Uptake, Metabolism, and Persistence of 3-Methylcholanthrene in Rat Embryo Cells Infected with Murine Leukemia Virus

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SUMMARY

The uptake and persistence of 3-methylcholanthrene have been followed in both uninfected rat embryo tissue culture cells and in cells infected with type C RNA virus. No significant differences in these parameters were observed as a function of viral infection or cell passage level. Moreover, neither binding of 3-methylcholanthrene to nucleic acids or proteins nor carcinogen metabolism were altered by the viral carrier state. Although transformation of rat cells by chemical carcinogens alone has been reported by us and other authors, the low-passage rat embryo cells used in this study will not transform unless cells are carrying exogenous type C RNA virus. We thus suggest that the virus must play a more direct role in the transformation process rather than affecting the ability of the cell to absorb, retain, or metabolize the chemical.

INTRODUCTION

Low-passage rat embryo cells infected with type C RNA viruses and treated with diethylnitrosamine (10), MCA (23), or 7,12-dimethylbenzanthracene (27) transformed in vitro within 15 population doublings (generations). In these cultures, treatment with either chemical alone or virus alone failed to produce transformation, although similar cells reportedly (26) undergo transformation by chemical alone. In addition, virus had to be present prior to, or concomitant with, chemical treatment for transformation to result (24). In these studies, it was suggested that the virus acted as the genetic determinant of the transforming event and that the chemical acted as the "derepressor." One of the alternatives is that the virus may accelerate and potentiate membrane permeability and metabolism of the carcinogen. In this study, we report that virus-infected cells are quantitatively similar to noninfected cells regarding the uptake metabolism and persistence of carcinogen, regardless of the passage level of the cells (number of generations).

1 This work was supported by Contract NIH-NCI-E-70-2068 B within the Virus Cancer Program of the National Cancer Institute.

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3 The abbreviations used are: MCA, 3-methylcholanthrene; EMEM, Eagle's minimal essential medium; RLV, Rauscher leukemia virus; BA, benzo(a)-pyrene; AHH, aryl hydrocarbon hydroxylase; BP, benzo(a)-pyrene.

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removed. Cells were washed twice and refed with medium containing no MCA. At periods from 15 min to 48 hr later, the cold medium was removed and the cells were digested and counted as described above.

**Protein Determination.** Total protein per culture was determined by assaying 0.5 ml of the NaOH digest according to the method of Oyama and Eagle (22).

**Binding of Carcinogen to Macromolecules.** Sixteen 32-oz flasks were seeded with $3 \times 10^8$ cells each. When the cell sheet was approximately 90% confluent (usually 24 to 48 hr), the cells were fed with fresh EMEM containing 0.4 μM [6-14C]MCA (5.47 mCi/mmmole) for 24 hr. The label-containing medium was then removed, the cell sheet was washed 3 times with EMEM, and the cells were removed from the flask with a rubber policeman and pooled. DNA, RNA, and protein were extracted according to the method of Diamond et al. (6). The amount of DNA recovered was determined by the diphenylamine reagent (3), RNA was determined by the orcinol reagent (17), and protein was determined by the biuret method (28). The amount of bound, labeled MCA was determined by counting an aliquot of each macromolecular fraction dissolved in Aquasol in the LS-250 liquid scintillation counter.

**Metabolism of Carcinogens.** The carcinogen-metabolizing system was monitored in 2 ways: (a) measurement of constitutive and BA-induced AHH activity as described by Nebert and Gelboin (21), and (b) measurement of the ability of the cell to convert BP from an organic soluble to an aqueous acetone-soluble form, as described by Nebert and Bausserman (19) and Diamond (5). This latter procedure was made quantitative by the following modifications. Experimentally growing cells were treated with 1.0 μg BP per ml (measured by fluorometric analysis) supplemented with 20 μCi [3H]BP per ml (specific activity = 2 μCi/μg). At 24 hr posttreatment, medium was added to 50-ml centrifuge tubes, cells were washed twice with 1-ml aliquots of cold Hanks’ balanced salt solution, and washes were pooled with medium. Cells were collected by scraping with a rubber policeman and total protein was determined as described above. Organic soluble chemicals were extracted by shaking the medium with 4 ml acetone, then two 5-ml aliquots of cold hexane. The BP converted to an aqueous acetone-soluble form was detected in a scintillation counter, and cpm of BP were converted to g BP with an external quench curve and an external 137Cs standard. Results were given in terms of g BP metabolized to aqueous acetone-soluble form per mg protein. More than 95% of the total counts added to the cultures were accounted for in the 2 phases (acetone-hexane and aqueous acetone).

