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Application of a Sensitive Immunoassay to the Study of DNA Adducts Formed in Peripheral Blood Mononuclear Cells of Patients Undergoing High-dose Melphalan Therapy

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The levels of DNA adducts formed in peripheral blood mononuclear cells of 13 patients undergoing high-dose melphalan therapy were determined 0–24 h after drug administration using a modification of a previously described immunoassay. This assay was validated for DNA extracted from drug-treated cells. Adduct levels in normal mononuclear blood cells 1 h after drug administration correlated well ($r = 0.846$) with drug dose (expressed as mg/m^2) and with area under the curve for plasma levels of melphalan during the first h ($r = 0.842$). 1 patient sustained a high degree of toxic side-effects from the melphalan treatment and showed a high level of adducts. Plasma cell leukaemia tumour cells from another patient showed a level of adducts approximately six times higher than those in the normal blood cells of the other patients. The levels of DNA adducts in normal peripheral blood mononuclear cells did not change markedly between 1 and 24 h after drug administration.
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INTRODUCTION

MELPHALAN IS a widely used anticancer drug [1] and an established human carcinogen [2]. It is generally held that the cytotoxic and anti-tumour effects of melphalan are due to its bifunctional alkylation of genomic DNA.

Melphalan continues to play a major and expanding role in the treatment of certain cancers [3] and chemically related drugs form the basis of new treatment protocols [4], however, very little is known about the pharmacology of these drugs at the molecular level, particularly in patients. The development of a sensitive immunoassay for melphalan–DNA adducts [5] has made it possible to measure the levels of DNA modification induced in patients as a result of therapy with this drug. Melphalan forms a number of mono- and bifunctional adducts on DNA ([6] and Lawley, Osborne and Tilby, unpublished data). The immunoassay appears to detect mainly adducts of melphalan on the N-7 position of guanine and shows the highest affinity for cross-linked nucleotides [6].

We report here melphalan–DNA adduct levels in peripheral blood mononculear cells removed from 13 patients at various times after the intravenous administration of high-dose melphalan and determinations of plasma levels of melphalan.

The three main objectives of this study were firstly, to determine the degree of interpatient variation in DNA adduct formation. Recent studies on cisplatin adducts have indicated large interpatient variation in adduct formation [7] apparently related to response to therapy [8]. Secondly, it was of interest to assess the extent and interpatient variability of DNA repair processs *in vivo*. The third objective was to provide a set of data with which to compare the levels of adducts formed in current *in vitro* studies of the effects of melphalan on clinical samples. We are aware of only one other publication reporting the absolute levels of DNA modification induced in patients by therapy with an alkylating agent [9].

It was shown previously that the immunoassay for DNA–melphalan adducts was many fold more sensitive when the DNA was denatured [5], however, denaturation of the DNA was difficult to achieve without destruction of the adducts which are chemically unstable. We show here that the immunoassay sensitivity is enhanced by enzymatic digestion of the DNA and that the improved assay accurately reflects the levels of DNA adducts formed in cells over a wide range of drug doses.

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MATERIALS AND METHODS

Solutions

Buffer B consisted of 50 mmol/l NaCl, 50 mmol/l sodium phosphate, pH 7.0 and 0.2 mg/l NaN_3. Buffer C was buffer B containing 5 mmol/l $MgCl_2$. Phosphate buffered saline (PBS) was 10 mmol/l Na/K phosphate, pH 7.5, 140 mmol/l NaCl and 0.2 mg/l NaN_3. Acid ethanol was ethanol: 5 mol/l HCl (49:1, v/v).

Alkylation of purified DNA and hydrolysis of DNA with DNAase I

DNA (calf thymus, Sigma) was reacted with radioactive melphalan (ring(n)-^{3}H, 3 GBq/mmol, Amersham) and the levels of alkylation were determined from the amount of radioactivity that became bound to the DNA [5]. To DNA (150 μg/ml in buffer C), DNAase I (Bovine, Sigma) was added (5 Kunitz units/μg DNA) and incubated at 37°C for 60 min. The enzyme was removed by ultrafiltration (10 000 M_r cut-off, Centricon, 5000 g, 4°C) and the ultrafiltrate stored at −20°C.

