Effects of Hyperthermia on Survival and Progression of Chinese Hamster Ovary Cells

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ABSTRACT

In general, the survival of Chinese hamster ovary cells exposed to hyperthermic temperatures of 42.5–46.0° decreases exponentially as a function of duration of heat exposure in a manner quite similar to survival as a function of radiation dose. The data indicate that above 43° a 1° change in temperature requires a 2-fold change in time to achieve the same degree of killing, whereas below 43° the same 2-fold change in time requires only a 0.5° change in temperature for the same effect. An Arrhenius-type plot of the rate of killing as a function of reciprocal temperature exhibits linearity with a change in slope at 43°. This change in slope suggests either a change in the mechanism of cell killing below this temperature or a manifestation of thermal tolerance that is readily observed when the duration of heating exceeds 4 to 5 hr.

Thermotolerance to 45.5°, as evidenced by a 3- to 4-fold increase in $D_n$, is observed in synchronous G1 cells exposed to heat 20 hr after an initial heat dose. This thermotolerance develops, although no progression of cells into S phase occurs during this period. In addition, thermotolerance develops in both asynchronous and synchronous G1 cells exposed to single heat doses between 41.5 and 42.5° for periods exceeding 4 to 5 hr, i.e., survival decreases exponentially as a function of duration of heating up to 4 to 5 hr, after which survival decreases very little. At 42.0–42.5°, survival is extremely sensitive to changes in temperature, with as much as a 10-fold difference in survival for a 0.1° difference in temperature with heat exposures greater than 4 hr. The above data indicate the importance of careful treatment design and precise temperature control if hyperthermia is to be used for cancer therapy.

No progression of synchronous G1 cells into S phase is observed for cells continuously exposed to temperatures of 42.0° and above. However, computer simulation of sequential DNA histograms from flow cytometry of synchronous cells continuously exposed to 41.5° indicates that cell cycle delays of 5.4 and 2.4 hr for G1 and S, respectively, occurred for cells which exposure began in G1, and delays of 0.5 and 5.4 hr for S and G2 plus mitosis, respectively, occurred for cells which exposure began in late S. Normal cell cycle phase transit times for G1, S, and G2 plus mitosis are 4.3, 7.1, and 2.4 hr, respectively.

In addition, mitotic indices and increases in cell number of asynchronous populations of cells continuously exposed to 41.5° indicate that entry into mitosis is delayed for approximately 2 hr. Following this delay, cells begin to enter mitosis and accumulate from 2 to 6 hr in metaphase; after about 6 hr, they begin to progress into G2. However, comparison of flow cytometry data and mitotic index data suggests that during the initial 6 hr of heating, the majority of cells accumulating in G2 plus mitosis are actually delayed in G2.

INTRODUCTION

The current interest in the effects of hyperthermia, especially as applicable to cancer therapy, is well documented (29). Hyperthermia both alone and as an adjunct to current therapeutic techniques is presently under investigation by many researchers, and its potential importance lies partly in its ability to increase selectively the killing of cells that are resistant to X-irradiation (7, 14, 17, 18, 34), i.e., S-phase cells, and poorly oxygenated cells that are frequently under thermal sensitizing conditions of poor nutrition and low pH.

As originally shown by Westra and Dewey (37) for CHO in vitro, an Arrhenius plot of the logarithm of the reciprocal of $D_n$ from heat survival curves plotted as a function of the reciprocal of absolute temperature suggests that a singular mechanism with an apparent inactivation energy ($\mu$) of 141 kcal/mole is responsible for heat inactivation at 43.5–46.5°. However, much of the research for the treatment of tumors by hyperthermia has been done at temperatures below 43° (29). Thus, additional information on the effects of heat at these lower temperatures is necessary.

The response of mammalian cells to hyperthermia is characterized by sigmoidal-shaped survival curves much like those observed for radiation exposures. While this relationship is well founded, certain instances of resistance to thermal killing have been observed as the duration of heating increased (14, 19, 28). Other examples of what appears to be thermotolerance have been shown with split-dose treatments in CHO and HeLa cell cultures (12, 21). In most cases, this hyperthermic resistance has not been genetically transmissible. Studies with pig kidney cells are an apparent exception, since thermally resistant cell lines have been isolated and maintained for extended periods of time (20).

