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Publication Date

1976-11-01

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November 16, 1976

DISCLAIMER

COMPARISON OF THE ACCUMULATION OF BENZO[a]PYRENE IN MOUSE

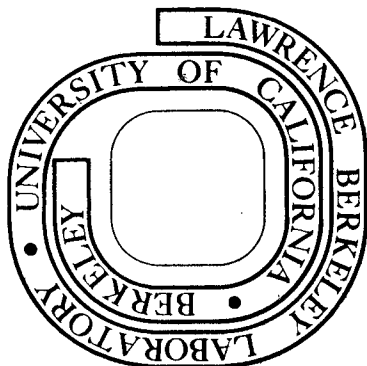EPITHELIAL CELLS SENSITIVE AND RESISTANT TO THE CYTOTOXICITY

OF THE HYDROCARBON[1]

Running Title: Uptake of Benzo[a]pyrene in Cultured Epithelial Cells

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FOOTNOTES

1

This study was supported by National Cancer Institute Contract
YOl CP 50203 and the Energy Research and Development Administration.

2

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The abbreviations used are: BaP, Benzo[a]pyrene; FMF, flow
microfluorometry.

SUMMARY

The accumulation of benzo[a]pyrene by two strains of mouse liver
epithelial cells in culture was measured. The cell strains studied differ
in their sensitivity to benzo[a]pyrene cytotoxicity, and in the inducibility
of aryl hydrocarbon hydroxylase, but are very similar in other respects.
Benzo[a]pyrene uptake was measured by a technique using radioactive compound,
and also by flow microfluorometry. Taken together these techniques suggest
that the accumulation process is only slightly different in these strains,
in spite of the significant difference in effect of this compound on the growth
of the two cell strains. It is concluded that differences in sensitivity
to benzo[a]pyrene must result from alterations in the induction or activity
of enzymes involved in benzo[a]pyrene metabolism.

INTRODUCTION

Benzo[a]pyrene (BaP[4]) is a common environmental carcinogen, which has been shown to be toxic to mammalian cells grown in culture (4,7,3). This cytotoxic response is dependent upon the metabolism of BaP by aryl hydrocarbon hydroxylase. The hydroxylase enzyme system is induced by the hydrocarbon, and converts the parent hydrocarbon into more oxidised derivatives (4). BaP has also been observed to cause the malignant transformation of cultured cells (3), and the covalent attachment of BaP metabolites to cellular macromolecules has been reported (13). All of these processes are dependent on the generation of reactive derivatives in the course of enzymic oxidation of BaP (7,8).

Recently, a mouse liver epithelial cell strain (NMuLi) has been described which is highly sensitive to the cytotoxic action of BaP (1). In addition, a derivative of NMuLi, NMuLi-BaP, was selected on the basis of its resistance to BaP cytotoxicity. This resistant derivative has a 10-fold decrease in the inducibility of the aryl hydrocarbon hydroxylase relative to the parent NMuLi. This loss of sensitivity of NMuLi-BaP may be due to the failure of BaP to induce an active hydroxylase, or the resistant cells may fail to accumulate BaP to levels necessary for enzyme induction.

In these experiments we have investigated the accumulation of BaP by these related sensitive and resistant cells, in the hope of identifying causes of the difference in response to BaP cytotoxicity.

MATERIALS AND METHODS

Cell Culture

The growth and properties of NMuLi and NMuLi-BaP have been described previously (1,10,12). Both cell strains were maintained and passaged in 100 mm

plastic dishes (Falcon Plastics, Oxnard, California) and incubated at 37°
under an atmosphere of 5% CO_2/95% air. The cells were grown in modified
Eagles's medium (14), containing 10% fetal calf serum (Grand Island Biological
Co., Grand Island, New York) and 10 μg/ml insulin. The cells were transferred
every seven days by removal from the dishes with 0.05% trypsin (1:250; Difco,
Detroit, Michigan) in 25 mM Tris buffer, pH 7.4, containing 140 mM NaCl,
5 mM KCl and 0.7 mM Na_2HPO_4 (isotonic Tris Buffer). The seeding density was
1/10 of the saturation density.

