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Original Articles

Repair of DNA Interstrand Crosslinks After Busulphan

A Possible Mode of Resistance

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Summary. *The technique of alkaline elution has been used to study the interaction between the antineoplastic drug busulphan and the DNA of cells derived from the transplantable rodent Yoshida sarcoma. A dose-dependent proteinase-resistant filter retention was observed after drug treatment, which indicated the presence of DNA interstrand cross-links. Such cross-links were removed after 6 h in cells resistant to busulphan but not in the busulphan-sensitive parent cells, even after 24 h. Such temporal differences in DNA cross-linking could be correlated with cell survival and also with the level of anaphase chromosome aberrations, which was found to be four-fold higher in the sensitive line than in the resistant line.*

Introduction

Busulphan (1,4-dimethanesulphonyloxybutane, Myleran) is the four-carbon chain member of the homologous series of dialkanesulphonic acid esters with the general formula: $H_3C\text{-}SO_2\text{-}O\text{-}(CH_2)_n\text{-}O\text{-}SO_2\text{-}CH_3$. The use of this agent in the clinical treatment of chronic myeloid leukaemia [11] was originally prompted by the optimal depression of the level of circulating neutrophils in rats and growth inhibition of the Walker carcinoma 256 by this agent when compared with the other members of the series [12]. Despite its being a difunctional alkylating agent [6], there is little or no evidence for *interstrand* cross-linking of DNA following treatment of cells. Chromatographic studies in which 2,3-^{3}H-labelled drug [4] was used directly on isolated DNA revealed an immobile 'spot' of zero Rf, which was attributed to the formation of 1,4-di-(guanin-7-yl) butane but was not confirmed by appropriate markers. The presence of this cross-linked DNA adduct following treatment of DNA directly with busulphan has recently been confirmed by ultrasensitive high-performance liquid chromatography [17], but it was noted that this adduct could be associated with either DNA *intra-* or *inter*strand cross-links, and no distinction was made. Previous physicochemical techniques, such as resistance to denaturation of DNA [15], caesium chloride gradients [18], and salt dissolution studies [1], failed to reveal the presence of DNA interstrand cross-links after busulphan. The combination of the low sensitivity of these techniques and the low reactivity of the drug with DNA [4, 17] may have accounted for the negative finding previously reported.

The more sensitive technique of alkaline elution [14] has been used to describe the interaction between busulphan and the DNA of cells derived from the transplantable rodent Yoshida sarcoma and the findings related to the in vitro cytotoxicity and chromosome aberrations in the anaphase of cell lines resistant and sensitive to the agent.

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Materials and Methods

1. Agents Used. Busulphan was synthesised according to the method of Timmis [16] by the anhydrous reaction of methanesulphonyl chloride with 1,4-butanediol in the presence of pyridine. The drug was purified by twice-recrystallisation from ethanol and its purity verified by melting point and infra-red spectroscopic determinations.

Acetic-orcein stain was obtained from G. T. Gurr, Manchester; Euparal Essence from GBI Labs., Manchester; USA; and ^{14}C-methyl- and 2-^{3}H-thymidine from the Radiochemical Centre, Amersham, GB. Polyvinyl chloride filters were obtained from Millipore Corporation, Mass., USA, sodium lauroylsarkosinate (Sarkosyl) from Geigy, and tetra-propyl ammonium hydroxide from Eastman-Kodak, NY, USA.

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2. Establishment of Cell Lines and Cell Culture. Wild-type, sensitive cells (referred to as YS) were obtained from an ascites form of the solid rodent Yoshida sarcoma as previously described [9] and busulphan-resistant cells (referred to as YR) were grown up from gently dispersed pieces of solid resistant tumour in Fischer's medium (Flow Laboratories) supplemented with 20% horse serum (Gibco, Bio-cult Ltd). Resistant tumours had previously been obtained by transplantation of recurring tumours in female outbred Wistar rats after a dose of 10 mg/kg IP had been given. Cells were grown in the above culture conditions in the presence of antibiotics and glutamine in an atmosphere of 5% CO_2 : 95% air. Cloned cells were routinely screened for mycoplasmal contamination prior to use.

