ABSTRACT

Four mouse hepatoma cell lines, a parent (Hepa-1c1c7) and three variants (MUL12, BPc1, and TAOc1BPc1) which had been derived from Hepa-1c1c7 by the fluorescence-activated cell sorter, were incubated with benzo(a)pyrene, and the metabolites were analyzed by high-pressure liquid chromatography. Among these four cell lines, Hepa-1c1c7 and MUL12 metabolized benzo(a)pyrene the most quickly and to the greatest extent, and BPc1 had the weakest metabolic activity for this substrate. TAOc1BPc1 had intermediate benzo(a)pyrene-metabolizing activity, depending on cell density and incubation time. At low cell density, the active variant TAOc1BPc1 resembled the parent clone Hepa-1c1c7 and the highly active variant MUL12. At short incubation times, TAOc1BPc1 also had low conjugating activity while, at longer incubation times, the conjugating activity approached that of Hepa-1c1c7 and MUL12. At low cell density, Bp c1 was able to produce phenols, but this variant did not seem to have this ability at high cell density. When the substrate concentration was 4 uM and the incubation time was 24 h, g-glucuronidase treatment of water-soluble metabolites released about 5.3 times more pmol of quinones compared with phenols. But when the substrate concentration was 25 nm, g-glucuronidase released about 2.0 times as many phenols compared with quiñones. The parent and the two more actively metabolizing variants showed differences in the peak times of accumulation of 9,10-diol and 7,8-diol of benzo(a)pyrene, which may have implications for binding to DNA and nuclear proteins. It was concluded that BPc1 has basal but not easily inducible aryl hydrocarbon hydroxylase activity, whereas Hepa-1c1c7, MUL12, and TAOc1BPc1 have basal and inducible aryl hydrocarbon hydroxylase activity. These results show that variants of a single parent cell line can exhibit significant differences in the rate and extent of metabolism of benzo(a)pyrene.

INTRODUCTION

Polycyclic aromatic hydrocarbons are common contaminants of the environment. Some of these compounds, including B(a)P, have been shown to cause cancer in laboratory animals after metabolic activation to reactive electrophilic reagents that alkylate cellular macromolecules (22). This activation is performed by various enzyme systems known as the monoxygenases which contain one or more forms of cytochrome P-450 (8, 23).

These biochemical conversions have been studied extensively in established cell lines in culture, in microsomes, and in tissue explants. These studies have shown differences in B(a)P metabolism by different species (28), cell types (11), and organs (29). It is often assumed that a given cell line is composed of a population of cells which is homogeneous with respect to the ability to metabolize a foreign compound (27). However, studies have shown that this might not always be true (33). Therefore, efforts are routinely made to increase the homogeneity of a cell population by subcloning.

In particular, a permanent cell line, designated Hepa, was isolated in 1970 from a mouse hepatoma, BW 7756, which had been produced in mouse strain C57L/J (4). A clonal line, Hepa-1, and 10 subclones were derived from Hepa in 1973 (4). Hepa-1 had high AHH activity and was very sensitive to B(a)P toxicity. The 10 subclones varied with respect to albumin secretion, a characteristic function of liver cells.

In 1979, Hankinson selected Hepa-1c1c7 and other variant subclones of Hepa-1 by resistance to the toxicity of B(a)P (9). Those B(a)P-resistant variants tested had much reduced AHH activities. After further study, the heterogeneity in AHH activity among cells of the variants was said to be "relatively slight" (10).

Miller and Whitlock (19) subjected the cell line Hepa-1c1c7 to fluorescence-activated cell sorting and obtained new variants in B(a)P metabolism, some with reduced, and some with elevated, AHH activity. In further flow cytometric studies, they used a B(a)P disappearance assay to show that the variants, which had been selected by the cell sorter, were heterogeneous in the rate of induction of B(a)P metabolizing activity (20). They also used fluorescence spectroscopy to measure relative differences in the production of 3-hydroxybenzo(a)pyrene.

We have extended these studies to determine the differences among these 4 derived subpopulations with regard to B(a)P metabolism. We have incubated these cells with B(a)P and analyzed the metabolites by HPLC. It was concluded that one of the subpopulations, BPC1, had a low basal AHH activity at low but not at high cell density, but it lacked inducible AHH activity. Two of the subpopulations, Hepa-1c1c7 and MUL12, were rapidly inducible. The fourth subpopulation, TAOc1BPc1, had activities intermediate between BPC1 and the other 2. Differences were observed among the 4 subpopulations in the overall accumulation of ethyl acetate-soluble metabolites, in the production of water-soluble conjugates, and in the rate of production of the 7,8- and 9,10-diols of B(a)P.

