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Concentration and Time-dependent Inter-relationships for Cytotoxicities of Nitrogen Mustard Drugs against Lymphoblasts In-vitro*

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Abstract—Peripheral lymphoblasts were exposed either to different initial concentrations of the alkylating agents (melphalan, chlorambucil or phenylacetic acid mustard) using a fixed incubation time or a constant initial concentration with variable exposure times. The cytotoxic activity was evaluated as the inhibition of [^{3}H]methylthymidine incorporation into the trichloroacetic acid-insoluble fraction of the cells. The concentration-time relationships were evaluated by calculating the amount of drug which had chemically reacted in the incubation system. Melphalan showed lower cytotoxicity at short exposure times and high drug concentrations, while chlorambucil exhibited higher cytotoxicity at longer exposure times. In the latter case the effect could be accounted for by the cytotoxic activity of monohydroxy chlorambucil which was formed in the incubation system.

The cytotoxic effects of alkylating antineoplastic drugs are supposed to be mediated by a complexity of chemical reactions. The formation of DNA-interstrand cross-links seems to play an important role for the cell-killing effect (Kohn et al 1966; Lawley & Brookes 1967; Parsons 1984; Roberts 1975; Ross et al 1978). Moreover, other chemical events such as DNA-intrastrand (Walker 1971), DNA-protein cross-links (Ewig & Kohn 1977; Klatt et al 1969), reaction with cyclic 3′,5′-nucleotide phosphodiesterase (Tisdale & Phillips 1975) and reaction with the plasma membrane (Baxter et al 1982; Grunicke et al 1979) have been suggested to contribute to the cytotoxic activity.

In general, the in-vitro cytotoxic effects of alkylating agents of the nitrogen mustard type have been studied at different initial drug concentrations using a fixed incubation time. Studies on the effect of variable incubation times are more sparse and the results obtained often confusing. Barlogie & Drewinko (1977) examined the cytotoxic effect of melphalan using human lymphoid cells and observed an enhanced cell-killing effect when the cells were exposed to high concentrations of melphalan for a short time (1 h) compared with the use of a low concentration for 48 h. Similar results were obtained by Brody (1979) exposing peripheral lymphocytes to melphalan for 4 and 48 h. The conclusions drawn concerning the effect of concentration-time inter-relationships on the cytotoxicity were based upon the assumption that melphalan was stable for the in-vitro system for at least 48 h. However, this assumption is incorrect since several studies have shown that melphalan is rapidly decomposed in aqueous solution (Ehrsson & Lönroth 1982; Bosanquet 1985; Stout & Riley 1985 and references therein). The low chemical stability of melphalan was taken into account by Parsons (1984) evaluating cell survival as a function of the integrated product (Cxt) of melphalan concentration and treatment time taking into account that melphalan had a half-life of 1 h in the incubation medium used.

Since the alkylating agents exert their pharmacological activity by forming covalent bonds with essential cell constituents, the disappearance of the intact drug by chemical reactions is a prerequisite for the cytotoxic activity. Therefore, it seems more rational to correlate the cytotoxic activity to the amount of drug which has chemically reacted during the incubation conditions used rather than to the integrated product of concentration of the intact drug and time since no pharmacological effect has been exerted by the intact drug in the incubation system. A similar approach has been used by Weinkam & Deen (1982) in their calculation of the concentration of the parent drug that reacted to form active alkylating intermediates during exposure of rat brain tumour cells to chloroethylnitrosoureas.

In the present study human lymphoblasts have been exposed to different initial concentrations of the alkylating agents (melphalan, chlorambucil and phenylacetic acid mustard) using a fixed incubation time or a constant initial concentration with variable exposure times. The cytotoxic activity was evaluated as the inhibition of tritium labelled methylthymidine incorporation into the trichloroacetic acid insoluble fraction of the cells (Hansson et al 1985). The concentration-time inter-relationships were evaluated by calculating the amount of drug which had chemically reacted in the incubation system. By this approach it was possible to obtain an evaluation of the significance of exposure time and concentration on the cytotoxic activity.

