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Induction of Chromosome Damage by Ultraviolet Light and Caffeine: Correlation of Cytogenetic Evaluation and Flow Karyotype[1]

C. Cremer[2], T. Cremer, and J. W. Gray
Biomedical Sciences Division, Lawrence Livermore National Laboratory, Livermore, CA 94550 (C.C., J.W.G.) and Institute of Anthropology and Human Genetics, University of Heidelberg, D-6900 Heidelberg, Fed. Rep. of Germany (T.C.)

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Asynchrononously growing cells of a M3-1 Chinese hamster line were ultraviolet (UV) irradiated (λ = 254 nm) with UV fluences up to 7.5 J/m². After irradiation, cells were incubated with or without 2 m*M* caffeine for 20 hr, then mitotic cells were selected by mechanical shaking. Their chromosomes were isolated, stained with Hoechst 33258 and chromomycin A3, and measured flow cytometrically. While the fluorescence distributions of chromosomes (flow karyotypes) from cells treated with UV alone or with caffeine alone were very similar to those of untreated controls, the flow karyotypes of UV + caffeine-treated cells showed a debris continuum that increased with increasing UV fluence suggesting an increased number of chromosome fragments. Visual evaluation of metaphase plates revealed that the percentage of cells with chromosome damage also increased steadily with increasing UV fluence. A high degree of correlation was observed between the relative magnitude of the debris level from flow karyotypes and the percentage of cells with chromosome damage and with generalized chromosome shattering, respectively, as determined from metaphase spreads.

Key terms: Flow cytometry, chromosomes, ultraviolet light, caffeine, chromosome damage

Recently, flow cytometry (12, 17) has been shown to be useful in chromosome classification (1, 2, 10, 11, 15, 22). In this approach, mitotic cells are collected and mechanically sheared to release the chromosomes. These chromosomes are then stained with a DNA specific fluorescent dye and analyzed flow cytometrically to produce a fluorescence distribution pattern showing peaks caused by chromosomes with the same stain fluorescence being superimposed on a continuum that is caused by chromosomes or chromosome fragments with abnormal DNA content. This debris continuum declines rapidly with increasing DNA content and its magnitude has been shown (3) to correlate with chromosomal damage caused by clastogenic agents.

In the present paper, we apply this method to investigate the flow karyotype of Chinese hamster cells after treatment with ultraviolet (UV) light and caffeine. In rodent cells and in a number of other cell types, the clastogenic action of ultraviolet light is known to be synergistically enhanced by caffeine (for review see [14]). An interesting characteristic of Chinese hamster cells is the frequent occurrence of cells with generalized chromosome shattering (GCS) in which all chromosomes are fragmented and/or pulverized (4–8, 13, 14, 16, 19). The incidence of GCS after UV plus caffeine has been studied in detail by microscopic analysis of metaphase spreads (5–7).

In this paper, we demonstrate that the magnitude of the debris in flow karyotypes correlates well with the frequency of cells with GCS and with chromosome damage. These data support the use of flow karyotyping as a quantitative tool for the study of chromosome damage by other clastogenic agents.

Materials and Methods

Cell culture: The present studies were conducted using a derivative (650 A) of the M3-1 Chinese hamster cell line (10). These cells have a well defined flow karyotype as established cytologically and by univariate (10) and bivariate flow cytometry (11, 15). The cell cultures were maintained under standard conditions in minimum essential medium (MEM) (Gibco Grand Island, NY) plus 10% fetal calf serum. The cell cycle transit time of asynchronously growing cells was determined to be approximately 11 hr (9).

UV + caffeine treatment: Asynchronously growing cells were inoculated into large (10 cm diameter) or small (5 cm diameter) plastic Petri dishes at 2 × 10^3 cells/cm². Two days later, the cells were

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[2] On leave from the Institute of Human Genetics, University of Freiburg, D-7800 Freiburg i, Br., Fed. Rep. Germany.

washed once with phosphate buffered saline solution (PBS) and then covered with a 1-mm layer of PBS. After this, the cells were irradiated from above with a germicidal lamp emitting predominantly at 254 nm (fluence rate 0.12 W/m^2). Other cultures were treated identically except that they received no irradiation. Immediately after the UV treatment, the cells were incubated at 37°C in MEM with 10% fetal calf serum with or without caffeine (2 m*M*). Fifteen hours later, Colcemid (Sigma Chem. Co., St. Louis, MO) (0.033 μg/ml) was added. Twenty hours after irradiation, chromosomes were prepared for cytogenetic analysis or cells were collected for flow cytometric analysis.

