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Cinemicrographic Determination of Cell Progression and Division Abnormalities after Treatment with 1, 3 Bis(2-Chloroethyl)-1-Nitrosourea*

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Abstract—*Time-lapse photography was used to study cell-cycle progression and cell-division abnormalities in rat 9L brain tumor cells after treatment in culture with* 2 μg/ml *and* 5 μg/ml *of* 1, 3 *bis*(2-*chloroethyl*)-1-*nitrosourea* (*BCNU*). *Cells treated in* G_1 *were extensively delayed in reaching the first mitosis after treatment, whereas cells treated in S and* G_2 *were not. However, cells treated in* G_1 *returned to nearly normal generation times in the first full post-treatment generation, whereas cells treated in S and* G_2 *had an abnormally long generation time in that generation. Most cells completed or at least attempted on or two divisions before dying, therby producing a* 3- *to* 4-*fold increase in cell number before any decrease was evident. Both the length of time required for cells to die and the number of divisions made before the cells died depended on the BCNU concentration to which they were exposed. Nearly all cells underwent abnormal divisions before dying, including tripolar divisions, unsuccessful divisions, divisions with the loss of a non-viable cytoplasmic mass, and unorganized divisions. The length of time that cells remained rounded at division depended on both the BCNU concentration and the generation after treatment. BCNU induced chromosomal aberrations that could be responsible for killing the cells or for the observed division delays and abnormalities.*

INTRODUCTION

IT IS generally accepted that knowledge of the effects of chemotherapeutic agents on tumor and normal cell growth and progression through the cell cycle could lead to more effective scheduling of drugs. Nevertheless, obtaining information for cell-cycle-nonspecific agents, such as the nitrosoureas, is not without attendant difficulties in interpretation. Of the nitrosoureas, BCNU has activity against a variety of human and animal tumors [1] and is currently the most effective single drug used in the management of malignant brain tumors [2]. Consequently, the age sensitivity and progression of cells treated with BCNU in the tissue culture have been studied previously [3–7]. Investigators have used cells which have been synchronized by chemical blocks [3, 5, 6], by nutritional deficiency [7], and by a combination of mechanical and enzymatic procedures [4]. The effects of BCNU on progression through the cell cycle have been evaluated primarily by the use of cells labelled with radioactive DNA precursors [3], or by measuring the DNA content of cells using flow-microfluorometric techniques [7]. Neither of these techniques distinguishes between cells which continue to proliferate by division into the second and third generations after treatment and those which do not. To overcome

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this difficulty, we used time-lapse photography to study the effects of BCNU on the progression of cultured rat 9L brain tumor cells.

MATERIALS AND METHODS

Rat brain tumor cells (9L) were grown in Eagle's Basal Medium, Earle's Salts (BME) supplemented with 10% fetal calf serum (FCS), vitamins, and essential amino acids as previously described [8]. For the time-lapse experiments, about 1.0×10^6 cells were seeded into a 75 cm^2 Falcon tissue culture flask; the flask was placed on a Reichert Biovert microscope equipped with a green filter, long working distance condenser, 16× objective, and 5× eyepiece. A Plexiglas cage surrounding the microscope contained two heating elements with Edison-type screw bases, type SCB-50 W, 110 V, which were controlled by an immersion tube thermoswitch with a range of −100°C–400°C, 110 V and 10 A (both from Montgomery Brothers, Burlingame, California, U.S.A.). A motor-driven Bolex 16 mm camera was controlled by an automatic time-lapse system (Bio-Optical Devices, White Plains, New York, U.S.A.). Pictures were taken with Kodak Plus-X Reversal Film at approximately 35 frames/hr with an exposure time of about 0.5 sec. A specimen-shielding shutter protected the cells from the microscope light between exposures, and a black cloth covered the entire Plexiglas cage to shield the cells from the room light.

A field of cells was selected and its location marked on the bottom of the flask with a diamond needle. The cells were then photographed for 18–20 hr before BCNU was added at concentrations of either 2 or 5 μg/ml. After BCNU treatment, time-lapse photography was continued for 75–92 hr. Because BCNU has a short half-life under these culture conditions (≈50 min), the medium was not changed after BCNU treatment; the treated cells therefore received an infinite exposure dose during the experiment [8]. In previous experiments, no increased 9L cell killing was observed with drug exposures of greater than 2 hr [8]. After an infinite exposure dose to BCNU at concentrations of 2 and 5 μg/ml, survival (measured by colony formation) was about 45% and 0.79%, respectively [8]. In a separate experiment, untreated cells were photographed for about 70 hr. In experiments where the photographed cells were treated with BCNU, a separate flask of untreated cells was always carried alongside for observation at various intervals to be sure that the cells reached confluency at the expected times to conform to proper growth of the cells.

After exposure, the film was commercially developed, and we analyzed cell activities by observing the film on a Kalart Pro-editor with a frame counter or on an LW International 16 mm movie projector with a frame counter (Woodland Hills, California, U.S.A.). The number of the frame in which each event was observed (e.g., cell rounding, cell division, cell fusion, or cell death) was recorded.

