Changes in the response of the RIF-1 tumour to melphalan in vivo induced by inhibitors of nuclear ADP-ribosyl transferase

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Summary The effect of inhibitors of nuclear ADP-ribosyl transferase (ADPRT) on the cytotoxicity of melphalan (L-PAM) in the RIF-1 tumour in vivo was investigated. A large single dose of nicotinamide (1000 mg kg⁻¹) enhanced the tumour cell killing by L-PAM as measured by tumour cell survival. This enhancement was maximum when nicotinamide was administered within 1 h before injecting the L-PAM. When given at this time, the nicotinamide had a dose-modifying effect on all L-PAM doses tested, giving rise to a mean enhancement ratio (ER) of 2.2. Nicotinamide did not appear to inhibit the recovery from L-PAM induced potentially lethal damage. L-PAM (6 mg kg⁻¹) produced a transient drop in mouse body temperature. This effect was both increased and prolonged by nicotinamide. In addition the inhibitor also delayed the clearance of L-PAM from the plasma of C3H mice, such that the half-life of the chemotherapeutic agent was extended from 41 min to 143 min. The effect of combining L-PAM with nicotinamide doses below 1000 mg kg⁻¹ was also investigated. The results showed that as the nicotinamide dose was decreased, the enhancement of the effects on body temperature, pharmacokinetics and white blood cell counts were reduced. However, a concomitant loss in the enhancement of tumour cell killing was also observed. Similar results were obtained using 3-aminobenzamide, a more efficient inhibitor of ADPRT.

A major factor affecting the response of malignant tumours to various chemotherapeutic agents may be their ability to repair potentially lethal damage (PLD). Recovery from PLD has been observed in tumour cells after drug treatment both in vitro and in vivo (for review see Bertrand & Deen, 1980). It remains a possibility that if this repair can be inhibited a better clinical response following drug therapy may be achieved.

There is now strong in vitro evidence that the enzyme, nuclear ADP-ribosyl transferase (ADPRT), participates in DNA excision repair after exposure of eukaryotic cells to radiation or alkylating agents, although the precise molecular involvement of the enzyme is not yet known (for review see Shall, 1982). The activity of this enzyme can be inhibited by several groups of compounds including thymidine, nicotinamides, benzamides and methylxanthines (Preiss et al., 1971; Shall, 1975; Davies et al., 1978). Combining these inhibitors with DNA damaging agents has been shown to produce a significantly greater degree of cell killing than seen with these agents in the absence of the inhibitor. This has been reported for the enhancement of drug-induced damage in vitro (Nduka et al., 1980; Jacobson et al., 1984) and to a lesser extent in vivo (Smulson et al., 1977; Sakamoto et al., 1983).

In order to investigate these effects in more detail, we have studied the effect of two inhibitors of ADPRT, nicotinamide and 3-aminobenzamide, on the activity of the bifunctional alkylating agent melphalan (L-phenylalanine mustard, L-PAM) against normal and malignant tissues in the mouse. A preliminary report of this work has been presented (Brown et al., 1984b).

Materials and methods

Tumour system

The RIF-1 tumour was used in all experiments. This tumor is a radiation-induced sarcoma which arose in the inbred female C3H/Km mouse. It is routinely maintained by passage in vivo and in vitro according to a published protocol (Twentyman et al., 1980). Solid tumours were produced in 3–4 month old female C3H/Km mice by inoculating 2 × 10⁵ cells into the gastrocnemius muscle in the right rear leg. All drug treatments were carried out when the tumour size was 300–600 mg. The experimental techniques have previously been described in detail (Horsman et al., 1984).
Drug treatments

All drug solutions were prepared immediately prior to injection. Nicotinamide (Sigma Chemical Co., St. Louis, MO) was dissolved in a sterile saline solution (0.9% NaCl). 3-aminobenzamide (Sigma) was prepared by dissolving 100 mg in 1 ml absolute ethanol and diluting in 9 ml saline. Subsequent dilutions were in saline. L-PAM (Burroughs Wellcome Co., Research Triangle Park, NC) was prepared by dissolving 10 mg in 1 ml 95% ethanol and 5% HCl. It was further diluted to the required concentration in a 60% solution of propylene glycol in saline. Prepared drug concentrations were varied so that a constant volume (0.01 ml g⁻¹ body weight) could be injected for both nicotinamide and L-PAM. The low solubility of 3-aminobenzamide, however, necessitated the injection of 0.01 to 0.04 ml g⁻¹ body weight depending on the required final drug concentration. All drugs were injected i.p.

