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Giant cell formation produced by laser microbeam irradiation of chromatin in Chinese hamster cells

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GIANT CELL FORMATION PRODUCED BY LASER MICROBEAM IRRADIATION OF CHROMATIN IN CHINESE HAMSTER CELLS

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SUMMARY

A pulsed laser microbeam of wavelength 532 nm was used to produce visible small lesions in the nucleoplasm or in the cytoplasm of V79 Chinese hamster cells. Transmission electron microscopy (TEM) of microirradiated nuclei showed that the lesions were produced within the nucleus and comprised between 0.2 and 0.5 % of the total chromatin. Serial sections above and below the lesion site did not reveal any detectable chromatin damage, indicating that a visible lesion was restricted to the focal point of the beam. Whereas cells microirradiated anywhere in the cytoplasm showed normal clonal growth with few exceptions, the cells containing nuclear lesions did not enter mitosis at the time of unirradiated controls. Instead they formed giant cells in a high percentage of cases (72/99). The DNA content of these cells was considerably increased suggesting polyploidization. In some cases, division of giant cells was observed resulting in non-viable daughter cells containing micronuclei. Further evidence that the induction of giant cell formation depends on chromatin damage was obtained by microirradiation of chromosomes in anaphase. Here, giant cell formation was observed in the daughter cell which received microirradiated chromatin, whereas microirradiation of cytoplasm between the moving sets of chromosomes did not affect subsequent divisions of both daughter cells. Our data point out that loss of reproductive integrity and giant cell formation can be induced by damage at many sites of the chromosome complement.

A microbeam gives the unique possibility for producing damage restricted to organelles in a small and selected part of the cell [1–9]. The type of damage can be modified by varying the physical parameters of the radiation, e.g., the use of different wavelengths, pulsed or continuous laser sources [2, 7, 8]. In the present study, we have investigated effects of microbeam lesions on cellular growth of V79 Chinese hamster cells. Lesions were produced in the nucleoplasm, in anaphase chromosomes or in the cytoplasm by a pulsed laser microbeam of wavelength 532 nm. The size and morphology of nuclear lesions was investigated by electron microscopy at different times after microirradiation. Lesions in chromatin inhibited clonal growth and triggered giant cell formation, whereas lesions in the cytoplasm did not. Our approach presents a new possibility to investigate the cellular distribution and the minimum number of targets which contribute to loss of reproductive integrity and giant cell formation.

MATERIALS AND METHODS

Cell culture

A V79 subline of Chinese hamster cells was used for the present experiments [8]. Stock cultures were maintained in plastic Falcon T-75 flasks in Eagle's MEM (Gibco) with Earle's salts and supplemented with 1 % glutamine, 1 % non-essential amino acids and 10 %

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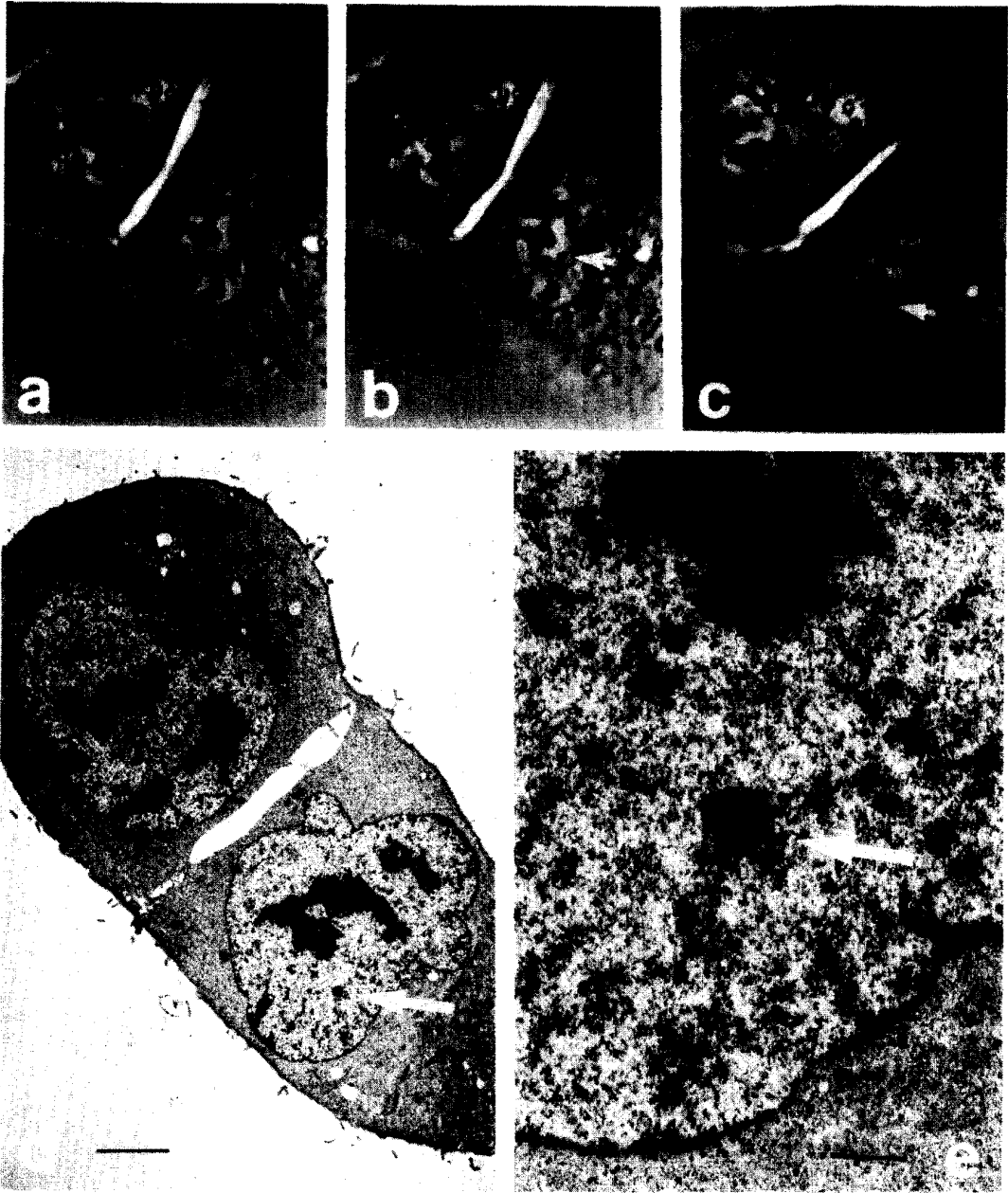[2] To whom offprint requests should be directed.