Live interphase cells characteristically extend and withdraw cytoplasmic protrusions which result in "crawling" across the plastic surface. Mitotic cells are round, luminescent, and unattached (or only lightly attached) to the plastic surface. They exhibit internal motion until just a few minutes before cytokinesis. Cell rounding was considered to have occurred when no visible cell processes were attached to the flask and the cell had acquired a round or nearly round shape. The time of cell division was defined as that time when the rounded cell became two or more definite lobes with a constriction between them. The exact times of cell fusion were difficult to determine because the process was a slow, progressive one, but fusion was considered to have occurred when two cells moved through the field as one body with only one surrounding membrane. The time of cell death was usually considered to be the time at which the cell lysed. Almost all cells which died detached from the plastic surface a few hours before death in a manner similar to that previously described for mitotic cells. The event of death could usually be seen as a precipitous change of the cell from a luminescent body with internal motion to a dark body without any motion. A few cells, however, shrank rapidly and ceased all motion but remained luminescent for several hours and sometimes for the remainder of the experiment. These cells were considered to be dead because they exhibited no growth, division, or movement. The time of each event relative to the time the BCNU was added to the cells was calculated and a family history (Fig. 1) was compiled for each cell observed.

We estimated the phase of the cell cycle in which a cell was treated with BCNU by calculating from the film the number of hours between the last cell division and the addition of BCNU. Then we determined how far the cell had progressed, assuming that the cell spent the average length of time in each phase of the cell cycle. The length of each phase of the cell cycle for the 9L cells grown in this

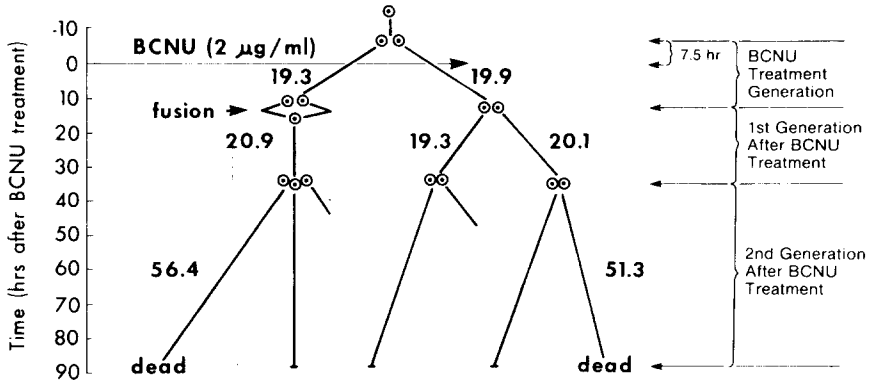

Fig. 1. The family histories of 2 sister cells treated 7.5 hr after division (G_1/S phase in this experiment) with 2 μg/ml of BCNU. Each circle represents 1 cell. The numbers represent times between 2 successive divisions (generation time) or between the last division and the death of one of the daughters. The scale on the left indicates the time of occurrence of events after BCNU treatment. The lines emanating from the daughter cells after the last observed division stop at the times (on the scale) that the cell either moved out of the field (2 cells) or died (2 cells). The horizontal bars on the lines stopping at about 90 hr after BCNU treatment represent cells that were alive (i.e., vegetative) when the filming ended.

laboratory was determined to be: $G_1 = 8.0$ hr, $S = 7.9$ hr, $G_2 = 3.2$ hr, $M = 0.5$ hr [9]. Where the time-lapse data of the cells prior to treatment indicated generation times which were longer or shorter than the usual total cell cycle time of 19.6 hr, we assumed a lengthening or shortening of the G_1 phase, since it is generally accepted that variation in the generation times is mostly a result of variation in the length of G_1.

To determine whether the rounded cells that began to appear in large quantities 40 hr after treatment were in mitosis or simply dying, and whether the abnormal divisions observed were the result of BCNU-induced chromosome aberrations, chromosome preparations were made from a culture treated with 5 μg/ml of BCNU. Forty-four hours after BCNU treatment, Colcemid (0.06 μg/ml) was added to the culture for 4 hr. The round and loose cells were then shaken off the flask and centrifuged for 6 min at about 1000 ***g***. After rinsing with saline and centrifuging, we added 0.075 M KCl to make the cells swell. After centrifuging the cells again and discarding the supernatant, we added methanol-acetic acid fixative (3 MeOH:1 HAc) and resuspended the cells with a Pasteur pipette. A drop of chromosome suspension was placed on a clean slide, flamed until dry, and stained with Giesma. The percentage of mitotic cells was determined by scoring 390 cells, and 25 mitotic cells were examined more closely for chromosome abnormalities.

RESULTS

Time-lapse photography revealed three pronounced effects of BCNU on rat 9L brain tumor cells: cell cycle-dependent alterations in the length of cell generation times (the time it takes for a cell to traverse the cell cycle from one mitosis to the next), mitotic abnormalities, and cell death.

We determined that the cells were growing normally before treatment with BCNU by observing them with time-lapse photography for a period of about one generation time (18–28 hr) before the BCNU was added to the culture flasks. A cell at any particular point in the cell cycle (e.g., early S-phase) would pass through mitosis once (and only once) during a period exactly equal to its generation time. For a population with an average doubling time of 18.7 ± 5.6 hr S.D.*, 50% of the cells would be expected to traverse at least one complete cell cycle (including one mitosis) in 18.7 hr. In 24.3 hr (18.7 + 5.6), 84% of the cells would be expected to traverse at least one complete cycle (including mitosis). Of the 71 cells originally in the fields of observation in our experiments, 85% (60/71) divided at least once and some of them twice during this pre-treatment period, which averaged about 24 hr for all of the films. Seven of the remaining cells (10%) divided within a few hours after treatment. This indicated that

*Standard deviation.

