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Combined effect of pH and sodium cyanate on the inhibition of tumor cell proliferation and metabolism by BCNU and hyperthermia

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Summary. In previous studies, we have found that combined treatment with BCNU and sodium cyanate could have a greater effect on the survival of mice bearing B16 melanoma than treatment with either agent alone. With rat hepatoma and human colon cancer cells in culture, we have obtained evidence that the inhibition of cell proliferation by sodium cyanate is greater at pH 6.6 than at pH 7.4. In the present work, the effects of combination treatments on the proliferation of cancer cells were studied with cyanate, pH, BCNU, and hyperthermia. With HT29 human colon cancer cells, the inhibitory effect of BCNU (50–100 μg/ml) was greater when the cells were treated at pH 6.6 than at pH 7.4. The influence of pH appeared to be absent or minimal at lower or higher concentrations of BCNU. We confirmed our previous observation that the inhibition of proliferation of LS174T human colon cancer cells is greater at pH 6.6 than at pH 7.4, and we observed an inhibitory effect of BCNU (50 or 200 μg/ml). However, no more than additive effects were seen with combination treatment. An inhibitory effect of hyperthermia was seen for the incorporation of [^{3}H]-leucine into protein of rat hepatoma cells (HTC) and for that of [^{3}H]-thymidine into DNA of human colon cancer (HT29) cells. In neither case was the effect of hyperthermia significantly enhanced by treatment with sodium cyanate beyond that seen with one of the treatments alone. The data confirmed that the inhibitory effect of sodium cyanate on cell proliferation can be enhanced by a low pH but did not provide evidence for synergistic effects in combination with BCNU or hyperthermia.

Introduction

Previous studies have shown that sodium cyanate has some degree of selective action on tumor metabolism [13]. Because of the relatively low toxicity of sodium cyanate in humans, its use in combination chemotherapy has been suggested, although it is not effective as a single agent against colon cancer [7]. In mice bearing P388 leukemia cells, i.p. injection of sodium cyanate (200–250 mg/kg) had no effect on life span. Synergistic effects were observed when sodium cyanate (250 mg/kg) and melphalan were injected simultaneously [1]. The treatment involved the combined action of a carbamoylating agent and an alkylating agent. This combination of activities is a feature of some nitrosoureas that have been of clinical value, including 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). The carbamoylating activity released by BCNU is attributable to 2-chloroethylisocyanate. The carbamoylating activity of this organic isocyanate differs from that of inorganic cyanate inasmuch as low pH increases the carbamoylating activity of cyanate by favoring the formation of isocyanic acid. In studies with mice bearing B16 melanoma, survival was not influenced by sodium cyanate given as a single agent [14]. However, combined administration of sodium cyanate and BCNU increased the survival of the mice more than treatment with BCNU alone. No synergism was observed with sodium cyanate and melphalan or 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.

It has been demonstrated that the cytotoxicity of many antineoplastic drugs can be potentiated by mildly elevated temperatures [5]. When whole-body hyperthermia was combined with chemotherapeutic agents in cancer patients, only a modest effect was observed [6]. Another line of study has indicated that the cytotoxicity of BCNU was increased when the drug was combined with low pH and hyperthermia in cultured RIF cells [17]. Since we have observed that inhibitory effects of sodium cyanate on macromolecular synthesis are enhanced at low pH [8], the possibility was considered in the present work that combined treatment with sodium cyanate and these modalities might contribute to effects on tumor cell proliferation.

