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DNA-Damage in Human Cells Treated with the Closely Related Alkylating Agents Peptichemio, *m*-L-Sarcolysin and Melphalan

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Abstract—In vitro *experiments were performed on human peripheral leukocytes to compare DNA repair synthesis induced by peptichemio (a multipeptide complex containing* m-L-*sarcolysin), m-L-sarcolysin and melphalan.*

The peptide bound m-L-*sarcolysin gave rise to maximum DNA repair synthesis at a concentration interval between* 1×10^{-5} *and* 3×10^{-5} M *as opposed to a concentration interval of* 3×10^{-4} *to* $1 + 10^{-3}$ M *for melphalan. The peptide bound* m-L-*sarcolysin induced DNA repair synthesis at similar molar concentrations to* m-L-*sarcolysin alone. The difference between melphalan on one hand, and* m-L-*sarcolysin and peptichemio on the other hand, may reflect a more rapid uptake of the latter substances. Inhibition of* ^{3}H-*uridine incorporation in human peripheral lymphocytes supports this hypothesis. The frequency of sister chromatid exchanges in human fibroblasts was significantly higher after treatment with* m-L-*sarcolysin compared to melphalan, a finding which also indicates that these chemically closely related substances modify the cellular DNA differently. When DNA repair synthesis was induced by u.v.-light and methyl-methanesulphonate (a monofunctional alkylating agent), no additional DNA repair synthesis was induced, if peptichemio,* m-L-*sarcolysin or melphalan was added. These results indicate that the repair of lesions induced by melphalan peptichemio, u.v.-light and methylmethane-sulphonate is mediated by at least one common controlling enzyme.*

INTRODUCTION

PEPTICHEMIO is a multipeptide complex synthesized by De Barbieri and his coworkers [1]. It consists of 6 peptides conjugated, by means of covalent bounds, at the amino-carboxyl groups of the *m* [di (2-chloroethyl) amino]-L-phenylalanine molecule. The drug possesses both alkylating and antimetabolic properties. Its alkylating action is due to the dichloroethylamino groups of phenylalanine, while its antimetabolic properties are due to the six peptides [1, 2]. Applied in clinical work the drug has exhibited oncolytic effects in a wide spectrum of neoplastic diseases [3–7]. There is some evidence from treatment of myeloma that peptichemio may be effective in patients that do not respond to melphalan or cyclophosphamide [8]. Further, its mode action seems to differ from that of melphalan: For example, it induces a more rapid reduction of the monoclonal immunoglobulin produced by the myeloma cell population, and its side effects on normal bone marrow cells seems to be less pronounced (Merlini, P., personal communication).

Lesions induced by alkylating agents tend to undergo enzymatic repair (for review, see reference [9]). Since peptichemio contains *m*-L-sarcolysin it was considered of interest to know whether its stimulatory effect on DNA repair synthesis was similar to that of *m*-L-sarcolysin alone or to that of melphalan, which has a closely related chemical structure. Peptichemio and *m*-L-sarcolysin were found to induce DNA repair synthesis at a lower molar concentration than when melphalan was used.

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MATERIALS AND METHODS

Chemicals

Peptichemio and *m*-L-sarcolysin were donated by Istituto Sieroterapico Milanese, S. Belfanti, Milan, Italy. Melphalan was received gratis from Wellcome Foundation. Methylmethane-sulphonate was obtained from Merck-Succhard, München, Germany, and hydroxyurea from Sigma Chemical Company, St. Louis, Mo. 63178.

Measurement of DNA repair and RNA synthesis

The buffy coat was isolated from freshly collected heparinized venous blood from healthy volunteers. The cells were washed in phosphate buffered saline, transferred to tubes, pelletted and resuspended in Parker 199 medium (Flow Laboratory, England) at a concentration of about 10^6 cells/ml. The medium was supplemented with 125 μg of streptomycin and 125 i.u. of benzylpenicillin/ml. Hydroxyurea was added to give a final concentration of 10^{-2} M. The drugs were placed in the same tubes at zero time. Hydroxyurea inhibits replicative DNA-synthesis without significantly affectin the DNA repair synthesis [10]. After incubation at 37°C for 30 min, ^{3}H-thymidine (5 Ci/mM, The Radiochemical Centre, Amersham, England) was added to give a final concentration of 10 μCi/ml. The incubation was then continued for 2 hr. For measuring the u.v.-induced DNA repair synthesis, leukocytes were suspended in phosphate buffered saline, placed in Petri dishes and irradiated with u.v.-light (19.2 J/m^2) as described elsewhere [11]. The incubation was then performed as above.

