Detection of DNA methylation adducts in Hodgkin's disease patients treated with procarbazine

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The aim of the present study was to assess the relationship between dose of the methylating agent procarbazine (PCZ), DNA methylation adduct formation and response to chemotherapy treatment in 23 Hodgkin's disease patients receiving MOPP/ABV combination therapy. The DNA adducts, 7-methyldeoxyguanosine (7-medG) and O^6-methyldeoxyguanosine (O^6-medG), were measured in leucocytes at the end of the first cycle of PCZ treatment (77–100 mg m^{-2} per day). 7-medG was detected in only two patients prior to treatment and O^6-medG was below the detection limit (0.08 μmole per mole dG) in all subjects prior to treatment. The mean levels after PCZ treatment were 12.55 μmole 7-medG per mole dG and 0.254 μmole O^6-medG per mole dG with a 2–3 fold variation between individuals. No correlation was observed between the levels of the two adducts suggesting inter-individual differences in formation and removal of the two adducts. Failure of treatment was observed in five patients and this was not correlated with higher or lower levels of 7-medG or O^6-medG. Other adducts formed as a consequence of treatment with PCZ or other MOPP/ABV components could have more relevance in this respect. The ability to measure DNA methylation adducts at the individual level following exposure to PCZ or other methylating chemotherapeutic drugs (e.g. dacarbazine) could be useful in prospective studies of secondary cancer in Hodgkin's disease patients.

Keywords: *DNA methylation adducts, chemotherapy, procarbazine, molecular epidemiology, Hodgkin's disease.*

Abbreviations: *ABV, doxorubicin, bleomycin, vinblastine; AGT, O^6-alkylguanine DNA alkyltransferase; DTIC, dacarbazine; MOPP, Nitrogen mustard, procarbazine, vincristine, prednisone; PCZ, procarbazine; O^6-medG, O^6-methyldeoxyguanosine, 7-medG, 7-methyldeoxyguanosine.*

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Introduction

Procarbazine [PCZ; *n*-isopropyl-*α*-(2-methylhydrazino)-*p*-toluamide hydrochloride] is used in the treatment of human neoplasms, including Hodgkin's disease, in combinations such as MOPP (nitrogen mustard, procarbazine, vincristine, prednisone) or MOPP/ABV (doxorubicin, bleomycin, vinblastine) (Carde *et al.* 1990). This multi-agent chemotherapy greatly improved the prognosis of Hodgkin's disease patients, although it has also been shown to induce long-term adverse effects, in particular acute leukaemias, non-Hodgkin's lymphoma and some solid tumours (Boffetta and Kaldor 1994). Combination therapy with ABVD (doxorubicin, bleomycin, vinblastine and dacarbazine [DTIC, 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide]) has also been widely used. PCZ is carcinogenic in experimental animals, with the mammary gland as the main target organ (Lee and Dixon 1978), and it has been suggested that the carcinogenic action of PCZ could be related to its ability to induce DNA methylation damage. PCZ, after initial oxidation to azoprocarbazine by cytochrome P450, is further metabolized to generate methylating ions (Prough and Tweedie 1988), which can react with DNA bases. Similarly, DTIC, used in the ABVD combination therapy, is carcinogenic in animals and induces the same DNA methylation adducts as PCZ (Souliotis *et al.* 1991).

The major DNA adduct following exposure to methylating agents, including PCZ, is 7-methyldeoxyguanosine (7-medG). This adduct represents approximately 70% of total initial level of methylation adducts (Singer and Grunberger 1983) and is repaired by the mammalian methylpurine DNA glycosylase, although at a much slower rate than 3-methyladenine (Chakravarti *et al.* 1991). Given the high level of formation and slow repair, 7-medG can accumulate in DNA upon repeated exposure to methylating carcinogens and it has been suggested as a possible marker of recent past exposure to methylating agents (Bianchini and Wild 1994a). O^6-methyldeoxyguanosine (O^6-medG) is formed at approximately 10 times lower levels than 7-medG and is generally subjected to rapid repair in mammalian cells by the O^6-alkylguanine DNA alkyltransferase (AGT) protein, which is inactivated as a consequence of accepting a methyl group from the O^6 position of guanine (Pegg 1990). O^6-MedG is directly mutagenic causing miscoding (GC to AT transition mutations) in DNA following cell replication, and has been suggested to be of major importance in the development of tumours in experimental animals following treatment with methylating carcinogens (Pegg 1984, Dumenco *et al.* 1993). While 7-medG does not appear to be directly mutagenic this DNA adduct could result in mutations indirectly through the formation of apurinic sites after chemical depurination or repair of the modified guanine residues (Loeb *et al.* 1985).