Fig. 1. (*a–c*) Phase micrographs of V79 daughter cells pre- and post-irradiation of nucleoplasm of the right cell with a 532 nm laser microbeam. (*b*) Two min post-irradiation (arrow indicates lesion); (*c*) same cells 100 min post-irradiation; (*d*) electron microscopy of the same cells; (*e*) detail of the right cell. Bar, (*a*) 10 μm; (*d*) 5 μm; (*e*) 1 μm. (Arrow in (*d*) and (*e*) indicates lesion site.)

heat-inactivated fetal calf serum (FCS). Cells were grown at 37°C in a 5% CO_2 incubator. The average generation time was 13 h. Subculturing was performed every 3–4 days by treatment with 0.125% Viokase and 0.1% EDTA solution. For each microirradiation experiment, the cells were harvested from the T-75 flasks by mitotic shakeoff and seeded into Rose tissue culture chambers at a density of 3000–4000 cells/chamber (in a total vol of 2 ml/chamber). The cells were grown in a conditioned medium that was ob-

tained from log phase growing cells. Daughter cells were chosen at different times after seeding (3–9 h corresponding to G1 and S, in some cases 11–13 h corresponding to late S and G2). In each experiment, one cell was microirradiated either in the nucleoplasm or in the cytoplasm, whereas the other cell served as a control. In other experiments, a chromosome or cytoplasm between the moving sets of chromosomes were microirradiated in anaphase. The cells were photographed prior and immediately following irradiation and subsequently followed and rephotographed for up to 50 h post-irradiation. Clonal growth was assumed when cells were able to complete at least two subsequent cell cycles after the first post-irradiation mitosis and thereby produce a minimum of five apparently healthy-looking cells [8].

Laser microbeam

In all the experiments, a pulsed, neodymium-YAG laser was used at the second harmonic wavelength of 532 nm. Laser output was 3–5 kW/pulse at 180 nsec duration. The laser beam was attenuated with neutral density filters, 0.9–1.1 density, prior to entry into a Zeiss photomicroscope modified as described earlier [10–12]. The combined attenuation of the microscope and neutral density filters resulted in an energy density of 45–117 μJ in the focal point of the microscope objective. A Zeiss Neofluar ×100 oil immersion objective was used. The laser focal spot was 0.25–1.0 μm in diameter. Some 2–5 laser pulses were used in each microirradiation experiment. The target site within the cell was selected by viewing on a closed circuit video system that was interfaced with a Zeiss photomicroscope III.

Autoradiography

A series of microirradiated cells were grown in the presence of [^{3}H]thymidine (0.2 $\mu Ci/ml$) to determine if DNA synthesis occurred following irradiation. The cells were prepared for autoradiography according to procedures described earlier [13].

DNA content

Cells were fixed in Carnoy's fixative (3:1, acetic acid:ethanol) 12 h and 24–48 h following irradiation and stained by the standard Feulgen-Schiff procedure [14]. A Nanometric Nanospec 10 microspectrofluorometer set to read fluorescence at 620 nm was used to record quantitatively the fluorescence produced per nucleus in microirradiated and control cells [15].

Electron microscopy

For EM analysis microirradiated cells were fixed immediately (within 1 min), 10 and 100 min after irradiation or after they had reached giant cell size. Cells were fixed in 3% glutaraldehyde, post-fixed in OsO_4 and stained with 10% uranyl acetate in methanol and lead citrate, as described in numerous earlier publications [3, 16]. Cells were flat embedded, relocated with light microscopy and serial sectioned as

described previously [3, 16]. Sections were examined and photographed using a JEOL 100C transmission electron microscope. The same procedure was performed with several cells at the indicated times.

RESULTS

Microirradiation experiments in interphase were routinely performed 3–9 h after mitosis when the cells were in G1 and S phase respectively. In all cases, one of two daughter cells was microirradiated. In some experiments, cells presumably in G2 were selected for microirradiation at a time when their corresponding sister cell had already entered or even completed mitosis. The effects to be described below could be observed after microirradiation at any time in interphase. Special reference to the time at which microirradiation was carried out during the cell cycle is only made where necessary.

Size, morphology and time-dependent changes of laser microbeam-induced nuclear lesions