For measurement of ^{3}H-uridine incorporation peripheral lymphocytes were isolated by differential centrifugation in Ficoll–Isopaque [12]. In order to obtain a clean lymphocyte cell population, the cell suspension was incubated in a plastic bottle for the selective adherence of monocytes. The lymphocytes were suspended in Parker 199 medium and incubation conditions were the same as above. ^{3}H-uridine (48 Ci/mM, The Radiochemical Centre, Amersham, England) was added to the medium to give a final concentration of 30 μCi/ml. Incubation times were the same as above.

The incubation was interrupted by immersing the cell suspensions in iced water. Trichloroacetic acid was added to give a final concentration of 5%. After washing twice more with trichloroacetic acid the precipitate was plated on glass fibre filters and washed three times with 70% ethanol. The filters were treated with solubilizer (Soluen, Packard) and the radioactivity was measured by liquid scintillation in a Packard Counter. The background radioactivity was 20 counts/min with an efficiency of about 30%. When the DNA repair synthesis was measured the values obtained were reduced by that of the hydroxyurea insensitive background radioactivity obtained from cells not exposed to the repair inducing agents.

Analysis of sister chromatid exchange (SCE)

Human skin fibroblasts were cultured in the dark for 48 hr in minimal essential medium containing Hepes buffer, 10% of new born calf serum, and 10 μm of bromodeoxyuridine. The medium was then discarded and replaced by medium containing the indicated concentrations of melphalan or *m*-L-sarcolysin. After 1 hr of treatment, the medium was renewed and cultivation was continued for 24 hr. Colcemid was then added and after an additional 4.5 hr the cells were harvested and chromosome preparations were made. The slides were stained with the FPG-technique according to earlier descriptions [13, 14], coded and analysed blindly. Sister chromatid exchanges were recorded only in cells with 46 chromosomes.

RESULTS

Incubation of peripheral leukocytes with melphalan at a range of concentrations, in the presence of hydroxyurea and ^{3}H-thymidine, resulted in a dose dependent incorporation of ^{3}H-thymidine (Figs. 1 and 2a). Maximum incorporation was found at a melphalan concen-

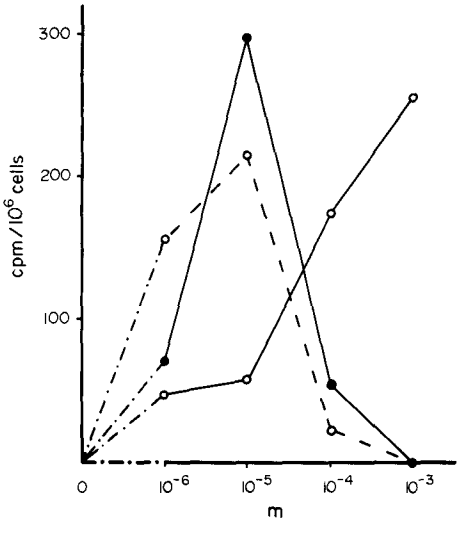

Fig. 1. DNA repair synthesis induced in human peripheral leukocytes by various doses of melphalan (open circles), peptichemio (closed circles) and m-L-*sarcolysin (dotted line). The cells were preincubated with various doses of the repair inducers and* 10^{-2} M *hydroxyurea for* 30 min *after which* ^{3}H-*thymidine was added and incubation continued for a further* 2 hr.

