A Proposed Operational Model of Thermotolerance Based on Effects of Nutrients and the Initial Treatment Temperature

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ABSTRACT

The phenomenon of thermotolerance in mammalian cells has been extensively documented in the literature. Because of its potential clinical importance as well as the fundamental biological interest, we pursued additional studies investigating pH and nutritional effects. Split-dose experiments were performed using plateau phase Chinese hamster HA-1 cells. The effects of the nutritional environment during the initial 43°C incubation and the second 43°C treatment on the induction of thermotolerance were studied by comparing survival in full medium at pH 7.4 or in Hanks' balanced salt solution at pH 6.7. In additional experiments, we examined the effect on thermotolerance of changing the temperature of the initial treatment to 41°C. Thermotolerance was induced independently of the nutrient conditions of the first treatment. However, survival at the time of maximum expression of thermotolerance depended primarily on the duration and temperature of the initial treatment. Temperatures of 45°C or higher inhibited the development of thermotolerance during the first heat exposure. In contrast, if the initial exposure was at 41°C, thermotolerance was almost fully expressed by the end of this initial treatment. Changing pH in Hanks' balanced salt solution from 6.7 to 7.4 did not affect survival.

On the basis of these and other data, we suggest that thermotolerance can be divided into three complementary and sometimes competing processes: an initial event ("trigger"); the expression of resistance ("development"); and its disappearance ("decay"). Trigger is induced at all hyperthermic temperatures while development requires a permissive temperature, i.e., less than 43°C for HA-1 cells. This model can provide plausible interpretations of several currently puzzling aspects of the survival kinetics of heat-exposed mammalian cells.

INTRODUCTION

The ability of mammalian cells to transiently acquire heat-induced resistance to subsequent exposures at elevated temperatures has been called thermotolerance (4, 5, 10, 11, 13, 14, 21, 23). For example, Henle et al. found that thermotolerance can be induced in CHO cells by a short 45°C heat treatment followed by incubation at 37°C. This was evidenced by the finding that the survival curve after a second 45°C exposure showed an increased D0. Their results also indicated that CHO cells incubated at 40°C up to 7 hr developed a somewhat different form of thermotolerance. The survival curve to a subsequent exposure at 45°C showed no change in D0 but an increase in the width of the shoulder. Below about 43°C, tolerance can also be induced during continuous heating (3, 6, 10, 21, 22); however, above this temperature, thermotolerance occurring during continuous heating has not been demonstrated. A recent thorough review discusses this subject in considerable detail (12).

The effect of thermotolerance can be quite dramatic; increases in survival levels by orders of magnitude are quite commonplace. However, this large increase in survival, when comparing responses of tolerant to nontolerant cells, corresponds to an equivalent decrease in temperature of only 1 to 2°C (1). Thermotolerance may also be of great importance clinically where it acts as a 2-edged sword. If induced in tumor cells, it may partially negate the anticipated cytotoxic effects of subsequent heat fractions. On the other hand, tolerance induced selectively and intentionally in treatment-limiting normal tissue could greatly enhance the therapeutic efficacy of localized or regional hyperthermia. Thus, for biological and clinical reasons, studies leading to a better understanding of this intriguing phenomenon appear warranted.

We report here on experiments which were designed to test the effects of the nutritional environment on the induction of thermotolerance in CHO HA-1 cells. Specifically, we compare survival of plateau phase HA-1 cells after split-dose treatments at 43°C in either full medium (pH 7.4) or a BSS (pH 6.7). These 2 combinations were chosen so as to maximize cellular resistance and sensitivity, respectively, while still maintaining physiologically meaningful conditions. Some control experiments were, however, performed in Hanks' BSS at pH 7.4. We also examined the nutrient effects of incubations at 37°C between the 2 treatments. Finally, we describe kinetics of inductions of thermotolerance if the initial treatment temperature is reduced to 41°C. We chose plateau phase cells because experiments with such cells are relatively free of the complications introduced by cell progression (9). Our definition of tolerance is based exclusively on survival increases, thus involving one or possibly more parameters of the survival curve.

