case if immortalization results from gene am-
plification (29, 30), or nondisjunction (7).

Another possibility is that the alteration is purely epigenetic. Also the nature of the activation is unknown. This could be a generalized stress response with concomitant genetic instability. Evidence that a stress response, induced by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, leads to genetic instability has been put forward (27). Therefore it is considered possible that treatment with a carcinogen leads to a stress response which is accompanied by elevated mutation rates. The level of such a response appears related to the inducing agent; the rate of immortalization per cell per generation can be calculated to be $4.7 \times 10^{-8}$ for B(a)P [32 events in 7.2 $\times 10^6$ cell generations; this paper and Ref. 1], $3.3 \times 10^{-8}$ for ethylnitrosourea [5 events in 1.5 $\times 10^6$ cell generations (1)]; and $9.4 \times 10^{-8}$ for X-ray [3 events in $3.2 \times 10^6$ cell generations (1)].

In 1990 LeBoeuf et al. (31) reported that cells derived from morphologically transformed SHE cells have a greater chance to become immortal than cells derived from clones with a normal morphology. The observation from our second experiment that all 3 immortal cultures were derived from clones with a morphologically transformed phenotype are in line with this finding. This implicates that the end point of the morphologically transformed phenotype after treatment with carcinogens could be of direct relevance for the process of multistep carcinogenesis. This until now not understood correlation between morphological transformation and carcinogenic potency would become understandable if both morphological transformation and immortalization (or transformation) are caused by a stress response of carcinogen treated cells. It also indicates that there is heterogeneity among carcinogen treated cells in degree of “activation.”

REFERENCES

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