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ACCUMULATION OF TWO ALKYLATING AGENTS, NITROGEN MUSTARD AND BUSULFAN, BY MURINE LEUKEMIA CELLS *IN VITRO**

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Abstract—The accumulation of two alkylating agents by murine leukemia cells *in vitro* was studied. Nitrogen mustard (HN_2) and busulfan rapidly penetrated cells; cell–medium drug distribution ratios of one were established within 3 min at 0° over a wide range of external drug levels. Accumulation of busulfan was not much affected by raising the incubation temperature. NH_2 binding was, however, a remarkably temperature-sensitive process. At 10^{-5} M levels, busulfan and HN_2 partly inhibited cellular incorporation of precursors into nucleic acids *in vitro*. When cell types differing widely in sensitivity to these alkylating agents were tested, no differences in capacity for drug accumulation or in subsequent inhibition of nucleic acid synthesis could be found.

NITROGEN mustard (HN_2, methyl-*bis*[2-chloroethyl]amine, NSC 762) and busulfan (Myleran, 1,4-dimethanesulfonyloxybutane, NSC 750) are clinically useful antitumor agents which are capable of alkylating amino, carboxyl and sulfhydryl groups of proteins and related molecules, phosphate groups of phospholipids and nucleic acids, and purine and pyrimidine nitrogens. The literature on these and related compounds has been extensively reviewed.[1–5] Resistance to alkylating agents in certain cell lines has been attributed to barriers to uptake,[6,7] but other examples of resistance did not involve such barriers.[8–10] Little seems known about modes of accumulation of these alkylating agents by tumor cells. The present study of uptake and retention of HN and busulfan is an extension of previous work[11–14] relating to the nature of cellular uptake of antitumor agents.

EXPERIMENTAL

Sources and maintenance of tumor lines[15] and methods of isolation and incubation of cells[11,12] have been described. Briefly, 200-μl portions of 5 per cent cell suspensions, in a buffered-salts medium, were incubated after addition of labeled drug. Incubations were terminated by collection of cells by centrifugation for 30 sec; the extracellular fluid was removed and accumulation of labeled drugs was measured by liquid scintil-

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lation counting of suspensions of cell pellets.[13] In some experiments, the cell pellets were resuspended in fresh medium for varying intervals, then the cells were collected and the drug accumulation was measured. In addition to providing data on drug exodus, this procedure also effectively eliminated contamination of pellets by extracellular radioactivity carried over from the initial incubation. Otherwise, an appropriate correction was made for trapped radioactivity. This procedure involved incubation in medium containing 1 mM labeled sulfate to which these cells are impermeable.[14] The cells were then collected by centrifugation, blotted dry, and resuspended in 0·9% NaCl for determination of total radioactivity associated with the pellet. Under the experimental conditions employed here, 200-μl aliquots of 5 per cent cell suspensions yielded pellets of 10 mg of cells which contained 4 μl of extracellular water.

To measure drug effects on incorporation of uracil or thymidine into nucleic acids, 5 per cent cell suspensions were prepared in a modified Eagle's medium[16] containing 20 per cent horse serum and 35 mM phosphate buffer, pH 7·4. After a 15-min exposure to the alkylating agent at 37°, the cells were collected, resuspended in fresh Eagle's medium, and incubated with 10 μM radioactive uracil or thymidine for 30 min at 37°. The cells were again collected and incorporation of radioactivity into nucleic acids was measured.[16] For control experiments, no drugs were added during the first incubation.

Labeled HN_2 (1,2-^{14}C, 29 mc/mM) was purchased from TracerLab; busulfan 2,3-^{3}H (800 mc/mM) from Schwarz Bioresearch; uracil-2-^{14}C (10 mc/mM) and thymidine-6-^{3}H (1·2 c/mM) from New England Nuclear Corp. Unlabeled HN_2 (Mustargen) was supplied by Merck, Sharpe & Dohme; busulfan (Myleran) by Burroughs, Wellcome & Co. HN_2 solutions were prepared each day and buffered with sodium acetate to pH 4·5; busulfan solutions were prepared in ethanol and stored at −20°. In general, incubation procedures involved addition of 2 μl (total radioactivity, 40,000 cpm) of labeled drugs, adjusted to the desired concentration by addition of carrier, to the 200 μl of cell suspensions.

Counting efficiencies of 60 per cent for ^{14}C and 25 per cent for ^{3}H were usually attained; these were not significantly changed when radioactive cell suspensions rather than cell extracts were counted.

RESULTS AND DISCUSSION

The following data refer to experiments done on suspensions of L1210 cells, although, as discussed below, the data were not significantly altered when other cell lines were employed.