*Table 1. Generation times of untreated control cells and cells before treatment**

Generation	No. of cells observed	Mean ± S.D. (hr)	Median (hr)
1st	30	18.7 ± 5.6	16.9
2nd	42	19.5 ± 5.2	17.8
3rd	52	19.5 ± 4.6	18.7
Before BCNU treatment	8	16.4 ± 2.8	16.8

*Two cells are not included in these data. One showed no mitotic activity for the entire length of the film (~70 hr); the other died 48 hr after the film began.

before BCNU treatment, the cells were growing at the normal rate of untreated control cells (Table 1). In cases where the cells divided twice before BCNU treatment, an estimate of the average generation time could be made. This estimate indicated that the cells were growing at a rate similar to that measured by us in untreated cell populations which were observed for several generations (Table 1) and to that of the average doubling time (determined by conventional methods) of 19.6 ± 0.5 hr measured by others for 9L cells grown under identical conditions in this laboratory [9]. The final indication that cells used for the experiment were growing normally was that the cells in the control flasks which were plated at the same time, but left untreated by BCNU, always reached confluency at the expected time for cells with a doubling time of 17–20 hr. Thus, the cells observed in the time-lapse studies were growing normally until they were treated with BCNU.

BCNU significantly altered the cell generation times. After treatment with BCNU, the time between two successive divisions of a cell was lengthened (Tables 1 and 2). The extent to which the generation time was lengthened depended on the phase of the cell cycle during which the drug was added (Table 2, Figs. 2 and 3). Cells treated with BCNU within the first few hours after a division (presumed G_1 cells) took longer to reach the next division: the mean time of the generation in which they were treated was 29.7 hr, compared to 16.4–19.5 hr for untreated controls (Tables 1 and 2, Fig. 2). However, the cells treated with BCNU in S or G_2 phases lagged only slightly in reaching the next mitosis: mean generation times of 19.6, 20.5 and 18.2 hr, respectively, were observed for cells treated in early S, late S and G_2 (Table 2, Fig. 2).

The effect of treatment on generation time in the first generation after BCNU was added

Table 2. Generation times of cells treated with 2 μg/ml of BCNU

Treatment phase	Generation	Mean ± S.D. (hr)	n_{mean}†	Median (hr)	n_{median}‡
G_1	BCNU treatment	29.7 ± 6.6	9	29.2	11
	1st after BCNU	17.9 ± 2.7	9	20.4	14
	2nd after BCNU	25.7 ± 4.0	12	25.0	15
Early S	BCNU treatment	19.6 ± 3.1	13	19.2	15
	1st after BCNU	25.8 ± 5.2	17	25.9	18
	2nd after BCNU	28.5 ± 6.8	3	35.5	10
Late S	BCNU treatment	20.5 ± 6.7	25	18.0	25
	1st after BCNU	32.1 ± 8.7	25	32.0	32
	2nd after BCNU	25.7 ± 7.3	12	23.2	28
G_2	BCNU treatment	18.2 ± 3.4	6	18.0	6
	1st after BCNU	39.2 ± 6.9	9	42.7	9
	2nd after BCNU	28.9	1	21.4	7

†Number of cells used to determine mean. (Cells that died during this generation are not included.)

‡Number of cells used to determine median. (Cells that died during this generation are included.)

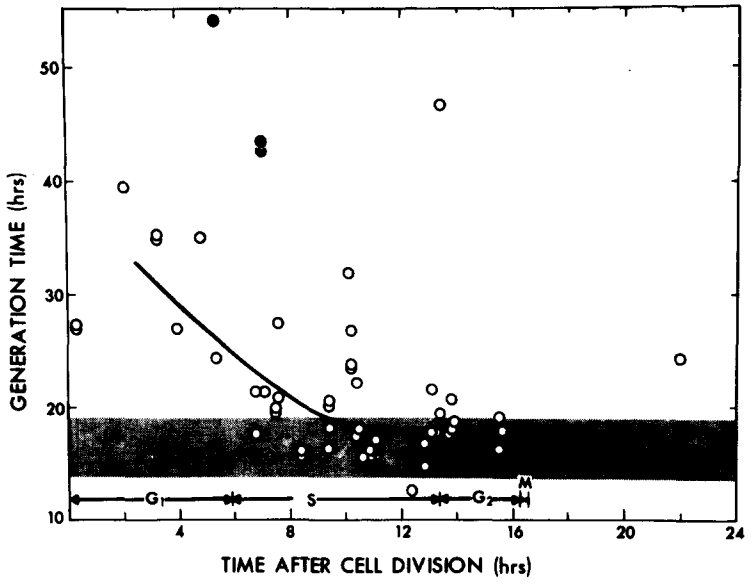

Fig. 2. Generation times of cells during BCNU treatment generation. The generation time is the number of hours *between the mitosis before treatment and the mitosis after treatment. The abscissa indicates the time after the pre-treatment division that* 2 μg/ml *of BCNU was added. The presumed phase of the cell cycle in which the cell was treated is indicated by the insert. The gray area encompasses the mean* ± 1 S.D. *of the generation times of untreated control cells (i.e., those dividing twice before BCNU treatment).* ○, *cells alive at the end of the BCNU treatment generation;* ●, *cells that died at the end of this generation.*