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S. A. Separeto et al.

The studies on thermotolerance mentioned above have all been performed on asynchronous populations of cells and, while thermotolerance is clearly evident, the effect is complicated by possible redistribution and progression of cells in the cell cycle. In the studies presented here, synchronous cells were used and were carefully monitored for cell cycle progression to ascertain the importance of this possible complication.

Evidence of thermotolerance also is suggested from studies in which hyperthermia was administered in vivo for the treatment of cancer. Crile (5) reported that both normal tissue and several types of tumors in mice became heat resistant at 24 hr after exposure to one-third of a lethal dose of heat, but by 72 hr this heat resistance was lost.

The technique of FCM analysis of cellular DNA content has been shown to be a useful tool for the rapid determination of cell cycle-age distribution (33). Several methods for the statistical analysis of DNA histograms obtained from FCM measurements have been demonstrated (1, 6). However, while effective in analyzing asynchronous cultures in exponential growth, these methods are not suited for the analysis of sequential DNA histograms for synchronous cells progressing through the cell cycle. A method of computer simulation of sequential spectra based on the input of various parameters has been developed to avoid this difficulty (15), and this technique has been applied to the analysis of cell cycle perturbations under various conditions (16). In the work shown here, this technique has been used to determine the cell cycle perturbations caused by continuous low-level hyperthermic treatment of synchronous populations.

MATERIALS AND METHODS

Monolayer cultures of CHO were maintained as described previously (8, 35) at 37° with the use of McCoy's Medium 5A supplemented with 10% calf and 5% fetal calf sera.

Asynchronous cells in log-phase growth (doubling time, 13 hr) were plated into Falcon T-25 flasks containing 4 ml of medium at room temperature and subsequently were returned to 37°. Synchrony was accomplished by mitotic shake selection as described previously (8, 31, 36), and mitotic cells were pooled and maintained at 4° until plated (no more than 3 hr). Mitotic indices varied from 87 to 97% as determined by scoring 200 squashed cells stained with acetoorcein as described previously (8). Maintaining mitotic cells in the cold for less than 4 hr causes about a 1-hr lengthening of G1, (26, 27) with no evidence of cytological damage and very little reduction in plating efficiency. Progression of cells was assumed to begin 15 min after they were placed in the 37° incubator, i.e., when the cells had reached 37°. After the synchronous cells were incubated for 1.5 hr, 98 to 99% of the cells had entered G1.

 Heating was accomplished by total immersion of paraffinsealed flasks in Precision-Freas water baths (Precision Scientific Co., Chicago, Ill.) capable of maintaining temperatures within ±0.01°. All thermometers (mercury-in-glass) were calibrated by standardization against a National Bureau of Standards-certified thermometer (Fisher Scientific Co., Pittsburgh, Pa.), and the standard error over the range of temperatures used was determined to be less than ±0.02°. In addition, freeze-point checks were periodically made, and corrections were made for emergent stem effects (3).

Cells were maintained at 37° at all times except for stated treatments. All flask volumes were maintained within 10%, and temperature transitions were accomplished by rapid transfer of flasks between water baths set at the appropriate temperatures to assure reproducibility of temperature equilibration. The t1 for temperature transition for 4 ml of medium in T-25 flasks with this system is approximately 20 sec, as determined through continuous measurement of the temperature during transition with a thermistor surface probe (Yellow Springs Instrument Co., Yellow Springs, Ohio) in contact with the surface of the flask where the cells are attached.

Following the treatments, cells were incubated at 37° for 7 to 11 days until macroscopic colonies could be counted. Plating efficiencies were between 60 and 100% in all experiments. Initial dilutions were calculated to obtain between 10 and 200 colonies, and only those colonies containing greater than 50 cells and less than one-third giant cells were scored. By counting at least 4 replicates, we determined a mean and S.E. for each point. All points were corrected for multiplicity at the time of treatment (32).

In asynchronous populations, mitotic indices were scored by counting at least 1000 cells/sample, with 2 replicates/point. Growth curves were determined with an inverted-phase microscope to count the number of cells within 8 microscopic fields/flask (0.25 sq mm/field); for maintenance of a constant temperature during thermal treatment, flasks were kept in a precision water bath, but the cells were counted in a walk-in incubator set at the same temperature (±0.2°).