Materials and methods

Cells. Rat hepatoma cells (HTC) and human colon cancer (HT29 and LS174T) cells were maintained in Chee's essential medium (B&B/Scott

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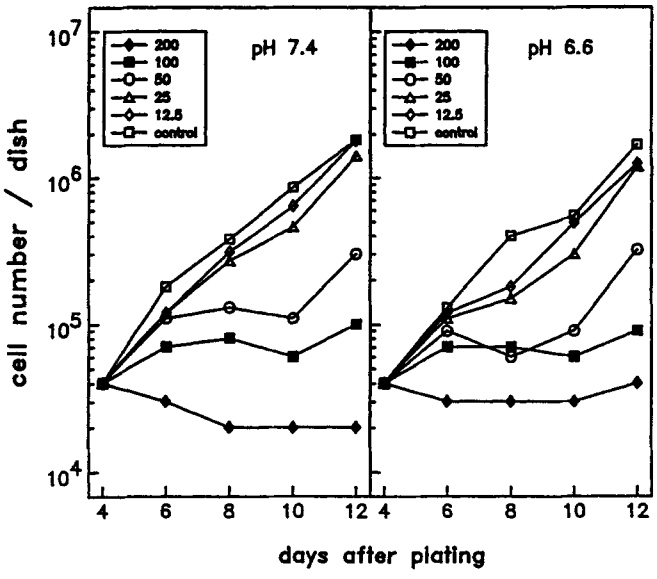

Fig. 1. Combined effect of pH and BCNU on the growth of HT29 cells. The HT29 cells (0.2×10^4) were cultured in Chee's essential medium containing 10% fetal bovine serum. On day 4, cells were treated with BCNU (12.5–200 μg/ml) in GF-3 serum-free medium (pH 7.4) for 1 h at 37° C. The medium was then replaced with Chee's essential medium containing 10% fetal bovine serum and 25 m*M* PIPES at either pH 7.4 or pH 6.6. The medium was changed every other day. The cell number per plate is given over the 12-day culture period. Each point represents the mean of two samples

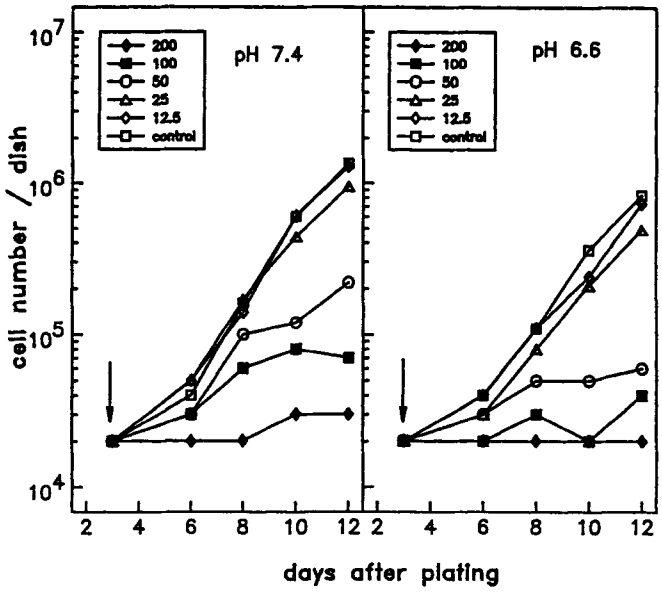

Fig. 2. Combined effect of pH and BCNU on the growth of HT29 cells. The experimental conditions and presentation of data were the same as those described in Fig. 1. The only modification was that treatments with BCNU were carried out in GF-3 serum-free medium at either pH 7.4 or pH 6.6

Laboratories, Fiskeville, R. I.) supplemented with 10% heat-inactivated fetal calf serum and antibiotics at 37° C in a humidified atmosphere (95% saturation) containing 5% CO_2. GF-3 medium was Chees's essential medium supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (5 ng/ml).

Chemicals. Sodium cyanate was obtained from ICN Pharmaceuticals (Plainview, NY). L-[4,5-^{3}H]-Leucine (44 Ci/mmol) and [*methyl*-^{3}H]-thymidine (62 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, Calif.) BCNU was supplied by Bristol Myers (Syracuse, N. Y.), and the drug was dissolved in ethanol before use.

Incorporation of isotope-labeled compounds. The incorporation of precursors into DNA and protein was determined following procedures described previously [12].

Statistical evaluation. Statistical significance of the results was determined by a paired Student's *t*-test. A probability of <5% was considered to be significant.

