Hydrogen Ion-mediated Enhancement of Cytotoxicity of Bis-Chloroethylating Drugs in Rat Mammary Carcinoma Cells in Vitro

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ABSTRACT

Aerobic glycolysis, a metabolic characteristic of malignant cells, can be exploited to increase the concentration of lactic acid selectively in tumor tissues in vivo by systemic administration of glucose (E. Jähde and M. F. Rajewsky, Cancer Res., 42: 1505–1512, 1982). To investigate whether a more acidic microenvironment can enhance the effectiveness of cytoidal drugs, we have analyzed the colony-forming capacity of M1R rat mammary carcinoma cells exposed to bis-chloroethylating agents in culture as a function of extracellular pH (pHe). At pHe 6.2 the cytotoxicity of 4-hydroperoxycyclophosphamide, as measured by inhibition of colony formation, was potentiated by a factor of ~200 as compared to pHe 7.4. Similar results were obtained with mafosfamide, nitrogen mustard, nor-nitrogen mustard, melphalan, and chlorambucil; not, however, with ifosfamide. As indicated by experiments using the ionophor nigericin for rapid equilibration of pHe and intracellular pH (pHi; measured with pH-sensitive microelectrodes), modulation of drug action by varying pHe, primarily resulted from the concomitant decrease in pHi. The acidic microenvironment enhanced cytotoxicity most effectively during the phase of cellular drug uptake and monofunctional alkylation of DNA. DNA cross-link formation appeared to be less affected by pHe, and lowering of pHe, during the phase of cross-link removal was only marginally effective.

INTRODUCTION

The therapeutic efficiency of anticancer drugs is severely limited by their toxic side effects on critical normal cell systems. The excellent therapeutic index of bactericidal as opposed to cytoidal chemotherapeutic agents is based on the exploitation of metabolic differences between bacteria and mammalian cells. In designing cytoidal drugs and strategies for cancer treatment, few investigators (1, 2) have thus far exploited aerobic glycolysis, a metabolic property distinguishing most malignant cells, of both animal and human origin, from their normal counterparts (3, 4; Table 1; for exceptions see Ref. 5).

The amount of lactic acid formed by malignant cells is highly sensitive to changes in the supply of glucose (6, 7). The glucose concentration in the interstitial fluid of malignant tumors is very low in comparison to arterial blood or the interstitial fluid of normal tissues (8). Therefore, the glycolytic rate of cancer cells in vivo is generally far below its potential maximum (6, 7) and may be increased by an elevated supply of glucose. Since lactic acid concentration is closely related to the concentration of H+ ions (9), an increased glycolytic rate of tumor cells leads to a reduction of intratumoral pHe (9, 10). Various studies have shown that the intratumoral concentration of H+ ions can indeed be increased up to 10-fold in primary and transplanted rodent tumors by systemic administration of glucose in vivo (mean pHe, ~6.2; Table 1). When the glycolytic activity of transplanted TV1A rat neuroinomas was increased by i.v. infusion of glucose, the mean intratumoral pHe decreased from about 6.9 to 6.1 in a tumor-selective manner (10). pH distributions in normal tissues of the tumor-bearing hosts were only marginally affected by the hyperglycemia (Table 2). Similar results were obtained with other tumors of epithelial and mesenchymal origin (Table 1), and confirmed by pH measurements in human tumors (11, 45).