Microirradiation at one site of the nucleoplasm with 2–5 pulses of a frequency-doubled, neodymium laser at 532 nm resulted in a small lesion which became clearly visible in phase contrast after 1–2 min (fig. 1a, b). Transmission electron microscopy (TEM) confirmed that these lesions were produced within the nucleus (fig. 2). Morphologically, the lesions appeared as electron-dense aggregates with a less dense, amorphous interior (almost vacuolar); see fig. 2c. Similar findings were obtained immediately and 10 min after microirradiation. The maximal lateral diameter of the lesion area as measured in phase contrast microphotographs and electron micrographs of serially sectioned cells varied between 0.5 and 1.0 μm. Evaluation of serially sectioned nuclei showed a limited ex-
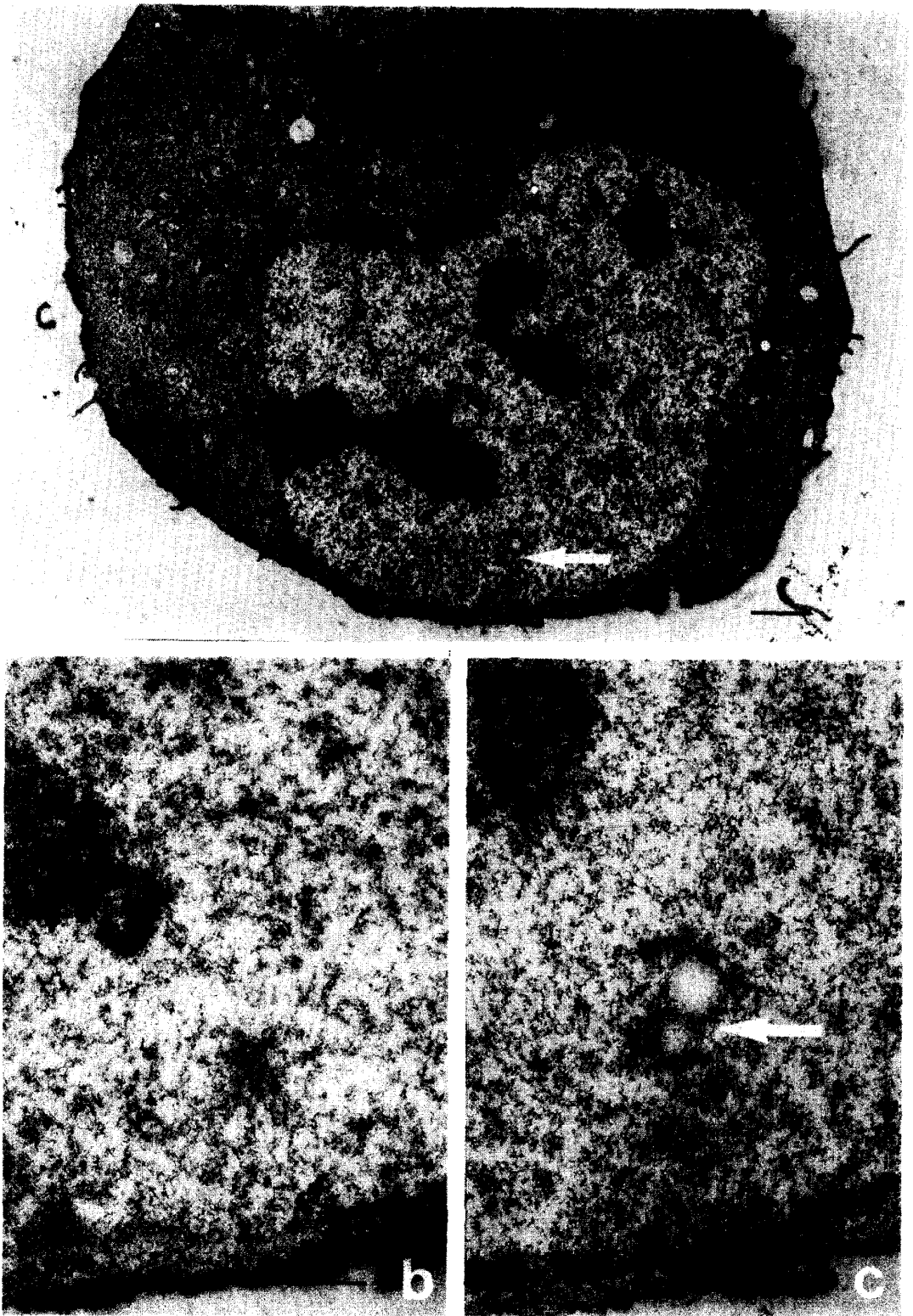
Fig. 2. EM sections of cell fixed 2 min post-irradiation. (*a*) Low magnification (arrow indicates lesion); (*b*) serial section just above plane of major lesion; (*c*) serial section directly through lesion (*arrow*). Bar, (*a*) 2 µm; (*b, c*) 0.5 µm.
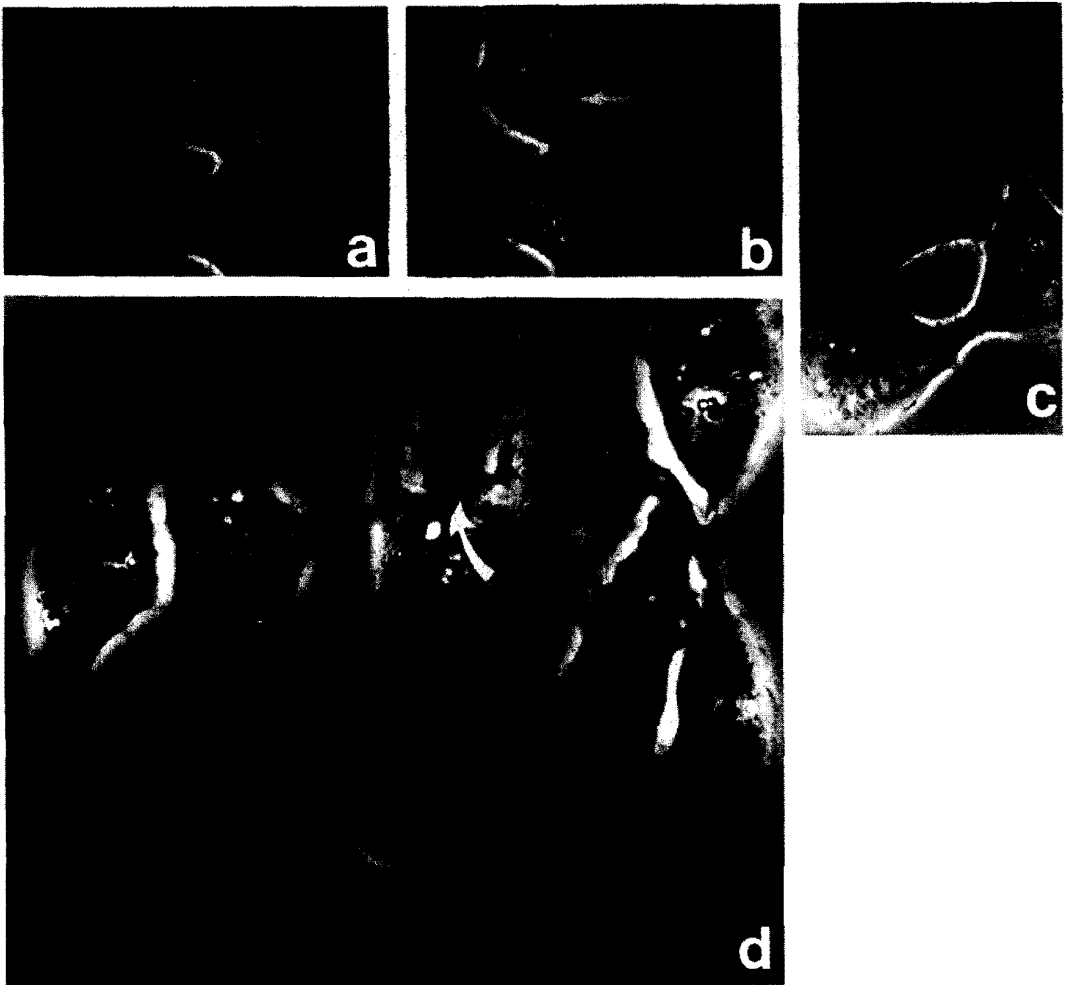
Fig. 3. Giant cell formation after microirradiation of the nucleoplasm in G1/early S 3 h after mitosis. (*a*) Daughter cells before irradiation; (*b*) 2 min after microirradiation of upper cell in the nucleoplasm; lesion is indicated by arrow; (*c*) 14 h after irradiation. Control cell has formed two normal daughters. Microirradiated cell has increased in size; (*d*) 42 h after irradiation. The microirradiated cell has formed a giant cell; the control cell has formed a clone of six cells. Arrow indicates the site of the giant cell nucleus shown in fig. 4*a* in the electron microscope. Magnification is the same in (*a–d*). Bar in (*d*), 10 μm.

