Modulation and Cellular Distribution of O^6-Alkylguanine-DNA-Alkyltransferase in Normal and Malignant Human Tissues

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by

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Abstract

There is substantial experimental evidence to indicate that the expression of the DNA repair protein, O^6-alkylguanine-DNA-alkyltransferase (ATase) is a major factor in cellular resistance to the cytotoxic efects of a number of anti-tumour methylating and chloroethylating agents and that resistance is achieved through the repair of the potentially toxic lesion, O^6-alkylguanine.

The first part of the thesis examines the effects of dacarbazine (DTIC), CB10-277, temozolomide, fotemustine and BCNU on ATase levels in peripheral lymphocytes and, in some regimen, O^6-methylguanine levels in total leukocyte DNA of patients undergoing chemotherapy for various malignancies. ATase depletion was observed in all regimen but there was a wide inter-individual variation in the initial levels, and rates and extents of depletion and recovery. For DTIC, the ATase nadir was coincident with the peak of O^6-methylguanine formation in leukocyte DNA. In the process of examining the effects of BCNU on ATase levels in peripheral lymphocytes, an unexpected cyclophosphamide-induced ATase depletion was seen and *in vitro* experiments using pure recombinant human ATase suggested that this may have been mediated by the production of acrolein *in vivo*.

In an attempt to increase the response rate in patients with metastatic melanoma, fotemustine was administered 4 h after various doses of DTIC. It appeared that escalation of the DTIC dosage may result in a higher response rate but at the expense of increased haematological and pulmonary toxicity which was probably related to a general depletion of ATase in both normal and tumour tissues.

The second part of the thesis examines the ability of a polyclonal antiserum raised against recombinant human ATase to detect this protein in human tumour sections and, having shown this to be the case the inter- and intra-cellular distribution of ATase in tumour tissues. The antiserum was initially shown to detect the human ATase protein in crude cell-free extracts of melanoma and ovarian tumours on western blots indicating

that the antiserum is highly sensitive and specific. In immunohistochemical staining, the reaction in positively staining cells was intense and predominantly located within the nucleus. Marked cellular heterogeneity of ATase staining could be discerned in many of the tumours examined. In a comparative study, ATase staining intensity was strongly positive in ovarian tumour sections but weak in Hodgkin's lymphoma sections suggesting that the generally observed low clinical response rate of ovarian cancer to the methylating and chloroethylating antitumour agents may be a consequence of high levels of ATase expression in the tumour cells. These studies have established the feasibility of *in situ* detection of ATase using an immunohistochemical technique and, if a relationship to tumour response is established, its use as a prognostic indicator working towards the individualisation of patient treatment.

Declaration

I declare that no portion of the of the work referred to the thesis has been submitted in support of an application for any degree or qualification of this or any other university or other institute of learning.

Signed.....

Date.....22nd April 1994

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Abbreviations

A	Adenine
ABMR	Autologous bone marrow rescue
ABVD	Adriamycin, bleomycin, vinblastine and dacarbazine
AIC	5-Aminoimidazole-4-carboxamide
ALL	Acute lymphoblastic leukaemia
Alk	Alkyl
3-AlkA	N3-alkyladenine
3-AlkG	N3-alkylguanine
7-AlkG	N7-alkylguanine
AlkPT	Alkylphosphate diesters
AML	Acute meyloid leukaemia
ANPG	Alkyl-N-purine-DNA glycosylase
AP	Apurinic
ATase	O^6-Alkylguanine-DNA-alkyltransferase
AUC	Area under concentration-time curve
BCNU	1,3-Bis(2-chloroethyl)-1-nitrosourea (carmustine)
BSA	Bovine serum albumin
C	Cytosine
CB10-277	1-p-Carboxyl-3,3-dimethylphenyltriazene
CCNU	1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine)
cDNA	Complementary DNA
ChlVPP	Chlorambucil, vinblastine, procarbazine and prednisolone
CML	Chronic myeloid leukaemia
CNU	Chloroethylnitrosourea
CPHMT	1-(4-Carboxylphenyl)-3-hydroxymethyl-3-methyltriazene
cpm	Counts per minute
CPMT	1-(4-Carboxylphenyl)-3-methyltriazene
CRC	Cancer Research Campaign
CXR	Chest x-ray
Da	Dalton
DAB	3',3'-Diaminobenzidine-4 HCl
DLCO	Total lung carbon monoxide transfer
DNA	Deoxyribonucleic acid
dG	Deoxyguanosine
dR	Deoxyribose
DTIC	5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (dacarbazine)
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetate
FEV1	Forced expiratory volume in one second
G	Guanine
HD	Hodgkin's disease
HENU	Haloethylnitrosourea
HMTIC	5-(3-Hydroxymethyl-3-methyl-1-triazenyl)imidazole-4 carboxamide
HYBRID	Vinblastine, procarbazine, chlorambucil, vincristine, etoposide, adriamycin, prednisolone
IHC	Immunohistochemistry
i.v.	Intravenous
KCO	Transfer coefficient
kDa	Kilodalton
MAG	3-Methyladenine-DNA glycosylase

MAO	Monoamine oxidase
Me	Methyl
3-MeA	N3-methyladenine
3-MeG	N3-methylguanine
7-MeG	N7-methylguanine
MMS	Methyl methanesulphonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MOPP	Mechlorethamine, vincristine, procarbazine and prednisolone
mRNA	Messenger RNA
MTD	Maximum tolerable dose
MTIC	5-(3-Methyl-1-triazenyl)imidazole-4-carboxamide
MVPP	Mechlorethamine, vinblastine, procarbazine and prednisolone
NBU	N-nitroso-N-butylurea
NDMA	N-nitrosodimethylamine
NEU	N-nitroso-N-ethylurea
NHL	Non-Hodgkin's lymphoma
Ni-DAB	Nickel-complexed diaminobenzidine
NMU	N-nitroso-N-methylurea
mRNA	Messenger ribonucleic acid
O^4-AlkT	O^4-alkylthymine
O^6-AlkG	O^6-alkylguanine
O^6-BeG	O^6-benzylguanine
O^6-MedG	O^6-methyldeoxyguanosine
O^6-MeG	O^6-methylguanine
P	Probability
PBS	Phosphate-buffered saline
R	Correlation coefficient
RNA	Ribonucleic acid
RV	Residual volume
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	Standard saline citrate
T	Thymine
TBI	Total body irradiation
TBS	Tris-buffered saline
TCNU	1-(2-Chloroethyl)-3-(2-(dimethyl-amino-sulphonyl) ethyl)-1-nitrosourea (tauromustine)
TEMED	NNN'N'-tetramethylethylethylenediamine
TLV	Total lung volume
Tris	Tris(hydroxymethyl)methylamine
UV	Ultra-violet
VAPEC-B	Vincristine, adriamycin, prednisolone, etoposide, cyclophosphamide, bleomycin
VC	Vital capacity
WBC	White blood cell
WHO	World Health Organisation

Publications arising from this thesis.

Copies are to be found in the Appendix

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This thesis is dedicated to

Monica, Lennard and Julian

One of the major obstacles to more effective chemotherapy for advanced malignancies is the presence of intrinsic or acquired drug resistance. Many specific mechanisms of cellular drug resistance have been described including reduced uptake of drugs into cells, defective metabolism of drugs to active compounds, increased intracellular degradation, alteration of target proteins and increased efficiency of DNA repair (Devita et al. 1989; Vendrick et al. 1992).

While the precise nature of the critical DNA lesions that primarily contribute to cytotoxicity has not been elucidated for many antitumour alkylating agents, a number of *in vitro* and *in vivo* experiments have demonstrated that an important determinant of alkylation resistance to antitumour methylating and chloroethylating agents is related to the cell's ability to repair alkylation damage incurred at the O^6-position of guanine in DNA. Alkylation occurring at this site can be very efficiently removed by a specific DNA repair enzyme, O^6-alkylguanine-DNA-alkyltransferase (ATase) which forms the basis of this thesis.