Uptake of HN_2 and busulfan proceeded rapidly, regardless of the incubation temperature; at 0°, equilibrium between medium and cellular drug levels was achieved within 1 min (Figs. 1 and 2). In other studies, we found that varying the external drug level between 10^{-4} M and 10^{-6} M did not alter this result. The major portion of the accumulated busulfan was immediately lost when cells were washed at 0°, but the remainder was not removed. If the initial incubation at 0° were prolonged or if incubation were carried out at 37°, the size of the pool of intracellular radioactivity stable to washing was slightly increased (Fig. 1). In other studies, we found that the latter fraction could not be extracted from cells, even by prolonged washing in medium at 37°, and was precipitated with the cellular material insoluble in cold 5% trichloroacetic acid. These results were not changed when the busulfan level was

varied between 10^{-4} and 10^{-6} M, nor when dinitrophenol (10^{-3} M) or glucose (10^{-2} M) was present during incubation. We interpret these findings to indicate that busulfan readily penetrates L1210 cells, that a portion of the drug is bound to cellular components and the remainder is freely lost upon subsequent washing, and that neither the uptake nor the binding process is particularly temperature-sensitive.

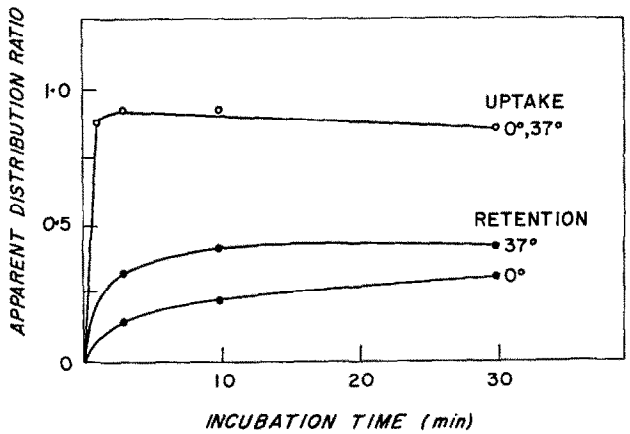

FIG. 1. Accumulation of labeled busulfan by L1210 cells. The upper curve represents drug concentration in diffusible cell pool/drug concentration in medium, after incubation at 0° or at 37°. The lower curves represent drug concentration in nondiffusible cell pool–concentration in medium. The extent of the latter ratio varied with the incubation time and temperature as shown.

Like busulfan, HN_2 was readily taken up by L1210 cells at 0°, leading to a cell–medium distribution ratio of 1 (Fig. 2). The drug accumulated at 0° could readily be washed from the cells.

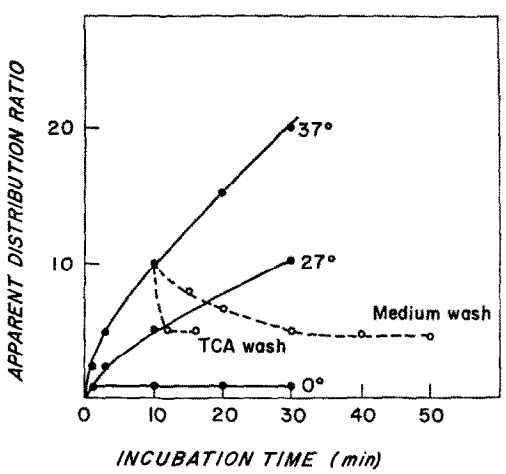

FIG. 2. Accumulation of labeled HN_2 by L1210 cells as a function of time and incubation temperature. The ratio shown is HN_2 concentration in cells–medium. The effects on HN_2 retention when previously loaded cells were washed in fresh medium at 37° or in 5% trichloroacetic acid at 0° are also shown.

At higher temperatures, apparently concentrative uptake of HN_2 was observed (Fig. 2), but this was traced to extensive binding of the drug to cell components. Unlike busulfan, HN_2 was not readily washed from cells. There was, however, a slow loss of a portion of accumulated cellular radioactivity upon washing. The rate of loss ($T_{1/2} = 20$ min) was not affected by the wash temperature. The same amount of radioactivity could be removed from cells either by 20–30 min of washing in fresh medium or by a 5-min treatment with 5% trichloroacetic acid at 0°. Representative data are shown in Fig. 2.