We obtained FCM measurements of cellular DNA content by trypsinizing cells and pooling them at 4°. The cells were vigorously resuspended in a hypotonic citrate solution containing propidium iodide (5 mg/100 ml of 0.1% sodium citrate) used as a fluorescent DNA stain (25). Cells were maintained at cool temperatures for a maximum of about 48 hr until analysis could be completed at the Biophysics and Instrumentation Group of the Los Alamos Scientific Laboratory.

To determine cell cycle progression times, we used computer simulation of sequential DNA histograms, as described by Gray (15), to fit the experimental data visually. This analysis was performed at the Biomedical Division of the Lawrence Livermore Scientific Laboratory.

RESULTS

Kinetics of Thermal Killing. To ensure that heat treatments would begin at the same point in the cell cycle, we placed synchronous G1 cells at 20° at 1.5 hr after plating and held them at 20° for up to 4.5 hr before heat treatments commenced. Progression ceased for the duration of time at 20°, as shown in Chart 1 by measurement of [3H]thymidine incorporation.

Following various periods at 20°, the synchronous cells in G1 were immersed in precision water baths at 42.0–46.0°
for various lengths of time and then were returned to 37° and incubated for colony formation. Similarly, asynchronous cells were plated in T-25 flasks, incubated for 4 to 6 hr at 37°, and then treated at 41.5-46° for various times (Chart 2). At 42.0-42.5°, a large change in sensitivity to heating was observed. A more exact determination of this effect is demonstrated by an Arrhenius plot, i.e., a plot of the inverse of the $D_0$ value (an indication of the rate of killing) as a function of the inverse of absolute temperature (Chart 3). The $D_0$ values used were obtained from the maximum slopes determined in Charts 2 and 5. Of particular importance is the apparent break in linearity observed in the region of 43°. Values for $\mu$, a measure of inactivation energy, can be calculated by entering the data in Chart 3 into the Arrhenius equation:

$$\ln k = \ln A - \frac{\mu}{RT}$$

where $k$ is replaced by $1/D_0$, $A$ is a constant, $R$ is the gas constant, and $T$ is temperature in °K (23, 37). The heat inactivation energy values obtained were 148 and 365 kcal/mole above and below 43°, respectively, and are in good agreement with the values reported in the literature (4, 22, 37). However, the rates determined are valid for G1 cells that are relatively heat resistant compared to S-phase cells; S-phase cells need not necessarily have the same values for $\mu$.

**Induction of Thermotolerance.** A split-heat dose experiment was performed with synchronous G1 cells 2 hr after mitotic cells were plated. An initial heat dose of 15 min at 45.5° reduced survival to an average value of 0.15. The first heat treatment was followed by incubation at 37° for various times until a series of second heat treatments at 45.5° were given. In Chart 4, the results of this experiment are shown with all initial survival values normalized to 1.0. The dotted curve represents a single heat dose survival curve, and the dashed line (labeled 0 hr) is the lower portion of the same curve beginning at 0.15 survival (from 15 min heat treatment), normalized to start at 0 time and 1.0 survival fraction. At intervals of 2, 4, 6, 10, and 20 hr, second heat doses were given to obtain the curves shown. Concurrent pulse-labeling of cells at 2-hr intervals after the initial 15-min heat dose showed no incorporation of [3H]thymidine by 20 hr (data not shown), which indicated that the cells remained in G1 after the first treatment. The results show that, by the end of 2 hr, survival is slightly greater than an additive level (0-hr dashed line) and that, somewhere between 6 and 10 hr, survival becomes significantly higher than the independent level (represented by the dotted single-dose line; for a more complete definition of additive and independent see Ref. 11). Although the shoulder is returning, the slope is...
also decreasing and by 20 hr, despite the lack of progress,
the $D_s$ value has increased by a factor of about 3
above the single-dose curve.

The survival curves shown in Chart 2 for temperatures
of 42.5° and below indicate that a thermotolerant effect may
become evident for heating times greater than 200 to 300
min. This phenomenon was studied in more detail by
heating asynchronous cells or synchronous G₁ cells at
42.0–42.5° (Chart 5). The lack of additional killing at times
beyond 4 hr is clearly evident.