In the present study we have investigated whether the cytotoxic effects of bis-chloroethylating drugs on malignant cells in culture can be modified by varying pHe over the range previously measured in malignant tumors with and without glucose perfusion. Specifically, the following questions were addressed: (a) is the cytotoxicity of alkylating drugs, in particular nitrogen mustard derivatives like CP and its analogs, dependent on microenvironmental pH; and (b) which molecular mechanisms may be sensitive to an H+ ion-mediated modulation of drug cytotoxicity? Bis-chloroethylating agents were chosen for two reasons: It is known that ethylenimines, a structurally different group of alkylating compounds, exhibit a higher cytotoxic activity at reduced pHe, probably due to protonation of the nitrogen atoms of the ethylenimmonium rings resulting in increased reactivity (12). Moreover, it has been suggested that chloroethylating agents like CP and its metabolites also react via formation of an immonium ion intermediate (13, 14), and a recent study has shown that the action of CP on malignant cells in culture may be dependent on pHe (15). In the latter analyses mouse serum containing CP and its various metabolites in undefined quantities was used as the source of aCP. We have attempted to quantitate the influence of H+ ion concentration on the cytotoxic action of CP and to evaluate the role of CP metabolites in pHe-dependent cytotoxicity, using chemically defined preparations of aCP and its metabolites. In addition, the effect of an acidic environment on the cytotoxic action of IFO, an analog of CP, and of bis-chloroethylating agents structurally different from oxazaphosphorines was investigated.

MATERIALS AND METHODS

Drugs

4-OOH-CP, MAFO, and 4-OOH-IFO were kindly provided by Dr. P. Hilgard (Asta-Werke, Bielefeld, FRG). Drugs were stored at ~20°C

3 The abbreviations used are: pHe, extracellular pH; pHi, intracellular pH; aCP, activated cyclophosphamide; BNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BIS-TRIS, 2-[bis(2-hydroxyethyl)iminoo]-2-(hydroxymethyl)-1,3-propanediol; HN2, mechlorethamine; IFO, ifosfamide; L-PAM, melphalan (L-phenylalanine mustard); MAFO, mafosfamide; mafosfamide; nor-HN2, nor-nitrogen mustard [bis-(2-chloroethyl)-amine-hydrochloride]; 4-OOH-CP, 4-hydroperoxycyclophosphamide; 4-OOH-IFO, 4-hydroperoxyifosfamide; PBS, phosphate buffered saline; pH-DMF, pH-dose modifying factor.

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cells were fixed, stained with Loeffler’s methylene blue, and colonies with a diameter >1 mm (~1000 cells) were counted. As shown in separate experiments, there was a linear relationship between the number of cells plated and the number of colonies formed over a range of 25–500 cells per plate. The colony-forming fraction of cells surviving drug treatment was calculated as the ratio of the number of colonies formed to the number of drug-exposed cells inoculated. Calculated values were normalized to the plating efficiency of untreated control M1R cells and corrections were made for the fraction of cells lysed during drug exposure according to the following formula:

\[ \text{Colony-forming fraction} = \frac{\text{plating efficiency exposed} \times \text{cells/dish exposed}}{\text{plating efficiency control} \times \text{cells/dish control}} \]

Results are presented as mean values of three to six separate experiments with the standard deviations (not shown), typically, being less than 30% of the means.

### Measurements of Intracellular pH

Microelectrodes. Fiber-containing borosilicate capillaries (Hilgenberg, Malsfeld, FRG), 1.0-mm outer diameter and 0.5-mm inner diameter, were cleaned by immersion (24 h) in chromic sulfuric acid followed by repeated rinsing in distilled H2O. Micropipets were pulled with a vertical puller (700°C; David Kopf Instruments, Tujunga, CA). Tip resistance varied between 10–20 MΩ when the pipets were filled with 3 M KCl solution. Micropipets for H+ ion-selective microelectrodes were dried at 180°C for 20 min. Their tips (diameter, ~0.5 μm) were silanized by dipping into a solution of 10% (v/v) trichloromethylsilane (Fluka, Buchs, Switzerland) in tetrachloromethane (Merck, Darmstadt, FRG) for 20 s and subsequently baked in an oven at 100°C for 30 min. Silanized micropipets could be stored over silica gel for several days without alterations of tip resistance. Micropipets were filled with a H+ ligand cocktail (Fluka) based on tridodecylamine (18) with the use of a syringe and a fine glass drain tube. The top of the microelectrode shank was filled with the ion-selective liquid [height, ~3 mm], followed by backfilling with internal filling solution [40 mM KH2PO4-23 mM NaOH-15 mM NaCl buffered to pH 7 (18)]. Conventional microelectrodes for measuring membrane potentials were backfilled with 3 M KCl solution.

