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Effects of Chronic pH 6.6 on Growth, Intracellular pH, and Response to 42.0°C Hyperthermia of Chinese Hamster Ovary Cells[1]

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ABSTRACT

Culturing Chinese hamster ovary cells in low pH (6.6) medium for several months altered the reproductive survival of these cells to combined low pH treatments and 42.0°C heating. We isolated new pH-resistant cells (identified as pHV-2) with enhanced ability to grow and divide under a low pH (6.6) environment. Their growth characteristics include (*a*) a plating efficiency of 70%, (*b*) a doubling time of 16 to 17 h, and (*c*) a steady state intracellular pH 0.12 pH units higher than for cells grown at a normal pH of 7.3. The pHV-2 cells had 100- to 200-fold increases in survival after 5 h of heating compared to cells incubated at low pH (6.6) for 4 h prior to and during the heat treatments. In addition, they developed a significant degree of thermotolerance. We measured a progressive decline in the intracellular pH as a function of time at 42.0°C. However, the decrease in the intracellular pH did not seem to be correlated with the increased heat sensitivity. The ability to select for low pH variants may have important implications in the extrapolation of *in vitro* hyperthermic data to the *in vivo* situation.

INTRODUCTION

An early observation made with certain solid tumors was that the extracellular medium bathing the tumor cells could be 0.4 to 1.0 pH units lower than the interstitial fluid of normal tissues (1). The importance of this acid environment was emphasized when it was discovered that *in vitro* cell cultures were sensitized to hyperthermic treatments under low pH (6.4 to 6.8) conditions (2–5). This low pH phenomenon is potentially an important factor in the response of tumors to hyperthermic treatments (6). Unfortunately, despite several years of intensive research, the mechanism of low pH-induced heat sensitization remains unknown.

A difficulty in relating *in vitro* studies to the *in vivo* situation is the length of time cells were exposed to low pH medium before heating. Generally, cells were incubated in low pH medium from 30 min to 24 h prior to heat treatments (2–5). However, cells within tumors may experience growth under low pH conditions for days, weeks, or even months at a time. Since low pH can be viewed as an environmental stress, continued growth under these conditions could potentially select for cells with enhanced pH regulatory mechanisms.

Numerous studies have established that cells actively regulate their pH_i[4] (7–9). One element of this regulation is thought to be the presence at the cell surface of a Na^+/H^+ antiporter (10, 11). This antiporter appears to be normally quiescent in cells (or at least operating at low levels) but can be stimulated with growth factors (12, 13), osmotic shock (14), and intracellular acidification (15). Reducing the pH from 7.3 to 6.6 could act as a stimulus for this pump since a reduction in the resting pH_i would be expected (and is observed with CHO and many other cell types (12, 16)) with the nearly 10-fold increase in the H^+ concentration. Recently Na^+/H^+ mutants have been identified (17), with one mutant in particular overexpressing an altered Na^+/H^+ pump which provided protection against intracellular acidification (17).

In this report we demonstrate that by culturing CHO cells in low pH (6.6) medium for extended periods of time, we were able to select for cells with altered pH_i characteristics. These cells are more resistant to 42.0°C heat at pH 6.6 than cells exposed to short-term low pH conditions.

MATERIALS AND METHODS

Cell Cultures and Conditions. CHO 10B2 cells were routinely subcultured in Ham's F12 medium containing 10% fetal bovine serum. The pH (7.3) of the stock cultures was maintained with 14 mM bicarbonate and 5% CO_2 in a humidified 37.0°C incubator. Chronic low pH cultures were established by removing normal pH medium (7.3) and replacing it with low pH medium (6.6). The low pH was obtained by reducing the medium bicarbonate from 14 to 2.4 mM while maintaining the CO_2 at 5%. The cells were routinely subcultured under these low pH conditions.

Heating and Survival Procedures. Stock cultures were trypsinized with 0.25% trypsin 14 to 16 h prior to heating and replated into normal pH 7.3 medium. Control cells were heated in this medium. For acute low pH hyperthermia, cells were placed in pH 6.6 medium 4 h prior to heating at 42.0°C. Immediately after heating, replicate survival plates were placed in a 37°C incubator for 5 to 15 min. The medium was then replaced with prewarmed normal medium and the plates were returned to the incubator for colony formation.