1.1 Alkylating Agents

Alkylating agents are defined as chemicals that transfer an alkyl group onto a nucleophilic site. They comprise of a diverse group of chemicals which can produce a wide range of biological effects in prokaryotes and eukaryotes including mutation, clastogenesis, teratogenesis, carcinogenesis and cytotoxicity and this latter effect has been exploited in the development of antitumour agents. In cancer chemotherapy, five major types of alkylating agents are used in the treatment of neoplastic diseases: the nitrogen mustards, the ethylenimines, the alkyl sulfonates, the nitrosoureas, and the triazenes. They have in common the property of forming a reactive, probably a positively charged carbonium ion which then attacks nucleophilic (electron-rich) sites in nucleic acids, proteins, and small molecules, such as sulfhydryl groups and amino

acids: the cytotoxic effects of these agents are thought to be related to alkylation of DNA. The N-nitroso compounds, from which the nitrosoureas are derived, will be discussed initially as they are the best studied and understood of the alkylating agents. They are therefore ideal model compounds for understanding the metabolism, activation and mechanisms of action of the antitumour alkylating agents that form O^6-alkylguanine as one of the DNA alkylation product, referred to in this thesis as O^6-alkylating agents.

1.1.1 The N-nitroso compounds

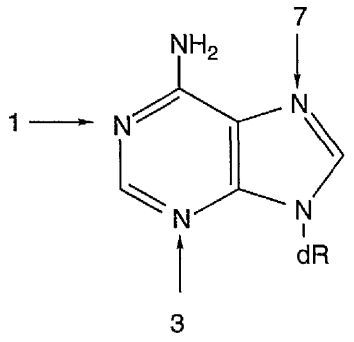
Under physiological conditions, the nitrosamides e.g. N-nitroso-N-methylurea (NMU) are unstable and undergo spontaneous, base catalysed hydrolysis in aqueous solution to generate the electrophilic and highly reactive intermediate. Nitrosamides cause most damage at the site of application, particularly when low doses are used, because of their spontaneous decomposition to generate the reactive chemical species. However, intravenous administration, in achieving a rapid distribution, can give rise to similar levels of alkylation of DNA in several different tissues (Goth & Rajewsky, 1974; Margison and Kleihues, 1975).

In contrast, the nitrosamines e.g. N-nitrosodimethylamine (NDMA) are chemically stable compounds under physiological conditions and require metabolic activation to generate an alkylating species. The initial step is α-carbon hydroxylation mediated by a cytochrome P_{450}-dependent mixed function oxidase system. The hydroxylated product is chemically unstable and spontaneously decomposes in a stepwise manner similar to nitrosamides to form the reactive carbonium ion (Druckrey 1972). The liver is the main site of alkylation following any route of administration since metabolism occurs predominantly in this organ. However, other tissues including lung, kidney and lymphocytes (Gupta et al. 1988) are also capable of metabolism and are therefore targets of alkylation damage.

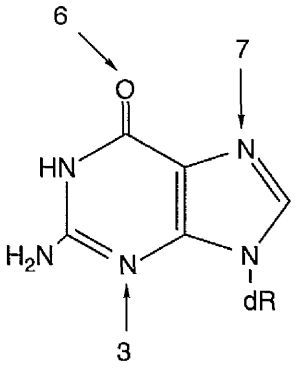
Certain antitumour alkylating agents, e.g. dacarbazine, CB10-277 and cyclophosphamide, also need to be metabolically activated by the cytochrome P_{450} system, while others agents are directly active compounds, e.g. nitrosoureas, temozolomide and nitrogen mustards.

1.1.2 Reaction products in DNA

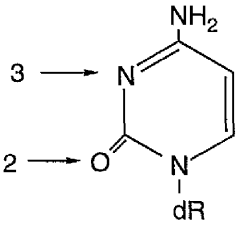
The reaction products in DNA treated with monofunctional methylating and ethylating agents have been characterised. When activated these agents attack a variety sites on DNA molecules (Singer and Grunberger, 1983; Saffhill et al. 1985; Beranek 1990), with alkylation occuring at the N1, N3, and N7 positions of adenine; the N3, N7, and O^6 of guanine; the N3 and O^2 of cytosine; the N3, O^4, and O^2 of thymine and 2 stereoisomers of alkylphosphate diesters (AlkPT) generating in total, 13 different alkylation products (Fig. 1.1). The relative amounts of each DNA adduct formed vary according to the alkylating agents used and examples of this are shown in Table 1.1. The overall degree of reaction generally declines with increasing molecular weight of the alkylating species; thus, N-nitroso-N-ethylurea (NEU) reacts with an order of magnitude less than NMU when present in the same concentration. The reactions of higher alkylating agents (e.g. propylating agents) with DNA have been examined in less detail than methylating and ethylating agents, but the spectrum of products formed resembles those produced by the ethylating agents. Among these reaction sites, the N7 position of guanine is one of the major targets of alkylation in *in vitro* experiments using several alkylating agents but with the higher alkyl groups much higher relative amounts of AlkPT are formed (see Table 1.1; Pegg 1983).



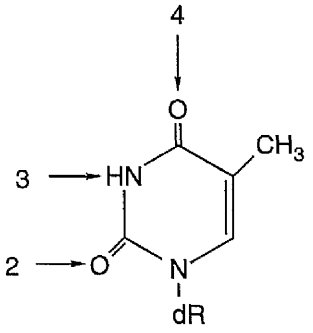
Adenine
N1
N3
N7



Guanine
N3
N7
O^6



Cytosine
N3
O^2



Thymine
N3
O^2
O^4

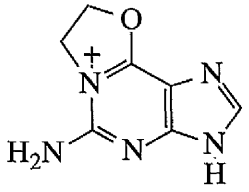
Figure 1.1. Sites of alkylation of DNA bases indicated by the arrows. dR represents the deoxyribose residue. The relative amounts of these products are shown in Table 1.

Table 1.1 Relative amounts of alkylation products formed in DNA by various N-nitroso-compounds

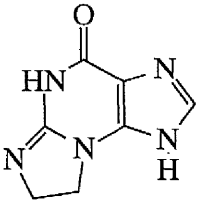
Alkylation site	NMU	NEU	NBU
		% of total alkylation	
Adenine			
N1	0.8	0.2-0.3	nd
N3	8.2	2.8-5.6	2.3
N7	0.2-0.8	0.3-0.6	0.7
Cytosine			
O^2	0.1	2.7-2.8	nd
N3	0.5	0.2-0.6	0.6
Guanine			
N3	0.8	0.6-1.6	0.9
O^6	5.9-7.7	7.8-9.5	6.1
N7	66-70	11.0-11.5	66.8
Thymine			
O^2	0.1	7.4-7.8	0.1
N3	nd	0.8	nd
O^4	0.7	1.0-2.5	0.4
AlkPT	12.1	55.0-57.0	12

Data from O'Connor, 1981; Singer and Grunberger, 1983; Den Engelse et al, 1986.
nd = indicates adduct below limits of detection.
NBU: *N*-nitroso-*N*-n-butylurea.

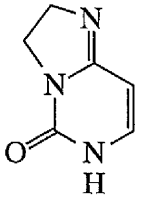
Progress has also been made in identifying the products obtained after reaction of DNA with bifunctional agents such as haloethylnitrosoureas (HENU) which include the anti-tumour chloroethylnitrosoureas (CNUs). The products are much more complex because under aqueous conditions, HENU decomposes to several reactive intermediates capable of alkylating DNA bases and the phosphate group (see review by Ludlum 1990). The N-7 position of guanine is the primary site of action resulting in the formation of hydroxyethyl and chloroethyl adducts (Gibson et al. 1985a; Bodell et al. 1988). HENUs react with other nucleophilic sites, such as O^6-guanine, N3-adenine and N3-cytosine (Ludlum 1990) and can also form ethano-derivatives with an additional heterocyclic ring (see Fig. 1.2). These are formed when an initial alkylation occurs at one of the positions involved in the attachment of the ethano bridge and ring closure takes place at the other position. This can occur with all DNA bases except thymine and three of the derivatives have an ethano bridge between a ring nitrogen and an exocyclic nitrogen whilst the fourth derivative, 1,O^6-ethanoguanine, has an ethano bridge between a ring nitrogen and the exocyclic oxygen (Fig. 1.2).



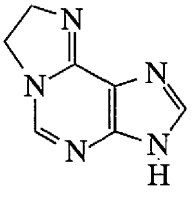
1,O^6-ethanoguanine



N^2,3-ethanoguanine



3,N^4-ethanocytosine

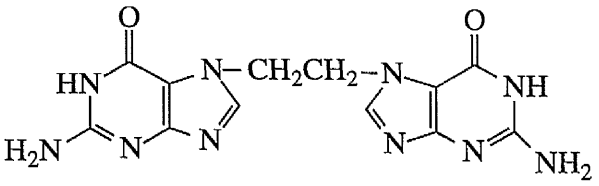


1,N^6-ethanoadenine

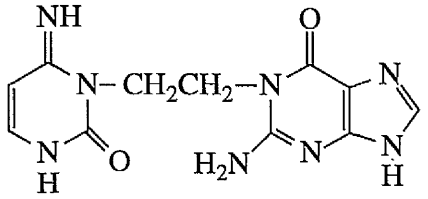
Figure 1.2. Heterocyclic DNA modifications formed by reaction with haloethylnitrosoureas.

