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Cell Killing and Chromosomal Aberrations Induced in Chinese Hamster Ovary Cells by Treating with Cisplatin at 41.5°C during G_1 or Late S Phase[1]

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ABSTRACT

Variation in sensitivity to cisplatin during the cell cycle was studied in synchronous Chinese hamster ovary cells treated during G_1 or late S for 1 h at 41.5°C with cisplatin (0.25–1.25 μg/ml, 0.8–4.2 × 10^{-6} M). The cells were assayed for cell killing and chromosomal aberrations. Either they were plated for colony survival, or colcemid was added from 12 to 40 h after plating followed by fixation 4 h later for analysis of chromosomal aberrations after the cells completed 1 or 2 cycles (*i.e.*, first or second mitosis). When the cells were treated either in G_1 or late S, the cells entering metaphase exhibited primarily chromatid-type deletions and exchanges. However, aberrations were observed primarily in the first mitosis when cells were treated in G_1 compared with aberrations being observed in both the first and second mitoses when cells were treated in late S. For a given amount of cytotoxicity or cytological damage, the cisplatin concentration at 41.5°C could be reduced 4–6-fold compared with treatment at 37°C. For low cisplatin concentrations of less than 0.5–0.7 μg/ml (survival, ~0.3), heat killing predominated, and cells treated in S phase were more sensitive than those treated in G_1. However, for cisplatin concentrations greater than 0.5–0.7 μg/ml, cisplatin cytotoxicity predominated, and for both cell killing and chromosomal aberrations, the cells treated in G_1 were ~1.5 times more sensitive than those treated in late S. Furthermore, the positive correlation between survival and aberration frequency was similar for cells treated at 37°C or 41.5°C in either G_1 or late S. These results suggest that cisplatin administered at 37°C or 41.5°C causes cell lethality primarily by the induction of chromosomal aberrations.

INTRODUCTION

Hyperthermia has been shown to enhance the cytotoxicity of cisplatin both *in vitro* (1–7) and *in vivo* (5, 8, 9). Furthermore, cisplatin treatment at 37°C causes more cytotoxicity when administered during G_1 than when administered during S phase (10–14). This increase in cytotoxicity during G_1 *versus* S was found to correlate with an increase in chromosomal aberrations when cells were treated at 37°C in G_1 *versus* S (15). In this study, we investigated the possibility that increased cytotoxicity caused by treating cells at the mild hyperthermic temperature of 41.5°C would be the same for treatment during G_1 and S and would be observed for both cell killing and chromosomal aberrations.

MATERIALS AND METHODS

Culturing Conditions and Treatment with Cisplatin. Chinese hamster ovary (CHO10B6) cell cultures maintained in McCoy's 5A medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum were grown at 37°C (pH 7.4) in roller bottles, and the mitotic cells were collected by mitotic shake-off (mitotic index of 95–97%) (16). Mitotic cells plated at a density of 5 × 10^5 cells/T-25 flask were allowed to progress into G_1 or S (90 min or 9 h after plating, respectively). By 90 min, the cells had divided and attached to the surface of the flasks. Cells were treated with various doses (0–1.25 μg/ml; 0–4.2 × 10^{-6} M) of cisplatin (molecular weight, 300; Sigma, St. Louis, MO) at 41.5°C for 1 h in a temperature-controlled water bath (YSI model 72; Yellow Springs Instruments, Yellow Springs, OH). Then the cells were washed and either trypsinized and plated for survival or incubated at 37°C until they progressed into mitosis at 37°C. Cells in replicate flasks were pulse labeled with [^{3}H]thymidine (4 μCi/ml; 25 Ci/mM; 1 mCi = 37 MBq) for 15 min immediately before cisplatin and heat treatment to obtain the percentage of cells in S phase at the time of treatment (94–96%); these same samples were plated for survival and analyzed for chromosomal aberrations (described below). Replicate samples without cisplatin or heat treatment represent control samples.

