OBSERVATIONS ON THE IN VIVO ALKYLATION OF EHRLICH TUMOR CELL DNA BY NITROGEN MUSTARDS

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Ascites tumor cells can be exposed to nitrogen mustards in vitro and the cytotoxic effects can be evaluated in terms of the increase in the life span of mice receiving inoculations of the treated cells as compared to controls receiving untreated inocula. By utilizing isotopically labeled alkylating agents correlations can then be sought between the observed cytotoxicity and the extent of alkylation of the tumor cell constituents. In this communication, we wish to report our observations on the alkylating action and biological effects of two agents, nitrogen mustard (HN2) and chloroquine mustard (CQM).

Methods

Ethyl-C^{14}- labeled HN2 (methyl bis-β-chlorothyl amine) was obtained from Merck, Canada; labeled CQM (7-chloro-4- 1'-methyl(4'-bis-N-(β-chloroethyl)-aminobutyl) -aminoquinoline) was prepared by Wilzbach tritiation (1, 2) of 4,7-dichloroquinoline followed by condensation with 1-N-bis(β-hydroxyethyl) amino-4-aminopentane and conversion to the bis-β-chloroethyl derivative (2).

Ehrlich ascites cells, freed of ascites fluid, were incubated with the labeled agents at 37°C for 60' with mild shaking in a medium composed of one part Locke-Ringers phosphate (pH7.0) and four parts of saline. Following incubation, the cells were separated by centrifugation, and washed free of excess alkylating agent. Aliquots containing 10^7 cells were transferred to 20-g. mice, which were observed up to 90 days for mean survival time, MST. Control experiments showed that incubation in the absence of alkylating agent did not alter the 'virulence' or growth performance of the cells; the MST after such incubations varied from 12 to 18 days as compared to an MST of 15 ± 2 days for freshly-harvested tumor cells.

The tumor cells were fractionated into protein, RNA and DNA fractions by phenol extraction following lysis in a 0.3M Na benzoate, IM NaCl and 1% Na laurylsulfate solution (3). The RNA was precipitated from the aqueous phase with 3M NaCl; the DNA by cold alcohol. Fractions were assayed for radioactivity by liquid scintillation procedures and analyzed for ribose, deoxyribose and U.V. absorption (3).

Results

The pertinent results are summarized in Fig. 1. and 2. Fig. 1 presents the relation between the concentration of alkylating agent used and the average life span of mice receiving an inoculum of $1.0\text{-}1.5 \times 10^7$ treated cells. At each concentration, the values for alkylation of DNA (in μμM./mg.DNA) by the particular agent appear in parentheses above the bar. (Alkylation continues to increase with concentrations above 10^{-5}M, but the DNA becomes more difficult to isolate and purify.) Fig. 2 shows the relation between the extent of alkylation of the DNA and the reduction of the growth potential, which bears a reciprocal relation to the relative increase in MST.

Collateral studies of CQM have shown its *in vivo* binding to be metabolically stable and its *in vitro* binding to be chemically stable (4). While the binding of HN2 is less stable, and metabolism of the C^{14}- ethyl side chain might indirectly label the DNA, this would only serve to increase the contrast between the two agents.

It is clear from Fig. 1 that HN2 is more effective in injuring the Ehrlich tumor cells than CQM; cytotoxic action commences at lower concentrations and increases more rapidly with concentration. It also can be seen that CQM alkylates the DNA more efficiently at each concentration level.

As a result of differences in the alkylation of DNA and the effect on cell viability, a plot of % Extension MST vs. the extent of attack on DNA shows the wide divergence indicated in Fig. 2.

Discussion

The use of the % Extension of MST as the index of cell damage is based on the simple assumption that the increase of the life span of the tumor bearing

