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DISCLAIMER

CARCINOGEN MODULATION OF CELL CYCLE PARAMETERS OF MOUSE LIVER CELLS[1]

Running Title: Carcinogen Modulation of the Cell Cycle

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FOOTNOTES

1. Work carried out under the auspicies of the Division of Biomedical and Environmental Research of the U. S. Department of Energy.

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5. The abbreviations used in this manuscript are: BaP, benzo[a]pyrene; 7,8-diol, ±(trans)-7α, 8β-dihydroxy-7, 8-dihydro-BaP; diol-epoxide, ±7α, 8β-dihydroxy-9β, 10β-epoxy-7, 8, 9, 10-tetrahydro-BaP; tetrol, 7, 8, 9, 10-tetrahydroxy-7, 8, 9, 10-tetrahydro-BaP; AHM, aryl hydrocarbon monooxygenase; EDTA, ethylenediamine tetraacetic acid; TCA, trichloroacetic acid; DMSO, dimethyl sulfoxide; FCM, flow cytometry; 3-HO-BaP, 3-hydroxy-BaP; Tdr, thymidine.

6. Pearlman, A.L., Navsky, B.N., and Bartholomew, J.C., Extraction of Information from Single DNA Histograms, Manuscript in Preparation.

SUMMARY

We have used flow cytometry (FCM) to monitor the alterations in cell cycle distributions caused by chemical carcinogens. Two closely derived mouse liver cell strains growing in culture have been studied with regard to the effect of benzo[a]pyrene (BaP) and derivatives of BaP on DNA synthesis. The derivatives tested were ±(trans)-7α, 8β-dihydroxy-7, 8-dihydro-BaP (7,8-diol); ±7α, 8β-dihydroxy-9 , 10 -epoxy-7, 8, 9, 10-tetrahydro-BaP (diol-epoxide); and 7, 8, 9, 10-tetrahydroxy-7, 8, 9, 10-tetrahydro-BaP. One cell strain used in this study, NMuLi cl 7, is not highly inducible for the enzyme system (Aryl Hydrocarbon Monooxygenase, AHM) that converts the parent compound into the derivatives listed above. The second strain, NMuLi cl 8, is highly inducible for AHM. Correlated with the high level of metabolic activity is an increased sensitivity to the cytotoxicity of the parent compound. However, both strains were equally sensitive to the diol-epoxide. FCM analysis and measurements of ^{3}H-thymidine incorporation into DNA showed that the diol-epoxide increased the number of cells involved in DNA synthesis, but that the rate of DNA synthesis was greatly reduced. BaP and 7,8-diol had this same effect on NMuLi cl 8, but not on NMuLi cl 7. Kinetic modeling studies indicated that the cell cycle perturbations caused by diol-epoxide are consistent with the rate of traverse of S being slower than other phases of the cell cycle, and that as cells move through S their rate of DNA synthesis decreases.

INTRODUCTION

BaP[5] is a common environmental contaminant produced by the combustion, liquifaction, or gasification of fossil fuels (5,6,45). The carcinogenicity, mutagenicity, and cytotoxicity of this hydrocarbon to cells of mammalian origin has been extensively studies (1,11,15,23,26,28, 29,31,33). All of these biological effects have been shown to involve metabolism of BaP by the AHM of the cell (15,23). This enzyme system converts BaP into many products (8,40), but recent studies have implicated the conversion into the (-)-t-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene via the combined enzymatic action of AHM and epoxide hydrase followed by a second epoxidation in the 9,10-positions to give r-7, t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene (diol-epoxide I) as the important pathway in carcinogenesis and mutagenesis (4,9,25,38,42).

Alterations in cellular physiology caused by BaP and/or the above metabolites have been described in the literature. Much of this research has been concerned with the inhibition of DNA synthesis (22,37,43). This focus on DNA synthesis has resulted from the observation that many, if not all, chemical carcinogens bind to DNA and interfere with DNA replication leading to transformation, mutation, or cell death (19,24,32). The mechanism of DNA synthesis in eukaryotic cells is only beginning to be understood (17,41), and little information exists as to how chemical carcinogens interfere with this process. A precise understanding of how these compounds alter the biochemistry of DNA synthesis coupled with an understanding of the chemistry of carcinogen DNA interaction would aid greatly in our ability to predict the carcinogenicity of individual compounds. As a prelude to studying the biochemistry of carcinogen inhibition of DNA synthesis we studied the effect of BaP and some of its carcinogenic

derivatives on the kinetics of cell movement through the DNA synthetic period of the cell cycle.

MATERIALS AMD METHODS

Cells and Culture Techniques

The cells used in this study were derived from NMuLi (36) mouse liver epithelial cells by the cloning technique of Puck et al. (39). The sensitivity of these clones to BaP cytotoxicity was first demonstrated using a clonal assay and shown to correlate with the presence of a highly inducible AHM activity (27). All cells were cultured in plastic dishes (Falcon, Oxnard, Calif.) and incubated at 37°C in a 5% CO_2 incubator. The medium used to grow the cells was Eagle's minimal medium (12) (GIBCO Grand Island, N.Y.) containing 10% donor calf serum (Flow Laboratories, Rockville, Md) and 10 µg/ml insulin (Schwarz/Mann, Orangeburg, N.Y.). The cells were judged free of mycoplasma by incorporation of ^{3}H-Tdr (20.1 ci/mM; New England Nuclear, Boston, Mass.) into the nucleus of cells and not the cytoplasm (18). Stock cultures were maintained by subculturing the cells twice weekly at a cell density of 1×10^4 per cm^2. BaP and the derivatives were dissolved in DMSO (Matheson, Coleman, and Bell, Los Angeles, Calif.) immediately before addition to the cultures. The final DMSO concentration in all experiments was 0.1%. Cell counts were determined using a Coulter Counter, Model ZBI (Coulter Electronics, Inc., Hialeah, Florida).