**Cell Cycle Progression.** For ascertainment of the move-
ment of cells through the cell cycle at hyperthermic tempera-
tures, synchronous G₁ cells were kept at 37° or were
raised to a temperature of 41.5° commencing 2 hr after the
mitotic cells were plated at a density of $4 \times 10^6$/sq cm. At
2-hr intervals continuing until 18 hr after plating, the cells
were sampled for FCM measurements of DNA content.

In fitting the data by computer simulation, coefficients of
variation for the dispersion of average phase transition
times and for instrumental dispersion were assumed to be
20 and 5%, respectively. In addition, the rate of DNA
synthesis through S phase was approximated by a bell-
shaped function, and approximately 4% of the population
was assumed to be noncycling with a DNA content of G₁
cells. By adjustment of the average phase transition times
by trial and error, a best visual fit was obtained with times
of 4.3, 7.1, and 2.4 hr for G₁, S, and G₂ plus mitosis,
respectively. Actual experimental DNA histograms and cor-
responding computer-simulated spectra are shown in Chart
6. These transition times for incubation at 37° are in excel-
lent agreement with published cell cycle times for our CHO
cell line synchronized by mitotic selection (27).

For synchronous G₁ cells incubated at 41.5°, beginning 2
hr after mitosis, the best computer fit was obtained with
average transition times of 9.7 and 9.5 hr for G₁ and S,
respectively (Chart 7). The time period for sampling (18 hr)
was not long enough to obtain an estimate for G₂ plus
mitosis. In addition, the number of noncycling G₁ cells was
increased from 4 to 15%, and the coefficient of variation
for the average phase transition times was increased from
20 to 25%. Thus, the average delays in phase transition
times were found to be 5.4 and 2.4 hr for G₁ and S,
respectively.

In the same experiment, synchronous cells in mid to late
S phase also were incubated at 41.5°, beginning 10 hr after
mitosis. Computer analysis of these data (Chart 8) was
performed with the use of a 10-hr cell cycle distribution
obtained with control parameters as initial conditions. Sim-
ulated histograms were then calculated at 2-hr intervals up
to 18 hr after mitosis, with the use of the cell cycle transition
time parameters of 9.5 and 7.8 hr for S and G₂ plus mitosis,
respectively. Since FCM is unable to distinguish between
G₂ and mitotic cells, delays are reported for G₂ and mitosis
combined. The cell cycle time for S phase is in agreement
with that determined for cells for which heat treatment was
initiated 2 hr after mitosis. However, since these cells were
allowed to progress most of the way through S before the
initiation of heat treatment, a total S-phase transition time
of 9.5 hr represents only a 0.5-hr delay of progression into
G₂ for these cells (Table 1). This agreement in average S-
phase transition times for initiation of heating in G₁ or S
suggests that there is no single point in S at which cells
are blocked but, rather, that progression is uniformly
slowed throughout S. Due to the delay in G₂ plus mitosis,
cells would not have reached the second-generation S
phase by 18 hr after mitosis, even with a normal second-
generation G₁. Thus, the G₁ parameter could not be deter-
mined.
Heat Effects on Survival and Progression

Chart 6. Sequential DNA distributions of cell number, plotted on the ordinate (arbitrary units), as a function of fluorescent output (DNA content) at 2-hr intervals after mitosis. On the abscissa, the DNA contents of $G_1$ and $G_2$ cells are approximated by the left and right tick marks, respectively. $E$, series of experimental spectra obtained from synchronous cells maintained at 37°; $S$, series of computer-simulated spectra with average cell cycle phase transit times of 4.3, 7.1, and 2.4 hr for $G_1$, $S$, and $G_2$, plus mitosis, respectively. The mitotic index at the time of synchrony was 97%. See text for further details.

Chart 7. Sequential DNA distributions of cell number as a function of fluorescent output at 2-hr intervals. $E$, series of experimental spectra from synchronous cells that were heated at 41.5° beginning 2 hr after mitosis and extending until the times of measurement; $S$, series of computer-simulated spectra with average cell cycle phase transit times of 9.7 and 9.5 hr for $G_1$ and $S$, respectively. The dotted curves at 16 and 18 hr on the simulated graphs represent spectra obtained with 9.7 and 8.3 hr for $G_1$ and $S$, respectively, and are shown as an indication of the sensitivity of fitting. Spectra before 16 hr do not show significant differences with these parameters. The control for this experiment is illustrated in Chart 6 for cells incubated at 37°.