Analysis of Chromosomal Aberrations. Colcemid (0.06 μg/ml) was added to accumulate cells in metaphase. Colcemid was added to the first flask 12 h after plating mitotic cells, and the cells were fixed at 16 h; Colcemid was added to replicate flasks at 4-h intervals, *i.e.*, at 16, 20, 24, 28, 32, 36, and 40 h through two cell cycles. After 4 h of colcemid treatment, cells were trypsinized and processed for analysis of mitotic indices and chromosomal aberrations (15, 17); for each fixation time, 50 metaphases were scored for chromatid and chromosome breaks and exchanges. Average values for a given treatment were obtained by weighting values from replicate samples (fixed at different times) by their mitotic indices. Only near-diploid (2*n*) cells (21–23 chromosomes) were analyzed for aberrations; about 90% of the metaphase cells were near-diploid. Tabulations in a format like that shown for cells treated with cisplatin at 37°C (15) are available for individual samples; these tabulations give for each dose and fixation time the number of normal cells, the mitotic index, the percentage of polyploid cells, the numbers of different types of chromosome and chromatid deletions and exchanges, and the aberration frequency.

Survival Determination. Cell survival was determined by trypsinizing the cells immediately after treatment with cisplatin and heat and plating them in T-25 flasks with irradiated feeder cells (18). Macroscopic colonies were counted after incubation for 8–12 days at 37°C. The plating efficiency for all of the experiments was 74–80%.

RESULTS

Traversal of Cells through the Cell Cycle after Treatment with Cisplatin in G_1 or Late S. Cells treated for 1.0 h at 41.5°C exhibited a delay of about 5 h in reaching the first and second mitoses, *i.e.*, division delay (Figs. 1 and 2). When the cells were heated in the presence of cisplatin, the delay increased as the concentration of cisplatin increased. Because of the large amount of asynchrony that developed after treating with cisplatin at 41.5°C, the second cycle could not be distinguished clearly from the first cycle. Therefore, we cannot say with certainty that when cells were treated in late S, the delay into the second mitosis [S(2)] was greater than the delay into the first mitosis [S(1)]. Cells treated in late S were followed into the second mitosis because treatment at 37°C with cisplatin during late S results in many more chromatid aberrations in the second mitosis than in the first mitosis (15). In contrast, treatment at 37°C during G_1 results in chromatid aberrations that appear almost exclusively in the first mitosis (15).

Chromosomal Aberrations Observed as the Cells Entered Metaphase after They Were Treated in G_1 or Late S with Cisplatin. When cells were arrested in metaphase with colcemid at different times after they had been treated with cisplatin in G_1 or late S, the frequency of chromosomal aberrations in the metaphase cells increased as they were fixed at later times in the first mitosis. (Fig. 3). Therefore, the individual aberration frequencies, observed as the metaphase cells were fixed at different times as the cells entered the

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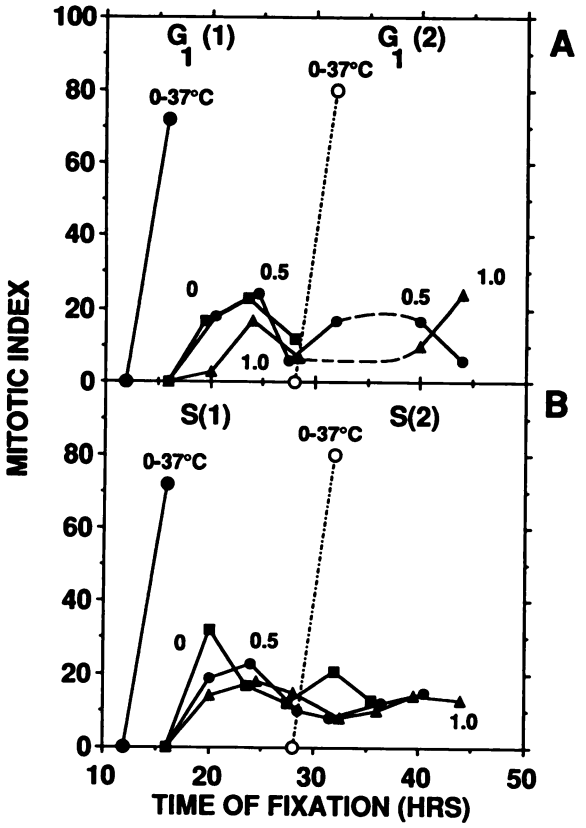