Formation of DNA methylation adducts has been demonstrated in rodents after treatment with PCZ (Wiestler *et al.* 1984, Fong *et al.* 1990, Valavanis *et al.* 1994). In addition, leucocytes of cancer patients treated with PCZ (MOPP therapy) or DTIC, have been shown to contain detectable levels of 7-medG or O^6-medG (Souliotis *et al.* 1990, 1991, 1994, Mustonen *et al.* 1991, van Delft *et al.* 1992, Lee *et al.* 1994). In the present study, the formation of both O^6-medG and

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7-medG in total blood leucocyte DNA of Hodgkin's disease patients following MOPP/ABV chemotherapy was investigated. Such an investigation has two important implications: on the one hand, if the level of DNA adducts following chemotherapy is a determinant of the efficacy of that therapy then the measurement of DNA adducts would be a useful parameter for optimizing treatment modalities. On the other hand, if DNA methylation is also correlated with risk of second cancer development, then the quantification of DNA adducts would enable clinicians to identify those patients at highest risk of therapy-induced malignancy soon after treatment, thus allowing development of the most appropriate follow-up. This second research question would require a long-term prospective investigation and is outside the scope of the present study.

MATERIALS AND METHODS

Patients

A total of 23 Hodgkin's disease patients (see Table 1) receiving MOPP/ABV chemotherapy in the context of the H7 (20881) and H34 (20884) European Organization for Research and Treatment of Cancer (EORTC) randomized clinical trials were enrolled from four treatment centres in Villejuif (12 patients), Lyon (2 patients), Brussels (4 patients) and Athens (5 patients). The EORTC regimen includes the following drugs: nitrogen mustard (mechlorethamine) (day 1, i.v. 6 mg m^{-2}); vincristine (day 1, i.v. 1.4 mg m^{-2}), procarbazine (days 1–7, p.o. 100 mg m^{-2}), prednisone (days 1–14, p.o. 40 mg m^{-2}, doxorubicin (day 8, i.v. 35 mg m^{-2}), bleomycin (day 8, i.v. 10 mg m^{-2}), and vinblastine (day 8, i.v. 6 mg m^{-2}) The patients from Athens received a similar therapy except that prednisone was administered daily at 48 mg m^{-2} rather than 40 mg m^{-2}). The period of inclusion was from March 1990 to April 1992. Complete clinical information was available for only 18 patients. Of these, eight had stage I or II (early) disease and the remaining 10 were stage III or IV (advanced). Information on response to therapy and follow-up was obtained from the EORTC Lymphoma Cooperative Group Trial Office. Response to therapy was evaluated after six cycles of chemotherapy, and classified as follows: complete remission (disappearance of all disease-related symptoms and all measurable disease, normalization of X-ray and CAT-scan), partial remission (decrease of at least 50% of the diameter of initial measurable lesions), progression (involvement of new sites or increase of at least 50% of tumour mass), no change (none of the above). Outcome of follow-up was classified as successful (no recurrence of disease, nor disease-related death) or failure (recurrence or disease-related death during follow-up).

Blood samples

Each patient provided two blood samples (i) before starting the first cycle of chemotherapy; (ii) during the first cycle of chemotherapy, within 2–3 h after the last dose of PCZ (100 mg m^{-2} daily; days 1–7 of the cycle). It should be noted that for the five patients from Athens (patients 9–13, Table 1) PCZ was administered daily as three tablets of 50 mg, one in the morning, noon and evening (corresponding to 77–91 mg m^{-2} per day) and that the second blood sample was collected on the morning of day 7 of the cycle, i.e. prior to the last two 50 mg tablets. If blood sample (ii) could not be taken on day 7, it was obtained between day 8 and 10. A 5 ml aliquot of whole blood was initially frozen (–20 °C) and then transferred to –70 °C until DNA extraction from leucocytes and analysis of O^6-medG (Souliotis *et al.* 1990). A 20 ml aliquot of blood was taken for isolation of leucocytes after precipitation of red blood cells by a gelatin solution, as previously described (Bianchini and Wild 1994b). Leucocytes were stored frozen at –70 °C prior to DNA extraction (Bianchini and Wild 1994b) and Rnase treatment (Bianchini *et al.* 1993) for analysis of 7-medG.