Table 1

<table>
<thead>
<tr>
<th>Av. cell cycle delay (hr)</th>
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<tr>
<td>Time heating began</td>
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<tr>
<td>$G_1$ (2 hr postmitosis)</td>
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<tr>
<td>Late $S$ (10 hr postmitosis)</td>
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</table>

$^a$ At 10 hr postmitosis, the beginning of heat treatment, the cells without heating would require 1.4 hr to reach $G_2$ [4.3 ($G_1$) + 7.1 ($S$) - 10]. With $S$ phase for heated cells increased from 7.1 to 9.5 hr, the heated cells required 1.9 hr to reach $G_2$ [1.4 (9.5/7.1)]. Thus, the cells were delayed 1.9 - 1.4 or 0.5 hr. This calculation assumes that heat reduces the rate of progression uniformly throughout $S$ phase.

Chart 8. Sequential DNA distributions of cell number as a function of fluorescent output are plotted at 2-hr intervals. $E$, series of experimental spectra for synchronous cells incubated at 41.5° beginning 10 hr after mitosis. $S$, series of computer-simulated spectra beginning from a normal (37°) 10-hr simulated spectra (see Chart 6) with average cell cycle phase transit times of 9.5 and 7.8 hr for $S$ and $G_2$, plus mitosis, respectively.

For confirmation of the results of FCM measurements and computer simulation, synchronous cells were similarly heated in $G_1$ (2 hr after plating of mitotic cells) at a cell density of 400/sq cm. At 2-hr intervals, the cells were returned to 37°C for 10 min, pulse labeled with $[^3H]$thymidine for 15 min, and fixed. Autoradiographic scoring (Chart 9) showed that, for temperatures of 42.0° and above, no incorporation of label was seen during the 14-hr observation period. In contrast, progression was seen at 41.5°, but the cells were delayed 5 hr behind controls. Furthermore, the maximum percentage of cells labeled was reduced by

Chart 9. Autoradiographic measurements of percentage of cells incorporating $[^3H]$thymidine as a function of time after mitosis are illustrated. Cells were either incubated at 37°C or, beginning 2 hr after mitosis, incubated at 41.5°C, 42.0°C, 42.5°C, or 43.0°C until the time of measurement.
incubation at 41.5° (50 versus 76% for controls). This delay in transition from G₁ into S is in agreement with the 5.4-hr lengthening in G₁ observed through FCM measurements, although overall cell cycle times were longer for the autoradiographic studies than for the FCM studies, presumably due to the lower cell density used for autoradiography (9, 10).

Since FCM analysis of the cell cycle cannot differentiate between delays in G₂ and mitosis, an experiment was performed to measure the change in the differential mitotic index of an asynchronous population of cells maintained at 41.5°. In addition, growth curves showing the increase in number of cells were determined under the same conditions. The mitotic index decreased within 30 min and reached a minimum after 2 hr of heating (Chart 10). When heating continued beyond 2 hr, however, the mitotic index increased above the control value. In addition, the growth curve (Chart 11) indicates that few cells at 41.5° were able to divide during the 10-hr period; thus, the return of the mitotic index to a level greater than the control level must result from an accumulation of cells in mitosis. The differential mitotic indices support this conclusion by showing that, as the mitotic index and metaphase index of heated cells increased above control levels, the anaphase and telophase indices remained at very low levels. Thus, after 2 hr of heating, cells were entering mitosis but were greatly delayed in completing metaphase and entering G₁. The slight increase in anaphase and telophase indices, beginning after 4 hr of heating, indicates that cells began to move slowly through mitosis into G₁. This resumption in division at a reduced rate after 4 hr of heating is also evident from the growth curve (Chart 11).

DISCUSSION

The survival curves in Chart 2 show that for a given duration of heat treatment the survival of synchronous cells in G₁ is slightly higher than the survival of asynchronous cells. This is due to a selective killing of a heat-sensitive subpopulation, namely, S-phase cells. However, the final slope of the survival curve for the asynchronous population is determined by the resistant G₁ cells.

