Anti-Cancer Drugs, **2**, pp. 565–570

Interaction of melphalan and dexamethasone in a human myeloma cell line

Jan-Olof Fernberg,[CA] Rolf Lewensohn and Sven Skog

J-O Fernberg and R Lewensohn are at the Department of Oncology, Radiumhemmet, Karolinska Hospital, S-104 01 Stockholm, Sweden. S Skog is at the Department of Medical Radiobiology, Karolinska Institute, Stockholm, Sweden.

The effects of a combination of melphalan and dexamethasone on cell growth, cell cycle flow, cell loss and DNA cross-links were studied on a myeloma cell line (RPMI 8226). At low concentrations melphalan reduced the cell growth by prolonging the S and G_2 stages. Steroid sensitivity of the cell line was characterized by dose-dependent *inhibition of cell growth* after exposure of up to 1 μm dexamethasone with no cell loss found even at 10-fold saturation concentration. Dexamethasone induced prolongation of all cell cycle phases without any preferences. In combined treatment with melphalan and dexamethasone, inhibition of cell growth was found after 24 h followed by *cell loss* after 48 h. This cell loss was obtained with concentrations of the drugs which by themselves are only growth inhibitory. Calculation of cell flow showed that cell loss is a delayed process occurring after the cells have left the G_1 phase. By alkaline elution it was found that dexamethasone treatment caused an increase in melphalan-induced DNA interstrand cross-links.

Key words: Cell cycle flow, dexamethasone, DNA damage, human myeloma cells, melphalan.

Introduction

The combination of the alkylating agent melphalan and the steroid prednisone has become standard therapy for multiple myeloma.[1] No multidrug therapy has shown better therapeutic results than this combination.[2] Clinical results with this combination have indicated an advantage of the addition of a steroid to melphalan.[3] It is, however, not known whether this clinical advantage in myeloma is based on a cellular interaction between the steroid and melphalan. Melphalan, being a bifunctional alkylating agent, has been shown to exert its main effect upon DNA.[4] Alkylating agents delay cell cycle progression in all cell cycle phases.[4] For corticosteroids the effect on the cell cycle has been described as an effect on early G_1 cells[5] or the G_1/S transition.[6] The combination of melphalan and a steroid has been shown to potentiate the cytotoxic effect of either drug given alone[7–9] and a synergism has been postulated in some systems.[10] The mechanism of interaction is, however, not clear.

In an attempt to find whether an interaction exists between melphalan and steroids in a human myeloma cell line (RPMI 8226)[11,12] we have analyzed the effects of the drugs on cell cycle flow and DNA cross-linking.

Materials and methods

Cells and cell culture

A well documented human myeloma cell line RPMI 8226[11,12] was used. Cells were maintained in a suspension culture of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 50 μg/ml of penicillin and 50 μg/ml of streptomycin. Cell viability was assessed by trypan blue exclusion.

Drugs

Melphalan (Burroughs Wellcome Foundation, Beckenham, Kent, UK) was obtained as a sterile powder in commercial vials and 100 mg was dissolved in 1 ml of 92% ethanol with 2% HCl and

This work was supported by a grant from 'The King Gustav V Jubilee Fund' (89:526), Stockholm.

[CA] Corresponding Author

then diluted with 9 ml of 60% propylenglycol in sterile water with 1.2% dipotassium hydrogen phosphate. Stock solutions were frozen at −70°C. Immediately before drug incubations melphalan was further diluted in cell culture medium to the desired drug concentration. Dexamethasone (Merck Sharp & Dohme International, Rathway, NJ, USA) was used from commercially available solutions in liquid form.

Cellular DNA content

DNA content of the cell nuclei was measured using a technique described previously.[13] Cells were fixed in 96% ice-cold ethanol. After fixation the cells were washed in Tris-EDTA buffer (0.1 M Tris, 0.07 M NaCl, 0.005 M EDTA, pH 7.5) together with 1 mg/ml of RNase. After pepsin treatment single cell nuclei were obtained and stained with ethidium bromide (25 μM). The DNA content of the cell nuclei was determined using a rapid-flow cyto-fluorometer ICP 11 (Ortho Instruments, West Wood, MA, USA). The output was sorted using a 256-channel analyzer. The number of cells in each of the cell cycle phases was calculated from the areas of the histogram assuming a Gaussian function for the G_1 and $G_2 + M$ maxima and attributing the remaining part of the DNA histogram to the cells of the S phase.