Fig. 1. Mitotic indices *versus* time of fixation show the progression of cells through two cell cycles. Cells treated with various concentrations of cisplatin (0–1.25 μg/ml; 0–4.2 × 10^{-6} M) for 1 h at 41.5°C were allowed to progress into mitosis. At 12 h after plating mitotic cells, colcemid was added to accumulate cells in metaphase for 4 h before the cells were fixed at 16 h. Then, colcemid was added at 4-h intervals to replicate flasks, and the cells were fixed 4 h later. These samples were scored for mitotic indices and chromosomal aberrations. *A*, representative data as cells progressed into the first mitosis (1) or second mitosis (2) after they had been treated in G_1 (■, 0; ●, 0.5; ▲, 1.0 μg/ml). ○, data for cells heated only; ●, 0–37°C for cells that were not heated. *B*, progression of cells treated in late S (9–10 h after plating of mitotic cells).

first metaphase, were weighted by the mitotic indices and added to obtain the mean aberration frequency for each treatment. These mean values are plotted in Fig. 4. As cells entered the first mitosis after heating only for 1.0 h at 41.5°C, the cells treated in late S [S(1)] had a higher frequency of chromosomal aberrations than cells heated in G_1 [G_1(1)]; in fact, the aberration frequency for the cells heated in G_1 was not much higher than that for the controls. However, as cells entered the first mitosis after treatment at 41.5°C with cisplatin at concentrations greater than 0.5 μg/ml, the cells treated in G_1 [G_1(1)] had a much higher frequency of chromosomal aberrations than cells treated in late S [S(1)]. In addition, when the cells had been treated in late S with concentrations greater than 0.5 μg/ml, the aberration frequency was higher in cells entering the second mitosis [S(2)] than in cells entering the first mitosis [S(1)]. This contrasts with the very low frequency of aberrations observed in the second mitosis when cells were treated in G_1 [G_1(2)]. These results suggest that the induction of chromosomal aberrations by cisplatin occurred as the cells traversed through S phase.

The results for treatment at 41.5°C agree qualitatively with the results for treatment at 37°C if the concentration of cisplatin at 41.5°C is reduced 4–6-fold (data not shown). The main difference between treatments at 37°C and 41.5°C is the induction of aberrations from heat alone when cells were heated in late S. In fact, if the curves in Fig. 4 are normalized to zero to subtract the effect from heat alone, the G_1(1) curve is much higher than the curve (Fig. 4, ×) for S(1) and S(2) combined. Therefore, both at 37°C (15) and at 41.5°C, cisplatin induced more chromosomal aberrations when cells were treated in G_1 than when they were treated in late S. Note that the points (×) for S(1) and S(2) combined are not simply the arithmetic mean between S(1) and S(2), because the fraction of the treated population entering the first mitosis is greater that the fraction entering the second mitosis (Fig. 1; mitotic indices not shown). As observed at 37°C (15), the ratio of breaks to exchanges generally decreased as the frequency of exchanges increased because the frequency of exchanges was propor-