Our results lead us to propose a new operational model of thermotolerance. This model has been tested on several levels, and it may clarify several currently puzzling aspects of the survival kinetics of heat-exposed mammalian cells.
**MATERIALS AND METHODS**

Cells and Culture Conditions. CHO HA-1 (25) fibroblasts were grown in Eagle's minimal essential medium supplemented with 15% fetal calf serum, penicillin, and streptomycin. The cultures were kept in a humidified incubator with a mixture of 95% air and 5% CO2 and routinely checked for mycoplasma. Density-inhibited cultures (plateau phase) were obtained by seeding 1 x 10^6 cells/sq cm in 60-mm Petri dishes. Beginning on the day when the cell number reached 1 x 10^5 cells/sq cm, media were changed daily. Experiments were performed on the second day after the cell number had started to stabilize; this was usually the ninth day after plating. The maximum cell density under these conditions was 1.2 to 1.5 x 10^6 cells/sq cm. At the completion of each final heating, cells were trypsinized and counted on a Coulter counter, and appropriate dilutions were plated to yield approximately 100 to 200 colonies/sq cm. At the completion of each final heating, cells were trypsinized and counted on a Coulter counter, and appropriate dilutions were plated to yield approximately 100 to 200 colonies/dish. Trypsinization has little or no effect on survival as demonstrated by published results (18, 19). After 10 days of incubation at 37°C, colonies were fixed, stained, and counted. Plating efficiencies were 60 to 80%. All experiments were performed at least twice; these yielded consistent results. Data from representative experiments are shown in Chart 2, a to d. Each set of experiments had its own control, i.e., 0-hr survival curve. Intraexperimental results were consistent with those predicted by Poisson statistics; for each data point, at least 100 colonies were counted.

**Heating.** Monolayers of cells on plastic Petri dishes were exposed to 43°C in specially designed hot water baths in incubators (8). The pH of the medium overlying the cells was maintained by a regulated gas flow of a mixture of air and CO2 to values between 7.2 and 7.4. The pH was measured immediately before and immediately after heating. Incubation for up to 26 hr did not result in pH changes greater than ± 0.2 in medium and ± 0.05 in Hanks' BSS. In those experiments in which the cellular response in Hanks' BSS was investigated, the cells were rinsed at least twice with Hanks' BSS before the experiment in order to minimize the possible influence of serum on cellular response. The pH of Hanks' BSS was 6.7 except when other values are specified. The temperature of the hot water baths was controlled to within ± 0.1°C. The time required to reach thermal equilibrium was about 3 min and is included in the total time of heating. Immediately before each treatment, either 43°C heating or 37°C incubation, the cultures were overlaid with 5 ml of fresh medium or 5 ml of Hanks' BSS. For simplicity, both in the text and charts, medium with 15% fetal calf serum is referred to as M*, and Hanks' BSS is referred to as H.

**Notation.** Because the complexity of some of the experiments would require excessively cumbersome word descriptions, we have adopted a hopefully clarifying notation. Each experiment involves 3 separate sets of environmental conditions, and each such condition involves 3 variables which need to be specified. These conditions occur during initial heating, during incubation between exposures, and during the second heating. The variables involved are: temperature; duration of treatment; and type of medium. An illustration of our notation is the description of Chart 2.

\[(43^\circ, 30\text{ min}, M^*) \rightarrow (37^\circ, t, M^*) \rightarrow (43^\circ, t_2, M^*)\]

The conditions of the initial heat exposure (43°C, fixed 30-min exposure duration, in full medium) are in the first set of parentheses. The incubation conditions are depicted above the arrow: 37°C; variable period of time, t; in full medium. The second set of parentheses indicates that the second heating was performed at 43°C, that the time duration of that treatment (t2) was a variable, and that exposure was in full medium also. The general notation is

\[(T_1, t_1, M^*) \rightarrow (T_2, t_2, M^*)\]

where parameters [(T1 and T2) and (t1 and t2)] are the temperatures and durations of the 2 treatments, respectively, and T1 and t1 the temperature and duration of the intervening incubation.

**RESULTS**

Chart 1a shows the survival responses of HA-1 cells exposed to 43°C in Hanks' BSS. Data are from 12 control experiments performed over a 30-month period. While considerable survival fluctuations are seen, these appear to result from relatively minor changes in the width of the shoulders of the survival curves. The steepness of those curves accentuates the survival variations. In Chart 1b, we demonstrate that incubation in Hanks' BSS for up to 28 hr did not change the cells' survival response to 43°C. Finally, in Table 1, we present data on the fraction of cells which lyse following heating. If incubation following a 43°C exposure was in full medium, little if any lysis was observed; the number of cells/dish does not change significantly over a 24-hr period. In Hanks' BSS, some of the cells do lyse; the number of cells/dish may drop as much as 30% in the 26-hr interval.

The results presented in Table 1 and Chart 1 are of little interest by themselves, but they aid in the interpretation of the body of the data.