Accumulation of HN_2 at 37° was not affected by dinitrophenol, glucose or cyanide; simultaneous addition of cysteine or the sulfhydryl-reactive agents, *N*-ethylmaleimide and iodoacetate, reduced the extent of drug accumulation (Table 1). Addition of

TABLE 1. EFFECT OF TEST COMPOUNDS ON ACCUMULATION OF HN_2*

Test compound†	Level (mM)	HN_2 distribution ratio (cell/medium)
None		10·3
Cysteine	10	6·0
NEM	1	3·9
DNP	10	11·2
IAA	5	5·6
Glucose	10	11·0
Cyanide	1	10·5

* Cells were incubated with test compounds and 10^{-5} M HN_2 for 10 min at 37°.

† Abbreviations used: NEM, *N*-ethylmaleimide; DNP, 2,4-dinitrophenol; IAA, iodoacetate.

either of the last three agents to the wash medium did not alter the rate of HN_2 loss from previously loaded cells. None of the data reported here was changed when HN_2 levels were varied between 10^{-4} and 10^{-6} M. The usual level employed was 10^{-5} M.

These data show that HN_2 readily enters L1210 cells, even at 0°, and is "fixed" therein by a highly temperature-sensitive process. The latter leads to an apparently concentrative accumulation of HN_2-^{14}C from the medium. Intracellular HN_2 was found in two fractions: one removed by extensive washing, the other stable to washing or to treatment with cold trichloroacetic acid. Whether this represents a distinction between drug bound to different functional groups remains to be established. Under conditions similar to those employed here, HN_2 was shown to alkylate cell proteins extensively, with lesser alkylation of DNA and RNA.[17]

The nature of HN_2 uptake at temperatures much above 0° was masked by the extensive binding of the drug to cell components. Uptake of busulfan was apparently temperature-insensitive. The rapid uptake of these drugs at 0° could argue against a specific transport process, which might be expected to exhibit the high degree of temperature sensitivity characteristic of, for example, amino acid transport. Wolpert and Ruddon[7] have reported data suggestive of an HN_2 transport process in Ehrlich cells, which was impaired in a drug-resistant subline. Other evidence indicating temperature sensitivity of the growth-inhibitory action of HN_2 has also been reported.[18] These findings could reflect the temperature sensitivity of binding of HN_2 rather than of HN_2 uptake. Both HN_2 and busulfan at 10^{-5} M inhibited incorporation

of uracil or thymidine into nucleic acids (Table 2). This experiment appears to rule out the possibility that the drug accumulation found represented only binding to the cell surface.* The experiments described here do not, however, argue against substantial surface binding of these agents.

TABLE 2. EFFECTS OF HN_2 AND BUSULFAN ON INCORPORATION OF URACIL AND THYMIDINE*

Drug	Uracil incorporation (%)	Thymidine incorporation (%)
HN_2	66	50
Busulfan	70	65

* Cells were incubated for 15 min at 37° in modified Eagle's medium containing 10^{-5} M HN_2 or busulfan. Control tubes contained neither drug. The cells were then washed and resuspended in fresh medium containing 10^{-5} M labeled uracil or thymidine for 30 min at 37°. The extent of incorporation of radioactivity into nucleic acids was measured; incorporation in control tubes = 100 per cent.

TABLE 3. DRUG-PROMOTED INCREASE OF LIFE SPAN OF TUMOR-BEARING HOSTS*

		Busulfan		HN_2	
Cell line	Host	Dose†	% ILS‡	Dose†	% ILS‡
L1210	BDF_1	12·5	22	0·25	52
P388	BDF_1	12·5	6	0·25	134
P1534Jj	DBA/2	6·25	0	0·13	18
Dunning/Schmidt	Fischer	25	125	0·25	175

* Animals received 10^6 tumor cells (L1210 = 10^5) by i.p. injection.

† Drug dose (in mg drug/kg animal wt.) was administered intraperitoneally from days 1 to 10 (Dunning–Schmidt = days 1 to 5) starting 24 hr after tumor inoculation.

‡ Mean drug-promoted increase in survival time of host as compared with untreated control animals.

The L1210 cell line used here was relatively insensitive to busulfan and moderately sensitive to HN_2 (Table 3). When we studied other cell lines, described in Table 3, which varied widely in natural sensitivity to these alkylating agents, the results obtained did not differ from those reported here for L1210.

The present data show that methods described here for the study *in vitro* of uptake of HN_2 and busulfan and of drug effects on nucleic acid synthesis cannot be used to predict for drug sensitivity inherent to spontaneous tumors. There is some evidence[7] that such measurements might predict for "acquired" drug resistance.

The present data suggest that HN_2 and busulfan might resemble certain other drugs, e.g. actinomycin D,[14] daunomycin[19] and the phthalanilides,[20] where only measurements of drug disposition *in vivo* were shown to have a correlation with drug sensitivity in different tumor systems.

* In other experiments, we found that treatment with HN_2 or busulfan did not affect transport of uracil or thymidine across cell membranes.

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