Cyclophosphamide decreases O\textsuperscript{6}-alkylguanine-DNA alkyltransferase activity in peripheral lymphocytes of patients undergoing bone marrow transplantation

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Summary O\textsuperscript{6}-alkylguanine-DNA alkyltransferase (ATase) levels were measured in extracts of peripheral blood lymphocytes taken at various times during chemotherapy from 19 patients with various haematological malignancies. Seven patients with advanced Hodgkin's disease received preparative treatment consisting of cyclophosphamide (1.5 g m\textsuperscript{-2}, daily) administered on days 1 to 4 and BCNU (600 mg m\textsuperscript{-2}) on day 5 prior to autologous bone marrow rescue (ABMR) delivered on day 7. Treatment in the remaining 12 patients consisted of cyclophosphamide (1.8 g m\textsuperscript{-2}, daily) given on days 1 and 2 followed at day 4 with total body irradiation (TBI) administered in six fractions over the subsequent 3 days to a total dose of 1200 cGy prior to bone marrow transplantation. In the Hodgkin's group, significant decreases in ATase activity were seen during the cyclophosphamide treatment, and the median ATase nadir was 32% (range 0% to 57%) of pretreatment levels following 4 days of cyclophosphamide. In one patient, no ATase activity was detectable following the 4th cyclophosphamide treatment. ATase activities decreased further after BCNU administration to a median of 19% (range 0% to 32%) of pretreatment levels. Extensive cyclophosphamide-induced reduction of lymphocyte ATase levels was also seen in the other group of 12 patients treated with cyclophosphamide/TBI. Post-cyclophosphamide median ATase nadir was 35% (range 12% to 78%) of the pretreatment levels. No ATase depletion was seen when cyclophosphamide (up to 10 mM) was incubated for 2 h with pure recombinant human ATase in vitro whereas ATase activity was reduced by 90% on preincubation with 100 μM acrolein with >1 mM phosphoramidemustard. This suggests that a cyclophosphamide-induced decrease in ATase levels in human peripheral lymphocytes in vivo may be due to depletion mediated by the production of intracellular acrolein. Since ATase appears to be a principal mechanism in cellular resistance to the cytotoxic effects of BCNU and related alkylating agents, these observations suggest that a cyclophosphamide-induced reduction in ATase activity may be an additional factor in the effectiveness of the combined sequential therapy.

Autologous Bone Marrow Rescue (ABMR) following ablative chemotherapy is being increasingly adopted for patients with high risk advanced Hodgkin's disease, who fail to obtain complete remission following primary induction chemotherapy, relapse within 1 year of completing chemotherapy or who are in second or subsequent relapse after receiving two or more standard chemotherapy regimens (Armitage et al., 1989). Clinical results from recent ABMR series indicate that despite achieving an initial high response rate (range 70–85%), long term cure is achieved in only about 30–35% with most relapse occurring at sites previously involved with disease (Ahmed et al., 1989; Bierman et al., 1988; Carella et al., 1988; Gribben et al., 1989). This indicates that inadequate chemotherapy is the prime reason for failure.

The most popular preparative treatment regimen used for ABMR in Hodgkin's disease involves Carmustine (BCNU) in combination with cyclophosphamide and etoposide. This was originally developed by the MD Anderson group (Jagannath et al., 1986; Spitzer et al., 1980) and several variants are currently in use (Ahmed et al., 1989; Bierman et al., 1988; Carella et al., 1988; Gingrich et al., 1990; Gribben et al., 1989; Reece et al., 1991; Teillet et al., 1987). The mechanism of cell killing by BCNU is initiated by the formation of the mono-adduct, O\textsuperscript{6}-chloroethylguanine which undergoes an intramolecular rearrangement to form O\textsuperscript{6}-N1-ethanoguanine. This then reacts with a cytosine residue in the opposite strand to form a lethal N1-guanine-N3-cytosine ethano DNA cross-link (Brent, 1985; D'Incalci et al., 1988; Gonzaga et al., 1990; Pegg, 1990; Tong et al., 1982). The principal mechanism of BCNU resistance involves the DNA repair enzyme, ATase (Pegg, 1990; D'Incalci et al., 1988) which can remove the chloroethyl group from O\textsuperscript{6}-chloroethylguanine and hence prevent the formation of DNA interstrand cross-links. However, many tumour cells and most tumour cell lines isolated so far have high ATase levels limiting the potential usefulness of BCNU (D'Incalci et al., 1988). One theoretical approach to increasing sensitivity to BCNU is to reduce the levels of ATase prior to administration of the chloroethylyating agents. This can be achieved in the case of ATase because of its autoinactivating stoichiometric reaction mechanism and its slow rate of resynthesis (Pegg, 1990). Indeed it has been shown in cultured cells that depletion of endogenous ATase by prior exposure to non-toxic doses of monofunctional methylating agents (Futschek et al., 1989; Zlotogorski & Erickson, 1984) or O\textsuperscript{6}-methylguanine (Dolan et al., 1985; Gerson et al., 1988; Yaresh et al., 1986) or O\textsuperscript{6}-benzylguanine (Dolan et al., 1990) rendered the cells more sensitive to subsequent treatment with chloroethylyating agents. Conversely, transfer and expression of ATase genes in ATase deficient cells renders them more resistant to chloroethylyating agents (Brennand & Margison, 1986; Margison & O'Connor, 1990).