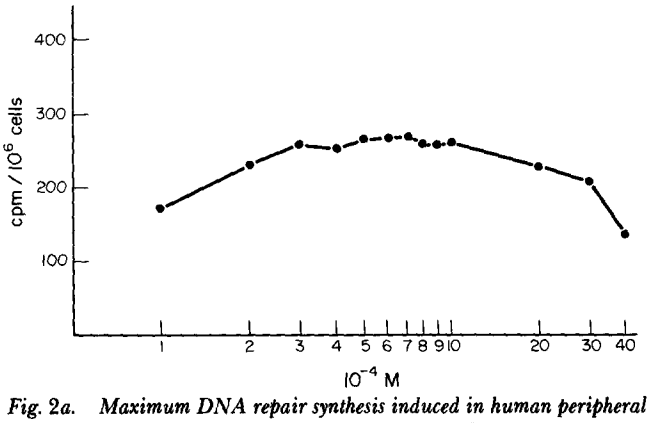

Fig. 2a. Maximum DNA repair synthesis induced in human peripheral leukocytes by melphalan. Registered data are mean values obtained from 3 different experiments. The cells were preincubated with various doses of melphalan and 10^{-2} M hydroxyurea for 30 min after which ^{3}H-thymidine was added and incubation was continued for a further 2 hr.

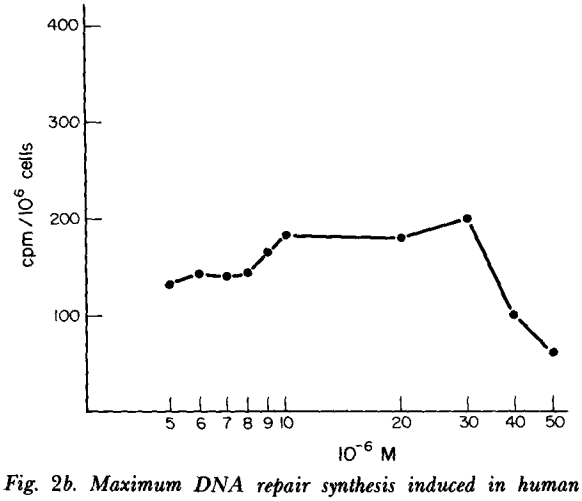

Fig. 2b. Maximum DNA repair synthesis induced in human peripheral leukocytes by peptichemio. Registered data are mean values obtained from 3 different experiments. The cells were preincubated with various doses of peptichemio and 10^{-2} M hydroxyurea for 30 min, and incubation was continued for a further 2 hr.

tration of 3×10^{-4} M, and no further incorporation could be obtained with increasing concentrations up to 1×10^{-3} M. In fact, at higher concentrations a gradual decrease in the incorporation was noted. When the cells were incubated with *m*-L-sarcolysin or petichemio in equimolecular doses, as calculated from the *m*-L-sarcolysin content of peptichemio, a dose dependent uptake of ^{3}H-thymidine was obtained and peak uptake occurred at 1×10^{-5} M (Fig. 1). With increasing concentrations of peptichemio no further increase could be noted, and at concentrations higher than 3×10^{-5} M the labelling of the cells decreased (Fig. 2b). To reach the maximum level of DNA repair synthesis, the concentration of peptide bound *m*-L-sarcolysin was 3% of that needed for melphalan. The difference found in the stimulation of DNA repair synthesis by *m*-L-sarcolysin and peptichemio on one hand and melphalan on the other, may reflect a more rapid uptake of the former substances. When the effect of these substances on RNA synthesis was studied in peripheral lymphocytes it was found that *m*-L-sarcolysin and peptichemio inhibited, more effectively than melphalan, the uptake of ^{3}H-uridine (Fig. 3). Human fibroblasts were treated for 1 hr with two different concentrations of melphalan and *m*-L-sarcolysin. Analysis of sister chromatid exchange (SCE) was carried out 24 hr later, i.e., one cell cycle after the treatment. As shown in Table 1, 10^{-7} M concentration of melphalan did not induce any change in the number of SCE, whereas the same concentration of *m*-L-sarcolysin gave rise to an increase of about 35% in the SCE frequency compared to the control cells. The effect of drug treatment was much more pronounced at the higher concentration. Treatment with 10^{-6} M of melphalan increased

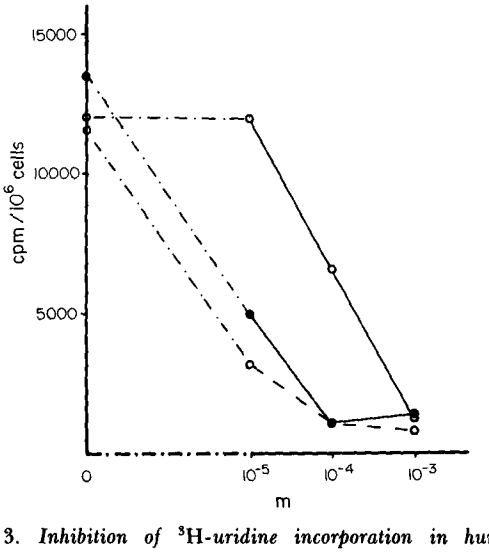

Fig. 3. Inhibition of ^{3}H-uridine incorporation in human peripheral lymphocytes by melphalan (open circles), peptichemio (closed circles) and m-L-sarcolysin (dotted line). The cells were preincubated with various doses of the three repair inducers for 30 min after which ^{3}H-uridine was added and incubation continued for a further 2 hr.

