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Differential DNA Cross-Linking and Cytotoxicity in PHA-Stimulated Human Lymphocytes Exposed to Melphalan, *m*-L-Sarcolysin and Peptichemio

R. LEWENSOHN,* J. HANSSON,* U. RINGBORG* and H. EHRSSON†

**Radiumhemmet, Karolinska Hospital, S-104 01 Stockholm, and †Karolinska Pharmacy, P.O. Box 60024, S-104 01 Stockholm, Sweden*

Abstract—*DNA interstrand, as well as DNA protein cross-linking, was more efficient in phytohaemagglutinin-stimulated human lymphocytes exposed to* m-L-*sarcolysin as compared to melphalan at equivalent concentrations of the drug. The cellular uptake of* m-L-*sarcolysin was more efficient as compared to melphalan. With peptichemio, a mixture of* 6 *peptides containing* m-L-*sarcolysin, an intermediate level of DNA cross-linking was found. The differences in DNA cross-linking between the* 3 *drugs were parallel to differences in cytotoxicity. Peptichemio has been ascribed anti-metabolic properties in addition to the alkylating properties conferred by its* m-L-*sarcolysin content. In the phytohaemagglutinin-stimulated lymphocytes, however, the effect of peptichemio seems linked to its capacity for DNA cross-linking.*

INTRODUCTION

ALTERATION of the melphalan molecule by shifting the di(2-chloroethyl) amino-group from the para- to the meta-position of the phenylalanine results in *m*-L-sarcolysin. By covalent conjugation of different amino acids at the amino and carboxyl groups of this molecule, a drug complex (peptichemio) consisting of 6 peptides, to which has been ascribed both alkylating and anti-metabolic properties is formed. In several clinical investigations, peptichemio has shown activity in a wide spectrum of human malignancies [1–5]. There is also some evidence from the treatment of human myeloma that peptichemio may show activity in patients that do not respond to melphalan [6].

In previous *in vitro* experiments it was found that lower concentrations of peptichemio and *m*-L-sarcolysin as compared to melphalan were required to induce DNA-repair synthesis, inhibit ^{3}H-uridine incorporation and elicit sister-chromatid exchanges [7]. These differences between the agents might be explained by their abilities to modify cellular DNA. In the present investigation DNA interstrand as well as DNA protein cross-links were measured in human phytohaemagglutinin-stimulated lymphocytes exposed to melphalan, *m*-L-sarcolysin and peptichemio using the alkaline elution technique. The cytotoxic effects of the drugs were evaluated using the Weisenthal dye exclusion technique [8]. Further, the cellular uptake of melphalan and *m*-L-sarcolysin was measured by liquid chromatography.

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Correspondence to: Dr Rolf Lewensohn, Radiumhemmet, Karolinska Hospital, S-104 01, Stockholm, Sweden.

MATERIALS AND METHODS

Chemicals

Melphalan was received as a sterile powder from The Wellcome Foundation Ltd, London, England. Peptichemio (PTC) was provided as a solution containing 80 mg/ml of the peptide preparation equivalent to 32 mg/ml of *m*-L-sarcolysin (*m*-L-SL). *m*-L-SL was obtained as a sterile power. PTC and *m*-L-SL were donated by Istituto Sieroterapico Milanese, S. Belfanti, Milan, Italy.

Preparation of cell suspensions

Lymphocytes were collected from heparinized fresh human blood from healthy donors by differential centrifugation in Ficoll-Isopaque [9]. The lymphocytes were stimulated for 48–72 hr with phytohaemagglutinin M (Difco Laboratories, Detroit, MI, U.S.A.) (PHA) in modified Parker 199 medium with Earle's salts (Flow Laboratories, U.K.) supplemented with 10% foetal calf serum (FCS), 125 IU benzyl-penicillin and 125 μg of streptomycin per ml of medium. The cell concen-

tration was 0.5–1 × 10^6 cells/ml at the beginning of stimulation.

Measurement of DNA cross-links with the alkaline elution technique

In order to study the formation of DNA cross-links by melphalan, *m*-L-sarcolysin and peptichemio in human PHA-stimulated lymphocytes the alkaline elution assay according to Kohn [10] was performed, with minor modifications as previously described [15].