tension of the lesion also within the longitudinal axis of the microbeam. There was no indication of any ultrastructural changes of chromatin above and below the lesion (fig. 2*b*). Possible changes of the nuclear envelope or the plasma membrane at the irradiation site were not investigated, but it can be noted that no significant ultrastructural differences between the irradiated and control cell could be detected both in the nucleus and the cytoplasm, except for the lesion site. Our data suggest that a detectable lesion was only produced within the focal region of the microbeam.

The volume of the damaged nuclear material was approx. 1–1.5 μm^3, whereas the total volume of the nuclei was estimated between 400 and 500 μm^3 in G1 and early

Table 1. *Increase of total cell area and nuclear area following laser microirradiation of the nucleus (3 h after mitotic selection)*

	Total cell area (μm^2 ± S.D.)		Nuclear area (μm^2 ± S.D.)	
	Control	Irradiated	Control	Irradiated
At time of irradiation	459±119	434±157	109±34	111±28
1–2 h before division of control	837±226	793±144	211±38	198±42
2–3 h after division of control	413±79	879±264	113±18	203±46
24–48 h after irradiation	657±288	3 590±1 363	157±46	495±134

A total of 36 cells (18 irradiated cells plus 18 control sister cells) were evaluated.

S phase. Phase contrast observations of nuclei in living microirradiated cells showed that after irradiation, nuclear lesions became less visible over time and were hardly detectable after 100 min (fig. 1*c*). Electron microscopy performed at this time still revealed a region of increased chromatin density at the irradiation site (fig. 1*d*, *e*), but the alteration was only very slight, compared with the changes observed immediately after irradiation. This difference was observed in all of the sections which contained the lesion and was confirmed for several cells processed for electron microscopy immediately and 100 min after microirradiation.

Formation of giant cells

In numerous experiments, both the microirradiated cell and the unirradiated sister cell were followed by phase contrast observation, and proliferation was documented by drawings and photomicrographs. Fig. 3 shows the typical result of an experiment performed 3 h after mitosis. Two minutes after microirradiation of the nucleus of the upper cell, a lesion became clearly visible (fig. 3*b*). Fourteen hours later, the unirradiated control had produced two normal daughters while the irradiated cell had approximately doubled in size (fig.

3*c*). The number of control cells increased further, indicating normal clonal growth of these cells. The irradiated cell did not divide but finally covered an area several times as large as the average area covered by control cells (fig. 3*d*, table 1). A similar increase was observed in the nuclear area of microirradiated cells (table 1). Cells which became at least twice the size of unirradiated sister cells in G2 were designated 'giant' cells (table 1). Notably, giant cell formation could be induced not only after microirradiation at an early stage of the cell cycle, but also at later stages corresponding to S and presumably to G2 in some cases. Giant cells generally contained one large nucleus, which was frequently lobed and, by electron microscopy, was shown to contain numerous infoldings of nuclear membrane (fig. 4*a*). Serial sections of giant cell nuclei did not show any lesion site detectable by electron microscopy. Increased numbers of centrioles of normal ultrastructure were found in the cytoplasm (fig. 4). Giant cells were occasionally observed in untreated cultures, but the percentage of spontaneous giant cell formation was low (≤1%). When microirradiated cells were grown in the presence of [³H]thymidine and processed for autoradiography, giant cells were heavily labeled
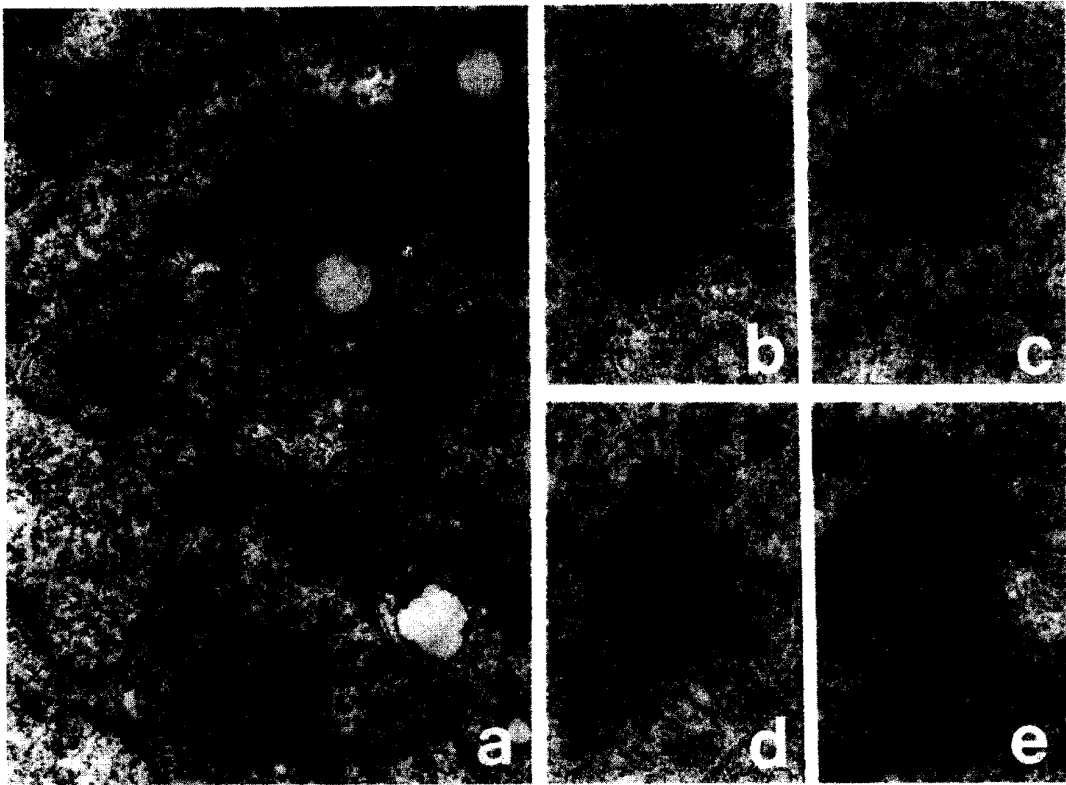
Fig. 4. (*a*) EM section of the giant cell shown in fig. 3*d*. A marked folding of the nuclear envelope is noted. Arrow indicates the site of a centriol duplex. (*b–e*)