Table 1. Sister chromatid exchange (SCE) in human fibroblasts treated with melphalan and m-L-*sarcolysin (M.V. = mean value; S.D. = standard deviation)*

Compound concentrations (mol/l)		SCE/cell M.V.S.D.	Range	No. of cells
Control	—	9.7 ± 3.2	5–17	28
Melphalan	10^{-7}	7.9 ± 2.4	4–13	20
	10^{-6}	23.8 ± 4.5	12–33	20
m-L-sarcolysin	10^{-7}	13.1 ± 4.6	6–26	20
	10^{-6}	49.6 ± 13.3	33–82	14

the SCE-frequency more than two-fold, and with *m*-L-sarcolysin more than five-fold, compared to the control cells. Thus, again there was a difference in the effect of the two drugs. The effect of *m*-L-sarcolysin was apparent at a lower concentration and was more pronounced at the higher concentration than the effect of melphalan.

A comparison was made of the effect of melphalan and peptichemio on the DNA repair synthesis induced by u.v.-light and methyl-methane-sulphonate, a monofunctional alkylating agent. The u.v.-light and methyl-methane-sulphonate doses known to give rise to maximum DNA repair synthesis in peripheral leukocytes were chosen. Figure 4a shows the results when peripheral leukocytes were irradiated with 19.2 J/m^2 u.v.-light and incubated with different concentrations of peptichemio or melphalan. It was found that

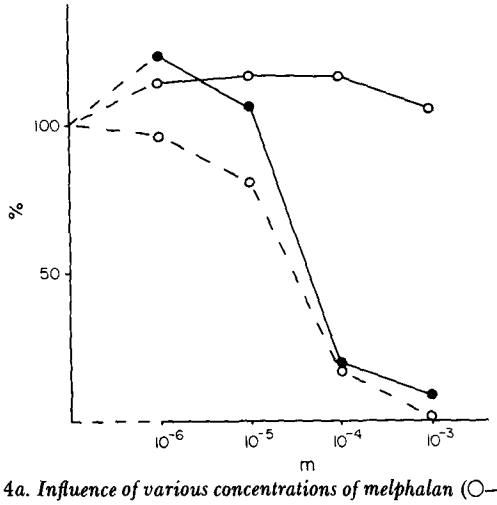

Fig. 4a. Influence of various concentrations of melphalan (O—O), *peptichemio* (●—●) *and* m-L-*sarcolysin* (O---O) *on DNA repair synthesis induced by u.v.-light* (19.2 J/m^2). *The cells were after u.v. irradiation preincubated with hydroxyurea* (10^{-2} M) *and melphalan, peptichemio or* m-L-*sarcolysin for* 30 min, *after which* ^{3}H-*thymidine was added and incubation was continued for a further* 2 hr. *Data is expressed as relative inhibition. The amount of* ^{3}H-*thymidine incorporation after* 19.2 J/m^2 *was* 579 *counts/min/*10^6 *cells.*