For the study of cross-links in DNA the cells were suspended in Parker 199 medium (0.5 × 10^6 cells/ml) supplemented with 100 IU of benzyl-penicillin and 100 μg of streptomycin per ml and incubated at 37° C for 24 hr in the presence of ^{3}H-methylthymidine 0.1 μCi/ml (5 Ci/mmol, The Radiochemical Center, Amersham, England). The cells were then incubated with different concentrations of the drugs (10^{-6}–10^{-4} M) for 30 min in serum free medium. Parallel controls without drugs were run. The drug incubation was followed by incubation in drug-free medium with 10% FCS for various times. The incubation was interrupted by placing the tubes on ice and the cells were resuspended in cold phosphate-buffered saline and kept on ice. For the analysis of DNA interstrand cross-links the cells were irradiated with 600 R. The cells intended for the study of DNA protein cross-links were irradiated with 3000 R. The radiation was performed in order to introduce random single strand breaks in the DNA allowing the occurrence of DNA cross-linking to be detected as an apparent reduction in the number of the X-ray-induced DNA breaks. The radiation was performed with the tubes placed on ice to prevent repair of the X-ray-induced single strand breaks. The cell suspensions were then collected on polyvinylchloride filters (pore size 2 μm, dia. 25 mm; Millipore Co, Bedford, Mass., U.S.A.) and washed with 10 ml cold phosphate buffered saline.

For the analysis of DNA interstrand cross-links, cells were lysed on the filters with 5 ml sarkosyl–EDTA solution (sarkosyl 20 g/l, 0.02 M EDTA 7.44 g/l, 5M NaOH added to pH 10.0), at room temperature. The lysis solution was allowed to flow through by gravity, after which 2 ml of lysis solution containing proteinase-K (0.5 mg/ml) (Merck, Germany) was added and allowed to remain on the filter for 1 hr. This treatment has been shown to eliminate 97% of cellular protein from the filters [10]. The DNA was then slowly eluted from the filters during 10 hr with a solution consisting of 0.02 M EDTA (acid form), tetraethylammoniumhydroxide (20% solution in water, Merck, Germany) added in the amount required to give a pH of 12.1 and with the addition of 0.1% sarkosyl. Elution was carried out in the dark with a Minipuls 2 peristaltic pump (Gilford Medical Electronics, Villiers–Le Bel, France) for 10 hr at a flow rate of approx. 0.035 ml/min. The eluate was collected for 2-hr periods in 5 separate scintillation vials using an automatic vial changer. The eluate was mixed with 1.4 vol. of Instagel (Packard) for scintillation counting. Radioactivity remaining on the filters was determined by treating the filters with 0.4 ml 1M HCl at 60° C for 1 hr followed by 2.5 ml of 0.4 M NaOH at room temperature after which 4 ml of Instagel were added (Packard Instrument Company, Downers Grove, IL, U.S.A.). Scintillation counting was performed with a Packard liquid scintillation spectrometer.

For the analysis of DNA protein cross-links, cells were lysed as described above. The lysis solution was allowed to flow through by gravity and the filters were washed with 0.02 M EDTA, pH 9.5. No proteinase-K was used. The eluting solution was identical to the one described above but without 0.1% sarkosyl. Elution, collection of eluate, removal of radioactivity from the filters and scintillation counting was carried out as described above.

Data analysis for comparative purposes

The assay with proteinase-K measures the DNA interstrand cross-links, whereas the assay without proteinase-K estimates the DNA protein cross-links. A rational model for the calculation of DNA interstrand cross-links and DNA protein cross-links has been proposed [11]. The model is based on the assumption that cross-linked DNA seems to retard elution rate. More DNA will be retained on the filters representing drug-exposed samples as compared to those not exposed to the drug. Results of different experiments may be compared by relating the elution rates to the irradiated controls and expressing frequencies of DNA interstrand cross-links in R equivalents using the formula:

$$\text{R equivalents} = \left(\sqrt{\frac{1 - r_o}{1 - r}} - 1 \right) \times 600 \text{ R}.$$

r_o and r are the fractions of DNA retained on the filters of the irradiated control- and drug-treated specimens respectively. The assay without proteinase-K measures DNA protein cross-links. By the use of the higher radiation dose determination of the fraction of the DNA bound to protein is permitted and calculation of the DNA protein cross-linking frequencies are given by the formula:

$$\text{R equivalents} = \left(\frac{1}{\sqrt{1 - r}} - \frac{1}{\sqrt{1 - r_o}} \right) \times 3000 \text{ R}$$

where r_o and r are the fractions of DNA retained on the filters in the slowly eluting component [10] for irradiated control- and drug-treated specimens respectively.

Cytotoxicity assay

Drug toxicity was determined by the dye exclusion technique described by Weisenthal *et al.* [8]. In summary, 5×10^5 cells were exposed to drugs for 30 min in serum-free medium. The incubation was then continued for 48 hr in fresh medium supplemented with 10% FCS. Parallel controls without drugs were run. The cells were then concentrated in 0.2 ml after which duck erythrocytes were added followed by the addition of 0.2 ml of 2% fast green dye in 0.15 M NaCl. The suspension was then shaken on a whirl mixer and after 10 min the suspension was sedimented onto microscope slides using a SCA Shandon cytocentrifuge (500 rpm, 10 min). The cells were then counterstained by a modified haematoxylin–eosin technique. 'Living' cells stained pink and 'dead' stained green. The ratio of 'living' cells over duck erythrocytes was determined on each slide and expressed as a percentage of the control.

Determination of cellular concentrations of melphalan and m-L-*sarcolysin*

For each drug concentration tested 7.5×10^6 lymphocytes were phytohaemagglutinin-stimulated. After stimulation the cells were incubated in serum-free medium with various concentrations of melphalan and *m*-L-sarcolysin for 30 min at 37°C. After terminating incubation by placing the cell suspensions on ice, the cell suspension was layered on top of a 0.25 M sucrose gradient and centrifuged 2 min at 3000 rpm. The supernatant (medium) and the cell pellet were collected separately and analyzed for drug concentration by reversed-phase liquid chromatography with fluorometric detection (260/360 nm) after derivatization with *N*-acetylcysteine as described earlier [11]. Briefly, the supernatant and the cells, resuspended in distilled water, were mixed with *N*-acetylcysteine (final concentration about 0.2 M) and the derivatization was carried out at pH 11 for 15 min at 70° C. After acidification and filtration part of the solution was injected into the liquid chromatograph using the same equipment and chromatographic conditions [12]. The capacity factor was 9.6 and 5.4 for the *N*-acetylcysteine derivative of *m*-L-sarcolysin and melphalan respectively.

To correct for possible variations in cell number the drug content of the separate specimens were related to the protein content of the samples, as determined by the method of Lowry *et al.* [13].

RESULTS

Our first objective was to determine whether *m*-L-sarcolysin and peptichemio give rise to DNA cross-links as has been described earlier for melphalan [13, 14]. Based on experiences of the build-up of DNA cross-links induced by melphalan [15] we exposed the cells to drugs for 30 min and then DNA cross-linking was measured immediately and after incubation of the cells for a further 5.5 hr in drug-free medium. The 3 drugs were always analysed in parallel. Results of measurements of DNA interstrand cross-links are presented in Fig. 1. There was an increase in DNA interstrand cross-linking from 30 min to 5.5 hr for all drugs investigated. At 5.5 hr of drug-free incubation significant differences in the levels of DNA cross-linking were observed. Peptichemio induced a significantly higher level of DNA interstrand cross-links compared to melphalan ($P < 0.01$) and *m*-L-sarcolysin was significantly more efficient when compared to peptichemio ($P < 0.02$).