### Calibration

H+–sensitive microelectrodes were calibrated in BIS-TRIS buffered DMEM (pH 6.15–7.9) before and after experiments. The corresponding microelectrode potentials were plotted as a function of pH. The mean slope and resistance of the pH-sensitive microelectrodes did not significantly alter during the experiments.

### Electrical Measurements

Measurements of membrane potentials and pH were performed under a phase-contrast microscope (Epipvert; Leitz, Wetzlar, FRG). The two recording microelectrodes were connected to the input of a high impedance amplifier (FD 223; WP Instruments, Sweden).
New Haven, CT). The reference electrode in 3 m KCl solution was connected to the sample solution via a Ringer agar bridge. Potential differences were recorded with a pen recorder (S 600; Gebr. Laumann, Selb, FRG). All electrophysiological measurements were performed in BIS-TRIS buffered DMEM at 37° ± 1°C. pH was adjusted to the required value by titration with 1 m HCl or 1 m NaOH, respectively. Micromanipulators with electrical drive (Gebr. Märzhäuser, Wetzlar, FRG) ensured controlled microelectrode movements.

RESULTS

Colony-forming Capacity of M1R Cells at Reduced pH. The effect of an acidic microenvironment per se on the colony-forming capacity of M1R cells is shown in Fig. 1. The fractions of colony-forming cells decreased with decreasing pH and as a function of the duration of exposure to reduced pH, respectively. However, this effect became significant only below pH 6.3 and after exposure times ≥24 h. At pH 6.3 the fraction of colony-forming cells still amounted to 65% of control (pH 7.4) after 24-h exposure. Even at pH 5.9 an incubation >24 h was required to lower this value to <10%. In order to minimize the influence of reduced pH per se on the results of the following experiments, drug exposure was limited to 24 h, unless otherwise stated.

Cytotoxicity of aCP on M1R Cells as a Function of pH. aCP is a prodrug requiring microsomal hydroxylation for the formation of its first reactive metabolite, 4-OH-CP. Since 4-OH-CP is highly unstable in aqueous solution, a number of self-activating CP derivatives were used instead. Among these were 4-OH-CP, a stabilized form of 4-OH-CP, and MAFO, a new oxazaphosphorine compound (20, 21). Both derivatives undergo rapid spontaneous hydrolysis in H2O, with liberation of 4-OH-CP (20, 21).

To determine whether a reduced pH influences the cytotoxicity of aCP, log phase M1R cells were incubated with 4-OH-CP at different pH (Fig. 2). At pH 7.4 the fraction of colony-forming cells (4-OH-CP concentration, 1.0 μg/ml) was ~8% of untreated controls; the corresponding value at pH 6.2 was ~0.04%, representing a 200-fold enhancement of aCP cytotoxicity at pH 6.2 as compared to pH 7.4. Cytotoxicity was further enhanced by lowering pH to 5.6. At this pH inhibition of colony-formation by aCP was potentiated by a factor of ~10^3 relative to control cells exposed to aCP at pH 7.4. The observed H+ ion-mediated enhancement of aCP cytotoxicity was independent of the kind of prodrug applied. Essentially the same results were obtained when MAFO (2.5 μg/ml) was used as a precursor of 4-OH-CP instead of 4-OH-CP (Fig. 3).