Calculation of cell cycle flow

The method for calculating cell flow through the various cell cycle phases has been described in detail elsewhere.[14] The advantage of this method is that cell cycle kinetics at disturbed cell growth can also be studied. The calculation is based on the total number of cells and the relative number of cells in G_1, S and $G_2 + M$ at different times. In this study we calculated *the outflow of cells* from the different cell cycle stages, related it to the total number of cells in the corresponding compartments and expressed the values as percent per hour (%/h). We also assumed that no resting cells were present.

Alkaline elution

Alkaline elution was used with some modifications according to the technique used by Kohn.[15,16] RPMI 8226 myeloma cells were labeled for 24 h with [^{14}C]thymidine (5 μCi; 61 mCi/mmol) and then exposed to melphalan at different concentrations for 30 min in serum-free medium. After this the cells were incubated in medium supplemented with 10% FCS ± dexamethasone for 24 h. The cells were then resuspended in ice-cold medium with 4% FCS and irradiated with 600 R with a Siemens Stabilopan ortovoltage X-ray apparatus (140 kV, 20 mA, 4 mm Al), collected on polyvinyl chloride filters (pore size 2 μm, diameter 25 mm; Millipore Corp. Bedford, MA, USA), washed with 10 ml ice-cold phosphate buffered saline (PBS) and lysed with 5 ml sarcosyl-EDTA solution (sarcosyl 20 g/l, 0.02 M EDTA, 5 M NaOH added to pH 9.5), which was allowed to flow through the filter by gravity. The DNA was then slowly eluted from the filters with a tetraethylammoniumhydroxide–EDTA solution [0.02 M EDTA (acid form), 5.845 g/l tetra-ethylammoniumhydroxide added to pH 12.1, for the analysis of DNA interstrand cross-links 0.1% sarcosyl was added] at a flow rate of 2.1 ml/h using a Minipuls 2 peristaltic pump (Gilford Medical Electronics, Villers-le Bel, France). For the analysis of DNA interstrand cross-links, protein was removed from the filter by adding another 2 ml of lysis solution containing 0.5 mg/ml of proteinase K to the filter and allowing it to remain in contact with the filters for 1 h. The lysis solution was removed by washing the filters with 5 ml 0.02 M EDTA (acid form), pH 9.5. The elution was continued for 16 h and the eluted DNA was collected in eight fractions. The ^{14}C activity in each fraction was analyzed by liquid scintillation counting after adding 1.33 volumes of Instagel (Packard Instrument Co., Downers Grove, IL, USA) to each sample. DNA remaining on the filters at the end of the elution was removed by hydrolysis in 1 M HCl at 60°C for 1 h and vigorous mechanical agitation. The DNA remaining in the funnels, filter holders and tubes was removed by pumping 2.5 ml of 0.4 M NaOH at room temperature through the system, and the ^{14}C activity thus obtained was added to the activity released from the filters.

The following formula was used to calculate the amount of DNA cross-links as described by Kohn *et al.*[16]

$$CF = \sqrt{\frac{1 - r_0}{1 - r}} - 1$$

where CF is the cross-linking factor, r_0 is the fraction of DNA remaining on the filter at the end of elution in irradiated control cells and r is the fraction of DNA remaining on the filter in melphalan-exposed, irradiated cells. To express the frequency of cross-links in R-equivalents the CF is

multiplied by the radiation dose used (600 R). The amounts of DNA–protein cross-links after melphalan exposure was calculated by subtracting the amounts of DNA interstrand cross-links from the total amounts of cross-links obtained without proteinase K.

Results

Effects of dexamethasone on cell growth

Continuous treatment with dexamethasone up to 72 h resulted in a cell growth delay of 24 h, followed by regrowth up to 48 h and then a further delay at concentrations of 1 μM or more (Figure 1). An increase in concentration of the drug up to 10 μM gave no additional effect on cell growth. No specific changes in the cell cycle composition were seen at any time after dexamethasone treatment (Figure 2); however, small changes as an increase of cells in early G_1/G_0 could not be estimated from the DNA histograms. The cell growth delay should be interpreted as a result of a general decrease in cell flow through the various phases of the cell cycle.