3. Cell Survival Studies. Exponentially growing, mycoplasma-free cells (1×10^5/ml) were treated with various doses of busulphan in dimethyl sulphoxide (DMSO). The level of DMSO never exceeded the previously determined non-toxic level of 2% v/v. Removal of drug-containing medium by centrifugation (200 g; 37° C; 5 min) was followed by suspension in fresh medium. Cell numbers were assayed electronically (Coulter Electronics, Herts.). After adjustment to constant cell density, cells from triplicate treatments were counted on alternate days (for 14 days) and diluted as necessary until parallel outgrowth of both drug-treated and untreated cells were observed. Cell survival was estimated by back-extrapolation of growth curves [2].

4. Estimation of Anaphase Chromosome Aberrations. Cells of each line (100 ml at 1×10^5/ml) were treated for 1 h with busulphan (50 μg/ml), followed by removal of drug-containing medium (200 *g*; 37° C; 5 min) and resuspension in fresh medium for 24 h. For the chromosome preparations, cells were centrifuged (150 *g*; 24° C; 5 min), the supernatant removed and KCl : sodium citrate buffer (0.0375 *M* and 0.034 *M*) added dropwise to give a final volume of 10 ml. After 10 min the cells were spun as before, and fixed by the dropwise addition of methanol : acetic acid (2 ml; 3 : 1 v/v) prior to application on pre-washed ice-cold slides. Acetic orcein staining was followed by destaining and dehydration in 45% acetic acid (5–10 s), absolute alcohol (10 min), fresh absolute alcohol (10 min) and Euparal Essence (30 min). Application of coverslips to euparal oil was followed by drying at 30° C and examination by phase contrast under a Zeiss photomicroscope. Cells (approx. 1,000) were scored for mitotic index and about 100 mitotic divisions were scored for anaphase aberrations, namely multipolar divisions and anaphase bridges with and without fragments.

5. Radioactive Labelling of Cells. Exponentially growing cells were template-labelled with ^{14}C-methyl thymidine (0.05 μCi/ml; 56 mCi/mmole) or 2-^{3}H-thymidine (1 μCi/ml, 4 Ci/mmole) for between 18 and 20 h. Cells were used for alkaline elution without further treatment.

6. Alkaline Elution of DNA. The method used was a modification of that described previously [14]. Briefly, ^{14}C-template-labelled cells were treated with busulphan for 1 h prior to drug removal in the usual manner. Cells were either resuspended in fresh warm medium for post-treatment incubation, or in ice-cold medium for immediate elution assay. Drug-treated ^{14}C-labelled cells (5×10^5) were mixed with ^{3}H-labelled untreated cells (2.5×10^5) at 0° C prior to the introduction of a controlled number of single-strand breaks by means of γ-irradiation over ice (5 grays from a ^{137}Caesium source delivering 0.15 grays/s).

The cells were layered onto pre-washed polyvinyl chloride filters (2.5 cm; 2.0 μ*M*), followed by washing in phosphate-buffered saline (PBS, 10 ml, pH 7.3, 0° C) and lysed in sarkosinate solution (5 ml; Sarkosyl; 0.02% w/v; sodium chloride; 2 *M*; disodium EDTA, 0.04 *M* adjusted to pH 10.00). In assays carried out to determine the extent of DNA-protein cross-linking, proteinase K (0.5 mg/ml, Sigma Chemicals) was included in the lysis solution and the flow stopped for 1 h to allow the enzymic destruction of the cross-links. After lysis the filters were washed with disodium EDTA (10 ml, pH 10.0). All washing and lysis stages were carried out under constant pulse-free suction from an eight-channel peristaltic pump (Gilson Minipuls II) at a flow rate of 1.5 ml/min, which was reduced to 0.05 ml/min for elution with a mixture of ethylene diaminetetraacetic acid, 0.02 *M* and tetrapropylammonium hydroxide (sufficient to give a pH of 12.1–12.2). Fractions of eluted DNA (2.0 ml) were collected and their radioactive content assayed in Aquasol (II) (12.0 ml New England Nuclear containing 0.0125 *M* acetic acid to prevent chemiluminescence) in conditions which would allow the separation of isotopes. The radioactivity was removed from pump-dried filters by incubation with 1 *M* HCl (1 ml, 60%, 1 h) followed by neutralisation with 1 *M* NaOH (1 ml, 21° C, 30 min) and assayed as above. Elution profiles were obtained by plotting the log-percentage ^{14}C activity against the log-percentage ^{3}H levels retained on the filter. The latter counts were used as an internal standard by which ^{14}C counts were normalised to compensate for differences in flow rate encountered between filters. From the elution profiles the percentage of ^{14}C-DNA retained at 50% retention of internal standard was determined and used to calculate the retention in drug-treated cells relative to untreated cells (relative retention) which had received 5 grays only. The number of cross-links per unit DNA were quantitated according to the method of Kohn [13]:

