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The Combination of Melphalan with Prednisolone

Anti-tumor Effect and Normal Tissue Toxicity in Laboratory Systems

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Summary. *The effect of prednisolone upon the therapeutic index of melphalan has been studied in a variety of laboratory systems. The anti-tumour action of melphalan was assessed for a human melanoma xenograft growing in immune-deprived mice, clonogenic cell survival and tumour growth delay being used as end-points. Normal tissue toxicity was assessed for human bone marrow colony-forming units, murine bone marrow colony-forming units, murine gastrointestinal crypt microcolony-forming cells, and mouse survival.*

Prednisolone had no anti-tumour effect when given alone, but increased the anti-tumour effect of melphalan significantly. No increase in the toxicity of melphalan to marrow or gut colony-forming cells could be demonstrated. However, mouse survival was significantly lower after treatment with the combination than with melphalan alone.

This study supports the view that steroids may enhance the anti-tumour effect of some alkylating agents, but this may be at the expense of increased normal tissue toxicity in some circumstances.

Introduction

Combinations of alkylating agents with steroids are widely used in cancer chemotherapy, and constitute an important part of many successful regimens employed to treat, in particular, haematopoietic and lymphoid neoplasms [1, 3, 4]. The use of steroids may be valuable because of a specific anti-tumour action (e.g., acute lymphoblastic leukaemia) or because of a stimulatory effect upon normal bone marrow [10]. However, steroids may improve the response of some tumours to combination treatments without apparently having very much direct cytotoxic effect alone [3].

Recent investigations have shown that prednisolone can increase the ability of some alkylating agents to inhibit the growth of ascites tumours in rats [22]. Some alkylating agent-resistant cell strains were rendered slightly sensitive by the addition of prednisolone [9]. This apparent cytotoxic effect upon the tumour cells was dependent upon the schedule of administration of some drugs. Prednisolone given 4 h after chlorambucil appeared to produce the maximal improvement in therapeutic index [9]. More recent studies have shown that the combination of prednisolone and chlorambucil or other alkylating agents could induce changes in nuclear chromatin morphology, nuclear protein phosphorylation, and DNA cross-linking in resistant tumour cells which were otherwise unchanged by the alkylating agents alone [23].

There has been recent interest at this Institute in the use of melphalan in high doses to treat human malignant melanoma [11]. In this study we set out to assess the likelihood that prednisolone could improve the therapeutic index of melphalan in this setting.

Materials and Methods

Tumours

Human melanoma xenograft HX34 was grown as an intramuscular implant in the hind legs of CBA/lac mice immune-deprived by thymectomy, cytosine arabinoside pretreatment, and whole-body irradiation according to the method of Steel et al. [17]. The tumour retained its human histology, chromosomes and antigenicity during the period of the study [15].

Cytotoxic Treatment

Melphalan (Alkeran, Wellcome) was dissolved according to the manufacturer's instructions and given by intraperitoneal (IP) or

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intravenous (IV) injection. Prednisolone phosphate (Codesol, Merck, Sharp & Dohme, GB) was diluted in saline and given by IV or IP injection.

Measurement of Tumour Response

a) Clonogenic Cell Survival. The survival of clonogenic cells after therapy was assayed by means of the agar diffusion chamber (ADC) technique described by Smith et al. [16].

Mice bearing tumours of similar size (1 cm diameter) were treated by IP injection of melphalan and prednisolone separately or in combination, and the tumours were excised 18 h after the time of the melphalan treatment. Single cell suspensions were prepared in Ham's F12 medium and plated in 0.3% agar in Millipore diffusion chambers which were incubated in the peritoneal cavity of pre-irradiated mice. Colonies were scored 21 days later under a dissecting microscope and compared with the plating efficiency of control tumours to calculate a surviving fraction. Full details of this method, including the evidence for the origin from human tumour cells of the colonies scored, have been previously described [14].