MATERIALS AND METHODS

Chemicals. Generally labeled tritiated B(a)P (Amersham/Searle Corp., Arlington Heights, IL; ≥25 Ci/mmol) was diluted with unlabeled B(a)P.
ethyl acetate, the aqueous phase was washed twice more with solvent. The aqueous and ethyl acetate phases were measured, and the volumes were measured, and aliquots were taken and counted, as described above for the samples of extracellular medium.

After incubation periods from 0.5 to 48 h, the extracellular medium was removed by aspiration, the cells were gently washed with phosphate-buffered saline, and the wash and medium were combined and frozen at -20°C for subsequent analysis. The cells were scraped from the 3 plates with a rubber policeman, the plates were washed with 10 ml of phosphate-buffered saline, and the wash and cells were combined and treated as above. Cell counts were performed by hemacytometer or on a Coulter Counter Model ZF (Coulter Electronics, Inc., Hialeah, FL 33010).

Analysis of Extracellular Medium. The samples of extracellular medium were thawed and twice extracted with 2.5 volumes of ethyl acetate. After extraction, the aqueous and ethyl acetate phases were measured, and aliquots were taken from each for liquid scintillation counting.

The ethyl acetate was dried over anhydrous magnesium sulfate and evaporated under vacuum, and the residue dissolved in methanol for analysis by HPLC. Metabolite separation was performed with a Spectra Physics 3500B high-pressure liquid chromatograph fitted with a DuPont 1-m ODS-Permaphase column. Elution was by reverse phase with a solvent gradient from 30 to 70% or from 10 to 70% methanol/water (26). Flow rate was 0.6 ml/min, and oven temperature was 50°C. Effluent was monitored by a DuPont 836 multiwavelength fluorescence and UV spectrophotometer with a 250- to 350-nm transmission filter. Gradient sweep time was 30 min for the 30 to 70 gradient or 60 min for the 10 to 70 gradient. Radiolabeled metabolite standards (\( \text{H} \)) were coinjected with each sample, and peaks were identified by coincident retention time. Fractions (0.2 ml) were collected and measured by radioactivity in a Searle Mark III scintillation counter with Aquasol (New England Nuclear, Boston, MA) as the counting solution.

Raw scintillation data were converted to picomolar quantities and plotted by a Tektronix 4051 Graphics Terminal.

Analysis of Cells. The harvested cells were thawed and extracted, the volumes were measured, and aliquots were taken and counted, as described above for the samples of extracellular medium.

\( \beta \)-Glucuronidase Treatment. After extraction of the medium with ethyl acetate, the aqueous phase was washed twice more with solvent and treated with bacterial \( \beta \)-glucuronidase in order to release metabolites which had been conjugated as glucuronides. After digestion, the aqueous phase was extracted with ethyl acetate, and the metabolite analysis was continued as described above for the medium.

## RESULTS

Distribution of Radioactivity. Between 65 and 93% of the radioactivity recovered from the plates was found to be in the medium, with the rest of the radioactivity remaining in the cells. The distribution of radioactivity from the medium and cells of a typical experiment into the ethyl acetate and aqueous phases is shown in Chart 1. It is seen that, for all 4 cell lines, the ethyl acetate-soluble radioactivity in the medium decreased with increasing incubation time. The aqueous-soluble radioactivity in the medium correspondingly increased for the parent, Hepa-1c1c7, and 2 of the variants, MUL12 and TAOc1BpC1, indicating that the parent and these 2 variants were capable of converting B(a)P to water-soluble conjugates. Time points on Chart 1B show that MUL12 was the fastest metabolizer, followed by Hepa-1c1c7 and TAOc1BpC1, in that order. Furthermore, at incubation times of 8 h or less, TAOc1BpC1 seemed to resemble BpC1 in the low level of formation of water-soluble conjugates but, at longer incubation times, this aspect of the behavior of TAOc1BpC1 approaches that of MUL12 and Hepa-1c1c7.

In contrast, the third variant, BpC1, did not show an increase in water-soluble radioactivity in the medium. However, this variant showed a slight increase of ethyl acetate-soluble radioactivity in the cells (Chart 1C), which indicates that B(a)P was entering the BpC1 cells but was not being efficiently metabolized.

