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ULTRAVIOLET LIGHT-INDUCED MUTATIONS IN SYNCHRONOUS CHO CELLS

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SUMMARY

Asynchronous and synchronous CHO cells were irradiated with germicidal UV light to determine the dose response curve for cell killing, and the induction of resistance to 6-thioguanine, ouabain, and diphtheria toxin. For asynchronous populations the data show a sigmoidal response for induced reproductive death, as has been seen by others, with a D_0 of 6 J/m^2 and an extrapolation number of 2.5. The induction of mutations appears to be a linear function for all three mutagenic markers up to a dose of 17 J/m^2.

Reproductive death induced in the synchronous populations is a function of the time at which exposure occurs in the cell cycle, with the late G_1 and early S being the sensitive stages. The induction of resistance to 6TG, ouabain, and diphtheria toxin (DT) all seem to depend on the time of exposure in the cell cycle. The more sensitive periods for mutation induction appear also to be the G_1 and early S period of the cell cycle. However, the degree of sensitivity in G_1 and early S appears to be most dramatic for induced DT resistance. These results suggest that estimates of the degree of mutagenesis expected from an environmental hazard based on log growth cells *in vitro* might yield data which do not always reflect the actual mutagenic hazard for cells not in logarithmic growth.

A comparison of the results reported here for UV exposure with exposures of synchronous cells to X-rays and ethylnitrosourea suggest that there are different age-specific responses to mutation induction for each agent, and that there are often different age responses for different mutagenic endpoints with the same mutagen.

ABBREVIATIONS

CHO	Chinese hamster ovary cells
HPRT	hypoxanthine-guanine phosphoribosyl transferase
$6TG^R$	6-thioguanine resistance
OUA^R	ouabain resistance
DT^R	diphtheria toxin resistance
UV	ultraviolet light
ENU	ethylnitrosourea
FMF	flow microfluorimetry

INTRODUCTION

Virtually all of the studies thus far of mutagenesis by UV light in mammalian cells have been performed using asynchronous populations. In recent years much progress has been made in understanding the processes leading to reproductive death by X-rays and UV through studying the effects of these agents on populations of cells highly synchronized by selective rather than inductive techniques. It seemed to us that similar studies of mutagenesis on synchronous cells might be helpful in elucidating the mutagenic process. Therefore, we have been performing mutagenic studies on synchronous cells using a variety of agents including X-rays, ultraviolet light, alkylating carcinogens, and bromodeoxyuridine. In addition to the use of the CHO/HPRT system, we use induction of resistance to ouabain, and induced resistance to diphtheria toxin. These studies are designed to discover "hot spots" for mutagenesis in the cell cycle, and to compare three different types of endpoints: chromosomal alteration, dominant, and recessive point mutations.

We report here studies of the effects of acute exposures to germicidal UV light which suggest that the G_1 period and the early S period are most important for mutagenesis, although mutations can be induced by UV exposure at any time in the cell cycle.

MATERIALS AND METHODS

Cell Culture

The CHO cell line was obtained from L. Kapp and R. Klevecz of the City of Hope Medical Center, Duarte, California, and had been selected

for use on the cell-cycle analyzer (referred to below). Stocks of this CHO cell line, which we call "CHO-KK," have been kept frozen in our laboratory since 1976. Fresh cultures are started about four times a year to insure that the genetic changes in the cell lines during growth at 37°C are minimal and reproducible from year to year.

This cell line has been shown to be free of PPLO, and has a modal chromosome number of 21. The G-banded karyotype has been determined, and shows four abnormal autosomes and only one complete X chromosome (Burki, unpublished). The population doubling time of the cells is about 12 hours under optimum conditions with a four-hour G_1 period, six-hour S period, and a G_2 plus M period of 1.5 hours.

The cells are grown at 37°C in a CO_2 incubator, in either closed tissue culture flasks or large glass roller bottles (Bellco) at 37°C in McCoy's 5A media, supplemented with 15% fetal calf serum and 100 units/ml of penicillin and 100 μg/m of streptomycin, and 1mM HEPES buffer to slow pH changes during experiments.

Synchronization Methods

Cells were grown for several days in logarithmic growth phase in roller bottles turned at 0.5 rev/min. They were subcultured into one or more roller bottles at 2 to 3 X 10^7 cells per bottle. After 24 hours, the cells were synchronized in a 37°C room using a "Cell Cycle Analyzer" (Talandic Research Corporation, Pasadena, Ca.). This apparatus is patterned after the instrument developed by Klevecz (ref.15). The mitotic cells were usually shaken off at one-hour intervals at the following speeds: 54 min at 0.5 RPM, 3 min at 180 RPM, 2 min for cell

collection, and 1 min for media replacement in the roller bottle. This apparatus thus gives continuous samples of cells spaced in these experiments one hour apart in cycle time. The typical yield per roller bottle is 2 X 10^6 cells in very early G_1 . More than 90% of the cells at harvest appear as small doublets. These cells are permitted to attach to 75 cm^2 plastic tissue culture flasks.

Cell Life Cycle Progress Analysis After Mitotic Detachment

(a) Cell volume spectroscopy: The modal size and the volume distribution of the trypsinized synchronous cells at different positions in the cell cycle were determined, using a Coulter Counter Model ZBI matched to a Coulter Channelyzer. This system was calibrated using 10-micron spheres and ragweed pollen provided by the Coulter Company. An X-Y plotter was used to monitor populations as a function of the cell-cycle time. The volume of the cells appeared to increase almost linearly until the cells divided after approximately 12 hours.