The inactivation energies (µ) in Chart 3 for both G₁ and asynchronous cells correspond approximately to the observation that, for the same level of survival, a 1° increase in temperature above 43° requires a 2-fold decrease in time. However, below 43°, only a 0.5° increase in temperature is required. These same relationships between time and temperature are seen for many in vitro and in vivo cellular systems, both normal and malignant (4, 7, 22), which suggests that these cells are killed by a similar mechanism although sensitivity to a given temperature may vary greatly between cellular systems.

Below 42.4°, the value determined for µ is uncertain, since the region of the survival curve used for determination of the slope (1/Do) did not extend much below the first decade; i.e., an exponential region could not be clearly differentiated from the shoulder region. Furthermore, although thermotolerance becomes evident only for exposures greater than 4 hr, a gradual development of tolerance between 2 and 4 hr could be causing an increase in survival that would decrease the slopes of the curves in this range of exposures. Thus, the change in slope of the Arrhenius plot below 43° (illustrated only by 3 points determined below the first decade for 42.4 and 42.5°) could actually be a manifestation of thermotolerance and not necessarily be due to different mechanisms of cell killing above and below 43°.

The sensitivity of cells to variation in temperature is illustrated dramatically (Chart 2) by the observation that a 0.5° change in temperature for a given time of heating changes survival by several decades. In fact, as shown in Chart 5, as much as a 10-fold difference in survival is observed for only a 0.1° increment at 42.4-42.5°, with heat exposures greater than 4 hr. Since these temperatures are in the range proposed for clinical treatments, precise temperature control may be an important consideration in the
use of hyperthermia for cancer therapy. On the other hand, an important benefit may be gained in terms of a sparing effect if a thermal gradient can be maintained between malignant and normal tissues.

As illustrated in Chart 4, thermotolerance was observed with split-heat doses administered to synchronous cells in G1, the most thermally resistant part of the cell cycle. Although similar studies with asynchronous cells have demonstrated thermotolerance by this method with temperatures as low as 43.5°C (12), the changes in slope shown in Chart 4 clearly demonstrate thermotolerance at 45.5°C without complications caused by cell progression. Even if progression had occurred, the G1 cells would have moved into the more thermosensitive S phase, thus reducing survival.

Below 43°C, thermotolerance also was observed for single doses of heat (Chart 5) as the duration of heating was extended beyond approximately 5 hr. Whether or not this phenomenon for single heat doses is the same as that observed with split-heat doses above 43.5°C is not known and will require further study. Indeed, thermotolerance with single doses of heat may occur at temperatures above 42.5°C but the survival level after 5 hr of heating, the time at which thermotolerance would be expected to appear, would probably be less than 10^-16.

While thermotolerance occurs at 45.5°C in synchronous G1 cells without progression into S phase (Chart 4), the relationship between the induction of thermotolerance and cell progression at temperatures below 43°C should be considered. For synchronous cells incubated in G1 at 41.5°C, the 5-hr period required for the induction of thermotolerance (Chart 5) was quite similar to the delay in progression into S phase (Chart 9; Table 1). However, the fraction of the population that progressed into S phase after 5 hr may or may not represent the thermotolerant cells. Although no progression was observed above 42°C (Chart 9), the fraction of the population that developed thermotolerance was less than 10^-2 (Chart 5); therefore, if this small thermotolerant population did indeed progress into S phase, its progression could not have been detected. At 42°C, however, approximately 15% of the synchronous G1 cells attained thermotolerance, yet no progression of G1 cells into S phase occurred for up to 14 hr after heating began. Thus, the induction of thermotolerance, as observed with either fractionated or single heat doses, occurs without cell cycle progression.

The observation that delay in G1 plus mitosis was longer than delay in S phase when cells were heated continuously at 41.5°C (Table 1) is generally consistent with reports in the literature for delays following short exposures to 41 or 43°C (24, 26, 30). Furthermore, since the maximum delays in G1 and G2 plus mitosis (5.4 hr) were longer than the delay in S phase (2.4 hr), heating an asynchronous population for several hr should reduce the number of cells in S phase, with a concomitant increase occurring predominantly in the number of cells in G2. This prediction was confirmed by heating an asynchronous population at 41.5°C for 6 hr. As the percentage of cells in G2 plus mitosis increased by 11% of the total population, there was a corresponding decrease in the percentage of cells in S (Chart 12; Table 2). No significant change was observed in the G1 populations.