^{3}H-Thymidine Incorporation

At each time point, cultures were pulsed with 10 µCi/ml ^{3}H-Tdr for 15 min. Autoradiography was carried out as previously described (2). For determination of ^{3}H-Tdr incorporated during the pulse, the cells on the dish were washed 2 times with Saline GM (1.5 mM Na_2HPO_4, 1.1 mM KH_2PO_4, 1.1 mM Glucose, 0.14 M NaCl, and 5 mM KCl at pH 7.5) and removed from the dish with DISPO (Saline GM containing 0.5 mM EDTA and 0.1 mg/ml

crystalline trypsin (GIBCO, Grand Island, N.Y.). The suspended cells were filtered onto Millipore HAWP filters with 0.45μ pore size (Millipore Filter Corp., Bedford, Mass.). The cells on the filter were washed 2 times with cold 5% TCA, 2 times with cold H_2O and 1 time with 90% ethanol. The filters were air-dried, placed in scintillation vials, and counted using a Permafluor/toluene cocktail (Packard Instrument Co., Inc., Downers Grove, Ill).

Flow Cytometry

FCM has been described in detail elsewhere (7,20,21) and in these experiments a fluorescent DNA probe was used to identify where individual cells were located in the cell cycle. FCM has many advantages over ^{3}H-Tdr incorporation for measuring cell cycle parameters (10,13,16), but most importantly for these studies is the fact that FCM measures total DNA content per cell and is therefore not affected by carcinogeninduced DNA repair.

Cells were stained with propidium iodide using the technique described by Crissman and Steinkamp (7). The DNA content of the stained cells was analyzed using a flow cytometer as described previously (2). Analysis of the resulting histograms was carried out using a program developed by Pearlman et al.[6]. In general, the program is based on the approach described by Fried et al. (13) and allows for interactive processing of data with a CDC6600 computer (Digital Equipment Corporation, Maynard, Mass.) after transforming the data to log space, allowing for variation in spacing between G_1 and G_2+M, and extraction of a representative G_1 spread function from samples having a low contribution due to cells in S. In test cases with or without carcinogens, this data analysis technique was within experimental error of standard autoradiography techniques for estimating the

proportion of a population in the S phase of the cell cycle. Kinetic modeling studies were carried out as described by Gray (16).

BaP Derivatives

The BaP derivatives used in these studies were synthesized as described by Meehan, et al. (30).

Aryl Hydrocarbon Monooxygenase Induction and Assay

AHM induction and assay was as described previously (26,27). The assay is essentially that of Nebert and Gelboin (34) as modified by Nebert and Gielen (35), and measures the production of derivatives of BaP with fluorescence properties equivalent to the 3-HO-BaP (a gift of Dr. H. V. Gelboin).

RESULTS

Growth Studies

The two clones used for these studies were isolated from a parent culture of NMuLi cells which had previously been shown to have an inducible AHM activity and be highly sensitive to the cytotoxic action of BaP (7). Neither clone has been exposed to BaP during isolation; however, NMuLi cl 7 cells in comparison to NMuLi cl 8 cells had considerably lower levels of basal and induced AHM activity (Table 1). The relative sensitivity of the two clones to growth inhibition by BaP and BaP derivatives reflected this difference in AHM activity. The LD_{37} values listed in Table 2 indicate that clone 8 was equally sensitive to BaP and 7,8-diol toxicity, while diol-epoxide was approximately 10 times more cytotoxic than BaP to this clone. Clone 7 was as sensitive as clone 8 to diol-epoxide toxicity and was 2.5 times less sensitive to BaP toxicity. The 7,8-diol did not reduce clone 7 growth below 37% of the control, and the tetrol was not toxic to either clone. DMSO, the solvent in which the compounds were added, was only slightly toxic at the 0.1% level used in these experiments.

The growth curves of cells in medium containing approximately 8 μM of the derivatives were determined as shown in Chart 1. This concentration of each derivative was chosen because it gave the maximum cell cycle effect in the studies reported below. The growth parameters of cells from these two clones in the absence of test compounds differed slightly as shown in Chart 1 and Table 3. The primary difference was that clone 8 had a reduced G_1 residence time relative to clone 7, and an increased saturation density. Growth of NMuLi cl 7 and cl 8 was completely arrested by the diol-epoxide. The other compounds did not affect significantly the doubling time of clone 7 cells, however, they did have an effect on the saturation density of this clone. The tetrol increased the saturation density relative to the control by 127%. BaP and 7,8-diol decreased the saturation density to 31% and 36% of control respectively. The growth of cells with an inducible AHM was not affected by this concentration of tetrol, but BaP and the 7,8-diol both greatly increased the doubling time of clone 8 cells and reduced the saturation density.