(b) Flow cytometry (flow microfluorimetry): Cells at different times after mitotic detachment were collected by gentle centrifugation and fixed at 0^oC, using a fixative containing three parts 200 m$\underline{M}$ $MgCl_2$ plus one part absolute alcohol, and kept at 4^oC. They were then stained with chromomycin A3 (100 μgm/ml, Cal Biochemical) for at least one hour (ref. 21). Analysis of the fluorescence was made, using an instrument according to the design of Steinkamp et al. (ref. 20), and described by Hawkes and Bartholomew (ref. 13).

UV EXPOSURE AND PLATING EFFICIENCY DETERMINATION

Cells attached to either 75-cm^2 tissue culture flasks (with the tops removed using a glass cutter) or cells in 90-mm tissue culture dishes were washed three times with Pucks Saline A (PSA) without phenyl red. The final wash was removed and the cells irradiated "dry" (thin film of PSA) at a fluence rate of 2.1 $J/(m^2 \cdot sec)$ by three germicidal lamps in a UV irradiation box constructed for cell irradiations. The exposure was determined by using a Yellow Springs Instrument Radiometer calibrated by the company against standard light sources, and was determined with and without the collector cone surrounding the thermister. In addition, the instrument was checked against a similar one used at the Laboratory of Radiobiology at the University of California, San Francisco. Both meters gave similar results.

After exposure at room temperature for up to 12 seconds, the cells were trypsinized and some cells plated in 90-mm dishes to determine the plating efficiency after treatment. After eight days of growth in normal medium at 37°C, the dishes containing the surviving colonies were stained with 1% methylene blue. The media containing the stain was poured off and after drying, the number of cells surviving irradiation was determined by counting visible colonies.

Selection for Drug and Toxin Resistance.

After exposure to UV and trypsinization, the cell number was determined and at least 2×10^6 cells were plated into a large roller bottle. The cells were then grown at 37°C in logarithmic growth for eight days. This required two subcultures of the bottle to 2×10^6

cells per bottle in control cultures and one subculture of the cells exposed to large doses of UV. After eight days, the cells were trypsinized, the cell number determined, and 10^5 cells exposed to 6TG (5 μg/ml), or 10^6 cells to either ouabain (3 mM), or diphtheria toxin (0.1 Lf/ml).

A number of studies of our own and others have established that an expression period of 7-9 days is required for maximum 6TG resistance (ref.17; Burki, unpublished). Although ouabain resistance is a co-dominant trait, it still requires an expression time of at least five days, especially at higher doses of mutagen (ref. 2,9,10). Diphtheria toxin resistance is associated with the alteration of elongation factor 2 (EF-2) necessary for protein synthesis (ref.16) and also requires an expression time of at least five days after mutagen treatment.

Resistance to 6TG is thought to be due to X chromosome alteration or chromosome deletion (ref. 6). Ouabain resistance is due to a point mutation that is co-dominant (ref. 1) while diphtheria toxin resistance is due to a point mutation that is recessive (ref.11) in somatic cell hybrids. These mutants retain their resistance to drugs or diphtheria toxin for 50 or more generations in non-selective media.

RESULTS

The dose response curve for the induction of reproductive death by germicidal UV for acute exposures is a sigmoidal survival curve. The slope of the linear portion of the curve is 6 J/m^2 and the extrapolation number is 2.5 (D_q=5 J/m^2) (Fig. 1). This result is quite similar to

that reported by others for irradiation under similar conditions. The induction of mutations to $6TG^R$, oua^R, and DT^R(Fig. 2 a,b,c) are all linear functions of the UV dose, at least for the low doses of UV and the conditions used for the expression time and irradiation. The results for the induction of $6TG^R$ mutations are similar to that reported by Hsie et al. (ref.14) for a similar system. It is important to note that under these conditions, curves are all linear and all quite similar functions of fluence, a situation that does not obtain with a different mutagen ENU and X-rays under the same conditions (refs. 2,9,10).

The degree of synchrony obtained by mitotic detachment at optimum growth conditions is monitored in every experiment by Coulter volume spectroscopy and flow microfluorimetry (see also ref. 3), and often incorporation of tritiated DNA precursors. The DNA content of cells is proportional to the fluorescence of chromomycin-stained cells and is one measure of the time in the cell cycle of a population. The FMF data from a typical UV experiment (in this case a 17 J/m^2 experiment) is given in Figure 3. From this and other data the stage times of the cell life cycle also given in the figure are derived. The G_1 period is 4 hours, the S period is almost 6 hours, and the G_2/M is 1.5 hours. These cell cycle parameters appear to vary slightly from experiment to experiment, and the degree of synchrony may vary slightly from day to day, as measured by the coefficient of variation of the DNA content in different experiments.

The age response of these CHO cells in the cell cycle, in terms of the survival measured at different times in the cell cycle after exposure to either 8.5 or 17 J/m^2, is given in Figure 4a. There is

maximum sensitivity to the UV at the end of the G_1 period and the beginning of the S period.

The induction of mutations to drug or toxin resistance during the cell cycle is shown in the data in Figure 4 b,c,d. The induction of 6TG resistance occurs throughout the cell cycle and does not appear to be very much a function of the time of the irradiation in the cycle. However, at the higher doses there is a greater sensitivity at the third hour of the G_1 stage and in the early S phase. This does not mimic the cell killing induction by UV.

The induction of mutants resistant to ouabain under these experimental conditions occurs throughout the cycle and does not show a dramatic age response at low fluences of UV. At the UV fluence of 17 J/m^2 there appears to be some increased sensitivity at the early S period.

The induction of mutations to diphtheria toxin resistance is a dramatic function of the time in the cell cycle. There appears to be a sensitive period near the end of the G_1 stage. The time for maximum induction is a small portion of the cell cycle at around three hours after mitosis. This result is quite different from the results for either cell killing or for mutation induction to 6-thioguanine or ouabain resistance.