For the chronic low pH cells, exponentially growing cultures were trypsinized from T-75 cm^2 tissue culture flasks and known numbers of cells were replated into T-25 cm^2 flasks 14 to 16 h prior to heating. The medium pH was kept at 6.6 at all times prior to and during heating. Replicate plates were used to monitor pH before and during the 42.0°C heating experiments. The pH was measured with either an Orion pH meter (Cambridge, MA) or a Beckman model 71 pH meter (Irvine, CA), both of which were accurate to within 0.001 pH units. During the 10 h of heating the pH remained within 0.05 to 0.10 pH units of the original pretreatment pH value. Immediately after heating, the low pH medium was removed and replaced with normal pH 7.3 medium for colony formation.

Three survival plates were utilized for each heat survival point. After 8 to 11 days growth, the colonies of cells were fixed with a 3:1 mixture of methanol:acetic acid and stained with crystal violet for counting. Only colonies containing at least 50 cells were counted. The number of cells per colony was determined prior to each heat experiment, and a multiplicity correction was made according to standard procedures (18). Multiplicity values were around 1.7 for normal cells and 1.4 for chronic low pH cells.

Growth Rate Determinations. CHO cells were trypsinized from stock flasks and 20,000 cells were inoculated into T-25 cm^2 tissue culture flasks for growth rate determinations. Growth occurred at either pH 7.3 (normal cultures) or pH 6.6 (acute or chronic cultures). Periodically over a 5-day interval two flasks from each culture were trypsinized and

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[4] The abbreviations used are: pH_i, intracellular pH; CHO, Chinese hamster ovary; ADB, 1,4-diacetoxy-2,3-dicyanobenzol.

Table 1 *Growth properties for CHO cells grown at normal pH 7.3, chronic pH 6.6, and acute pH 6.6*

Acute pH 6.6 was defined for heating as 4 h of pH 6.6 prior to and during 42.0°C heating. However, for growth measurements the cells were necessarily kept at pH 6.6 from 4 to 90 h.

Cell line	pH	Doubling time (h)	Plating efficiency (%)
10B2	7.3 (normal)	12–13	67 ± 9
10B2	6.6 (acute)	24–25	68 ± 10
10B2	6.6 (chronic)	24–25	39 ± 9
pHV-2	6.6 (chronic)	16–17	69 ± 14

the cells were resuspended in saline buffer for counting. The total number of cells for each flask was determined with an electronic cell counter (Particle Data Inc., Elmhurst, IL).

pH_i Measurements. The technique for pH_i measurements has been described in more detail elsewhere.[5] The sample preparation for the chronic low pH cells was identical to that previously described for normal and acute low pH cells (19), but a brief description is given below. Immediately after heating, the chronic low pH cells were trypsinized from plates, washed once with saline buffer at pH 6.6, then resuspended in 1 ml of saline buffer (pH 6.6). Five μl of ADB (Molecular Probes Inc., Junction City, OR) were added to the single cell suspension to give a final concentration of 42 μM. The cells were incubated with ADB for 19 to 20 min at room temperature to allow for hydrolysis of ADB to the fluorescent molecule 2,3-dicyanohydroquinone. The fluorescence from each cell was measured with a Coulter EPICS V cell sorter (Hialeah, FL), using 200 mW UV excitation from an argon ion laser. Fluorescence from each cell was collected and integrated over two spectral bandwidths: 418 to 440 nm (F1) and 469 to 485 nm (F2). The analog ratio of the two signals (F1:F2) was calculated by the computer and displayed as a histogram in real time. A calibration curve of the ratio signal in channels *versus* pH was generated prior to each pH experiment using nigericin, and the slope was generally 58 to 60 channels/pH increment (19). Saline buffer contained 145 mM NaCl, 5 mM KCl, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM $NaHPO_4$, 5 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 10 mM piperazine-*N*-*N*'-bis(2-ethanesulfonic acid).