Patient	Stage HD[a]	Sex	Age	Response[b]	Failure[c]
1	Adv	F	35	CR	No
2	Early	F	32	CRU	Yes
3	Adv	M	62	CR	Yes
4	Adv	M	46	CR	No
5	Adv	M	33	CR	No
6	Adv	F	29	CR	No
7	Early	M	43	CR	No
8	Early	F	24	PR	No
9	NA	NA	27	CR	No
10	NA	NA	43	CR	Yes
11	NA	NA	60	CR	No
12[d]	NA	NA	38	CR	Yes
13	NA	NA	47	CR	Yes
14	Adv	F	30	CR	No
15	Early	F	24	CR	No
16	Adv	M	25	CR	No
17	Early	M	42	CR	No
18	Early	M	27	CRU	No
19	Adv	F	60	PR	No
20	Early	M	21	CR	No
21	Early	M	25	CR	No
22	Adv	M	24	CR	No
23	Adv	M	32	CR	No

Table 1. Patient characteristics.

[a] Stage HD: Adv = advanced.

[b] Response to initial treatment. CR = complete response, CRU = complete response unconfirmed, PR = partial response (see Materials and Methods for further details).

[c] Median time of failure after treatment was 16 months (range 9–34 months).

[d] This patient was the only recorded death during the study protocol.

NA, not available.

DNA adduct measurement

7-medG was analysed by a method involving the release of the free base 7-methylguanine (7-meG) from DNA by thermal hydrolysis, followed by immunopurification of the adduct and quantitation using HPLC with electrochemical detection (Bianchini *et al.* 1993, Bianchini and Wild, 1994b). This method can detect as low as 100 fmole adduct in any quantity of DNA. The detection limit is thus dependent on the amount of DNA available, being approximately 0.6 μmole 7-medG per mole G with 250 μg DNA, the amount generally available from 20 ml blood. 7-medG was analysed in a total of 17 samples before chemotherapy and 15 samples after chemotherapy, the other samples yielding insufficient DNA for sensitive analysis of this adduct.

O^6-MedG was analysed by a method involving the suicide inactivation of a known quantity of AGT protein when repairing alkylated bases in a DNA sample of unknown O^6-medG content (Souliotis and Kyrtopoulos 1989). Only 10 μg DNA is required for the analysis, the detection limit being 0.08 μmole O^6-medG per mole G. O^6-medG was analysed in 23 samples before and after chemotherapy. The data for the five patients from Athens (patients 9–13) have been reported previously (Souliotis *et al.* 1994).

Statistical analysis

The non-parametric Kruskal–Wallis test was used for testing for differences between mean adduct levels with respect to the day of the second blood sample collection, the centre of treatment, age (as a categorical variable < 30, 30–39

or ≥ 40 years), gender, stage of Hodgkin's disease, response to therapy and treatment failure.

Linear regression analysis was performed to examine the dependency of 7-medG and O^6-medG levels on age (as a continuous variable) and the correlation between 7-medG and O^6-medG levels. Two patients were sampled on days 12 and 43 after the beginning of the cycle of chemotherapy and these were not included in data analysis because of the lateness of sample collection compared with the other subjects.

