Title

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Publication Date

1975-09-01

Submitted to Cancer Research

LBL-4262
Preprint c.1

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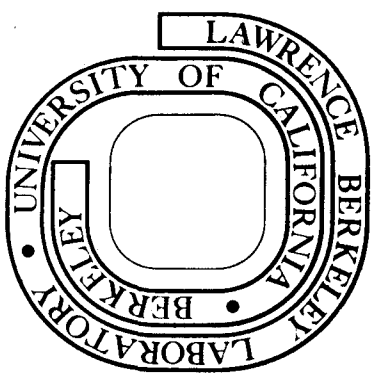
September 1975

Prepared for the U. S. Energy Research and Development Administration under Contract W-7405-ENG-48



DISCLAIMER

KINETICS OF THE TOXICITY OF BENZO(a)PYRENE TO A LIVER EPITHELIAL CELL LINE DERIVED FROM NAMRU MICE AND THE PRODUCTION OF RESISTANT VARIANTS[1]

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Running Title: Toxicity of Benzo[a]pyrene to a Liver Epithelial Cell Line

3 Tables

7 Charts

[1]This study was supported by NCI Contract FS-(71)-58, NCI Grant Number CA14828-02, and the U. S. Energy Research and Development Administration.

SUMMARY

An investigation was undertaken on the toxic effects of the carcinogen benzo(a)pyrene (BaP) to an epithelial cell strain derived from the livers of Namru mice (NMuLi). Growth curves of NMuLi in BaP showed that the carcinogen markedly depressed the saturation density and growth rate of the cells. These effects were dose-dependent. The toxicity of BaP to NMuLi was much greater (27 x) on logarithmically growing cells than on confluent cells. There was also a slight lag (1/2 day) before the toxic effects of BaP became apparent. These results indicated a necessity for cell division in the expression of the toxicity.

When cells surviving the initial BaP treatment (BaP-NMuLi) were again cycled in BaP, it was found that the population was enriched 8-fold in cells resistant bo BaP relative to the parent, untreated population (NMuLi). The effects of BaP in depressing the growth rate and saturation density of BaP-NMuLi were less than its effects on NMuLi.

Survival curves for clones of NMuLi and BaP-NMuLi treated with BaP possessed slight shoulders, indicating a multi-hit process for cell death. The D_0 for BaP-NMuLi was greater than that for NMuLi.

A clonal analysis of NMuLi for resistance to the cytotoxicity of BaP showed heterogeneity in the levels of resistance among the clones. The same analysis of BaP-NMuLi showed that clones derived from it appeared enhanced in their resistance relative to those from NMuLi.

INTRODUCTION

The action of certain chemicals on the differentiated epithelial cells of various organs to produce carcinomas is presumed responsible for the

occurrence of many tumors in man (3). Statistics from a study carried out in Denmark show that 92% of human cancer incidence is a result of carcinomas (epithelial origin) while the remaining 8% is due to leukemias and sarcomas (connective tissue tumors) (4).

It is difficult to culture epithelial cells free of fibroblasts, and consequently only a small amount of work has been conducted on them (22,23,24). Recently, Owens et al. have isolated a pure strain of epithelial cells from the livers of Namru mice and characterized these cells as to epithelial morphology and ultrastructural characteristics (20).

While the cytotoxic effects of carcinogens have been studied in fibroblastic and mixed fibroblastic-epithelial systems (7,8,11,12,21), these effects have not been well characterized in pure epithelial systems. Such epithelial tissues as skin and lung, which initially come into contact with carcinogenic agents, and liver, and which possesses a wide variety of enzyme systems which metabolize these compounds, would be of particular relevance in toxicity studies. Investigations on the kinetics of toxicity in these systems should give information on the mechanism of the toxicity.

Of equal importance is the mechanism by which variants resistant to the cytotoxic action of these chemicals arise and the biological consequences of production of these variants. Some organ systems susceptible to the cytotoxicity of carcinogens, such as liver, possess a regenerative capacity. It is conceivable that a high rate of regenerative cell division coupled with spontaneous mutation frequencies of the order of 10^{-6} per cell (5,12) could result in the production of new clones of cells lacking those properties necessary to the integrity of the organ. This effect would be in addition to the direct malignant transformation of cells in culture by chemical carcinogens (2,9,16,17,19,23).

Parallel cases regarding the induction of variants resistant to the toxicity of certain chemicals have been studied in a number of systems. In the

investigations of bromodeoxyuridine resistance in frog cells (18) and resistance to 8-azaguanine in Chinese hamster cells (13,25), the lack of a ploidy effect on 'mutation' rates had led to doubts as to whether the variants arise by a simple nuclear genetic mutation. Explanations such as epigenetic processes or extranuclear mutations have been suggested as possibilities to explain the effects (13). Investigation of another case may serve to generalize these results.

On a more general level, studies of the production of variants resistant to BaP should lead to a better understanding of the process of de novo variation of cells in culture. Such an understanding is vital if cell culture results are to be extrapolated with any confidence to whole animals.

Consequently, it was decided to investigate the cytotoxic effects of BaP, a common environmental carcinogen, on Owen's strain of epithelial cells. Bartholomew et al. have found these cells to be extremely sensitive to the cytotoxic effects of BaP even after many passages in culture (1). They also found that the production of a resistant population correlates with a loss in inducibility of the enzyme system which metabolizes BaP to cytotoxic derivatives.

We now extend these results by defining the time dependence of expression of the toxicity and its kinetics, the effects of BaP on the saturation density and population doubling time of the cells, and the degree of resistance induced in a population of cells that has been exposed to BaP.