Treatment of cells with drug in vitro

Cells for *in vitro* drug treatments were a human lymphoblastoid line (Hmy) [10] obtained from Dr M. O'Hare (Institute of Cancer Research, Sutton, Surrey, U.K.) and cultured in Dulbecco's modified Eagle's medium. For treatment with melphalan, cells were washed twice with PBS and resuspended in PBS at 4×10^6/ml. Melphalan was dissolved in acid ethanol at 100 times the final concentration and then diluted into PBS prior to addition to the cells. After incubation for 1 h at 37°C, the cell suspensions were cooled in ice and washed three times with PBS (0°C) by centrifugation. For time course experiments, cells were washed twice with and resuspended in medium (2×10^6/ml) and then incubated (37°C) for the indicated time before washing twice with PBS (0°C). Cells were stored at −20°C.

Patients

The details of the patients studied are given in Table 1. The 11 multiple myeloma patients were treated as part of the evaluation of high-dose melphalan for the therapy of this disease. This study was carried out with the approval of the ethics committee of the Royal Marsden Hospital.

Drug administration

Melphalan (Alkeran, Wellcome) was reconstituted according to the manufacturers instructions and was then injected as soon as possible as a rapid bolus dose into a fast running intravenous infusion via the central line of a subclavian cannula [11]. Frusemide and 2 l of fluid were given during the first 3 h following the injection [12].

Collection of blood and purification of cells for DNA extraction

Blood samples (20–40 ml) were collected into tubes containing heparin (without preservative, final concentration = 10 U/ml). Samples were processed immediately except for three which were removed from patients in the night and kept in ice until the morning. Mononuclear cells were isolated using lymphoprep (Nyegaard) (centrifugation: 700 *g* for 15 min, 20°C), were washed with PBS (0°C) and stored at −20°C.

Table 1. *Patients' characteristics*

Patient number	Disease	[^{51}Cr] EDTA clearance (ml/min/1.73m^2)	Melphalan dose (mg)	(mg/m^2)
1	Myeloma	39	130	70
2	Plasma cell Leukaemia	89	240	140
3	Myeloma	91	240	140
4	Myeloma	96	240	140
5	Myeloma	92	250	140
6	Myeloma	101	266	140
7	Myeloma	84	340	200
8	Myeloma	110	320	200
9	Myeloma	132	430	200
10	Myeloma	101	350	200
11	Myeloma	120	360	200
12	Myeloma	135	420	200
13	Ewing sarcoma	n.a.	n.a.	220

n.a.; Data not available.

Extraction of DNA and DNAase I hydrolysis

DNA was extracted from the cells by a method described previously [13] except that after the final reduction of volume of the DNA solution to 50 μl, the Centricon units were inserted into clean filtrate collection tubes and 400 μl of DNAase I solution (1250 Kunitz units/ml buffer C) was added above each membrane. After incubation (1 h × 37°C), the units were centrifuged to collect all the solution as ultrafiltrate. This was stored at −20°C. DNA concentration was calculated from optical density $(O.D.)_{260}$ using an experimentally determined extinction coefficient for this type of hydrolysate (1 O.D. unit = 37.7 μg/ml DNA).

In order to check that platelets contaminating the cell preparations did not interfere with the assays on lymphocyte DNA, on three occasions cell-free platelets were prepared from blood samples removed 1 or 2 h after melphalan administration. The washed cells were layered over 15 ml of serum (heat inactivated newborn calf serum) and centrifuged (400 *g* for 5 min) to collect cells and the supernatants centrifuged (2000 *g*, 10 min) to collect platelets. These platelets were devoid of detectable levels of DNA and of material reactive in the present immunoassay.

Immunoassay method

The monoclonal antibody was MP5/73 [5, 6]. The ELISA technique was as described previously [5] except that the solution containing the competing antigen and primary antibody contained, in addition to the previously reported components, 10 mmol/l EDTA, to inhibit the activity of any DNAase I in the DNA hydrolysates. The logistic equation was fitted to the data using three parameters: maximum, K and slope. K was the concentration of competitor that reduced the ELISA signal to 50% of the maximum. Slope was essentially independent of the nature of the competing antigen and was usually between 0.8 and 1.5, as described previously [5].

Collection of blood for pharmacological measurements

Blood samples (10 ml) were collected into heparinised tubes immediately prior to and then at 0.09, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8 and 24 h after injection of melphalan. The samples were immediately placed in ice, centrifuged (1800 *g*, 15 min, 4°C) and the plasma samples frozen on solid CO_2 before storage at −40°C.