Tumour studies

Tumour response was assayed by survival of tumour cells. Survival was determined, as previously described (Horsman et al., 1984), by excising tumours at various times, up to and including 24 h, after injecting L-PAM. Three tumours were combined for each datum point. They were subsequently minced, enzymatically disaggregated and single cell suspensions produced. The enzyme mixture was removed by centrifugation (1500 r.p.m.; 10 min) and the cells resuspended, counted, serially diluted and plated in Waymouth's medium + 15% foetal calf serum (Gibco, Santa Clara, CA). The colony-forming ability of these cells was then determined. Survival was expressed as surviving fraction g⁻¹ tumour. This is the product of the plating efficiency and cell yield g⁻¹ of treated tumours relative to that for untreated tumours.

Normal tissue studies

Previous experiments have shown that the number of white blood cells (WBC) in the peripheral blood declines for several days after treatment with cyclophosphamide (Law et al., 1981) and L-PAM (Hirst, unpublished) reaching a minimum at ~4 days before recovery begins. In our experiments 5 μl blood samples were taken from the tails of tumour-bearing mice four days after injecting drugs. The blood was diluted with 95 μl of 2% glacial acetic acid to lyse the erythrocytes. The resulting suspension of leucocytes was counted using a haemacytometer. Six mice were used for each treatment group.

Measurement of plasma L-PAM levels

Plasma L-PAM levels were determined as described previously (Horsman et al., 1984). Briefly, at various times after drug injection, mice were bled by cardiac puncture under diethyl ether anaesthesia and plasma separated by centrifugation (3,000 r.p.m.; 5 min). Drug concentrations were determined by reverse phase high-performance liquid chromatography (Waters Associates, Milford, MA) using the procedure of Furner et al. (1976). Results were collected on a data module chart recorder (Waters Associates). Quantitation of drug concentration was by peak area with reference to linear calibration curves.

Body temperature measurements

For the RIF-1 tumour grown i.m. in the leg of C3H mice, body temperatures are a good indicator of tumour temperatures (Horsman et al., 1984). Drug-induced temperature changes were therefore determined by measuring mouse body temperatures at various times after drug injections using a rectally inserted thermocouple (Bailey Instruments, Saddle Brooke, NJ).

Results

When tumour-bearing C3H mice were given nicotinamide (1000 mg kg⁻¹) and L-PAM (6 mg kg⁻¹) in combination, there was an enhancement of cell killing above that obtained with L-PAM alone (Figure 1). The effect was maximal when nicotinamide was given between 1 h before L-PAM and at the same time as the alkylating agent. This enhancement was reduced as the time interval between the drugs increased. Nicotinamide alone caused a small amount of cell killing. As a consequence of the results shown in Figure 1, all further experiments were carried out with the nicotinamide being administered immediately before the L-PAM.

The effect of this large single dose of nicotinamide on the L-PAM dose-response curve, as measured by tumour cell survival at 24 h after drug treatment, is shown in Figure 2. Increasing doses of L-PAM caused increasing amounts of cell kill. Nicotinamide alone had a small effect on the tumour response. However, when combined with L-PAM it significantly enhanced the response to the alkylating agent as demonstrated by a steeper dose response curve for the two agents combined. The data shown in Figure 2 suggests that nicotinamide had a dose-modifying effect for all L-PAM doses, giving a mean enhancement ratio (ER; calculated as ratio of drug concentration for L-PAM alone to
Figure 1 The effect of drug timing on tumour cell survival. Nicotinamide (1000 mg kg\(^{-1}\)) was injected at various times before or after L-PAM (6 mg kg\(^{-1}\)) and survival of RIF-1 tumour cells assayed 24 h later: (○) nicotinamide + L-PAM diluent; (●) nicotinamide + L-PAM. The shaded area represents L-PAM only. Means ± 1 s.e. are shown for 3 to 4 data points.

Figure 2 The effect of L-PAM ± nicotinamide (1000 mg kg\(^{-1}\)) in the RIF-1 tumour as measured by tumour survival at 24 h after injection: (○) saline + L-PAM; (●) nicotinamide + L-PAM. Means ± 1 s.e. are shown for 4 separate experiments.

Figure 3 Survival of RIF-1 tumour cells as a function of time between drug administration and tumour removal: (▲) nicotinamide (1000 mg kg\(^{-1}\)) + L-PAM diluent; (○) saline + L-PAM (6 mg kg\(^{-1}\)); (●) nicotinamide (1000 mg kg\(^{-1}\)) + L-PAM (6 mg kg\(^{-1}\)). Means ± 1 s.e. for 4 separate experiments are shown.

Figure 4 shows plasma levels for L-PAM as a function of time after drug injections. Nicotinamide (1000 mg kg\(^{-1}\)) given immediately before a dose of 6 mg kg\(^{-1}\) L-PAM produced a marked slowing in the elimination of L-PAM from the blood. The half-

that obtained with L-PAM and nicotinamide to give the same response) of 2.2 (corrected for nicotinamide toxicity).