Development of thermotolerance in plateau phase HA-1 cells is shown in Chart 2. In these experiments, monolayers of cells were first exposed to 43°C for 30 min. Some of these cells were challenged immediately by a second treatment consisting of graded periods at 43°C. The other cells were incubated at 37°C for 4, 8, 16, or approximately 24 hr, and the cells were then given the second exposure at 43°C. The survival after the first treatment was 85 to 100%. The parameter varying in these experiments, in addition to incubation time, was the nutrient environment: during the first heat treatment; during the 37°C incubation; and during the second heat challenge. Four combinations of the sequences of nutrient conditions are shown in Chart 2.

\[(43^\circ, 30\text{ min}, M^*) \rightarrow (37^\circ, t, M^*) \rightarrow (43^\circ, t_2, M^*)\] (a)

\[(43^\circ, 30\text{ min}, M^*) \rightarrow (37^\circ, t, M^*) \rightarrow (43^\circ, t_2, H)\] (b)

\[(43^\circ, 30\text{ min}, H) \rightarrow (37^\circ, t, M^*) \rightarrow (43^\circ, t_2, H)\] (c)

\[(43^\circ, 30\text{ min}, H) \rightarrow (37^\circ, t, H) \rightarrow (43^\circ, t_2, H)\] (d)

In (a) and (b), the experimental conditions of the initial treatment as well as of the 37°C incubation were identical. Thus,
An Operational Model of Thermotolerance

Chart 1. Survival responses of plateau phase HA-1 cells exposed to a single dose at 43° in Hanks' BSS. In a, monolayers of cells were exposed to 43° for various lengths of time before trypsinization and plating for colony formation. Data are from 12 control experiments (as indicated by various symbols). These results were obtained over a 30-month period. In b, plateau phase HA-1 cells were incubated in 5 ml Hanks' BSS for up to 28 hr before the 43° exposure. This preincubation is seen not to affect survival.

Table 1

<table>
<thead>
<tr>
<th>Duration of 37° incubation, t (hr)</th>
<th>0</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>26</th>
</tr>
</thead>
<tbody>
<tr>
<td>(43°, 30 min, M+)</td>
<td>23-30</td>
<td>26-30</td>
<td>22-28</td>
<td>22-27</td>
<td>20-26</td>
</tr>
<tr>
<td>(43°, 30 min, H)</td>
<td>23-30</td>
<td>21-26</td>
<td>18-24</td>
<td>18-20</td>
<td>18-20</td>
</tr>
</tbody>
</table>

Numbers shown are millions of cells/60-mm Petri dish. Variations are extreme values seen in about 30 experiments; unheated controls show similar changes in Hanks' BSS.

examination of the survival results depicted in these 2 panels compares the cytotoxic effect of varying the medium during the second heat treatment. Similarly, comparing results from (c) and (d) permits study of the survival kinetics of thermotolerant cells during 37° incubation in either H or M+. Finally, to study the effect on thermotolerance of having H or M+ present during the first heat treatment, the results from (b) and (c) should be compared. While thermotolerance developed in each of the experiments represented by Chart 2, there were clear quantitative differences in the survival curves. After 30 min of heating at 43° followed by a 4-hr incubation at 37°, 100% of the cells survived 3 hr of reheating at 43° provided the treatment was carried out in M+. This 100% survival was seen even after 24 hr of incubation at 37° (Chart 2a). However, if the cells were in H during the second heat treatment, results demonstrated that a partial, gradual disappearance of the acquired tolerance had occurred within 24 hr of 37° incubation (Chart 2b). If the second treatment was in M+, survival was 100%, and therefore, the data provide no information regarding the possible decay of the tolerant state within the 24 hr examined (Chart 2a). For this reason, in most other experiments reported in this communication, a second treatment (43°, 120 min, H) was used. This treatment resulted in some cell lethality even in cells in their most tolerant state (except for 2 points, one on Chart 2b and the other on Chart 3b where survival was 100%). We recognize that other combinations of time and nutrient conditions might yield a set of data showing quantitatively different kinetics. In fact, it is possible that the lesions induced or recognized in H might even be somewhat different from those in M+ even at similar exposure temperatures.

Kinetics of the development of thermotolerance in plateau phase HA-1 cells following an initial treatment at 43° for 30 min are shown in Chart 3. Four combinations are presented there.

\[(43°, 30 \text{ min, } M^+) \rightarrow (43°, 120 \text{ min, } M^+) \rightarrow (43°, 120 \text{ min, } H) \] (a1)

\[(43°, 30 \text{ min, } M^+) \rightarrow (37°, t, M^+) \rightarrow (43°, 120 \text{ min, } H) \] (a2)
Chart 2. Development of thermotolerance in plateau phase HA-1 cells. a. (43°, 30 min, M*) → (43°, t2, M*); b. (43°, 30 min, M*) → (43°, t2, H); c. (43°, 30 min, H) → (43°, t2, H); and d. (43°, 30 min, H) → (43°, t2, H). Durations of incubations at 37° between the 2 challenges are as indicated (0 to 26 hr).