MATERIALS AND METHODS

Cells. The NMuLi cell strain is an epithelioid derivative obtained from the livers of Namru mice by Owens (20). BaP-NMuLi is a derivative of NMuLi obtained by exposing NMuLi to 5 ug/ml of BaP, as described in a previous report (1). Both cell strains were cultured in minimal Eagle's

Medium (10) fortified with 10% fetal calf serum (GIBCO, Grand Island, N.Y.), 250 units/ml of penicillin G (Calbiochem., San Diego, Calif.), and 50 ug/ml of Streptomycin sulfate (Mann Research Labs., New York, N.Y.).

Cells were routinely verified to be Mycoplasma-free by autoradiography using a modification of the method of Culp and Black (6). Cells were seeded at 1 x 10^5 per 100 mm dish, allowed to recover for one day, and then treated for 24 hr with 5.0 or 0.1 uCi/ml of ^3H thymidine (20.1 Ci/nmole; New England Nuclear, Boston, Mass.). The medium was then aspirated off, the cells were rinsed for 6 minutes with hypotonic KCl (0.075 M), and fixed two times for fifteen minutes each with acetic acid-methanol 3/1 (v/v). The plates were allowed to dry overnight, and then Kodak Nuclear Emulsion (Eastman Kodak, Rochester, N.Y.) was applied for 3 days at room temperature.

Toxicity Studies. For all cell counting toxicity studies, cells were seeded at 3 x 10^4 per 35 mm dish (Falcon, Oxnard, Calif.) in 1.5 ml of medium. Twenty-four hours post-plating, cells were treated with freshly prepared solutions of BaP in acetone (10 ul, 0.66% in the medium). After BaP treatment, the medium was aspirated off and the cells were washed with 0.25 mM Tris buffer, pH 7.4, containing 140 mM NaCl, 5 mM KCl, and 0.7 mM Na_2HPO_4 (isotonic Tris buffer). The cells were then removed from the plates by treatment with 0.01% trypsin (Difco, 1:250, Detroit, Mich.) in isotonic Tris buffer for 20 minutes at 37°C. An aliquot of the trypsin solution was counted in a model Fn Coulter counter (Coulter Electronics, Hialeah, Fla.).

For growth curve determinations, cells were plated and treated with BaP as above. They were maintained in BaP (no fluid changes) and counted each day as necessary. For both growth curve and toxicity studies, four dishes were averaged per experimental point.

In clonal survival determinations, cells were plated at 2 x 10^2 per

60 mm dish in 5 ml of medium. One day post-plating, cells were treated with 10 ul of BaP (final concentration 5 ug/ml) in acetone. BaP treatment lasted for 3 days, at which time the medium was removed, the cells were washed with isotonic Tris buffer, and fresh medium was added. On day 9-13 post-plating, the medium was removed, the clones were stained and fixed for 5 minutes with 1% crystal violet in 25% ethanol, and the dishes were rinsed gently 3 times with tap water.

Cloning. Cloning experiments were performed by the glass cylinder isolation procedure (15).

Chemicals. Acetone was distilled. Benzo(a)pyrene (Aldrich, San Leandro, Calif.) was purified by column chromatography on neutral alumina (Woelm, Eschewege, Germany) with benzene as eluant. It was then recrystallized from benzene-isopropanol, and its purity was verified by thin-layer chromatography on 6060 silica gel plates (Eastman Kodak, Rochester, N.Y.) with 19/1 (v/v) benzene-ethanol as developing solvent.

RESULTS

Description of the Toxic Effects. Growth curves of NMuLi and BaP-NMuLi in benzo(a)pyrene show that the toxic effects of the carcinogen appear to be amplified by cell growth (Charts 1 and 2). The saturation densities and growth rates of both cell strains are lowered as the concentration of BaP is raised, those of NMuLi to a much greater extent than those of BaP-NMuLi.

This data can be replotted to show the effect of time on the expression of toxicity more clearly. For cells in logarithmic growth phase, $N = N_0 e^{at}$. For cells growing in BaP in log phase, a death term due to the effects of the carcinogen must be added: $N_B = N_0 e^{a't} e^{-bt}$, where b is a function of BaP concentration. Taking the ratio of a BaP-treated culture to a mock-treated culture, $\log(N_B/N) = (a' - a - b)t$, and a plot of $\log(N_B/N)$ versus t

should be linear as long as the cells are growing logarithmically.

If the growth rate a is the same for cells in BaP and in the control medium, then the plot will show the effect of carcinogen-induced cell death directly. However, if $a \neq a'$, then the plot will still be linear, but will not distinguish between true cell death and a decrease in the growth rate of the BaP-treated cells. More sophisticated cell cycle analyses are needed to differentiate between these two cases.

The replot of the data in Chart 3 shows that there is a slight delay in the expression of toxic and/or cell-cycle lengthening effects of BaP, which are then exponential until the cells reach confluence.

Kinetics of Toxicity. An investigation of the kinetics (BaP dependence) of the toxicity is shown in Chart 4. At high concentrations of BaP, the curves level off, suggesting that a sub-population of cells resistant to the toxicity of the carcinogen exists in both cell strains.

Upon subtraction of the resistant fraction of cells from the curves, normalization of the controls to 1.0, and replotting, exponential curves with a shoulder are obtained (Chart 5), indicating a multi-hit mechanism for the toxicity process.

The toxicity of BaP to confluent populations of NMuLi was also investigated, and the slope of the confluent killing curve is 27 times less than that of the log phase killing curves (Chart 6 versus Chart 5). Since few cells are dividing at confluence, the results are consistent with a requirement for cell division in the expression of the toxicity.

Survival Curves in Clonal Analyses. The death of clones of NMuLi and BaP-NMuLi was also investigated to confirm the cell counting survival plots. In two experiments, the clonal survival curves paralleled those of the cell counting assays in qualitative shape. Subtraction of the fraction of resistant clones, renormalization, and replotting also yielded an exponential curve for NMuLi with a small shoulder (unpublished data).