Determination of plasma levels of melphalan

Plasma melphalan was determined by the HPLC method of Chang *et al.* [14], except that calibration was by external standard curve. The standard curve was on all occasions linear over the range 0.2–10 μg/ml melphalan ($r \geq 0.999$). For a 5 μg/ml melphalan standard the intra-assay coefficient of variation was < 5% and over the duration of the study the interassay coefficient of variation for the same concentration was 2.5%. Pharmacokinetic parameters were calculated using a two-compartment model. The two-compartment model was fitted to the data by non-linear least squares analysis (Graph-Pad, Sandiego, California) using unweighted plasma concentration vs. time data. The correlation coefficient for the fitted equation $C_t = Ae^{-\alpha t} - Be^{-\beta t}$ was > 0.99 for all patients. The area under the plasma concentration vs. time curve (AUC) (0–60 min) was calculated by the equation:

$$AUC_{0-60} = \left(\frac{A}{\alpha} + \frac{B}{\beta}\right) - \left(\frac{C_{60}}{\alpha} + \frac{C_{60}}{\beta}\right),$$

where A, B and α, β are the concentrations and rate constants, respectively, calculated from the biexponential fit and C_{60} is the plasma melphalan concentration 60 min after melphalan administration. The accuracy of this method of calculating the AUC was confirmed by also calculating this parameter by the trapezoidal rule (ratio of $AUC_{trapezoidal}$:$AUC_{integrated}$ = 1.04, coefficient of variation = 9.2%).

RESULTS

Assay sensitivity after DNAase I hydrolysis of alkylated DNA

Purified native DNA was reacted with radioactively labelled melphalan to give levels of alkylation ranging from 200 nmol–24 μmol melphalan/g DNA (7×10^{-5}–8×10^{-3} melphalan residues/DNA base). The immunoreactivities of these preparations were found to be consistent with our previous observations, i.e. K values in the range 700–1000 fmol adduct/assay well [5]. When these preparations were assayed after digestion with DNAase I, the mean K value was 53.0 ± 5.8 (S.E.) fmol/well which was almost as low as that for adducts on denatured DNA (30 fmol/assay well).

The presence of excess DNAase I during these digestions was confirmed by digesting the alkylated DNA with and without the prior addition of a 10-fold excess of control DNA, using the same quantity of enzyme. In other experiments the amount of enzyme per μg of alkylated DNA was increased or decreased by 5-fold. The immunoreactivities of the resulting hydrolysates prepared from DNA alkylated at high or low levels were not significantly affected by these variations.

In order to establish the validity of this assay for DNA extracted from drug-treated biological samples, DNA was extracted from Hmy that had been exposed for 1 h to radioactively labelled melphalan. The level of DNA alkylation, determined by measuring the bound radioactivity, was essentially linearly related to the exposure concentration of melphalan (Fig. 1a). The sensitivity of the immunoassay for melphalan adducts on these DNA preparations (Fig. 1b) is similar to that for samples produced by reacting melphalan with purified DNA and is independent of alkylation level over several orders of magnitude.

Completeness of hydrolysis of DNA extracted from drug-treated cells was confirmed by shortening the digestion time to 15 or 30 min or increasing the DNAase I concentration 5-fold. These variations made no significant difference to the immunoreactivities, the DNA recoveries or specific radioactivities of the final hydrolysates (data not presented). The overall mean assay sensitivity for melphalan adducts was 45.5 ± 2.3 (S.E.) fmol/assay well.

DNA adducts formed in peripheral blood cells

Mononuclear cells were isolated from blood samples taken from patients before and at various times after intravenous administration of a single dose of melphalan. DNA was isolated from these cells and the level of melphalan adducts determined by immunoassay of DNAase I hydrolysates. Typical competitive ELISA data for clinical samples are shown in Fig. 2. As reported previously [5], control DNA cross reacted slightly in this assay. DNA from all samples removed before melphalan administration behaved as expected for control DNA. The levels of DNA adducts in cells removed 1 h after drug administration are shown in Fig. 3. In two cases sufficient blood was obtained to permit the samples to be divided into two portions. The DNA extraction and immunoassay procedures for the duplicated samples were carried out on completely separate occasions. The results indicate a good degree of reproducibility (Fig. 3).