The kinetics of inhibition of colony-forming capacity by aCP were investigated using MAFO as a source of 4-OH-CP. The fraction of colony-forming cells decreased as a function of the time of drug exposure both at pH 7.4 and at pH 6.2 (Fig. 4). After drug exposure for 12 h (24 h) about 65% (100%) of the respective maximum inhibitory effect was obtained at both pH values. Incubation of cells with MAFO for periods >24 h did not result in additional cytotoxicity at either pH in comparison to 24-h exposure.
pH as a Dose Modifying Factor. From the data obtained with MAFO as the precursor of 4-OH-CP, a “pH-dose modifying factor” (pH-DMF) was calculated. The pH-DMF indicates the drug concentration required at acidic pH to achieve a given level of cytotoxicity (e.g., colony-forming fraction 10% or 1% of untreated control) divided by the drug concentration resulting in an identical fraction of colony-forming cells at pH 7.4. As shown in Fig. 5, only 20% of the MAFO concentration required to reduce the fraction of colony-forming cells to 1% at pH 7.4 are sufficient to achieve the same effect at pH 6.2 (pH-DMF = 0.2).

Effect of Acidic Microenvironment on the Cytotoxic Action of Chloroethylating Drugs Structurally Different from CP. In addition to CP a number of chloroethylating agents have gained wide clinical application. Among these are HN2, L-PAM, and chlorambucil. As with CP, the action of these drugs resides in their bis-chloroethylamine group. In IFO, an oxazaphosphorine analog of CP, one of the chloroethyl side chains is shifted from the amino nitrogen to the ring nitrogen. The results of exposure of M1R cells to these agents in normal and acidic culture media are demonstrated in Figs. 6–9. The cytotoxic action of HN2, L-PAM, and chlorambucil was markedly enhanced when pH was lowered from 7.4 to 6.2. At a concentration of 0.2 µg/ml, the cytotoxic effect of HN2 at pH 6.2 was potentiated by about a factor of 200 as compared to pH 7.4. In contrast, the colony-forming capacity of cells treated with IFO remained essentially unaffected by variations of pH over a range of 7.4 to 5.6. The bis-chloroethylamine group may thus be a critical determinant for H⁺ ion-mediated enhancement of cytotoxicity in this group of agents.

Molecular Mechanisms Sensitive to H⁺ Ion-mediated Modulation of Alkylating Drug Cytotoxicity. Nor-HN2 is one of the terminal metabolites of aCP (14). In a cell free in vitro assay system, the alkylating potency of nor-HN2 has been shown to be highly sensitive to alterations of pH (14, 22). To evaluate the role of CP metabolites in H⁺ ion-mediated enhancement of CP cytotoxicity, the effect of pH on the cytotoxic action of nor-HN2 was investigated. At pH 6.2 the cytotoxic effect of nor-HN2 on M1R cells (10 µg/ml) was potentiated by a factor of about 5 × 10³ as compared to pH 7.4 (Fig. 10).

In all aforementioned experiments drug cytotoxicity was analyzed as a function of pH. However, the critical molecular targets of alkylating drugs are located intracellularly, in particular in the nucleus (DNA). We therefore investigated whether alterations of pH are accompanied by corresponding shifts of pH. For these experiments the culture medium of M1R monolayers, previously grown at pH 7.4, was changed to medium of
pH 5.8–7.8. Two h later pH i in single cells was measured with the use of H⁺ ion-sensitive microelectrodes (see "Materials and Methods"). In separate experiments (not shown) it had been demonstrated that a 2-h incubation at pH c different from 7.4 was sufficient for generation of a new steady state relationship between pH e and pH i. As demonstrated in Fig. 11, the pH i of M1R cells varied with pH c; however, pH i shifts were generally less pronounced than the variations of pH e. For example, a pH c of 7.8 corresponded to pH i of 7.4, and a pH c 6.2 resulted in a shift of pH i to 6.7.