Four sites of centrioles detected in this giant cell. Bar, (*a*) 1 μm; (*b–e*) 0.1 μm.

over the whole nucleus in seventeen cases followed up to the giant cell stage. Continued DNA synthesis after microirradiation was confirmed by measurements of the DNA content in Feulgen-stained cells at different times after irradiation of cells in G1 (table 2). The relative DNA content in G1 was measured from control cells in telophase. Approx. 12 h after irradiation, i.e., at a time when division of controls had occurred, the relative DNA content of the microirradiated cells had doubled. A further increase was observed in giant cells in which the DNA content was estimated 24–48 h after irradiation. In a total of 99 experiments in which a nuclear lesion was produced at a random site, the irradiated cell did not enter mitosis in the time range typical for control cells, although progress in the cell cycle was indicated by both the increase in cell size and DNA content. Giant cell formation was observed in 72 of these experiments (table 3).

Many giant cells degenerated during interphase. A minority of these cells, however, entered mitosis after a delay corresponding to at least one additional cell cycle in the control cells. Evidence of grossly abnormal mitotic events in giant cells was obtained by the observation of abnormal cytokinesis (fig. 5*b–e*), the formation of micronuclei (figs 5*f*, 6, 7) and the occasional

Table 2. *DNA content (Feulgen) of cells grown for different times after microirradiation of the nucleus in G1*

	n	DNA content (rel. units ± S.D.)
Control G1	80	1.0±0.34
12 h after irradiation	13	2.0±0.75
Giant cells 24–48 h after irradiation	36	3.1±1.08

observation of a chromatin bridge between the daughters of a dividing giant cell (fig. 7). In a few cases in which Feulgen measurements of giant cell daughters could be performed, the DNA content suggested a near tetraploid state of these cells, whereas V79 control cells were near diploid (modal chromosome number, 21). Since a considerable number of giant cells were fixed prior to the onset of degeneration, the percentage of giant cells which had retained the capability to enter mitosis cannot accurately be estimated from our data. In one case, clonal growth of a giant cell was observed up to the formation of ten large cells. The typical observation, however, was degeneration of the microirradiated cells, either as a giant cell with one large nucleus or after one abnormal mitosis. Normal clonal growth of control cells was observed in 97% of these experiments (table 3).

Proliferation of cells after microirradiation of the cytoplasm

The ability of cells to proliferate after microirradiation of the cytoplasm is illustrated in fig. 8. Immediately after irradiation, paling was observed at the irradiation site. Alterations obtained were not characterized by electron microscopy in the present experiments, but it has been shown previously that microirradiation of the cytoplasm results in damage restricted to certain cell organelles present within the focus of the microbeam [3], as well as in transient changes of the exposed part of the plasma membrane [17]. Follow-up studies of the cells microirradiated in the cytoplasm did not reveal any delay of the onset of mitosis when compared with the unirradiated sister cells. Both microirradiated and control cells contributed to the same extent to formation of clones composed of cells with normal size and morphology. In 33/34 experiments performed in the way illustrated by fig. 8 normal clonal growth of both the irradiated and control cell was observed (table 3). The formation of a giant cell which was observed in one case was possibly the result of unintended damage of the nucleus in this particular experiment. Occasional irradiation of centrioles may be another possible explanation. These experiments confirm that the nucleus was the target site for giant cell formation in the large majority of microirradiation experiments.