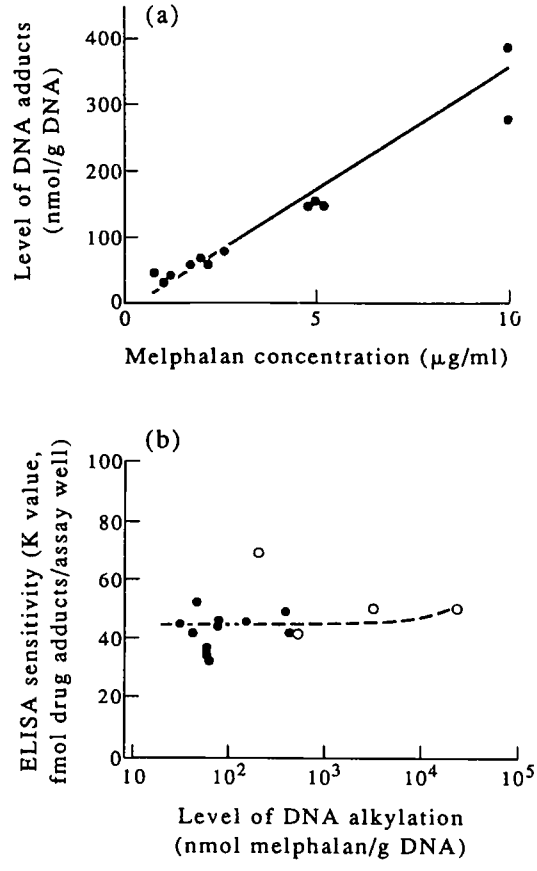


Fig. 1. (a) Dose–response data for formation of melphalan-DNA adducts in human lymphoblastoid line cells. Cells were exposed to [³H]melphalan in phosphate buffered saline for 1 h at 37°C. The levels of DNA alkylation were calculated from the radioactivity that bound to the DNA. Results from three separate experiments. *Line*: linear regression fit, slope =37 (nmol/g)/(μg/ml), correlation coefficient = 0.964. (b) Relationship between ELISA sensitivity for melphalan–DNA adducts and level of DNA alkylation. ●, DNA extracted from drug-treated cells; ○, DNA purified before reaction with melphalan. Line: Linear regression fit of K value on level of alkylation. Slope = 2.5×10^{-4} (fmol/well)/(nmol/g), intercept = 45.0 fmol/assay well.

At each drug dose given to the patients, most samples showed similar levels of alkylation, however, among the patients given 200 mg melphalan/m², one sample (from patient 7) gave a particularly high value. This high level was also seen in a sample removed from the same patient the following day (see below and Fig. 4a). After all the samples had been analysed, it was learnt that this patient had experienced an exceptionally high degree of toxic side-effects from the therapy, including very serious mouth ulceration (WHO grade 4) necessitating extended inpatient care.

Among the patients given 140 mg/m², one was suffering from plasma cell leukaemia and, at the time of treatment, > 90% of the peripheral blood mononuclear cells were tumour cells. The DNA isolated from this sample was, therefore, derived mainly from tumour cells. This DNA exhibited an extremely high level of alkylation (Fig. 3), approximately six times that observed in DNA from the normal mononuclear cells of the other patients who received the same dose of melphalan. Three aliquots of cells from the plasma cell leukaemia patient were frozen at the same time. Two of these were analysed by different DNA preparation and immunoassay methods, both of which indicated levels of alkylation above 80 nmol melphalan/g DNA and therefore

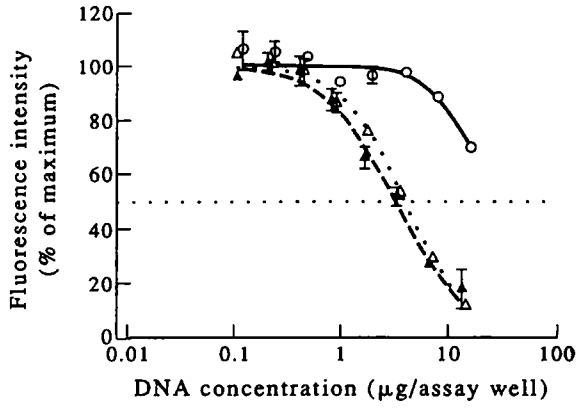


Fig. 2. Typical competitive ELISA data obtained using DNA extracted from peripheral blood mononuclear cells from patient no. 6 who received melphalan at 140 mg/m². — — and ..... represent logistic equations fitted to the data. The fitted parameters are indicated below. ○——○, blood removed before melphalan administration △....△, blood removed 1 h after melphalan administration (*K* value = 3.9 μg DNA/well, slope = −1.5, 11.6 nmol melphalan/g DNA); ▲---▲ blood removed 20 h after melphalan administration (*K* value = 3.3 μg DNA/well, slope = −1.3, 13.5 nmol melphalan/g DNA). Points, mean readings from four assay wells. Bars, standard errors. Standard errors lie within symbols unless shown by bars.

corroborate the very high level of DNA damage observed using the standard methods.