Petrurbation of Actively Growing Cells

Perturbations in the cell cycle distributions caused by a compound were studied by FCM analysis of the DNA content per cell and ^{3}H-Tdr incorporation into the DNA during 15 min pulses to logarithmically growing cultures. Chart 2 shows that the amount of DNA synthesized as indicated by ^{3}H-Tdr incorporation decreased to 40% of control 2 hr after addition of diol-epoxide to the culture medium of NMuLi cl 8 cells; however, by 8 hr the amount of DNA synthesis during the pulse returned to nearly control levels. FCM analysis of the diol-epoxide treated cultures indicated that as the ^{3}H-Tdr incorporation returned back to near control levels a wave of cells was moving out of G_1 into S. After 8 hr, although FCM indicated that the

the number of cells in S in the diolepoxide treated cultures was still high relative to the control, the amount of DNA synthesis measured by ^{3}H-Tdr incorporation in the cultures dropped considerably, suggesting that as the wave of cells from G_1 moved through S, the rate of DNA synthesis decreased. Autoradiography of ^{3}H-Tdr treated cultures (data not presented) confirmed that beginning at 4 hr after the diol-epoxide exposure a greater proportion of the exposed population was making DNA than the controls. At no time during the experiment, however, did the grain counts in the labeled cells exceed that of the 2 hr sample indicating that those cells that were in G_1 during the initial exposure and then moved into S did not synthesize DNA any faster than cells that were in S at the time of the exposure to diol-epoxide.

The alterations in the cell cycle distributions for all cell cycle phases are shown in Chart 3. Diol-epoxide caused an increase in the proportion of the cells in S both in clones 7 and 8 cultures beginning at about 4 hr after adding the compounds. NMuLi cl 8 cells accumulated in S faster than clone 7 cells presumably due to the shorter residence time in G_1 (Table 3). BaP caused similar alterations in cell cycle distributions in NMuLi cl 8 cultures, but the increase in fraction of the population in S began between 6 and 8 hr after BaP addition instead of at 4 hr as for diol-epoxide. This time lag corresponds to the time necessary to induce AHM in these cells (3). BaP had essentially no effect on the cell cycle distribution of clone 7 cells. 7,8-Diol had similar effects on both cell types as BaP both in the extent of perturbation and the time of appearance of cells in S. The tetrol did not alter the cell cycle distribution of either clone.

Chart 4 presents the DNA histograms from clone 8 cells prepared for analysis daily - beginning 24 hr after treatment with BaP, diol-epoxide,

or DMSO (solvent controls). In the controls, the cell cycle distributions progressed toward a higher proportion of the population in G_1 as the cultures approached saturation density. In these cells that have an inducible AHM, BaP [as well as 7,8-diol (data not presented)] prevented this accumulation of cells in G_1. Diol-epoxide also prevented this G_1 accumulation and maintained even more cells in S and G_2+M than BaP. ^{3}H-Tdr incorporation (15 min pulse) on days 3 or 4 of the experiment followed by autoradiography of the cells indicated that even though cells were in S as indicated by FCM analysis and by counting the number of cells incorporating ^{3}H-Tdr in the BaP and diol-epoxide treated cultures, their DNA synthesis rate during the pulse determined by counting grains per labeled nucleus was less than 1% controls. These observations suggest that after the initial wave of cells move from G_1 through S and into G_2+M in the diolepoxide treated cultures, movement around the cycle becomes extremely slow or arrested. The effects are not just on a single cell cycle phase since at no time after exposure do all the cells accumulate in one particular cell cycle phase.

Cell Cycle Perturbations of Serum Stimulated Cells

The alterations in cell cycle distribution caused by diol-epoxide and diol-epoxide generating compounds described in Chart 3 could be due to a carcinogen-induced stimulation of cells from G_1, an inhibition of DNA synthesis, or some complex combination of these two phenomena. To obtain information on these alternate descriptions of carcinogen effects on the cell cycle, NMuLi cl 7 and cl 8 cells were stimulated from saturation density cultures with fresh medium and serum in the presence or absence of BBaP. NMuLi cl 7 and 8 cells become distributed in G_1 upon reaching saturation densities in culture. As seen in Chart 5, cells from both clones can be stimulated by subculturing to move from G_1 into S and G_2+M. The period between

subculturing and the appearance of cells in S is composed of time necessary to recover from being at saturation density as well as the normal G_1 period (44,46). Clone 7 cells were approximately 2 hr slower than clone 8 cells in moving into and through S, again reflecting the 2 hr difference in the G_1 period of these clones. BaP added to these stimulated cells did not affect the time it takes cells from either clone to begin appearing in S. However, NMuLi cl 8 cells, but not clone 7 cells traversed S slower in the presence of BaP than in its absence. Also, with clone 8, the number of cells moving through S at any particular analysis time in the presence of BaP is greater than in the controls. This increase number of cells in S was not due to BaP increasing the number of cells stimulated by serum since in an experiment where colchicine (0.2 µg/ml) was added to trap in mitosis cells stimulated by serum, the proportion of the population building up behing the colchicine block was not affected by BaP. This experiment suggests that BaP does not alter the ability of serum to promote the G_0 to G_1 transition.