Total Accumulation of Ethyl Acetate-soluble Metabolites in Extracellular Medium. The ethyl acetate phase from extraction of extracellular medium was analyzed by HPLC. A typical plot of the total accumulation of ethyl acetate-soluble metabolites in the extracellular medium as a function of incubation time is presented in Chart 2. With increasing incubation time, Hepa-1c1c7, TAOc1BpC1, and MUL12 showed an increasing accumulation, while BpC1 did not.

The dependence of this parameter on cell density is shown in Table 1. At low cell number, BpC1 and TAOc1BpC1 were similar in this parameter but, at high cell number, TAOc1BpC1 showed a higher accumulation of ethyl acetate-soluble metabolites than did BpC1, resembling Hepa-1c1c7 and MUL12.

Accumulation of Individual Ethyl Acetate-soluble Metabolites in Extracellular Medium. HPLC analysis showed that, of the ethyl acetate-soluble metabolites in the medium, the ones that had been accumulated to the greatest extent were the 9,10-diol and the 7,8-diol. As shown in Chart 3A, the parent and the 2 “faster-metabolizing variants” produced 9,10-diol, although peak times differed: MUL12 seems to have built up 9,10-diol first, followed by Hepa-1c1c7 and TAOc1BpC1. Furthermore, for MUL12 and Hepa-1c1c7, the arnassment of 9,10-diol appears to have dropped back to baseline by 8 h, whereas this metabolite was still at an elevated level in the medium from TAOc1BpC1 cells at 12 h.

The accumulation of 7,8-diol is shown in Chart 3B and appears to be less than the amount of 9,10-diol shown in Chart 3A. MUL12 seems to have accumulated very little 7,8-diol, compared with Hepa-1c1c7 and TAOc1BpC1. The amount of 7,8-diol in the medium from Hepa-1c1c7 cells seems to have reached a peak at 5 h and then dropped back to baseline by 8 h, whereas the 7,8-diol peak occurred at 10 h for TAOc1BpC1.

A cell density effect on the accumulation of individual metabolites was observed. As shown in Table 2, at high cell density, cell lines Hepa-1c1c7, MUL12, and TAOc1BpC1 accumulated more diols than phenols after 6 h of incubation with 4 \( \mu \)M B(a)P. However, at low cell density, there was a shift toward greater accumulation of phenols than diols. Even variant BpC1 accumulated an appreciable amount of phenols when a lower number

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METABOLISM OF B(a)P BY VARIANT MOUSE CELLS

Chart 1. Distribution of radioactivity in phases after extraction of cells and extracellular medium. Cells and extracellular medium were extracted separately as described in "Materials and Methods." After extraction, the volume of each phase was measured, 3 aliquots were counted for radioactivity, and the dpm were converted to nmol. The amount of radioactivity in the phase, expressed as nmol of B(a)P plus metabolites and conjugates per 10^6 cells, is plotted as a function of the incubation time. A, ethyl acetate fraction of medium; B, aqueous fraction of medium; C, ethyl acetate fraction of cells; D, aqueous fraction of cells. The concentration of B(a)P at the beginning of the experiment was 4 μM, and cell densities ranged from 5.8 to 19.5 × 10^6 cells per 100-mm dish. ∆, BPc1; ▲, TAOc1BPc1; ○, Hepa-1c1c7; ■, MUL12.

of cells was incubated with 4 μM B(a)P. The production of B(a)P 3-phenol by BPc1 was also observed in other experiments in which a small number of cells per dish was incubated with 200 or 1000 nm B(a)P (data not shown).

It is also seen in Table 2 that, at high cell density, the metabolic profile of TAOc1BPc1 resembles that of Hepa-1c1c7 but, at low cell density, TAOc1BPc1 looks more like BPc1.

Release of Radioactivity by β-Glucuronidase. The experiments described above were done at a B(a)P concentration of 4 μM. However, the parent and 3 variant cell lines were also incubated with 25 nm B(a)P for 12 and 24 h, since it has been shown that substrate concentration can influence kinetic measurements of metabolism (21). At both concentrations, there were only low levels of phenols in the ethyl acetate phase from extraction of the medium. When the aqueous phase from the medium which had contained 4 μM B(a)P was treated with β-glucuronidase, radioactivity was released which eluted at the polar end of the chromatogram, and in the quinone region, but only low levels of phenols were released. In contrast, when the aqueous phase from 25 nm B(a)P-containing medium was treated with β-glucuronidase, more phenols were released than quinones (Table 3). The data indicated that variant BPc1 conjugated very small amounts of quinones and phenols to glucuronides, compared with the other 3 cell lines. These results agree with the lack of accumulation of aqueous-soluble radioactivity in the medium from BPc1 cells, shown in Chart 1B.