DISCUSSION

I. Asynchronous Populations

Although the survival curve found after exposure of the cells to UV light is sigmoidal, the induction of mutations appears to be a linear function of fluence to at least 17 J/m^2. This difference in response

between killing and mutagenesis suggests that the mechanisms leading to the two processes are different.

The induction of 6-thioguanine resistance by UV is similar to results reported by Hsie et al. (ref. 14) and by others for Chinese hamster V79 cells (ref. 8). In the same way, the induction of ouabain resistance in a linear fashion with fluence has been reported (ref. 5).

It is important to realize that the methods used in these experiments involve mutagenizing the cells and then replating them for the subsequent drug challenge. An alternate method is the *in situ* or minicolony assay whereby surviving colonies (rather than single cells) are challenged in the same location in which they were mutagenized. For 6-thioguanine resistance, this method has been shown to yield non-linear UV mutation induction curves in Chinese hamster cells, whereas linear curves are obtained by replating under otherwise identical conditions (ref. 8). Thus, the non-linear oua^R induction curves with UV reported by Chang et al. (ref. 4) are probably due to their use of the *in situ* method, as opposed to the replating method which results in the linear response reported here.

With identical expression times and procedures in identical cells, the results for X-ray and ENU-induced mutation to $6TG^R$ are unlike those found here for UV and are in fact curvilinear (refs. 2,9,10). X-rays do not seem to induce ouabain resistance in these cells at detectable frequencies, while ENU gives a curvilinear dose response curve for oua^R. Mutation induction response for these two loci is thus quite mutagen-specific.

The frequency of the induction of diphtheria toxin resistance is similar, in its fluence response to UV, to results obtained for oua^R. This is not surprising since both are effects due to point mutations, although oua^R is dominant and DT^R is recessive in somatic cell hybrids. Based on asynchronous studies alone, one might conclude that the effects of UV on the two loci are quite similar. However, the synchronous studies show that this is not the case.

II. Synchronous Populations

The efficiency of producing ultraviolet-induced reproductive death is quite variable during the cell life cycle. The maximum sensitivity occurs near the G_1-S boundary (see Fig. 1) as reported previously for other mammalian cell lines (refs. 12,19, and 22). This characteristic age response for UV is similar to that found for ENU exposure, suggesting similar mechanisms for UV and ENU-induced reproductive death.

The induction of ouabain resistant clones occurs throughout the cycle and has a maximum in the early S period when higher fluences are used. The results for ENU-induced ouabain resistance in the cell cycle are simlar to UV at lower concentrations of ENU; at higher concentrations of ENU, however, mutagenesis is not cell-cycle-position dependent (refs. 9,10), suggesting that there is a difference at this locus in the way mutations are induced by ENU and UV.

The induction of clones resistant to 6-thioguanine during the cell cycle is a more complicated function of fluence, showing two peaks: one in G_1 and the other in early S, a type of result recently reported by Riddle and Hsie (ref. 18). The situation is different with ENU.

With low ENU concentrations, where survival is high, $6TG^R$ mutants are induced throughout the cycle. However, an age response for mutations is not seen with higher concentrations of ENU. In addition, the results with UV are completely different from the results for X-rays (ref. 2) in the same cell system since a dramatic effect for the induction of $6TG^R$ occurs in the G_1 period just before the replication of the gene or genes associated with 6-thioguanine resistance (ref. 3). These results suggest different mechanisms for both cell killing and mutation to 6-thioguanine resistance between X-rays and UV.

The induction of diphtheria toxin resistance by UV is dramatically different from the killing effects or the effects at the other drug-sensitive sites in that very few mutations are induced in the cycle outside of the G_1 period. The maximum induction of mutants resistant to diphtheria toxin occurs in late G_1 when the DNA is being prepared for replication.

In simple eucaryotic cells, genetic recombination repair has been reported to be involved in mutational processes in G_1 (ref. 7). The gene determining DT^R (presumably that coding for EF-2) may be particularly accessible to immediate error-prone recombination repair leading to mutagenesis if damaged during G_1.

The results reported here suggest that the effects of UV in the induction of mutation are both locus and cell-cycle specific. When the results are compared with similar experiments using X-rays or an alkylating carcinogen in the same system, one is forced to conclude that "random mutagens" like X-rays, UV, and ENU may in general be

expected to have both locus and life-cycle-specific effects. This makes efforts to evaluate the mutagenic hazards more difficult to evaluate than previously imagined.

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FIGURE LEGENDS:

Figure 1. Percent survival (normalized to control plating efficiency) of CHO-KK cells exposed to various ultraviolet light fluences. ○, □, △, indicate separate experiments.

Figure 2. Induction of mutations in CHO-KK cells by ultraviolet light.

a) Mutants resistant to 3 mM ouabain (per 10^6 viable cells). ● and ⊙ indicate separate experiments.

b) Mutants resistant to 0.1 Lf/ml diphtheria toxin (per 10^6 viable cells). Δ, ▼, and ★ indicate separate experiments.

c) Mutants resistant to 5 μg/ml 6-thioguanine (per 10^5 viable cells). □, ■, and Δ indicate separate experiments.

Figure 3. Flow microfluorimetry data from a typical experiment. Peak channel FMF (proportional to DNA content) is plotted against time (in hours) in the cell cycle.