Kinetic cell cycle modeling (16) of the NMuLi data presented in Chart 5 revealed that the apparent increase in cells in S can be explained by a greater effect of the carcinogen on S relative to G_1 (Table 4). Also, these modeling studies indicated that the dispersion of the cell cycle times is not affected by BaP, but that the rate of DNA synthesis slows down as the cells exposed to BaP move through S. The maximum rate of DNA synthesis in the cultures treated with BaP occurred when the serum stimulated cells had traversed 0.2 of the S period. The culture without BaP had a maximum DNA synthetic rate as the stimulated population was at 0.5 S.

DISCUSSION

These experiments demonstrated that BaP or the 7,8-diol when they could not be metabolized to the diol-epoxide had very little effect on cell cycle progression. When these compounds could be metabolized, they gave a cell cycle response identical to the diol-epoxide. At the concentrations tested they primarily slowed the progress of cells through S. They had no effect on the G_0 to G_1 transition probability (46), nor on the lag time from serum stimulation to the appearance of cells in S. All of these processes, however, are inhibited at concentrations above 40 μM (data not presented). The wave of cells moving through S beginning 4 hr after treatment of a randomly growing population, and the increased proportion of the population in S at individual analysis times after serum stimulation of BaP (or diol-epoxide) treated cultures were probably both due to the effect of diol-epoxide on slowing progress through S. Kinetic modeling studies of logarithmically growing populations indicates that when progress through a compartment in the cell cycle is inhibited and the dispersion in the transit times is not changed, the proportion of the population in the inhibited cell cycle phase will increase (16). Modeling of the population distribution as a function of time after serum stimulation in the presence of BaP, as well as the results from the incorporation of ^{3}H-Tdr in diol-epoxide treated logarithmic cells indicated that the rate of DNA synthesis was continually decreasing as cells progressed through S.

The events described here take place in the first generation after adding the toxic compounds. Under the conditions of these experiments there does not appear to be a second generation. The wave of cells passing through S does not return and the population dies with cells distributed in all phases of the cell cycle.

The simplest interpretation of the relationship between the cell cycle perturbations reported here and the initiation of carcinogenesis is that some cells survive the toxic effects of these chemicals with alterations in their genome that reflect changes in growth control. This interpretation suggests that all chemical carcinogens would inhibit DNA synthesis, and that inhibition of DNA synthesis could be used as an assay for chemical carcinogens (37). The results of this study point to the importance of looking at the detailed effect of test compounds on the population dynamics rather than by measuring DNA synthesis at some arbitrary point after application of a compound.

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TABLE 1. Induction of Aryl Hydrocarbon Monooxygenase in NMuLi cl 7 and cl 8

clone	pmoles 3-HO-BaP/min/mg protein uninduced	induced
7	0.08	0.65
8	0.40	14.80

All AHM measurements were made on 5 x 10^6 cells grown in roller flasks. Induction was with 40 μM BaP for 12 hr on day two post seeding.

TABLE 2. Toxicity of BaP and Some Derivatives to NMuLi Clones

Compounds	LD_{37} (μM) cl 7	cl 8
BaP	3.33	1.32
7,8-Diol	-	1.86
Diol-Epoxide	0.02	0.02
Tetrol	-	-

Cells were seeded in culture dishes as described in MATERIALS AND METHODS at a density of 6.25×10^3 per cm^2. After 24 hr, different concentrations of the compounds to be tested were added from a DMSO solution. The final DMSO concentration in all plates was 0.1%. After 3 days incubation, the number of cells was determined for each concentration of compound and compared with DMSO control. The LD_{37} was taken as that concentration that reduced the cell number to 37% of the control. (-) indicates that the cell number did not drop below 37% of controls at any concentration tested up to approximately 80 uM.

TABLE 3. Growth Parameters of NMuLi cl 7 and cl 8

clone	doubling time (hr)*	saturating density ($cells/cm^2$)	length of cell cycle phase (hr)** G_1	S	G_2+M
NMuLi cl 7	16.50 ± 0.21	1.1×10^4	6.20	7.79	2.51
NMuLi cl 8	14.98 ± 0.52	2.4×10^4	3.90	7.63	3.45

*The doubling times were computed from the cell counts using a least squares program. The values reported had correlation coefficients greater than 0.995.

**The length of the cell cycle phases was calculated from the FCM DNA histograms and the doubling times (2).

TABEL 4. Kinetic Modeling of BaP Perturbations in Cell Cycle Parameters of NMuLi cl 8 cells

	Minus BaP	Plus BaP
G_1 (hr)	3.1	4.4
S (hr)	6.3	18.8
G_2+M (hr)	2.8	6.0
Dispersion in Cycle Times (CV)	0.35	0.35
DNA Synthetic Rate Max.	0.50	0.20

CHART LEGENDS

Chart 1. Growth of NMuLi cl 7 and 8 in the Presence of BaP Derivatives.
The compounds were added to the culture medium from a solution in DMSO (1 mg/ml) 24 hr after seeding the cells. The final concentrations were: BaP (8.0 μM); 7,8-diol (7.0 μM); diol-epoxide (6.7 μM); and tetrol (6.4 μM).

