DNA content of cells with generalized chromosome shattering induced by ultraviolet light plus caffeine

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Summary

Asynchronously growing Chinese hamster cells (M3-1) were UV-irradiated (λ = 254 nm) and then incubated with/without caffeine (2 mM) for 20 h. Microscopic evaluation of metaphase spreads revealed that after UV-irradiation alone (5.0 J/m²) and caffeine treatment alone, the percentage of cells with condensed chromatin appearing fragmented and/or pulverized ('GCS-like' cells; GCS, Generalized Chromosome Shattering) was very low while it was high following the combined treatment. Cytogenetic and flow cytometric analysis of cells obtained by mechanical shaking cultures treated with UV and caffeine indicated that 'GCS-like' cells have the same DNA content as untreated cells in G2 phase and mitosis.

In a number of cell strains, especially in rodent cells, the clastogenic action of a variety of agents (e.g. UV light or alkylating substances) is synergistically enhanced by posttreatment with caffeine (Kihlman et al., 1974; Kihlman, 1977; Roberts, 1978). A particular characteristic of Chinese hamster cells following UV-irradiation (λ = 254 nm) plus caffeine posttreatment is the frequent appearance of cells in which the nuclear material condenses and takes on a 'shattered' appearance (Love-lace, 1954; Chu, 1965; Nilsson and Lehmann, 1975; T. Cremer et al., 1978). Cells with such characteristics are not found immediately following irradiation but are

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Abbreviations: GCS, generalized chromosome shattering; PCC, premature chromosome condensation.
observed after a delay of several hours the frequency being dependent on UV fluence and caffeine concentration (C. Cremer et al., 1980, 1981); dose–effect curves (C. Cremer et al., 1981) indicate the existence of a certain 'threshold'.

The formation of cells with a 'shattered' appearance of the nuclear material is thought to be due to the fragmentation/pulverization of chromosomes of mitotic cells and is referred to as 'generalized chromosome shattering' (GCS). However, GCS is not the only possible explanation of cells with condensed, fragmented/pulverized chromatin. Studies of cell fusion between mitotic and interphase cells (Johnson and Rao, 1970; Nichols, 1970; Sperling and Rao, 1973) have shown that interphase nuclei can be induced to form condensed chromosomes (premature chromosome condensation, PCC). The cells in S phase at the time of fusion form chromosomes that appear pulverized and which are morphologically very similar to cells displaying GCS after treatment with UV light and caffeine. A 'GCS-like' appearance was also observed in prematurely condensed chromosomes of UV-irradiated cells in G1 phase (Waldren and Johnson, 1974; Schor et al., 1975). Chromosome fragmentation/pulverization has also been obtained without cell fusion following a treatment associated with a breakdown of DNA synthesis (Kihlman et al., 1963; Hsu et al., 1964; Freed and Schatz, 1969; Newsome and Littlefield, 1975; Kihlman, 1977). These facts indicate that cells with 'GCS-like' appearance might not be mitotic cells, but instead might be interphase cells in G1 or S with condensed/pulverized chromatin. If so, then their DNA content should be considerably lower than the DNA content of 'normal' untreated metaphase cells.

Flow cytometry (for review, see Horan and Wheeless, 1977; Melamed et al., 1979) has provided a reliable approach for assessing relative DNA contents of various cell types. Compared with microspectrophotometry, its advantages are rapidity, statistical precision, and quantitative accuracy. Here, we present the result of flow cytometric measurements of the DNA content of UV- and caffeine-treated Chinese hamster cells that indicate that 'GCS-like' cells have the same DNA content as untreated cells in G2 phase and mitosis (referred to as 'normal' G2M content). This suggests that the 'pulverized' appearance of cells treated with UV and caffeine is indeed due to generalized chromosome shattering of mitotic cells as commonly supposed and not to condensation of nuclear material in interphase cells with DNA content markedly lower than G2M.