$$Px = [\sqrt{(1 - Ro)/(1 - R)} - 1] \times Pb$$

where Px is the number of cross-links per unit DNA, Ro the fraction of ^{14}C radioactivity remaining on the filter at 50% retention of ^{3}H radioactivity (internal standard) for irradiated-only controls, R, the fraction of ^{14}C radioactivity remaining on the filter at 50% retention of ^{3}H radioactivity (internal standard) for drug-treated and irradiated samples, and Pb the frequency of strand-breaks induced by the assay dose of radiation (i.e., 1.515 per 5 grays for Yoshida cells [5]). The calculation assumes that cross-links are randomly distributed.

Results

Cell Survival

Survival curves for both cell lines are shown in Fig. 1. The doses of busulphan producing 50% cell death were 7.5 and 54.5 μg/ml for YS and YR, respectively.

Chromosome Aberrations

Table 1 shows the mitotic indices and chromosome aberrations after 50 μg busulphan/nl. Drug treatment in both cell lines caused a greater than two-fold depression in the mitotic index after 24 h post-treatment incubation. If the total aberration frequencies for both cell lines were modified for the control aberration frequencies it could be seen that there was a four-fold greater number of aberrations in the sensitive line than in the resistant line.

Table 1. Mitotic indices and anaphase chromosome aberrations in YS and YR cells 24 h after busulphan (50 μg/ml)

Sample	Interphase cells scored	Mitoses	M.I.[a]	Normal anaphase cells scored	Abnormal anaphase	
					B ± F[b]	M[c]
YS control	1,012	33	0.033	69	28	3
YS treated	1,064	13	0.012	32	66	2
YR control	1,019	37	0.036	84	15	1
YR treated	1,007	16	0.016	76	23	1

[a] Mitotic index
[b] Bridges (B), fragments (F) or bridges and fragments
[c] Multipolar anaphase divisions (M)

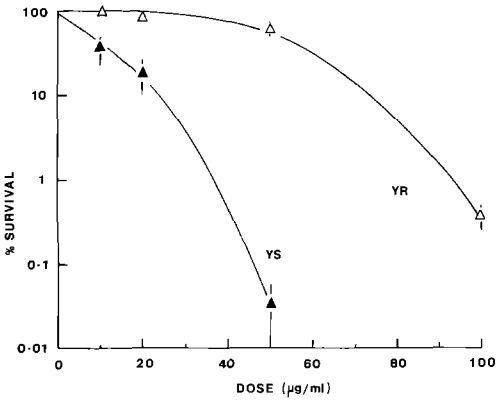

Fig. 1. Cell survival curves of Yoshida cells after busulphan. Treatment was for 1 h at 37° C

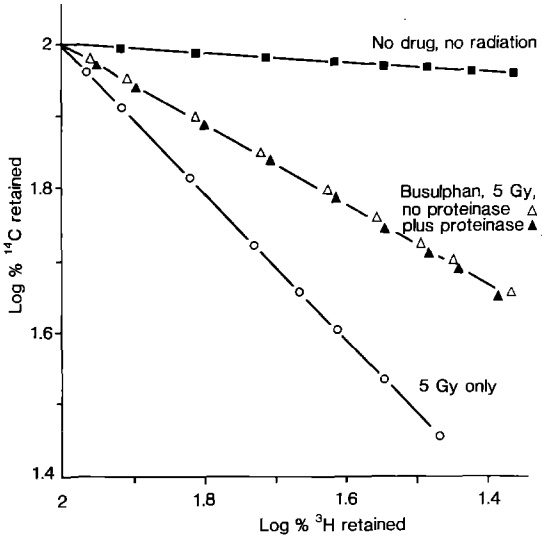

Fig. 2. Elution profiles of YS DNA after busulphan (100 μg/ml). Elution assays were carried out immediately after drug removal. Each sample received 5 Gy of radiation prior to alkaline elution