Chart 2. Alterations in DNA Synthesis in Logarithmically Growing Cultures of NMuLi cl 8 Caused by Diol-Epoxide.
Each measurement was made as described in the text. The diol-epoxide (6.7 μM) was added at 0 time and the proportion of the population in S determined by FCM or the amount of DNA synthesis measured in a 15 min pulse of ^{3}H-thymidine was compared to controls.

Chart 3. Perturbations in the Distribution of Cells in the Cell Cycle Phases Caused by BaP or Diol-Epoxide.
The BaP (8.0 μM) or diol-epoxide (6.7 μM) was added to the culture medium of logarithmically growing cells at 0 time. The DNA histograms were obtained and the data were reduced as described in the text.

Chart 4. Long Term Effects of BaP and Diol-Epoxide on the Cell Cycle Distribution of NMuLi cl 8 cells.
The compounds were added as described in the legend to Chart 1, and cells were prepared for DNA analysis each day thereafter.

Chart 5. BaP Effects on the Movement of Serum Stimulated Cells Through the Cell Cycle.

Cells were grown to saturation density in medium containing 10% serum. At saturation density the cells were distributed with DNA contents representing G_1 (71.3%), S(11.9%), and G_2+M(16.8%). The cells were stimulated to move out of G_1 into the rest of the cell cycle by replating them at 1/4 the saturation density in the presence of 20% serum. BaP was added as described in the legend to Chart 1.