b) Tumour Growth Delay. Groups of five or six immune-deprived CBA/lac mice bearing bilateral hind-leg tumours (mean volume 0.5 cm^3 in each group) were treated with melphalan with or without prednisolone and volume growth curves were plotted and compared with those for untreated controls. Volumes were estimated from $\pi/6\ d^3$, where d is tumour diameter. Median times to double size (TD) after treatment were estimated and treatment growth delay estimated as:

$$\frac{TD_{treated} - TD_{control}}{TD_{control}}.$$

Measurement of Normal Tissue Toxicity

a) Normal Human Bone Marrow Colony-forming Units. The sensitivity of human bone marrow progenitor cells which form colonies in ADCs [8] was measured by the method of Gordon et al. [7]. Normal bone marrow cells obtained during routine assessments of patients with solid, non-haematopoietic neoplasms at the Royal Marsden Hospital were suspended in soft agar and injected into Millipore diffusion chambers. The ADCs were implanted into the peritoneal cavities of normal C57Bl mice, which received IV injections of melphalan and prednisolone, separately or in combination. Eighteen hours later the ADCs were transferred into pre-irradiated mice and incubated for 9 days, when colonies were scored and results compared with those in control chambers.

b) Normal Murine Bone Marrow. The influence of prednisolone on the recovery of murine bone marrow spleen colony-forming units (CFU-S) after treatment with melphalan was examined by the spleen colony assay described by Till and McCulloch [19]. The numbers of CFU-S in each femur of CBA mice were estimated 6 days after treatment. Previous work has shown that at this time recovery is 10%–20% of the complete recovery, which usually takes about 10 days [12, 13] after melphalan.

c) Normal Murine Gastrointestinal Crypt Stem Cells. The survival of cells in murine small-intestinal crypts which are capable of forming microcolonies was measured by a modification of the microcolony assay of Withers and Elkind [13, 24]. This assay made use of the ability of melphalan to reduce crypt stem cells to a level low enough to allow examination of regenerating microcolonies at 4 days. Microcolonies are assumed to arise from a single surviving stem cell [13].

Melphalan was given IP to groups of five male C57B mice. After 4 days, IP injections of tritiated thymidine (20 μCi/mouse) were given and the mice were sacrificed 40 min later. Four transverse sections of jejunum were taken from each mouse for autoradiography and H & E staining. Uptake of tritiated thymidine by proliferating cells facilitated identification of regenerating crypts, which were counted and expressed as a proportion of the number of crypts/circumference in an untreated mouse.

d) Mouse survival was observed in groups of ten normal CBA/lac mice treated with melphalan ± prednisolone.

Results

Tumour Response

Figure 1 shows a clonogenic cell survival curve for human melanoma xenograft HX34 treated with melphalan alone, compared with a similar curve for

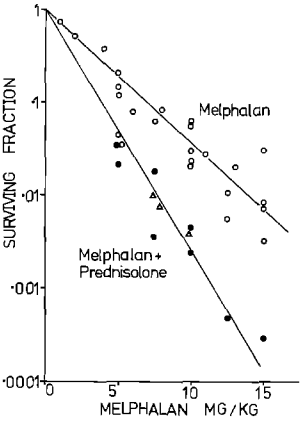

Fig. 1. Clonogenic cell survival after IP treatment in vivo of human melanoma xenograft HX34 with melphalan (○), with melphalan plus prednisolone 15 mg/kg 4 h later (●), or with melphalan plus simultaneous prednisolone 15 mg/kg (△). In three experiments, the treatments were compared directly. In each of these experiments, prednisolone 15 mg/kg alone did not reduce cell survival. Data from other experiments with melphalan alone are included. *Points* represent the mean of at least four agar diffusion chambers and the SEM was less than 10% in most cases

Table 1. Measurement of tumour growth delay

Treatment	Median time to double (days)	Growth delay
Control	6.25	–
Melphalan 10 mg/kg	18	2.88
Melphalan 10 mg/kg + prednisolone 15 mg/kg 4 h later	23	3.68
Melphalan 10 mg/kg + prednisolone 15 mg/kg simultaneously	23.5	3.76

melphalan followed 4 h later by prednisolone phosphate 15 mg/kg. The data were fitted by linear regression analysis and the slopes of the derived lines differed significantly ($P < 0.005$). The ratio of doses required to produce similar levels of cell kill was 1.8. Cell survival measurements when prednisolone was given simultaneously did not appear to differ from those when it was given with 4 h delay (Fig. 1). Prednisolone alone produced no reduction in clonogenic cell survival.