Table 2. Mean relative retention values for the DNA of YS cells treated with busulphan (100 μg/ml)

Sample	Mean relative retention ± SD
Irradiated control	1.000
Drug + radiation; no proteinase	1.278 ± 0.08
Drug + radiation; + proteinase	1.201 ± 0.16
No drug; no radiation	4.448 ± 1.59

Alkaline Elution Studies

Figure 2 shows typical elution profiles in YS cells after a single 1-h dose of busulphan (100 μg/ml). A small but reproducible proteinase-resistant retention was observed immediately after drug treatment, indicating the presence of DNA *interstrand* cross-links. Table 2 shows the relative retention values (logarithmic fraction of ^{14}C retained for drug-treated cells/logarithmic fraction of ^{14}C retained for control cells) in quadruplicate drug treatments. As the dose of busulphan was increased, an increasing filter retention was observed (Fig. 3), from which the number of cross-links per unit DNA could be assessed (see *Materials and Methods* section). The relationship between drug dose and the frequency of DNA interstrand cross-links is shown in Fig. 4. The appearance of a plateau in the shape of the curve at higher doses may indicate that there are a limited number of sites for cross-linking, or that the higher doses used could be directly denaturing the DNA, thus preventing increased filter retention.

A temporal study of DNA *interstrand* cross-linking after a single dose of busulphan (50 μg/ml; 1 h)

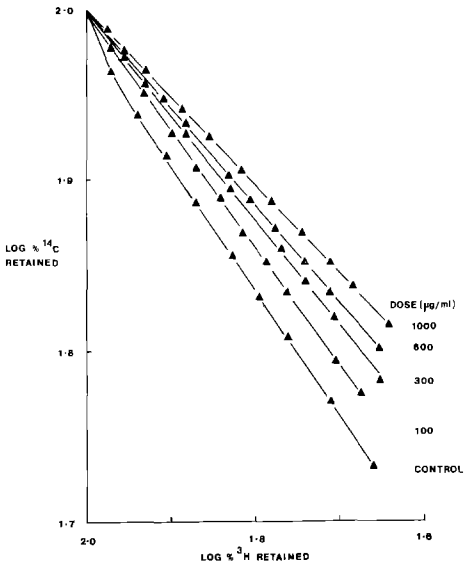

Fig. 3. Elution profiles of YS DNA after increasing doses of busulphan. Assays were carried out immediately after drug removal

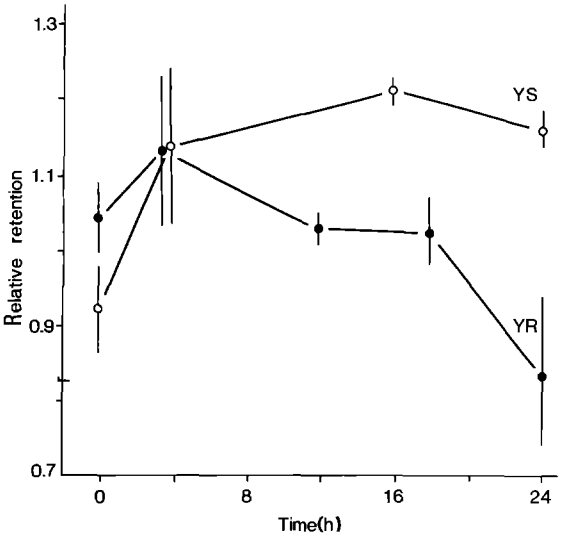

Fig. 5. Time course of DNA interstrand cross-linking in Yoshida cells after a single dose of busulphan (50 μg/ml/1 h). Values are the means ± SD of up to five determinations