Figure 3 shows the effect on survival of varying the time of removal of tumours from the animals following an L-PAM injection. When mice were given saline immediately before a single injection of L-PAM (6 mg kg\(^{-1}\)), significant toxicity was seen even when the tumours were removed within 2 h following the L-PAM dose. The nadir in survival was reached at 4 h and was followed by repair of L-PAM-induced potentially lethal damage (PLD), shown by an increasing surviving fraction from 4 to 24 h. Nicotinamide alone (1000 mg kg\(^{-1}\)) reduced survival to ~60% over the 24 h period. In combination with L-PAM, it not only enhanced the degree of cell killing, but also moved the nadir to 12 h. After this time cell survival increased by about the same amount as seen with L-PAM alone, although the final survival level was between 1½ to 2 decades lower.
The temperature within a tumour-bearing C3H mouse was monitored after L-PAM administration. Each point on the graph represents a time point after injection, with the results from 3 separate experiments shown. The shaded area represents the body temperature of untreated mice. Means ± 1 s.e. for 3 to 6 mice are shown.

In order to test whether an enhancement of L-PAM tumour cell killing could occur in the absence of any effect on L-PAM pharmacokinetics or body temperature, experiments were performed with reduced nicotinamide doses. As illustrated in Figure 6, when the nicotinamide dose injected immediately before a single dose of L-PAM (6 mg kg⁻¹) was decreased, there was a concomitant reduction in the fall in mean body temperature (measured over 6 h after drug injection) and the change in plasma levels of L-PAM (determined 3 h after injection). Below 125 mg kg⁻¹ nicotinamide these changes were virtually eliminated. This change in nicotinamide dose also produced a similar effect on the L-PAM-induced tumour cell killing, such that at the doses where no modification of body temperature or pharmacokinetics were observed, no significant enhancement of tumour response could be obtained. The effect of nicotinamide dose on a normal tissue is shown in Figure 6(d). White blood cell (WBC) counts were selected because haemotological effects are the major dose-limiting problem with alkylating agents. These counts were determined 4 days after injecting the drugs, when the WBC nadir is achieved following L-PAM administration (Hirst, unpublished). Any possible effect of nicotinamide dose on the subsequent recovery of WBC counts was not investigated. As shown in Figure 6(d), the L-PAM-induced
reduction in WBC counts were similarly affected by changes in nicotinamide dose as reported for the other endpoints shown in Figure 6.

Since 3-aminobenzamide is a more efficient inhibitor of ADPRT than nicotinamide (Purnell & Whish, 1980), we repeated these experiments with this compound. The results are shown in Figure 7. Although 3-aminobenzamide was more effective than nicotinamide on a concentration basis, its effects on all the end points studied were similar to those obtained with nicotinamide.

Discussion

The present studies demonstrate that an enhancement of tumour response to the bifunctional alkylating agent L-PAM can be achieved with the ADPRT inhibitors nicotinamide and 3-aminobenzamide. Most of this effect appears to be the result of an increased plasma half-life of the chemotherapeutic agent and may not involve any inhibition of the repair of L-PAM-induced PLD.

Nduka et al. (1980) demonstrated that in L1210 cells the cytotoxicity of methylnitrosourea (MNU) could be potentiated if these cells were exposed to non-toxic concentrations of various ADPRT inhibitors during exposure to the cytotoxic agent and the subsequent cloning period. Similar results have been reported for dimethyl sulphate (Durkacz et al., 1980), streptozotocin (Shall, 1982), methyl methane sulphonate (Boorstein & Pardee, 1984) and for recovery from N-methyl-N'-nitro-N-nitrosoguanidine damage (Jacobson et al., 1984). In addition, the in vitro cytotoxicity of the bifunctional alkylating agents nitrogen mustard (Das et al.,
1982) and L-PAM (Brown et al., 1984b) can be enhanced by these enzyme inhibitors.

In vivo the results have been far less dramatic. Smulson et al. (1977) showed that the mean survival time for L1210 tumour bearing mice could be increased by treatment with various doses of MNU. For each MNU dose there was an additional enhancement of survival time when treatment was combined with 500 mg kg\(^{-1}\) nicotinamide. Sakamoto and colleagues (1983) reported similar effects for bleomycin and benzamide in Ehrlich ascites tumour bearing mice. While the effects of these inhibitors in vitro may be attributed to an inhibition of PLD repair (Shall, 1982), this may not be the case in vivo.