Based on our recent observations of progressive depletion of ATase activity in human peripheral blood lymphocytes of patients with malignant melanoma treated with sequential dacarbazine and ifosfamide (Lee et al., 1991a), we began to examine the kinetics of ATase depletion following BCNU prior to ABMR. However, results in the first patient to receive sequential cyclophosphamide and BCNU showed an unexpected decrease in ATase activity in the post-cyclophosphamide samples. This observation was pursued and we report here a marked decrease in ATase activity following cyclophosphamide treatment in seven patients with advanced Hodgkin's disease receiving cyclophosphamide and BCNU and in a further group of 12 patients with various haematological malignancies undergoing preparative treatment with cyclophosphamide and total body irradiation (TBI).
Materials and methods

Chemicals

Cyclophosphamide was obtained from Farmitalia Carlo Erba Ltd; acrolein and glutathione were from Sigma Chemical Co Ltd. and phosphoramid mustard was a generous gift from Dr A. McCown (Paterson Institute, Manchester, UK). Freshly prepared stock solutions (100 mM) in distilled water were used to examine the effects on ATase as described below.

Patients and blood samples

The clinical characteristics of the 19 patients with various haematological malignancies studied are outlined in Table I. All the patients with Hodgkin’s disease had failed front line chemotherapy (including MOPP: mechlorethamine, vincristine, procarbazine and prednisolone; CHLPP: Chlorambucil, vinblastine, procarbazine and prednisolone or HYBRID: Vinblastine, procarbazine, prednisolone, chlorambucil, vincristine, etoposide and Adriamycin) and/or salvage chemotherapy (including VAPEC-B: Adriamycin, cyclophosphamide, vincristine, bleomycin, etoposide and prednisolone or HYBRID) or had relapsed less than 12 months after chemotherapy. They received preparative treatment consisting of cyclophosphamide (1.5 g m⁻² i.v., daily) administered on days 1 to 4, BCNU (600 mg m⁻² i.v.) on day 5 and autologous bone marrow rescue on day 7. The remaining 12 patients (Table I) presented with a variety of haematological diseases and were treated with cyclophosphamide/TBI in which cyclophosphamide (1.8 g m⁻² i.v., daily) was given on day 1 and 2 followed by six fractionated doses of total body irradiation (200 cGy twice daily) to a total dose of 1,200 cGy from day 4 to 6 prior to allogeneic or autograft marrow transplantation. Patients with acute myeloid leukaemia who had previously received combination chemotherapy comprising cytosine arabinoside, daunorubicin and thioguanine and patients with acute lymphoblastic leukaemia had combination chemotherapy with vincristine, daunorubicin, prednisolone, high dose methotrexate followed by intensification with vindesine, asparaginase, cytosine arabinoside, prednisolone and oral maintenance with 6-mercaptopurine and methotrexate. Patients with chronic myeloid leukaemia had previously received hydroxyurea treatment. Serial blood samples were collected at various times during the two preparative regimens. For the cyclophosphamide/BCNU group, blood samples were taken just before chemotherapy and approximately 3, 18, 24, 36, 45, 63, 75, 85, 98, 108, 124 and 132 h after administration of the first dose of cyclophosphamide. For the cyclophosphamide/TBI group, blood samples were taken before and approximately 3, 6, 15, 22, 29, 50, 64, 70, 88, 94, 112, 120 h after chemotherapy. Bloods were drawn into a 20 ml universal container containing 0.5 ml of 0.5% EDTA and stored at 4°C before isolation of lymphocytes.