Results

In the study presented in Fig. 1, HT29 cells were treated with BCNU at pH 7.4 for 1 h at 37° C. Inhibition of cell proliferation in a subsequent incubation in fresh medium was most noticeable after preincubation with BCNU at concentrations of >25 μg/ml. No consistent effect of pH was apparent when the medium was changed after the preincubation with BCNU. However, if the cells were preincubated with BCNU for 1 h at either pH 6.6 or pH 7.4 and then incubated at the same pH, the data suggested that the inhibitory effect of BCNU was enhanced at pH 6.6 as compared with that at pH 7.4 (Fig. 2). This trend was most apparent after treatment with BCNU at a concentration of 50 or 100 μg/ml.

The data in Fig. 3 indicate that the proliferation of LS174T cells was also inhibited by pretreatment with BCNU (50 or 200 μg/ml) and by incubation with sodium cyanate. After incubation with sodium cyanate at a concentration of 0.1 mg/ml, the inhibitory action on cell proliferation was greater at pH 6.6 than at pH 7.4. There were some additive effects of BCNU (50 μg/ml) and sodium cyanate (0.1 mg/ml), but this was not seen at all times. Lower concentrations of BCNU (20 μg/ml) and sodium cyanate (0.02 mg/ml) were examined in the next study (Fig. 4). The data suggested that no more than additive effects were obtained with BCNU and sodium cyanate. Cell proliferation was lower at pH 6.6 than at pH 7.4 with the different drug treatments.

A study using HTC cells indicated that the incorporation of [^{3}H]-leucine into protein was greatly decreased by increasing the temperature of incubation from 37° C to 43° C (Table 1). Once again, the effect of lowering the pH from 7.4 to pH 6.6 was to increase the inhibitory effect of sodium cyanate. A change in pH and the addition of sodium cyanate did not further affect the decrease in incorporation seen after raising the temperature of the incubation from 37° to 43° C. Less response to hyperthermia was observed in studies on the incorporation of [^{3}H]-thymidine into DNA of HT29 cells (Table 2). When the cells were incubated at pH 6.6, there was a 79% inhibition of the incorporation of [^{3}H]-thymidine caused by sodium cyanate (0.25 mg/ml). However, hyperthermia (43° C) in combination with sodium cyanate did not significantly affect the magnitude of the inhibition seen with sodium cyanate alone.

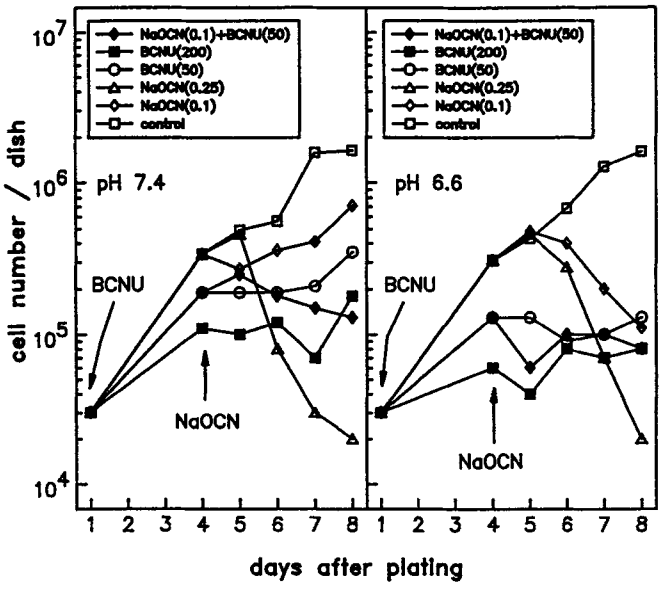

Fig. 3. Combined effect of BCNU, pH, and sodium cyanate on the growth of LS174T cells. The LS174T cells (1×10^4) were cultured in Chee's essential medium containing 10% fetal bovine serum. On day 1, cells were treated for 1 h at 37° C with BCNU (50 or 200 μg/ml) at the indicated pH. The medium was then replaced with fresh Chee's essential medium containing 10% fetal bovine serum at the stated pH. Treatment with sodium cyanate (0.1 or 0.25 mg/ml)) was started from day 4. Medium with or without sodium cyanate was changed daily. The cell number per plate is given over the 8-day period. Each point represents the mean of two samples