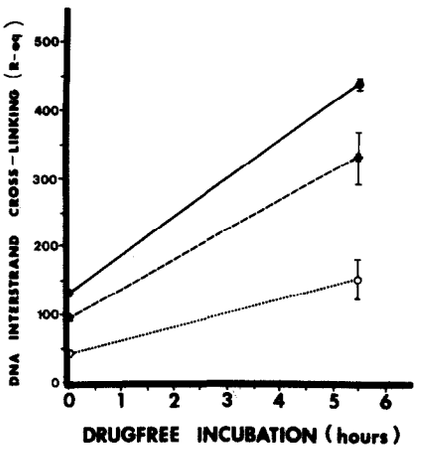

Fig. 1. DNA interstrand cross-linking in human phytohaemagglutinin-stimulated lymphocytes after treatment with 1×10^{-5}M *of melphalan* (O—O), *peptichemio* (*—*) *and* m-L-*sarcolysin* (●—●). *Cells were exposed to the alkylating agent for* 30 *min and analysed for DNA interstrand cross-links immediately or after further* 5.5 hr *incubation in drug-free medium. Mean values ± S.E.M. are plotted. At* 30 min *a single experiment, and at* 6 *hr* 5 *experiments were performed. DNA cross-linking was analysed by the alkaline elution assay.*

DNA protein cross-links were measured in order to find whether the drug-related differences in interstrand cross-linking should be regarded as specific or not. The measurements of DNA protein cross-links were performed at 5.5 hr of drug-free incubation. The results of these experiments are presented in Fig. 2. Again, similar differences were found for the 3 alkylating agents. The lowest level of DNA protein cross-links was obtained for melphalan, which was significantly different from that obtained for peptichemio ($P < 0.01$). A higher level of DNA protein cross-links was induced by *m*-L-SL as compared to PTC. The difference was, however, not statistically significant. When the free cellular concentration of the drugs, as related to protein content of the specimen, was plotted against the drug concentration of the supernatant (Fig. 3), an increase in cellular uptake was noted with increasing concentration of the 2 drugs. As can be seen, there

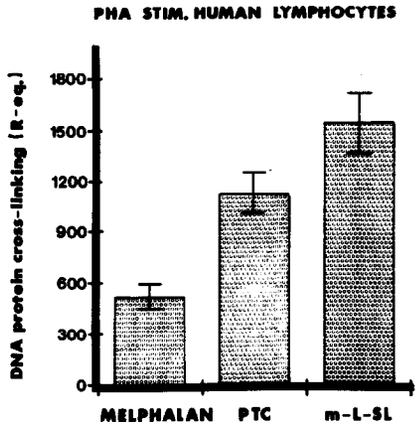

Fig. 2. DNA protein cross-linking in human phytohaemagglutinin-stimulated lymphocytes after treatment with 1×10^{-5}M *of melphalan, peptichemio (PTC) or* m-L-*sarcolysin* (m-L-*SL). Cells were treated with alkylating agents for* 30 min *after which incubation was continued for a further* 5.5 hr. *DNA protein cross-links were estimated by the alkaline elution assay after radiation of the cells with* 3000*R. Mean* ± *S.E. of* 4 *experiments are shown.*

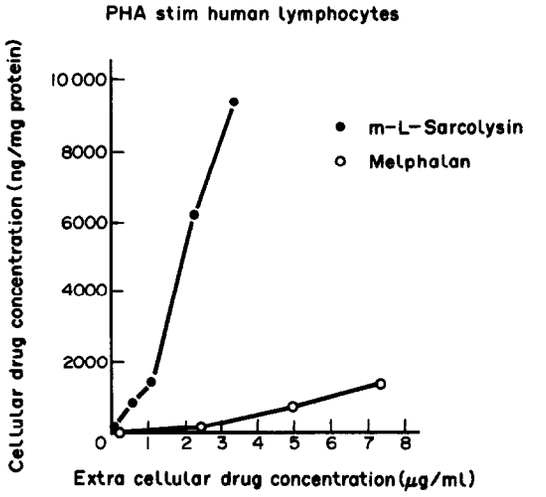

Fig. 3. Intracellular concentration of free melphalan and m-L-*sarcolysin in phytohaemagglutinin-stimulated lymphocytes following exposure to different drug concentrations for* 30 min. *Cellular concentration is expressed as ng/mg protein and related to the extracellular concentration* (ug/ml*medium*).

was a higher intracellular concentration of free *m*-L-SL as compared to melphalan.