Biological Significance of Resistance to the Cytotoxicity of BaP

Levels of Resistant Cells in NMuLi and BaP-NMuLi. Two interesting conclusions arise from the three-day kinetics curves for NMuLi and BaP-NMuLi (Chart 4). First, cycling the NMuLi population in 5 ug/ml of BaP has enriched it 8-fold for cells resistant to the cytotoxic effects of BaP. Both cell and clonal survival assays lead to the same values for the fractions of the populations (Table I). This property is stable, since both NMuLi and BaP-NMuLi maintain their percentages of resistant cells as a function of passage (Table II). Secondly, the skew of resistance appears wider in BaP-NMuLi relative to NMuLi, suggesting that there is a heterogeneity of cell types sensitive to BaP cytotoxicity in the BaP-NMuLi population. This point will be treated later.

The BaP-NMuLi population has the same doubling time and saturation density as the NMuLi population (Table III) and hence it should possess no selective advantage over NMuLi. When 4×10^6 cells of either cell strain were injected sub-cutaneously into the isogeneic host, no malignant tumors were produced (unpublished data).

Degrees of Resistance and Population Heterogeneity - Clonal Analysis. The death of clones and cells sensitive to the cytotoxic effects of BaP is essentially complete after a 3-day treatment with 5 ug/ml of the compound (Chart 4). The clonal death procedure was adopted for assessing the levels of resistance to BaP in isolated clones derived from NMuLi and BaP-NMuLi.

The results, plotted as histograms (Chart 7), show that both cell strains are composed of clones of varying sensitivities to the cytotoxic effects of BaP. Cycling of NMuLi in BaP to produce BaP-NMuLi has apparently caused the fully sensitive clones of NMuLi to die and/or develop higher levels of resistance. This is a confirmation of the results of Chart 6,

showing that the D_0 for the sensitive cells in NMuLi is only 0.06 ug/ml versus 0.10 ug/ml for the sensitive cells in BaP-NMuLi. The mean level of clonal resistance is also much higher in BaP-NMuLi, 0.7 $\pm$ 0.2 versus 0.4 $\pm$ 0.3 in NMuLi.

The clonal histogram also confirms the results of Chart 4 in showing that there is a greater heterogeneity of sensitive cell types in BaP-NMuLi relative to NMuLi. Further, cells susceptible to BaP-induced cytotoxicity in BaP-NMuLi are not those from NMuLi which fortuitously escaped the first round of toxicity, but are more resistant than their progenitors in NMuLi.

It is also of interest that there are fully resistant clones in NMuli itself. This may be a result of culturing, or it may reflect a previous exposure of the Namru mice to carcinogens in vivo. This question can only be answered by performing the experiment immediately upon isolating these cells.

DISCUSSION

Due to its extreme sensitivity to the cytotoxic effects of BaP, it appears that the NMuLi cell strain will serve as a good epithelial model for studying toxicity due to carcinogenic polycyclic aromatic hydrocarbons.

Of interest was the fact that the kinetics of toxicity manifested a shoulder or lag in the expression of toxicity as does the time dependence of the toxicity. Both effects may be due in part to the induction of the enzyme that metabolizes BaP to cytotoxic products, aryl hydrocarbon hydroxylase (AHH) (11), and/or to the existence of DNA repair enzymes. Efforts are in progress to characterize the induction parameters for AHH induction in sensitive clones derived from NMuLi, and to study the loss of this inducibility as the cells become more resistant to BaP-induced cytotoxicity.

The use of clonal histograms has shown that both NMuLi and BaP-NMuLi are heterogeneous in their responses to the cytotoxicity of BaP. Furthermore cloning experiments on isolated clones of NMuLi have shown that the subclones all breed true and that the production of variants resistant to the cytotoxicity of BaP is not an all-or-none, one-step process, but a sequential, multi-step process (manuscript in preparation). It is also of interest that the D_0 values for NMuLi and BaP-NMuL suggest that resistant variants may be due to a production of new variants with different levels of resistance as well as a selection for pre-existing resistant variants.

Finally, experiments are being conducted to test whether the process of formation of resistant variants is a spontaneous or induced process via fluctuation analyses, and to measure the mutation rate of this process.

ACKNOWLEDGEMENTS

We thank Helen Hesser for her technical assistance in culturing the cells used in these experiments. The cells were obtained through the courtesy of Robert Owens of the Naval Biomedical Research Laboratory. In addition, we would like to thank Dr. Morgan Harris for invaluable discussions. This research was supported in part by the U.S. Energy Research and Development Administration.

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Table 1

Fractions of the NMuLi and BaP-NMuLi Populations Resistant to 5 ug/ml of BaP

	% Resistant Cells	
	Cellular Assay	Clonal Assay
NMuLi	7±4 (6 expts.)	9±6 (2 expts.)
BaP-NMuLi	57±13 (4 expts.)	59±14 (2 expts.)

Table 2

Constancy of the Fraction of Cells Resistant to the Cytotoxic Effects of BaP in the NMuLi and BaP-NMuLi Populations as a Function of Passage Number

Experiment #	% Resistant Cells		Passage Number
	NMuLi	BaP-NMuLi	
1	12	-	27
2	4	67	29
3	4	38	30
4	7	60	33
5	12	63	34
6	5	-	35
NET	7±4	57±13	

Table 3

Growth Parameters for NMuLi and BaP-NMuLi Cell Strains

	Doubling Time, Hrs.	Saturation Density, x 10^6
NMuLi	16±1 (4 expts.)	1.8±0.4 (3 expts.)
BaP-NMuLi	20±6 (2 expts.)	1.1±0.6 (2 expts.)

CHART LEGENDS

Chart 1. Growth curves of NMuLi in BaP. Cells were seeded at 3 x 10^4 and allowed to grow for one day before treatment with BaP in acetone.

Chart 2. Growth curves of BaP-NMuLi in BaP.

Chart 3. Curves from Chart 1 were all divided by the acetone controls and replotted as survival curves.