Materials and methods

Cell culture

In this investigation, a derivative (650A) of the M3-1 Chinese hamster cell line (Gray et al., 1975) was used. The cells were grown in minimum essential medium (α-MEM) plus 10% fetal calf serum (FCS). The cell cycle transit time of asynchronously growing cells was determined to be approx. 11 h (Gray, 1980).

Asynchronously growing cells were inoculated into large (d: 10 cm) or small (d: 5 cm) plastic petri dishes at $2 \times 10^3$ cells/cm². 2 days later, the cells were irradiated (5 J/m²) with a germicidal lamp emitting predominantly at 254 nm. Prior to
irradiation the medium was removed and the cells were washed once with phosphate-buffered saline solution (PBS) and then covered with a 1-mm layer of PBS. The cells were irradiated from above at a fluence rate of 0.12 W/m². Other cultures were treated identically except they received no irradiation. Immediately after the UV treatment, the cells were incubated at 37°C in α-MEM with 10% FCS with or without caffeine (2 mM). 15 h later, colcemid (0.033 μg/ml) was added. 20 h after irradiation, cells were prepared for cytogenetic analysis or for DNA-content analysis.

**Cytogenetic analysis**

The cells in the small petri dishes were exposed for 30 min to cold (4°C) hypotonic solution (0.075 M KCl). Then fixative (acetic acid:methanol, 1:3) was slowly added with a syringe, and the hypotonic solution was gradually replaced by adding fixative. Under these conditions, the frequency of burst mitotic cells was very low. The preparations were air-dried and stained with acetic orcein. The cells grown in large petri dishes were mechanically detached by shaking, spun down, resuspended in cold (4°C) KCl (0.075 M) for 30 min, again centrifuged and resuspended in acetic acid:methanol (3:1) for 60 min. The cells were then centrifuged, resuspended in 0.3–0.5 ml acetic acid:methanol (1:3), dropped onto the bottom of a petri dish, air-dried and stained with acetic orcein. These preparations were scored microscopically to determine the fraction of cells displaying a ‘GCS-like’ morphology (all chromosomes fragmented and/or pulverized) (a) as a percentage of the total number of cells (%GCSₜ), and (b) as a percentage of cells with condensed, chromosome-like chromatin (%GCSₘ). The morphology of the ‘GCS-like’ cells as observed here was the same as previously observed in Chinese hamster V79 cells following UV plus caffeine treatment (C. Cremer et al., 1980, 1981).

**Preparation and staining for flow cytometry**

Cells in the large petri dishes were mechanically detached by the mitotic shake off procedure, spun down, resuspended for 30 min in 70% ethanol, again centrifuged and dispersed for 30 min in Tris-buffered isolation medium (Gray et al., 1979). The DNA-specific dyes Hoechst 33258 (HO; Latt and Wohlleb, 1975) and chromomycin A3 (CA3; Behr et al., 1969) were added to about 5 × 10⁵ cells in 1 ml isolation buffer; final concentrations of HO and CA3 were 2 and 80 μg/ml, respectively. Preparations were stored at 4°C prior to flow cytometric analysis.

**Flow cytometry and cell sorting**

The HO-fluorescence (DNA content) distributions of ethanol-fixed cells were measured with the Livermore Flow Cytometer (single beam flow cytometer) utilizing a Spectra Physics argon ion laser (171-05) operating at 351.1 plus 363.8 nm (referred to as UV-excitation) with a power of 0.6 W. Fluorescence was collected through a 450-nm-long wavelength pass filter. The percentages of cells in the G1, S and G2M parts of the fluorescence distributions were determined by computer analysis using a procedure similar to that reported by Dean and Jett (1974).