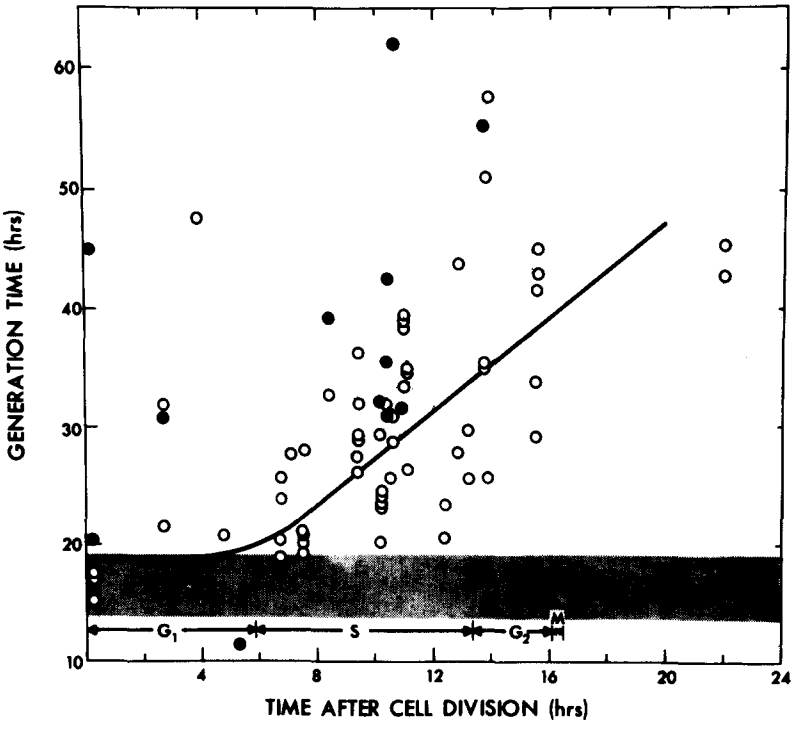

Fig. 3. Generation times of cells in the first full generation after BCNU treatment. The generation time is the number of hours *between the first mitosis after treatment with* 2 μg/ml *of BCNU and the second mitosis after treatment. The abscissa indicates the presumed phase in the cell cycle of each cell at the time of treatment. The gray area indicates the range of generation times of untreated control cells (i.e., the mean* ± 1 S.D. *of the generation times of cells that divided twice before BCNU treatment).* ○, *cells alive at the end of the first post-treatment generation;* ●, *cells that died at the end of the first post-treatment generation.*

was opposite to the effect observed in the BCNU treatment generation: cells treated in G_1 reverted to nearly normal generation times in the first post-treatment generation, whereas cells treated in S or G_2 phase have substantially longer generation times in that generation (Table 2, Fig. 3). In general, the nearer a cell was to mitosis at the time of BCNU treatment, the shorter was its BCNU treatment generation and the longer was its first generation after treatment (Table 2, Figs. 2 and 3). For example, cells treated with BCNU in G_2 had an average BCNU treatment generation time (18.2 hr) that hardly differed from that of the untreated controls, but these same cells spent an average of 39.2 hr in the generation after BCNU treatment (Table 2). The length of the second post-treatment generation was similar for all cells, regardless of where they were in the cycle when they were treated. All treated cells had second post-treatment generation times of 25–29 hr, significantly longer than those of untreated control cells (Tables 1 and 2).

The growth curves derived from pooled cell histories also reflect the lengthened generation times (Fig. 4). Cell populations treated in G_1 did not increase until at least 20 hr after treatment, whereas cells treated in G_2 showed an increase almost immediately (Fig. 4). In one respect, the growth curves for cells treated in all parts of the cycle were similar. Ninety hours after treatment, all the cell populations had increased roughly 3- to 4-fold over the original number (total live and dead cells) (Fig. 4). This 3- to 4-fold increase was also apparent when a composite growth curve was constructed by pooling the data from all parts of the cycle. For comparison, 90 hr after seeding, the total number of untreated control cells was 25–30 times the original number of cells seeded in the flask.

The observation that BCNU lengthens the generation times of cells was supported by the observation that some cells did not divide or attempt to divide within the time course of the experiments, but merely remained alive and moving (i.e., vegetative). (See Materials and Methods section of a description of the live cells.) No 9L cells remained vegetative in the generation during which they were treated with 2 μg/ml of BCNU, but many cells in the first and second post-treatment generations were in this category. Whether the vegetative cells divided after the end of the photographic observation or died could not be determined. If they divided, the mean and median generation times of table 2 would be substantially longer. If they died, only the median generation time of Table 2 would be longer. Therefore, generation times for the daughters and granddaughters of the BCNU-treated cells in Table 2 are shorter than the true generation times.

Many cells died during the film observation period. About 7% of the cells treated with 2 μg/ml of BCNU died before completing the first mitosis, and larger numbers died in subsequent generations (Fig. 5). Of cells treated with a higher concentration of BCNU (5 μg/ml), about 55% died before completing the first mitosis and almost 90% died before completing the second mitosis (Fig. 5). In other words, the higher the dose, the earlier the generation of death. However, because a higher dose of BCNU lengthened the cell generation time, the average time of death for cells during the treatment generation was later than that of cells treated with a lower dose (Fig. 5b). These plotted values were corrected for cell multiplication. Thus, if each treated cell divided into two cells, one of which died and the other of which lived through the next mitosis, 50% of the originally treated cells were considered to have