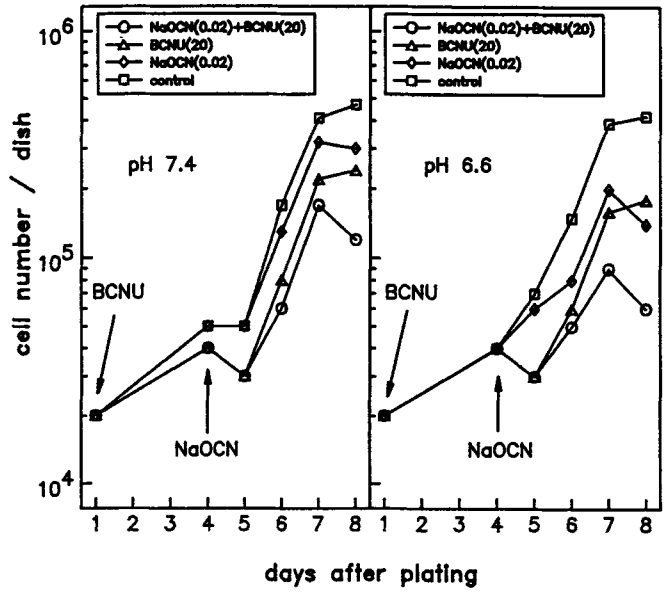

Fig. 4. Combined effect of BCNU, pH, and sodium cyanate on the growth of LS174T cells. The experimental conditions and presentation of data were the same as those described in Fig. 3. The concentration of sodium cyanate in this study was 0.02 mg/ml and that of BCNU was 20 μg/ml

Table 1. Combined effect of sodium cyanate, pH, and hyperthermia on the incorporation of [^{3}H]-leucine into protein in HTC cells

Sodium cyanate (mg/ml)	pH	Incorporation as % control at:	
		37° C	43° C
0	7.4	100	22 ± 7*
0.25	7.4	88 ± 14	26 ± 4*
0.50	7.4	70 ± 14*	19 ± 5*
0	6.6	100	21 ± 2*
0.25	6.6	62 ± 26*	18 ± 3*

HTC cells were preincubated at 37° C for 10 min with sodium cyanate in 1 ml Eagle's minimum essential medium containing 50 m*M* piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES) buffer at the stated pH and temperature. There was a further incubation for 30 min after the addition of 5 μCi [^{3}H]-leucine in 10 μl water. Incorporation of isotope into protein is expressed as a percentage of the incorporation in control cells incubated at pH 7.4 (493 ± 141 cpm/10^5 cells) or pH 6.6 (610 ± 143 cpm/10^5 cells). Each point represents the mean ± SD for three experiments in which incubations were carried out in triplicate

* $P < 0.05$ (control vs NaOCN-treated cells)

Table 2. Combined effect of sodium cyanate, pH, and hyperthermia on the incorporation of [^{3}H]-thymidine into DNA in HT29 cells

Sodium cyanate (mg/ml)	pH	Incorporation as % control at:	
		37° C	43° C
0	7.4	100	68 ± 37
0.25	7.4	75 ± 54	51 ± 27*
0.50	7.4	53 ± 50	49 ± 42*
0	6.6	100	65 ± 28*
0.25	6.6	21 ± 19*	20 ± 11*