Growth delay measurements are shown in Table 1. Growth delay was greater when prednisolone (4 h delay or simultaneous) was added to melphalan than with melphalan alone ($p < 0.05$ according to a Mann-Whitney U-test).

Normal Tissue Toxicity

Figure 2 shows the survival of CFU-ADC from normal human bone marrow treated in ADCs with melphalan or with the combination of melphalan and prednisolone. Prednisolone did not alter the survival of CFU-ADC after melphalan. The exposure of cells to melphalan in these circumstances differs radically from that used for whole xenograft tumours. To assess whether the effect of added prednisolone could be detected in the ADC exposure system, one experiment was performed in which tumour cells were plated in ADC and treated in the same way as bone marrow cells. Melphalan 10 mg/kg IV reduced the surviving fraction of tumour cells to 5.5 ($\pm$ 3) $\times 10^{-3}$, while the addition of prednisolone reduced the surviving fraction to 1.6 ($\pm$ 0.6) $\times 10^{-3}$, a significant difference.

The method of treatment of human bone marrow cells in ADCs cannot be adapted to assess repopulation of marrow after therapy. To assess the possible influence of prednisolone on this process, studies were performed on CFU-S and peripheral blood white cell counts of CBA/lac mice, and the results are outlined in Table 2. No significant influence of prednisolone was demonstrated.

The plot of surviving fraction for gut microcolonies after treatment with melphalan shows a wide shoulder, but is steeply exponential over the dose range 15–20 mg melphalan/kg [13]. Comparisons of microcolony survival were therefore performed over this range and the results of four experiments are shown in Fig. 3. These data must be interpreted cautiously because of their wide scatter. No consistent influence of prednisolone is shown, but a moderate effect cannot be excluded.

In experiments involving animal survival, mice were given either 17 mg melphalan/kg alone or this dose of melphalan followed immediately or 4 h later with a dose of 15 mg prednisolone/kg (Fig. 4). With the use of a contingency table and the χ^2-test it could be shown that survival was significantly reduced when

Table 2. Murine CFU-S 6 days after therapy

Treatment	CFU-S/femur	Peripheral WBC
Control	3,450 ± 213	10,003 ± 215
Melphalan 15 mg/kg	795 ± 48	2,128 ± 281
Melphalan 15 mg/kg + prednisolone 15 mg/kg 4 h later	966 ± 61	2,269 ± 316

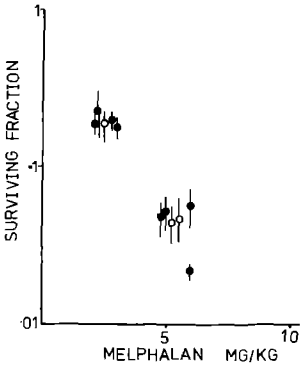

Fig. 2. Survival of normal human bone marrow ADC-CFU treated IV in ADC with melphalan (●) or melphalan plus prednisolone 15 mg/kg simultaneously (○). Prednisolone 15 mg/kg alone did not reduce cell survival

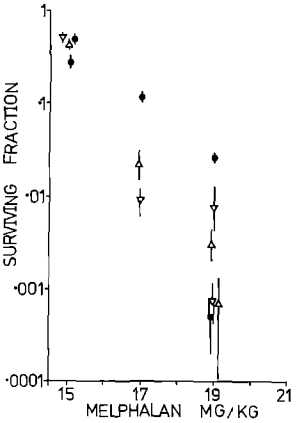

Fig. 3. The survival of normal murine jejunal crypt microcolony-forming cells after the treatment of the mouse IP with melphalan alone (●), melphalan plus prednisolone 15 mg/kg 4 h later (▽), and melphalan plus simultaneous prednisolone 15 mg/kg (△) ± 1 SEM