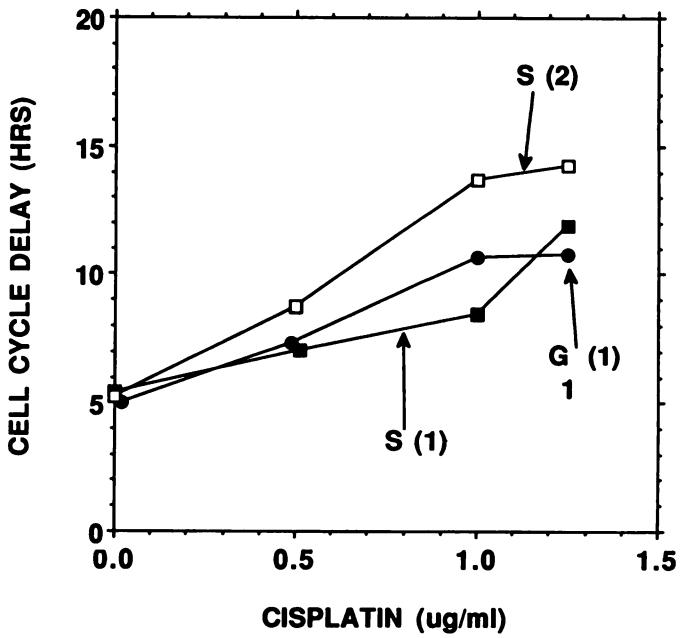

Fig. 2. Cell cycle delay (division delay) was estimated from cumulative mitotic indices (adding mitotic indices values over 3–4 4-h intervals). The delay is the time difference between untreated cells (0–37°C) and treated cells at 30% cumulative mitotic index. ■, ●, first mitosis (1); □, second mitosis (2). Linear regression analyses gave values with SEMs for the intercepts and slopes, respectively, of: 5.0 ± 0.5 and 5.0 ± 0.6 for G1(1); 5.0 ± 1.0 and 4.8 ± 1.2 for S(1); and 5.3 ± 0.6 and 7.6 ± 0.8 for S(2). Only S(2) was significantly different (P = 0.05) from the others.

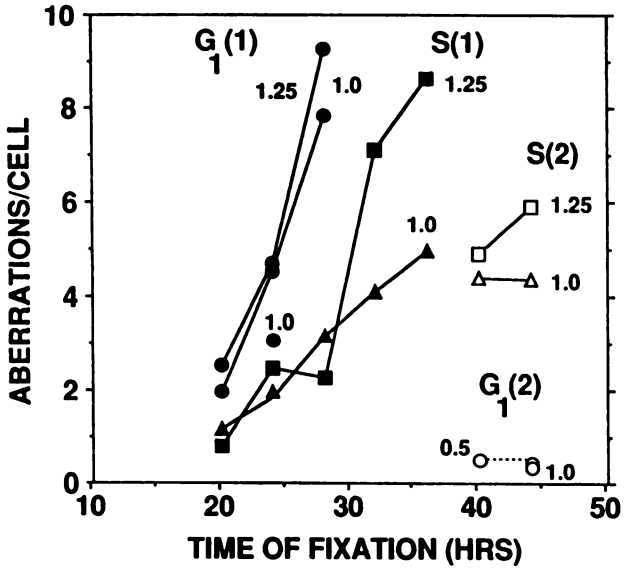

Fig. 3. Aberrations per cell *versus* time of fixation show that chromosomal damage increased as the cells sustained an increase in cell cycle delay. The phase in which the cells were treated is indicated, and the numbers in parentheses indicate whether the cells were scored in the first mitosis (1) (●, ■, ▲) or second mitosis (2) (○, □, △). The concentrations (μg/ml) of cisplatin used are indicated next to each line and/or symbol. The SEMs for individual points were about ±0.2 for 1–3 aberrations/cell, about ±0.3 for 4–5 aberrations/cell, and about ±0.4 for 6–8 aberrations/cell.

tional to a higher power of the cisplatin dose than the frequency of breaks (data not shown). Also, as observed at 37°C (15), the frequency of breaks relative to the frequency of exchanges for cells scored in the first mitosis was higher for cells treated in late S than for those treated in G_1 (data not shown).

Data in Table 1 indicate that primarily chromatid-type aberrations were induced in cells scored in the first or second mitosis after they were treated in G_1 or late S. Because chromatid-type aberrations in the first mitosis should be converted into chromosome-type in the second mitosis (19), the failure to observe a large number of cells with chromosome-type aberrations when cells were scored in the second mitosis suggests that many of the cells having chromatid-type aberrations in the first mitosis failed to enter the second mitosis. Although most of the aberrations scored in the second mitosis [$G_1(2)$] after cells were treated in G_1 were of the chromosome type, the frequency of aberrations was only one-tenth of that observed in cells entering the first mitosis [$G_1(1)$] (Figs. 3 and 4).