Chart 4. Cytotoxicity of BaP to NMuLi (o----o) and to BaP-NMuLi (●---●). Cells were seeded at 3 x 10^4, allowed to recover for one day, and then treated with BaP in acetone for 3 days.

Chart 5. The fraction of cells resistant to 5 ug/ml of BaP for 3 days was subtracted from the toxicity curves of Chart 4. The data was then normalized by multiplying the entire curves by the factor necessary to correct the controls to a survival fraction of 1.0. a) ●, BaP-NMuLi data. b) o, NMuLi data.

Chart 6. The kinetics of toxicity of BaP to confluent NMuLi. Cells were seeded at 1.5 x 10^6/35 mm dish, and the control plates were monitored until they had reached confluence on day 2, as shown in a). BaP was then added, and the cells were counted 3 days later. These results are plotted in b).

Chart 7. Clonal analysis of resistance to the cytotoxicity of BaP. The NMuLi and BaP-NMuLi populations were cloned, and each clone was

tested for its ability to survive a three-day treatment with 5 ug/ml of BaP. The abscissa represents the survival of 100 BaP-treated clones relative to the untreated controls from the same isolated clone. The black arrows represent the calculated mean clonal survival for each population. a) NMuLi. b) BaP-NMuLi.

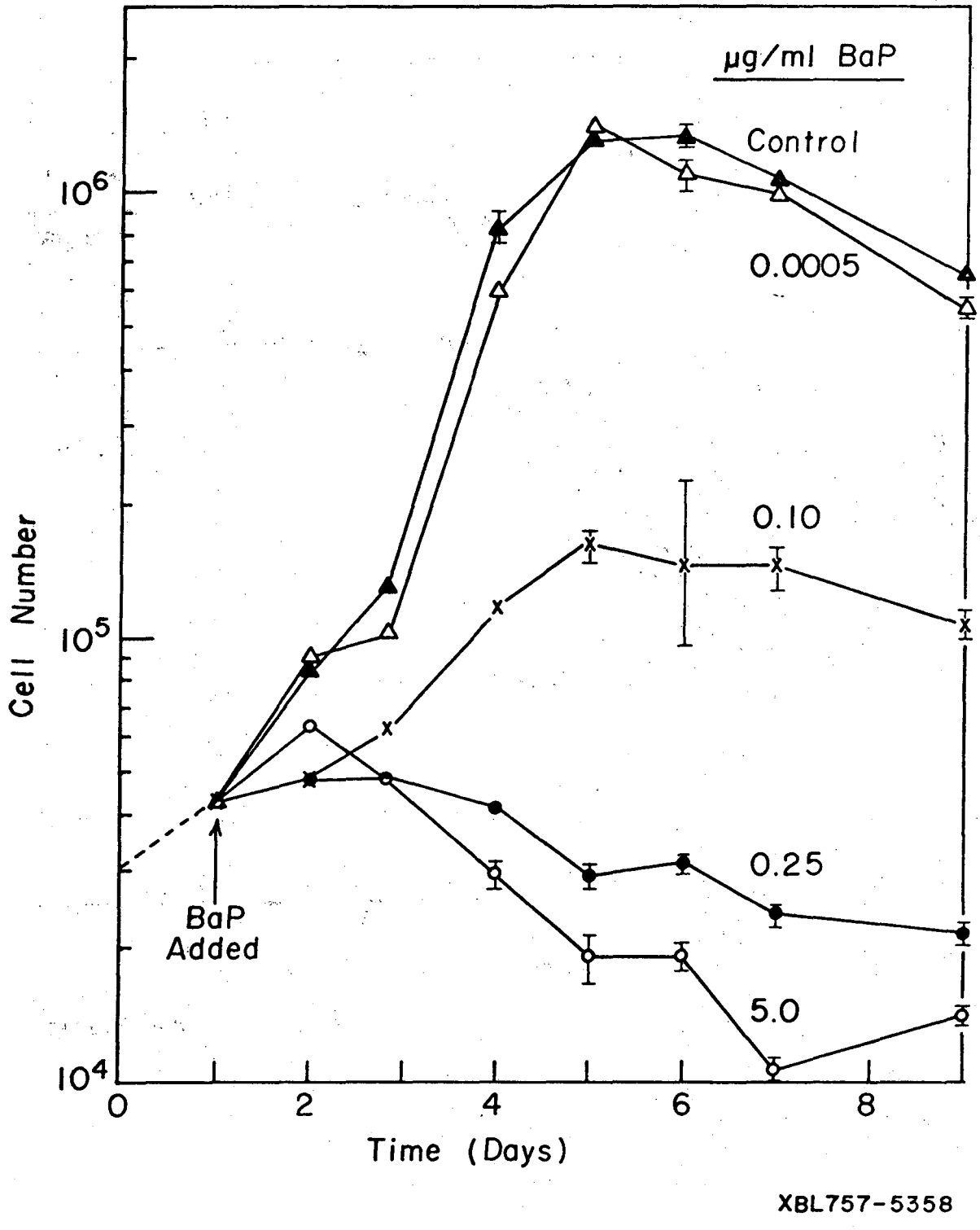

Chart 1

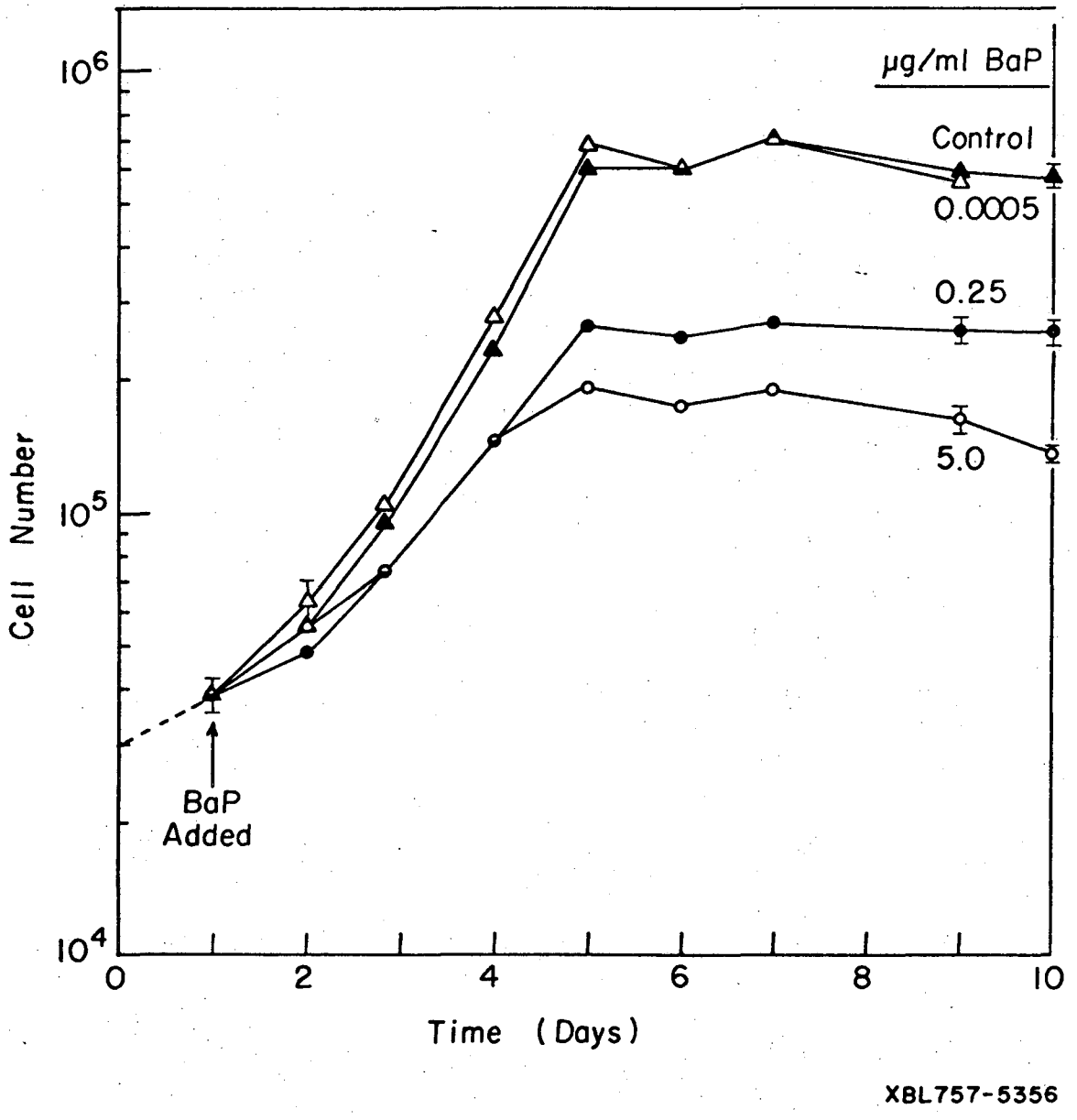
μg/ml BaP
Control
0.0005
0.25
5.0
Cell Number
10^6
10^5
10^4
BaP
Added
0
2
4
6
8
10
Time (Days)
XBL757-5356