Pharmacokinetic analyses

For 6 patients, plasma concentrations of melphalan were determined at 0.09–24 h after drug administration. In all patients, melphalan pharmacokinetics could be described by a two

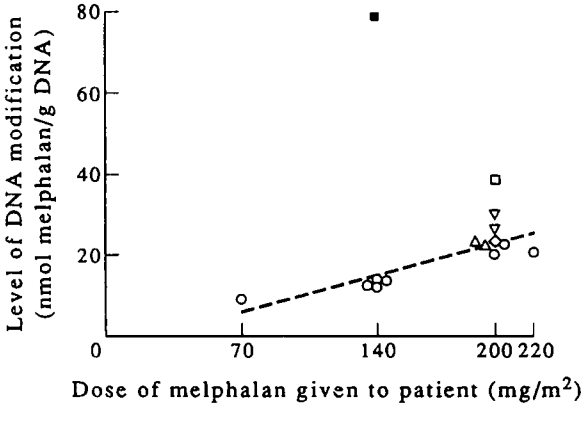
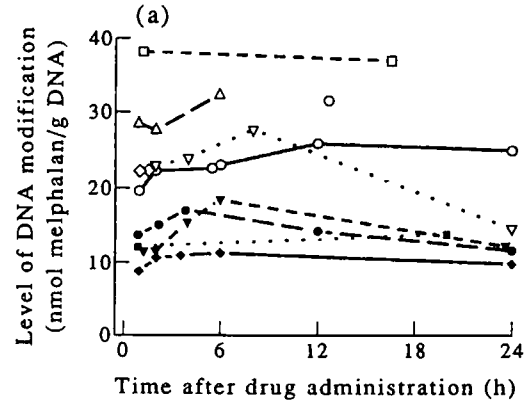


Fig. 3. Levels of DNA adducts formed in peripheral blood mononuclear cells 1 h after intravenous administration of the indicated doses of melphalan. △ and ▽ show results from pairs of replicate samples which were processed on completely separate occasions. Each point or pair of *points* represents a different patient. ○, △, ▽, ◇ and □, normal cells; ■, predominantly plasma cell leukaemia tumour cells (patient 2). ◇, sample removed 2 h after melphalan administration. This data is included because a 1 h sample was not available for this patient and there is very little change in adduct levels between 1 and 2 h (see Fig. 4a). □, This patient (number 7) experienced an unprecedented degree of toxic side-effects from the melphalan treatment. The line shows the linear regression fit to the data, excluding points ■ and □ (slope = 0.126 (nmol/g)/(mg/m²), correlation coefficient = 0.846).



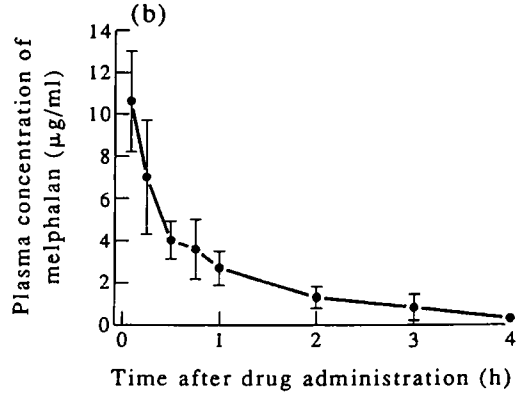


Fig. 4. (a) Levels of DNA adducts in peripheral blood mononuclear cells removed at various times after administration of melphalan for 9 patients. ◆, drug dose = 70 mg/m² (patient 1); ●, ▼ and ■, drug dose = 140 mg/m², (patients 3, 5, 6); □, △, ▽, ◇ and ○, drug dose = 200 mg/m² (patients 7, 8, 9, 10, 12). (b) Mean plasma concentrations of melphalan (± S.D.) during the first 4 h after melphalan administration for the 6 patients of Fig. 5.

compartment open model ($t_{1/2}$ α = 6.7 ± 4.0 min, $t_{1/2}$ β = 57 ± 16 min) and plasma levels were below the limit of detection (0.2 μg/ml) 6 h after drug administration. Areas under the curve (AUC) of concentration vs. time plots were calculated for the first hour for each patient. The correlation between the adduct level at 1 h and 0–60 min AUC is shown in Fig. 5a. The relationship between DNA adduct level and drug dose for this subset of patients is shown in Fig. 5b. For both analyses, a significant linear relationship was observed (P = 0.02, 0.01).