Cytological Damage Compared with Cell Killing. Results in Fig. 5*A* indicate that cells treated in late S at 41.5°C with low concentrations of cisplatin (<0.7 μg/ml) were more sensitive than cells treated in G_1. However, cells treated in G_1 at higher concentrations of 1.0 or 1.5 μg/ml were more sensitive than cells treated in late S. This difference between the high and low concentrations is due to selective killing of S-phase cells by heat alone (17). When the curves are normalized (Fig. 5*B*) to eliminate the difference in heat killing between G_1 and late S, the data show that the G_1 cells were more sensitive at 41.5°C to all concentrations of cisplatin than the late S cells. In fact, the results at 41.5°C were qualitatively similar to those observed at 37°C (Fig. 5*B*, *dotted curves*) if the concentration of cisplatin at 41.5°C is reduced by ~4-fold.

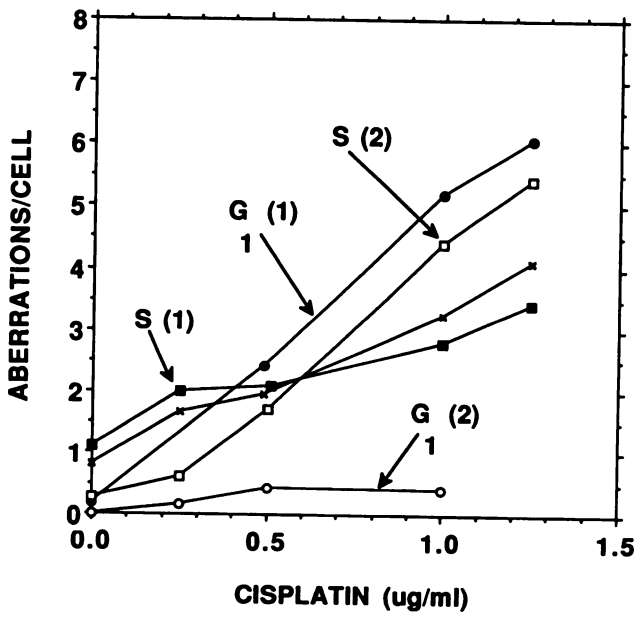

Fig. 4. Aberrations per cell *versus* cisplatin concentration for cells treated for 1 h at 41.5°C in G_1 or late S and analyzed in the first (1) or second (2) mitosis after treatment. The × points are the values for S(1) and S(2) combined. The experimental protocol and times of fixation were as described in Fig. 1. Each point represents the mean aberration frequency obtained from 3–4 samples (5–7 samples for S(1) and S(2) combined; ×), with the individual frequencies weighted by the mitotic indices; one exchange was counted as two aberrations. For each sample, 50 metaphases were analyzed for breaks and exchanges; 95% of the exchanges were of the chromatid type. Linear regression analyses gave values with SEMs for the intercepts and slopes, respectively, of: 0.15 ± 0.17 and 4.8 ± 0.2 for $G_1(1)$; 1.3 ± 0.2 and 1.6 ± 0.2 for S(1); −0.16 ± 0.28 and 4.4 ± 0.4 for S(2); and 0.85 ± 0.12 and 2.5 ± 0.2 for S(1) and S(2) combined (×). These results show that the largest number of aberrations were induced by cisplatin after the cells traversed through an entire S phase, *i.e.*, $G_1(1)$ and S(2); after normalizing for the aberration frequency for heat alone, the curves for G1(1) and S(2) are significantly different (P = 0.01) from the curves for S(1) and $G_1(2)$.

Table 1 *Percentage of aberrations due to chromosome type*

Sample	Deletions	Exchanges
$G_1(1)$ 0.5–1.25 μg/ml	9.0	2.9
$G_1(2)$ 0.5–1.0 μg/ml	83	63[a]
S(1) 0.25–1.25 μg/ml	7.4	2.5
S(2) 0.25–1.25 μg/ml	15.5	3.3

[a] Note in Fig. 3 that the aberration frequency was only 0.4 and about one-tenth of that for $G_1(1)$.