Chromosome isolation for flow cytometry: Cells in large Petri dishes were mechanically detached by vigorous shake off. The mitotic fraction as determined by microscopic examination of cell suspensions varied considerably (mean value 66%, range ± 16%). However, no marked alterations of the flow karyotype were observed between samples with high and with low mitotic fraction if the same pretreatment was applied. After shake off, the cells were resuspended in hypotonic solution (0.075 *M* KCl) for 30 min and then resuspended in 0.5 ml Tris buffered isolation medium (25 m*M* Tris-HCl, pH 7.5, 0.75 *M* hexylene glycol, 0.5 mM $CaCl_2$, and 1 m*M* $MgCl_2$). The cells were mechanically ruptured in a VirTis homogenizer (VirTis Co., Gardiner, N.Y.) (21,500 rpm) to release the chromosomes and were stained in suspension by adding Hoechst 33258 plus Chromomycin A3 in chromosome isolation buffer to a final concentration of 2 μg/ml Hoechst 33258 and 16 μg/ml Chromomycin A3. Chromomycin A3 was added in all cases because of its presumptive stabilizing effect on chromosome morphology. Prior to flow cytometry, chromosome suspensions were examined by fluorescence microscopy. After UV treatment (5.0–7.5 J/m^2) alone or caffeine treatment alone, most chromosomes appeared to have a normal morphology. Only a few morphologically intact chromosomes were observed, however, after the combined treatment while the vast majority of fluorescing particles were small chromosome fragments. To investigate the possibility that these chromosome fragments were due to the mechanical fragmentation of abnormally fragile chromosomes during chromosome isolation, chromosome suspensions obtained from cells homogenized for different times T (20–200 sec) were examined. The time of homogenization was found to have little effect: chromosomes released from UV plus caffeine treated cells appeared fragmented after 20 sec homogenization time (shorter times were not investigated since chromosomes were not released from the cells at shorter times). On the other hand, normal chromosomes were obtained even at T = 200 sec from cells treated with UV alone or caffeine alone. This showed that if chromosomes are mechanically fractured during chromosome isolation, the fragmentation is complete even after the lightest possible shearing. For the flow cytometric experiments described in this paper, a homogenization time of 60 sec was used.

Flow cytometry: The Hoechst fluorescence distributions (flow karyotypes) of the Hoechst-Chromomycin A3 stained chromosome suspensions were measured with the Livermore Flow Cytometer (single beam flow cytometer) (Lawrence Livermore Ntl. Lab., Livermore CA) 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. Typical flow karyotypes showed several peaks superimposed on a debris continuum (Fig. 1). The relative area *C* of the continuum underlying the peaks in each flow karyotype (C = "debris level") was estimated as indicated in Figure 1. This procedure was chosen because the grossly distorted flow karyotypes obtained after the combined treatment with UV + caffeine (see "Results") could not be fitted in a satisfying way using the normal least squares best-fit techniques (11, 18) which approximate the fluorescence distribution as the sum of several normal distributions plus a smooth "continuum" function.

Chromosome preparation for cytogenetic analysis

The cells in the small Petri dishes were exposed for 30 min to 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. The preparations were air dried and stained with acetic orcein. Microscopic scoring of cells was performed as described (5–7). Briefly, all cells with condensed, chromosome-like chromatin were scored as mitotic. These cells were divided into three classes: normal mitotic cells, cells with some chromosome abnormalities but at least one normal chromosome, and cells with GCS. The percentages of mitotic cells in each class were designated %intact, %aberrant and %GCS, respectively. For each treatment, 200 mitotic cells were scored.