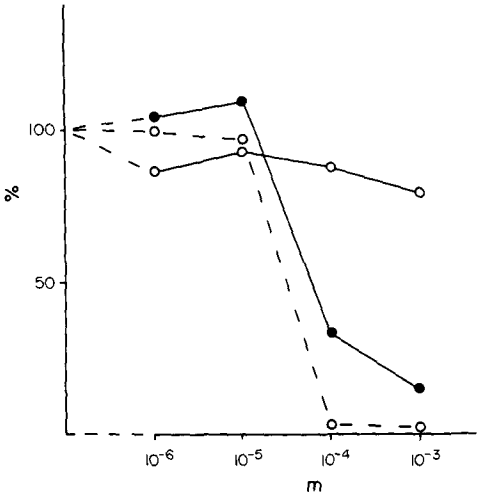

Fig. 4b. Influence of various concentrations of melphalan (O—O), *peptichemio* (●—●) *and* m-L-*sarcolysin* (O---O) *on DNA repair synthesis induced by methyl-methane-sulphonate. Cells were preincubated with methyl-methane-sulphonate* (10^{-3} M) *and hydroxyurea* ((10^{-2} M) *together with melphalan, peptichemio or* m-L-*sarcolysin for* 30 min, *after which* ^{3}H-*thymidine was added and incubation was continued for a further* 2 hr. *Data is expressed as relative inhibition. The amount of* ^{3}H-*thymidine incorporation after treatment with* 10^{-3} M *methyl-methane-sulphonate was* 258 *counts/min/*10^6 *cells.*

neither peptichemio nor melphalan added any DNA repair synthesis in the u.v.-treated leukocytes. A similar result was obtained when peripheral leukocytes were incubated with 10^{-3} M methyl-methane-sulphonate (Fig. 4b).

DISCUSSION

Melphalan, *m*-L-sarcolysin and peptichemio stimulate human peripheral leukocytes to DNA repair synthesis. The fact that the maximum DNA repair synthesis was obtained at an *m*-L-sarcolysin and peptichemio concentration of 1×10^{-5} M as opposed to 3×10^{-4} M for melphalan suggests that the former substances may enter the leukocytes more rapidly than melphalan. The fact that melphalan is less effective than *m*-L-sarcolysin and peptichemio in its ability to inhibit ^{3}H-uridine incorporation supports this hypothesis. Alternatively, *m*-L-sarcolysin and melphalan, although being closely related chemically, may give rise to DNA damage with different biological consequences. It was expected that *m*-L-sarcolysin and peptichemio would differ in the ability to induce DNA repair synthesis and inhibit ^{3}H-uridine incorporation. This was, however, not the case. The similarity between these two substances may indicate that *m*-L-sarcolysin is liberated rapidly from the peptide complex by the cells.

The frequency of SCE has been shown to increase in a dose-dependent way when mammalian cells are treated with mutagens and carcinogens[15]. The differences found be-

ween *m*-L-sarcolysin and melphalan in ability to nduce SCE are consistent with the results on DNA repair synthesis and ^{3}H-uridine incorporation.

It is known from the treatment of myeloma patients that peptichemio differs from melphalan in its oncolytic properties; for example, it seems to produce a more rapid decrease of the monoclonal immunoglobulin produced by the myeloma cells and causes less severe side effects on the normal bone marrow ([8]; Merlini, P, personal communication). One possible explanation of this disparity may thus be a difference in the uptake of peptichemio or released *m*-L-sarcolysin and melphalan by different types of cells.

We did not find any differences between *m*-L-sarcolysin and peptichemio in their ability to induce DNA repair synthesis or to inhibit ^{3}H-uridine incorporation in peripheral lymphocytes. This was not expected since De Barbieri *et al.* [16] have earlier shown that *m*-L-sarcolysin more effectively inhibits DNA synthesis than peptichemio in mitogen transformed lymphocytes. On the other hand, the uptake of substances may vary in different phases of the cell cycle.

It is evident from the experiments that DNA repair synthesis was not increased when using a combination of two repair inducing agents. This suggests that so far as peripheral leukocytes are concerned the repair of lesions induced by melphalan, peptichemio, u.v.-light and methyl-methane-sulphonate is mediated by at least one common, controlling enzyme. The combination of repair inducing agents was not examined systematically in this study; however, in the study of repair damage induced by u.v.-light and *n*-acetoxy-2-acetylaminofluorene, conducted in human fibroblasts, evidence of different rate limiting steps was obtained [17]. The similarity in decrease of the repair synthesis induced by u.v.-light and methyl-methane-sulphonate by high concentrations of peptichemio and *m*-L-sarcolysin while melphalan had low or no effect is consistent with our data indicating a lower level or different type of DNA-damage by melphalan compared to *m*-L-sarcolysin and peptichemio. Once again, we were not able to demonstrate any differences in action between *m*-L-sarcolysin and peptichemio.

To our knowledge no comparison between *m*-L-sarcolysin and peptichemio in clinical studies is available in the literature.

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