O'-Alkylguanine-DNA alkyltransferase assay

Lymphocytes (mononuclear cell fraction) were isolated by centrifugation on Ficoll (Pharmacia, Uppsala, Sweden), washed with PBS and centrifuged again into a pellet and stored at −20°C. The ATase extraction and assay procedure was carried out as described previously (Lee et al., 1991a) with slight modifications. Briefly, cells were disrupted by sonication in 1 ml of buffer I (50 mM Tris–HCl, 3 mM diethiothreitol, 1 mM EDTA, pH 8.3) and centrifuged to prepare cell extracts. Varying amounts of cell extract were incubated with ³H-methylmitrosourea-methylated calf thymus substrate DNA (specific activity, 19 Ci mmol⁻¹) at 37°C for 2 h in a total volume of 500 µl of 1 mg ml⁻¹ bovine serum albumin in buffer I. After incubation, bovine serum albumin (1 mg ml⁻¹ of a 10 mM stock of bovine serum albumin and perchloric acid (200 µl of a 4 M solution) were added in that order to precipitation. A further 2 ml of 1 M perchloric acid was added and the mixture heated at 75°C for 45 min. Samples were clarified by centrifugation and the precipitates were washed with 4 ml of 1 M perchloric acid before being resuspended in 300 µl of 0.01 M sodium hydroxide and dissolved in 3 ml of aqueous scintillation fluid (Ecocount A; National Diagnostics). Counting efficiency was approximately 28%. Specific activity measurements were based on a minimum of three points on the linear part of the curve. ATase activity was expressed as femol methyl transferred to protein per mg of total protein in the extract, protein concentration being measured using the Bradford method with bovine serum albumin as the standard (Bradford, 1976). Statistical analysis was based on repeated measurement analysis using the BMDP statistical software program and was done on the pre-chemotherapy and pre-TBI assays.

In order to assess the ability of cyclophosphamide, acrolein and phosphoramid mustard to inhibit the ATase in vitro, varying concentrations of these agents were incubated with 70 femol of pure recombinant human ATase (Santibanez-Koref et al., 1992 in press) for 2 h at 37°C in buffer I without diethiothreitol. Residual ATase activity was then measured after incubation with excess substrate DNA. The effect of glutathione on the inhibition of ATase by acrolein was monitored by incubating various amounts of glutathione for 2 h with 50 µM acrolein, a concentration that caused a 95% depletion in ATase activity in vitro. Following this, 70 femol of recombinant ATase was added and the experiment continued as above.

Results

Decrease in ATase in vivo following cyclophosphamide and BCNU

Pretreatment ATase levels in the seven Hodgkin’s patients ranged from 33 to 183 (mean 141) fmg⁻¹ total protein. In all seven patients, decreases in ATase activity were seen following cyclophosphamide administration. Wide variations were noted in the rates and extents of ATase reduction between various individuals (see Figures 1a and 1b). In three patients the first cyclophosphamide treatment caused reduction to 52%, 64% and 67% of their pretreatment levels (patients RD, SH and ST, Figures 1a and 1b) while in two other patients the first treatment caused only a 10% loss in activity (patients SJ and JC, Figure 1b). Following four cyclophosphamide treatments the median ATase nadir was 32% (range 0% to 57%) of pretreatment levels. In one patient (ST,

| Patient | Age/sex | Disease | Treatment | ATase activity (fmg⁻¹) | Initial | Nadir |
|---------|---------|---------|-----------|------------------------|--------|-------|
| ST      | 17/F    | HD      | IVB       | Cyclo/BCNU             | 33     | BD*   |
| SH      | 20/M    | HD      | IVB       | Cyclo/BCNU             | 148    | 47    |
| MR      | 50/F    | HD      | IVB       | Cyclo/BCNU             | 130    | 25    |
| MS      | 23/M    | HD      | IVB       | Cyclo/BCNU             | 170    | 55    |
| RD      | 21/M    | HD      | IVB       | Cyclo/BCNU             | 162    | 12    |
| SJ      | 34/M    | HD      | IVB       | Cyclo/BCNU             | 163    | 53    |
| JC      | 39/F    | HD      | IVB       | Cyclo/BCNU             | 183    | 34    |
| AW      | 26/M    | AML     | 1st CR   | Cyclo/TBI              | 107    | 19    |
| OD      | 43/M    | AML     | 1st CR   | Cyclo/TBI              | 168    | 10    |
| LW      | 39/M    | AML     | 1st CR   | Cyclo/TBI              | 101    | 13    |
| SA      | 44/F    | AML     | 1st CR   | Cyclo/TBI              | 405    | 138   |
| MC      | 18/F    | AML     | 1st CR   | Cyclo/TBI              | 148    | 18    |
| HR      | 30/F    | AML     | 1st CR   | Cyclo/TBI              | 103    | 19    |
| TC      | 21/F    | AML     | 3rd CR   | Cyclo/TBI              | 168    | 37    |
| SC      | 28/F    | CML     | 1st CR   | Cyclo/TBI              | 160    | 23    |
| LB      | 38/M    | CML     | 1st CR   | Cyclo/TBI              | 102    | 45    |
| CN      | 38/M    | CML     | 1st CR   | Cyclo/TBI              | 200    | 18    |
| RB      | 54/M    | NHL     | 1st PR   | Cyclo/TBI              | 107    | 12    |