Figure 4. Age response of CHO-KK cells.

a) Percent survival of cells after 8.5 J/m^2 (Δ) or 17 J/m^2 (○) of ultraviolet light for different times after mitotic selection.

b) Induction of 6-thioguanine (5 μg/ml) resistance after 8.5 J/m^2 (□, ○) or 17 J/m^2 (■, ●) of ultraviolet light for different times after mitotic detachment. Different symbols indicate separate experiments.

c) Induction of ouabain (3 mM) resistance after 8.5 J/m^2 (○, □) or 17 J/m^2 (●, ■) of ultraviolet light for different times after mitotic selection. Different symbols indicate separate experiments.

d) Induction of diphtheria toxin (0.1 Lf/m) resistance after 8.5 J/m^2 (△) or 17 J/m^2 (○, ●) of ultraviolet light for different times after mitotic selection. C indicates control (background) frequencies. Different symbols indicate separate experiments.

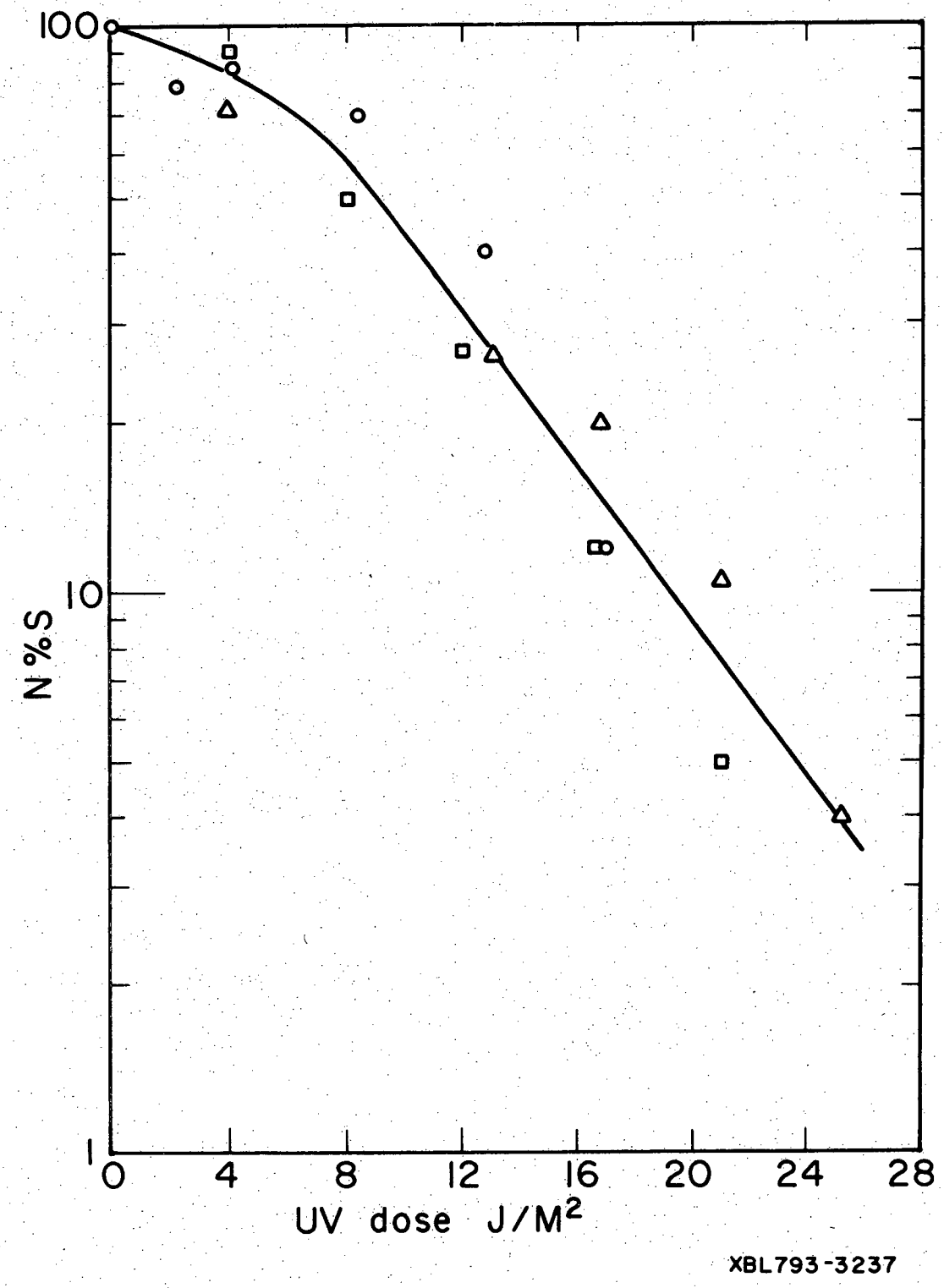


Figure 1

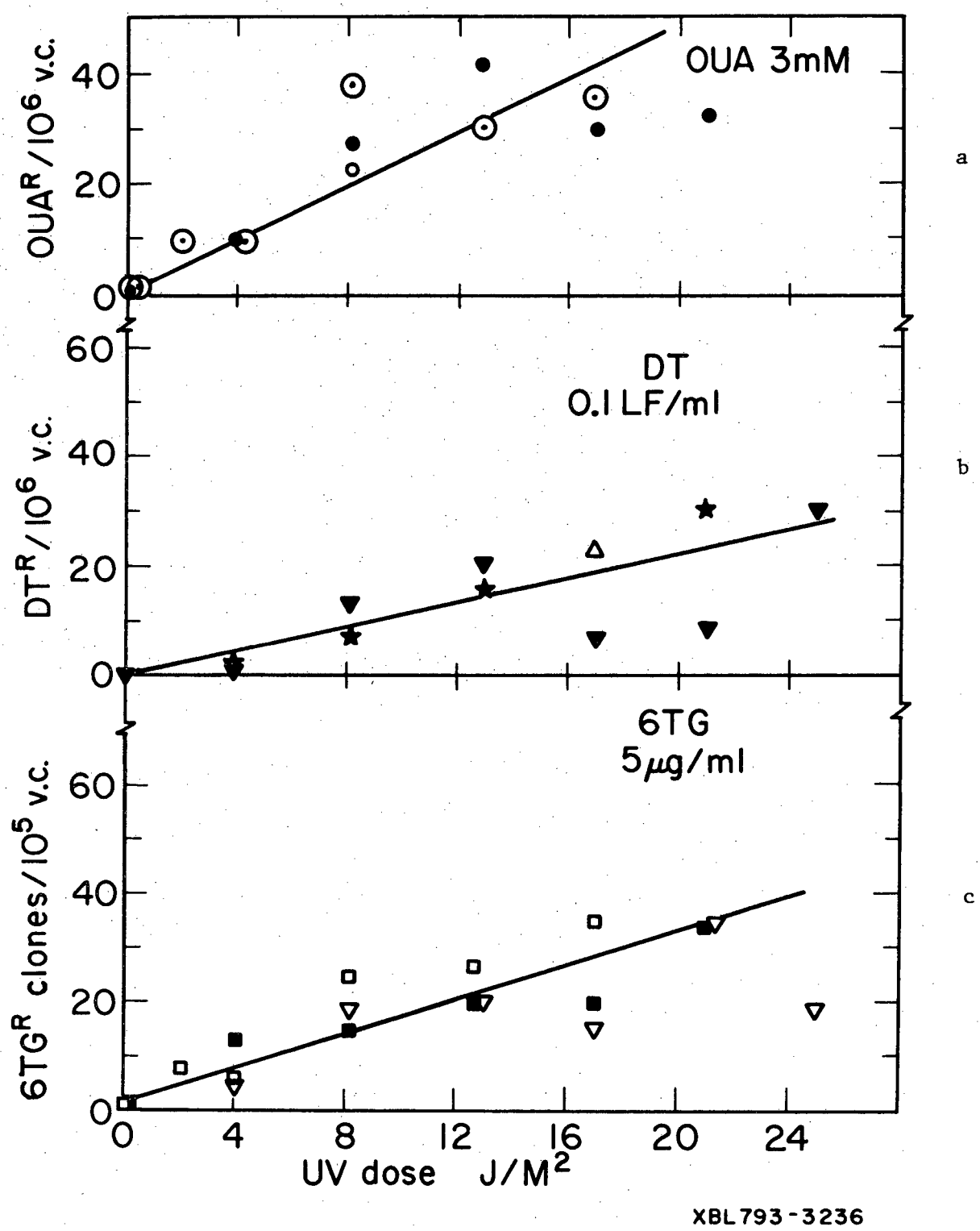
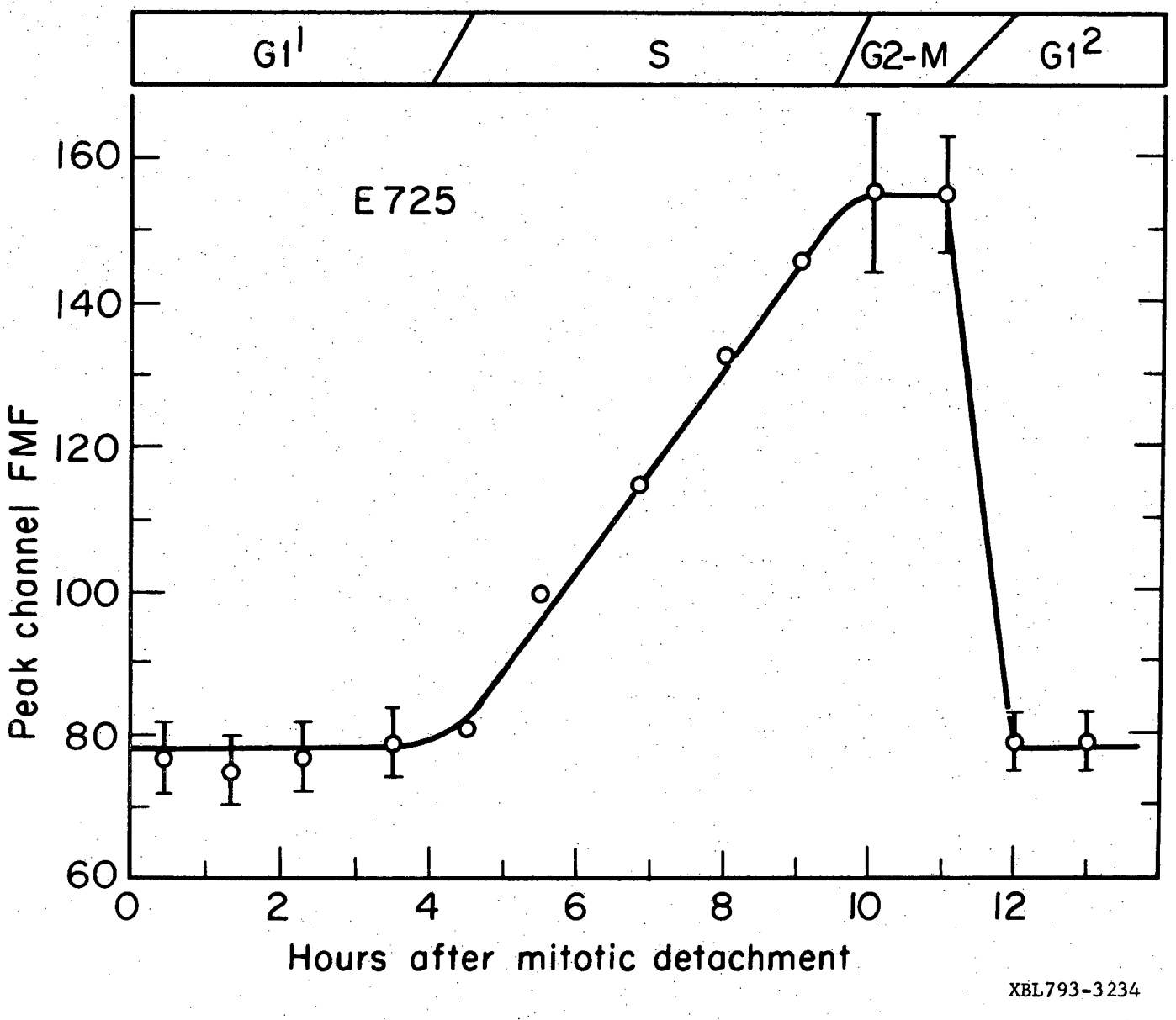