DISCUSSION

Our results indicate that subpopulations of cells separated from a parent clone can have markedly different metabolic abilities. The parent, Hepa-1c1c7, and one of the variants, MUL12, are similar in overall activity, both having high ability to metabolize B(a)P both in terms of accumulation of ethyl acetate-soluble metabolites and water-soluble conjugates. The variant BPc1 has low B(a)P metabolizing activity at low cell density, and TAOc1BPc1 has intermediate activity, depending on cell density, incubation time, and type of metabolism.

Analysis of individual metabolites of B(a)P is important, because evidence has been obtained that some metabolites contribute more than others to the carcinogenicity of a compound. In particular, it has been shown that the 7,8-dihydrodiol-9,10-epoxide of B(a)P is the metabolite that is mainly responsible for binding to DNA (31). Studies have shown that the metabolic conversion of B(a)P to its 7,8-dihydrodiol-9,10-epoxide occurs in 3 steps via the 7,8-oxide and the 7,8-diol (7, 12, 32, 34). In addition, more recent studies have indicated that the 9,10-diol of B(a)P may be converted to a metabolite (presumably the 9,10-diol-7,8-epoxide) that covalently binds to a nuclear protein(s) having electrophoretic mobility similar to that of histone H1 (15, 16). Thus, it was of interest to determine the levels of 7,8-diol and 9,10-diol produced by these 4 cell lines.

Our results indicate that MUL12 and Hepa-1c1c7 are more active in the further metabolism of 9,10-diol than is TAOc1BPc1. This could have implications for the binding of B(a)P metabolites to nuclear proteins. We might speculate that the conversion of the 9,10-diol to the 9,10-diol-7,8-epoxide may be slower in TAOc1BPc1 than in MUL12 or Hepa-1c1c7. If further metabolism of the 9,10-diol is necessary before binding to a nuclear protein can occur, then perhaps TAOc1BPc1 would show less binding of B(a)P to this nuclear protein than would the other 2
METABOLISM OF B(a)P BY VARIANT MOUSE CELLS

**Table 1**
Accumulation of total ethyl acetate-soluble metabolites in extracellular medium from high- or low-density cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Low (pmol/10^6 cells)</th>
<th>High (pmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP'c1</td>
<td>9.52</td>
<td>7.82</td>
</tr>
<tr>
<td>Hepa-1tc1c7</td>
<td>20.22</td>
<td>21.39</td>
</tr>
<tr>
<td>MUL12</td>
<td>26.50</td>
<td>22.56</td>
</tr>
<tr>
<td>TAOc1BP'c1</td>
<td>10.39</td>
<td>19.49</td>
</tr>
</tbody>
</table>

* "High cell density" refers to apparent confluence, which ranged from 6.5 to 9.0 x 10^6 cells per 100-mm dish, and "low cell density" refers to 1.1 to 1.5 x 10^6 cells per 100-mm dish.

**Table 2**
Accumulation of individual ethyl acetate-soluble metabolites in extracellular medium from high- or low-density cells

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>BP'c1</th>
<th>Hepa-1tc1c7</th>
<th>MUL12</th>
<th>TAOc1BP'c1</th>
</tr>
</thead>
<tbody>
<tr>
<td>9,10-diol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>9</td>
<td>33</td>
<td>42</td>
<td>120</td>
</tr>
<tr>
<td>High</td>
<td>6</td>
<td>110</td>
<td>11</td>
<td>207</td>
</tr>
<tr>
<td>4,5-diol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>9</td>
<td>41</td>
<td>14</td>
<td>102</td>
</tr>
<tr>
<td>High</td>
<td>6</td>
<td>110</td>
<td>11</td>
<td>207</td>
</tr>
<tr>
<td>7,8-diol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>6</td>
<td>69</td>
<td>34</td>
<td>263</td>
</tr>
<tr>
<td>High</td>
<td>6</td>
<td>110</td>
<td>11</td>
<td>207</td>
</tr>
<tr>
<td>Quinones</td>
<td>42</td>
<td>520</td>
<td>48</td>
<td>497</td>
</tr>
<tr>
<td>9-phenol</td>
<td>9</td>
<td>149</td>
<td>13</td>
<td>437</td>
</tr>
<tr>
<td>3-phenol</td>
<td>16</td>
<td>468</td>
<td>14</td>
<td>21</td>
</tr>
</tbody>
</table>

* "High cell density" refers to apparent confluence, which ranged from 6.5 to 9.0 x 10^6 cells per 100-mm dish, and "low cell density" refers to 1.1 to 1.5 x 10^6 cells per 100-mm dish.