HT29 cells were preincubated at 37° C for 10 min with sodium cyanate in 1 ml Eagle's minimum essential medium containing 50 m*M* PIPES buffer at the stated pH and temperature. There was a further incubation for 30 min after the addition of 2.5 μCi [^{3}H]-thymidine in 10 μl water. Incorporation of isotope into DNA is expressed as a percentage of the incorporation in control cells incubated at pH 7.4 (167 ± 56 cpm/10^4 cells) or pH 6.6 (124 ± 7 cpm/10^4 cells). Each point represents the mean ± SD for three experiments in which incubations were carried out in triplicate

* $P < 0.05$ (control vs NaOCN-treated cells)

Discussion

Carbamoylating agents do not have an established role in cancer chemotherapy [13]. Several nitrosoureas of clinical value have carbamoylating activity in addition to their alkylating activity, but the latter is generally considered to be of primary significance. Inhibition by carbamoylating agents of the repair of DNA damage resulting from alkylating activity provides a possible rationale for combined action. The carbamoylating agent released from BCNU, namely, 2-chloroethyl-isocyanate, differs in its action from sodium cyanate. Rates of carbamoylation are slower with cyanate, but this compound is much longer-lived in aqueous solution. The pH of the medium is likely to have a greater effect on carbamoylation by cyanate due to the equilibrium with isocyanic acid.

The effect of BCNU in prolonging the life of mice bearing B16 melanoma was enhanced by combined treat-

ment with sodium cyanate [14]. Neilan et al. [17] found that the cytotoxicity of BCNU against cultured RIF tumor cells was enhanced when the drug was combined with low pH and hyperthermia. Our rationale in studying the combined effects of cyanate and BCNU was that cyanate would not only increase the carbamoylating activity but also make the treatment more sensitive to differences in tissue pH. Low pH in tumors would favor the conversion of cyanate to the carbamoylating agent, isocyanic acid. The present data suggested that the effect of BCNU on HT29 cell proliferation was increased when the treatment was carried out at a low pH. Additive effects were seen with LS174T cells that were incubated with sodium cyanate for 3 days after treatment with BCNU at pH 6.6 or 7.4.

When HTC cells were incubated at a high temperature (43° C), the incorporation of [^{3}H]-leucine into protein was decreased significantly. No synergistic effect of hyperthermia was observed with either low pH or sodium cyanate under these experimental conditions. A lower response to hyperthermia was seen for the incorporation of [^{3}H]-thymidine into DNA of HT29 cells, and the effect was not influenced by either low pH or sodium cyanate. A lack of synergism under these conditions may be contrasted with several reports of synergism between hyperthermia and radiation or chemotherapy [19, 20]. Conditions have also been reported under which hyperthermic cytotoxicity was enhanced by a more acidic environment [2–4].

A relationship between glutathione levels and the development of thermotolerance has been suggested. Depletion of glutathione was suggested to be related to the increase in thermal sensitivity in an acidic environment [4, 16]. Our previous studies [9, 10] have shown that sodium cyanate decreases cellular glutathione to a greater extent at pH 6.6 than at pH 7.4, and cyanate can affect the regulation of intracellular pH in normal and neoplastic cells. The mechanisms of thermal cell killing are not clear, but it has been suggested that hyperthermia might cause cell death by affecting cell membranes, lysosomal vesicles, or nuclear proteins or by a nonspecific immunologic response [18]. Carbamoylation of sulfhydryl groups is less stable than the modification of amino groups, but with sodium cyanate the reaction with sulfhydryl groups is much more rapid than that with amino groups. There is evidence for carbamoylation of sulfhydryl groups in vivo [11]. Stahl et al. [21] have provided evidence for the formation of mutagenic derivatives by the reaction of 2-chloroethyl-isocyanate with glutathione. On this basis, they have questioned the clinical use of BCNU. On the other hand, we have obtained evidence for selective effects of carbamoylating agents on tumor metabolism [13, 15]. The basis for this specificity requires greater clarification before the use of carbamoylating agents in cancer chemotherapy can be discounted.