Two BCNU-induced DNA cross-linked structures have been identified from *in vitro* studies (Fig. 1.3): one cross-linked structure, 1,2-bis(7-guanyl)ethane is thought to be

formed by initial attachment of the chloroethyl group to the 7 position of guanine and this subsequently reacts with a neighbouring guanine, probably within the same strand of DNA (Tong et al. 1981). The other cross-linked structure, 1-(3-cytosinyl)-2-(1-guanyl)ethane occurs by an initial chloroethylation at the O^6-position of guanine in DNA (see Fig. 1.4, step 1). This rapid step is followed by internal cyclization leading to the formation of an unstable intermediate, 1,O^6-ethanoguanine (Fig. 1.4, step 2) and then by a slower reaction of the intermediate with the paired cytosine on the opposite DNA strand to form an ethano bridge between the two bases (Fig. 1.4, step 3) (Tong and Ludlum 1982, Tong et al. 1983).



1,2-bis-(7-guanyl)-ethane



1-(3-cytosinyl)-2-(1-guanyl)-ethane

Figure 1.3. Cross-linked DNA bases formed by reaction with haloethylnitrosoureas.

This proposed scheme is supported by observations that human extracts containing ATase can suppress the formation of BCNU-induced interstrand cross-links (Robins et al. 1983; Brent 1984) and of 1-(3-cytosinyl)-2-(1-guanyl)ethane in isolated DNA treated with chloroethylating nitrosoureas (Ludlum et al. 1986). It has recently been shown that ATase can also react with 1,O^6-ethanoguanine (Gonzaga and Brent 1989; Brent et al. 1991; Gonzaga et al. 1992) but the biological significance of this has yet to be established. It may be that the ATase would repair the initial O^6-chloroethylguanine

adduct so rapidly that very little of the cyclic adduct would be formed in ATase-expressing cells.

Recently, it has been shown that alkylation of the N7 position of guanine is not completely at random; alkylation by chloroethylating agents and triazeno compounds occurs preferentially at the central guanine in runs of three or more guanine residues (Hartley et al. 1986, 1988).

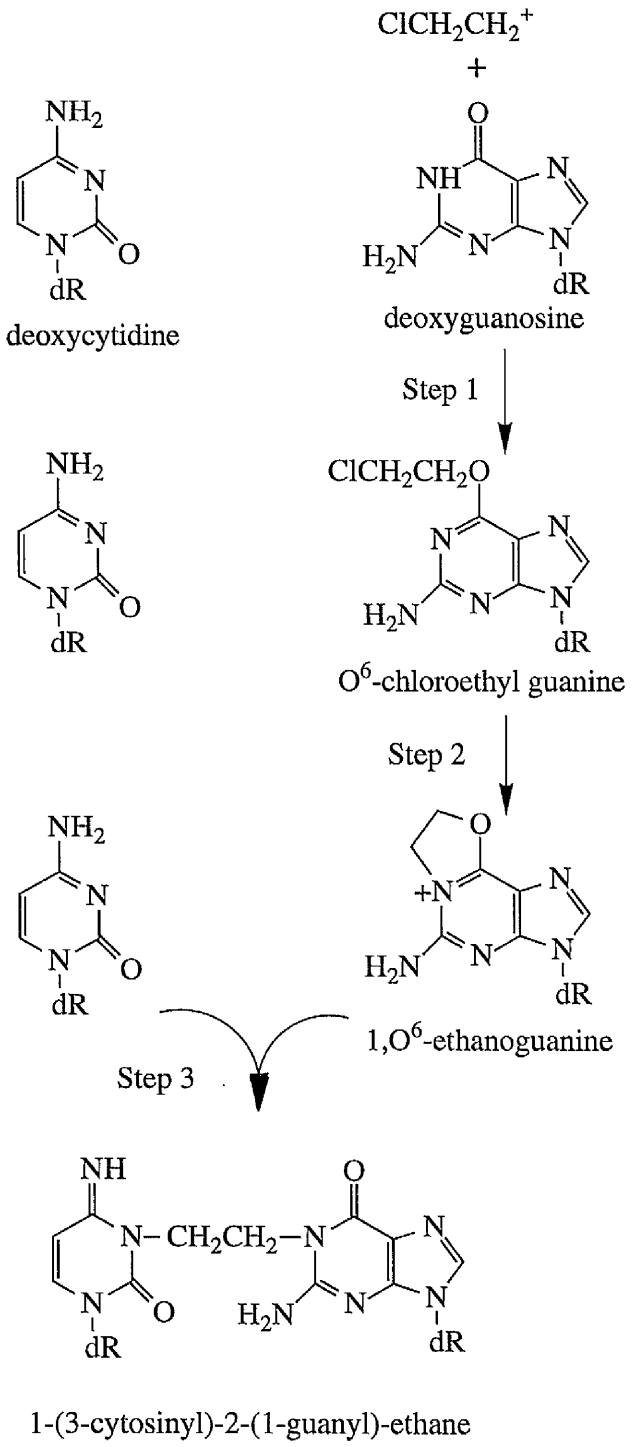


Figure 1.4. Mechanism of formation of the cross-link, 1-(3-deoxycytidyl)-2-(1-deoxyguanosinyl)ethane in DNA.

1.1.3 Biological consequences of DNA alkylation

Starting with the pioneering work of Swann and Magee (1968), Loveless (1969) and Goth and Rajewsky (1974), there is now a considerable amount of evidence to indicate that of the 13 DNA products formed by the alkylating agents, O^6-alkylguanine (O^6-AlkG) and O^4-alkylthymine (O^4-AlkT) are of the greatest biological importance. Loveless (1969) proposed that alkylation at the O^6-position of guanine should cause a mutation during DNA replication resulting from mispairing with thymine instead of with the normal complementary base cytosine. This was later confirmed in studies using bacterial and mammalian cells showing that the presence of O^6-methylguanine (O^6-MeG) in DNA leads to the induction of mutations which are G:C → A:T transitions (reviewed in Saffhill et al. 1985; Margison and O'Connor 1990a). Similarly, the presence of O^4-AlkT leads to the misincorporation of guanine and the production of A:T → G:C transitions (Saffhill et al. 1985; Margison and O'Connor 1990a). The miscoding ability of these lesions was initially thought to be a consequence of increased H-bond stability between O^6-AlkG:T and O^4-AlkT:G base-pairs as compared to O^6-AlkG:C and O^4-AlkT:A, respectively but recent results suggest that conformational restraints imposed by the DNA double helix structure which favour the pairing of the abnormal base-pairs are responsible for miscoding (Swann 1990).

Evidence in support of the carcinogenic potential of O^6-AlkG in DNA was first produced in rodent models showing that the induction of brain tumours by nitrosamides correlated with the poor ability of this tissue to repair O^6-AlkG (Goth and Rajewsky 1974; Margison and Kleihues, 1975). In addition, comparative studies in rat liver showed that the increased persistence of O^6-AlkG in non-parenchymal versus parenchymal cells correlated with the induction of angiosarcoma and not hepatocellular carcinoma by dimethylhydrazine (Bedell et al. 1982). Chronic administration of N-nitrosodiethylamine which results in O^4-ethylthymine accumulation in hepatocyte DNA was associated with liver cell carcinoma; in contrast, O^6-ethylguanine was apparently rapidly repaired probably by ATase, the activity of which was increased by chronic exposure (Swenberg et al. 1984; Dryoff et al. 1986). More extensive correlations of

carcinogenicity following single or chronic treatments with a variety of N-nitrosocompounds are covered in the review by Saffhill et al. (1985).

The mechanism of tumour initiation by alkylating agents may in some case be related to the activation of oncogenes or inactivation of tumour suppressor genes and this is best exemplified by the Ha-*ras* gene in NMU-induced mammary tumours where activating G to A transitions occur exclusively in the second G of codon 12 (Sukumar et al. 1983; Zarbl et al. 1985). A number of similar mutation involving K-*ras* or Ha-*ras* oncogenes has also been seen with other tumour types in experimental rodent models following treatments with a number of N-nitroso-compounds (Topal 1988; Belinsky et al. 1989; Wang et al. 1990; Pegg 1990). A high incidence of G to A transition mutations in the *p53* gene clustered in codons 204 and 213 was recently reported in a variety of rat tumours induced by N-nitroso compounds (Ohgaki et al. 1992). It is therefore not unreasonable to suggest that some of the second malignancies seen in patients treated with O^6-alkylating agents may be related to *ras* or *p53* mutations.