Chart 2

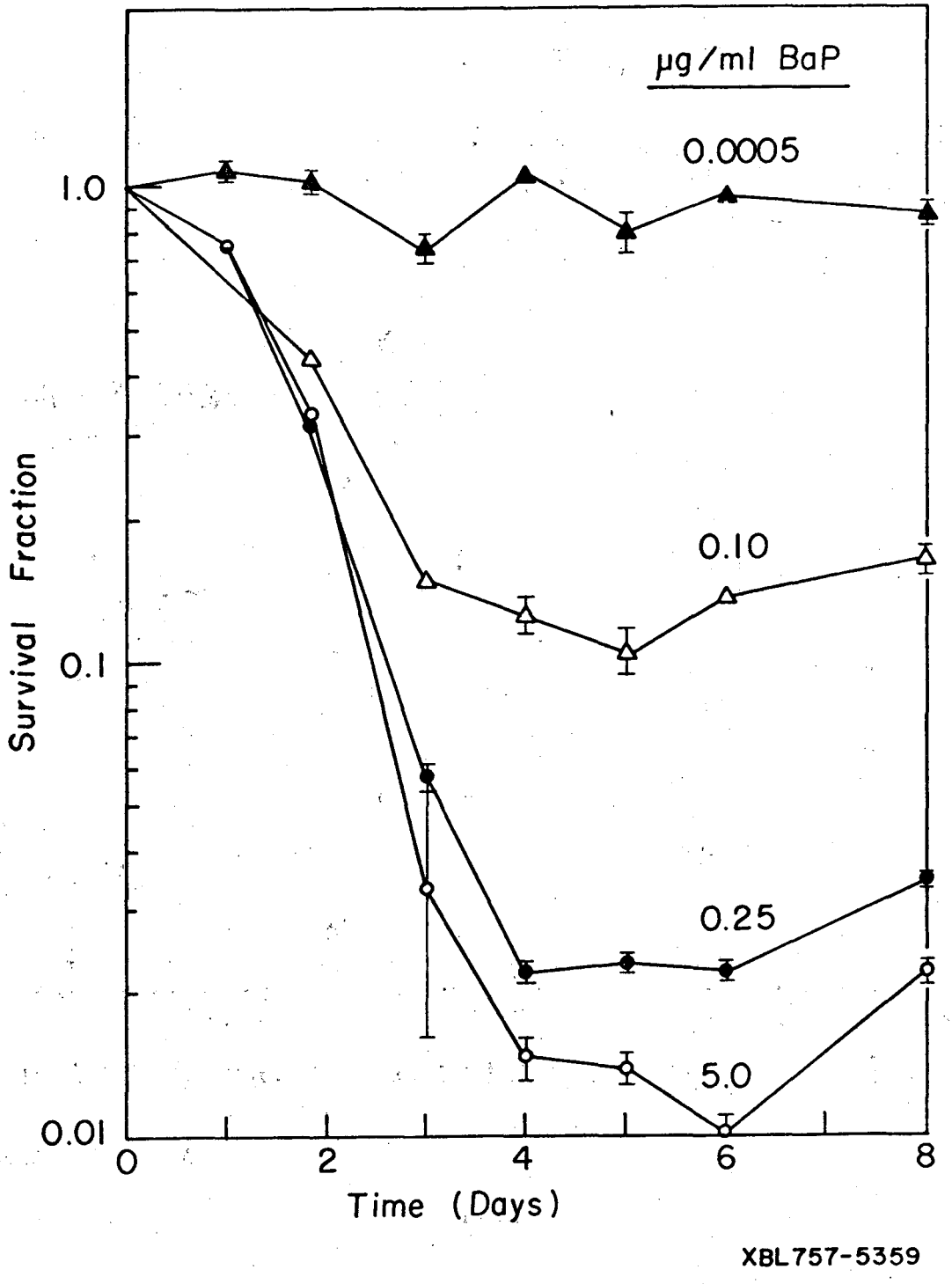

Chart 3

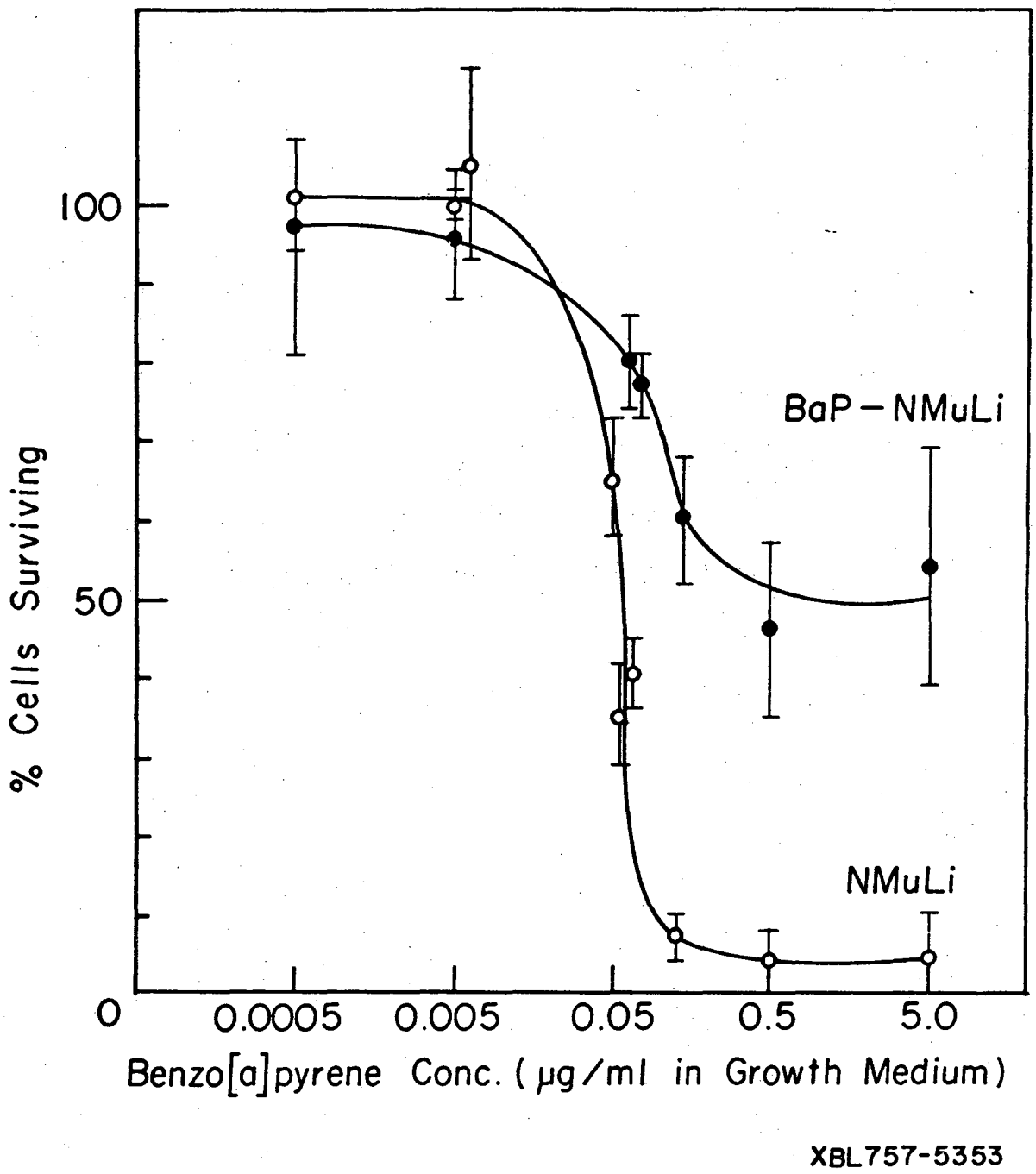
100
50
0
% Cells Surviving
BaP – NMuLi
NMuLi
0.0005
0.005
0.05
0.5
5.0
Benzo[a]pyrene Conc. (µg/ml in Growth Medium)
XBL757-5353