Sorting of cells was performed using a modified FACS II system equipped with a
### TABLE I

**PROPERTIES OF CHINESE HAMSTER M3-1 CELLS RELATING TO THE ESTIMATION OF THE FRACTION OF CELLS WITH G2M DNA CONTENT DISPLAYING 'GCS-LIKE' CHROMATIN**

<table>
<thead>
<tr>
<th>Undisturbed cultures</th>
<th>Cells selected by mechanical shaking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>%GCS, %GCS&lt;sub&gt;m&lt;/sub&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>0.0 0</td>
</tr>
<tr>
<td>Caffeine alone 2 mM</td>
<td>0.0 0</td>
</tr>
<tr>
<td>UV alone 5 J/m²</td>
<td>0.1 1</td>
</tr>
<tr>
<td>UV+caffeine 5 J/m², 2 mM</td>
<td>10 86</td>
</tr>
</tbody>
</table>

%GCS<sub>m</sub> = (number of cells with 'GCS-like' appearance/number of cells with condensed, chromosome-like chromatin) $\times$ 100, as determined from chromosome preparations. At least 200 cells were scored in each case.

%GCS = %GCS<sub>m</sub> × MI, where MI = (number of cells with condensed, chromosome-like chromatin)/(total number of cells). For determination of MI, approx. 500 cells were scored.

<sup>a</sup> relative G2M/G1 ratio = (mean of peak B (Fig. 1) divided by the mean of peak A (Fig. 1))/(average mean of peak B for controls divided by the average mean of peak A for controls). Listed are average ratios for n independent experiments. The numbers in parentheses indicate the range of observed ratios.

<sup>b</sup> %G2M = (number of cells with G2M content)/(total number of cells in the distribution) $\times$ 100. %G2M was determined by computer evaluation of flow histograms with a procedure similar to that of Dean and Jett (1974). Listed are average percentages. The numbers in parentheses indicate the range of percentages observed in n independent experiments (see *).

<sup>c</sup> %G2M<sub>sort</sub> = (number of cells with condensed, chromosome-like chromatin sorted from the right half of peak B, Fig. 1)/(total number of cells sorted from right half of peak B) $\times$ 100. In each case, at least 200 sorted cells were scored.
Spectra Physics 171-05 argon ion laser. Again, the HO-fluorescence induced by UV excitation (power 0.6 W) was measured through a 450-nm-long wavelength pass filter. Cells with fluorescence intensities appropriate for G1, S, or G2M DNA contents were sorted onto separate microscope slides ($1 \times 10^4$ cells each) and mounted with cover slides. Microscopic evaluation of the sorted cells was made using a fluorescence incident light microscope (Zeiss) equipped with phase contrast facility. For each treatment regimen and sorting region, 100–200 sorted cells were evaluated.

Results

1. Cytogenetic analysis

   The percentages of Chinese hamster cells in undisturbed cultures showing 'GCS-like' morphology are listed in Table 1. Very few 'GCS-like' cells were observed for control cultures or for cultures exposed to UV alone or to caffeine alone. However, the $\%GCS_t$ and $\%GCS_m$ increased sharply to 10 and 86, respectively, after treatment with UV and caffeine. Likewise, in cells selected by mechanical shaking, the incidence of 'GCS-like' cells is near zero for all cultures except those treated with UV and caffeine where $\%GCS_t = 38$ and $\%GCS_m = 87$.

2. DNA distribution measurement and cell sorting

   Fig. 1 shows HO-fluorescence distributions obtained from M3-1 cells. In all cases, 2 peaks labeled A and B are visible. As Fig. 1a (untreated controls) shows, peak A and peak B correspond to cells with G1 and G2M DNA content, respectively. The fluorescence distributions for control, UV-treated, and caffeine-treated cells are very similar (Fig. 1a–c). The fluorescence distribution for cells treated with both UV-irradiation and caffeine (Fig. 1d) is similar to the other distributions except it shows an increased continuum caused by particles with less than a G2M fluorescence.

   The cells sorted from peak A (see Fig. 1) consisted for 96–99% of cells with interphase nuclei for all treatment regimens. The same result was obtained if cells with S-phase DNA content (up to mid-S phase) were sorted. The percentages of cells in peak B (G2M), the relative G2M/G1 peak mean ratios, and the percentages of cells with condensed chromosome-like chromatin are listed in Table 1. For all treatment regimens, the difference in the relative G2M/G1 peak mean ratios are not significant or small ($\leq 6\%$). In particular, no significant difference was observed between cells treated with caffeine alone ($\%GCS_t = 0$) and cells treated with UV plus caffeine ($\%GCS_t = 38$). It is concluded that peak B in fluorescence distributions of UV- and/or caffeine-treated cells corresponds to a 'normal' G2M content.