The comparison between surviving fraction and frequency of chromosomal aberrations is shown in Fig. 6. The values not normalized for heat killing show that for low aberration frequencies, where the main effect is due to heat alone, the correlation between aberrations per cell and survival is better between S(1) and $G_1(1)$ than between S(2) and $G_1(1)$. However, for high aberration frequencies (>2 for about 0.5 μg/ml; Fig. 4), where the main effect is due to cisplatin cytotoxicity, the correlation between S(2) and $G_1(1)$ is quite good. In general, the correlation between surviving fraction and aberrations per cell is similar for cells treated in G_1 or late S at either 41.5°C or 37°C (Fig. 6, *curves without data points*, taken from Ref. 15). In particular, the × points for the aberration frequencies for S(1) and S(2) combined correlate well with the other points plotted for $G_1(1)$ for 41.5°C and for $G_1(1)$ and S(2) for 37°C. This positive correlation between cell killing and cytological damage suggests that cisplatin administered at 37°C or 41.5°C to cells in G_1 or late S causes cell lethality primarily by the induction of chromosomal aberrations that result from lethal intrastrand and/or interstrand cross-links in the DNA.

DISCUSSION

The variation between G_1 and late S in sensitivity to cell killing and induction of chromosomal aberrations when cells are treated with cisplatin at 41.5°C is essentially the same as that observed for treating at 37°C if the selective heat killing during S phase is subtracted (Figs. 4–6). Cisplatin is more cytotoxic when administered during G_1 than during late S; however, administration at 41.5°C compared with 37°C causes an ~4-fold reduction in the concentration of cisplatin required for an isoeffect (Fig. 5). In addition, heating late S-phase cells for 1.0 h at 41.5°C without cisplatin reduces the survival to ~50% and induces ~1 chromosomal aberration/cell. In contrast, heating G_1 cells for 1.0 h at 41.5°C has little effect on survival (80%) and induces an aberration frequency of only 0.22 (0.07 for controls). Because of this selective heat killing of S-phase cells compared with G_1 cells, late S cells are killed more readily than G_1 cells for treatments at 41.5°C with cisplatin concentrations less than ~0.7 μg/ml that kill less than ~70% of the cells. Then, as the cisplatin concentration is increased above ~0.7 μg/ml, the cell cycle response for cisplatin cytotoxicity predominates, and the G_1 cells are killed more readily than late S cells. Note that if the data for cell killing are normalized for the heat effect alone (Fig. 5*B*), cells in G_1 are more sensitive than those in late S to treatment at 41.5°C with all concentrations of cisplatin, *i.e.*, essentially the same as that observed at 37°C if the cisplatin concentration at 37°C is increased ~4-fold. This increase in cisplatin cytotoxicity at 41.5°C has clinical implications, and the use of heat to selectively kill the S-phase cells exposed to very low concentrations of cisplatin also may have clinical significance.

The reason that cells treated with cisplatin during G_1 are more sensitive that those treated during late S has been discussed previously (15). Briefly, the hypothesis was presented (11, 15) that this increase in sensitivity when cells are treated in G_1 is due to damage in the DNA as replication of the genome occurs on a template containing cisplatin