RESULTS

Chronic Low pH Cultures. We established chronic low pH cultures by culturing CHO cells in pH 6.6 medium for periods of several months in order to test their heat sensitivity. The growth characteristics of cells under these conditions are listed in Table 1. In general, the cells grew slowly under chronic low pH conditions and had a relatively low plating efficiency. Growth characteristics under acute low pH conditions were similar, except that the plating efficiency was higher. The doubling times measured for both the normal and acute low pH cells were consistent with the results obtained by Gerweck and Rottinger (2). In one case, we maintained the chronic low pH cells in culture for more than a year. We discovered a change in their growth under low pH conditions, resulting in a near normal plating efficiency and a doubling time only about 25% longer than for cells at pH 7.3.

We measured the pH_i of these chronic low pH cultures, identified as pHV-2, using the ADB technique in conjunction with flow cytometry. Fig. 1*A* shows the pH_i histograms obtained with cells grown at normal pH 7.3 or under acute low pH (6.6) conditions. The acute low pH cells had a steady state pH_i decreased by 0.14 to 0.19 pH units from cells at normal pH 7.3. The pHV-2 cells had a mean steady state pH_i approximately 0.12 pH units higher than cells under normal pH conditions (Fig. 1*B*). It should be emphasized that the pHV-2 cells were grown at pH 6.6 and, in addition, processed for pH_i analysis in pH 6.6 buffer. A direct comparison of the pH_i histograms for both acute low pH and pHV-2 cells is given in Fig. 1*C*. The resting pH_i of the pHV-2 cells was approximately 0.31 pH units higher than the acute low pH cells when measured in buffers at pH 6.6.

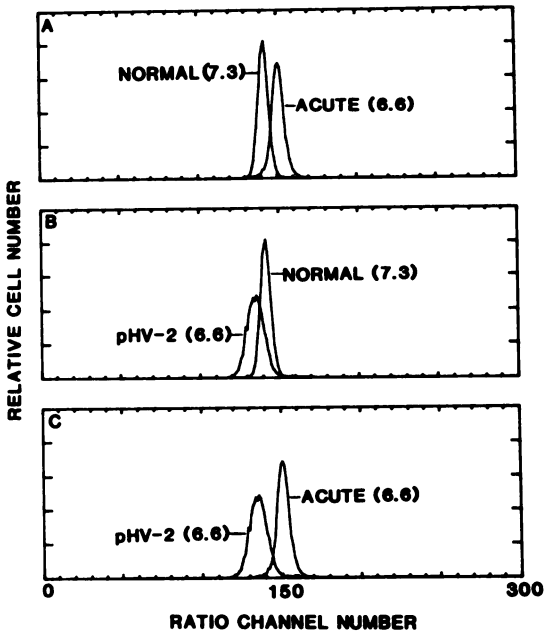

Fig. 1. Ratio (pH_i) histograms of CHO cells grown under either normal pH (7.3) or low pH (6.6) conditions. *A*, acute low pH cells *versus* normal pH cells. *B*, pHV-2 cells *versus* normal pH cells. *C*, pHV-2 cells *versus* acute low pH cells. All samples were processed and analyzed at similar times. Increasing pH is indicated by decreasing ratio channel numbers. 10,000 cells were collected and analyzed for each histogram shown.

Another interesting feature of the pHV-2 cultures was an increased population pH_i heterogeneity (Fig. 1). This heterogeneity was determined for each pH_i histogram by calculating the coefficient of variation of the peaks. The pH_i coefficient of variation was 3.9% for the pHV-2 cells, 2.6% for acute low pH cells, and 2.3% for cells grown at normal pH 7.3. Based on a calibration curve with a slope of 58 channels/pH unit, this corresponds to a pH_i standard deviation of 0.09, 0.068, and 0.056 pH units, respectively. We have monitored the pH_i of these chronic cells for several passages and the pH_i changes noted were quite stable.