HD: Hodgkin’s disease; AML: acute myeloid leukaemia; ALL: acute lymphoblastic leukaemia; CML: chronic myeloid leukaemia; NHL: non-Hodgkin’s lymphoma; CR: complete remission; IVB: stage IVB disease; PR: partial remission; CP: chronic phase; 1st: first; 3rd: third.

*BD* = below detection.
Figure 1. O⁶-alkylguanine-DNA-alkyltransferase (ATase) specific activity (fmole mg⁻¹ protein) in extracts of peripheral lymphocytes of patients treated with cyclophosphamide and BCNU. (a) Patients ST (Δ), SH (▲), MR (●), and MS (●). (b) Patients RD (Δ), SJ (▲), and JC (●).

Figure 2. O⁶-alkylguanine-DNA-alkyltransferase (ATase) specific activity (fmole mg⁻¹ protein) in extracts of peripheral lymphocytes of patients treated with cyclophosphamide and BCNU. (a) Patients AW (▲), GD (Δ), CW (●), and DHI (●). (b) Patients SA (▲), MR (Δ), HR (●), and TC (●). (c) Patients SK (▲), LB (Δ), CN (●), and RB (●).

Figure 3. Consensus data for O⁶-alkylguanine-DNA-alkyltransferase (ATase) specific activity (fmole mg⁻¹ protein) in extracts of peripheral lymphocytes of patients treated with cyclophosphamide and BCNU. (a) Patients treated with cyclophosphamide and BCNU (see Figure 1). (b) Patients treated with cyclophosphamide and TBI. (c) Patients treated with cyclophosphamide and BCNU. Figures show mean values (●), upper (▲) and lower (●) 95% confidence intervals.
in bone

In acrolein-induced depletion of ATase activity in peripheral lymphocytes of patients receiving cyclophosphamide/TBI, the mean pretreatment leucocyte count was 7.04 × 10^9 l⁻¹ and mean post-cyclophosphamide leucocyte count was 3.18 × 10^9 l⁻¹.

ATase depletion in vitro following incubation with cyclophosphamide and its metabolites

The direct effects of cyclophosphamide, acrolein and phosphoramide mustard on ATase were also assessed by incubating the drugs with a fixed amount of pure recombinant human ATase for 2h at 37°C in vitro. Figure 4a shows the dose-response curves for ATase depletion following incubation with the above drugs. No ATase depletion was seen when recombinant human ATase was incubated with cyclophosphamide. By contrast, acrolein was a highly effective inactivator of the enzyme, in that under the conditions used, only 100 μM caused 90% depletion. ATase depletion was also seen with phosphoramide mustard but this was with a concentration far in excess of that achievable in patients receiving the drug (>1 mM) (Jardine et al., 1978; Sladek et al., 1984; Juma et al., 1979).

Increasing concentrations of glutathione were also incubated with 500 μM acrolein which caused 95% depletion of ATase activity in the competition assay above. As shown in Figure 4b, 1 mM glutathione was able to completely prevent acrolein-induced depletion of ATase.

Discussion

In the present study we have shown extensive decreases in ATase activity in peripheral blood lymphocytes of 19 patients receiving cyclophosphamide preparative treatment prior to bone marrow transplantation. Wide interindividual variations in the pretreatment levels and in the rate of ATase loss was noted. In the Hodgkin's patients the effect was so marked that after the 4th cyclophosphamide treatment the median ATase nadir was 32% (range 0 to 57%). In one patient (Figure 1a) no ATase activity was detected following the 4th cyclophosphamide administration. The two patients that showed least overall decrease immediately prior to BCNU demonstrated partial recovery of ATase activity during the cyclophosphamide treatments and this may have contributed to the overall lower ATase reduction (Figures 1a and 1b). Although in some cases, the loss of ATase was minor after administration of BCNU, overall there was a substantial decrease in ATase and this was highly statistically significant with maximal loss occurring between the second and fourth dose of cyclophosphamide (P = 0.0013) and after BCNU administration (P = 0.0018) (Figure 3a). The significant reduction of ATase observed, agrees with that of another study (Gerson, 1989) using 350 mg m⁻² of BCNU.

