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CYTOTOXICITY AND DEOXYRIBONUCLEIC ACID DAMAGE ASSOCIATED WITH BROMOACETATE*

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Abstract—Bromoacetate, one of the hydrolysis products of bromoacetylcholine, has been shown previously to inhibit the growth of neuroblastoma cells in culture. Its mechanism of action is unknown. In this work we have further characterized the cytotoxic effect of bromoacetate in C-1300 neuroblastoma cells in culture and extended it to a cell line not of neural origin, the mouse leukemia L-1210 line. Doses required to inhibit the growth of L-1210 cells in culture were similar to those that inhibited the C-1300 line. Cytotoxicity depended on concentration and duration of exposure. L-1210 colony formation in soft agar was also inhibited by bromoacetate. Bromoacetate was shown to produce DNA single strand breaks in L-1210 cells by the alkaline elution assay. These breaks continued to form after the drug was removed from the medium. Taken together, the toxicity demonstrated toward L-1210 cells and the evidence of DNA damage following drug exposure indicate that the anti-tumor action of bromoacetate may be based on monofunctional alkylation of DNA and not related to inhibition of the cholinergic receptors.

Bromoacetate (BrAc) is a hydrolysis product of bromoacetylcholine [1]. The latter compound was studied as a specific inhibitor of neuroblastoma (NB) because of its irreversible binding to nicotinic receptors. Bromoacetate is a much less potent cholinergic antagonist than bromoacetylcholine or alpha-bungarotoxin, but it is more cytotoxic to NB cells in tissue culture experiments [1]. Bromoacetate has further been shown to produce regressions of C-1300 NB tumors in A/J mice following intratumoral injection [2]. The spectrum of anti-tumor activity and the mechanism upon which it is based remain unknown. As an alkyl halide, BrAc should be capable of monofunctional alkylation of nucleophilic molecules within the cell such as DNA, RNA, and protein. In this regard, Chapman *et al.* [3] have documented inhibition of uridine and leucine incorporation by BrAc.

In this report we further characterize the cytotoxicity of bromoacetate to C-1300 neuroblastoma cells and to a cell line not of neural origin, mouse leukemia L-1210. Using the DNA alkaline elution technique, we demonstrate DNA strand breakage following treatment of L-1210 cells with BrAc, suggesting a possible mechanism of action.

MATERIALS AND METHODS

Materials. Bromoacetic acid (Aldrich Chemical Co., Milwaukee, WI) was used. It was dissolved in deionized water and sterilized by filtration before addition to the cell cultures.

Cell cultures. Murine C-1300 NB cells, originally obtained from the American Type Culture Collection (Rockville, MD), were maintained in modified Eagle's medium supplemented with 10% fetal calf serum.

Cultures used for experiments were grown in 100 × 20 mm round dishes at 37° under 95% air/5% CO_2, with 100% humidity. Under these conditions, a doubling time of approximately 24 hr was observed.

L-1210 mouse leukemia cells were grown in suspension culture in RPMI 1630 medium supplemented with 20% heat-inactivated fetal calf serum plus penicillin and streptomycin at 37° and ambient relative humidity and O_2/CO_2 concentrations. Stock cultures were maintained in static bottles in the same medium without antibiotics and were used to initiate suspension cultures at weekly intervals. Cultures used for experiments were in exponential growth with a doubling time of approximately 12 hr. One hour prior to drug treatment, cells were resuspended in fresh warm medium at a cell density of 5×10^5 cells/ml. For alkaline elution experiments, cells were radiolabeled by 18-hr incubation with [^{14}C]thymidine (0.01 μCi/ml) prior to drug treatment.

Growth curves. NB cells were maintained in several 75 cm^2 flasks; these stock cultures had their medium changed every other day and were split approximately every fourth day. For experiments, approximately 5×10^4 cells in fresh medium were transferred to 100 × 20 mm petri dishes. Once transferred, the cells were allowed to attach and grow for 36 hr under conditions stated above. After 36 hr, the medium was replaced with fresh medium, and the drug treatment was started. At the end of drug treatment the cells were trypsinized, and metabolically viable cells were counted with a hemocytometer. Metabolic viability was determined by exclusion of trypan blue. Drug treatment consisted of exposure for 1, 4, or 24 hr, and the cells were counted at various times after the drug had been removed.

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Survival curves. Drug-treated and control L-1210 cells were assayed for colony survival in soft agar by the method of Chu and Fisher [4]. Following drug treatment, cells were spun and resuspended in fresh medium twice. At this point, serial dilutions of the cells were added to a prepared solution of agar in the normal medium of the cells. After 14 days of incubation at 37°, the number of colonies was counted and compared to controls. Cloning efficiency of control cells was 40–60 per cent.