Time course studies

For certain patients, blood samples were obtained at a number of times after drug administration. The results (Fig. 4a) show that the levels of DNA adducts reach almost their maximum value during the first hour. After 24 h the apparent adduct level is usually similar to that observed after 1 h. The plasma levels of melphalan over the initial 4 h period are given in Fig. 4b.

A number of factors could have contributed to the observed changes in melphalan–DNA adduct levels with time (see below). Chemical and biological processes might alter the nature of the adducts on the DNA such that those adducts remaining at extended times might have different overall immmunoreactivities to the adducts formed initially. Therefore, the influence of incubation time after drug treatment on adduct immunoreactivity was investigated by treating Hmy cells with radioactively labelled melphalan, washing off drug after 1 h and then incubat-

ing the cells for various periods of time before extraction of the DNA. Levels of melphalan adducts on these DNA preparations were determined from specific radioactivity and the immunoreactivities of the adducts were determined by competitive ELISA. The results (Fig. 6) show that the overall immunoreactivity of the DNA adducts did not change significantly during this period. The quantity of DNA recovered from the cells was constant for all time points (data not shown). This indicates an absence of overall DNA synthesis or degradation during the period of the experiments.

DISCUSSION

The objectives of the work described in this study were to define interpatient variability in melphalan–DNA adduct formation and repair and to provide *in vivo* clinical data for comparison with *in vitro* results. In addition, as an improvement to the ELISA assay, we have established that the hydrolysis of DNA samples with DNAase I provides a reliable method for improving the immunoreactivity of melphalan–DNA adducts.

Measurements of DNA adduct levels in peripheral blood normal mononuclear cells at 1 h after drug administration showed a generally low degree of interpatient variation and a good correlation with the dose of melphalan administered, expressed as mg/m^2. This contrasts with the marked variation in levels of cisplatin–DNA adducts detected in patients [7, 8]. In the subset of patients for whom pharmacokinetic measurements

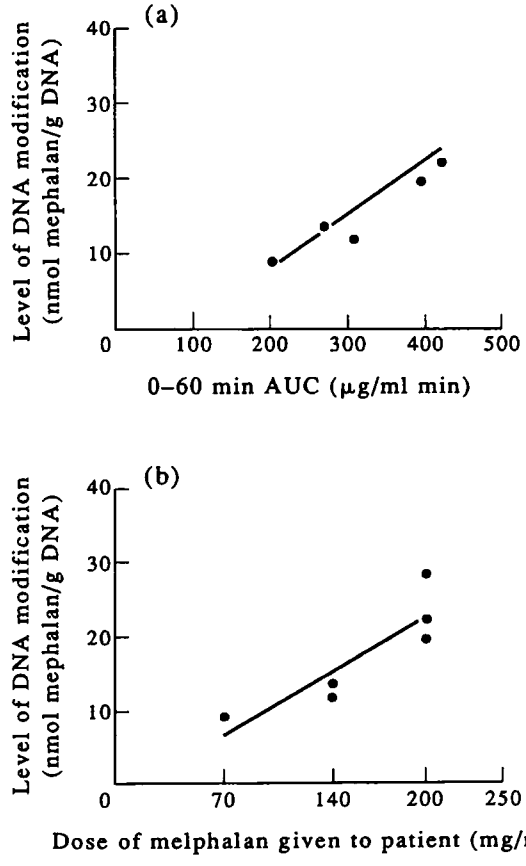


Fig. 5. (a) Relationship between DNA adduct level in peripheral blood mononuclear cells at 1 h after drug administration and melphalan serum 0–60 min area under curve. Correlation coefficient = 0.842. (b) Relationship between DNA adduct level and dose of melphalan administered to the patient for the subset of patients represented in (a). Correlation coefficient = 0.876 (patients 1, 5, 6, 10, 11, 12).