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References

1. Dufour M, Germain J, Skalski V, Dorata A, Lazarus P, Panasci LC (1984) Effect of administration of sodium cyanate and melphalan on the lifespan of P388 tumor-bearing CD2F1 mice. Cancer Chemother Pharmacol 12: 94
2. Freeman ML, Malcolm AW, Meredith MJ (1985) Decreased intracellular glutathione concentration and increased hyperthermic cytotoxicity in an acid environment. Cancer Res 45: 504
3. Gerweck LE, Richards B, Michaels HB (1982) Influence of low pH on the development and decay of 42° C thermotolerance in CHO cells. Int J Radiat Oncol Biol Phys 8: 1935
4. Goldin EW, Leeper DB (1981) The effect of low pH on thermotolerance induction using fractionated 45° C hyperthermia. Radiat Res 85: 472
5. Hahn GM (1979) Potential for therapy of drugs and hyperthermia. Cancer Res 39: 2264
6. Herman TS, Zukoski CF, Anderson RM (1982) Review of the current status of whole-body hyperthermia administered by water circulation techniques. Natl Cancer Inst Monogr 61: 365
7. Herrera-Ornelas L, Petrelli NJ, Madajewicz S, Mittelman A, Allfrey VG (1985) Phase I clinical trial of sodium cyanate in patients with advanced colorectal carcinoma. Oncology 42: 236
8. Hu JJ, Luke A, Chellani M, Zirvi KA, Lea MA (1988) pH-related effects of sodium cyanate on macromolecular synthesis and tumor cell division. Biochem Pharmacol 37: 2256
9. Hu JJ, Dimaira MJ, Zirvi KA, Dikdan G, Lea MA (1989) Influence of pH on the modification of thiols by carbamoylating agents and effects on glutathione levels in normal and neoplastic cells. Cancer Chemother Pharmacol 24: 95
10. Hu JJ, Zirvi KA, Lea MA (1989) Interrelationship between sodium cyanate and pH in the regulation of tumor cell division. Cancer Biochem Biophys 10: 269
11. Koshiishi I, Shibayama R, Morimoto Y, Imanari T (1988) Studies on metabolic pathways of cyanate in rats. J Pharmacobiodyn 11: 730
12. Lea MA (1983) Decreased sensitivity to colchicine-mediated inhibition of metabolite uptake in isolated hepatoma cells. J Natl Cancer Inst 71: 1073
13. Lea MA (1987) Effects of carbamoylating agents on tumor metabolism. CRC Crit Rev Oncol Hematol 7: 329
14. Lea MA, Luke A, Velazquez O, Carpenter L, Martinson CV, Hill HZ, Hill GJ (1986) Effects of sodium cyanate in mice bearing B16 melanoma. Cancer Chemother Pharmacol 17: 231
15. Lea MA, Luke A, Hu JJ, Velzquez O (1987) Action of carbamoylating agents on the uptake of metabolites in hepatomas and liver. Biochem Pharmacol 36: 2775
16. Mitchell JB, Russo A (1983) Thiols, thiol depletion, and thermosensitivity. Radiat Res 95: 471
17. Neilan BA, Jenkins TL, Henle KJ, Moss AJ, Nagle WA (1988) Interaction of BCNU, low pH, glucose and hyperthermia in cultured RIF cells. Cancer Lett 39: 275
18. Reddy EK, Kimler BF, Cytaki EP, Evans RG (1986) Hyperthermia in cancer therapy. Kans Med 87: 163
19. Reinhold HS, Zee J van der, Faithfull NS, Rhoon G van, Wike-Hooley J (1982) Use of Pomp-Siemens hyperthermia cabin. Natl Cancer Inst Monogr 61: 371
20. Song CW, Kang MS, Rhee JG, Levitt SH (1980) The effect of hyperthermia on vascular function, pH, and cell survival. Radiology 137: 795
21. Stahl W, Denkel E, Eisenbrand G (1988) Influence of glutathione on the mutagenicity of 2-chloroethylnitrosoureas. Mutagenic potential of glutathione derivatives formed from 2-chloroethylnitrosoureas and glutathione. Mutat Res 206: 459