Results

7-medG and O^6-medG were not detected in white blood cells obtained before treatment, except for two samples where 1.42 (patient No. 5) and 1.53 (patient No. 6) µmole 7-medG per mole dG were found. Figure 1 shows the levels of DNA adducts measured after chemotherapy according to the day of blood sample collection. 7-MedG ranged from 7.91 to 20.80 µmole per mole dG and O^6-medG from 0.173 to 0.349 µmole per mole

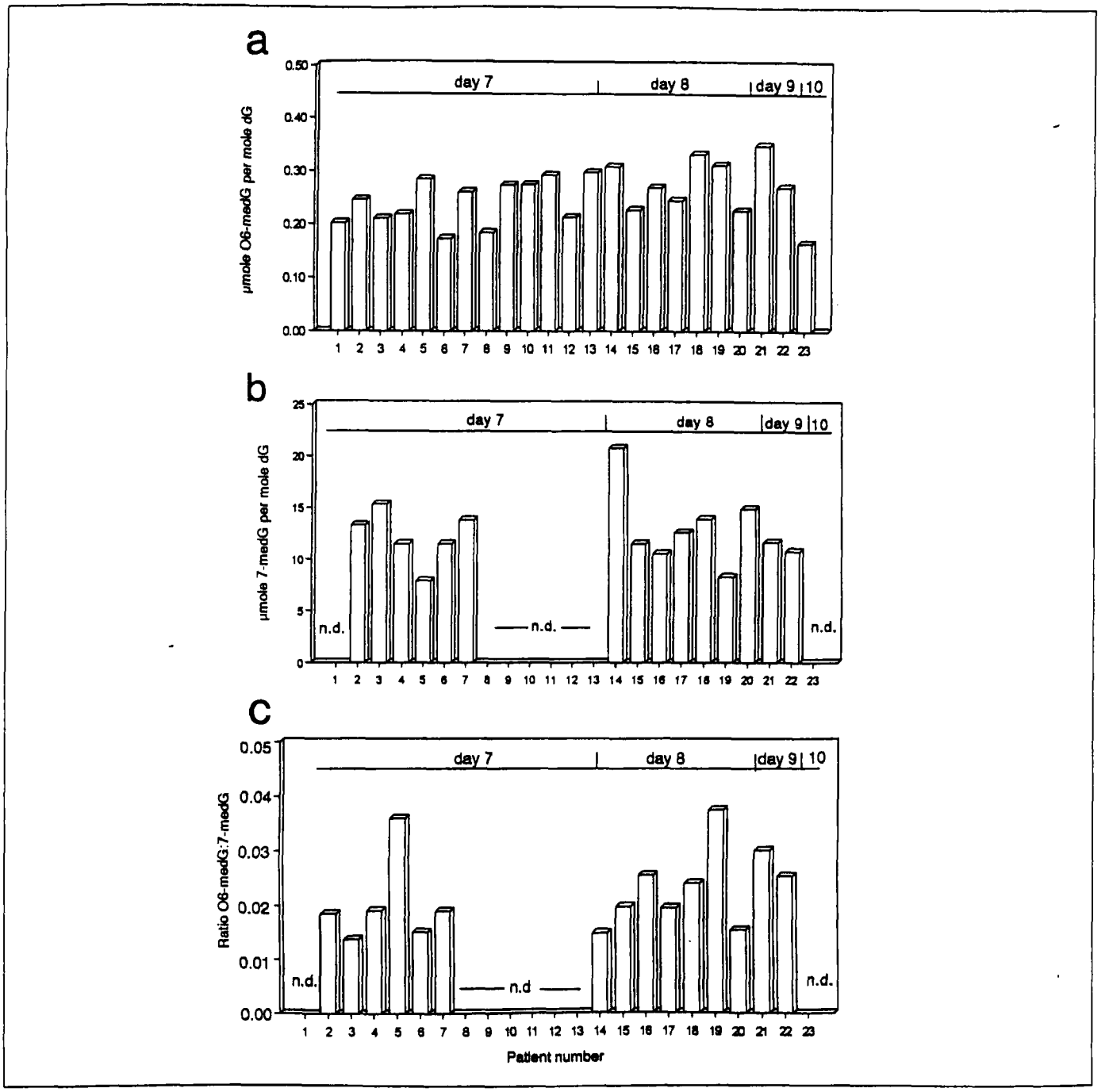

Figure 1. Levels of O^6-medG (panel a), 7-medG (panel b) and their ratio (panel c) according to the day of blood sample collection in Hodgkin's disease patients following chemotherapy with PCZ. n.d., not determined due to insufficient DNA.

dG with mean levels of 12.55 and 0.254 respectively. Thus, there was a narrow range of inter-individual variation (2–3-fold) for both adducts. No correlation between the levels of the two adducts for a given individual was observed ($r = 0.01$, $p = 0.96$). Figure 1 also shows the ratio between O^6-medG and 7-medG for each individual. This value ranges between 0.014 and 0.038 again with a 2–3-fold variation between individuals. Two additional patients were sampled at later time points, one on day 12 (i.e. 5 days post-PCZ treatment) who did not have detectable O^6-medG but still had detectable 7-medG (13 µmole per mole dG), and the other patient (sampled on day 43) had no detectable adducts.