In a further set of experiments two different techniques were applied, each of which lowered the pH i of MAFO-treated cells to approximately the same value (pH i 6.5–6.6) while the corresponding pH e values were different (5.9 versus 6.5, respectively). If pH i was the primary determinant for MAFO-induced cytotoxicity, then both modalities would be expected to give similar results. First, pH i was lowered solely by reduction of pH e. M1R cells were exposed to MAFO at pH c 5.9, corresponding to a pH i of ~6.6 (Fig. 11). Alternatively, pH i was equili-

brated with pH c (6.5) prior to drug exposure, using the H⁺ ionophor nigericin (23). Fig. 12 demonstrates that after exposure to MAFO at pH c 5.9 (pH i ~6.6) the fraction of colony-forming M1R cells did not differ significantly from the fraction measured after exposure to MAFO at pH c 6.5 in the presence

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of nigericin (pH 6.5). These results support the view that the
cytotoxic effect of aCP is increased primarily by raising the
intracellular concentration of H+ ions (which is sensitive to
variations of pH). At the concentration used (0.25 μg/ml)
nigericin per se was only slightly cytotoxic: the fraction of
colonies-forming cells at pH 6.5 was reduced by 60% as
compared to a reduction by 20% at pH 6.5 without nigericin.

The cytotoxic action of bifunctional chloroethylating agents
is considered to be mainly dependent on the formation of DNA
monoadducts (O-alkylguanine), with the varying cellular
capacity for their removal and repair as a modulating determinant,
and the subsequent formation of cytotoxic DNA interstrand
cross-links (diadducts; 24, 25). DNA diadducts are thus formed
in a two-step process. Following covalent binding of one of the
chloroethyl groups to a single nucleophilic site in one DNA
strand the second chloroethyl group is attached to the comple-
mentary strand after a time interval which appears to be drug
specific (26–28). DNA interstrand cross-links may subsequently
be removed by enzymatic repair mechanisms (26–28). To in-
vestigate the relative sensitivities of these reactions to altera-
tions of pH, MIR cells were treated with L-PAM. The forma-
tion and removal of DNA cross-links following exposure to this
drug have been studied in detail (26). During the 1-h period
of exposure to L-PAM mainly DNA monoadduct formation takes
place; at the end of drug exposure the "cross-linking index" is
low. This index increases after removal of the drug, reaching a
maximum 12 h later. The amount of cross-links detectable in
the DNA of surviving cells then begins to decrease, reaching
pretreatment values at about 40 h after the end of drug expos-
ure. Similar observations have been reported for aCP, 4-OH-
CP, and for 4-sulfido-cyclophosphamides (27–29). During the
phase predominated by DNA monoadduct formation, as ana-
alyzed after a 1-h exposure of MIR cells to L-PAM, an acidic
microenvironment (pH 5.6) resulted in a marked enhancement of
cytotoxicity as compared to exposure at pH 7.4 (Fig. 13).

The effect of reduced pH during the period mainly character-
ized by DNA cross-link formation was investigated by incubat-
ing MIR cells previously exposed to L-PAM for 1 h at pH 7.4
for a further 14 h at either pH 7.4 or pH 5.6 in drug-free
medium before determining the fractions of colony-forming
cells. To insure complete removal of free L-PAM, the cells were
washed three times with complete culture medium. The result-
ing increase in drug cytotoxicity at reduced pH was less pro-
nounced than in the foregoing experiments. At pH 5.6 the
fraction of colony-forming MIR cells was 0.007 as compared to
0.04% at pH 7.4 (L-PAM concentration, 6 μg/ml). For analysis of
the effect of reduced pH on aCP-treated cells during the
subsequent phase (DNA cross-link removal and repair), MIR
cells were again exposed to L-PAM for 1 h (pH 7.4), washed,
and incubated in drug-free medium (pH 7.4) for 14 h. There-
after, pH in part of the culture plates was lowered to 5.6 for
28 h. Then the cells were plated to determine the respective
fractions of colony-forming cells. As shown in Fig. 13, a reduc-
tion of pH during this phase did not result in additional toxicity
in comparison to the counterpart cells kept at pH 7.4 during
the entire posttreatment period.