Materials and Methods

Lymphocytes were isolated from freshly collected heparinized blood obtained from healthy volunteers by Ficoll-

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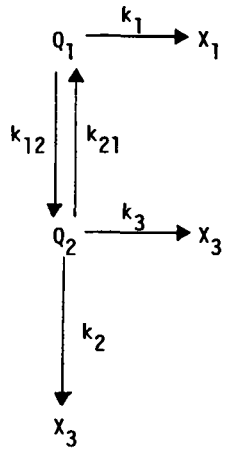
Isopaque centrifugation (Bøyum 1968). The cells (10^6) were cultured in tubes containing 1·0 mL of Parker medium 199 with Hepes buffer supplemented with 10% foetal calf serum (Flow Laboratories, Irvine Ayrshire, UK), 125 IU benzylpenicillin and 125 μg streptomycin. Stimulation with phytohemagglutinin M (Difco laboratories, Detroit, MI) was carried out for 72 h. The cell suspension was centrifuged and the supernatant discarded. The cells were reconstituted in medium (2·00 mL, protein free) and equilibrated at 37·0°C. Chlorambucil (Sigma, St Louis, MO) and phenyl-acetic acid mustard (synthesized by AB Leo, Helsingborg, Sweden) were dissolved in ethanol, and melphalan (The Wellcome Foundation Ltd, London, UK) in the acid-alcohol solvent supplied by the manufacturer. The degradation product of chlorambucil, 4-[p-(2-chloroethyl-2-hydroxy-ethylamino)-phenyl] butyric acid, was prepared according to Ehrsson et al (1980a) and was dissolved in ethanol. The cells were either exposed to different initial drug concentrations using a fixed incubation time (30 min) or varying incubation times (5–100 min) using a fixed drug concentration. Control experiments were performed to establish that the solvents used did not affect the cytotoxic effect observed. The time-concentration studies were always run in parallel using lymphocytes from the same donor. The cytotoxicity was evaluated according to Hansson et al (1985). Briefly after incubation (37·0°C) the cell suspensions were immersed in ice, centrifuged (4°C) and the supernatant was discarded. The cells were resuspended in medium (2·00 mL) and [^{3}H]methyl-thymidine (5 Ci mmole^{-1}, The Radiochemical Centre, Amersham, UK) was added to a final concentration of 10 μCi mL^{-1}. After incubation (2 h, 37·0°C) foetal calf serum (0·1 mL) and trichloroacetic acid were added to give a final concentration of 5%. After two further washings with 5% trichloroacetic acid the precipitate was collected on glass fibre filters and washed 3 times with 70% ethanol. The radioactivity of the precipitate was measured by liquid scintillation (1217 Rackbeta, LKB Wallac, Bromma, Sweden) after the filters had been treated with the solubilizer (Solven, Packard Instrument Company, Inc., Downers Grove, IL). The pseudo-first-order rate constants for the degradation of the alkylating agents were evaluated by following the decomposition of the compounds in the incubation medium by liquid chromatography with photometric detection (Ehrsson & Lönroth 1982; Ehrsson et al 1980a).

Results and Discussion

A thorough understanding of the effect of variations of concentration and exposure time of anticancer drugs on cytotoxicity is of paramount importance for the efficient use of the drugs in clinical settings. These inter-relationships have been addressed in several studies in-vitro using cell culture systems (Barlogie & Drewinko 1977; Brody 1979; Matsushima et al 1985; Rupniak et al 1983). Anticancer drugs of the alkylating type exert their cytotoxic effect by binding covalently to essential cell constituents. Thus, a prerequisite for the pharmacological effect is the disappearance of the intact drug molecule by chemical reactions. An evaluation of the relative importance of exposure time and concentration on the cytotoxic effect can be based on the following simplified model:



where Q_1 and Q_2 are the amounts of extra and intracellular drug, respectively; k_1 is the pseudo first-order rate constant for the elimination of the drug by alkylation reactions outside the cell; k_2 and k_3 the rates of alkylation within the cell. X_1, X_2 and X_3 are the amounts of alkylated products formed. X_3 constitutes the amount which is responsible for the cytotoxic effect observed. Under the assumption that the rate of influx and eflux are rapid as compared to the degradation rate constants i.e. k_{12}, $k_{21} >> k_1$, k_2, k_3 and parallel first-order kinetics are applicable X_3 will constitute a constant fraction of the total alkylated amount i.e.:

$$\frac{X_3}{X_1 + X_2 + X_3} = F \qquad (1)$$

Furthermore,

$$X_1 + X_2 + X_3 = Q_0 - Q_1 - Q_2 \qquad (2)$$

where Q_0 is the initial amount of alkylating agent. In the incubation system used the cell volume only is a minor fraction (< 1%) i.e. $Q_2 << Q_1$. Combination of Eq. 1 and 2 gives:

$$X_3 = F(Q_0 - Q_1) \qquad (3)$$

which can be rewritten as:

$$X_3 = F \cdot Q_0(1 - e^{-k_1 t}) \qquad (4)$$

where t is the exposure time.