**RESULTS**

**Effect of Viral-Carrier State on Uptake of MCA.** Both in virally infected (F2304V) and control (F2304C) cells, uptake of labeled carcinogen in terms of concentration per unit protein is rapid and linear for the 1st hr after treatment, after which the rate of uptake levels off, reaching a maximum at 4 to 6 hr (Chart 1). In the following 42 hr, the level of cell-associated labeled material falls to approximately one-half of its maximum value. This drop is probably not caused by cell loss, since total cellular protein measurements either remained steady or rose slightly during this period. The viral carrier state appears to affect neither the maximum cell-bound label per unit protein nor the rate kinetics of this binding. Readings taken after 96 and 168 hr, compared with the 48-hr level on chemical, show little change in the level of bound carcinogen and essentially no difference between virus-infected and control cells (Table 1). Uptake of [6-14C]MCA was measured in high-passage F1706C (control) and F1706V (RLV-carrier) over a period of 48 hr (Chart 1). Rate kinetics and absolute amount of intracellular carcinogen were similar to each other and also to those of the corresponding low-passage cells. Carcinogen levels in control and RLV-carrier cultures were assayed 6 hr after refeeding with fresh [6-14C]MCA-containing medium at 48 and 96 hr (Table 1). In each case, uptake of label rose between 3.3 ± 0.5 and 4.6 ± 0.5 nmoles/mg. Again, the presence of virus did not significantly alter the increase in incorporation at any time.


**Table 1**

Cell-associated \[^{14}C\]MCA (nmoles/mg protein)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Low-passage (Passage 20)</th>
<th>High passage (Passage 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F2304 control</td>
<td>F2304V + RLV</td>
</tr>
<tr>
<td>48</td>
<td>2.2</td>
<td>1.7</td>
</tr>
<tr>
<td>54</td>
<td>6.4</td>
<td>6.4</td>
</tr>
<tr>
<td>96</td>
<td>3.1</td>
<td>2.5</td>
</tr>
<tr>
<td>102</td>
<td>7.4</td>
<td>7.1</td>
</tr>
<tr>
<td>168</td>
<td>3.3</td>
<td>2.9</td>
</tr>
</tbody>
</table>

**Uptake and Persistence of MCA**

Persistence of Carcinogen as a Function of Virus Production and Passage Level. After removal of the labeled medium, the level of carcinogen associated with low-passage cells dropped sharply for 2 hr to less than 50% of its original value (Chart 2). By 6 hr after switching to “cold” medium, the amount of label bound to the cells had decreased to less than 10% of its initial amount, and remained at essentially this value for 48 hr. Once again, the presence of virus did not alter these kinetics. Very similar results were obtained when high-passage cells were used (Chart 2).

Incorporation of Carcinogen into Cellular Macromolecules in the Presence of Virus. In the low-passage F2304 cell system, persistent virus infection has no effect on the amount of MCA bound to either nucleic acids or proteins (Table 2), compared with the amount incorporated into those macromolecules in uninfected cultures. The high-passage system was not tested.

Carcinogen-metabolizing Activity as a Function of the Viral Carrier State. Constitutive and BA-induced AHH activities were similar in these rat cell lines, regardless of passage level or viral-carrier state (Table 3). However, with this assay, the low levels of AHH in these cells proved to be very difficult to measure reproducibly (note the wide ranges of AHH activity observed). The assay designed to measure conversion of BP to an aqueous acetone-soluble form proved to be much more reproducible and also demonstrated no differences between these rat cells regardless of passage level or viral-carrier state (Table 3).

**DISCUSSION**

Since, under certain conditions, there is a requirement for either antigenic or infectious type C RNA virus expression in these rat embryo cell cultures prior to transformation of cells by hydrocarbon carcinogens (24), this system provides an excellent model for studying the relative roles of virus and carcinogen in the transformation process (9). Several possible major roles for the virus can be envisioned: (a) virus alters cellular permeability and/or persistence of hydrocarbons; (b) the viral carrier state modifies cellular metabolism so as to favor more efficient conversion of the hydrocarbon to the ultimate carcinogenic form; or (c) the hydrocarbon affects cellular control so that oncogenic information contained within the virus becomes expressed. This study tends to indicate that the transformation in rat cells cannot be ascribed to changes in permeability or increase in overall hydrocarbon metabolism, although the actual levels of critical intermediates (e.g., epoxides) may differ in these cells.

The data clearly indicate that no such difference in
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Table 3
Carcinogen-metabolizing activity as a function of passage level and viral-carrier state in rat cells in vitro

All values are mean ± range of 3 separate experiments, each run in duplicate.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>AHH* (units/mg protein)</th>
<th>BP metabolized/mg protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Induced</td>
</tr>
<tr>
<td>F1706C</td>
<td>0.6 (0.2-1.3)</td>
<td>1.1 (0.7-1.8)</td>
</tr>
<tr>
<td>F1706V</td>
<td>0.8 (0.2-1.4)</td>
<td>1.2 (0.7-1.8)</td>
</tr>
<tr>
<td>F2304C</td>
<td>1.0 (0.5-1.3)</td>
<td>0.8 (0.4-1.2)</td>
</tr>
<tr>
<td>F2304V</td>
<td>1.5 (1.0-1.7)</td>
<td>1.1 (0.7-1.2)</td>
</tr>
</tbody>
</table>

* A unit of AHH activity is that amount of enzyme causing the fluorescent equivalent of 1 pmole of 3-hydroxybenzo(a)pyrene per min at 37°C.
* Units are μg BP metabolized to an aqueous acetone-soluble form per mg protein.
* Virus titers ranged from 10^4 to 10^5 tissue culture infectious doses of culture supernatant fluid (48 hr). No titer-related differences in AHH activity were noted.