Table 3. *Microirradiation of Chinese hamster cells in interphase*

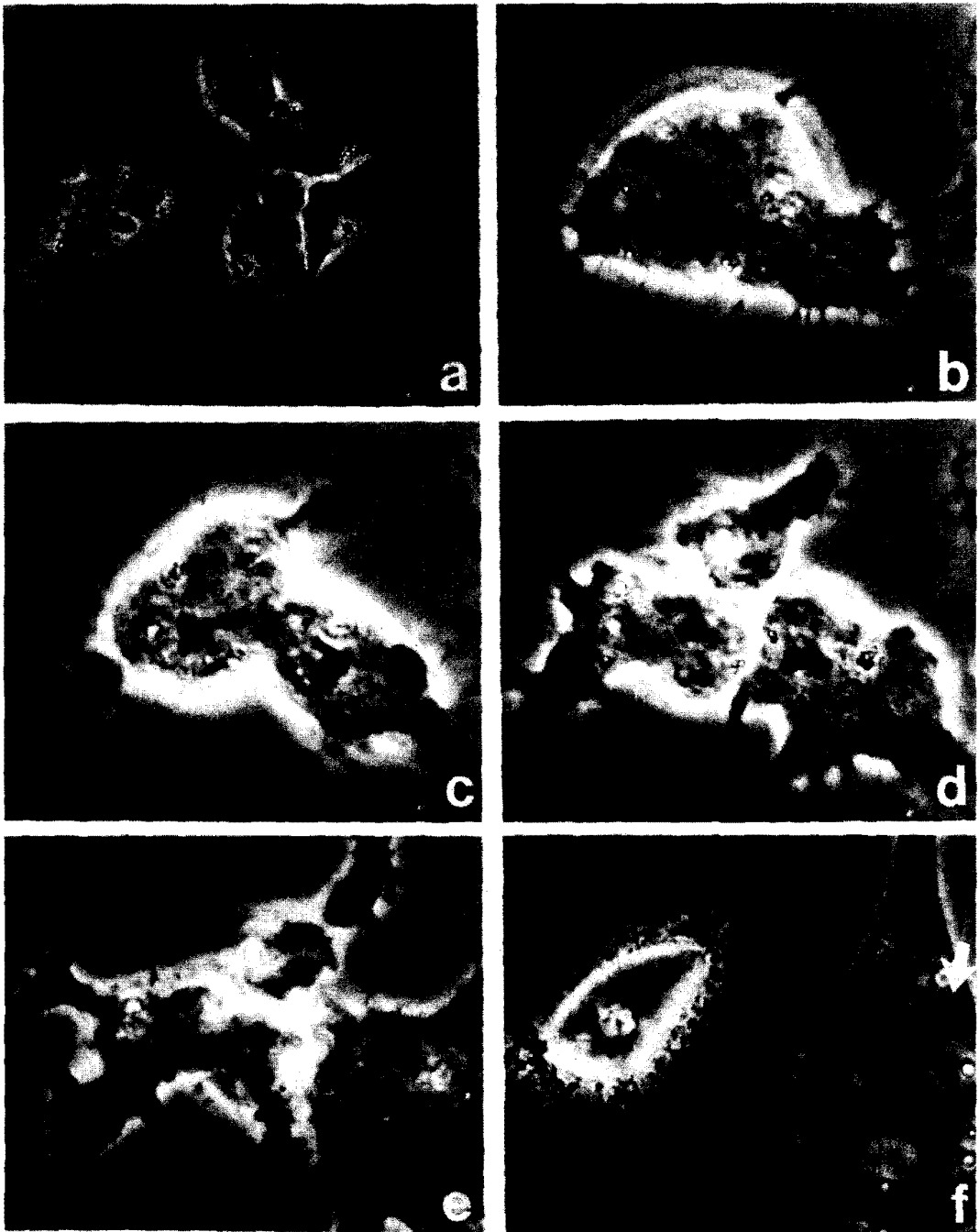| Site of microirradiation	Mode of microirradiation	No. of expts	Giant cells	Clonal growth
Nucleus	Defocused	35	0	35
	Focused	99	72	1
Cytoplasm	Defocused	9	0	9
	Focused	34	1	33
Control	–	177	0	172

Fig. 5. Abnormal mitosis of a giant cell. (*a*) Giant cell (*left*) 28 h after microirradiation of the nucleus and shortly before onset of mitosis. Control cell has formed four normal cells; (*b–e*) different stages of cytokinesis of this giant cell; (*f*) degeneration of the division products of the giant cell (arrow indicates a micronucleus). Bar, 10 μm.
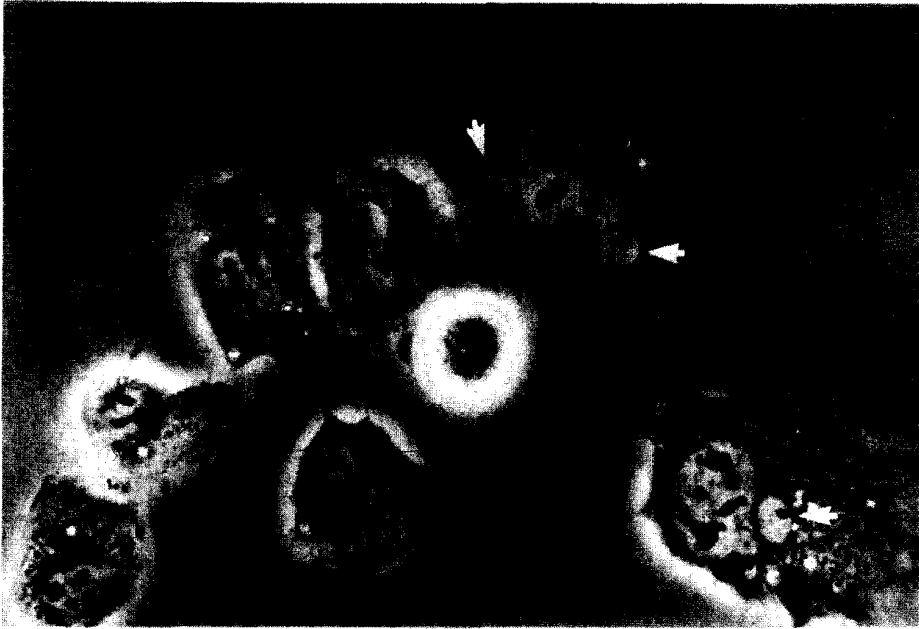
Fig. 6. Micronuclei (*arrows*) in the daughter cells of a giant cell 41 h after microirradiation of the nucleus. Giant cell had entered mitosis 32 h post-irradiation. Control cell has formed eight normal cells. Bar, 10 μm.

Focus–defocus experiments

To investigate whether effects of stray light were important in the induction of giant cell formation, the microbeam was defocused so that the focus was placed below the cell (fig. 9). Under these conditions, the total incident energy applied to the cell nucleus was the same as in the experiments in which the microbeam was focused in the cell nucleus. The distribution of energy applied to the nucleus was, however, largely different. By the defocused mode, approxi-