For comparison of the cytotoxic effect, equimolar concentrations of the drugs were studied by the dye exclusion technique described by Weisenthal *et al.* [8]. The results of these experiments are presented in Fig. 4. *m*-L-Sarcolysin was found to be most toxic to PHA-stimulated human lymphocytes. In comparison to *m*-L-sarcolysin the concentration of melphalan had to be increased about 5 times to obtain similar toxicity. The toxic effect of peptichemio was intermediate between *m*-L-sarcolysin and melphalan.

It has been suggested that peptichemio has both alkylating and anti-metabolic properties [15, 16]. The relationship between the toxicity and DNA

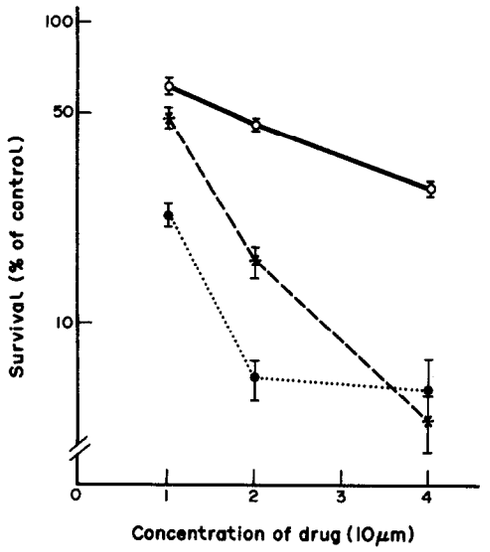

Fig. 4. Cytotoxicity of melphalan (○—○), *peptichemio* (*—*) *and* m-L-*sarcolysin* (●—●) *in phytohaemagglutinin-stimulated lymphocytes as determined by the dye exclusion technique according to Weisenthal* et al. [8]. *Cellular survival calculated as the % of the number of living cells in control samples, is shown as mean values* ± *S.E.M., based on* 4–7 *experiments.*

cross-linking obtained for melphalan, peptichemio and *m*-L-sarcolysin in the PHA-stimulated lymphocytes indicates that the cross-linking of DNA is sufficient to explain the toxicity obtained by peptichemio. This is illustrated by Fig. 5(a), in which DNA interstrand cross-linking and cytotoxicity obtained after treatment with 10^{-5} M of the 3 drugs are plotted. An almost linear relationship between cytotoxicity and DNA interstrand cross-linking was seen for melphalan, peptichemio and *m*-L-sarcolysin. A similar relationship was also obtained when DNA protein cross-linking was plotted against cytotoxicity (Fig. 5b).

DISCUSSION

A shift of the di(2-chloroethyl)amino-group in melphalan from the *para-* to the *meta-* position results in a compound (*m*-L-sarcolysin) which displays a higher antitumoral activity as compared to the *para*-compound (melphalan) when tested on different species of experimental tumors [17]. In clinical practice *m*-L-sarcolysin has been utilized in the multipeptide complex known as peptichemio. The main rationale for synthesizing peptichemio was that of creating a substance which is simultaneously endowed with cytotoxic activity due to the alkylating group and a selective uptake in neoplastic cells. It has also been suggested that the introduction of the amino acid complex on the molecular carrier (L-phenylalanine) in addition to the alkylating effect would give the drug anti-metabolic properties [16–18].