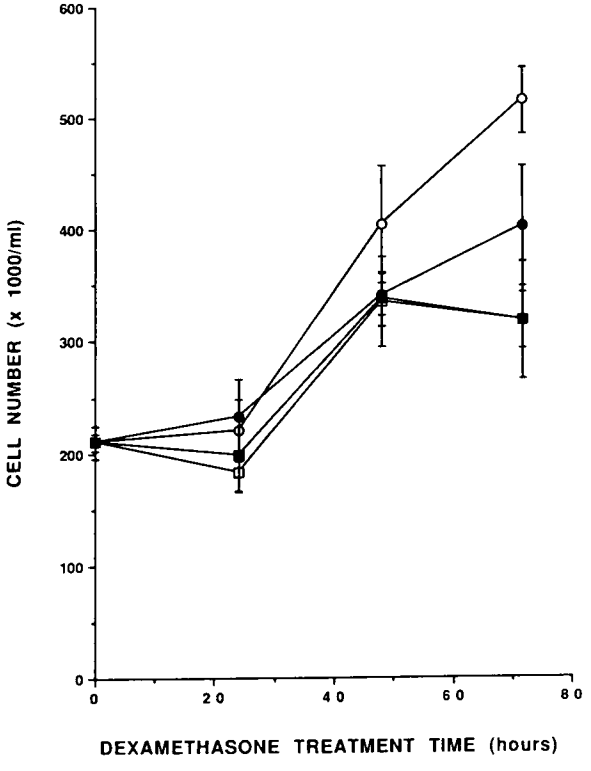

Figure 1. The growth of RPMI 8226 cells following a continuous incubation with dexamethasone. Number of untreated (○), and dexamethasone treated cells at 0.1 μM (●), 1 μM (□) and 10 μM (■). Data points are mean of triplicate determinations.

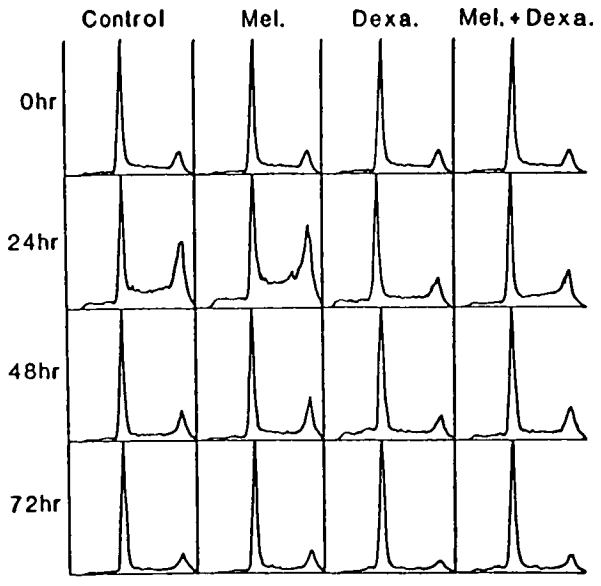

Figure 2. DNA histograms of RPMI 8226 cells at different time points after treatment with 5 μM of melphalan and 1 μM of dexamethasone, respectively, and in combination. Analyses were performed on cells pooled from triplicate specimens from the experiment shown in Figure 3. The first peak represents the G_1 phase. The second peak represents the G_2/M phase and cells in between are attributed to the S phase.

Effect of combined treatment with melphalan and dexamethasone on cell growth and cell cycle flow

A combination of a 30 min pulse treatment of cells with melphalan, followed by continuous treatment up to 72 h with dexamethasone resulted in a lower number of cells as compared with dexamethasone treatment (Figure 3). This effect was statistically significant at 72 h ($p = 0.001$). The early characteristic cell cycle effects seen after exposure to melphalan alone (accumulation in S and G_2 phase) were not found. We interpret this as a result of a general dexamethasone-induced inhibition of the cellflow through all cell cycle stages (Figure 2).

Calculation of cell outflow from the various cell cycle phases up to 24 h post-treatment shows that cells located in G_1 at the time of melphalan treatment in dexamethasone-exposed cells were found in S and G_2/M 24 h later (Table 1). Cells located in S and G_2/M at the time of treatment pass through mitosis during the first 24 h post-treatment resulting in an increase of G_1 cells (Table 1). We