Table 3 includes data on the AHH activity and BP metabolized/mg protein for various cell lines in vitro. The table is organized to show the AHH activity in control and induced conditions, as well as the BP metabolized/mg protein. All values are reported as the mean ± range of 3 separate experiments, each run in duplicate.

Cellular permeability or persistence of MCA exists in virally infected or uninfected rat cells of either high or low passage. The kinetics of incorporation showing a sharp rise for the 1st 4 hr after exposure to MCA, followed by a slow decline in intracellular levels, is similar to that found in hamster cells treated with BA (19) or BP (16). It is interesting that Dr. P. J. Price (personal communication) has observed that treatment of virally infected rat cells with MCA for only 2 hr is sufficient to cause transformation. It thus appears that the quantity of carcinogen taken up during this 2-hr period can trigger the events leading to transformation. Essentially similar kinetics of carcinogen uptake has been observed with autoradiographic (4) and fluorometric (1, 2, 29) techniques.

Duncan et al. (7) have noted that, although both carcinogens and noncarcinogens can be metabolized by mouse embryo cells, most carcinogens are more highly bound to nucleic acids than noncarcinogens. Using an assay which has been shown to produce fractions relatively free of other subcellular contaminants (6), we observed that the presence of virus does not alter the level of binding of MCA to either nucleic acids or proteins. Although equivalent amounts of chemical are bound to the nucleic acids, transformation occurs only in the presence of virus so that, at least in this system, quantitative covalent binding of the chemical, in itself, cannot be the critical transforming event. However, it is impossible to determine whether there is some qualitative difference in the binding in the 2 cases.

In agreement with these binding studies was the observation that the carcinogen-metabolizing ability of these rat cells was not affected by the presence of type C RNA viruses (Table 3). Neither of the 2 methods used could detect any viral-induced alteration in carcinogen metabolism. The assay that proved more reproducible in detecting the rather low levels of enzyme activity in these cells was the quantitative detection of the conversion of BP to an aqueous acetone-soluble form. This type of assay represents a very crude analysis of the hydrocarbon-metabolizing activity of these cells. Conversion to water-soluble metabolites (mainly carboxycyclic acids or conjugated derivations of dihydridols, phenols, and glutathione) (30) gives only the vaguest indication of how the systems in question attack the molecule and, regardless of the quantity of hydrocarbon metabolized, the types of intermediates (especially epoxides) may be very different for these various cell lines. Nonetheless, recent information (14) derived from the use of human lymphocytes suggests that this conversion to water-soluble forms is in fact correlated with levels of AHH inducibility and is also host gene regulated. Therefore, measurements of the ability of cells to convert BP to a water-soluble form may be a valid way to compare the hydrocarbon-metabolizing activity of various cell lines (15).

In studying the interferon-directed antiviral activity in mouse cells stimulated by benz(a)anthracene (BA), Nebert and Friedman (20) have likewise noted no effect on the rate of BA uptake, binding to subcellular fractions, or cellular AHH activity.

There are several reports of transformation of rat embryo cells by chemical carcinogens. In certain cases, these cells were transformed without the addition of exogenous virus (18, 26). In at least 1 case (26), the rat cell line that could be transformed without addition of exogenous virus was the exception out of many other rat embryo sublines studied by these authors. It should be noted that these cell lines were often not monitored for possible contaminating viruses and/or for expression of their own endogenous virus. We have reported that certain rat embryo cultures undergo an aging process wherein low-passage cells cannot and high-passage cells can be (8, 11, 12) transformed by chemicals. In this system, expression of the endogenous rat virus occurs concomitantly with chemical transformation of the cells (8).

Our current concept is that most rat embryo cell cultures are refractory to the transforming action of polycyclic aromatic hydrocarbons. Cells can become transformable by the addition of exogenous type C RNA virus or through expression of their own endogenous viral genome, an event that occurs randomly as the cells age (13, 25). In this study, we have used both low-passage cells refractory to chemical transformation and high-passage cells which are transformable. Regardless of cell-passage level, carcinogen permeability, metabolism, and binding are similar. The evidence presented thus suggests that the virus itself may be playing a direct role in transformation, perhaps by offering oncogenic information that can be derepressed due to...
the effects of chemical on the cell, rather than acting in an indirect role affecting permeability or metabolism of the chemical.

REFERENCES

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