Effect of pH and Elevated Temperatures on the Cytotoxicity of Some Chemotherapeutic Agents on Chinese Hamster Cells in Vitro¹

George M. Hahn² and Esther C. Shiu
Department of Radiology, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT

The cytotoxicity of some drugs is a function of extracellular pH. We show that this dependence is greatly enhanced at 43°C. At this temperature, we have examined the cell-killing ability of four drugs in the pH range of 6.5 to 8.5. For 1,3-bis(2-chloroethyl)-1-nitrosourea, cytotoxicity is minimum at alkaline pH and increases in monotonic fashion as the milieu becomes more acidic. There is little or no effect of pH on the cytotoxicity of methotrexate. Bleomycin is most effective at acidic pH (<7.5). Above that pH, its cytotoxicity remains unchanged. Amphotericin B is least cytotoxic at the pH of normal tissue, 7.4. At higher or lower values, its cell-killing efficiency increases symmetrically. These results may have some relevance in designing thermochemotherapeutic treatment protocols.

INTRODUCTION

It has been clearly established in vitro that many, although certainly not all, anticancer drugs kill cells more efficiently as the ambient temperature is increased above 37°C (5). In parallel, it has also been shown that in most cases this enhanced effectiveness can be translated into increased antitumor activity when systemic chemotherapy is combined with local hyperthermia (7). For whole-body hyperthermia, however, no rationale exists that supports that the combination of drug and heat should be more effective than either modality alone. The problem is that, while cell killing is still accentuated, both cancer cells and critical normal tissue (usually bone marrow or gastrointestinal crypt cells) may well be equally affected. If this were the case, there would appear to be no therapeutic gain from adding heat to drug.

We have initiated a study to see if we could identify chemotherapeutic agents that take advantage of the lower pH associated with portions of the interior of most solid tumors (9). In this initial report, we show that at 43°C the rate of cell killing for some drugs is a function of extracellular pH and that the combination of elevated temperature and particularly of low pH can dramatically increase cytotoxicity. While we show only data obtained at this temperature, we have also obtained preliminary results at a lower temperature (41°C). These results, while not as striking as the ones shown, are in the same direction, i.e., increased cytotoxicity at low pH. Our results provide at least some justification for the clinical use of whole-body thermochemo therapy. In addition, they point out again the possible advantages of localized hyperthermia in combination with chemotherapy.

MATERIALS AND METHODS

Cells and Culture Conditions. The experiments were performed using exponentially growing monolayer cultures of Chinese hamster cells (HA-1). The cells, routinely checked for Mycoplasma, were grown in Eagle's minimal essential medium supplemented with 15% fetal calf serum, penicillin (200 units/ml), and streptomycin (200 μg/ml). Neither of the antibiotics used is cytotoxic at these concentrations over the temperature range 37–45°C. The culture conditions were standard and have been described previously (4, 11). Exponential cultures were obtained by seeding 1 to 2 x 10⁶ cells onto 60 x 15-mm Falcon tissue culture dishes and using the cultures at a cell number of 1 to 3 x 10⁶ cells.

Heat and Drug Exposure at Various pH Conditions. Heat exposure was in specially designed hot-water baths with a temperature control accuracy within ±0.1°C (4). Various pH values of the medium overlying the cells during exposure were obtained by adjusting the amount of sodium bicarbonate used in the medium in the concentrations ranging from 0 to 4000 mg/liter. At the maximum concentration, the osmolarity, assuming complete dissociation, changed by about 85 mosm/liter/1, a change that would not be expected to affect survival even at 43°C (6). The adjusted pHs were then maintained by continuous gassing with 5% CO₂-air. The pH values were reproducible from experiment to experiment and accurate to ±0.1 unit. Control plating efficiencies were independent of pH over the range tested. The drugs were initially made up in concentrated form, and dilutions were made to the desired concentration using the pH-adjusted medium.

At the beginning of each experiment, the medium was replaced with appropriate drug-containing medium at the correct pH. The dishes were then placed in hot-water baths at the designated temperature for 1 hr. At the end of the heat exposure, the drug-containing medium was removed, the pH was measured, and the cells were rinsed 3 times, rapidly trypsinized, counted, and plated to assay for survival (Puck’s cloning technique). Four drugs were used. Bleomycin (Blencathex, bleomycin sulfate), methotrexate (Methexate, methotrexate sodium hydride), and BCNU were all obtained from Bristol-Myers, Syracuse, N. Y. Amphotericin B was provided by Sigma Chemical Co., St. Louis, Mo. All drugs were initially dissolved in their concentrated form: bleomycin in Dulbecco’s phosphate-buffered saline (Grand Island Biological Co., Santa Clara, Calif.); methotrexate in 0.9% sodium chloride solution (Abbott Laboratories, North Chicago, Ill.); BCNU in absolute ethanol (Bristol Laboratories, Syracuse, N. Y.); and amphotericin B in dimethyl sulfoxide (T. T. Baker Chemical Co., Phillipsburg, N. J.). Appropriate survival experiments at elevated temperatures were done to test for possible cytotoxic effects of the solvents (Charts 1 to 4). Drug solutions and dilutions were freshly made on the same day just before the beginning of each experiment.