Fig. 7. Formation of a chromatin bridge between nuclei of daughter cells of a giant cell. Phase contrast picture was taken after fixation with Carnoy's fixative. (*Inset*) Positive Feulgen fluorescence of the bridge obtained by illumination with a continuous HeCd laser. Arrows indicate micronuclei. Bar, 10 μm.
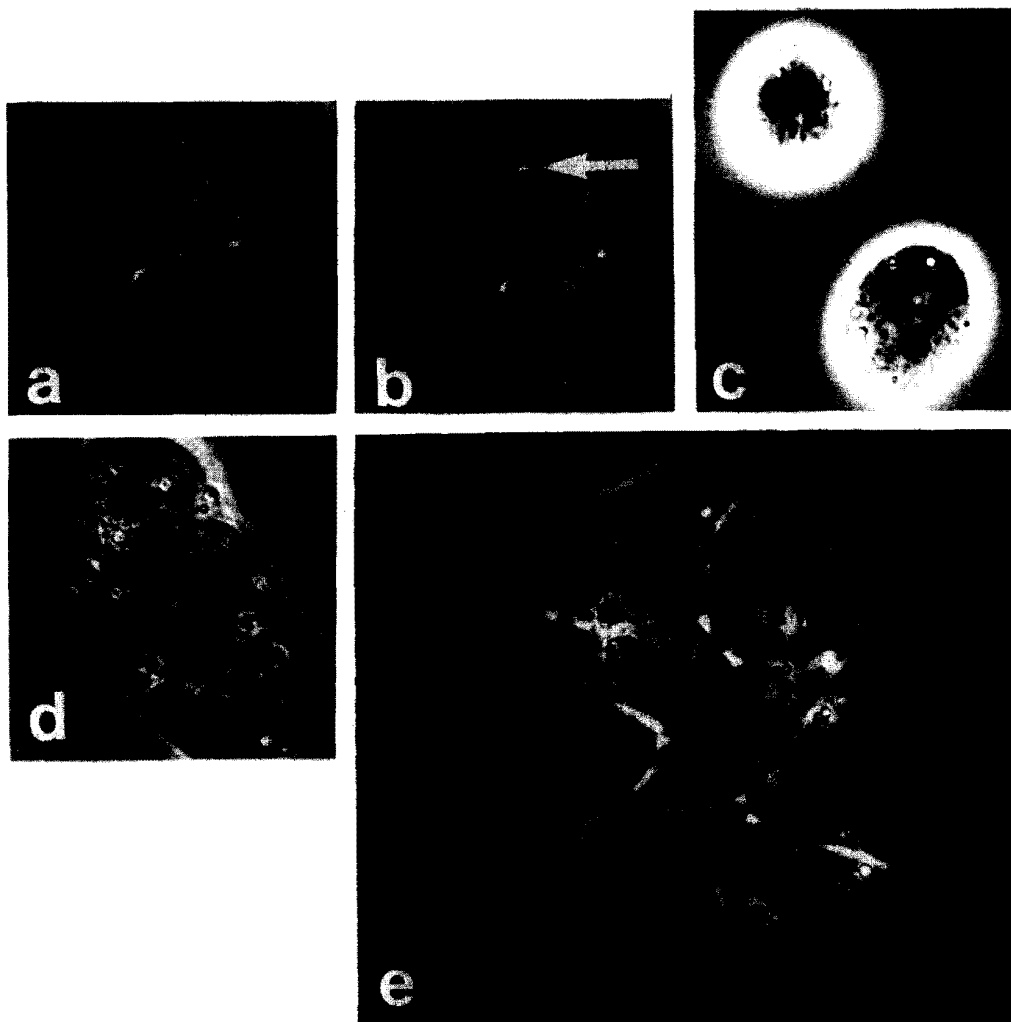
Fig. 8. Normal clonal growth after microirradiation of the cytoplasm. (*a*) Phase contrast picture of daughter cells before irradiation; (*b*) 1 min after microirradiation of the upper cell. Arrow indicates paling at the irradiation site; (*c*) 5 h after microirradiation. Irradiated cell and control cell are in mitosis; (*d*) 8 h after microirradiation; microirradiated cell and control cell have produced two normal daughter cells each; (*e*) 23 h after microirradiation. Microirradiated cell and control cell have produced four cells each. Bar, 10 μm, indicates magnification in (*a–e*).

mately the whole nucleus was exposed rather uniformly to laser light, and no lesion visible in phase contrast could be produced. If exposure of the unirradiated part of a nucleus by stray light would play a significant role in the induction of giant cell formation obtained after the focused mode, one would also expect giant cell formation after the defocused mode of microirradiation. In all of the 35 experiments in which the defocused mode was used for microirradiation of the nucleus, normal clonal growth was observed. The same was true for nine defocus experiments, in which the cytoplasm was irradiated (table 3). We conclude from these experiments that stray
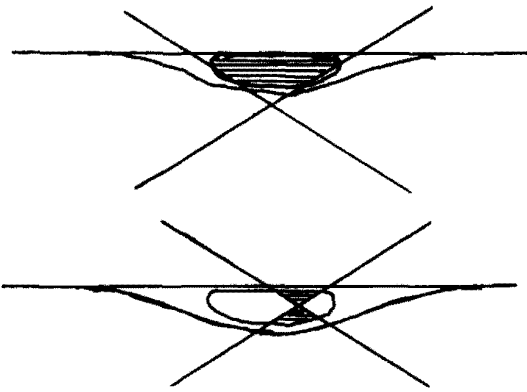
Fig. 9. Schematic diagram of microirradiation in focus–defocus experiments. In the 'focused' mode (*lower*), the focus of the microbeam is placed within the nucleus. In the 'defocused' mode (*upper*), the focus is placed somewhat below the nucleus. The same total incident energy is applied to the nucleus by both modes of microirradiation. The distribution of the energy, however, is largely different.

light is not effective in the induction of giant cell formation.

Microirradiation of Chinese hamster cells in anaphase

Finally, we have considered the question of whether induction of giant cells was due to damage of the chromatin or some other component of the nucleus, such as the nuclear envelope. This question was approached by microirradiation of mitotic cells when the nuclear envelope was not present. In a series of experiments summarized in table 4, either a chromosome during anaphase movement or a cytoplasmic region between the two anaphase chromosome sets was microirradiated. This approach was made difficult by the fact that V79 cells round up considerably in mitosis and thus obscure the direct observation of chromosomes. In spite of this problem, it was possible to find cells in anaphase suitable for microirradiation experiments. Both the daughter which received the microirradiated chromosome(s) and the con-

trol daughter were able to form normal looking nuclei after division of the microirradiated mitotic cell. In 8 of 39 experiments in which the cells were further observed for 48 h after microirradiation, giant cell formation was observed in the cell which had received microirradiated chromatin. Control daughters which received the unirradiated set of chromosomes showed normal clonal growth in all cases. Sixteen anaphase cells were microirradiated in a cytoplasmic region between the chromosomes. In 15 cases, clonal growth of both daughter cells was observed. One experiment resulted in the formation of a binucleate cell which subsequently degenerated and did not form a giant cell.