42.0°C Survival of the pHV-2 Cells. Because of the high pH_i of the pHV-2 cells, we measured the survival response of these cells to 42.0°C heating. Fig. 2 shows the survival as a function of 42.0°C heating for pHV-2 cells at pH 6.6, and normal cells at pH 7.3 or acute low pH (6.6). The pHV-2 cells were substantially more heat sensitive at pH 6.6 than normal cells at pH 7.3. However, in contrast to cells heated at acute low pH, the pHV-2 cells were capable of developing thermotolerance. We have repeatedly measured this development of thermotolerance for chronic low pH cells heated at 42.0°C, even when the cells were grown for only a few weeks at pH 6.6.

pH_i Response to 42.0°C Heating. Fig. 3 depicts the pH_i response of the pHV-2 population to 42.0°C heating. Unheated cells grown at pH 7.3 were analyzed along with these cells in order to quantitate the pH_i changes which occurred. One h of heating at 42.0°C induced an immediate decrease in the steady state pH_i, and this decrease continued progressively with heating out to 10 h. For heating times longer than 5 h, the pH_i distribution broadened and was skewed toward acidic pH_i val-

[5] Cook, J. A., and Fox, M. H. Intracellular pH measurements using flow cytometry with 1,4-diacetoxy-2,3-dicyanobenzene, submitted for publication.

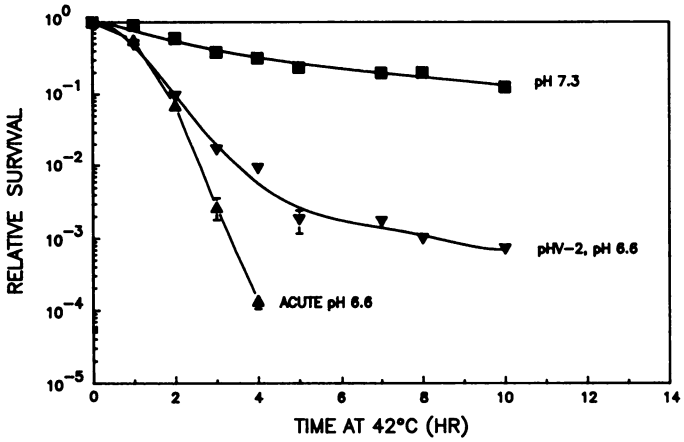

Fig. 2. Survival of normal pH (■), acute low pH (▲), and the pHV-2 (▼) cells as a function of time at 42°C. CHO cells were heated under either pH 7.3 (■) or pH 6.6 (▲, ▼) conditions. The pHV-2 cultures were grown continuously in pH 6.6 medium while the acute cultures received 4 h of low pH (6.6) medium prior to heating. SEMs were not plotted when smaller than the symbol size.

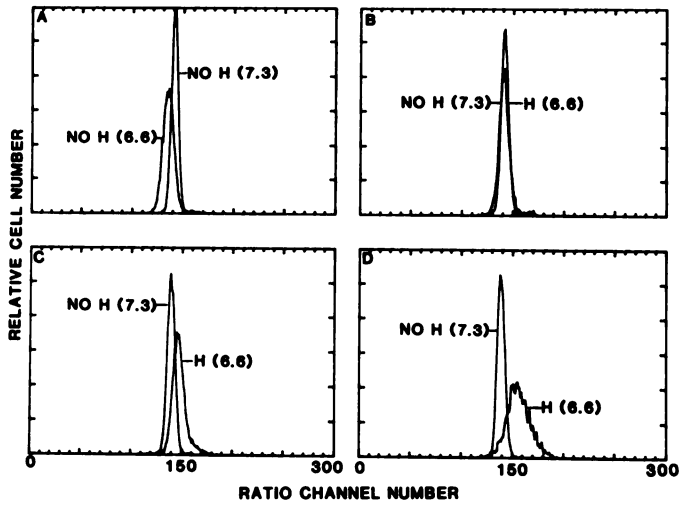

Fig. 3. Ratio (pH_i) histograms of pHV-2 cultures heated at 42°C for various times. Heated pHV-2 cells (*H*) were analyzed with unheated cells grown at pH 7.3 (*No H*). *A*, unheated pHV-2 cells *versus* unheated normal pH cells. *B*, after 1 h heat. *C*, after 5 h heat. *D*, after 10 h heat. Increasing pH is indicated by decreasing ratio channel numbers. The pHV-2 histogram in *D* resulted from the collection and analysis of 5000 cells. 10,000 cells were collected and analyzed for all other histograms shown.

ues. There was considerable overlap in the pH_i of the pHV-2 cells with the pH_i of normal cells, even for heating times as long as 10 h, when survival was about 10^{-3}.