OUA 3mM
OUA^R/10^6 v.c.
a
DT
0.1 LF/ml
DT^R/10^6 v.c.
b
6TG
5μg/ml
6TG^R clones/10^5 v.c.
c
UV dose J/M^2
XBL793-3236

Figure 2


G1¹
S
G2-M
G1²
E 725
Peak channel FMF
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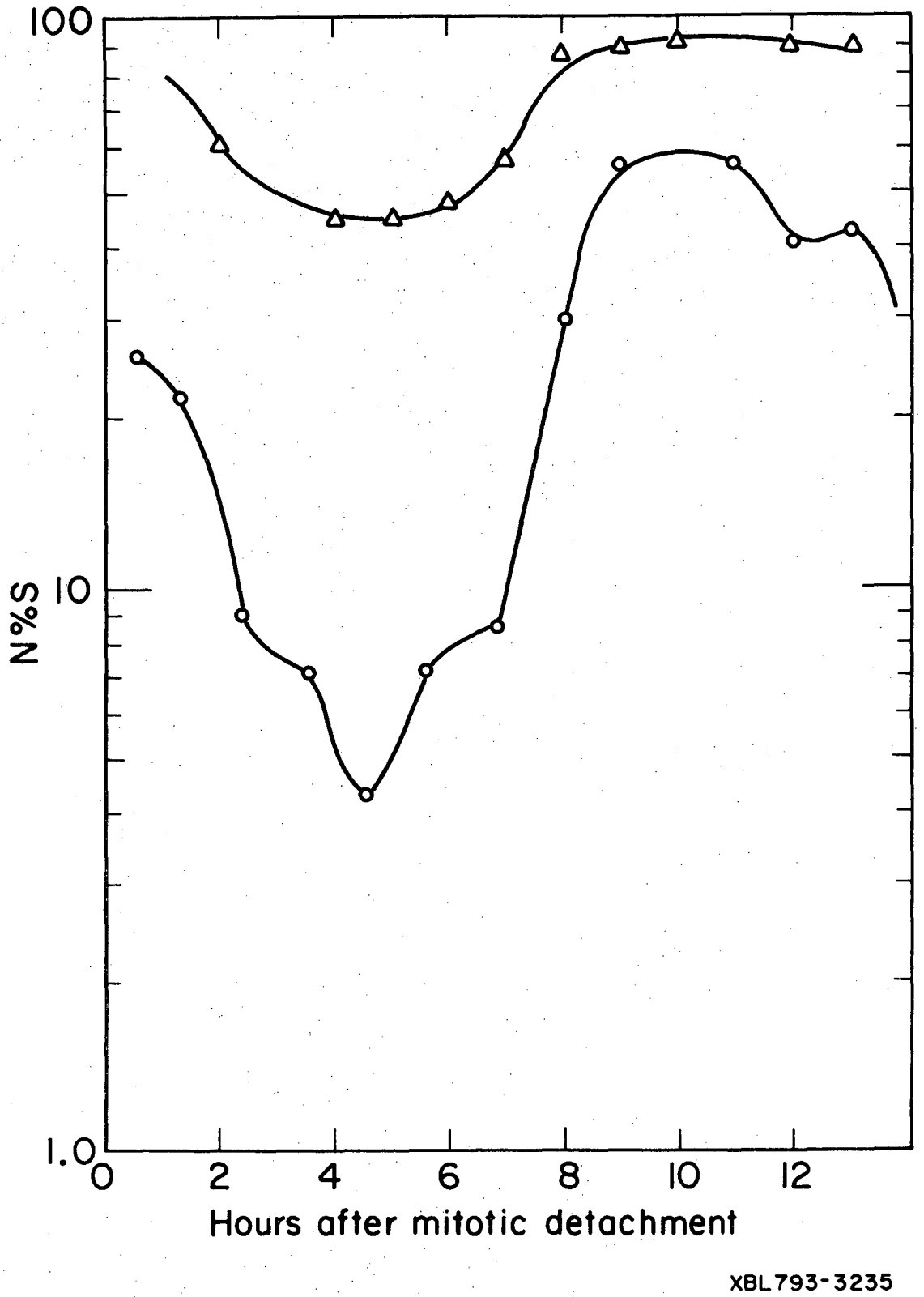
2
4
6
8
10
12
Hours after mitotic detachment
XBL793-3234

Figure 3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10
1.0
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8
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12
Hours after mitotic detachment
XBL793-3235