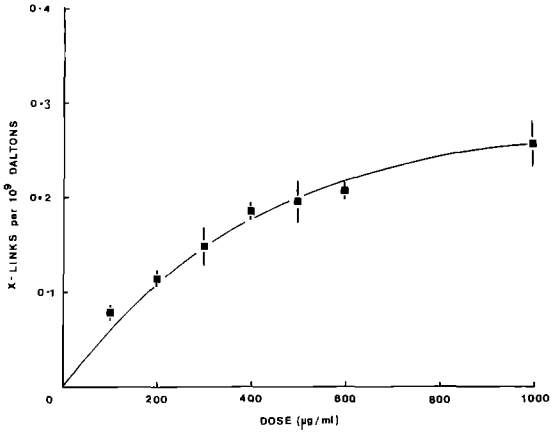

Fig. 4. Relationship between interstrand DNA cross-link frequency and dose of busulphan in YS cells. Assays were carried out immediately after drug removal. Values are the means ± SD of up to five determinations

revealed a gradual increase in cross-linking over the first 4 h, followed by a disappearance over 24 h in the YR line. This was not the case in the sensitive YS line, where there was an increase in cross-linking over 4 h but very little, if any, loss was observed 24 h after drug removal. The time-course of cross-linking in both lines in terms of the relative retention is shown in Fig. 5.

Discussion

The results reported here indicate that the difunctional alkylating agent busulphan is capable of inducing cross-links between adjacent strands of DNA in Yoshida sarcoma cells. It is unlikely that the proteinase-resistant retention demonstrated after busulphan is residual DNA-protein cross-linking that has not been destroyed by proteolytic treatment, since as has been shown elsewhere [3] with the same technique, complete removal of DNA-protein cross-links induced by formaldehyde could be achieved. Previous attempts to verify the presence of DNA *interstrand* cross-links suggested by the chromatographic evidence of Brookes and Lawley [4] may have failed due to the low reactivity of the drug with DNA and the relative insensitivity of the techniques used. Despite the identification of a diguanyl product by HPLC [17] it is difficult to envisage a cross-link between the N7 atoms of guanine on diagonally opposite guanine bases of adjacent DNA strands. The nearest possible proximity of these two nucleophilic centres is 8.0 Å (by models) and the extended structure of the butylene alkylating moiety of busulphan could only span a distance of 6.0 Å. It is possible, therefore, that the diguanyl product isolated by Tong and Ludlam was the result of *intrastrand* cross-links, and that different sites may be involved in the *interstrand* cross-linking demonstrated above.

Evidence is also presented to suggest that interstrand cross-linking may be an important factor determining cytotoxicity, since at a dose allowing the survival of over 50% of the resistant cells cross-links were removed over 24 h, yet at the same dose, which was supralethal to the wild-type sensitive line, cross-link removal did not occur even up to 24 h after treatment. Initial temporal studies with a dose of busulphan producing 50% cell death in the YS line have also shown a similar pattern of cross-linking, with little or no removal after 24 h, suggesting the absence or inefficiency of a DNA interstrand cross-link removal system. Such a lack of cross-link repair has been demonstrated by alkaline elution in L1210 mouse leukaemia cells treated with L-phenylalanine mustard or cis-diamminedichloroplatinum (II) [19] and in normal or transformed human embryo cells after various antitumour nitrosoureas [7]. In both cases, cells exhibiting sensitivity to the agents were unable to remove DNA-DNA interstrand cross-links, whereas comparatively resistant lines could do so over 24 h. The inability to repair DNA cross-links efficiently has also been suggested for cells taken from a patient with Fanconi's anaemia. These cells have been shown to repair interstrand DNA-DNA cross-links at an eight-fold slower rate than in normal human fibroblasts after mitomycin C [10]. This observation was not, however, supported by alkaline elution studies on a similar but not identical line [8], and the authors concluded that the cell lines may have differed and suggested the existence of Fanconi's anaemia complementation groups, similar to those found in Xeroderma pigmentosum cells.

A four-fold increase in anaphase chromosome aberrations was also observed in YS cells when compared with the YR cells. This may be a direct consequence of the ineffective repair of the interstrand cross-links, but the exact mechanism of formation of such aberrations is not known, and especially their relationship to cytotoxicity.

In conclusion, busulphan was found to induce a dose-dependent DNA interstrand cross-linking in cells derived from the transplantable Yoshida lymphosarcoma. Studies involving a comparison between cells sensitive and resistant to the agent revealed a four-fold increase in anaphase chromosome aberrations in the YS line, which may reflect the inability of the wild-type sensitive line to remove the interstrand DNA cross-links which have been shown to occur.

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