Alkylation at the O^6-position of guanine also appears to be involved in the cytotoxicity of both the methylating and chloroethylating agents. The earliest evidence comes from studies showing that cell lines deficient in the repair of O^6-AlkG and in the reactivation of methylated adenovirus (termed Mer^- or Mex^-) were hypersensitive to the killing effects of these agents in contrast to repair proficient cell lines (termed Mer^+ or Mex^+) (Day et al. 1980 a, b; Erickson et al. 1980 a, b; Sklar and Strauss 1981). Subsequently, Yarosh et al. (1983) showed that the repair proficient cell lines expressed high levels of ATase. There is general acceptance that O^6-chloroethylguanine produced in DNA by the CNUs exerts its cytotoxic effect by the formation of DNA interstrand cross-links (see Section 1.1.2). Thus, Erickson et al. (1980 a,b) demonstrated there was a good correlation between the number of DNA interstrand crosslinks formed and the cytotoxicity produced by BCNU in cell culture studies. The toxic effects of the DNA cross-links are probably the result of the inability of the strands to separate during DNA replication (Kohn 1983).

However, the molecular mechanism by which O^6-MeG mediates its cytotoxic effect is not understood. It has been proposed that it may block the binding of transcription factors resulting in the disruption of gene expression (Bonfanti et al. 1991). Another suggestion that has been put forward is that the O^6-MeG:C and O^6-MeG:T base pairs are recognised by the mismatch repair proteins resulting in DNA strand breaks: supporting this is the finding that in human fibroblasts and HeLa cells, alkylation sensitivity is correlated with increased rates of strand breakage, sister chromatid exchange and intrachromosomal recombination (Kalamegham et al. 1988; Maher et al. 1990).

N3-alkyladenine (3-AlkA) is another potentially toxic lesion produced in DNA by alkylating agents (Lawley and Warren 1976; Laval 1977; McCarthy et al. 1984). The strongest evidence for the cytotoxic effects of 3-AlkA in DNA comes from studies which showed that the expression of a transfected 3-methyladenine DNA glycosylase cDNA in various mammalian cell lines has been shown to confer resistance to cell killing by methylating and chloroethylating agents (Klungland et al. 1992; Habraken and Laval 1993; Matijasevic et al. 1993; see Section 1.1.4 below).

With regard to other DNA adducts there is insufficient experimental evidence to conclude that they may be promutagenic or cytotoxic and hence their potential biological importance cannot be assessed at this time (Saffhill et al. 1985).

1.1.4 Repair of DNA Alkylation Adducts

Two major pathways for the repair of DNA damaged by simple alkylating agents have been identified: one involving the release of the damaged base by a glycosylase and the other involving the simple removal of the alkyl group.

In mammalian cells, 3-MeA is removed from DNA by a multifunctional DNA glycosylase (MAG) also known as as alkyl-N-purine-DNA glycosylase (ANPG;

O'Connor and Laval (1991). cDNAs and coding sequences of the MAG cDNA from various mammalian sources including rat (O'Connor and Laval 1990) and human (O'Connor and Laval 1991) have been isolated. The proteins share a significant homology and are substantially different from the bacterial and the yeast enzymes. Mammalian MAG behave more like the inducible *E. coli* AlkA protein rather than the constituitive *E. coli* Tag protein in that the mammalian protein has a broader substrate range (including 7-AlkG, 3-AlkA and 3-AlkG) than the *E. coli* Tag which is specific for 3-AlkA in DNA. The preferred substrate for MAG is double stranded DNA (Gallagher and Brent 1984) and 3-MeA is more efficiently repaired than 7-MeG or 3-MeG (Singer and Brent 1981). The alkylated base is removed from DNA by the enzyme by hydrolysis of the N-glycosylic bond between the deoxyribose and the base leaving an apurinic (AP) site. This is then subjected to base excision repair by the action of an AP endonuclease which excises a region of DNA containing the AP site, producing a gap which is then filled in by the action of DNA polymerase and ligase to form a repair patch (Montesano 1981; Saffhill et al. 1985; Helland et al. 1987).

The second repair process involves the DNA repair protein ATase. As this forms the principal topic of this thesis, it is considered in detail in the Section which follows.

The larger DNA adducts may also be substrates for the nucleotide excision repair system and this may be the predominant pathway of repair for longer and branched chain adducts. Boyle et al. (1987) demonstrated that O^6-butylguanine can be repaired in ATase-deficient cell lines and they attributed this observation to nucleotide excision repair. A similar process appears to act on O^6-ethylguanine (Bronstein et al. 1992).

1.2 O^6-Alkylguanine-DNA-Alkyltransferase (ATase)

O^6-MeG was first demonstrated to be removed from the DNA of *E. coli* by an active process by Lawley and Orr (1970) and it was not until 1980 that Olsson and Lindahl demonstrated that the repair occurred by the transfer of the methyl group from the O^6-

position of guanine to a cysteine residue of an acceptor protein. The first ATase to be isolated and characterized was that derived from the *ada* gene of *E. coli.* The amino-acid sequences of ATases from a variety of species including bacteria, yeast, rodent and human have now been determined (see Section 1.2.3). Both *E .coli* and *B. subtilis* contain a constitutive ATase (*ogt* and *dat* respectively) and an inducible ATase (*ada* and *adaB* respectively). In eukaryotes, only one ATase has been detected. The ATase found in mammalian cells and bacteria have many properties in common, although they differ somewhat in their substrate specificity (reviewed in Pegg 1990). ATase stochiometrically transfers the alkyl group from the O^6-position of guanine and at a lower rate from the O^4-position of thymine to a cysteine residue in the repair protein itself. In the process the protein undergoes irreversible auto-inactivation and the damaged guanine or thymine residue is restored. The fate of the mammalian alkylated ATase protein has not yet been studied in detail, but recent studies using antibodies indicate that human protein is very unstable both *in vivo* and in cell extracts (Pegg et al. 1991a), suggesting that the alkylation of the cysteine acceptor site leads to a conformational change in the protein that renders it very susceptible to proteolytic degradation.

The ATase has a greater activity on double stranded DNA than on denatured or single stranded DNA (Pegg et al. 1983; Harris et al. 1983) and exhibits greater affinity towards short rather than long chain alkyl adducts. Its preferred substrate is O^6-MeG in double stranded DNA but longer chain alkyl groups can also be removed. The rate of repair decreases with the size of the alkyl group along the series ethyl-, n-propyl-, and n-butyl-. The repair of the branched chain isopropyl-, isobutyl-, and 2-hydroxyethyl groups are very much slower than the linear groups (Morimoto et al. 1985; Pegg et al. 1985). As discussed earlier, O^6-chloroethylguanine and 1,O^6-ethanoguanine, the cross-links precursors formed by CNUs are also repaired by ATase (sec Section 1.1.2).

The mammalian ATase requires no cofactors and has an optimal pH of about 7.8-8.5 (Pegg et al. 1983; Boulden et al. 1987). The ATase is strongly inactivated by a number

of compounds that might be expected to interact with the reactive cysteine at the acceptor site, including metals such as Zn^{2+}, Cd^{2+}, and Hg^{2+} and by the direct action of alkylating agents and aldehydes (reviewed in Pegg 1990). The extent to which these reactions might occur in cells exposed to anti-tumour agents and enviromental toxic agents such as cigarette smoke is not known.

1.2.1 Cellular and tissue distribution of ATase

ATase activity is expressed at vastly different levels. Variations in excess of 1000-fold are seen in a wide variety of mammalian systems and human tissues and cells generally contain considerably higher amounts of ATase than the comparable rodent cells (Gerson et al. 1986). There is also a striking difference in the content of the ATase between different organs and cell types therein. In general the highest ATase activities are found in the liver and the lowest in brain, bone marrow, bladder and small intestine. Other organs such as kidney, spleen and lung have intermediate levels (Gerson et al. 1986; D'Incalci et al. 1988; Badawi et al. 1994). In rat liver repair capacity is much greater in the hepatocytes than in non-parenchymal cells (Swenberg et al. 1985) and similar differences are present in different cell types isolated from the lungs, with Clara cells having particularly low ATase activity (Belinsky et al. 1988). Such differences may be of great importance in the sensitivity to carcinogenesis or killing by alkylating agents.