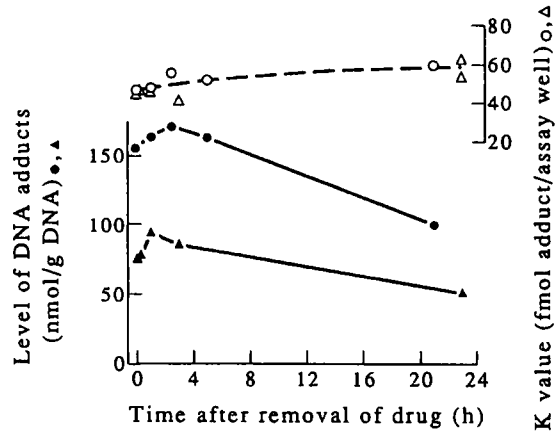


Fig. 6. Effect of incubation time after exposure to melphalan on the level and immunoreactivity of melphalan–DNA adducts in human lymphoblastoid line cells. In separate experiments, cells were treated with [^{3}H]melphalan at 2.6 (△, ▲) or 3.5 (○, ●) μg/ml for 1 h, washed free of drug and then incubated in medium for the indicated times. ▲, ●, levels of DNA alkylation determined from the radioactivity bound to DNA; △, ○, sensitivity of ELISA (*K* value) for the DNA adducts in DNA hydrolysed with DNAase I.

were obtained, the correlation between DNA adduct levels at 1 h and plasma melphalan AUC was similar to that for adduct level and dose to the patient. Although a superior correlation between AUC and adduct level might have been anticipated, the patients were pharmacokinetically a homogeneous group. Hence, the dose of melphalan administered was the major determinant of whole body exposure to active drug.

The levels of adducts observed in this study are 10–40-fold higher than the highest level of procarbazine-induced 7-methylguanine in DNA of peripheral blood leucocytes reported by Mustonen *et al.* [9] and several fold higher than the highest levels of DNA adducts in leucocytes from patients receiving haematologically non-toxic doses of cisplatin [7].

The observation that, in 1 patient (patient no. 7), the level of melphalan–DNA adducts in normal mononuclear cells was markedly higher than in other patients receiving the same or a slightly higher dose of drug appears to be significant, especially since this observation was repeated for a sample removed 20 h after drug administration (Fig. 4a) and since it was learnt, after the immunological analyses of the DNA samples were complete, that this particular patient had experienced a uniquely high degree of toxic side-effects from the melphalan therapy. Unfortunately, blood levels of melphalan were not determined for this patient so we do not know if the elevated level of adducts reflected atypical pharmacokinetics or an unusual susceptibility of the cells to DNA adduct formation. This patient had relatively poor renal function (Table 1) which would tend to increase the plasma melphalan AUC. However, this reduced renal clearance might have had only a limited effect on AUC during the first 60 min period [15].

The observation of a much higher level of DNA modification in the one case (patient no. 2) where primarily tumour cells were analysed needs to be confirmed in further studies. This observation is similar to data from Fichtinger-Schepman *et al.* [7] where levels of DNA adducts induced in patients by platinum-based drugs were higher in tumour cells than in normal peripheral white blood cells.

The invariance of the immunoreactivity of the melphalan adducts in Hmy cells with time was surprising in view of the

possibility of differential chemical stabilities and/or rates of repair of the different products formed when melphalan alkylates DNA [16, 17]. This finding enables us to conclude that the immunologically determined levels of DNA adducts in blood cells of patients several hours after drug administration probably reflect the actual levels of total melphalan adducts present at those times. In patients the levels of adducts declined only slightly during the first 24 h after drug administration. This slight decline could have been due to spontaneous hydrolysis and/or enzyme catalysed repair processes. Dilution of drug exposed cells with newly formed cells seems unlikely to have occurred because of the myelosuppressive effects of melphalan at the doses used here. However, we cannot exclude the possibility that the cell population being analysed is influenced by recruitment from a pool of cells that were protected from exposure to melphalan. Repair of DNA inter-strand cross-links was not apparent in lymphocytes from untreated chronic lymphocytic leukaemia patients following treatment of the cells with melphalan *in vitro* [18]. Attempts to characterise better the adducts recognised by the antibody are in progress.

This study demonstrates that melphalan adduct level monitoring is feasible in the clinical setting and represents a first clinical analysis of the effects of a widely used alkylating-type anticancer drug at the level of its ultimate target of action. This work is currently being extended to studies of drug resistance in adult lymphocytic leukaemia and lymphoma and to a more detailed investigation of the relationship between adduct level and toxicity.

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