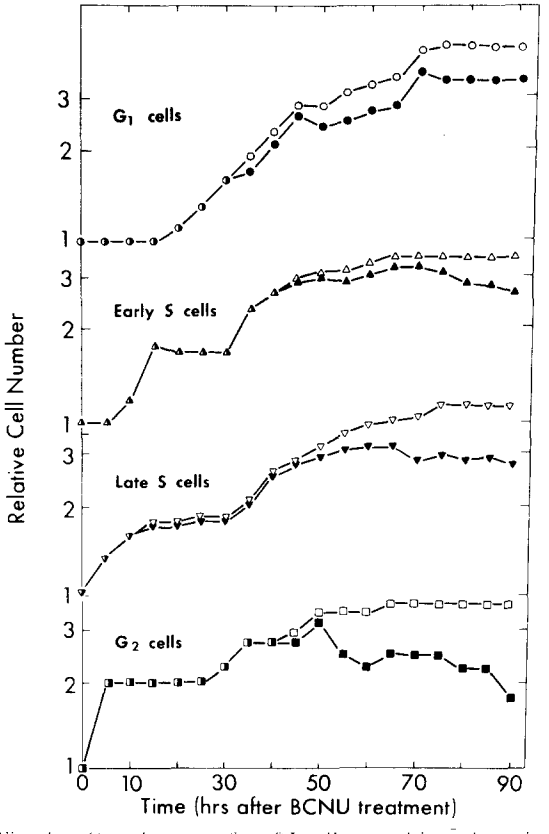

Fig. 4. Growth curves of rat 9L cells treated in various phases with 2 μg/ml of BCNU. ○●, cells treated in G_1 △▲, cells treated in early S; ▽▼, cells treated in late S; □■, cells treated in G_2; ○△▽□, total cell number; ●▲▼■, number of live cells.

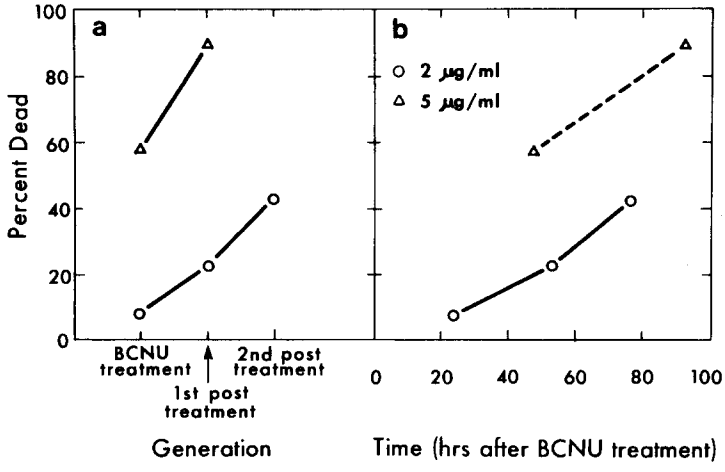

Fig. 5. Summary of the percentage of dead cells after each post-treatment generation, including both BCNU-treated cells and their progeny. The cells that died in the BCNU treatment generation are those that died before completing the first division after treatment. The cells that died in the first post-treatment generation are daughters of cells that survived the BCNU treatment generation and divided once before dying. Likewise, the dead cells in the second post-treatment generation are the progeny of cells that divided twice after BCNU treatment before dying. ○, 2 μg/ml of BCNU; △, 5 μg/ml of BCNU. A dashed line is used for the time of death after 5 μg/ml treatment of BCNU because the times of death of some of the cells during the first post-treatment generation had to be estimated owing to the large amount of dead cell debris that cluttered the field of vision.

died in the first generation after BCNU treatment.

The growth curves also show that significant numbers of cells treated with 2 μg/ml of BCNU began to die about 50 hr after treatment (Fig. 4). The number of cells that remained alive 90 hr after treatment was slightly larger for G_1 and early S cells than for late S and G_2 cells, although we cannot be sure that these differences are significant because of the small number of cells used in the analysis. It was also observed that cells which died during a particular generation (e.g., the first generation after BCNU treatment) had longer generation times than cells which survived that generation (Figs. 2 and 3).

BCNU induced a large number of abnormal divisions, including: (a) tripolar divisions (Figs. 1 and 6, Table 3); (b) attempted divisions in which the cell rounded as if for division and pulled in several directions but did not split into two or more cells (Table 3); (c) divisions in which the parent cell lost a small, non-viable piece of itself (Table 3); (d) unorganized divisions in which the cell rounded and pulled in many directions (instead of two) and finally divided, one daughter cell often being obviously larger than the other (Table 3); and (e) divisions into two or more cells followed closely by fusion of two or more of the resulting daughter cells (Figs. 1 and 6, Table 3). These were all in contrast to a normal cell division, in which the cell detached from the flask to become symmetrically round and divided easily into two equal-sized daughters without many contortions, gyrations, or loss of cellular material. The number of abnormal divisions increased with dose from 7.3% for control cells to 24.8% for cells treated with 2 μg/ml of BCNU and 70% for cells treated with 5 μg/ml of BCNU (Table 3). (The figures in Table 3 include pooled data from all generations after treatment.)

Besides morphologic abnormalities as-

*Table 3. Abnormal divisions observed during the 70–92 hr of filming**

BCNU dose μg/ml	Total No. of divisions observed	Tripolar divisions	Uncompleted divisions	Abortive piece	Unorganized divisions	Fusions	Total No. of abnormal divisions
0	150 (100.0)†	3 (2.0)	1 (0.7)	2 (1.3)	4 (2.7)	1 (0.7)	11 (7.3)
2	149 (100.0)	11 (7.4)	6 (4.0)	4 (2.7)	13 (8.7)	3 (2.0)	37 (24.8)
5	23 (100.0)	4 (17.4)	5 (21.8)	3 (13.0)	4 (17.4)	0 (0.0)	16 (70.0)

*Pooled data from all generations after treatment.
†Numbers in parentheses, percentage.

sociated with divisions of BCNU-treated cells, the length of time needed for cells to complete divisions was longer than that for the untreated cells (Table 4). Most untreated cells spent less than one-half hour from the time the cell was rounded to the time two or more definite, nearly separated lobes emerged. In the first division after BCNU treatment, 16% of the cells spent more than one-half hour in the rounded state, but 63.2 and 30.8% spent more than 0.5 hr in the rounded state in the second and third divisions, respectively (Table 4).