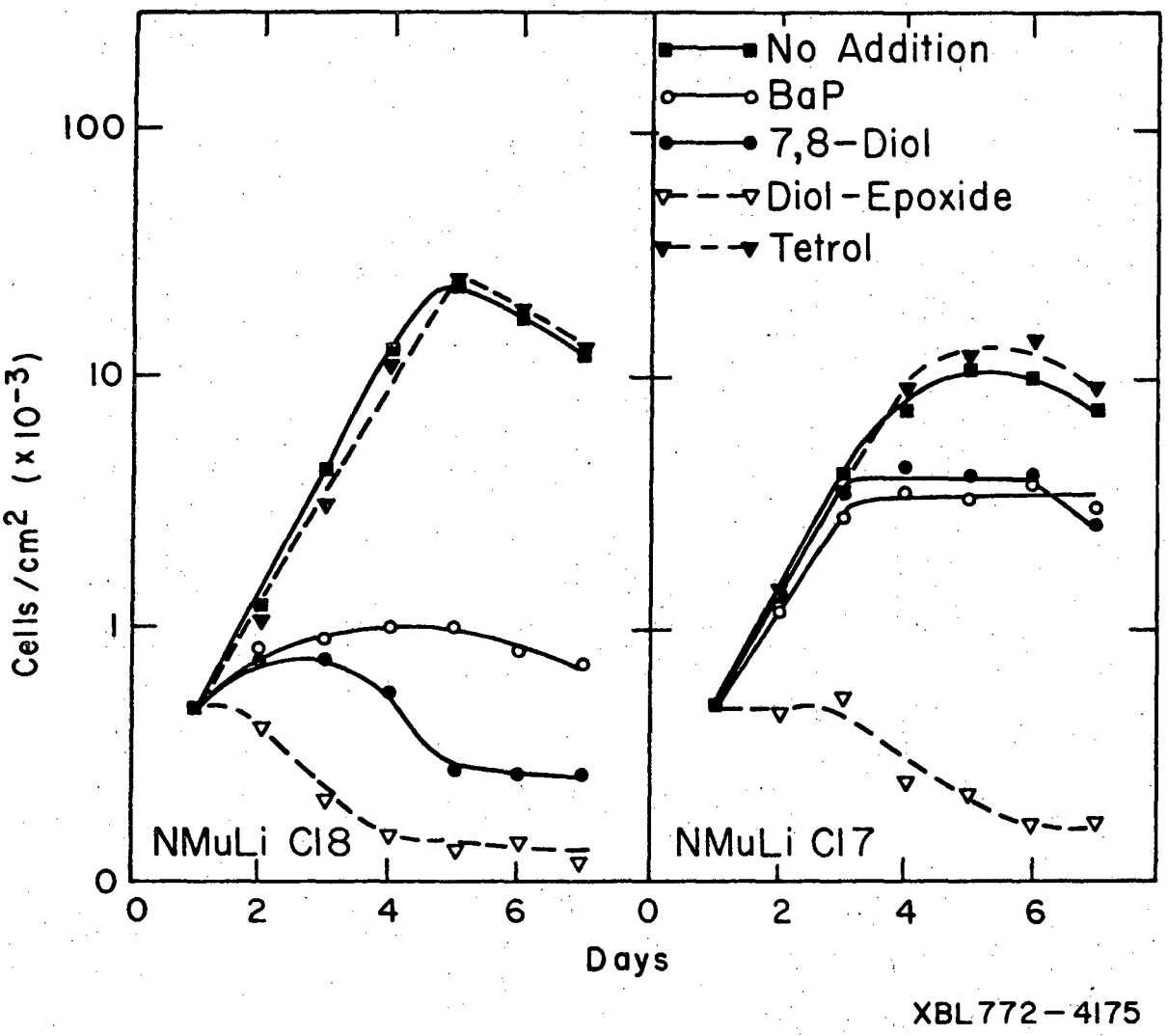

Chart 1

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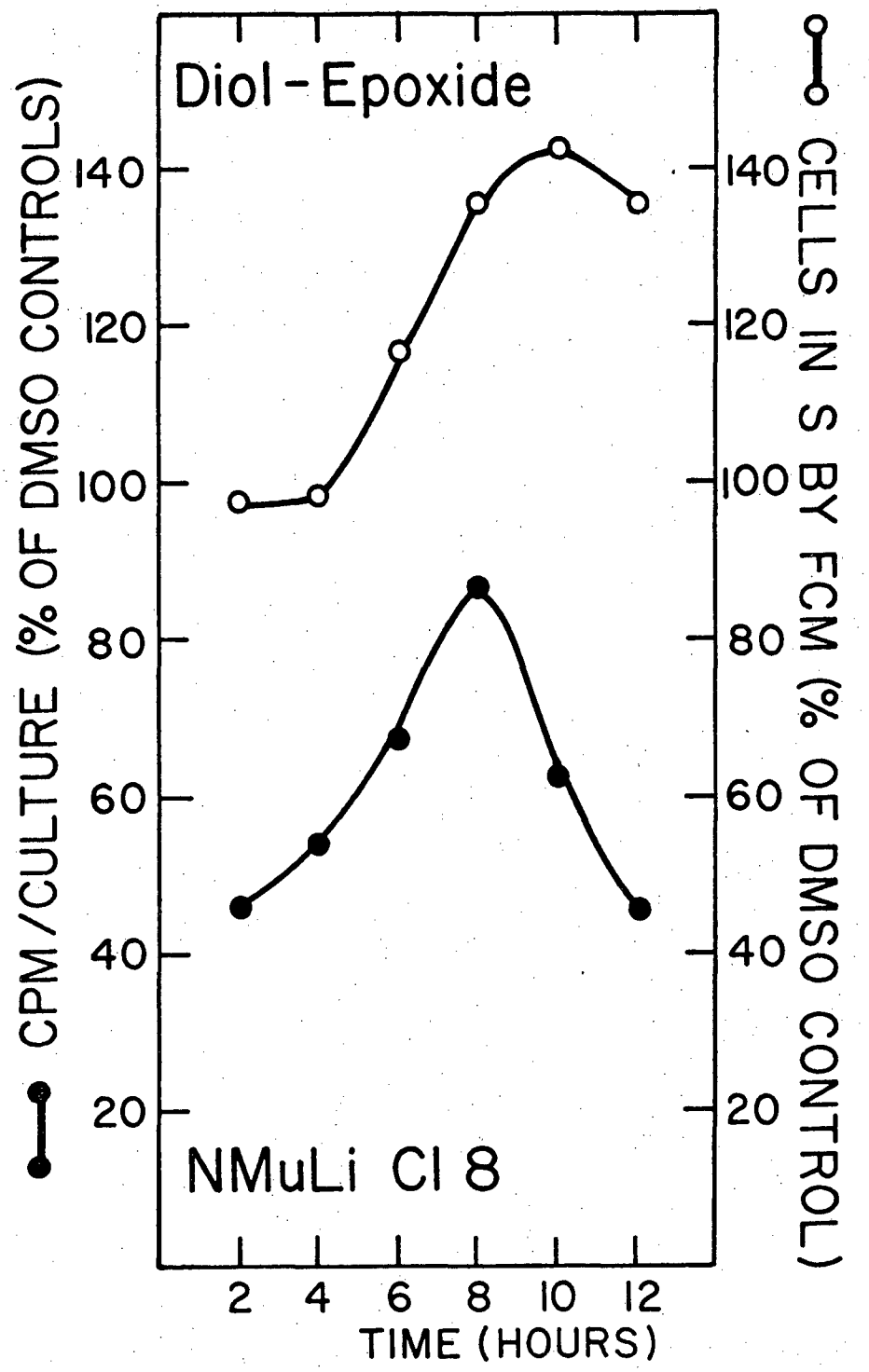