The mean pH_i of the profiles in Fig. 3 (and additional time points) was calculated. The differences between these values and the pH_i of unheated cells at pH 7.3 were plotted as a function of heating time at 42.0°C (Fig. 4). Additionally, Fig. 4 shows how the mean pH_i changed when cells were heated at a normal pH of 7.3, or under acute pH 6.6 conditions. In contrast to the response of acute low pH cells (19), the pHV-2 cells underwent a continuous acidification for the entire heating time, whereas the acute low pH cells initially increased their pH_i. After 2 h of heating, however, the pHV-2 and acute low pH cells had virtually identical pH_i values. By 10 h of heating, the pH_i was approximately 0.30 pH units lower than for unheated cells at pH 7.3 (Fig. 4). While the mean pH_i was much lower after 10 h of heating, 20 to 30% of the pHV-2 cells still had pH_i values which overlapped the unheated normal pH controls (Fig. 3*D*).

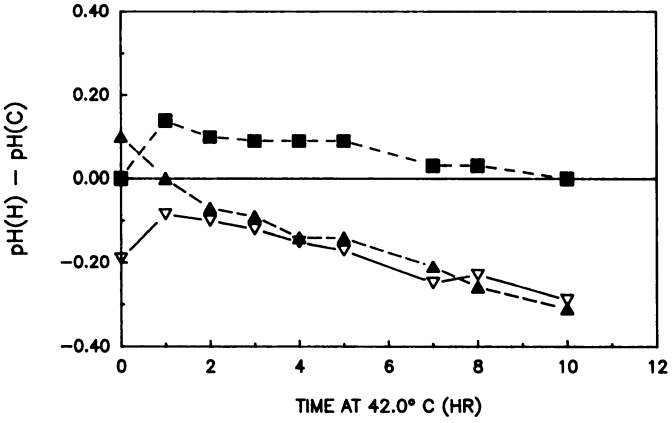

Fig. 4. Relative pH_i changes of normal cells at pH 7.3 and acute pH 6.6, and the pHV-2 cells at pH 6.6 as a function of time at 42°C. These results represent the mean pH_i changes compared to unheated normal pH cells. Heated cells were processed and analyzed along with unheated normal pH cells in order to obtain relative pH_i changes. When pH(H) − pH(C) = 0.00, no difference in the pH_i was measured with respect to the unheated normal pH cells. Positive changes on the ordinate represent increases in the pH_i, while negative changes represent decreases in the pH_i. Normal cells heated at pH 7.3 (■); normal cells heated at acute pH 6.6 (▽); pHV-2 cells heated at pH 6.6 (▲).

DISCUSSION

The evidence presented in this report indicates that selection and/or adaptation pressures under low pH (6.6) conditions can lead to cell populations with altered growth characteristics and heat responses. Our initial experiments demonstrated that CHO cells grown for several weeks or months in low pH medium (chronic low pH) showed enhanced resistance to 42.0°C heating compared to cells exposed to low pH conditions for a few hours (acute low pH) (20). A similar result has also been obtained by Hahn and Shiu (21). The heat sensitivity of these chronic low pH cells was nearly identical to that of the pHV-2 cells shown in Fig. 2 (data not shown). Whether adaptation or selection was responsible for the increased heat resistance is not known. Growth properties outlined in Table 1 suggested adaptation as the primary mechanism. These properties included a low plating efficiency and a long doubling time. However, because of the length of time in culture for these cells, selection of a pH-resistant clone cannot be ruled out. Selection of a pH-resistant clone appeared to be the mechanism responsible for obtaining the pHV-2 cultures used for survival in Fig. 3. This conclusion was based on the following evidence: (*a*) the plating efficiency of these cells was not significantly different from that of acute low pH or normal cells; (*b*) the doubling time for this culture was 16 to 17 h under the low pH conditions; (*c*) the resting pH_i was substantially increased (0.30 pH units) compared to either the acute low pH cells or to chronic low pH cells measured at an earlier time in culture.