Data analysis

   The measurements reported in this paper are sufficient to estimate the fractions of 'GCS-like' cells with G2M DNA content in the cells mechanically detached from cultures treated with UV plus caffeine. To accomplish this, we assume the cells to be divided into 5 subpopulations: (1) 'GCS-like' cells with G2M DNA content, (2)
Asynchronously growing M3-I cells were UV-irradiated with 0, and 5.0 J/m², and postincubated with or without caffeine (2 mM) for 20 h. Thereafter cells obtained by the mitotic shake-off procedure were fixed, stained with Hoechst 33258, and chromomycin A3, and the fluorescence (Hoechst) was measured in a flow cytometer. Ordinate: number of cells with a given fluorescence intensity. Full scale: 1000 cells per channel for Fig. 1a–c, and 300 cells per channel for Fig. 1d. Abscissa: channel number (proportional to HO fluorescence). Shown is a linear display; the scale is the same for all distributions (full scale: 256 channels). (a) no UV, no caffeine; (b) no UV, 2 mM caffeine; (c) 5.0 J/m², no caffeine; (d) 5.0 J/m², 2 mM caffeine. The left (G1) and right (G2M) peaks are designated A and B, respectively.

'GCS-like' cells with interphase DNA content (< G2M), (3) mitotic cells of normal metaphase chromosome morphology, (4) G2-phase cells and (5) interphase cells (DNA content < G2M) that are not 'GCS-like'. 4 experiments provided the information necessary to calculate the fractions of cells in each subpopulation. These measurements and the equations used to solve for the subpopulation fractions are listed in Table 1 and the Appendix, respectively. These calculations showed the fraction of 'GCS-like' cells with interphase DNA content (< G2M) to be approximately zero (actually −0.03). Thus, all 'GCS-like' cells were found to have the G2M DNA content. For these estimates, mean values (Table 1) were used. The upper limit for the fraction of 'GCS-like' cells with less than the G2M DNA content was determined as the largest fraction of such cells compatible with the range of experimental data in Table 1. This fraction was estimated to be 0.15 so that at least two-thirds of 'GCS-like' cells have G2M DNA content. In fact, the estimates made using the mean values of Table 1 suggest that all 'GCS-like' cells have G2M DNA content.
Discussion

A number of studies has been performed concerning the effect of caffeine on the rate of DNA synthesis ([13H]thymidine incorporation) following treatment with chemical or physical agents (for review see Roberts, 1978); in addition, the effect of UV plus caffeine treatment on cell cycle parameters has been studied (C. Cremer et al., 1980, 1981). So far, however, no direct measurements of the DNA content of cells after UV-irradiation (λ ≈ 254 nm) and caffeine postincubation have been reported.

In this investigation, flow cytometry and cell sorting, in combination with microscopic examination of sorted cells, and of chromosome preparations, respectively, were used to estimate the DNA content of 'GCS-like' Chinese hamster M3-1 cells following UV-irradiation (λ ≈ 254 nm) and posttreatment with caffeine. Since an exact determination of G2M/G1 ratios and of the fraction of cells in G2M may be considerably complicated in a cell population of highly heterogeneous DNA content (Dean and Jett, 1974; Gray, 1976), it was of advantage to select cells by the shake-off procedure instead of using trypsinized cultures. This approach resulted in essentially bimodal distributions and greatly facilitated evaluation. A comparison of undisturbed cultures and suspensions of cells selected by mechanical shaking showed (Table 1) that the number of cells with 'GCS-like' appearance divided by the number of all cells with condensed, chromosome-like chromatin (%GCSm) was practically identical. Thus, the shake-off technique employed did not potentially select for or against 'GCS-like' cells. A quantitative comparison (Table 1) of the bimodal fluorescence distributions indicated that for all treatment regimens, peaks A and B (Fig. 1) correspond to cells with 'normal' G1 DNA content and 'normal' G2M DNA content (= DNA content of untreated cells in G1 and G2M).