DISCUSSION

Any strategy to be considered for improving the effectiveness
of cancer chemotherapy must rely on the exploitation of phe-
notypic differences between normal and malignant cells. One
such difference is the ability of most malignant cells, as opposed
to unperturbed normal cells, to aerobically convert glucose to
lactic acid at a rate dependent on the microenvironmental
acidity. In a previous study we have shown that parental administration of glucose to tumor-
bearing rats increases the rate of aerobic glycolysis in malignant
cells, which in turn leads to an elevated concentration of lactic acid selectively in the tumor tissue (10).
This observation is consistent with results reported by other investigators. In tu-
mors of different histological type and of both animal and
human origin, the concentration of H+ ions can thus be in-
creased by a factor of up to 10 (Table 1), while pH distributions
remain essentially unchanged in normal tissues of hypergly-
cemic (nondiabetic) tumor-bearing hosts (Table 2).

Nitrogen mustard derivatives like CP and its analogs rep-
resent one of the most effective classes of anticancer agents.
The cytotoxic effects of bis-chloroethylating agents are concen-
tration-dependent both in vitro and in vivo (30). Theoretically,
therefore, the therapeutic efficiency of these agents could be
improved by either increasing their concentration or their re-
activity (on a molar basis) selectively in malignant tissues. The
work presented here is an example of the latter approach:
extracellular, and the subsequent intracellular reduction of pH
strongly increases the cytotoxicity of various bis-chloroethyl-
ating agents. The average pH in normal tissues is in the range
of 7.0 to 7.4 (Table 2). After exposure to aCP at pH 7.4 in
culture, the fraction of colony-forming MIR cells decreased to
8% of untreated control cells. However, when pH was lowered
to 6.2 (approximately the mean "aggregated" pH in malignant
tumors of hyperglycemic hosts; 10; Table 1), the fraction of
colony-forming cells decreased to 0.04%. This corresponds to
a more than 100-fold potentiation of aCP cytotoxicity at pH 5.6.

The cytotoxic effect of aCP was even more enhanced when
phosphate was lowered to the minimum level measured in different
areas of transplanted tumors in glucose-perfused rats (5.3–6.1;
10). At pH 5.6 the cytotoxic effect of aCP was potentiated by
a factor of 10. Similar results were obtained with HN2, nor-
HN2, L-PAM, and chlorambucil.

DNA is considered a critical molecular target of bifunctional
alkylating drugs (24, 25). Thus pH, in particular intracellular
phosphate, may play a critical role in the H+ ion-mediated potentiation
of drug cytotoxicity. This notion is supported by the results of

Fig. 13. Cytotoxic effect of simultaneous or sequential exposure of M1R cells
to L-PAM and reduced pH. ○, L-PAM (1 h, pH 7.4); ●, L-PAM (1 h, pH 5.6);
△, L-PAM (1 h, pH 7.4, followed by 14-h incubation in drug-free medium at pH
5.6); □, L-PAM (1 h, pH 7.4, followed by drug-free incubation at pH 7.4 for
14 h, and subsequently for 28 h at pH 5.6).
the present experiments, including those using the ionophor nigericin to equilibrate pH$_i$ with pH$_e$. The fraction of colony-forming M1R cells decreased to the same level irrespective of whether pH$_i$ was lowered to 6.5–6.6 by reduction of pH$_e$ to 6.5 in the presence of nigericin, or by decreasing pH$_i$ to 5.9 in the absence of the ionophor. Within the pH$_e$ range studied (5.8–7.8), the corresponding shifts of pH$_i$ were somewhat smaller than the changes of pH$_e$, indicating the cells’ ability to keep pH$_i$ closer to the physiological value in the presence of a lower pH$_e$, in agreement with data published by other investigators (23, 31, 50). pH$_i$ values may be nearer pH$_e$ in the case of malignant cells actively producing lactic acid in the presence of high concentrations of glucose (as opposed to the present experiments where the lowering of pH$_i$ was achieved by reducing pH$_e$). In our analyses, pH$_e$ 6.2, a pH$_e$ value frequently used in this study, lead to a pH$_i$ of 6.7 in M1R cells. This value is higher than the pH$_i$ reported for actively glycolyzing cells both in vitro and in vivo (32, 33). When the glucose supply in vitro to malignant cells previously starved of glucose was sharply raised, a procedure resembling the elevation of glucose concentration in tumor interstitial fluid after parenteral administration of glucose (8), pH$_i$ decreased from 6.9 to 6.3 (32). Conversely, when glucose was injected i.p. into tumor-bearing mice, the pH$_i$ of Rif-1 tumor cells, as measured by nuclear magnetic resonance spectroscopy, decreased to 6.6 (33). The potentiation of aCP cytotoxicity at reduced pH$_e$ (6.2) reported here, thus understimates the enhancement expected at an “aggregated” acidic pH$_e$ of 6.2 in malignant tumors in vivo (for detailed reviews of pH distributions in different compartments of mammalian tissues, see Refs. 10 and 34, and literature cited therein).