Flow microfluorometry

The amount of BaP per individual cell was quantified by measuring the
amount of fluorescence per cell using flow microfluorometry (FMF). The
FMF technique was also used to determine the amount of DNA per cell after
mithryamycin staining. This technique has been described previously (2,6).
Briefly, the cells were passed individually through the beam of an argon-ion
laser (Spectra-Physics, Mountain View, California) tuned to an appropriate
wavelength for excitation of the fluorophore of interest (363 nm for BaP,
457 nm for mithramycin). The pulse of fluorescence emitted was filtered to
reduce scattered light, and detected by a photomultiplier tube positioned at
right angles to the laser beam. The resulting signal is amplified electronically,
and recorded in the memory of a pulse height analyser (Northern Scientific,
Middletown, Wisconsin). The data in the form of a histogram was stored on
magnetic tape and processed by a program written for a Sigma 2 computer (Xerox,
Rochester, New York).

Accumulation of Benzo[a]pyrene

Epithelial cell strains grow with a patchy morphology making precise
reproduction of cell counts per dish difficult. In order to make the deter-
mination of the amount of BaP accumulated per cell as accurate as possible,

we divised a technique to determine the cell count and levels of BaP simultaneously.

The cells to be used in the experiment were prelabeled with ^{3}H-thymidine by plating 3×10^5 cells in 100 mm dishes with medium containing 0.05 µCi/ml ^{3}H-thymidine. After six days, the cells were replated at 1×10^5 cells in 35 mm dishes with medium containing 0.05 µCi/ml ^{3}H-thymidine. Each dish contained two sterile coverslips. After 24 hrs, U-^{14}C-BaP was added to a final concentration of 0.1 µCi/ml (1 µg/ml), as a solution in 10 µl of DMSO (final concentration equals 0.50%. After exposure to the BaP, the coverslips with cells attached were washed twice with isotonic Tris buffer and fixed for 24 hrs in 3.7% formaldehyde solution containing 0.1 M NaCl and 0.1 M Na_2SO_4. In addition to killing the cells, this treatment allows thymidine which has not been incorporated into macro-molecules, but merely entered the metabolic pool, to diffuse out of the cell. The amount of ^{3}H detected is therefore a measure of the amount of DNA in the sample, and thus the number of cells. This procedure does not signifi-cantly alter the amount of ^{14}C-BaP per cell, as estimated by comparison of fixed and unfixed cell samples.

The amount of ^{14}C-BaP per cell was measured as the ratio between counts per minute of ^{14}C and ^{3}H detected, converting the ^{3}H values to cell number by an independent determination of the amount of ^{3}H-thymidine per cell. The change in the ratio of ^{14}C to ^{3}H with time reflects the uptake of label by the cells. By assuming that this curve represents a single exponential, the asymptote (r_∞) may be estimated as:

$$r_\infty = \frac{r_b^2 - r_a r_c}{2r_b - (r_a + r_c)}$$

where r_a, r_b, and r_c represent equally spaced points taken from the curve. Having obtained this value, the rate constant of the exponential uptake curve may be obtained by plotting $\ln(r_\infty/r_\infty - r_t))$ versus time. The r_t in this equation is the value of r at any time, t. The slope of this line represents the first-order rate constant. It should be noted that this analysis may only be applied over the first 70% of the curve, since after this point the error in the logarithmic ratio becomes very large for a small error in the r_∞ or r_t. The success of this analysis does not prove that the curve represents a true first-order kinetic process. It may be possible to fit the curve equally well by certain other equations, and also there may be other factors which are not important under pseudo-first order conditions.