RESULTS

We have examined 4 drugs: a nitrosourea, BCNU; an antime tabolite, methotrexate; the glycopeptide, bleomycin; and the polynucle antibiotic, amphotericin B. In each case, we exposed cells at 43°C for 1 hr in the presence of the appropriate drug concentration. The pH was varied as described in "Materials and Methods." Results are shown in Charts 1 to 4. In each chart, 2 controls are shown, heat alone and drug alone. Cell killing by heat shows the pH dependence demonstrated by others, a

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² To whom requests for reprints should be addressed.

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plateau in the pH range of about 7.5 to 7.0, and a rather sharp increase in cytotoxicity at lower pH values (3, 8, 10).

Chart 1 deals with BCNU. Cell killing by this nitrosourea was only mildly pH dependent at 37°. At alkaline values, the effectiveness of the drug was minimized. As the pH was reduced, its cytotoxicity was enhanced. At 43°, there is clear evidence of synergism, and this is true at all pH values examined by us. For example, if cell killing were only additive, the expected survival at pH 7.5 would have been 0.35 (0.7 × 0.5). In fact, the observed survival is $3.5 \times 10^{-2}$. At pH 6.8, it would have been 0.22, while we measured a survival value of $2 \times 10^{-4}$. Very striking was the extremely low survival at the low pH values.

Methotrexate presents a very different picture. In Chart 2, we show results of survival experiments at 37 and 43°. At these two temperatures, the antimetabolite was relatively inefficient against the HA-1 cells. No pH effect was noticeable. At 43°, cytotoxicity was less than additive. In fact, at all pH values, cell-killing rates with and without drug were indistinguishable.

A different picture appears for bleomycin (Chart 3). Cell killing at 37° by this drug also showed little if any pH dependence. At 43°, there again was evidence of more than additive cell killing and, just as in the case of BCNU, this was accentuated at low pH values. However, in contrast to the behavior of the nitrosourea, cell killing by bleomycin plateaued at a pH value of about 7.4.

Chart 4 shows results obtained with amphotericin B. At the control temperature, there was only moderate killing, particularly at pH 7.4. At alkaline pH, a minor increase in cell killing was seen. At the higher temperature, the drug was an efficient cytotoxic agent, confirming our earlier data obtained at pH 7.4.
DISCUSSION

There is good evidence in the literature showing that tumors tend to occur at a pH lower than that of normal tissue (9). A priori, there are at least 2 ways in which one could use this finding to improve the chemotherapy of tumors. It might be thought that drugs could be designed that are activated only at the pH values associated with tumor tissue but that would be nontoxic at the pH of normal tissue. Unfortunately, the difference in proton concentrations between these tissues is not large, typically a fraction of a pH unit (1). It is difficult to think of a mechanism whereby small changes in pH could change the stereochemistry of a drug sufficiently to accomplish activation. The other approach is to recognize the stress that low external pH puts on the cell and then to look for drugs that are particularly cytotoxic under such conditions. This is the approach that we tried. Unfortunately, we found that while indeed some difference in cytotoxicity could be demonstrated at lower pH, the effect was hardly very impressive (Charts 1, 3, and 4, 37° data). Therefore, we looked for ways to enhance the stress, particularly with methods currently being developed for clinical use. Hyperthermia appeared to be a good candidate. Clearly, the data shown indicate that our approach was reasonable.

BCNU and bleomycin are currently used as antineoplastic agents, and therefore their use in thermochemotherapy protocols needs little discussion. Amphotericin B is not currently used for anticancer therapy. It is prescribed as an antifungal agent, but its severe kidney toxicity severely limits clinically usable concentrations even in that application. Our data suggest that, because of the increased cytotoxicity at elevated temperatures and low pH, its use as part of a multidrug, thermochemotherapy regimen might still be profitable. This would be particularly true if the other drugs constituting the combined treatment were chosen so that normal tissue side effects did not overlap with those of the polyene antibiotic.

We offer no explanation for our findings. The modes of cytotoxic action of BCNU, bleomycin, and amphotericin B are very different, but for each of these agents cell killing is much more effective at low than at “normal” pH. This would suggest that in an acidic milieu, an important cellular function is working at reduced efficiency. Energy metabolism, or at least availability of ATP, owing to its ubiquitous importance, is of course an obvious candidate, but in the absence of supporting data this is strictly speculation.

Even though we cannot give explanations for our findings, we can suggest some clinical applications. A temperature of 43° is too high for whole-body hyperthermia. It is, however, a reasonable and achievable temperature for localized or regional hyperthermia. Furthermore, as we indicated in the “Introduction,” our preliminary data indicate that low pH appears to be effective in increasing cytotoxicity also at lower temperatures, at least down to 41°, a temperature known to be safe for whole-body heating. This suggests that at least bleomycin and BCNU could be useful in conjunction with such heating. For localized heating in combination with systemic drug therapy, the results also offer some clinical directions. Several methods have been shown to be effective in increasing the acidity of murine tumors. Perhaps the most prominent of these is hyperthermia itself (1), and the induction of high blood sugar levels (2, 10). Pharmacokinetic studies need to be made to determine the effect of reduced blood flow on drug delivery, but we suspect that, at least for lipophilic agents such as BCNU, sufficient drug would reach tumors even with reduced blood flow. In any case, the increased cytotoxicity of drug that does enter the tumor might more than compensate for any reduction in tumor drug concentration. For these reasons, we suggest that methods of acidifying tumors, in conjunction with local heating, might increase the efficiency of thermochemotherapy of solid lesions.

REFERENCES

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