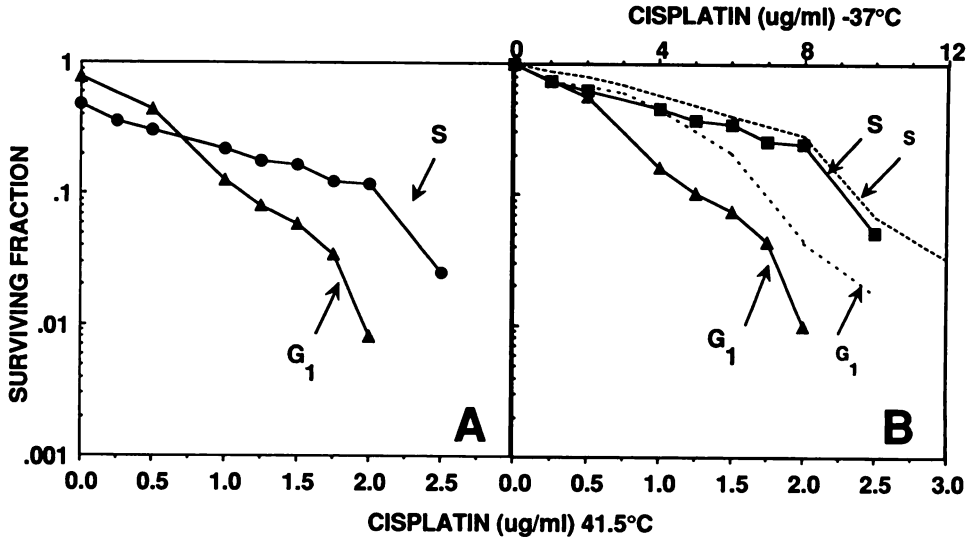

Fig. 5. Surviving fraction *versus* cisplatin concentration for cells treated during G_1 or late S at 41.5°C for 1 h. In *A*, survival has not been normalized for killing from heat alone. In *B*, survival has been normalized for heat killing; the *upper abscissa and dashed curves without data points* are for cells treated for 1 h at 37°C (15).

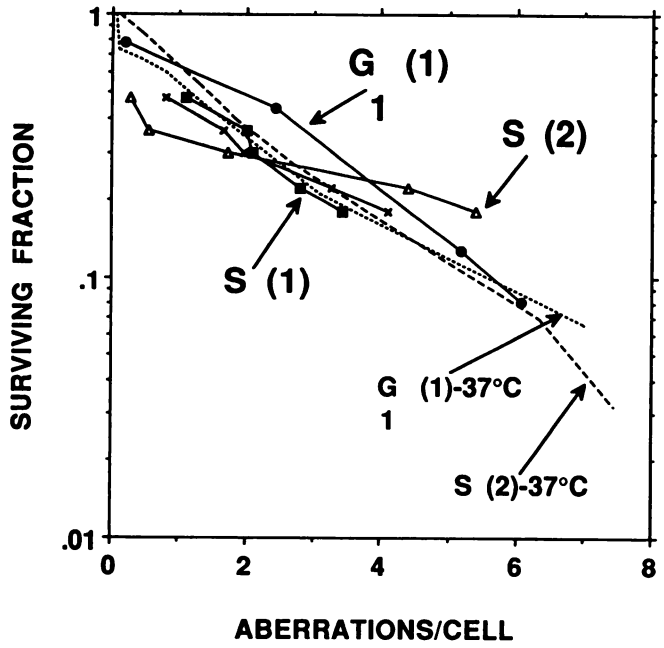

Fig. 6. Surviving fraction *versus* aberrations per cell for cells treated for 1 h at 41.5°C with cisplatin in G_1 or late S and scored for aberrations in the first (1) or second mitosis (2). ×, average values for S(1) and S(2) combined when 5–7 individual aberration frequencies for each point were weighted by their mitotic indices and averaged. Data were taken from Figs. 4 and 5 without any normalization for killing or aberrations induced by heat alone. A surviving fraction of 0.37 corresponds to about one aberration per cell if an exchange is counted as one aberration. For comparative purposes, *dotted and dashed lines without data points* (plotted in Ref. 15) are shown for cells treated in G_1 or late S at 37°C for 1 h and then scored in the first mitosis [G_1(1)] or the second mitosis [S(2)].

interstrand or intrastrand cross-links that were formed before the initiation of DNA synthesis. By the time cells treated in late S begin a whole round of DNA replication, many of the cross-links should have been removed (12, 20, 21); therefore, as observed (Ref. 15; Fig. 5; Fig. 4, G_1(1) *versus* × *points*), there should be less chromosomal damage and correspondingly less cytotoxicity when cells are treated in late S compared with G_1. The same phenomenon probably occurs at 37°C and 41.5°C, and the reduction in the cisplatin dose required for an isoeffect is probably attributed to heat causing an increase in cisplatin cross-links in the DNA (22). An increase in cross-links at 41.5°C could occur because of (*a*) an increase in the transport of cisplatin into the cell and/or a decrease in efflux from the cell (23, 24) and/or (*b*) an increase in reactivity at 41.5°C of cisplatin with the DNA. Since the ability of cells to remove or repair cisplatin cross-links in the DNA appears to be important in cisplatin cytotoxicity (25–29), heat which inhibits repair of radiation-induced DNA strand breaks (30, 31) may also reduce the ability of the cells to remove or repair cisplatin cross-links. Further studies are needed to address these questions.

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