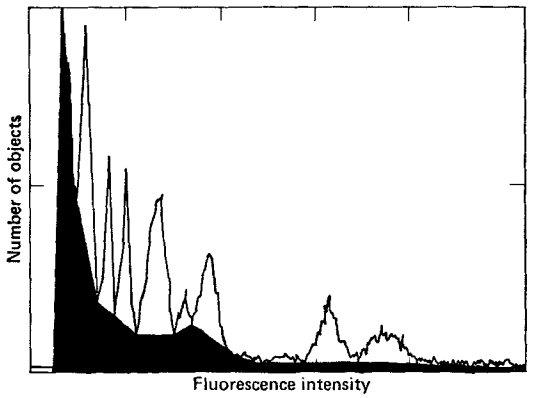

FIG. 1. Illustration of the procedure used to estimate the "debris level" (*C*) underlying the flow cytometric distribution. The minima of the peaks of the distribution were connected by a straight line. The area F_B underneath (*black*) and the total area F_o underlying the distribution was graphically determined and $C = F_B/F_o$ was calculated. This simple method can be applied both to normal and to grossly distorted flow karyotypes (Fig. 3). *C* as estimated here is a function of the coefficients of variation and of the amount of decreasing debris continuum. *C* increases with an increase of either of these parameters which are assumed to be correlated with the amount of chromosome aberrations present (3, 20).

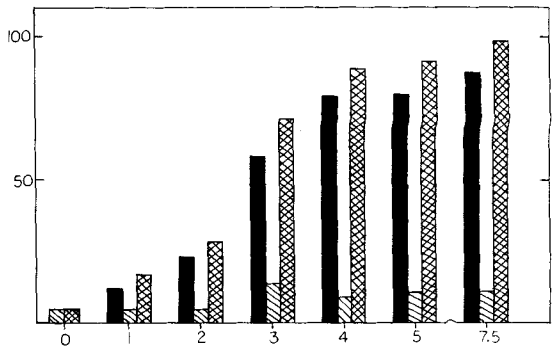

FIG. 2. Percentage of mitotic cells with chromosome alterations following UV irradiation and posttreatment with 2 m*M* caffeine (20 hr). *Ordinate:* percentage of mitotic cells with ■, generalized chromosome shattering (GCS) (all chromosomes fragmented and/or pulverized; % GCS); ▧, aberrations other than GCS (at least one chromosome intact; % aberrant); ▩, any kind of chromosome damage (% GCS + % aberrant). *Abscissa:* UV fluence (J/m^2). Each datum is the mean of an evaluation of duplicate cultures. For each treatment, 200 mitotic cells were scored.

Other experiments (Cremer and Gray; Mutat. Res., in press) show that the cells scored as GCS in the present experiments have a normal G_2M amount of DNA, supporting the notion that they are truly mitotic cells and not interphase cells expressing premature chromosome condensation.

Results

The increase in the frequency of mitotic cells showing GCS and other aberrations with increasing UV exposure is shown in Figure 2. The percentage of mitotic cells with GCS was very low after caffeine treatment alone. However, it increased drastically to almost 90% as the UV fluence increased to 7.5 J/m^2. At this dose, the percentage of mitotic cells with GCS

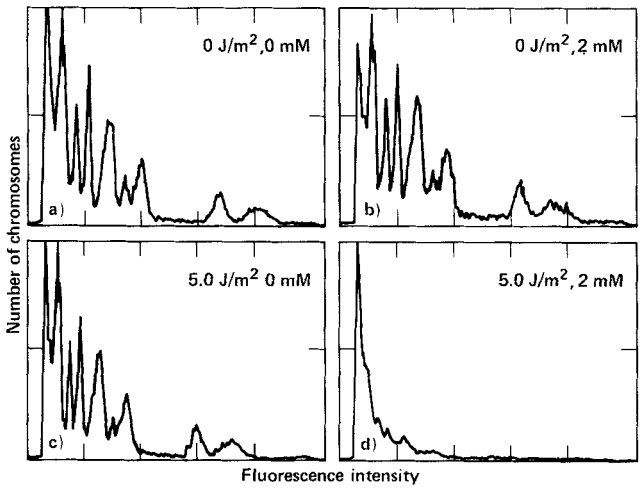

FIG. 3. Hoechst 33258 flow karyptypes of chromosomes from M3-1 Chinese hamster cells following UV irradiation (λ = 254 nm) and posttreatment with 2 m*M* caffeine for 20 hr. *a*, untreated control; *b*, caffeine incubation alone; *c*, UV irradiation alone (5 J/m^2); *d*, UV irradiation (5 J/m^2) plus posttreatment with caffeine.