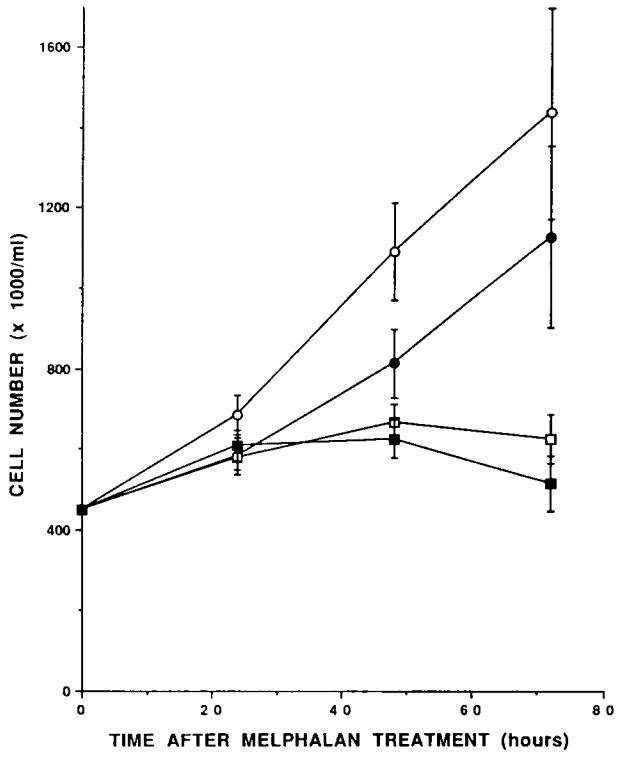

Figure 3. Growth of RPMI 8226 cells over time after drug exposure. Number of untreated (○), and cells treated with 5 μM of melphalan (●), 1 μM of dexamethasone (□) and both drugs in combination (■). Data points are means of five experiments ± SEM. Melphalan was given in a 30 min time pulse and dexamethasone was present during the entire observation period.

Table 1. Number of cells ($\times 10^3$/ml) in the various cell cycle phases and cell outflow up to 24 h after combined treatment with 5 μM melphalan and 1 μM dexamethasone[a]

Time (h)	G_1 phase	S phase	G_2/M phase	Total cell number
0	238	153	59	450
24	318	204	78	600
	+80→	+51→	+19→	+150→
Cell outflow/24 h:	220	169	150	300

[a] The cell flow between different cell cycle phases in the time interval 0–24 h is given below. The total cell number increases by 150 ($\times 10^3$/ml) cells which if no cell death was present would mean that 150 ($\times 10^3$/ml) cells have passed mitosis giving 300 ($\times 10^3$/ml) new G_1 cells. The number of G_1 cells is only increased by 80 ($\times 10^3$/ml) which means that 220 ($\times 10^3$/ml) cells have passed into S. The outflow from S and G_2/M phases can be calculated to be 169 ($\times 10^3$/ml) and 150 ($\times 10^3$/ml) cells, respectively, in the same way. Of the 220 ($\times 10^3$/ml) G_1 cells which have passed into S, 70 ($\times 10^3$/ml) are still in S and G_2/M at 24 h. It is also possible to conclude that these cells in S and G_2/M were in G_1 24 h earlier when treated with melphalan, since about 90% of these melphalan treated G_1 cells have passed into S and G_2/M.

suggest that most of the cells lost between 48 and 72 h were damaged in G_1 at the time of treatment. The elevated cell loss seen at 72 h in the combined treatment was from S and G_2 phase cells lost before mitosis and/or lost as G_1 cells after mitosis (Table 2). The cell loss was 20–40% depending on if the cells died before or after mitosis, respectively. Values of cell death were calculated from Table 2 where the reduction in cell number between 48 and 72 h is 20%. If cell death occurred after mitosis the number of dead cells would have been increased by 100% giving a cell loss of 40%. A further incubation up to 96 h showed no additional cell loss (data not shown).

DNA cross-linking after combined treatment with melphalan and dexamethasone

Interstrand and DNA–protein cross-links were determined by the alkaline elution technique. There was an increase of melphalan-induced interstrand DNA cross-links by the addition of dexamethasone at a time point after maximum build up of cross-links, i.e. 24 h post-melphalan treatment (Figure 4a). The amount of DNA–protein cross-links was not increased in the presence of dexamethasone at the time point measured (Figure 4b).

Discussion

Melphalan gives rise to DNA cross-linking and cell cycle progression delay. Several investigators have combined steroids with an alkylator but it is not known whether the addition of a corticosteroid like dexamethasone may interact with these melphalan-induced effects.