Alkaline elution. The theoretical basis for the alkaline elution technique is described in recent reviews [5, 6]. Following a 1-hr drug treatment, the ^{14}C-labeled cells were centrifuged twice and resuspended in cold medium. Approximately 5×10^5 cells were layered onto a polyvinyl chloride filter (pore size 2.0 μm) and lysed with a solution containing 2 M NaCl, 0.02 M Na_2EDTA, 0.2% sarkosyl, and proteinase K (0.5 mg/ml), at pH 10.0. The labeled DNA was then eluted from the filter with tetrapropylammonium hydroxide, pH 12.1, at a rate of 0.04 ml/min. The eluted DNA was collected in fractions over 15 hr and assayed by liquid scintillation counting. Prior to scintillation counting, DNA remaining on the filter was treated with 0.4 ml of 1 N HCl at 60° for 1 hr followed by 2.5 ml of 0.4 N NaOH. The fraction of DNA remaining on the filter at each point in the elution process was then calculated. DNA elutes from the filter at a rate which is dependent on strand size. Thus, an increasing frequency of single strand breaks results in smaller single strand lengths and faster elution from the filter.

RESULTS

C-1300 neuroblastoma. The effects of continuous exposure of NB cells to concentrations of BrAc between 10 and 100 μM are known [1, 7]. It was important, however, to determine if continuous exposure was required for cytotoxicity. Neuroblastoma cell growth following exposure to different concentrations of BrAc for various intervals is shown in Fig. 1. The broken line of open circles represents the response of untreated cells to the replacement of their medium with conditional medium. Exposure of cells to 10 μM bromoacetate for less than 24 hr had no effect on cell growth. Continuous exposure to this drug concentration for 24 hr, however, produced a stasis in cell growth that persisted for at least 24 hr beyond drug removal. Recovery beyond this point could not be ascertained without further time points. A bromoacetate concentration of 100 μM for 1 hr produced a decline in cell number which progressed for 48 hr beyond drug removal. The longer periods of drug exposure produced a precipitous and complete loss of cells from the culture plates. Thus, inhibition of cell growth was dependent on both concentration and duration of exposure.

Inhibition of colony formation. Survival of L-1210 cells following a 1-hr treatment with BrAc was determined by a colony forming assay (Fig. 2). The relationship between survival and drug dose was log-linear up to approximately 250 μM BrAc. There was no shoulder apparent, indicating little ability of

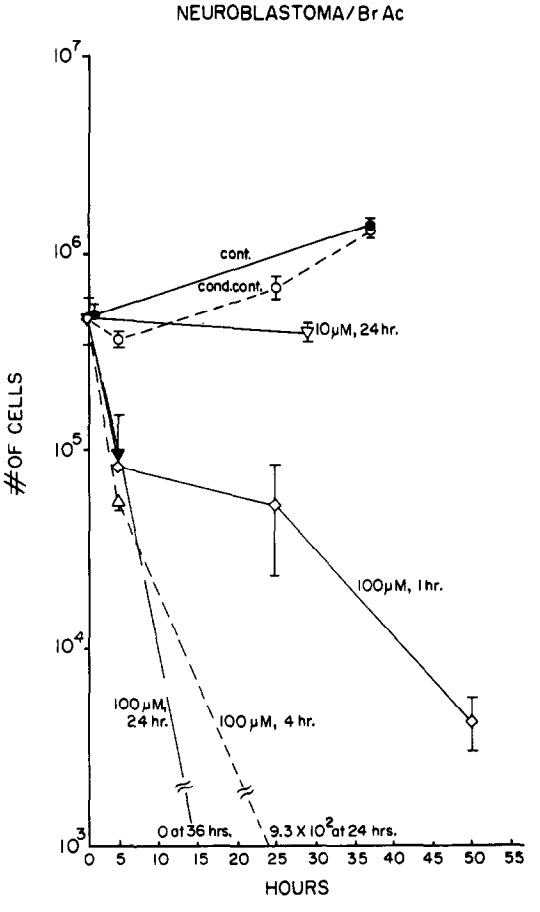

Fig. 1. Growth curves for NB. Concentrations of BrAc and length of exposure to each concentration are indicated. The abscissa refers to the number of hours following drug removal. Only viable cells were counted. Each point is the mean ± 1 S.E.M.

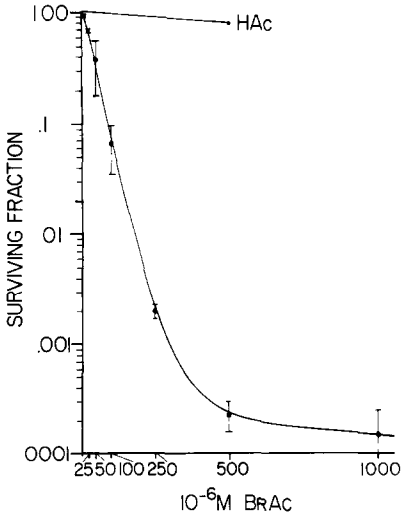

Fig. 2. Survival curve for L-1210. HAc = acetic acid. Following a 1-hr drug exposure, cells were washed, resuspended, and seeded in soft agar. Each point is the mean ± 1 S.E.M.