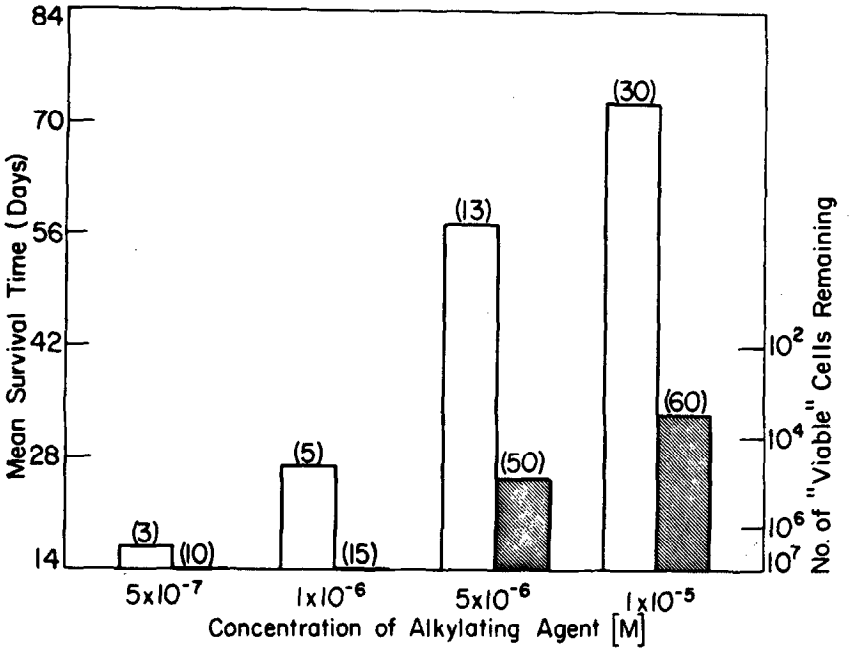

Fig. 1. Clear bar - HN2; cross-hatched bar - CQM. ( ) μμM alkylating agent/mg DNA. MST obtained upon inoculation of $1.0\text{-}1.5x10^7$ treated cells into each of 10 DBA/2XC3H hybrid mice.

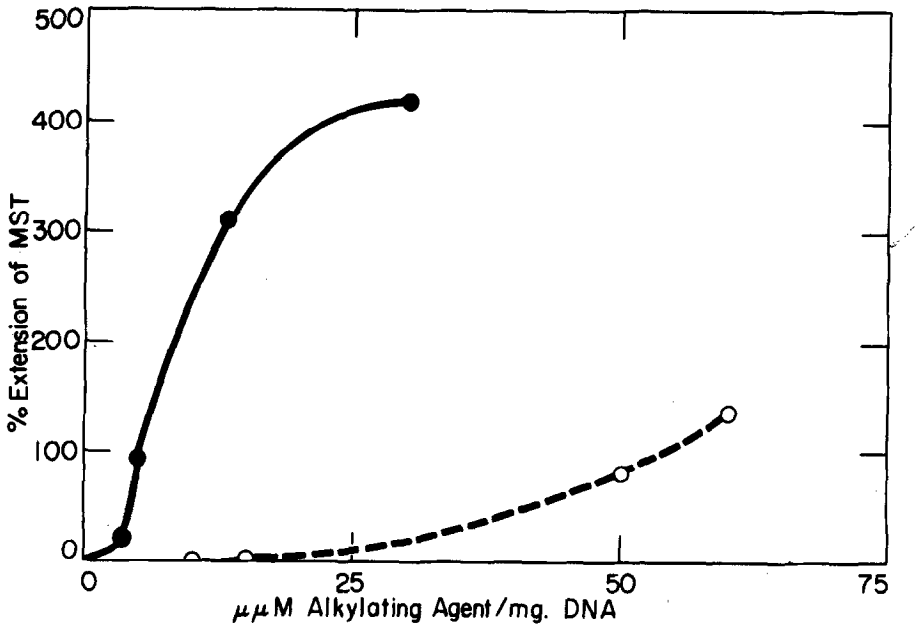

Fig. 2. HN2 - ● ; CQM - O, % Extension relative to incubated but untreated control cells.

host quantitatively reflects the reduction in the number of viable cells caused by the alkylation. It has been shown that there is a straight line semi-logarithmic relationship between the increase in life span of the host and the decrease of the inoculum size in the range from 10^7 down to 10^3 cells, and that a similar relationship holds with respect to the in vitro concentration of alkylating agent in the range $5x10^{-7}$ and $1x10^{-4}$M(3). Thus, the effect of the alkylating agent can be quantitatively related to "sterilization" of a certain % of the cells originally present.

Since the experimental data presented in this communication shows that the gross extent of alkylation of DNA, within limits, bears no direct quantitative relation to cytotoxicity, one might be led to conclude that the sensitive sites related to cell division are to be found elsewhere than in the DNA per se. However, a vast body of biological data argues against the non-involvement of the DNA, so that the more likely inference seems to be that cytotoxicity due to chemical attack on DNA is a function of the kind rather than the extent of alkylation. While there is as yet no direct evidence on this question, our studies also suggest that the extent of intra- or intermolecular crosslinking of the DNA is also not the critical factor, since CQM is far superior to HN2 in this respect (4). We are thus led to the view that differing abilities to produce specific kinds of alkylations under in vivo conditions, resulting from chemical differences in the alkylating agents (4), are responsible for the differences in effectiveness. In this connection, our observations (3, 5) suggest that cross-linking between nucleic acids and proteins is a significant feature of the in vivo process, and it is not impossible that this mixed cross-linking is the more important feature of the anti-tumor and cytotoxic activity of bi-functional agents. An alternative possibility is that there could be marked differences in the selection of target sites such as the phosphate, purine or pyrimidine groups (6, 7) of the DNA by different alkylating agents. Since alkylation of different target sites might not produce equivalent cell damage, the extent of alkylation by different agents would bear no direct relation to cytotoxicity or chemotherapeutic activity.

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