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Chart 2

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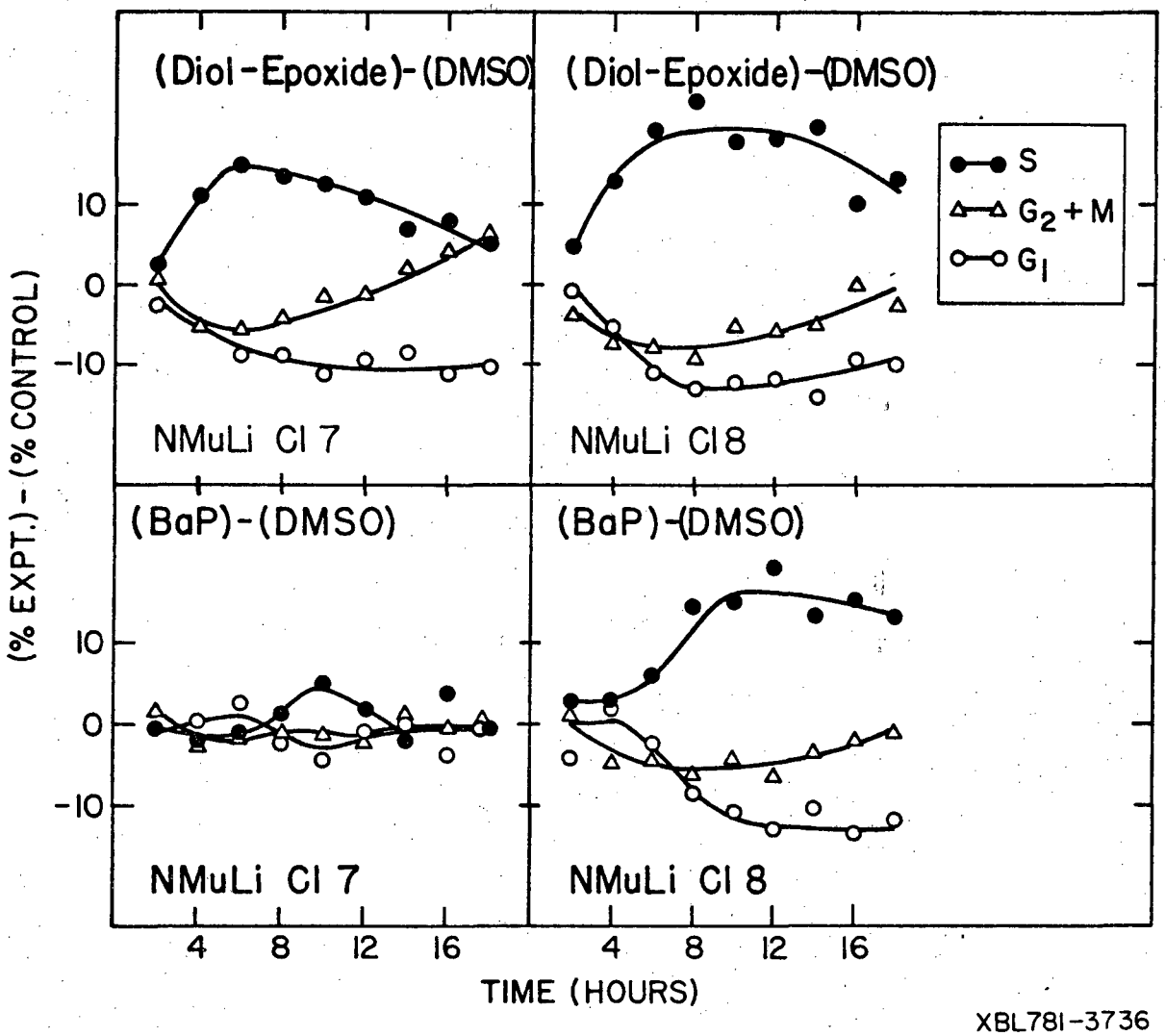