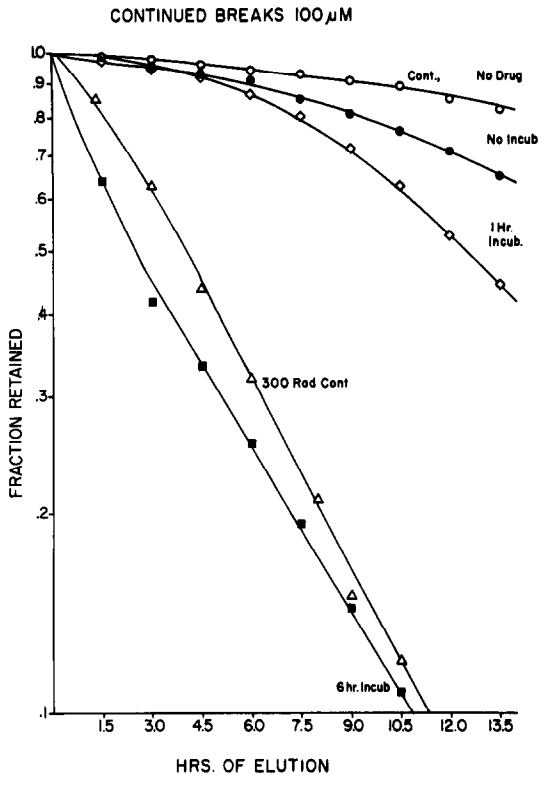

Fig. 3. DNA strand breaks after treatment of L-1210 cells with BrAc. Cells were exposed to 100 μM BrAc for 1 hr washed, and examined by alkaline elution immediately (●), or at 1 (◇) and 6 (■) hr of post-treatment incubation at 37°. The fraction of DNA remaining on the filter during a 13.5-hr elution is shown.

the cells to accumulate or repair sublethal damage. At a BrAc concentration greater than 500 μM, there was little increase in cell kill with increasing concentration. This plateau may represent a subpopulation of L-1210 cells that were resistant to the actions of BrAc for biochemical or kinetic reasons. The D_0 value obtained by inspection was 50 μM; this represents the dose that, on an average, produced one lethal event per cell or, more directly, reduced the colony forming ability to 1/*e* of the control. Cell survival was unaffected by 500 μM acetic acid.

DNA damage in L-1210 cells. To determine if the cytotoxicity of BrAc is related to DNA damage, cells were treated with 100 μM BrAc for 1 hr, washed free of drug, and examined by the alkaline elution assay (Fig. 3). Immediately after drug treatment there was a small increase in the rate of elution of the DNA from the filter, indicating strand breakage. If the cells were incubated at 37° in drug-free medium for 1 or 6 hr after drug treatment, however, many more strand breaks were present, as shown by the increasing elution rate. For purposes of comparison, the elution of DNA from untreated cells that had received 300 rads of gamma radiation prior to elution was included.

DISCUSSION

Bromoacetate is cytotoxic to both neuroblastoma and L-1210 cells. Both of these cell lines have been in passage for over 30 years but have very different origins. L-1210 cells were induced with 3-methylcholanthrene in a female DBA/2 mouse in 1949 [8]. C-1300 neuroblastoma cells arose spontaneously in the body cavity of an albino male A/J mouse in 1940 [9]. Neuroblastoma is a monolayer culture that, in our hands, could not be induced to grow in soft agar. L-1210 is a suspension culture that grows quite well in agar. Because of fundamental assay differences, it is impossible to quantitatively compare drug sensitivity of the two cell lines, but they appear to be similar. The data in L-1210 cells further support the contention of Chiou *et al.* [1] that the mechanism of BrAc cytotoxicity is unrelated to interactions with the cholinergic receptor.

If bromoacetate is not cytotoxic because of interactions with the cholinergic receptor, what is the mechanism by which it kills cells? The ability of bromine to act as a leaving group suggests the possibility that bromoacetate can alkylate nucleophilic macromolecules within the cell. Bromoacetate reacts at physiological pH and temperature with sulfhydryl groups, imidazoles, and amines [10]. Monofunctional alkylating agents have been well studied with regard to their cytotoxic effects, and there appears to be general agreement that these effects principally relate to binding to DNA with resultant inhibition of template function. Our data (Fig. 3) indicate that this is a plausible mechanism of action for BrAc. Following monofunctional alkylation of DNA by BrAc one would expect depurination to occur over a period of time [11–16]. This results in sites in the DNA that are vulnerable to cleavage by alkali (e.g. eluting buffer) or apurinic endonucleases. The continued formation of DNA strand breaks after drug removal (Fig. 3) is consistent with such a sequence of events. Further work will be required to prove that the observed DNA damage actually results from alkylation by BrAc and that it is causally related to cytotoxicity.

In summary, it is concluded from these data that bromoacetate is able to inhibit the growth of both neuroblastoma cells and L-1210 cells in culture. This cytotoxicity does not result from a specific interaction with cholinergic receptors. Rather, the observation of DNA strand breaks following treatment of L-1210 cells with BrAc suggests that the drug may act as an alkylating agent.

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