We have analyzed the influence of dexamethasone on cell cycle progression and DNA damage in the dexamethasone-sensitive RPMI 8226 cell line.[17,18]

Table 2. Number of cells ($\times 10^3$/ml) in the various cell cycle phases 48–72 h after combined treatment with 5 μM melphalan and 1 μM dexamethasone

Time (h)	G_1 phase	S phase	G_2/M phase	Total cell number
48	261	213	132	606
72	293	129	81	503
	+32	−84	−51	−103

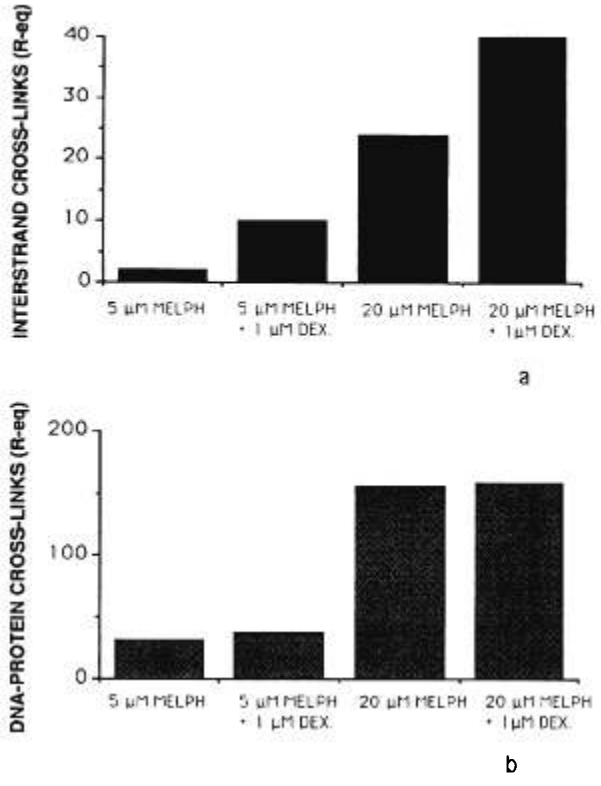

Figure 4. DNA interstrand cross-links (a) and DNA–protein cross-links (b) as measured by alkaline elution 24 h after a 30 min pulse treatment with 5 and 20 μM of melphalan. Dexamethasone at a concentration of 1 μM was added after the melphalan treatment and was present for 24 h. Results are means of duplicate determinations.

Cell growth retardation after melphalan treatment (5 μM) was found to be dependent on accumulation of cells in the S and G_2/M phases of the cell cycle, a finding in accordance with investigations on other alkylating agents.[4] The growth delay effect of dexamethasone analyzed by DNA flow cytometry showed that the cell cycle progression delay was not phase specific but rather caused by a similar, general delay of progression in all cell cycle phases.

With the incubation system used we were able to note that combination of 'growth inhibitory' concentrations (1 and 5 μM) of dexamethasone and melphalan resulted in a 'cytotoxic effect'. We have used a 30 min exposure to melphalan followed by continuous exposure to dexamethasone. The cytotoxic effect is delayed to the time interval 48–72 h. Calculations of the outflow of cells reveal that cells may be lost from the S and G_2/M phases in the 48–72 h time interval. This result is compatible with our previous experience with 'cytotoxic' concentrations of melphalan only.[19] There is, however, an increased number of G_1 cells between 48 and 72 h, indicating that cells have divided and cell death of cells after mitosis in the next cell cycle is probably also present (Table 2). In the present investigation a similar cytolytic effect was obtained at one-eighth of the melphalan concentration in the previous study.[19] Our finding of increased DNA cross-linking may serve as at least one explanation for the increased toxicity of melphalan upon exposure to dexamethasone. The effect is especially interesting since it was limited to an increase of DNA interstrand cross-links and not to total cross-linking. Dexamethasone may alter the chromatin structure to a transcriptionally active form facilitating DNA interstrand cross-linking. Pretreatment with steroids has increased the toxicity of alkylators[20] and the mechanism of increased cross-linking has been discussed though not shown.[10]

Conclusions

Dexamethasone, which in itself is only growth inhibitory at high concentrations, induced substantial cell death (20–40% of the cell population) when combined with 'low' growth inhibitory concentrations of melphalan.

The cell loss was delayed and seen between 48 and 72 h post-melphalan treatment. The cells lost were damaged in G_1 and died in S/G_2 phases and probably also during the following G_1 phase. An increase in melphalan-induced DNA interstrand cross-links by dexamethasone may in part explain the cytolytic effect of the drug combination.

Acknowledgments

The skilful technical assistance of Anna-Lena Wåhlin is acknowledged.