Chart 3

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NMuLi C18

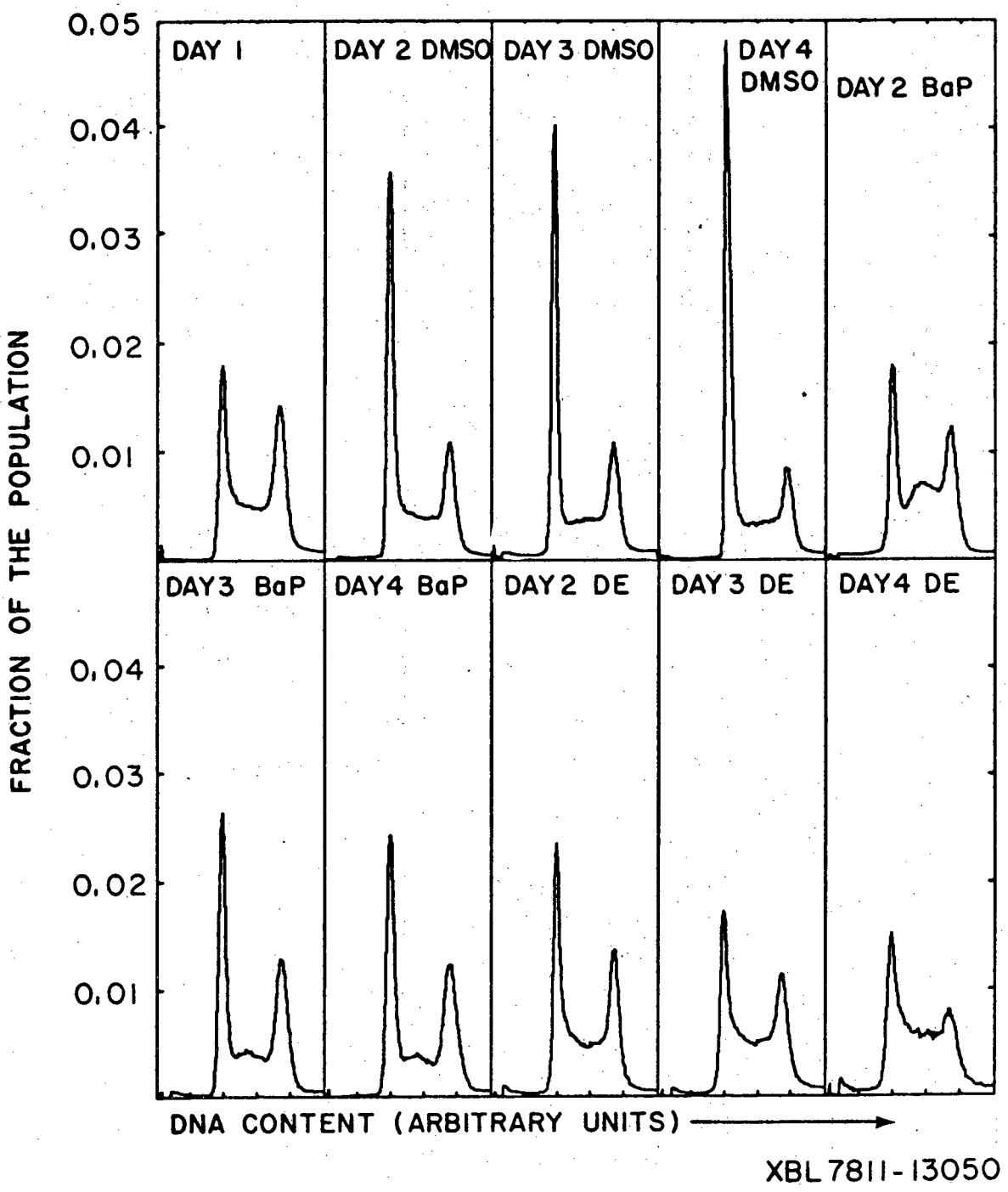

Chart 4

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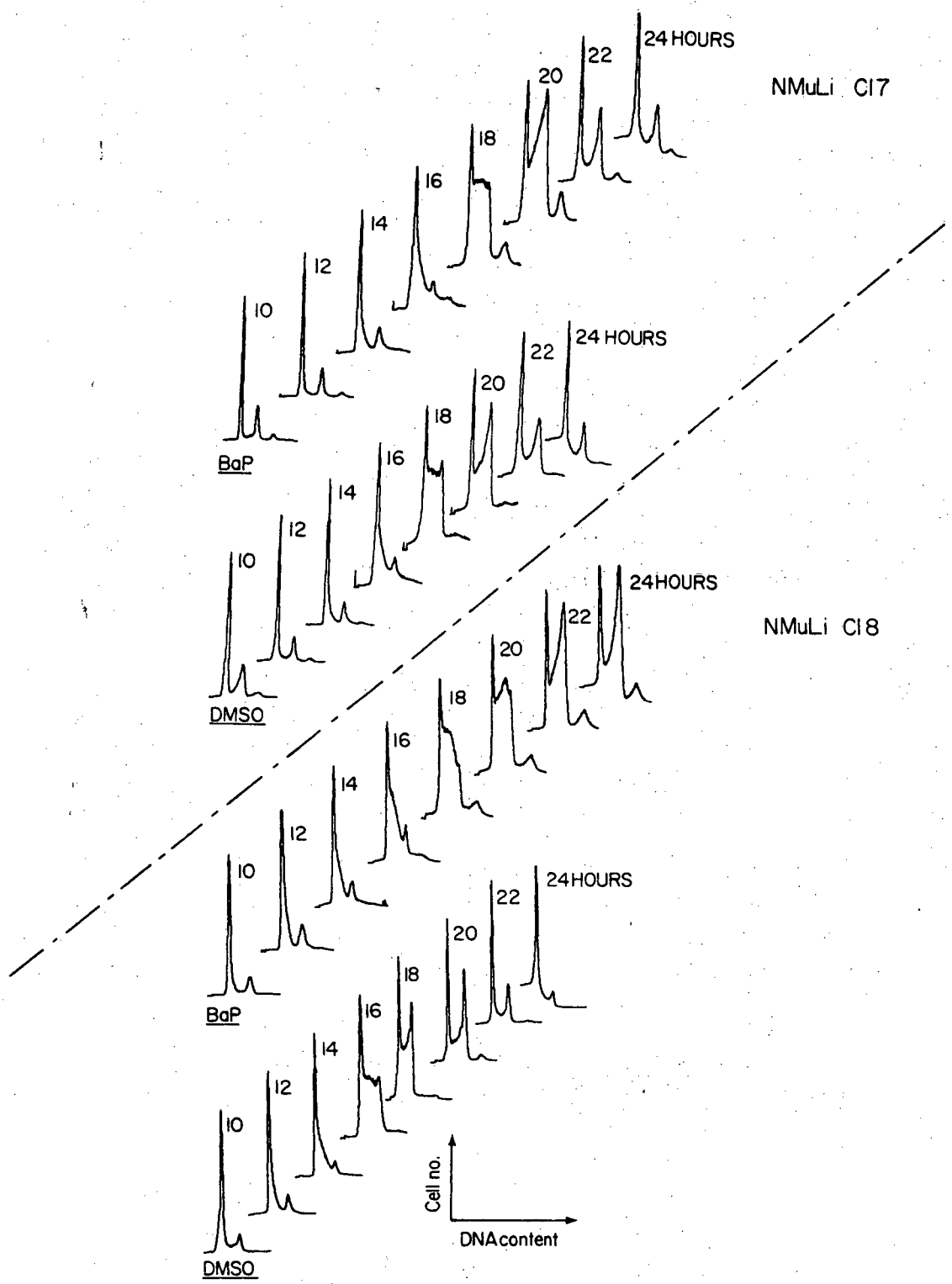

Chart 5

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