Under the chromatographic conditions used in these experiments, the most polar peak is eluted last on the chromatogram. The most polar peak at the beginning of the chromatogram probably contains triols and tetrols of B(a)P. These metabolites are not usually conjugated to glucuronides by rodent cells, although conversion of tetrols to glucuronides by human cells has been reported (1).

In early experiments with microsomes, in the absence of...
NADPH, quinones were not conjugated to glucuronides (24). However, several more recent reports (1, 2, 6, 14, 25) on the metabolism of B(a)P by intact cells have shown that the release of quinones upon treatment with β-glucuronidase. In addition, it has been shown that quinones can be conjugated to glucuronides in organ culture (17). It has also been observed (13) that B(a)P-3,5-quinone can be glucuronylated by microsomes if NADPH is present, suggesting that glucuronolysis is most likely preceded by reduction of the quinone, probably by the action of DT-diaphorase (13). However, other studies suggest that this reduction may be performed by NADP+-cytochrome P-450 (c) reductase rather than DT-diaphorase (5, 30).

Our data have indicated that there is a substrate concentration effect on the conversion of phenols and quinones to glucuronide conjugates: as shown in Table 3, at high substrate concentration, the ratio of quinone glucuronides to phenol glucuronides was higher whereas, at low substrate concentration, this ratio was low. A possible reason for this substrate concentration effect may be that, at the high substrate concentration, there is a greater opportunity for phenols to be oxidized to quinones whereas, at the low substrate concentration, the phenols are conjugated to glucuronides too rapidly for much oxidation to occur.

Our data indicate that, if the cell density is sufficiently low and if metabolism is studied with a sensitive radioisotope assay, it can be shown that BPc1 is able to metabolize B(a)P to phenols and quinones but is not able to conjugate these to glucuronides. These results indicate that BPc1 has a low basal AHH activity, which may be turned off at high cell density, but AHH activity may not be inducible by B(a)P in this variant.

From our results, we conclude that Hepa-1c1c7, MUL12, and TAOc1BPc1 have inducible and basal AHH activity, but that TAOc1BPc1 is more slowly induced than is Hepa-1c1c7 or MUL12. Thus, our rank order of inducible AHH activity for Hepa-1c1c7, TAOc1BPc1, and BPc1 agrees with that published by others (18).

Miller et al. (18) have suggested that BPc1 lacks detectable basal or inducible AHH activity, because this variant is unable to translocate an inducer-receptor complex from the cytosol to the nucleus, whereas TAOc1BPc1 has low levels of basal and inducible AHH activity, because this variant has cytosolic receptors which are decreased either in number or binding ability. Our observation of a lack of basal and inducible AHH activity in BPc1 at high cell density agrees with the results of these workers, but the observation of some production of phenols by this variant at low cell density differs from their results. Also, the dependence of AHH activity in TAOc1BPc1 on cell density and incubation time is a departure from their results.

The cell density effect which we have observed in this study also agrees with another report (3) in which confluent hamster or rat embryo cells metabolized B(a)P mainly to dihydrodiols, whereas low-density cells showed an increased accumulation of free phenols.

In conclusion, this study has shown that subpopulations of cells, separated from a parent cell line by flow cytometry, need not be biochemically homogeneous. In particular, the parent cell line and 3 variants derived from it, which have been studied here, show differences in the rate and extent of overall metabolism of B(a)P and in the production of individual metabolites. These differences probably influence the level of ultimate carcinogen formed and subsequent biological consequences in a given cell. These results suggest that subpopulations of cells, within a tissue or in a mass culture, may differ in their metabolic activation capability and therefore their susceptibility to transformation to a neoplastic state by a chemical carcinogen such as B(a)P.

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Metabolism of Benzo(a)pyrene by Variant Mouse Hepatoma Cells

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