References

1. Alexanian R, Bergsagel DE, Migliore PJ, *et al.* Melphalan therapy for plasma cell myeloma. *Blood* 1968; **31:** 1–10.
2. Woodruff R. Treatment of multiple myeloma. *Cancer Treat Rev* 1981; **8:** 225–70.
3. Bergsagel DE. Plasma cell myeloma. In: Williams JM, Beutler E, Erslev AJ, *et al.*, eds. *Hematology*, 4th edn. New York: McGraw-Hill, 1990: 1114–41.
4. Meyn RE, Murray D. Cell cycle effects of alkylating agents. *Pharmacol Ther* 1984; **24:** 147–63.
5. Smets LA, Bout B, Brouwer M, *et al.* Cytotoxic effects of dexamethasone restricted to noncycling, early G_1 phase cells of L1210 leukemia. *J Cellular Physiol* 1983; **116:** 397–403.

6. Gehring U, Gray JW, Tomkins GM. Corticosteroid effect on the cell cycle. In: Büchner T, Gölde W, Schauman J, eds. *Pulse-cytophotometry*. Ghent: European Press Medikon 1976: 284–9.
7. Selby PJ, Millar JL, Phelps TA, *et al.* The combination of melphalan with prednisolone. Antitumor effect and normal tissue toxicity in laboratory systems. *Cancer Chemother Pharmacol* 1981; **6:** 169–73.
8. Wilkinson R, Birbeck MSC, Harrap KR. Enhancement of the nuclear reactivity of alkylating agents by prednisolone. *Cancer Res* 1979; **39:** 4256–62.
9. Shepherd R, Harrap KR. Modulation of the toxicity and antitumour activity of alkylating drugs by steroids. *Br J Cancer* 1982; **45:** 1–8.
10. Benckhuijsen C, Osman O-M, Hillebrand MJX, *et al.* Glucocorticoid effect on melphalan cytotoxicity, cell-cycle position, cell size and [^{3}H]uridine incorporation in one of three human myeloma cell lines. *Cancer Res* 1987; **47:** 4814–20.
11. Moore GE, Kitamura H. Cell line derived from a patient with myeloma. *NY State J Med* 1968; **68:** 2054–60.
12. Moore GE, Marbell JW, Woods LK, *et al.* RPMI 8226, a human myeloma cell line: an update. *Proc Am Assoc Cancer Res* 1982; **33:** 126.
13. Tribukait B, Moberger G, Zetterberg A. Methodological aspects on rapid-flow cytofluorometry for DNA analysis of human urinary bladder cells. In: Haanen CAM, Hillen HFP, Wessels JMC, eds. *Pulse-cytometry*. Ghent: European Press Medikon 1975: 50–60.
14. Skog S, Tribukait B. Analysis of cell flow and cell loss following X-irradiation using sequential investigation of the total number of cells in the various parts of the cell cycle. *Cell Tissue Kinet* 1985; **18:** 427–44.
15. Kohn KW. DNA as a target in cancer chemotherapy: Measurement of macromolecular DNA damage produced in mammalian cells by anticancer agents and carcinogens. In: De Vita VT, Busch H, eds. *Methods in Cancer Research*. New York: Academic Press 1979; **16:** 291–345.
16. Kohn KW, Ewig RAG, Erickson LC, *et al.* Measurement of strand breaks and cross-links by alkaline elution. In: Friedberg EC, Hanawalt PC, eds. *DNA Repair. A Laboratory Manual of Research Procedures*. New York: Marcel Dekker 1981: 379–401.
17. Dalton WS, Durie BGM, Alberts DS, *et al.* Characterization of a new drug-resistant human myeloma cell line that expresses P-glycoprotein. *Cancer Res* 1986; **46:** 5125–30.
18. Gomi M, Moriwaki K, Katagiri S, *et al.* Glucocorticoid effects on myeloma cells in culture: correlation of growth inhibition with induction of glucocorticoid receptor messenger RNA. *Cancer Res* 1990; **50:** 1873–8.
19. Fernberg J-O, Lewensohn R, Skog, S. Interphase cell death as related to the cell cycle of melphalan-treated human myeloma cells. *Med Oncol Tumor Pharmacother*,1991; **8**: 63–67.
20. Tew KD, Schin PS, Lindner DJ, *et al.* Influence of hydrocortisone on the binding of nitrosoureas to nuclear cromatin subfractions. *Cancer Res* 1980; **40:** 3697–703.

(Received 19 September 1991; accepted 2 October 1991)