Most studies with human tumours have indicated that, although there was a wide variation in the activities, most samples did contain measurable ATase activitity. Wiestler et al. (1984a) in a survey of 23 brain tumours found ATase activity in all samples examined and this was also the case in the tumours examined by Myrnes et al. (1983) and D'Incalci (1988). Frosina et al. (1990) found two ATase "deficient" samples in a survey of 27 brain tumours and a relationship was seen between different histological subtypes and ATase activity; meningiomas had the highest ATase activity, glioblastomas, astrocytomas and oligodendrogliomas, intermediate activity and

lymphomas, the lowest activity. In a larger survey of 63 human tumours, Citron et al (1991) found six ATase "deficient" tumours out of 27 brain tumours (20%) and two ATase "deficient" tumours out of 15 lung tumours. There was no correlation in the same tumours with the absence of another DNA repair enzyme, MAG, suggesting that the lack of measurable ATase activity was not due to deterioration of tissue samples.

Another study examining ATase activity in extracts of liver tumours and adjacent normal livers from 21 patients found that all normal liver samples and most liver tumours contained a high level of ATase activity (Isowa et al. 1991). In a more recently reported survey of 74 human tumours, only 6 samples were found to have undetectable ATase activity (1/24 non-small cell lung cancer, 3/5 oesophageal cancer; 1/14 brain tumours and 1/5 colon carcinoma) whereas ATase activity was found in all breast cancer, small cell lung cancer, renal cell carcinoma and malignant melanoma examined (Chen et al. 1992).

In a comparison of ATase activity between normal and colorectal tumour tissue from 38 human samples, it was found that ATase activity was significantly greater for cancers than normal tissues (Citron et al. 1992) but this was not borne out in the Manchester study with 68 colon and stomach tumour samples (Margison et al. 1990b). In another comparative study between normal and neoplastic breast tissues, no significant difference in ATase activity was detected between the non-neoplastic and neoplastic breast tissues (Cao et al. 1991).

In contrast to the low incidence of ATase-deficient phenotypes in both normal and tumour tissues, ATase activity was absent in about 20% of the cultured human tumour cell lines in one study (Day et al. 1980b; Day et al. 1987) and in about one-third of human lymphoblastoid cell lines immortalized by Epstein-Barr viruses (Sklar and Strauss 1981). Transformation by other DNA and RNA viruses, such as SV40, Rous sarcoma virus and adenovirus have also to be reported to downregulate ATase expression in human cells (Day et al. 1980a; Heddle and Arlett 1980; Yarosh 1983;

Sklar and Strauss 1983). SV40 transformation of some murine cells also led to the appearance of cells lacking ATase activity (Yagi et al. 1984). The mechanisms by which these changes in ATase activity occur are not known.

1.2.2 Cytoprotective role of ATase

There is increasing evidence to suggest that endogenous ATase expression can confer cellular resistance to the O^6-alkylating anti-tumour agents. This evidence is summarised as follows.

a) As discussed earlier, resistance to cell killing by methylating or chloroethylating agents in a number of tumour cell lines has been shown to correlate with high ATase activity (Day et al. 1980b; Scudeiro et al. 1984a, b; Gibson et al. 1986a, b; Catapano et al. 1987; Tisdale 1987; Maher et al. 1990; Gerson et al. 1992; Morten et al. 1992). It has also been reported that ATase activity correlates with the therapeutic response to the O^6-alkylating agents of human tumour xenografts in nude mice (Brent et al. 1985; Watatani et al. 1985; Fujio 1989; Schold et al. 1989; Foster et al. 1990).

b) ATase proficient cells can be rendered sensitive to treatment with O^6-alkylating agents by pretreatment with non-toxic doses of methylating agents (e.g. streptozotocin) presumably by the production of an amount of O^6-MeG that depletes cellular ATase, such that on subsequent treatment with another O^6-alkylating agent, little or no repair of the toxic O^6-MeG or the O^6-chloroethylguanine lesions occurs (Zlotogorski and Erickson 1984; Gibson et al. 1986c; Zeller et al. 1986; Erickson et al. 1988; Futscher et al. 1989; Gerson 1989).

c) Exposure of ATase-proficient cells to the free base, O^6-MeG (Dolan et al. 1985a, b; Karran 1985) or O^6-benzylguanine (O^6-BeG) (Dolan et al. 1990) can also render the cells sensitive to the O^6-alkylating agents (Dolan et al. 1985a, 1991; Yarosh et al. 1986; Gerson et al. 1988; Baer et al. 1993). In this case the free base acts as a direct substrate

for the ATase protein causing its inactivation thereby leaving less ATase available for the repair of the lesions introduced by the O^6-alkylating agents (Dolan et al. 1985b; Yarosh et al. 1986; Gerson et al. 1988). More recently, it was demonstrated that depletion of ATase-proficient tumour xenografts by prior exposure of nude mice to O^6-BeG sensitised the xenografts to subsequent treatment with chloroethylating agents (Friedman et al. 1992; Mitchell et al. 1992a; Dolan et al. 1993; Felker et al. 1993; Gerson et al. 1993).

d) The strongest evidence for the cytoprotective role of ATase comes from ATase cDNA transfection experiments which show that expression of prokaryotic or eukaryotic ATase cDNAs in mammalian cells protects them against the toxic effects of these agents (Brennand and Margison 1986; Ishizaki et al. 1986; Kataoka et al. 1986; Samson et al. 1986; Jelinek et al. 1988; Kaina et al. 1991; Wu et al. 1991).

1.2.3 Mammalian ATase genes

The human ATase gene has been cloned as a cDNA by three independent groups (Hayakawa et al. 1990; Rydberg et al. 1990; Tano et al. 1990). The cDNA was used to map the human ATase gene to chromosome 10 (Rydberg et al. 1990) and subsequently to the tip of the long arm (Gardner et al. 1991; Natarajan et al. 1992). The genomic sequence for human ATase is composed of five exons, intervened by long introns and spans at least 170 kb (Shiraishi et al. 1992). The mRNA for human ATase is about 0.95-1.0 kb long (Tano et el, 1990; Fornace et al. 1990; Pieper et al. 1990). The molecular weight of the product protein is 22 kDa (Hayakawa et al. 1990; Rydberg et al. 1990; Tano et al. 1990). The promotor region of the human ATase has also been characterized (Harris et al. 1991).

More recently, ATase cDNAs have been cloned from rat (Potter et al. 1991; Sakumi et al. 1991; Rahden-Staron and Laval 1991), mouse (Santibanez-Koref et al. 1992; Shiota et al. 1992; Shiraishi et al. 1992) and Chinese hamster (Rafferty et al. 1992).

Alignment of the deduced amino acid sequences of the various cloned prokaryotic and eukaryotic ATase cDNAs has revealed that there are extensive sequence homologies between the mammalian but not bacterial ATase proteins. The derived amino acid sequence from rat (Potter et al. 1991) and mouse (Santibanez-Koref et al. 1992) ATase cDNA demonstrated 81% and 70% similarity respectively with the human ATase. Even higher homologies were found in the mouse and rat sequences which were 91.5% identical. In contrast to this homology among the mammalian proteins, homology with the bacterial ATases is mostly limited to the region which spans the alkyl-acceptor cysteine residue; in particular, the amino acid sequence, -Pro-Cys-His-Arg-Val- is conserved in all except the yeast ATases (Xiao et al. 1991). The methyl acceptor site is located at the cysteine residue in position 145 of the human ATase (Tano et al. 1990; von Wronski et al. 1991). Despite a close similarity in the active site in the amino-acid sequences, the *ada* ATase from *E. coli* is not inactivated whereas mammalian proteins are inactivated by very low concentrations of O^6-BeG (Dolan et al. 1991; Elder et al. 1994). *E. coli ogt* ATase is only inactivated by O^6-BeG at high concentration (Elder et al. 1994).

The sequence similarity among the mammalian proteins may anticipate a high degree of antigenic cross-reactivity. Indeed, two polyclonal antibodies raised against human ATase and rat ATase recognise all mammalian ATases so far tested by western analysis (Santibanez-Koref et al. 1992). Similar findings were noted by another group using three monoclonal antibodies raised against human ATase which recognise the mouse ATase in immunoblots (Shiota et al. 1992). Other anti-peptide antibodies which detect human ATase on westerns have not been assessed in this respect (Pegg et al. 1991b; Ayi et al. 1992). In liquid hybridization experiments, anti-human ATase polyclonal antibodies inhibited human but not rat or mouse ATase, whereas anti-rat polyclonal antibodies inhibited rat and mouse but not human ATase (Santibanez-Koref et al. 1992).