A similar picture emerged in the other group of 12 patients treated with cyclophosphamide/TBI, with a median post-cyclophosphamide nadir of 39%. In two patients partial recovery of ATase was seen during the cyclophosphamide treatments and prior to TBI (Figures 2b and 2c). In two patients there was some indication that TBI itself was associated with a transient (Figures 2b and 2c), and in one patient, continued (Figure 2c) suppression of ATase activity. Clearly this effect requires substantiation with a large number of patients receiving only TBI. It is interesting to note that in rodents, ATase activity in a number of tissues was increased by a single dose of ionising radiation (Margison et al., 1985; Stammberger et al., 1990).

Changes in the specific activity of peripheral lymphocyte ATase might be the consequence of cyclophosphamide-induced changes in the lymphocyte population: transient increases in ATase activity were seen in some patients but the changes were slight and may have been due to variation in the lymphocyte population. However, in the latter case it has been shown that most individuals have characteristic lymphocyte ATase levels over a short term period and this is therefore unlikely to contribute to the overall picture (Gerson et al., 1985; Sagher et al., 1988). It is also possible that clonal selection may have occurred as a consequence of cytolysis of a lymphocyte population(s) with relatively low ATase specific activity. The possibility that continued cytolysis may have contributed to the consistent decrease in ATase by affecting those lymphocytes with the highest specific activity cannot be excluded since white cell counts had decreased by approximately 50% post cyclophosphamide. Whilst the relative contribution of T and B lymphocytes to the overall ATase measurements was not assessed in this study, previous reports have shown that T and B cells contribute specific activity of 140 fm mg⁻¹ respectively (Gerson et al., 1985). However, the overall changes we have observed are unlikely to be attributable to T or B specific effects since B lymphocytes make up only a small proportion of the total population and the proportion of T and B cells is similar in Hodgkin's, non-Hodgkin's lymphoma and controls (Herrmann et al., 1983).

Another possible explanation for ATase loss is that there is a direct depleting effect on the ATase itself: as far as we are aware, O⁶-alkylguanine lesions have not yet been identified in DNA in vivo after administration of cyclophosphamide or its metabolites. There are two reports (Kleihues & Margison, 1976; Meier et al., 1989) which showed that cyclophosphamide is able to increase the amount of O⁶-methylguanine in DNA following a chosing dose of methylating agent in rodent liver and both authors attributed this to an unidentified O⁶-alkylation product of guanine in DNA which is repaired by ATase and results in ATase depletion. Alternatively, there may be a direct reaction of the cyclophosphamide metabolite acrolein with ATase: when given systemically, cyclophosphamide is metabolised by the hepatic mixed-function oxidases, to 4-hydroxy-cyclophosphamide, the 'transport' form which enters cells and eventually decomposes intracellularly to phosphoramide mustard, the ultimate cross-linking metabolite of cyclophosphamide, and acrolein (Brock, 1989; Sladek, 1987). We have shown that 100 μM acrolein is
able to deplete ATase activity when it was incubated in vitro with pure recombinant human ATase. This may be the result of the affinity of acrolein for sulphydryl groups including, possibly, the alkyl-accepting cysteine residue of the ATase protein. The peak concentration of phosphoramidate mustard achieved in the serum following high dose cyclophosphamide (60 and 75 mg kg⁻¹) was 50–100 μM (Colvin & Chabner, 1990; Jardine et al., 1978; Juma et al., 1979) indicating that the concentration of intracellular acrolein that depletes recombinant human ATase in vitro is potentially attainable in vivo. Phosphoramidate mustard was also able to deplete ATase activity but the concentration required (≥1 mm) was far in excess of that achievable in patients receiving the drug (Colvin & Chabner, 1990; Jardine et al., 1978; Sladek et al., 1984).

The variation in ATase decreases seen in the 19 patients studies following cyclophosphamide treatment may be due to the differential metabolism of cyclophosphamide or related to variations in cellular glutathione and glutathione transferase levels in different individuals, as both are responsible for the intracellular metabolism and detoxification of various cyclophosphamide metabolites (Christe et al., 1990; Draeger et al., 1976; Lee, 1991b; McGown & Fox, 1986). It has previously been demonstrated that the amount of the ultimate active metabolites formed intracellularly is dependent on the intracellular glutathione concentration and its interaction with the toxic metabolites (Lee et al., 1991c). Cyclophosphamide has also been shown to be able to deplete serum glutathione (Michel et al., 1989). High-dose potentially myeloablative chemotherapy and autologous bone marrow transplantation for patients with advanced Hodgkin’s disease. Leukemia, 3, 19–32.

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