By what physiological mechanism these cells were able to maintain the pH_i at such a high steady state value is unknown. However, the advantages of maintaining a high steady state pH_i with regard to the growth kinetics is understandable. It has been reported that increases in the steady state pH_i (by growth factor or NH_4Cl stimulation) can activate and increase cellular protein synthesis rates, thus stimulating growth and division (22, 23). Hence, the increased growth rate under low pH conditions may be a consequence of the ability of these cells to maintain a higher steady state pH_i. While the growth rate of these cells was enhanced compared to the acute low pH cells, the doubling time was still 4 to 5 h slower than CHO cells grown at normal pH 7.3. This combination of high steady state pH_i levels and slower growth kinetics suggests that low extra-

cellular pH can inhibit cellular growth through a mechanism which is not totally dependent upon a reduction in the internal pH. Supporting evidence for this idea is work which demonstrated that transport systems for certain amino acids are extremely pH sensitive (24, 25). These results indicated that transport and accumulation of amino acids (especially glutamine) can be severely reduced below pH 6.8 to 6.9.

Recent work by Pouysségur showed that selection of cells from *in vitro* cultures with enhanced pH_i regulatory properties was possible (17). Whether the selection and amplification of pH-resistant cells can occur under the harsher environmental conditions of tumors is not known. However, the phenotypic heterogeneity and the loss of growth regulatory properties of tumors strongly suggest that selection and amplification can occur. In addition, it has been reported that tumor cells have pH_i levels similar to cells from normal tissues, even though the extracellular pH may be lower (26). This indicates the presence of an active pH_i regulatory system which can be operational even under suboptimal conditions.

Heating the pHV-2 cells at 42.0°C induced an immediate decline in the pH_i (Fig. 4). By 10 h of heating, the mean pH_i had decreased by approximately 0.41 pH units (from the unheated pHV-2 cultures). While this large decrease in pH_i could certainly have contributed to the heat sensitivity, at least 20 to 30% of the cells still had pH_i values which were in the range of the unheated cells at pH 7.3 (Fig. 3*D*). However, because survival after 10 h of heating was approximately 10^{-3}, virtually all of the cells in the 10-h pH_i profile were reproductively dead. Therefore, since pH_i regulation appeared to be quite resistant to heat-induced damage, we conclude that intracellular acidification is not directly responsible for the heat sensitivity caused by the low pH conditions.

The pH_i changes we observed with the acute cells and 42.0°C hyperthermia indicate that beyond 2 h of heating the pH_i of both the pHV-2 and acute low pH cells responded to heat in an identical manner. The primary difference between the two cultures was seen in the first hour of heating when the pH_i of the acute low pH cells increased slightly. We have argued that this increase, which is also observed for cells at normal pH (Fig. 4), may be important for thermotolerance development (19). The observation that the pHV-2 cells had increased pH_i levels prior to heating, and can develop thermotolerance during heating, supports this suggestion. Hahn and Shiu (21) also reported that their pH-conditioned HA-1 cells (pH 6.8) had a greater ability to develop thermotolerance than normal HA-1 cells under acute pH 6.8 conditions. They did not measure pH_i, however.

In summary, we have examined the heat survival and pH_i response of CHO cells incubated under normal pH (7.3) and both acute and chronic low pH (6.6) conditions. Chronic incubation at low pH resulted in a variant population which showed enhanced thermal resistance compared to acute low pH cells. However, these pHV-2 cells were still heat sensitive when compared to cells heated at normal pH. This sensitivity did not appear to be the result of a collapse in the pH_i regulatory ability of the pHV-2 cells.

These results have implications for clinical hyperthermia, since it is likely that cells in some tumors may be under chronic low pH conditions. If cells in the tumor are able to adapt to the low pH, the surviving fraction after hyperthermia would be modified. Recent work by Leith et al. (27) shows that clones derived from a single tumor biopsy and heated under both normal and low pH conditions had heterogeneous survival responses. A possible cause for this heterogeneous response could be adaptation of the tumor cells to the chronic low pH conditions, similar to what we have reported in this paper. Our data suggest that extrapolation of *in vitro* results with acute low pH and hyperthermia to the *in vivo* situation should be done with extreme caution.

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