Results from experiments following Protocols (a1) and (a2) are shown in Chart 3a; those from Protocols (b1) and (b2) are in Chart 3b. If the initial 43° exposure was in H, the cells acquired maximum tolerance which resulted in a 105- to 106-fold increase in survival as shown by the survival values at the 4-, 6-, or 8-hr points. The rate of induction of thermotolerance was not significantly affected by nutrient condition of the initial treatment, although heating in H resulted in a delay of maximum tolerance by 1 to 2 hr. This may be because the increase in relative survival was always greater if the initial heating was carried out in H. On the other hand, decay of thermotolerance depended primarily on the milieu of the initial treatment; the
rate of its disappearance was faster if initial heating was in $M^*$. Because the experiments were limited to 24 hr, no attempt was made to quantify the rate constants. Finally, if the only parameter that varied was the nutrient environment during the $37^\circ$ incubation, then cells incubated in $M^*$ acquired more thermotolerance than did those incubated in $H$.

The effect on development of thermotolerance of varying the duration of the initial treatment was tested next (Chart 4). Cells were exposed to $43^\circ$ for 10, 20, and 30 min in $H$. The second treatment in all experiments was a 120-min exposure at $43^\circ$ in $H$. The magnitude of induced thermotolerance, manifested as usual by increased survival, clearly depended on the duration...

![Chart 3](chart3.png)

Chart 3. Kinetics of the development of thermotolerance in plateau phase HA-1 cells following an initial exposure at $43^\circ$ for 30 min. The second treatment was $(43^\circ, 120$ min, $H)$. The surviving fractions are plotted as a function of duration of $37^\circ$ incubation between the initial and second treatments. Note the very low survival at 0 time; in b, survival at 0 time is estimated to be $10^{-6}$ based on extrapolation of the survival curve (Chart 1).

![Chart 4](chart4.png)

Chart 4. Thermotolerance induced by initial heat treatments at $43^\circ$. Cells were exposed for 10, 20, and 30 min in 5 ml fresh $H$ as indicated. The second treatment in all experiments was a 120-min exposure at $43^\circ$ in $H$. The survival at 0 time was so low that no colonies were observed. We have estimated survival from its possible upper limit. Broken lines, lack of precise data at 0 time.
of the initial 43° treatment. However, neither the rate of induction nor the rate of decay of thermotolerance was influenced significantly by the length of the initial heat dose. Some of these data are presented in somewhat different fashion in Chart 5. Here, we plot the survival values after 4 hr of incubation versus the duration of the initial treatment. The 4-hr point was chosen because survival was then at or close to the maximum value independent of the initial treatment. This plot demonstrates that an initial exposure of 20 to 30 min is required for the development of maximum thermotolerance.

Finally, the kinetics of the development of thermotolerance in plateau phase HA-1 cells following an initial treatment at 41 or 43° for 60 min are shown in Chart 6. In this experiment, the final treatment was a 45-min exposure at 45° in M+. The survival value after this heat exposure (but without the 41° pretreatment) was $8 \times 10^{-5}$. Thermotolerance is seen to be essentially at its maximum by the end of the 41° priming treatment. This is evidenced by the increase from $8 \times 10^{-5}$ to $5 \times 10^{-3}$ in the 0-hr survival value and the very minor increase in survival as the interval between the 2 doses is increased. This is in very marked contrast to the survival kinetics following initial treatments at 43° where survival at 0 time is very much lower.