Table 1
Effects of UV plus caffeine treatment on the debris level of Hoechst 33258 flow karyotypes

Treatment	Debris Level (%)
No UV, no caffeine	47.1 ± 4 (n = 4)
No UV, 2 m*M* caffeine	49.1 ± 5 (n = 3)
5.0 J/m^2, no caffeine	49.0 ± 6 (n = 3)
7.5 J/m^2, no caffeine	57.7 ± 3 (n = 4)
5.0 J/m^2, 2 m*M* caffeine	89.6 ± 5 (n = 3)
7.5 J/m^2, 2 m*M* caffeine	91.9 ± 7 (n = 4)

M3-1 cells were UV irradiated and post-treated with/without caffeine for 20 hr as indicated. In flow karyotypes, the "debris level" (given as a percent) as defined in Figure 1 was determined. Given is the mean ± range; n, number of independent experiments.

or other aberrations was almost 100%. UV irradiation alone, however, did not induce GCS (% GCS <1%; % aberrant ≤5%) in the dose range investigated. These results are similar to those described for Chinese hamster V79 cells (5, 6).

Other experiments (Cremer and Gray, Mutat. Res., in press) showed that the percentages of mitotic cells with GCS in cultures prior to shake-off and in chromosome preparations made from cell suspensions selected by the mitotic shake-off procedure were practically identical. Thus, the mitotic selection technique employed during the chromosome isolation procedure did not potentially select for or against cells with GCS.

Figure 3 shows the effect of UV + caffeine treatment on the flow karyotype. The flow karyotypes of cells exposed to UV irradiation alone (Fig. 3*c*) and of cells treated only with caffeine (Fig. 3*b*) are very similar to those for untreated control cells (Fig. 3*a*). However, the debris continuum in the flow karyotype of UV irradiated cells (5 J/m^2) postincubated with caffeine is increased so drastically (Fig. 3*d*) that no clear chromosome peaks are obtained. Table 1 gives a quantitative evaluation of the "debris level" of flow karyotypes after treatment with 5.0 and 7.5 J/m^2 and posttreatment with and without caffeine, respectively. To examine the reproducibility, a number of independent experiments was carried out and isolation and flow cytometric measurements were performed on different days. The results show that the "debris level" is not or is only slightly enhanced compared with controls (no UV, no caffeine) after UV treatment alone or caffeine treatment alone while it is drastically increased after the combined treatment. For a given treatment, the relative variation in the "debris level" was observed to be ≤10%. This variation includes differences in preparation quality or optical alignment of the flow cytometer. Figure 4 shows the relative debris level in the flow karyotypes (defined to be the ratio of the "debris level" (C) for sample flow karyotypes (Fig. 1) to the "debris level" of the flow karyotypes for untreated controls) as a

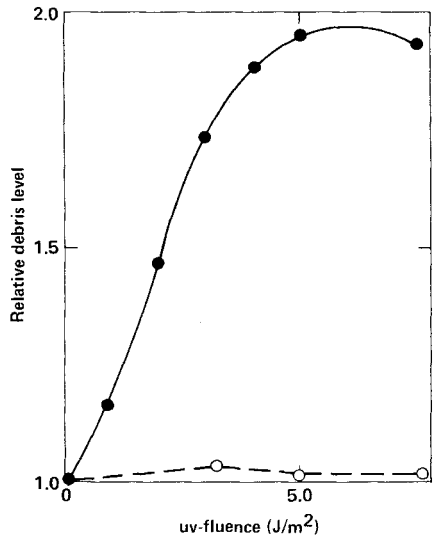

FIG. 5. Correlation between the relative debris level of flow karyotypes of cells irradiated with UV and posttreated with caffeine (Fig. 4), and the percentage of cells with GCS and with chromosome damage (% GCS + % aberrant, Fig. 2) as measured microscopically in chromosome preparations of identically treated cell cultures (same experimental setup). *Ordinate*, relative debris level; *abscissa*, percentage of cells with chromosome alterations. *A*, correlation for cells with GCS; *B*, correlation for all cells with chromosome damage (% GCS + % aberrant).