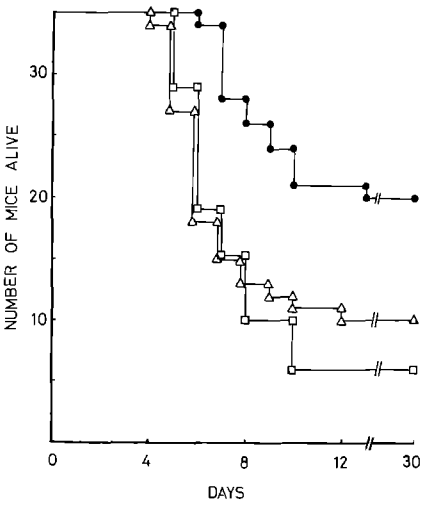

Fig. 4. Mouse survival after treatment IP with melphalan 17 mg/kg, (●) melphalan plus prednisolone 15 mg/kg 4 h later (□), and melphalan plus prednisolone 15 mg/kg simultaneously (△). Groups of 35 mice were ear-marked and randomly distributed throughout cages

prednisolone was added immediately after melphalan ($P < 0.03$) or 4 h after melphalan ($P < 0.001$). There was no significant difference between the two groups that received prednisolone, however. A similar effect has been observed by other investigators [22].

Discussion

Clonogenic cell survival in a solid human melanoma xenograft after treatment with melphalan was significantly reduced when prednisolone was given either simultaneously or 4 h later, and this reduction in cell survival was reflected in a greater tumour growth delay. The place of measurement of clonogenic cell survival in the assessment of drug effect on human tumour xenografts has been discussed elsewhere [14, 16]. The effect was not due to a direct cytotoxic effect of prednisolone alone. This observation is in keeping with the observations of Harrap and co-workers [9, 22, 23] and with the clinical value of combinations containing these agents [1, 3, 4], and supports the hypothesis that the role of steroids in cytotoxic combinations is not always dependent upon their direct action on tumour cells.

It is not clear, however, that the therapeutic index of melphalan was increased by the addition of prednisolone. We employed a series of end-points which seemed relevant to the prediction of human normal tissue toxicity. In no case was the toxicity of melphalan reduced. In human and murine bone marrow and murine crypt stem cells no increased toxicity was demonstrated but the mortality of mice after treatment was significantly increased. This may imply that the assays for marrow and particularly for gut stem cell damage were insufficiently sensitive to detect the additional damage caused by prednisolone or that the additional toxicity to whole mice was not mediated via marrow and gut stem cell death.

The mechanism of enhanced anti-tumor effect of melphalan by prednisolone is not clear, although it is known that melanomas may carry receptors for glucocorticoids [2]. The effects of the combination upon tumour cell nuclei do not differ qualitatively from those of melphalan alone, but the extent of loss of condensed nuclear chromatin, nuclear protein phosphorylation, and DNA cross-linkage is increased [23]. Whether the steroid acts at cellular level or on the whole animal is unclear. Both prednisolone [5] and melphalan [18] are bound to plasma proteins, and enhanced tumour toxicity could occur by way of an interaction at this level or a similar pharmacokinetic interaction. However, the difference between tumour and some normal tissues would argue for an effect at cellular level. Melphalan levels in mouse plasma have fallen considerably by 4 h, and studies in this department have shown that melanoma xenograft cells may be removed from the mouse 3 h after treatment with melphalan without altering the cell kill [14]. This seems to suggest that influence on plasma binding by prednisolone given 4 h after the melphalan is unlikely to explain the increased cell kill which is observed. An interaction at the cellular level seems more likely. An interesting possible cellular mechanism proposed by Wilkinson et al. [23] is increased activity of cyclic AMP-dependent protein kinase, since both chlorambucil [20] and steroids [6] may influence this system. Nuclear protein phosphorylation is probably a major regulatory mechanism in nucleic acid synthesis [21].

This study perhaps illustrates how a variety of laboratory model systems using human tissue where possible and employing a wide range of end-points, may be used to seek preclinical information on a putative method of improving the therapeutic index of a cytotoxic treatment.

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