DISCUSSION

Giant cells have been described in many cell systems and may occur either spontaneously [18–22] or after a variety of experimental treatments including X-irradiation [23, 24], administration of DNA cross-linking agents ([25] and our unpublished data) or exposure to sublethal temperatures [20]. Their formation may depend on fusion of several small cells [22, 26] or result from continued growth of single cells under conditions where amitosis, failure of mitosis or endoreduplication take place [27–29]. In all these instances the term 'giant' has been used to designate the strikingly large multinuclear or uninuclear cells which arise from

Table 4. *Microirradiation of Chinese hamster cells in anaphase*

Site of microirradiation	No. of expts	Giant cells
Chromosome	39	8
Cytoplasm	16	0

considerably smaller progenitor cells by the variety of mechanisms mentioned above. It has been suggested that in some cases division of giant cells may yield viable daughter cells with new genetic arrangements and altered cellular properties, as compared with the progenitor cells [20, 22]. However, in most cases giant cells appear to be terminally differentiated cells or 'end stage' cells, which have lost their reproductive integrity [18, 20, 26]. In this respect it is noteworthy that an increased percentage of polyploid cells and a several-fold increase in cell size are also well known phenomena in senescent fibroblast strains [30, 31].

Microirradiation experiments open a new possibility to examine the distribution and minimum number of subcellular targets important for the induction of giant cell formation in single irradiated cells. Our experiments show that lesions induced at randomly selected sites of the chromatin by pulsed laser light of wavelength 532 nm and comprising 0.2–0.5% of the total chromatin are sufficient to inhibit or strongly delay the onset of the first post-irradiation mitosis and trigger giant cell formation in a high percentage of V79 cells. We have observed this phenomenon after microirradiation of chromatin in anaphase and at different times in interphase, but not after microirradiation of cytoplasm. There is an obvious contrast, which we can not explain at present, between the capability of cells with microirradiated chromatin to increase their DNA content and cell size at a rate not detectably different from their unirradiated sister cells and their inability to undergo mitosis at the same time as the controls. Feulgen measurements suggest that these cells are able to enter at least one additional round of DNA replication. During this time period, the lesion disappears and can no longer be detected at the ultrastruc-

tural level. The mechanisms involved in the restoration of a normal ultrastructural morphology are unknown, but it seems unlikely that the damaged chromatin can be repaired to a full extent (compare fig. 2).

The physicochemical events by which the lesion is produced in living unstained cells by microirradiation with pulsed green laser light are presently not well understood. No naturally occurring chromophore with absorption at 532 nm is known to exist in V79 cells. Damage is possibly induced by non-linear effects of laser light at high intensities [32, 33]. If the intensity was decreased below a certain threshold, neither a lesion nor inhibition of clonal growth and giant cell formation was observed, even if the nucleus was exposed to many subthreshold pulses. If the intensity was increased above a certain range, microirradiation often resulted in an 'explosion' of the whole cell. Within a critical range of intensities, however, induction of small compact lesions could be obtained in an easily reproducible manner. Within this range an intensity resulting in chromatin damage was obviously only achieved at the focal region of the microbeam (fig. 2) and the severity of the lesion as judged by phase contrast observations depended on the number of pulses. Above and below the focal region the intensity decreased strongly due to the large aperture angle of the objective used for focusing the laser beam. Accordingly, electron microscopy did not reveal any chromatin damage in this part of the nucleus.

The idea that a diffusible product of the microirradiation damage inhibits nuclear division has been ruled out by further experimental work [36]. In these experiments, we have observed giant cell formation when chromatin of V79 cells was microirradiated in interphase or mitosis with 365 nm laser

light in the presence of psoralen (PUVA microirradiation) [34]. By this treatment monofunctional and bifunctional psoralen photoadducts can be produced in DNA and RNA respectively [35]. The specificity of the photoadducts for the induction of giant cell formation was demonstrated by the fact that neither psoralen alone nor microirradiation of chromatin in the absence of psoralen prevented normal clonal growth [34]. Giant cell formation was not observed after PUVA microirradiation of the cytoplasm besides the nucleus or mitotic chromosomes [36] and was a rare event after microirradiation of the nucleus at wavelength 257 nm [8].

We suggest that some permanent damage of chromosomal DNA produced at the lesion site may be essential for inhibition of mitosis associated with giant cell formation. Our results indicate not only that chromatin represents the target(s) for the induction of this effect but also that the minimum number of chromatin sites within the nucleus which can serve as targets must be rather high. For a rough estimate, we may assume that there are at least 100 different sites in the chromatin of V79 cells which can be independently damaged by a microbeam of 0.25–1 μm focal diameter. We may further assume that giant cell formation depends on whether or not a certain chromatin site which is hit by the microbeam contains one or several targets, e.g., certain genes. Since giant cell formation was found in 72 out of 99 experiments after microirradiation of the nucleus, a minimum number between 60 and 83 targets can be calculated from the binomial distribution for a confidence level of 99%. The percentage of giant cell formation was smaller after microirradiation of chromatin in anaphase (table 4) resulting in a minimum estimate between 7 and 41 targets.

The reason for this difference in the sensitivity of cells microirradiated in interphase and anaphase is not known. The number of chromatin sites which represent possible targets of giant cell induction might be considerably larger than the minimum numbers estimated above. We have noted that even in those experiments in which, by our definition, cells with microirradiated chromatin had not reached giant cell size, many cells had considerably enlarged after microirradiation.

The idea that damage of specific sites in the chromatin is responsible for giant cell formation may be questioned in the light of the numerous chromatin sites which can be involved as targets. Instead we may consider the possibility that lesions anywhere in chromatin may inhibit the subsequent mitosis and trigger giant cell formation under conditions, which await further clarification. If so, a general mechanism may be involved by which clonogenic survival of cells can be inhibited by the presence of lesions in chromatin of V79 cells.

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