In an earlier investigation on human lymphoblast cells it was found that lower concentrations of *m*-L-

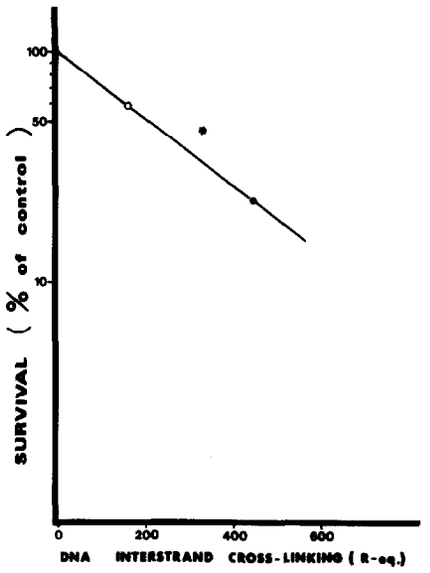

Fig. 5. Relationship between DNA interstrand cross-linking and cytotoxicity in human phytohaemagglutinin-stimulated lymphocytes after treatment with 10^{-5}M *of melphalan* (○), *peptichemio* (*) *and* m-L-*sarcolysin* (●) *for* 30 min. *The DNA interstrand cross-linking was measured with the alkaline elution assay and cytotoxicity with the dye exclusion technique by Weisenthal* et al. [8].

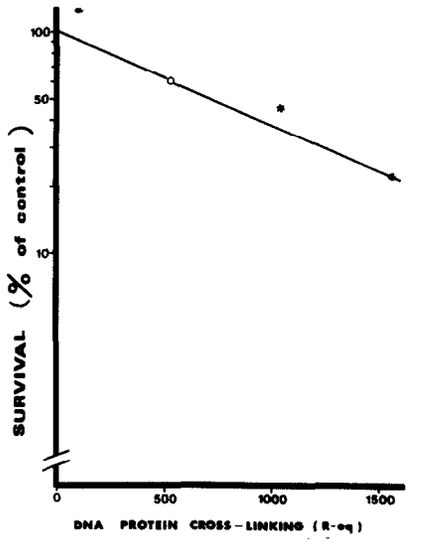

Fig. 5(b). Relationship between DNA protein cross-linking and cytotoxicity in human phytohaemagglutinin stimulated lymphocytes after treatment with 10^{-5}M *of melphalan* (○), *peptichemio* (*) *and* m-L-*sarcolysin* (●) *for* 30 min. *The DNA protein cross-linking was measured with the alkaline elution assay and cytotoxicity with the dye exclusion technique by Weisenthal* et al. [8].

sarcolysin and peptichemio were needed for the induction of DNA-repair synthesis and for the inhibition of ^{3}H-uridine incorporation compared to melphalan [7]. This may indicate a differential modification of cellular DNA by these drugs as compared to melphalan. To find whether these effects could be attributed to a difference in the frequency of DNA lesions, DNA cross-linking was investigated by the alkaline elution technique. The DNA cross-linking effect produced by *m*-L-sarcolysin was significantly higher than that by melphalan. The repeated experiments at 5.5 hr of drug-free incubation at an extracellular concentration of 10^{-5}M of the drugs confirm that the relative DNA cross-linking capacities of the drugs are *m*-L-sarcolysin > peptichemio > melphalan. The similarity in the inter-drug relations regarding both DNA interstrand as well as DNA protein cross-links probably reflects differences in drug concentration at the chromatin target. Measurements of the cellular uptake of melphalan and *m*-L-sarcolysin favours this possibility in the case of non-peptide bound drugs, since there was a much higher uptake of *m*-L-sarcolysin as compared to melphalan. The result is remarkable since the only difference between melphalan and *m*-L-sarcolysin is the position of the di-(2-chloroethyl)amino groups. The finding of an almost linear relationship between cytotoxicity and DNA cross-linking for melphalan, *m*-L-sarcolysin and peptichemio indicates that the cytotoxic effect by peptichemio may be solely explained by its ability to cross-link DNA. When equimolar concentrations of *m*-L-sarcolysin and peptichemio were compared, less DNA interstrand cross-linking was seen for the peptide-bound drug as compared to the free drug. The lower cytotoxic effect of the peptide-bound drug as compared to the free drug was paralleled by a similar difference in DNA cross-linking. This finding favours the possibility that the peptide-bound *m*-L-sarcolysin is less well taken up by the cell as compared to the free drug. The data also put the idea of peptichemio as a drug with alkylating, as well as anti-metabolic, properties in question. At least in PHA-stimulated human lymphocytes the cytotoxic effect may be merely a result of DNA cross-linking.

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