1.2.4 Regulation of ATase gene expression

Several reports indicate that control of ATase expression lies at the transcription level as ATase activity in general correlates with mRNA levels (Fornace et al. 1990; Ostrowski et al. 1991; He et al.1992; Citron et al. 1992). The ATase gene appears to be present in Mer$^-$ mammalian cells, and no gross deletions, rearrangements or amplifications have been detected (Pieper et al, 1990; Tano et al. 1990; Vlahos et al. 1990; Ostrowski et al. 1991; He et al. 1992). The high frequency with which the ATase-deficient lines arise and the occasional instability of the phenotype in culture (Strauss, 1990; Arita et al. 1990; Karran et al. 1990) suggests that silencing might be an epigenetic process. One potential factor that may contribute to the control of transcription of the human ATase gene is DNA cytosine methylation status. Recent studies showed that ATase expression was associated with CpG hypermethylation in the ATase gene in several tumour cell lines (Pieper et al, 1991a; Cairns-Smith and Karran 1992). However, another group showed no overall correlation between methylation and ATase expression but methylation of a specific cytosine in the ATase promoter was observed in the ATase deficient cell lines (von Wronski et al. 1992).

1.3 Anti-tumour Methylating Agents

As outlined above (Section 1.1.2) methylating agents produce a number of lesions in DNA including the cytotoxic product, O^6-MeG. This product is formed by the methylating antitumour agents dacarbazine, CB10-277, temozolomide, procarbazine, streptozotocin and NMU.

1.3.1 Dacarbazine (DTIC)

The first report of anti-tumour activity in a triazene compound was the demonstration almost 40 years ago by Clarke et al. (1955) that the carcinogen 3,3-dimethyl-1-phenyltriazene inhibits the growth of the mouse sarcoma 180. Later, dacarbazine [5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide, DTIC, NCS 45388] was synthesized at the Southern Research Institute (Shealy et al. 1962) in an attempt to

design antagonists of 5-aminoimidazole-4-carboxamide (AIC) and DTIC was subsequently found to have marked inhibitory activity against L1210 leukaemia and other rodent solid neoplasms (Montgomery 1976). Clinically, it is used widely in the treatment of malignant melanoma and is the single most active agent available for the control of this disease with a response rate of approximately 20% (Comis 1976; Balch et al. 1989). It is also used in treatment of Hodgkin's disease, soft tissue sarcomas, primary brain tumours, childhood neuroblastoma and malignant neuroendocrine tumours (Averbuch 1993). As a single agent, DTIC has produced temporary remissions in 56% of Hodgkin's disease patients (Spassova & Golovinsky 1985) and 18% of soft tissue sarcoma patients (Gottlieb et al. 1976).

The mechanism of action of DTIC involves metabolic N-demethylation *via* N-hydroxymethyltriazene [5-(3-hydroxymethyl-3-methyl-1-triazenyl)imidazole-4-carboxamide (HMTIC)] to generate the cytotoxic monomethyltriazene [5-(3-methyl-1-triazenyl)imidazole-4-carboxamide (MTIC)]; this hydrolyses spontaneously to AIC and the methyldiazonium ion which methylates DNA (Fig. 1.5). Thus it has been shown that in rats, intraperitoneal injection of [*methyl*-^{14}C] DTIC resulted in the formation of 7-[*methyl*-^{14}C]guanine and O^6-[*methyl*-^{14}C]guanine in DNA of selected tissues (Meer et al. 1986). Parsons et al. (1982) and Hayward et al. (1984) produced a human melanoma cell line that was resistant to MTIC after a single high dose exposure and found that the resistant line was able to remove O^6-MeG much more rapidly than the parent sensitive line. Another study examining the cytotoxic effects of MTIC and related triazenes on BE (Mer^-) and HT (Mer^+) human tumour cell lines (Gibson et al. 1986a) found MTIC was more cytotoxic towards the Mer^- cell line compared to the Mer^+ cell line and a similar finding was reported with the analogous triazenes in the phenyl series (Gibson et al. 1986b) and by 3 other groups studying different melanoma cell lines (Dempke et al. 1987; Lunn and Harris 1988; Maynard et al. 1989).

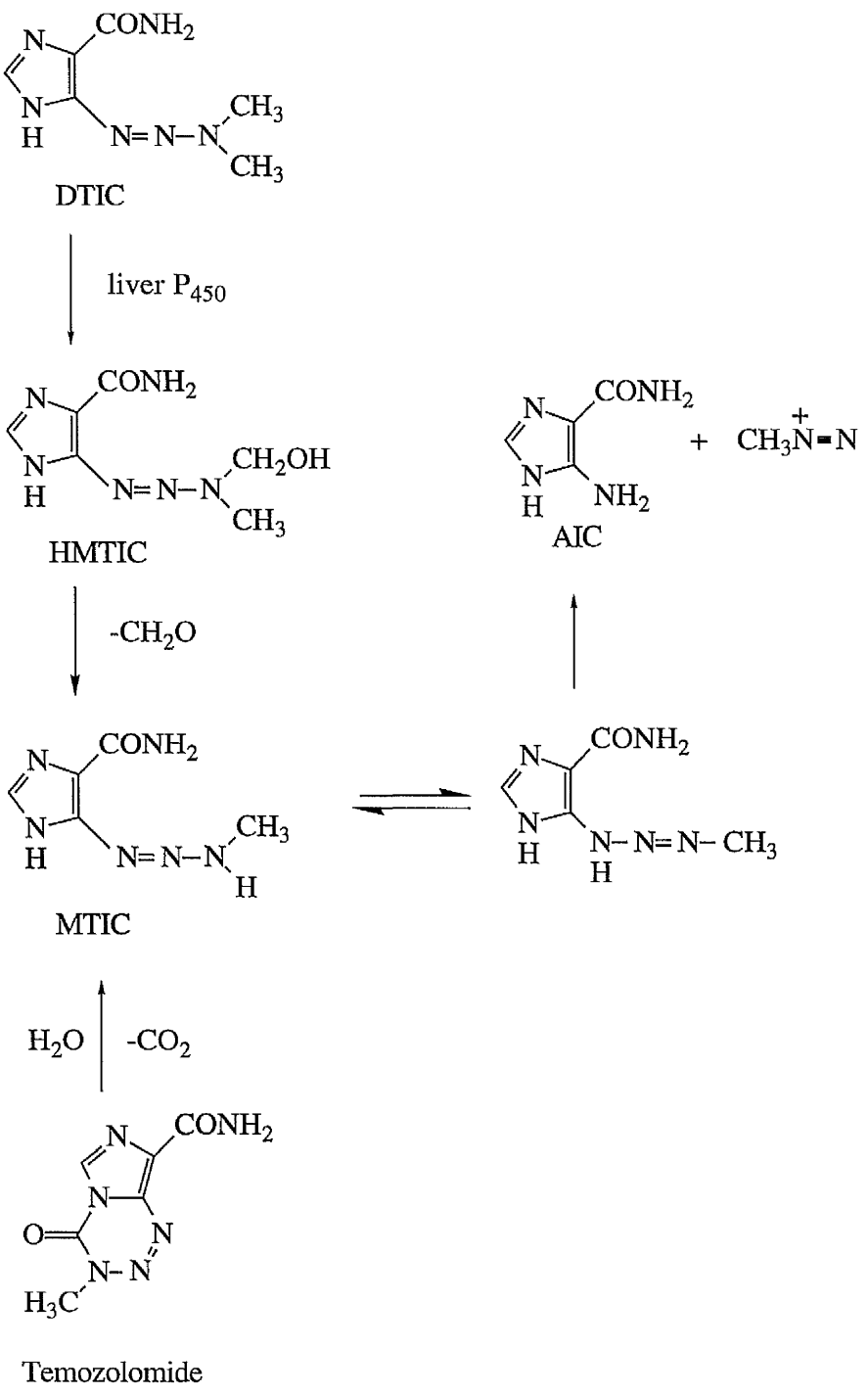


Figure 1.5. Metabolism of DTIC and decomposition pathway of temozolomide.