The H$^+$ ion-mediated enhancement of aCP cytotoxicity is not due to a more rapid formation of the reactive alkylating metabolites from 4-OH-CP at reduced pH$_e$ as compared to physiological culture medium. Wagner et al. (35) have shown that in aqueous solutions the decomposition of 4-OH-CP to phosphoramid mustard and acrolein is in fact prolonged at pH 5.5 as compared to pH 7.3. This does, however, not imply that the increased cytotoxicity of aCP at low pH$_e$ can be explained by extended exposure of cells to the more slowly generated reactive metabolites, since at both pH$_e$ 7.4 and pH$_e$ 6.2 maximum inhibition of colony-formation was observed after 24-h exposure to the drug.

Phosphoramid mustard is considered to be the major reactive metabolite of aCP (14, 22). Using the nitrobenzylpyridine assay for measuring the alkylating activity of CP metabolites, it has been shown that the reactivity of phosphoramid mustard was only slightly increased when the pH$_e$ of the assay solution was lowered below physiological values (14, 22). In contrast, the reactivity of nor-HN$_2$ was highly sensitive to variations of pH$_e$ (14, 22). At pH 7.4 alkylation of nitrobenzylpyridine by nor-HN$_2$ was marginal or not detectable. Under acidic conditions, however, the alkylating activity of nor-HN$_2$ was strongly enhanced and exceeded that of phosphoramid mustard. We propose, therefore, that the H$^+$ ion-mediated potentiation of aCP cytotoxicity mainly resides with this metabolite. This is supported by our finding that the cytotoxic action of 4-OH-IFO is not H$^+$ ion-dependent. Primary metabolites of IFO with structural similarities to those of CP have been identified (36). However, ifosfamide mustard does not, as phosphoramid mustard, further decompose to yield metabolites analogous to nor-HN$_2$, since one of the chloroethyl side chains of IFO is linked to the phosphoate group and not to the amino nitrogen as in CP and its metabolites.

In the present experiments, the cytotoxicity of HN$_2$ was also pH$_e$-dependent. In the nitrobenzylpyridine assay, on the other hand, HN$_2$ reactivity was not significantly enhanced when the pH$_e$ value was shifted from 7.4 to 5.6 (14). This discrepancy can be explained by the known enzymatic demethylation of HN$_2$ to the H$^+$ ion-sensitive nor-HN$_2$ in living cells (37).