Direct sorting of cells with G1 DNA content and of cells with DNA content up to mid-S phase showed that practically all cells with this DNA content were cells with interphase nuclei, independent of the treatment regimen. Thus, the possibility of a G1-PCC-related phenomenon (Waldren and Johnson, 1974; Schor et al., 1975) or of PCC occurring in the first half of S phase (Johnson and Rao, 1970; Sperling and Rao, 1973) may be ruled out as an explanation for the induction of cells with GCS under the conditions used. Furthermore, the results presented in Table 1 and in the Appendix indicate that under these conditions all or at least the large majority of cells with 'GCS-like' appearance have a G2M DNA content. Instead of PCC of cells in G1 or S phase, a more subtle process might be considered which results in a failure of normal chromosome condensation after most or all of the DNA has been replicated (T. Cremer et al., 1981).

It is interesting to note that cells with 'GCS-like' morphology were recently observed also following near-UV irradiation (λ ≈ 365 nm) of V79 cells in the presence of psoralen (PUVA treatment) and postincubation with caffeine (T. Cremer et al., 1981). In this investigation, preliminary results of Feulgen measurements were presented which suggested that also in this case 'GCS-like' cells have a 'normal' G2M DNA content. This lends further support to the notion (T. Cremer et al., 1981) that the induction of cells with GCS by PUVA plus caffeine treatment and by
far-UV light (\(\lambda = 254 \text{ nm}\)) plus caffeine postincubation may be closely related to each other.

Acknowledgement

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References


Appendix

The cell population selected by mechanically shaking a Chinese hamster M3-1 culture previously treated with UV and caffeine can be considered to have 5 components:

A = fraction of 'GCS-like' cells with G2M DNA content;
B = fraction of mitotic cells with normal chromosome morphology;
C = fraction of G2 interphase cells;
D = fraction of 'GCS-like' cells with less than G2M DNA content;
E = fraction of cells with less than the G2M DNA content excluding 'GCS-like' cells.

Experimental measurements of various combinations of these components allow development of 5 equations containing the components to be estimated. The experimental results and the corresponding equations are listed below.
Measurement or approximation *  Equation

1. The G2M peak makes up 68% of the total DNA distribution  \( \frac{(A + B + C)}{(A + B + C + D + E)} = 0.68 \)
2. 'GCS-like' cells make up 87% of the cells with condensed chromatin  \( \frac{(A + D)}{(A + B + D)} = 0.87 \)
3. The 'GCS-like' cells make up 38% of the total population  \( \frac{(A + D)}{(A + B + C + D + E)} = 0.38 \)
4. Cells with condensed chromatin make up 69% of the cells with G2M DNA content  \( \frac{(A + B)}{(A + B + C)} = 0.69 \)
5. All cells in the population are included in the DNA distribution  \( A + B + C + D + E = 1.00 \)

* Mean values as obtained from Table I. These equations were solved simultaneously to yield the following: \( A = 0.41, B = 0.06, C = 0.21, D = -0.03, E = 0.37 \). However, experimental uncertainty was associated with each measurement. The effect of varying the experimental values on the solutions was studied, using the observed range in case of %G2M (see Table 1). For %GCS, %GCSm, and %G2M ort, the limits of the 95% confidence intervals (C. Cremer et al., 1980), respectively, were used. These studies showed that the largest possible value for D, the fraction of 'GCS-like' cells with less than the G2M content, was 0.15 and that the minimum fraction of 'GCS-like' cells with G2M content was 0.30.

Erratum


In this paper, two sentences are missing in the legend to Fig. 6 (p. 341). These two sentences have to be added at the end of this legend and read as follows:

Two out of a total of 181 control cells (0 nJ) showed shattered chromosomes in this experiment (1 class D, 1 class E). Note that in the control distribution (left column) the scale for damage classes D and E is magnified by a factor of six.