Table 4. Rounding of 9L cells for division before and after treatment with 2 μg/ml of BCNU

Division	Cells rounding > 0.5 hr	
	Fraction	Percentage
Before BCNU treatment (control)	1/34	2.9
1st after BCNU	8/50	16.0
2nd after BCNU	36/57	63.2
3rd after BCNU	8/26	30.8

Because many of the cells in the viewing field were rounded for such a long time beginning at about 40 hr after treatment with 5 μg/ml of BCNU and because many of these subsequently died, we questioned whether the rounded cells were in fact attempting to divide or were only rounding to die. Consequently, the rounded cells were removed by shaking and were prepared for chromosome analysis. Of the 390 BCNU-treated cells examined after a 4 hr Colcemid treatment, 53.3% had mitotically condensed chromosomes, compared to 52.8% of the cells similarly obtained from an untreated culture. Thus, a prolonged state of roundness in BCNU-treated cells probably represented attempts by these cells to divide. Twenty-five of the stained cells were observed more closely for chromosomal aberrations. Each of these cells had at least two definite and gross aberrations, and most were so complex that accurate quantification was not possible.

DISCUSSION

It is possible that BCNU kills cells in one phase of the cycle more efficiently than in another. For example, killing of cultured human lymphoma cells seems more efficient if the cells are treated with BCNU in the G_2 phase of the cell cycle [5, 6], but Don cells seem more sensitive to BCNU in early G_1 phase [4] and Chinese hamster ovary (CHO) cells in late-mid S phase [3]. However, the sensitivity at various stages in the cell cycle of 9L cells could not be determined exactly in these studies because the cells could not be observed long enough for us to obtain absolute survival values. Nevertheless, the cell growth data for the first few days after treatment indicate that the generation in which the cells lag in reaching cell division is different for cells treated in different stages in the cycle. Because G_1-treated cells delay in the generation during which they are treated and G_2-treated cells delay in the next generation, all cells going through two divisions after treatment have approximately the same total delay, no matter in what stage they were treated. Whether this equalization of growth carries over to survival could not be determined. Our data differ from those of Barranco and Humphrey [3] who observed that CHO cells were significantly delayed in reaching the first mitosis no matter in what phase of the cell cycle they were treated. We suggest that the difference between our data and theirs may be due to differences in the drug dose, cell lines, or analytic method used.

Other data (K. T. Wheeler, unpublished) [7] indicate that the predominant delay induced by BCNU occurs in the G_2 phase of the cell cycle, although a transient G_1/S block has also been observed. Cells treated in G_1, therefore, carry the BCNU-induced injury through the remainder of G_1, through S, and into G_2 where the delay occurs. However, G_2-treated cells and most S-phase cells, especially those treated in late S phase, continue to progress normally into mitosis without any delay. This suggests that the damage is not fixed ina recognizable form for several hours and that the closer a cell is to mitosis at the time of damage, the better are its chances of progressing through mitosis undelayed by that damage. If DNA–DNA crosslinks were the damage responsible for the 9L cell G_2 delay, our observations would be consistent with those of Kohn [10], who found that the chloroethyl moeity of BCNU, although bound rapidly, produced DNA–DNA crosslinks with a half-time of a few hours. Thus, a cell treated in late S phase might move into its first G_2 without fixed damage, then into mitosis and G_1 of the next cycle before the damage is in a form that causes it to stop in the next G_2.

In this way, the G_2 block induced by BCNU differs from that induced by irradiation. Irradiated cells also become blocked

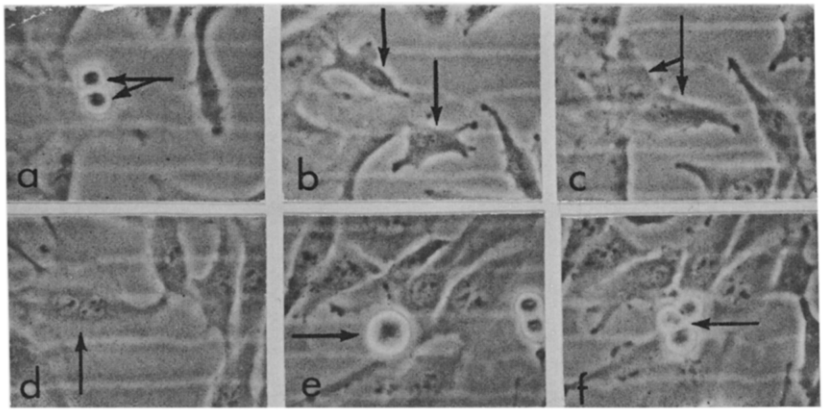

Fig. 6. *Time course for a successful cell division of a cell treated in* G_1/S *phase with* 2 μg/ml *of BCNU with subsequent fusion followed by a tripolar division* (*see Fig.* 1 *for cell history*). (*a*) 11.8 hr, *cell completing the first division after treatment;* (*b*) 14.5 hr, *daughter cells resulting from first division;* (*c*) 15.8 hr, *the* 2 *daughter cells fusing;* (*d*) 20.0 hr, 1 *binucleate cell resulting from the fusion of* 2 *daughters;* (*e*) 32.2 hr, *the binucleate cell rounded for the second division;* (*f*) 32.8 hr, *tripolar division of the binucleate cell. The horizontal striations are inherent markings on the tissue culture flask approximately* 18 μm *apart.* × 308.