DTIC is mutagenic in eukaryotic and prokaryotic cells and following chronic oral administration is carcinogenic in rats inducing tumours in the mammary gland, spleen and the thymus (Beal et al. 1975; Kolar 1986). Intraperitoneal administration to mice produced lung tumours and lymphomas (Weisburger et al. 1975; Weisburger 1977). DTIC and procarbazine have been implicated as potential human carcinogens; Sagher et al. (1988) found that patients who developed acute myeloid leukaemia following DTIC-

or procarbazine-based treatment had lower ATase activity in the peripheral lymphocytes, in comparison to normal controls, and postulated that the therapy-induced leukaemia may be the consequence of unrepaired promutagenic O^6-MeG lesions introduced in the bone marrow cells (Sagher et al. 1988). In support of this, O^6-MeG was detected in human leukocyte DNA from patients receiving DTIC and procarbazine (Souliotis et al. 1990, 1991).

1.3.2 CB10-277

1-(4-carboxylphenyl)-3,3-dimethyltriazene (CB10-277) (Fig. 1.6) was synthesized in an attempt to improve the clinical response rate of DTIC. CB10-277 has marked activity against experimental murine tumours (Colombo et al. 1984) and melanoma xenografts (Foster et al. 1990), and is significantly more effective than DTIC in inhibiting the growth of the Walker tumour in the rat (Rutty et al. 1986). CB10-277 requires metabolic activation to generate the putative active monomethyl metabolite, 1-(4-carboxylphenyl)-3-methyltriazene (CPMT), via 1-(4-carboxylphenyl)-3-hydroxymethyl-3-methyltriazene (CPHMT) (Fig. 1.6). In a comparative study, it was found in the rat that the plasma level of the active monomethyl metabolite of CB10-277 was 15-fold greater than that of DTIC (Rutty et el 1986). Furthermore, CB10-277 and its monomethyl metabolite were more active against tumour cell lines and melanoma xenografts with low ATase activity indicating the involvement of O^6-MeG lesion in cytotoxicity (Gibson et al. 1986b; Foster et al. 1990).

The possibility of improved metabolic activation and also improved *in vitro* stability and solubility as the result of substituting the imidazole moiety of DTIC with a phenyl ring and carboxylic acid prompted the selection of CB10-277 for clinical evaluation as a DTIC analogue. Clinical studies showed that it was effective against melanoma, sarcoma and lung cancer (Newell et al. 1990; Foster et al. 1993).

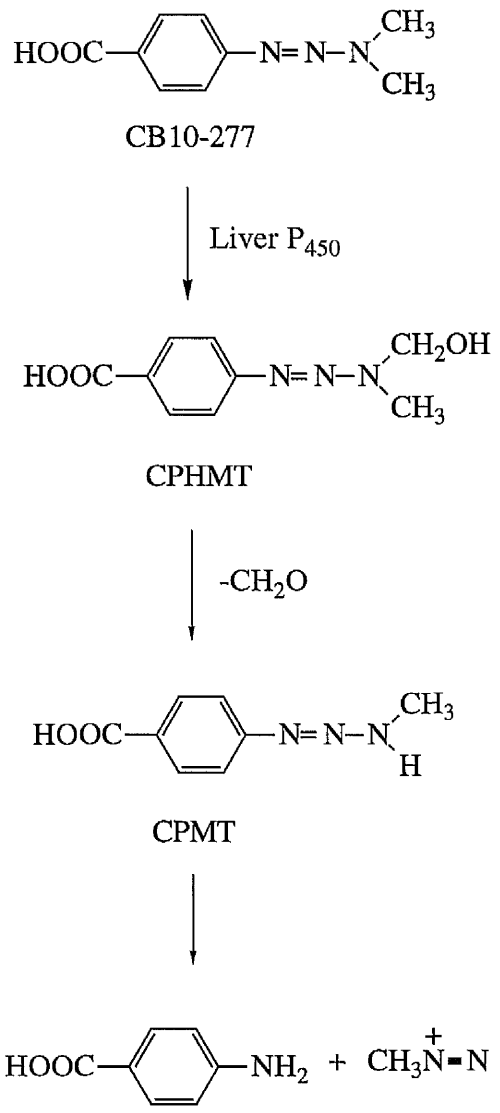


Figure 1.6. Metabolism of CB10-277. CPHMT: 1-(4-carboxylphenyl)-3-hydroxymethyl-3 methyltriazene, CPMT; 1-(4-carboxylphenyl)-3-methyltriazene.

1.3.3 Temozolomide

Temozolomide (8-carbamoyl-3-methyl-imidazole [5,1-d]-1,2,3,5-tetrazin-4(3H)-one, CCRG 810045, M&B 39831; see Fig. 1.5) was synthesized as the result of structural-activity studies of a series of imidazo[5,1-d]-1,2,3,5-tetrazine derivatives (Stevens et al. 1984; Stevens et al. 1987). It was selected for clinical trial due to its good anti-tumour activity against murine P388 and L1210 leukaemias, M5076 sarcoma and B16 melanoma (Stevens et al. 1987). Unlike DTIC and CB10-277, temozolomide rapidly

undergoes spontaneous decomposition in physiological solutions to form the same reactive methylating species as DTIC, i.e. MTIC (Tsang et al. 1991) (Fig. 1.5).

Temozolomide has recently completed a phase I studies and was found to be active against melanoma (17% response), mycosis fungoides and, of most clinical interest, it induced 33% response in patients with high grade glioma who had previously received radiotherapy (Newlands et al. 1992; O'Reilly et al. 1993). It is of interest to note that little clinical activity occurred when the drug is given as a single dose intravenously as opposed to a 5 day oral schedule (Newlands et al. 1992).

Temozolomide is more toxic to ATase-deficient than ATase-proficient cell lines (Catapano et al. 1987; Tisdale 1987) and pretreatment of ATase-proficient cell lines with O^6-MeG (Tisdale 1987) or O^6-BeG (Baer et al. 1993) to produce ATase depletion was associated with increased sensitivity to temozolomide, again indicating the cytotoxic role of O^6-MeG lesions introduced by this agent. Temozolomide is currently undergoing phase II trial in metastatic melanoma, brain gioma and lymphoma under the auspices of the Cancer Research Campaign (UK) Clinical Trial Committee.

1.3.4 Procarbazine

Procarbazine [*N*-isopropyl-α-(2-methyhydrazino)-*p*-toluamide hydrochloride] (Fig. 1.7), a 1,2-disubstituted hydrazine, is a commonly used chemotherapeutic agent particularly in combination chemotherapy regimens for Hodgkin's disease and non-Hodgkin's lymphoma. As a single agent, procarbazine induced a response rate of 53-69% of patients with Hodgkin's disease and 36-40% of patients with non-Hodgkin's lymphoma (Spivack 1974). It is also active against polycythaemia rubra vera, malignant melanoma, bronchogenic carcinoma, mutiple myeloma and brain tumours (Prough and Tweedle 1988).

The precise mechanism of the antitumour effect of procarbazine is uncertain but it is believed to be related to the chemical methylation of DNA (Kreis 1970; Weinkam and Shiba 1978). In rodent models, procarbazine administration results in DNA methylation and analysis revealed the ratio of O^6-MeG to 7-MeG is identical to that typified by nitroso-compounds such as NMU (Wiestler et al. 1984a; Meer 1989; Fong et al. 1990). The metabolism of procarbazine is complex (Fig. 1.7) but is thought to involve an initial oxidation to azoprocarbazine by a process mediated by cytochrome P_{450} and monoamine oxidase (MAO) (Prough and Tweedle 1988). The subsequent conversion of this intermediate to two isomeric azoxy derivatives, benzylazoxyprocarbazine and methylazoxyprocarbazine is catalysed exclusively by cytochrome P_{450} (Prough and Tweedle 1988). Methylazoxyprocarbazine is the predominant circulating azoxy metabolite *in vivo* (Shiba and Wienkam 1982) and is the intermediate from which the putative DNA methylating fragment is derived. It has been suggested that the conversion of the methylazoxyprocarbazine to a methylating species may proceed by spontaneous chemical decomposition as well *via* the activity of cytosolic enzymes (Erickson et al. 1989).

Evidence that the cytotoxic action of procarbazine is mediated by a methylating species is that methylazoxyprocarbazine, an intermediate on this pathway, is more cytotoxic than procarbazine, while benzylazoxyprocarbazine (the corresponding arylating metabolite) lacks cytotoxic activity (Erickson et al. 1989; Swaffar et al. 1989). Procarbazine is more active against glioma xenografts with low ATase activity than high ATase activity supporting the cytotoxic role of O^6-MeG produced in DNA by this agent (Schold et al. 1989).