Figure 4a

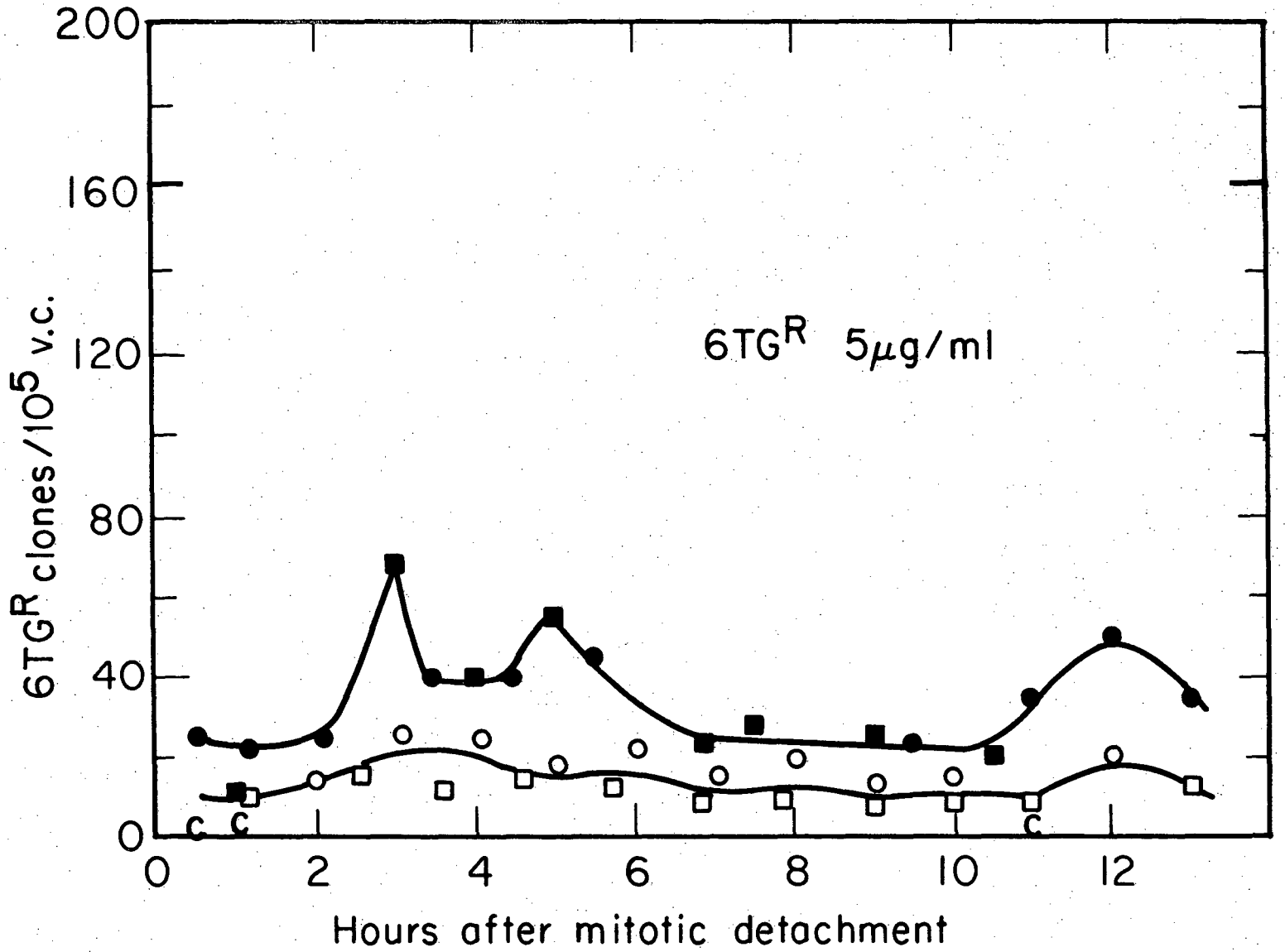

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Figure 4b

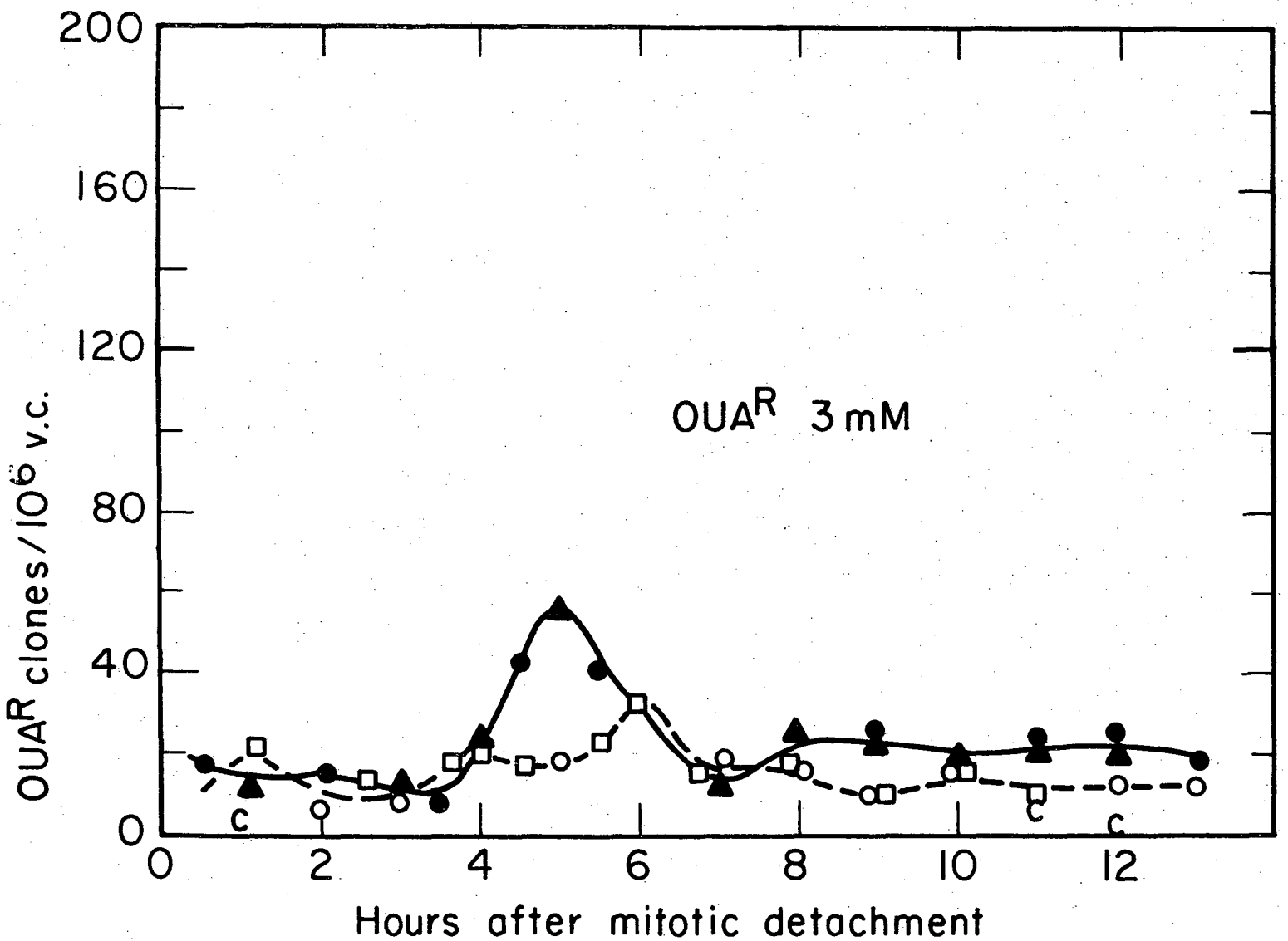

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Figure 