Chart 4

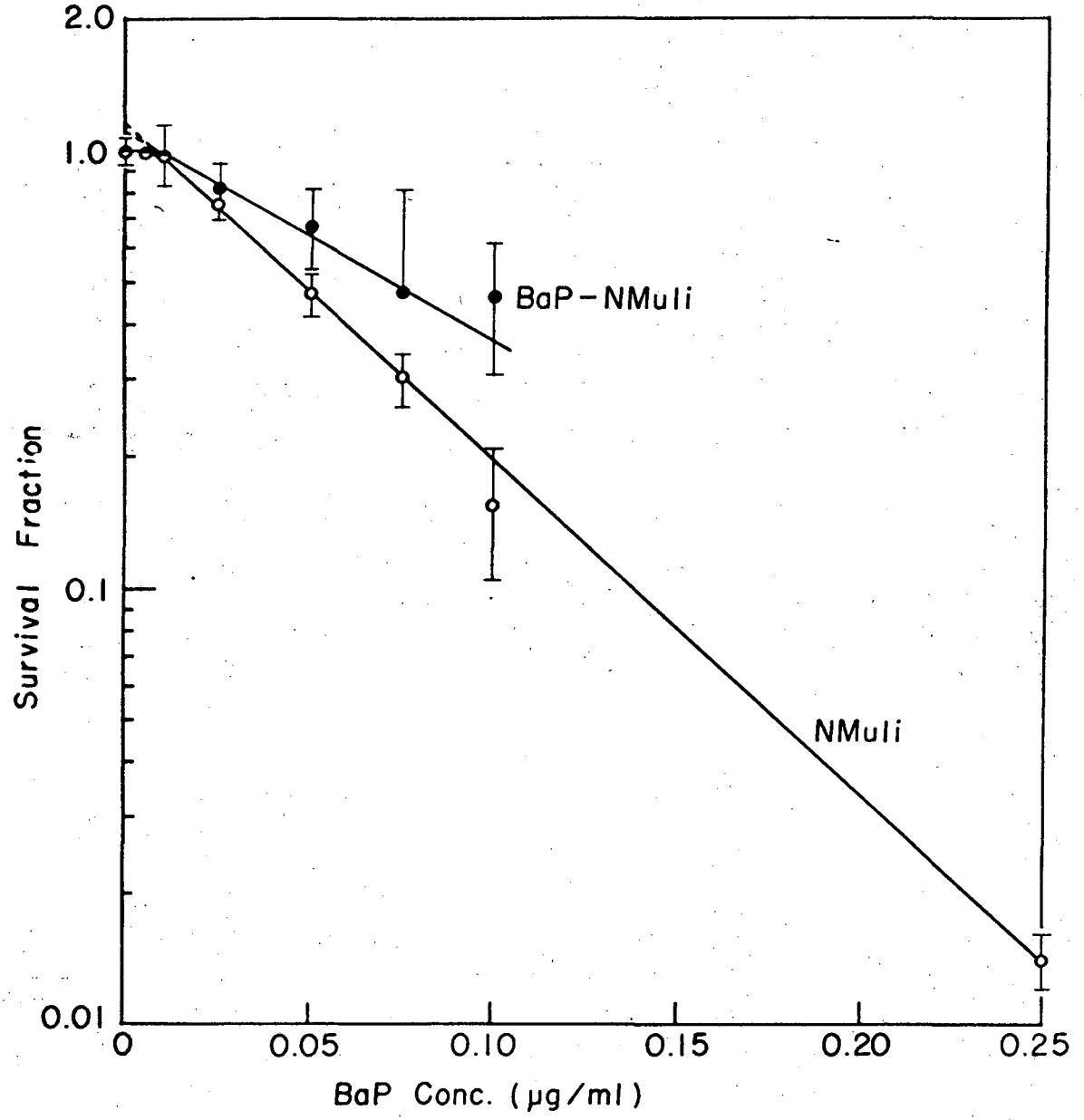

Chart 5

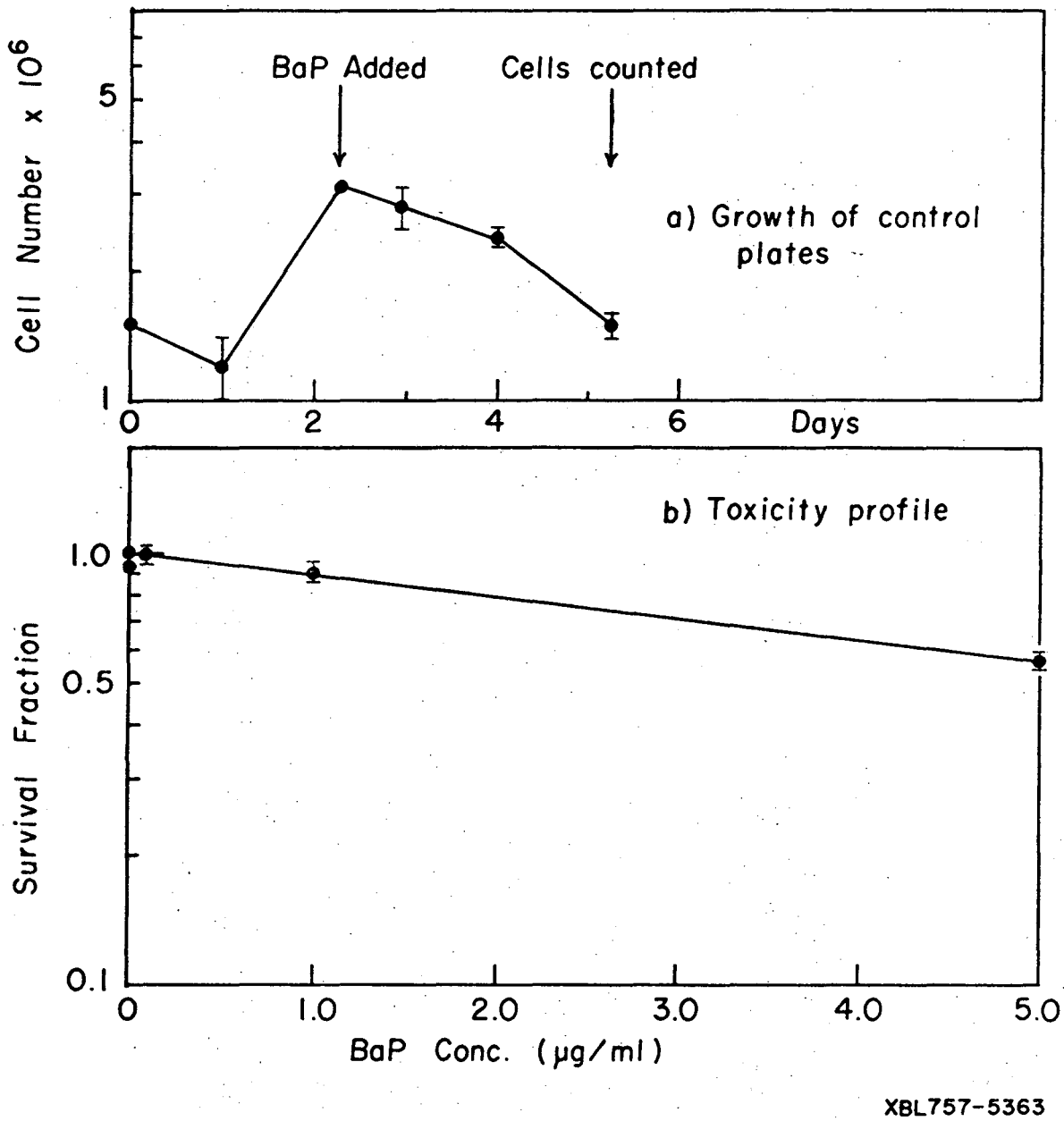
Cell Number x 10^6
5
1
BaP Added
Cells counted
a) Growth of control plates
0
2
4
6
Days
b) Toxicity profile
Survival Fraction
1.0
0.5
0.1
0
1.0
2.0
3.0
4.0
5.0
BaP Conc. (μg/ml)
XBL757-5363