Radioactivity Measurements

All samples for radioactivity measurements were burnt in a Packard Model 306 sample oxidiser, providing separate sampling of ^{3}H and ^{14}C with less than 1% cross contamination between samples or channels. The samples were counted in a Packard Model 2450 scintillation counter. Sample recovery and counting efficiency were measured by calibration with toluene standards, using an external standardization ratio to assess sample quenching individually.

Radioisotopes

The U-^{14}C-BaP (21 mCi/mM) was obtained from Amersham/Searle (Arlington Heights, Ill.). The purity was estimated by thin layer chromatography on silica plates with benzene-ethanol (19:1) as solvent. In the autoradiogram of the plate the main area of activity corresponded to the only visible fluorescent spot. This spot had the same r.f. as an authentic BaP standard. When the spot, the origin and the area of track other than the spot were scraped off the plate and radioactivity measured by scintillation counting,

at least 99% of the total counts were in the area of the spot. The ^{3}H-thymidine (20.1 Ci/mM) was obtained from New England Nuclear (Boston, Mass.).

RESULTS

Chart 1 shows the variation in ^{14}C-BaP/^{3}H-thymidine ratio with time for NMuLi and NMuLi-BaP cells exposed to labeled BaP. The uptake in NMuLi is fairly rapid, the asymptote of 0.4 being effectively reached within 100 min. The uptake by NMuLi-BaP was very similar although the particular values of the exponential parameters vary with cell type (Table 1). In each case a semi-logarithmic plot of the ratios measured during the first 10 min of uptake give a straight line, as illustrated for NMuLi and NMuLi-BaP in Chart 2. The positive intercept on the "Y" axis of the plot probably represents an error in the timing of the initial point, since the manipulation of each sample takes a small but finite time during which the cells are exposed to labeled BaP. Table 1 shows the values of the asymptote and the pseudo-first order rate constant for the two cell strains studied.

Flow Microfluorometric Analysis of BaP per Cell

BaP shows a fluorescence excitation spectrum with maxima near 390, 370, and 350 nm, and fluorescence emission maxima near 410, 430, and 433 nm (9). These wavelengths vary only slightly depending on the environment of the fluorophore. This fluorescent property enables the amount of BaP per cell to be measured using the technique of flow microfluorometry (6). The machine used in these experiments was able to measure BaP fluorescence when the laser was tuned to the 363 nm line, and the emitted light filtered through Corning 1-64 filter.

Charts 3 and 4 show flow microfluorometry measurements with NMuLi and NMuLi-BaP cells which were exposed to BaP (5 µg/ml) for 1 hr. The hydrocarbon

was added in acetone and control measurements were made on cells exposed to the same concentration (0.5%) of acetone only. Results are presented as histograms of cell number for varying DNA or BaP content. In the experiment shown in Chart 3, DNA content was determined by mithramycin staining. BaP had no effect during the course of these experiments on DNA content or the proportion of cells in any particular part of the cell cycle. However, there was a difference between NMuLi and NMuLi-BaP in the latter respect, NMuLi-BaP having a slightly higher proportion of cells in G_2+M, i.e. cells having twice the normal minimum complement of DNA. This difference reflects the slightly different growth properties of the two strains. Chart 4 shows measurements of fluorescence from the BaP contained in the cells. Cell fluorescence in the absence of added BaP was weak, and could only be measured at very high gain; it was similar for both cell lines. BaP accumulation was very similar for both cell lines, the NMuLi-BaP cells having a slightly higher proportion of cells with a higher BaP content, as measured by fluorescence. The position of the mode channel of the NMuLi-BaP population was slightly higher than that of NMuLi.

DISCUSSION

Both NMuLi and NMuLi-BaP accumulated BaP when exposed to this material in the medium, at rates which are the comparable within the accuracy of the measurement. However, the saturation level of BaP per cell is higher in NMuLi-BaP. The average value of this parameter measured by the radioactivity method is 2.75 times greater for NMuLi-BaP, although the exact degree of difference may be uncertain due to inaccuracies of the various measurements, particularly the cell number calibration with tritium labeling. The FMF measurements also show an increase in the amount of BaP per cell in the

population of NMuLi-BaP. The mode of the BaP content distribution as well as the proportion of cells having a BaP content greater than the mode is higher for NMuLi-BaP.