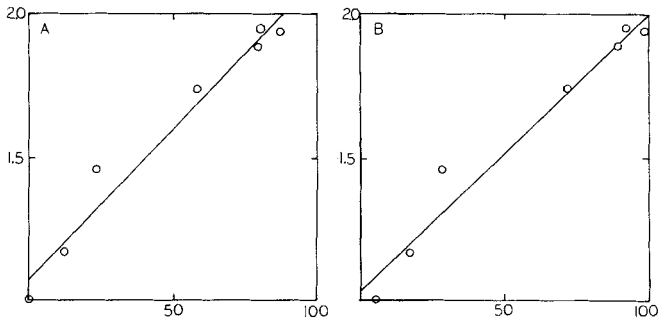

FIG. 4. Quantitative evaluation of flow karyotypes of M3-1 cells (Hoechst 33258 fluorescence) after UV irradiation and posttreatment (20 hr) with 2 m*M* caffeine. *Ordinate:* Relative debris level (see text). Each datum shown is based on fluorescence measurements of several thousand chromosomes. *Abscissa:* UV fluence (J/m^2). ○, Postincubation without caffeine (UV irradiation only); ●, postincubation in the presence of 2 m*M* caffeine. The chromosomes were isolated on the same day and the same apparative setup was used for all measurements.

function of the UV fluence for cells treated with UV and postincubated with or without caffeine.

While no marked increase of the relative debris level was observed following UV irradiation alone or caffeine treatment alone, the relative debris level was increased strongly with UV fluence after the combined treatment.

In Figure 5*A* the relative debris level following UV plus caffeine treatment is compared to the percentage of cells with GCS as obtained by microscopic examination of chromosome preparations of cells after an identical treatment. The two measurements are linearly correlated relative debris level: = $1.055 \times 10^{-2} \times$ % GCS + 1.081; correlation coefficient 0.98). Very similar results are obtained if the relative debris level is plotted as a function of the percentage of all cells with chromosome damage (% GCS + % aberrant; Figure 5*B*). In this case, the linear regression curve was found to be: relative debris level: = $0.97 \times 10^{-2} \times$ (% GCS + % aberrant) + 1.035; correlation coefficient 0.98. If only cells with aberrations other than GCS (% aberrant) were considered, the degree of correlation was observed to be considerably lower (correlation coefficient 0.82). This might be ascribed to the fact that under the conditions investigated, GCS was the predominant event (Fig. 2).

Discussion

The results presented here show that UV irradiation (up to 7.5 J/m^2) of Chinese hamster M3-1 cells and posttreatment with caffeine (2 m*M*) for an appropriate time (20 hr) results in a drastic change of the flow karyotype while no marked alterations were observed following UV irradiation alone or caffeine treatment alone. The relative debris level underlying the flow karyotype of caffeine treated cells increased steadily with UV dose suggesting an increasing number of mitotic cells with abnormal chromosomes (3). Microscopic examination of chromosome suspensions revealed a high frequency of chromosome fragments.

These results do not allow determination of whether the effects observed are due to a high number of chromosome breaks existing prior to chromosome isolation or to an increased chromosomal fragility. They do show, however, that if an increased chromosome fragility is the predominant factor, the chromosomes are so fragile that the disruption is complete after the minimum shearing time required to release normal chromosomes. Both possibilities are consistent with models for the induction of chromosome damage or GCS, by ultraviolet light and caffeine (7, 8, 14, 21). The high degree of correlation observed between the flow karyotype debris level and the percentage of cells with chromosome damage and with GCS following UV plus caffeine treatment suggests that flow cytometry might be used as a semiquantitative tool for rapid monitoring of effects of UV plus caffeine treatment. Since a flow cytometric measurement can be performed within a small fraction of the time required for conventional cytogenetic analysis, this might facilitate investigations on the action of ultraviolet light (or other mutagens) and caffeine on chromosomes.

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