Chart 6

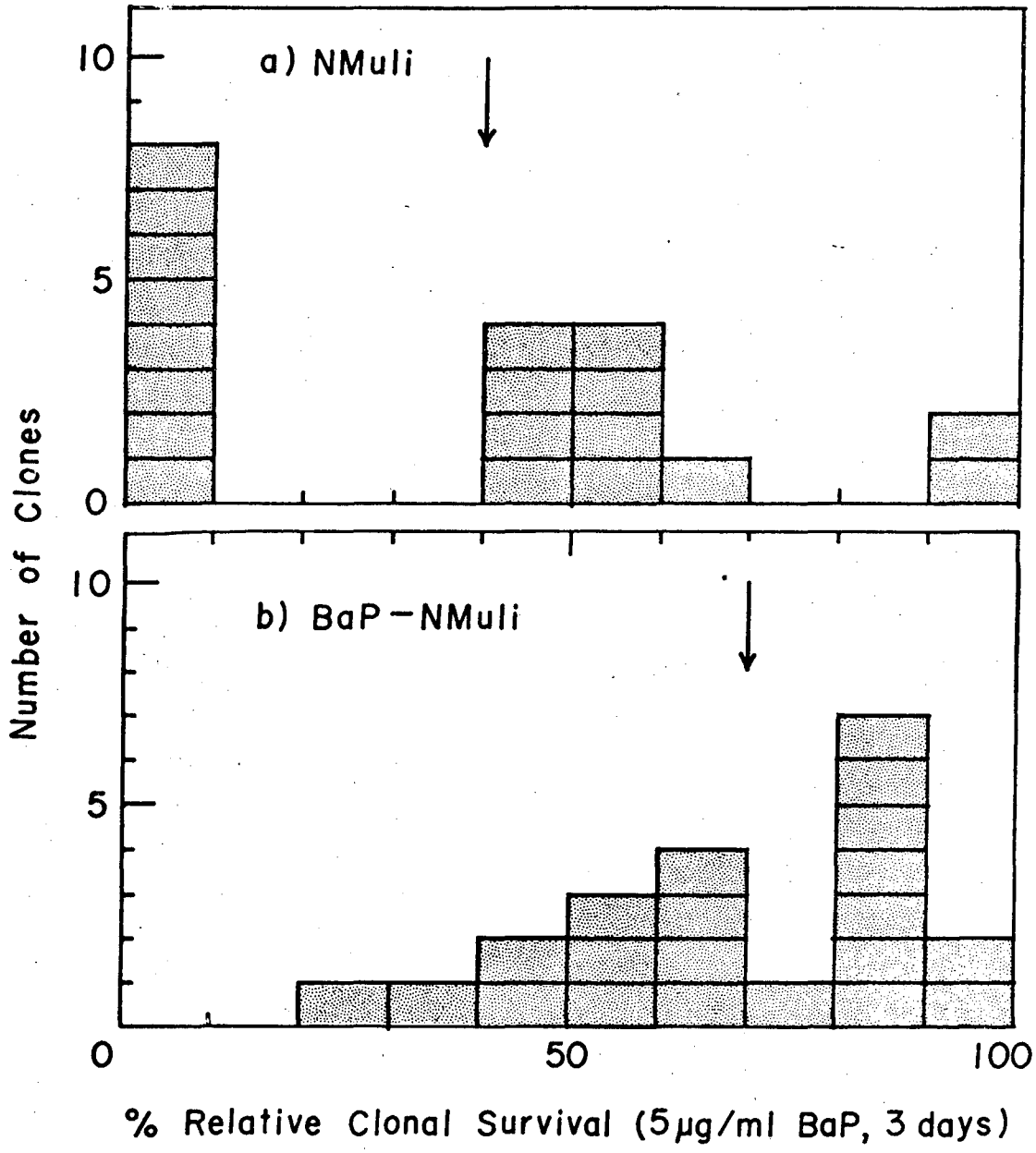

Chart 7

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