The analysis of DNA content by FMF enables these observations to be explained. In both NMuLi and NMuLi-BaP the majority of cells are in G_1. However, NMuLi-BaP is seen to have a greater proportion of cells with a double complement of DNA; i.e., in the G_2 phase of the cell cycle, or in the process of mitosis. Since G_2 and mitotic cells have a larger volume than cells in other parts of the cell cycle, the amount of BaP accumulated by these cells should be greater. Therefore, the increased amount of BaP taken up by populations of NMuLi-BaP is due at least in part to a redistribution of cells in the cell cycle. However, as seen in Fig. 4, the mode of the BaP content distribution of NMuLi-BaP is slightly greater than for NMuLi. This increase in BaP per cell in the resistant population may be due to the inability of the cell to metabolize and get rid of the hydrocarbon, or may be due to a difference in volume of cells.

It is clear from these studies that the observed 10-fold reduction in enzyme inducibility (1) is not due to the inability of BaP to accumulate in NMuLi-BaP. In fact, the level of BaP in NMuLi-BaP is higher than for NMuLi. Accumulation of BaP by these cells is a passive process; the rate is constant, and probably limited by diffusion, while the saturation level probably depends on cell size rather than cell type. Furthermore, the rate of accumulation is not dependent on the availability of ATP (AGS, unpublished observations). This leads us to suggest that, at least in these populations, cells showing resistance to BaP do so generally as a result of alterations in structural or regulatory genes of enzymes involved in BaP metabolism (11), rather than changes in transport of the hydrocarbon.

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TABLE 1

Accumulation of ^{14}C-Benzo[a]pyrene* by NMuLi and NMuLi-BaP

Cell Line	K_1 moles BaP/cell/min	r_∞ moles BaP/cell
NMuLi	0.35×10^{-15}	1.70×10^{-15}
NMuLi-BaP	0.24×10^{-15}	3.87×10^{-15}

* ^{14}C-benzo[a]pyrene was at a concentration of 1 µg/ml.

LEGENDS TO CHARTS

Chart 1   Accumulation of ^{14}C-BaP by mouse liver epithelial cells. •,
          NMuLi; o, NMuLi-BaP. Cells were exposed to 1 μg/ml ^{14}C-BaP, after
          prelabeling with ^3H-thymidine. The ratio $^{14}C/^3H$ is a measure
          of the BaP level per cell. The extent of ^3H labeling of the
          two cell labeling of the two cell lines was similar but not identical.

Chart 2   Accumulation of ^{14}C-BaP by mouse liver epithelial cells, analyzed
          as pseudo-first order rate process. •, NMuLi; o, NMuLi-BaP. The
          lines drawn are linear regression calculated from the date points.

Chart 3   Histogram of cell number for varying DNA content for populations
          of mouse liver epithelial cells. The measurements were made
          by FMF, using mithramycin staining. Cell populations are NMuLi
          and NMuLi-BaP, exposed to 5 μg/ml BaP for 1 hour, and control
          (acetone).