**DISCUSSION**

The thermotolerance literature and the present studies provide us with what appears to be a bewildering array of data describing a complex series of biological observations. Many of the data in the literature have been reviewed by Henle and Dethlefsen (12). These authors define 3 types of thermotolerance based on phenomenological characteristics of survival curves and calculated differences in thermodynamic parameters as obtained by Arrhenius analysis. For Arrhenius analysis to be valid, the existence of at least quasi-steady state conditions is required, and it is difficult to be certain that such a situation existed in all the experiments analyzed by them. We suggest that there exists only one phenomenon of thermotolerance. It can be analyzed in terms of 3 complementary and competing processes. These are: an initial event ("trigger"); the expression of resistance ("development"); and the gradual disappearance of resistance ("decay"). Each of these may have its own temperature dependence as well as dependence on other factors such as pH and nutrition. Conceptually, the 3 components of thermotolerance may be thought to be independent processes. However, independent measurement of each component is not always possible. For example, to measure the kinetics of triggering, development must be permitted to proceed. This is illustrated in Chart 5 where we have plotted survival to a second heat dose given 4 hr following completion of short (5 to 30 min) exposures of cells to 43°. We suggest that the very substantial increases in survival with increasing priming interval are a measure of trigger kinetics at 43°. We further suggest that, during exposure of HA-1 cells to temperatures of 43° or higher, development of tolerance is prevented; subsequent incubation at lower temperatures is required for development. Below 43°, both trigger and development occur simultaneously as is illustrated in Chart 6. In the experiments depicted there, the priming exposure was at 41°. In contrast to the 43° results, development is seen to be essentially complete by the end of the priming treatment. For this reason, the survival...
kinetics are more or less flat between 0 and 4 hr. From the data shown in Chart 5, we can estimate, for 43° exposures, the time it takes for the trigger to reach 50% of its maximum value. This is seen to be about 5 min; this very short interval strongly suggests that the process involving the trigger signal is physical or chemical (as opposed to enzymatic) in nature and probably does not involve gene activation. Certainly, 5 min is far too short a time for any synthesis of new proteins. Somewhat similar kinetics have been reported in vivo by Law et al. (17) who measured induction of thermotolerance in the mouse ear.

From our data, we can also make the following additional generalizations. Any initial heat treatment, either in H or M⁺, will induce the trigger; the magnitude of this trigger is a function of temperature, duration, nutrient environment, and possibly pH of the treatment. It is, however, not possible to separate completely pH and nutritional effects. In experiments in which cells were exposed to 43° in Hanks' BSS but at pH 7.4, survival values were identical to those seen at pH 6.7 (19). The survival response to a second treatment administered immediately following completion of the initial treatment depends very much both on temperature and nutrient conditions of the first treatment. On the other hand, survival at the time of maximum expression of development depends primarily on the magnitude of triggering event. At any one temperature, this is governed by the duration of the first exposure. The nutrient conditions during development are of less importance. The time course of decay of tolerance is largely determined by the nutrient conditions during the second treatment. Here H favors more rapid decay (compare Charts 2 and 3). However, the milieu during incubation is also of some importance. There M⁺ favors somewhat more rapid decay (Chart 3).

As indicated in the introduction, our model might aid in the interpretation or reinterpretation of some of the existing data in the literature. Our model would suggest that survival data following continuous exposures of cells to temperatures below 43° always include effects of development of thermotolerance and, to some extent also, of decay. Thus, activation energies obtained at these temperatures usually do not reflect steady-state conditions. Hence, the fundamental assumptions of Arrhenius theory may not be satisfied, and the "break" in Arrhenius plots observed at 43° (2, 3, 24) may be difficult to interpret. If this analysis is correct, it would have to suggest that current experimental evidence does not support the statement frequently made in the literature, that cells are killed via different mechanisms below and above 43°. It may very well be that the only difference between the two temperature ranges with respect to cell killing is that the former is permissive for development of thermotolerance while the latter is not. Sapper et al. (22) have recently speculated that the change in slope of Arrhenius plots below 43° could be a manifestation of thermotolerance.

The model would suggest that continuous heating below 43° is the most difficult experimental situation to analyze. If we define intrinsic heat sensitivity as that which would be observed in the absence of thermotolerance, then we would suggest that survival during continuous heating in the lower temperature range is a combination of expression of that heat sensitivity modified continuously by trigger, development, and decay of thermotolerance. On the other hand, above 43°, the absolute sensitivity is measured directly.

Our model clearly implies that an initial exposure of cells to, for example, 41° would protect survivors against a subsequent exposure to 45°, since 41° is permissive for both induction and development of the transient resistant state. The model might also explain the reverse: an apparent sensitization to 41° by a prior exposure to 45° (16). This latter temperature is nonpermissive for development of thermotolerance. We now make the reasonable (and testable) assumption that, in addition to being nonpermissive during 45° exposure, this temperature also induces a lag in development of thermotolerance at the permissive temperature of 43°. Then, during the lag, one would measure the intrinsic 41° sensitivity (as defined in the previous paragraph) of the cells. The absence of development of thermotolerance would be interpreted as sensitization. Our explanation would not require such concepts as persistence of lesions or interaction of different lesions (7, 13, 15, 16, 20). This hypothesis has indeed been tested, and data which will be presented later are shown to be consistent with the ideas discussed here.

ACKNOWLEDGMENTS

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G. C. Li and G. M. Hahn


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