The age and sex of the patient, the stage of disease, the centre of treatment and the day of blood collection were all examined in relation to the two DNA adduct levels, but none of these parameters were found to influence either adduct level. No difference was found in either adduct level according to response to therapy and outcome of treatment (Table 2). For 17 of the patients blood cell profiles were available but none of the parameters, including the number of leucocytes per ml blood, were associated with either DNA adduct level (data not shown).

	O^6-MedG		7-MedG	
	No.	µmole per mole dG	No.	µmole per mole dG
Response to therapy				
Complete remission	12	0.26	12	12.7
Complete remission unconfirmed	2	0.29	2	13.6
Partial remission	2	0.26	1	8.3
p-value	0.61		0.26	
Outcome of treatment				
No failure	18	0.26	13	12.3
Failure	5	0.25	2	14.3
p-value	0.82		0.17	

Table 2. Mean DNA adduct levels by response to therapy and outcome of treatment.

Discussion

In the present paper, 7-medG and O^6-medG were measured in leucocytes of Hodgkin's disease patients before and after treatment with MOPP/ABV combination chemotherapy. Assays used for measurement of 7-medG or O^6-medG were sensitive enough to detect the levels of adduct induced by therapeutic doses of PCZ. In addition to treatment with PCZ, DNA methylation adducts can result from environmental exposure to methylating agents or their precursors (Umbenhauer *et al.* 1985). The measurement made prior to therapy was a control for this environmental exposure while the measurement after the first cycle of therapy provided a measure of PCZ-induced adducts. In this series of patients only two had detectable 7-medG adduct levels prior to therapy and these levels were an order of magnitude lower than that induced by the PCZ treatment. Levels of 7-medG post-treatment (7–20 µmole per mole dG) were in agreement with the values (10–40 µmole per mole dG) reported after treatment with total doses of PCZ and DTIC of 1050 mg to 2800 mg and 1200 mg respectively (Mustonen *et al.* 1991); another study (van Delft *et al.* 1992) however reported higher levels (40–120 µmole per mole dG) after infusion with 200–800 mg m^{-2} DTIC. Levels of O^6-medG (0.16 to 0.35 µmole per mole dG) were in the same range as previous studies where a similar dose of PCZ was administered daily over a 10 day cycle (Souliotis *et al.* 1990, 1994). Higher levels (0.71–14.3 µmole per mole dG) were reported in metastatic melanoma patients infused with 400 mg m^{-2} DITC (Lee *et al.* 1994). The competitive repair assay used to measure O^6-medG in the present study is indirect (Souliotis and Kyrtopoulos 1989) and thus if other chemotherapeutic agents used in the combination therapy induce lesions which inactivate AGT then an overestimation of O^6-medG levels would result. In the current study nitrogen mustard, vincristine and prednisone were administered during the PCZ treatment. None of these are expected to yield significant levels of O^6-medG or other lesions which inactivate AGT. For example, in both Chinese hamster cells, selected for high levels of AGT, and haemopoietic stem cells transfected with *Escherichia coli* AGT there was no increased resistance to the cytotoxic effects of nitrogen mustard or vincristine (Jelinek *et al.* 1988, Morten and Margison 1988), something which would have been expected if lesions recognized by AGT were induced by these drugs. Therefore we currently think that the O^6-medG measurements are unlikely to have been interfered with in this way.