4c

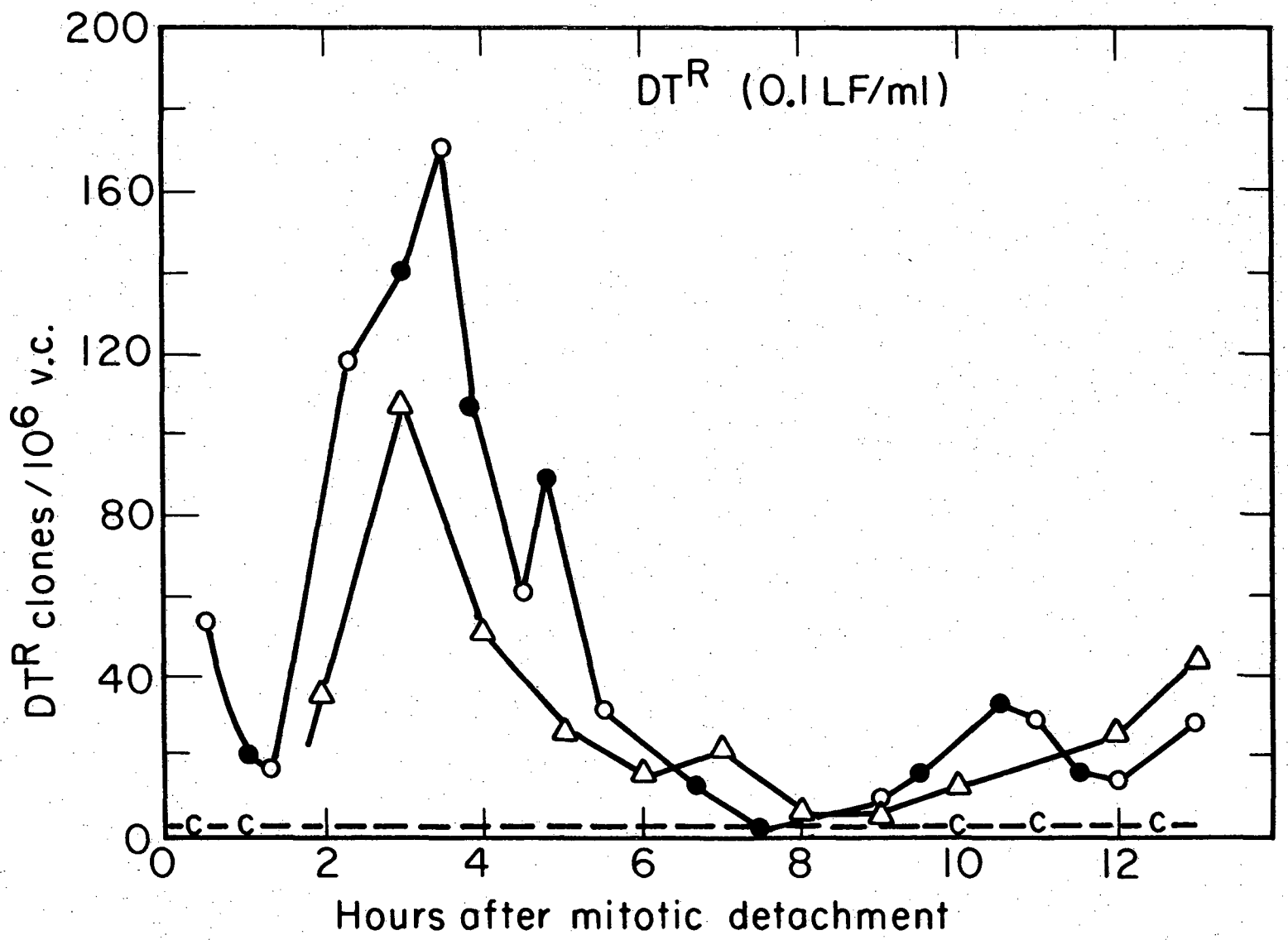

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Figure 4d

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