As can be seen from formula 4 the amount of drug reacting with the essential target can be varied both by changing Q_0 (using a constant incubation time) and t (using a constant initial amount of drug). The rate constants for the degradation of chlorambucil, phenylacetic acid mustard and melphalan used in the calculations are given in Table 1. Phenylacetic acid mustard was included in this study since it plays an important role in the cytotoxic effects observed in man after

Table 1. Pseudo-first-order rate constants for degradation of nitrogen mustard compounds.

Compound	k, h^{-1} (± s.e., n = 10)
Chlorambucil	1·21 ± 0·01
Phenylacetic acid mustard	0·97 ± 0·02
Melphalan	0·39 ± 0·01

Parker Medium 199; 37·0°C

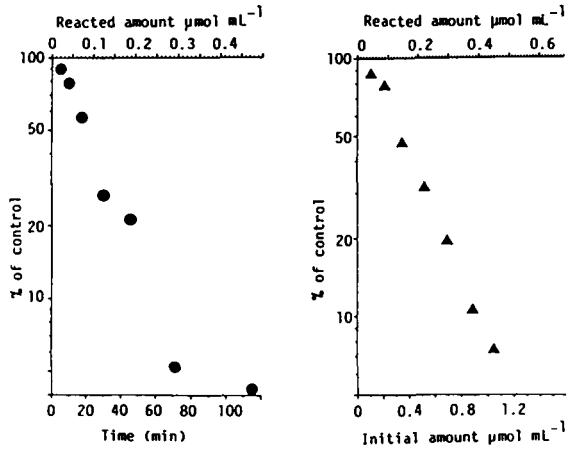


FIG. 1. Influence of initial concentration of chlorambucil (▲) and exposure time (●) on the cytotoxic activity against lymphoblasts. The initial concentration of chlorambucil was varied using an incubation time of 30 min. The effect of exposure time was studied using an initial concentration of 0·527 μmol mL^{-1}. The reacted amount was calculated using the pseudo-first-order rate constant for the degradation of chlorambucil in the incubation medium ($k = 1·21$ h^{-1}). Each value is a mean of 5 determinations.

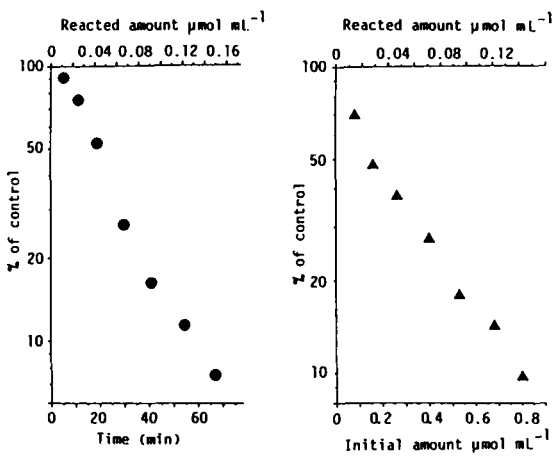


FIG. 3. Influence of initial concentration of melphalan (▲) and exposure time (●) on the cytotoxic activity against lymphoblasts. The initial concentration of melphalan was varied using an incubation time of 30 min. The effect of time was studied using an initial concentration of 0·400 μmol mL^{-1}. The reacted amount was calculated using the pseudo-first-order rate constant for the degradation of melphalan in the incubation medium ($k = 0·39$ h^{-1}). Each value is a mean of 5 determinations.

administration of chlorambucil (Ehrsson et al 1984). As can be seen (Table 1) melphalan is more stable than chlorambucil with the degradation half-lives of 1·78 and 0·57 h, respectively. The incubation studies were carried out in protein-free medium since the presence of albumin significantly decreases the rate of degradation of chlorambucil, phenylacetic acid mustard (Ehrsson et al 1980b) and melphalan (Ehrsson & Lönroth 1982). The presence of the lymphoblasts (5×10^5 mL^{-1}) did not affect the degradation rate of the compounds (data not shown). The concentration and time-dependent interrelationships for chlorambucil cytotoxicity, evaluated as the decrease of incorporation of [^{3}H]methylthymidine into the trichloroacetic acid insoluble fraction in peripheral lymphoblasts (Hansson et al 1985) are given in Fig. 1.