DNA adducts accumulate in leucocytes during chemotherapy and maximum levels occur at the end of the cycle; thereafter, the adducts are lost with differing half-lives (Souliotis *et al.* 1990, 1991, 1994, Van Delft *et al.* 1992). The half-life of 7-medG *in vitro* at pH 7 is approximately 150 h and has been reported to be somewhat shorter, between 50 and 85 h, *in vivo* in white blood cells of cancer patients administered DTIC by i.v. infusion (Van Delft *et al.* 1992). A half-life for O^6-medG of approximately 20 h was reported for patients treated with PCZ (Souliotis *et al.* 1990, 1994). In our study it was interesting that there were no significant differences between 7-medG or O^6-medG levels on day 7, immediately post-treatment and those on days 8 and 9 (Figure 1). One possibility is that repeated doses of PCZ could have led to accumulation of adducts in cells where repair is particularly slow, thus extending the apparent half-life when measures of total adducts in all cell populations are measured. Nevertheless, a patient who was not sampled until day 12 (i.e. 5 days post-PCZ treatment) did not have detectable O^6-medG but still had detectable 7-medG (13 µmole per mole dG), while another patient only sampled on day 43 had no detectable adducts, as expected. The O^6-medG : 7-medG ratio varied about three-fold and ranged between 0.014 and 0.038. The theoretical ratio after a single dose of PCZ is 0.12 (Wiestler

et al. 1984) suggesting, as expected, that 7-medG accumulates to a greater extent than O^6-medG in leucocyte DNA upon repeated exposure.

The inter-individual variation in both 7-medG and O^6-medG levels was approximately 2–3-fold. This is consistent with previous studies of O^6-medG in PCZ treated patients (Souliotis *et al.* 1990, 1994). Nevertheless a study of 21 patients followed for up to 12 cycles of MOPP therapy (Souliotis *et al.* 1994) showed that the inter-individual, rather than the intra-individual, variation accounted for the majority of variance in O^6-medG levels (84.5 versus 8.9%). Thus, significant inter-individual variability in DNA adduct formation does occur in response to MOPP and this could eventually be associated with outcome of therapy or risk of development of a secondary, therapy-induced cancer.

Previously a negative association between pre-therapy AGT level and accumulated O^6-medG level was observed in PCZ treated Hodgkin's patients (Souliotis *et al.* 1990). In experimental and human studies it has been shown that AGT is inactivated by PCZ and DTIC (Meer *et al.* 1989, Lee *et al.* 1991, Fong *et al.* 1992). In addition, AGT levels in rat mammary gland, the target organ for PCZ carcinogensis, are lower than in other organs, including liver and kidney, suggesting that repair capacity affects PCZ carcinogenicity (Fong *et al.* 1990). AGT activity was also lower in lymphocytes of therapy associated acute non-lymphocytic leukaemia (ANLL) patients compared with those in *de novo* ANLL patients and healthy volunteers (Sagher *et al.* 1988). This is consistent with the hypothesis that a low repair capacity could result in a higher risk of secondary cancer, although the repair activites in the latter study were measured at the time of relapse and may not be related to the repair activity at the time of therapy. In the current study we did not measure AGT prior to chemotherapy but the inter-individual variations in the ratio of O^6-medG : 7-medG (Figure 1) could possibly be accounted for by inter-individual differences in AGT levels. Additionally the variation in 7-medG levels, presumably less affected by DNA repair than O^6-medG, indicates that other factors such as PCZ metabolic activation, could also play a role.

Failure of therapy, occurring in five patients, was not related to the levels of either 7-medG or O^6-medG. In addition, no association between DNA adduct levels and age, gender, stage of disease and response to treatment was found. The small number of patients in this study should however be noted.

Results presented here confirm the formation of 7-medG and O^6-medG in Hodgkin's disease patients treated with PCZ. The measurement of the two adducts is complementary, one (7-medG) giving a measure of initial total DNA methylation damage and the other (O^6-medG) being a promutagenic adduct whose level is partially determined by a DNA repair process which could influence individual susceptibility to DNA damage.

The methylating agents PCZ and DTIC are generally administered as part of a combination chemotherapy. As a result the association between initial DNA damage and response to therapy or risk of secondary cancer should include a consideration of the role of the other chemotherapeutic agents involved. To date there is less progress in the determination of DNA adducts resulting from the other agents used to treat Hodgkin's disease. Alternatively biomarkers of early effect, such as cytogenetic alterations or mutations in oncogenes or tumour suppressor genes, would be informative.

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