A number of studies have clearly shown PLD repair in vivo following alkylating agent treatment. This was reported for cyclophosphamide in the RIF-1 and WHFIB tumours (Law et al., 1981; Martin et al., 1981), for L-PAM in the KHT, MT, and RIF-1 tumours (Siemann & Mulcahy, 1982; Sheldon & Batten, 1982; Horsman et al., 1984), and for the nitrosoureas CCNU and BCNU in the KHT sarcoma (Siemann & Mulcahy, 1982). In our studies we were able to enhance the tumour response to L-PAM by injecting nicotinamide (1000 mg kg\(^{-1}\)) immediately before L-PAM (Figure 2). However, the data of Figure 3 suggest no inhibition of PLD repair, although an inhibition of L-PAM-induced PLD repair in vitro has been seen (Brown, D., personal communication). The degree of recovery after alkylating agent damage in vivo is known to be greater as the nadir of survival goes lower (Sheldon & Batten, 1982; Horsman et al., 1984).

Figure 7 The effect of different 3-aminobenzamide doses on the response to L-PAM (6 mg kg\(^{-1}\)); (a) Mean mouse body temperature measured over 6 h after drug injection, (b) Plasma L-PAM levels determined at 3 h after drug administration, (c) Tumour survival assayed 24 h after L-PAM, (d) WBC counts measured 4 days after injection of drugs. (●) 3-aminobenzamide + L-PAM (6 mg kg\(^{-1}\)). Means ± 1 s.e. are shown for 3 to 6 data points.
Thus, while the similar PLD repair factors, shown in Figure 3, for L-PAM alone and L-PAM +nicotinamide do in fact imply some PLD repair inhibition by nicotinamide, the results are complicated by at least two factors. First, repair following L-PAM treatment is complete by 24 h (Horsman et al., 1984). This may not be true for nicotinamide+L-PAM. Second, the survival nadir in the combined treatment occurs at 12 h after drug injection, presumably a consequence of the L-PAM pharmacokinetic changes induced by nicotinamide, as shown in Figure 4. The continued cell-killing seen between 6–12 h for the combined treatment may mask any repair occurring during this time period. In other words, the true survival nadir may be lower that that actually seen at 12 h.

The pharmacokinetic effect of nicotinamide and 3-aminobenzamide may partially be a consequence of the induced hypothermia shown in Figure 5. The breakdown of L-PAM in vivo is believed to be primarily by hydrolysis and alkylation (Evans et al., 1982). Furthermore, the hydrolysis of L-PAM has been shown to be temperature sensitive with slower rates being obtained at lower temperatures, both in water (Chang et al., 1978) and in plasma (Hinchliffe et al., 1983). Alternatively, these inhibitors may actually interfere with these processes or affect some other metabolic route. Evidence exists showing that both benzamide and nicotinamide are capable of changing the levels of various rat liver enzymes (Blake et al., 1967; Griffin et al., 1984).

Our attempt to show a tumour effect by lowering the nicotinamide or 3-aminobenzamide doses so that the pharmacokinetic effect was eliminated proved generally unsuccessful (Figures 6 and 7). It is known that the inhibition of repair following both drug and radiation treatment is dependent upon the concentration of the inhibitor (Nduka et al., 1980; Brown et al., 1984a). Decreasing the inhibitor concentration as shown in Figures 6 and 7, coupled with the metabolism of the inhibitors in mice, may have reduced the levels to a value too low to cause an inhibitory effect. By using an inhibitor with a longer plasma half-life or by multiple dosing with low levels of nicotinamide or 3-aminobenzamide it may be possible to show PLD repair inhibition of L-PAM-induced damage, if indeed it occurs, in the absence of any pharmacokinetic effects.

While much of the enhancement of L-PAM killing in this tumour model by ADPRT inhibitors may be explained in the absence of any PLD repair inhibition, this may not be true for other drugs, inhibitors or tumour systems. Inhibition of drug-induced PLD repair has been thought to occur after treatment with a number of modalities, including hyperthermia (Braun and Hahn, 1975), nitroaromatic radioisensitizers (Law et al., 1981; Martin et al., 1981) and nucleoside analogues (U et al., 1982; Nakatsugawa et al., 1984). Regardless of the mechanisms involved, we have found that the enhancement of drug damage by either ADPRT inhibitors (Brown et al., 1984b) or nucleoside analogues (Horsman et al., 1986) can give rise to a therapeutic gain, albeit at drug doses which are probably not achievable in humans. Combinations of anti-tumour drugs and repair inhibitors may therefore have a clinical role to play. In fact, phase II trials with the nucleoside analogue Ara-A both as a radio- and chemosensitizer are currently in progress in Japan (Nakatsugawa, 1984). However, the experiments reported in the present study do suggest that a greater understanding of the interactions in the in vivo situation are necessary before such clinical trials are undertaken.

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