in G_2, but the block occurs in the first G_2 after irradiation no matter in what stage of the cycle they were irradiated [11–23]. In addition, cells irradiated in late S and G_2 phases suffer a longer delay than those irradiated in other parts of the cycle [14, 15, 21, 22]. The only cells that enter division without delay in G_2 are those that are irradiated while in the late G_2 phase, 1 hr or less from mitosis [24]. Thus, although superficially the cells seem to respond in the same way to damage by either X-rays or drugs such as BCNU (both cause cells to stop in G_2), there must be a fundamental difference in the damage causing the delay, in the way this damage is perceived, or at least in the time at which it is recognized.

The ultimate cause of death of cells treated with BCNU is not known, but we suggest that many of them die because they lose part of their genetic material during faulty divisions and are, therefore, not equipped to carry on all of the necessary functions for maintaining viability. The fact that almost every cell divides or at least makes one attempt at cell division before death supports this hypothesis. For example, if a cell divides into three and the chromosomes are divided equally among the daughters, then no daughter cell would have its entire genome because there is only enough DNA for two cells. Likewise, if a cell loses one or more chromosomes in a small aborted piece of cytoplasm that is cleaved off during division, neither daughter cell resulting from the division will carry a complete genetic complement. It is even probable that some cells that complete a bipolar division have not received exactly half of the entire genome. This is supported by the observation that many of the cells resulting from division after BCNU treatment do not have the normal amount of DNA for a G_1 cell (Wheeler and Hoshino, unpublished fluorometric data) [7]. Such an unequal division of DNA is certainly possible, considering the chromosome aberrations induced by BCNU.

If two chromosomes or parts of chromosomes become abnormally attached to each other as a result of BCNU treatment, then they both are likely to migrate to the same pole during division. If one of these chromosomes (or pieces) would normally have migrated to the other pole, then such a division must result in deficient genetic information for one of the daughters. In addition, if the abnormal chromosome(s) migrate improperly and do not become enclosed within the main nucleus of the daughter cell to which they migrate, but rather become part of the micronucleus (as happens often after X-irradiation of cells), then that chromosome may not function at all; micronuclei formed after X-irradiation disappear with time, presumably by degradation [25]. This event would result, of course, in the loss of genetic information to the daughter cell that received that chromosome at mitosis.

Although the chromosome aberrations themselves could account for some of the difficult divisions, such as those that take an abnormally long time, there may be secondary causes of faulty division. For example, multipolar divisions could occur as a result of the long G_2 delay in much the same way that they occur after a long arrest in mitosis with Colcemid. With the Colcemid arrest, two normally functioning centrioles may mature and divide into four functioning centrioles during the mitotic block because their growth and division cycle is not coupled closely to the cell cycle [26]. When the cell is then released from the mitotic block, not only two, but three or four centrioles are available centers for chromosome organization for division. The cell may then divide into three or four cells rather than two. Alternatively, the division may be so disorganized that either the chromosomes are not divided equally between daughters, or cytokinesis cannot be completed at all (Table 3).

Whatever the ultimate cause of death of cells treated with BCNU, death does not occur immediately after BCNU treatment. If tumor cells *in vivo* respond similarly to BCNU as do 9L cells *in vitro*, these studies have implications for chemotherapy. Because BCNU-treated cells do not die and lyse immediately after treatment, a tumor would not be expected to regress shortly after treatment. In fact, since some division usually occurs before the cells die, a treated tumor would be expected to grow before shrinking. Whatever the distribution of cells in the cell cycle, the number of cells in the tumor would probably multiply by a factor of 2 or 3 before significant numbers of cells died and caused growth to cease and regression to begin (Fig. 4). For a cell population with a doubling time of about 18 hr, the growth curve does not plateau until 2 or 3 days after treatment with 2 μg/ml of BCNU. For cells with longer generation times, the growth curve would likely take a proportionately longer time to plateau if the cells always need to go through division before expressing the lethal effects of BCNU. If a higher concentration of the drug is used,

the advantages are not only that fewer cells survive, but that cells die sooner after treatment with less increase in tumor size before stasis or regression. This may be particularly important for the treatment of brain tumors because the rigid skull does not allow much increase in tumor size without damage of normal tissues caused by the resulting pressure.

The size of a tumor depends not only on the number of live cells in the tumor, but also on both the removal rate of dead cell debris and the volume of the remaining live cells. Because dead cells are apparently not removed rapidly from the cranial cavity [27], dead brain tumor cells can contribute significantly to the tumor mass. In addition, an increase in the volume of clonogenically sterilized but as yet not dead BCNU-treated cells could contribute significantly to the tumor mass. The time-lapse pictures from which the data here are taken suggest that BCNU-treated cells become abnormally large after treatment and that therefore a tumor containing such cells would increase in size proportionately. Thus, a tumor treated with BCNU might increase in size even before any cell division occurred within it and grow with each newly added cell formed at division. Even as the cells die, the tumor size might not decrease rapidly if the debris is not removed quickly.

We hope that the data here will be useful in understanding the response of tumors treated *in vivo* when BCNU or other nitrosoureas are used as chemotherapeutic agents. At least, the data provide a basis for explaining why patients with brain tumors often get clinically worse before they get better after nitrosourea therapy. The data also caution against using the time of improvement of symptoms after therapy as the criterion for selecting which agent in a combined modality regimen is responsible for the clinical response.