The lengthening of mitosis when the cells are heated continuously at 41.5°C (Chart 10) is due to an accumulation of cells in metaphase. In fact, this accumulation of cells in metaphase appears quite similar to that seen with Colcemid treatment. This similarity suggests that heat may be delaying cells in metaphase by interfering with normal spindle formation. This hypothesis is further supported by the report of the appearance of tetraploid cells following heat treatment of mitotic cells (37). However, comparison of the increase in G2 plus mitosis (11% of the total population) (Table 2) with the increase in the fraction of cells in mitosis (2 to 3% of the total population) (Chart 10) indicates that the delay in cell progression is primarily associated with a lengthening of G2 instead of a prolongation of mitosis.3

An interesting comparison can be made between cell

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**Table 2**

<table>
<thead>
<tr>
<th>Cell cycle distribution (%)</th>
<th>G1</th>
<th>S</th>
<th>G2 + mitosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (37°C)</td>
<td>36.9</td>
<td>55.3</td>
<td>7.8</td>
</tr>
<tr>
<td>Heated for 6 hr (41.5°C)</td>
<td>38.2</td>
<td>43.3</td>
<td>18.5</td>
</tr>
</tbody>
</table>

3 The data in Table 1 and Charts 10 and 11 indicate that G2 cells cease entering prophase within 15 min after the start of heating and do not begin entering prophase again at any appreciable rate until at about 2 hr. During the 2-hr interval when cells are not entering mitosis, the few cells (about 0.8%) in anaphase and telophase at the initiation of heating apparently complete division within 15 min, and the cells in prophase and metaphase (about 3.4%) slowly move into anaphase and telophase. However, once the cells are in anaphase and telophase, they rapidly move into G1, as evidenced by a differential anaphase and telophase mitotic index of only about 0.01%. Then, at about 2 hr, G2 cells again begin progression through mitosis with transit times of about 13.8 min for prophase (9.5 min at 37°C), 101 min for metaphase (25.6 min at 37°C), and 5.8 min for anaphase and telophase (6.2 min at 37°C); transit times were calculated (31) for a cycle time of 24 hr at 41.5°C and the differential mitotic indices plotted (Chart 10) for heated cells at 8 to 10 hr. Thus, the total delays are about 5 hr for G2 plus mitosis (Table 1; Chart 11) with about 1.25 hr during metaphase and negligible delays during prophase, anaphase, and telophase (Chart 10).
cycle progression occurring during continuous hyperthermic exposure and cell cycle progression occurring during continuous low-level radiation exposure (2). A synchronous population of HeLa cells exposed continuously to 38 rads/hr and Chinese hamster V79 cells exposed to 90 rads/hr beginning in G1 progressed normally until reaching G2, in which the cells were delayed for 5 to 10 hr. Thus, while the continuous radiation exposure caused cell killing at any given time to about the same degree as that observed for our CHO cells receiving continuous heat exposure at 41.5°, the resultant cell cycle perturbations were quite different. In other words, continuous heating caused significant lengthening of G1 and S phase in addition to lengthening of G2. For both treatments, however, the cells do accumulate in G1, a phase that has been reported to be radiosensitive (32); therefore, this redistribution in the cycle may have implications in cancer therapy involving radiation and/or heat.

In summary, the survival of cells treated at 42-46° is extremely sensitive to variations in temperatures; i.e., a 0.1° increment in temperature can change survival by as much as a factor of 10. In addition, thermotolerance occurs with extended single doses of heat at 41.5-42.5°. Thermotolerance also can be seen with split-heat doses at 45.5° without cell cycle progression (Chart 4). Other investigators have observed tolerance for split-heat doses at 43.5-45.5° (12, 21). Furthermore, hyperthermia causes redistribution of cells in the cell cycle, either when heated continuously at 41.5° (Chart 12; Tables 1 and 2), or following a 1-hr heat treatment at 42 or 43° (24, 30). Since these temperatures are in the range being considered for therapeutic use, these observations stress the importance of both careful control of temperature and consideration of the duration of heating and the interval between heat fractions when hyperthermia is utilized for the treatment of cancer.

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