Chart 4   Histogram of cell number for varying BaP fluorescence. Cells
          were NMuLi and NMuLi-BaP, exposed to 5 μg/ml BaP for 1 hour,
          after which fluorescence was measured by FMF. Control populations
          were exposed to acetone only: the fluorescence was measured
          at a gain of 100 in these cases. BaP fluorescence was measured
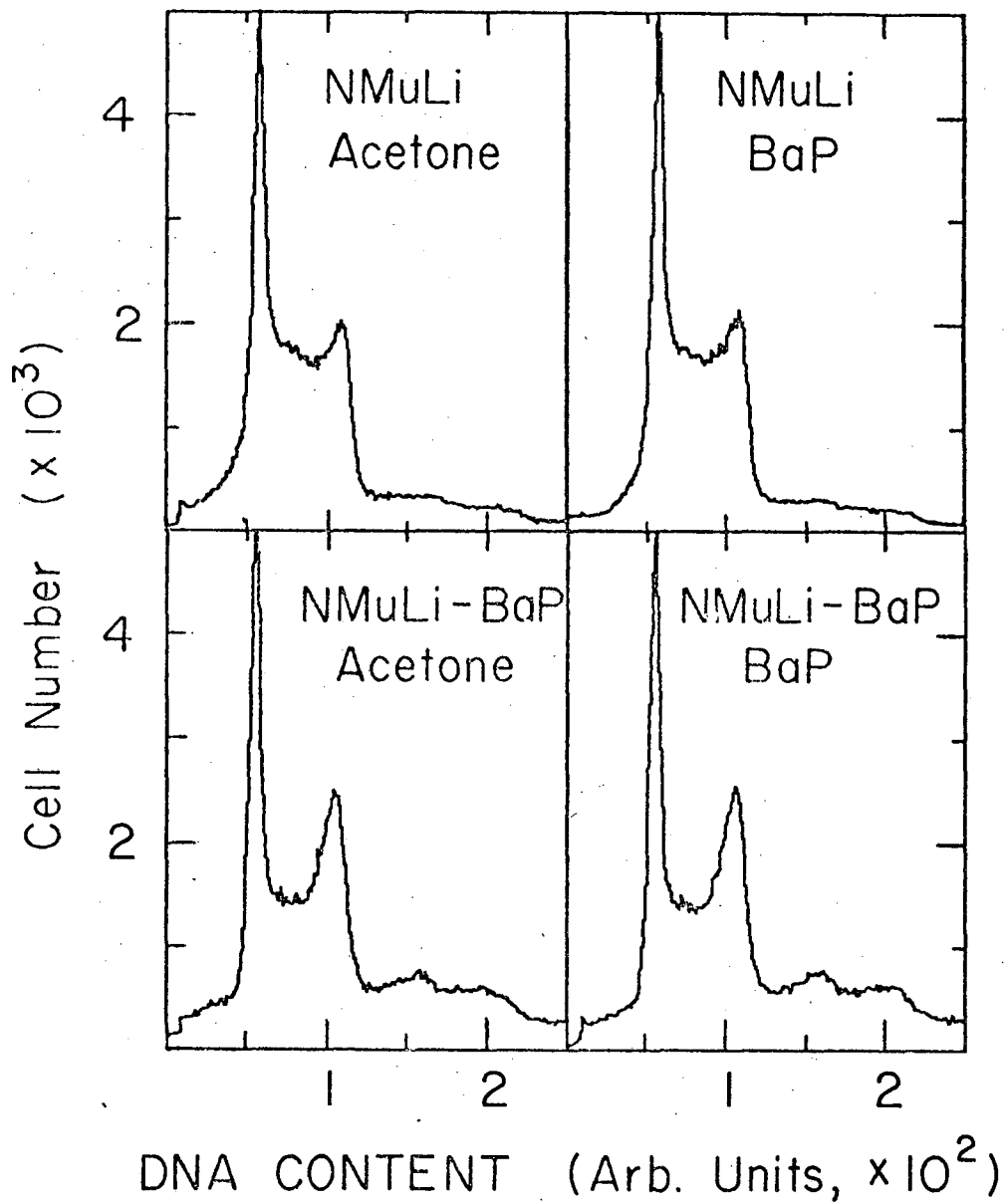   at a gain of 50.
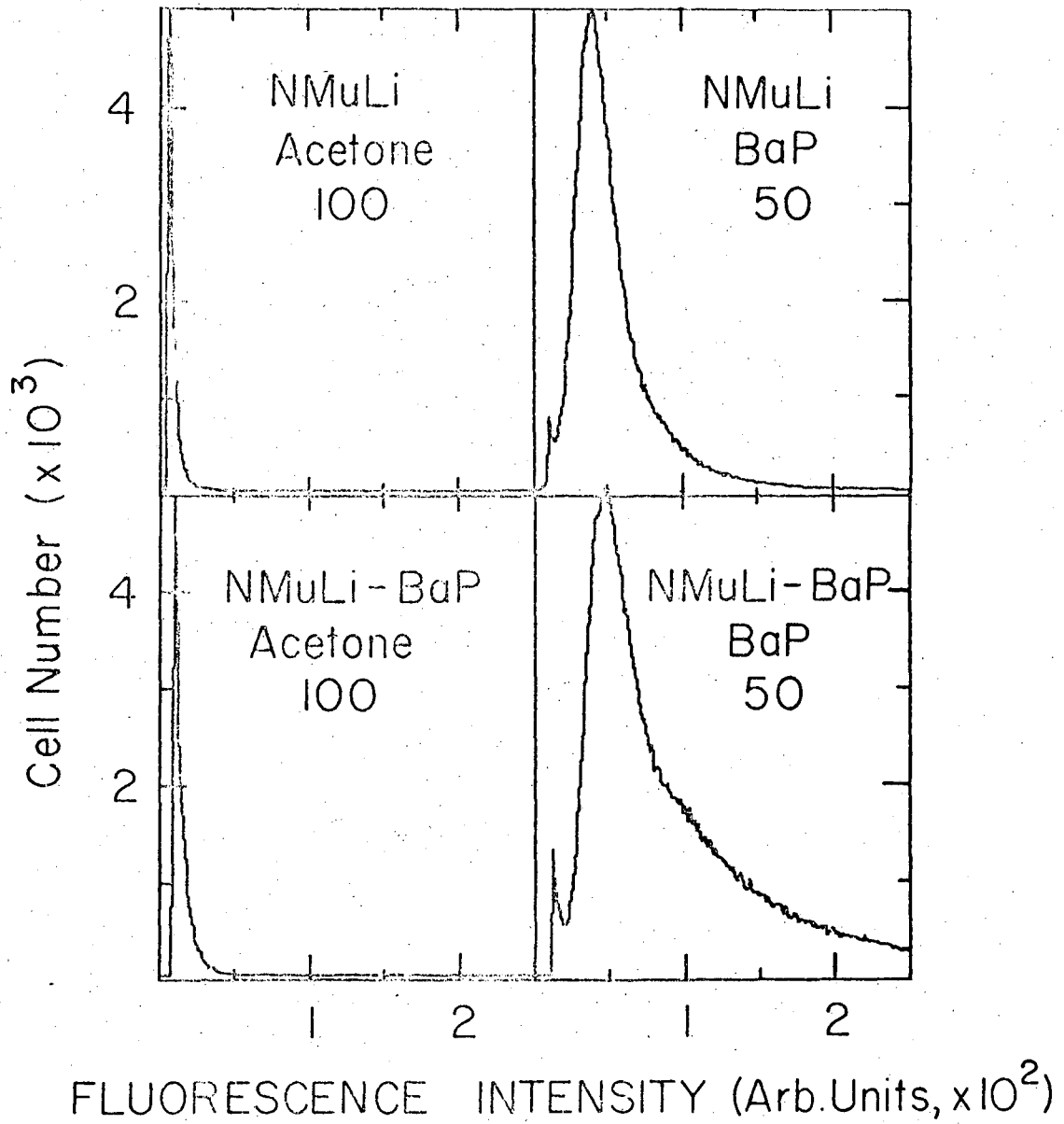
Fig. 1

XBL7510-8713

XBL754-5179

Fig. 2

Fig. 3

XBL 7611-9657

FLUORESCENCE   INTENSITY (Arb.Units, $\times 10^2$)

XBL 7611-9658

Fig. 4