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REFERENCES

1. S. K. Carter, Current status of new agents. *Cancer Chemother. Rep.* **3,** 33 (1972).
2. V. A. Levin and C. B. Wilson, Chemotherapy: the agents in current use. *Sem. Oncology* **2,** 63 (1975).
3. S. C. Barranco and R. M. Humphrey, The effects of 1, 3 bis (2-chloroethyl)-1-nitrosourea on survival and cell progression in chinese hamster cells. *Cancer Res.* **31,** 191 (1971).
4. B. K. Bhuyan, L. G. Scheidt and T. J. Fraser, Cell cycle phase specificity of antitumor agents. *Cancer Res.* **32,** 398 (1972).
5. B. Drewinko, B. W. Brown and J. A. Gottlieb, The response of synchronized human lymphoma cells to bleomycin and 1, 3 bis(2-chloroethyl)-1-nitrosourea. *Cancer Res.* **33,** 2732 (1973).
6. B. Drewinko, T. L. Loo and J. A. Gottlieb, A comparison of the lethal effects of three nitrosourea derivatives on cultured human lymphoma cells. *Cancer Res.* **36,** 511 (1976).
7. R. A. Tobey and H. A. Crissman, Comparative effects of three nitrosourea derivatives on mammalian cell cycle progression. *Cancer Res.* **35,** 460 (1975).
8. K. T. Wheeler, N. Tel, M. E. Williams, S. Sheppard, V. A. Levin and R. M. Kabra, Factors influencing the survival of rat brain tumor cells after *in vitro* treatment with 1, 3 bis(2-chloroethyl)-1-nitrosourea. *Cancer Res.* **35,** 1464 (1975).
9. K. Nomura, T. Hoshino, K. Knebel and M. Barker, Effect of dibuturyl cyclic AMP on cell cycle progression of rat brain tumor cells. *In Vitro* **14,** 174 (1978).
10. K. W. Kohn, Interstrand cross-linking of DNA by 1, 3 bis(2-chloroethyl)-1-nitrosourea and other 1-(2-haloethyl)-1-nitrosoureas. *Cancer Res.* **37,** 1450 (1977).

11. T. Caspersson, E. Klein and N. R. Ringertz, Cytochemical studies on some effects of X-radiation on three ascites tumors. *Cancer res.* **18,** 857 (1958).
12. W. Dittrich and W. Göhde, Phase progression in two dose response of Ehrlich ascites tumour cells. *Atomkernenergie* **3,** 174 (1970).
13. U. K. Ehmann, H. Nagasawa, D. F. Petersen and J. T. Lett, Symptoms of X-ray damage to radiosensitive mouse leukemic cells: asynchronous populations. *Radiat. Res.* **60,** 453 (1974).
14. G. Froese, Division delay in HeLa cells and chinese hamster cells. A time-lapse study. *Int. J. Radiat. Biol.* **10,** 353 (1966).
15. S. Okada, Cells. In *Radiation Biochemistry.* (Edited by K. I. Altman, G. B. Gerber and S. Okada) Vol. 1, p. 188. Academic Press, New York (1970).
16. J. Seed, The sysnthesis of DNA, RNA and nuclear protein in normal and tumor strain cells. I. Fresh embryo human cells. *J. Cell Biol.* **28,** 233 (1966).
17. J. Seed, The synthesis of DNA, RNA and nuclear protein in normal and tumor strain cells. II. Fresh embryo mouse cells. *J. Cell Biol.* **28,** 249 (1966).
18. J. Seed, The synthesis of DNA, RNA and nuclear protein in normal and tumor strain cells. III. Mouse ascites tumor cells. *J. Cell Biol.* **28,** 257 (1966).
19. J. Seed, The synthesis of DNA, RNA and nuclear protein in normal and tumor strain cells. IV. HeLa tumor strain cells. *J. Cell Biol.* **28,** 263 (1966).
20. W. K. Sinclair, Cyclic X-ray responses in mammalian cells *in vitro. Radiat. Res.* **33,** 620 (1968).
21. T. Terasima and L. J. Tolmach, Variations in several responses of HeLa cells to X-irradiation during the division cycle. *Biophys. J.* **3,** 11 (1963).
22. L. H. Thompson, and H. D. Suit, Proliferation kinetics of X-irradiated mouse L cells studied with time-lapse photography. II. *Int. J. Radiat. Biol.* **15,** 347 (1969).
23. G. F. Whitmore, C. P. Stanners, J. E. Till and S. Gulyas, Nucleic acid synthesis and division cycle in X-irradiated L-strain mouse cells. *Biochem. biophys. Acta (Amst.)* **47,** 66 (1961).
24. R. A. Walters and D. F. Petersen, Radiosensitivity of mammalian cells. I. Timing and dose-dependence of radiation-induced division delay. *Biophys. J.* **8,** 1475 (1968).
25. S. J. Grote, Radiation-induced genetic damage and lethality in mammalian cells. Ph.D. thesis, University of London (1971).
26. B. R. Brinkley and E. Stubblefield, Ultrastructure and interaction of the kinetochore and centriole in mitosis and meiosis. *Advanc. Cell Biol.* **1,** 119 (1970).
27. A. R. V. Kumar, T. Hoshino, K. T. Wheeler, M. Barker and C. B. Wilson, Comparative rates of dead tumor cell removal from brain, muscle, subcutaneous tissue, and peritoneal cavity. *J. nat. Cancer Inst.* **52,** 1751 (1974).