No difference in cytotoxicity was observed when the cells were exposed to a high initial concentration of chlorambucil for times less than 30 min compared with a lower initial concentration for 30 min at equivalent reacted amounts of chlorambucil ($P > 0·1$). However, exposure times of 70 and 115 min gave a significantly ($P < 0·001$) higher cytotoxic activity. Since chlorambucil in aqueous solution decomposes to the corresponding monofunctional alkylating agent 4-[*p*-(2-chloroethyl-2-hydroxyethylamino)-phenyl] butyric acid

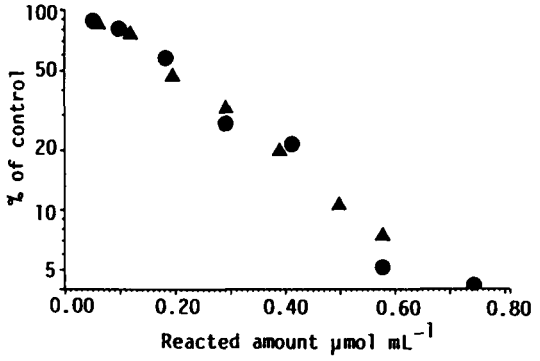


FIG. 2. Influence of total reacted amount of chlorambucil and monohydroxy chlorambucil on cytotoxicity. ▲ Concentration variation. ● Time variation.

(Ehrsson et al 1980a) and calculations based on consecutive first-order rate kinetics using the rate constant for chlorambucil (Table 1) and the monofunctional alkylating agent ($k = 1·38$ h^{-1}, 37·0 °C, Parker medium 199) showed that a considerable amount of the monofunctional alkylating agent had reacted at the longest incubation times studied, the cytotoxic activity of the compound was evaluated separately. The D_0 values (inverse slope of the linear portion of the curve obtained when plotting ln per cent radioactivity of control versus the initial amount of drug using an incubation time of 30 min) for chlorambucil and the corresponding intermediate were $0·37 \pm 0·02$ μmol mL^{-1} (s.e., $n = 18$) and $0·35 \pm 0·03$ μmol mL^{-1} (s.e., $n = 18$), respectively. The contribution of the intermediate was neglected in the calculations of the D_0 value for chlorambucil since only about 10% of the intermediate had reacted after 30 min. A replot of the data in Fig. 1, taking into account the total amount of alkylating agents which had reacted, is given in Fig. 2. The amount of the intermediate that had reacted was calculated from the degradation rate constants considering that 75% of chlorambucil was converted to the monochloromonohydroxy compound as determined by liquid chromatography. The good agreement between the concentration and time curves establishes that the hydrolysis product of chlorambucil contributes to the cytotoxic activity at the longest incubation times. The high cytotoxic activity of the monofunctional alkylating agent seems to be correlated to the method of cytotoxicity evaluation, since in a clono-genetic assay using Chinese hamster V79 cells the activity of the intermediate was < 10 compared with the parent compound chlorambucil. The concentration-time curves for the metabolite of chlorambucil, phenylacetic acid mustard, were similar to those of chlorambucil showing a significantly ($P < 0·001$) higher cytotoxicity at the longest incubation time (90 min). The corresponding monochloromonohydroxy degradation product was not isolated in this case but it is reasonable to assume that it contributes to the cytotoxicity at the longest incubation time due to its structural similarity to chlorambucil.

The concentration and time-dependent inter-relationships for melphalan are given in Fig. 3. Melphalan shows a lower degree of cytotoxicity at incubation times shorter than 30 min ($0{\cdot}001 < P < 0{\cdot}01$) while there was no significant difference at the longest incubation time studied (70 min). The difference between melphalan and chlorambucil with regard to the cytotoxic effect at the short incubation times might be due to the fact that chlorambucil enters the cells by a rapid passive diffusion while the uptake of melphalan is slower, mediated by an active carrier mechanism (Goldenberg & Begleiter 1980). The cytotoxic effect of the monochloromonohydroxy degradation product of melphalan was not evaluated. However, even if we assume that it has similar cytotoxicity to that of the parent compound, it should not contribute significantly to the cytotoxicity at the longest incubation time studied (70 min) since calculations showed that $< 10\%$ of the intermediate had reacted at that time. The principles outlined in the present study should also be applicable to other types of studies of alkylating agents such as the influence of hypo- and hyperthermy on cytotoxicity.

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