From the results of simultaneous or sequential exposure of M1R cells to L-PAM and to an acidic microenvironment, we conclude that the phase of drug uptake and the formation of DNA monoadducts is most pH$_e$-sensitive. To a lesser extent, DNA interstran cross-link formation also appears to be enhanced at acidic pH. The overall results are in agreement with data reported for EMT6 tumor cells exposed to mitomycin C (38), where an increased rate of DNA cross-link formation was observed when pH$_e$ was lowered from 7.5 to 5.7, 1 h prior to and during a 2-h drug exposure. In the present study, a reduction of pH$_e$ during the phase of DNA cross-link removal and repair did not significantly influence the cytotoxic effect of L-PAM. The molecular processes occurring during this phase may thus be the least H$^+$ ion-sensitive mechanisms in the sequence of events following the interaction of bis-chloroethylating agents with target cells.

Hahn and Shiu have analyzed the response of Chinese hamster cells to antineoplastic drugs different from oxazaphosphorines as function of pH$_e$, either alone or in combination with hyperthermia (51, 52). Whereas the cytotoxic effect of methotrexate, BCNU and bleomycin was not H$^+$ ion-dependent in the pH$_e$ range of 6.5 to 8.5 at 37°C, both BCNU and bleomycin cytotoxicity exhibited a marked pH$_e$-sensitivity in combination with simultaneous heat exposure (43°C).

When M1R cells were exposed to aCP at pH$_e$ 6.2, the “pH-dose modifying factor” was 0.2 relative to exposure to aCP at pH$_e$ 7.4. Solely by lowering pH$_e$, a cytotoxic effect can thus be exerted which, under physiological conditions, would require a five times higher dose. The magnitude of this dose-sparing effect makes a tumor-selective downshift of pH$_e$ by glucose perfusion a candidate procedure as an adjunct to systemic chemotherapy with alkylating agents; in particular, since the “drug” (glucose) used for sensitization is nontoxic. The feasibility of this approach will, however, depend on whether the pH$_e$ in primary human tumors can be shifted to the same level and for the same duration as the pH$_e$ in transplanted rodent tumors. We are aware of only two reports on pH measurements in human tumors following glucose administration (11, 45). In these studies hyperglycemia was generated only by either oral or short term (~1 h) i.v. glucose administration. Mean blood glucose did not exceed 23 and 16 mmol/liter, respectively, and the mean intratumoral pH$_e$ was only lowered to 6.6–6.7. Studies on animal tumors indicate, however, that tumor pH$_e$ may be inversely correlated with serum glucose up to 50 mmol/liter (10). In addition, there is evidence that the duration of glucose administration may be an important determinant for the pH$_e$ response of neoplastic tissues (10, 41). It remains to be investigated, therefore, whether intratumoral pH$_e$ values of 6.2–6.5 can be generated also in humans by intensification of i.v. glucose administration. In particular, it has to be analyzed whether in human tumors an acidosis can be maintained long enough to allow for H$^+$ ion-mediated drug activation (~12 h). It is of interest, therefore, that in transplanted rat tumors mean pH$_e$ values of 6.1–6.2 could be maintained up to 48 h by continuous glucose administration (10). Conversely, in healthy volunteers serum glucose concentrations of ~30 mmol/liter have been generated for up to 24 h solely by i.v. glucose infusion (39, 40). Apart from osmotic diuresis no serious side effects were observed.

Some conflicting results have been reported regarding the effect of combinations of alkylating drugs with parenteral ad-
ministration of glucose in vivo. Whereas Osinsky et al. (41) reported partial and complete remissions of Guerin carcinomas following treatment with thiophosphamide and i.v. glucose administration, Urano and Kim (42) noted no significant enhancement of cytotoxicity against a murine fibrosarcoma when CP treatment was combined with i.p. glucose administration; however, no information on intratumoral pH was given. The discrepancy between the latter data and the results presented here may be explained by the short duration of intratumoral acidosis following single i.p. injections of glucose into tumor-bearing mice (43), a technique applied in the study by Urano and Kim (42). Investigations on the treatment of transplanted tumors with chloroethylating drugs combined with tumor-selective acidification are in progress in our laboratory.

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