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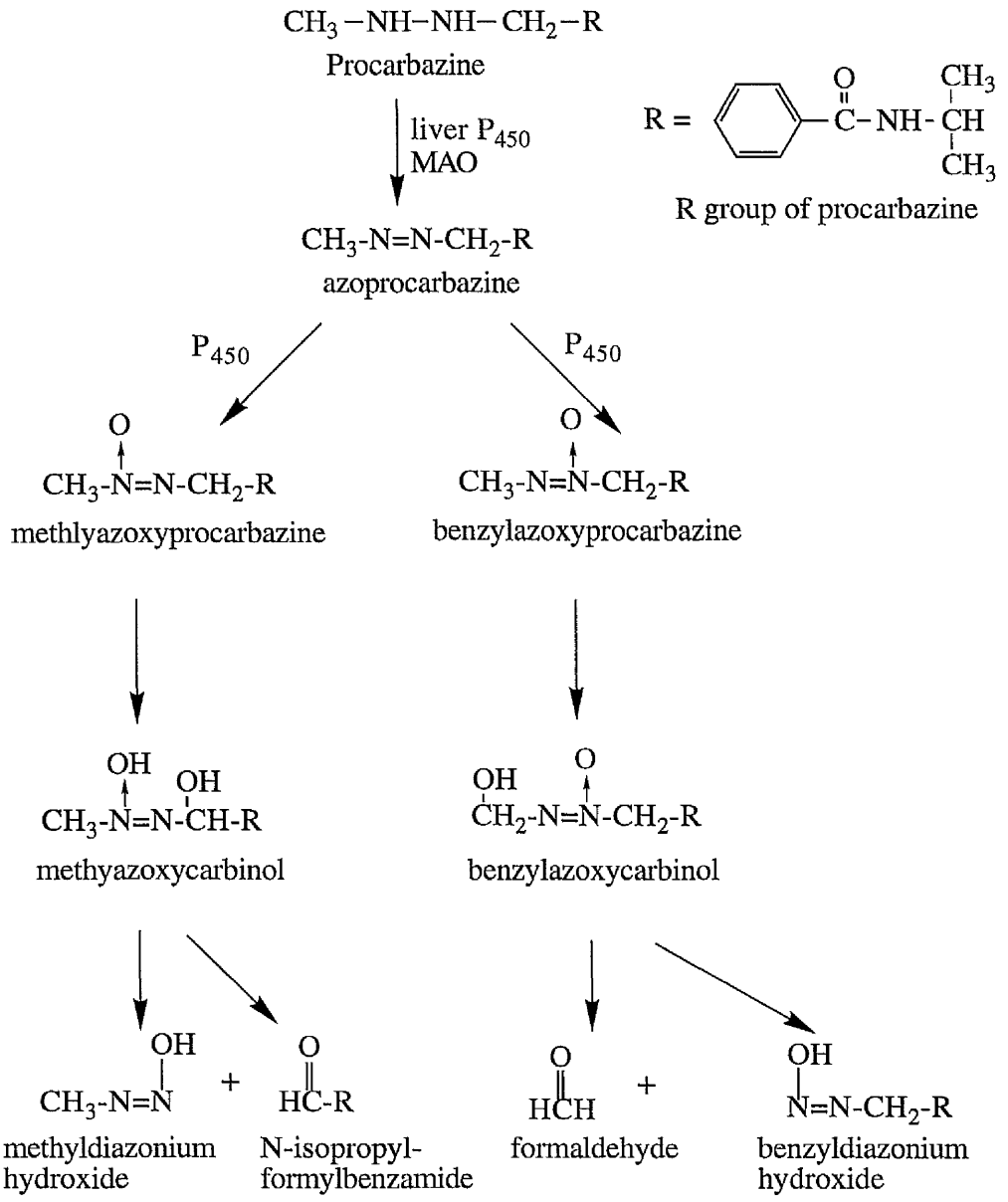


Figure 1.7. Metabolism of procarbazine. MAO represents monoamine oxidase.

In experimental models, procarbazine is carcinogenic inducing pulmonary tumours and leukaemia in mice and mammary carcinoma in rats (Kelly et al. 1964; O'Gara et al. 1971; Lee and Dixon 1978; Sieber et al. 1978, Bacci et al. 1982; Kelly et al. 1986; Fong et al. 1990).

1.3.5 Streptozotocin

Streptozotocin (Fig. 1.8) is a naturally occuring agent produced by fermentation of *Streptomyces achromogenes.* It has a methylnitrosourea moiety attached to the C-2

position of glucose and is used mainly in the treatment of islet cell carcinomas and carcinoid tumours (Weiss 1982).

Streptozotocin has been shown to generate O^6-MeG in rat tissue DNA (Bennett and Pegg 1981). In addition, streptozotocin resulted in ATase depletion in several tumour cell lines studied, due presumably to the repair of this adduct (Gibson et al. 1986c; Erickson et al. 1988; Futscher et al. 1989; Pieper et al. 1991b). A similar effect occurred in peripheral blood lymphocytes of patients treated with streptozotocin (Gerson 1989; Panella et al. 1992). In an attempt to increase the therapeutic effects of BCNU, streptozotocin was administered before BCNU; it was anticipated that ATase activity would be depleted in repairing the O^6-MeG lesions and there would be increased formation of the cytotoxic cross-links following administration of BCNU. In two reported phase 1 studies (Panella et al. 1992; Micetich et al. 1992), this treatment schedule was associated with reduction of the maximum tolerated dose (MTD) of BCNU to about 50-70% that of MTD of BCNU administered alone and a trend towards an earlier platelet nadir was seen.

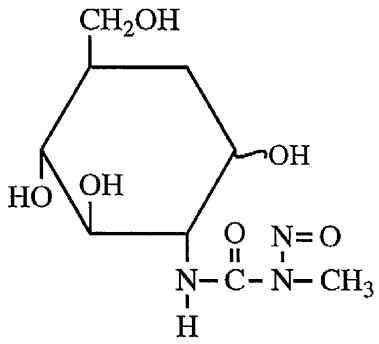


Figure 1.8. Structure of streptozotocin.

1.3.6 N-nitroso-N-methylurea

NMU (Fig 1.9) was developed at Southern Research Institute in an attempt to increase the antitumour activity of the carcinogen, N-methyl-N'-nitro-N-nitrosoguanine (MNNG) (Johnston et al. 1963) after it was found that MNNG was active against leukaemia L1210 cells in mice (Greene and Greenberg 1960). As discussed above

(Section 1.1.1) NMU undergoes spontaneous decomposition to generate a reactive methyl carbonium ion and has good clinical activities against lung cancer and previously treated HD (Emanuel et al. 1974).

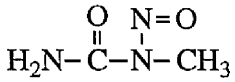


Figure 9. Structure of N-nitroso-N-methylurea.

In all animal species tested, MNU induces tumours in almost every organ, depending on the dose, route and schedule of administration (see review by Berger 1986).

1.4 Anti-tumour Chloroethylating Nitrosoureas

In an attempt to increase antitumour activity and to decrease carcinogenic activity, over 200 congeners of NMU were synthesized and it was found that only one specific structural type, N-(2-chloroethyl)-N-nitrosourea proved superior to NMU (Montgomery 1981). The 2-iodoethyl compounds were found to lack antitumour activity, while the 2-bromoethyl derivatives were less effective than the 2-chloroethyl compounds. Although the 2-fluoroethyl compounds showed activity equal to the 2-chloroethyl compounds, decomposition to fluoroacetate *in vivo* and the resultant central nervous system toxicity limited their clinical usage (Johnston et al. 1963). Further studies of alterations of the 2-chloroethyl and the nitrogen group lead to the synthesis of 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine, BCNU); 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine, CCNU); 1-2(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea (semustine, methyl CCNU) and fotemustine (Fig. 1.10). Chlorozotocin was synthesized by replacing the methyl group of a streptozotocin with a chloroethyl group in an attempt to increase the antitumour activity of streptozotocin, (Anderson et al. 1975). Clomesome was synthesized following studies of a series of 2-haloethylmethanesulfonates (Shealy et al. 1984) which although analogous to the CNUs as far as chloroethylation reactions are concerned, lacks two

prominent side reactions of the CNUs; hydroxyethylation and carbamoylation (Gibson et al. 1985b; 1986d; Dyke et al. 1989). Mitozolomide was synthesized by Stevens et al. (1984) as a prodrug of the cytotoxic triazene, 5-(3-(2-chloroethyl)triazen-1-y)imidazole-4-carboxamide.

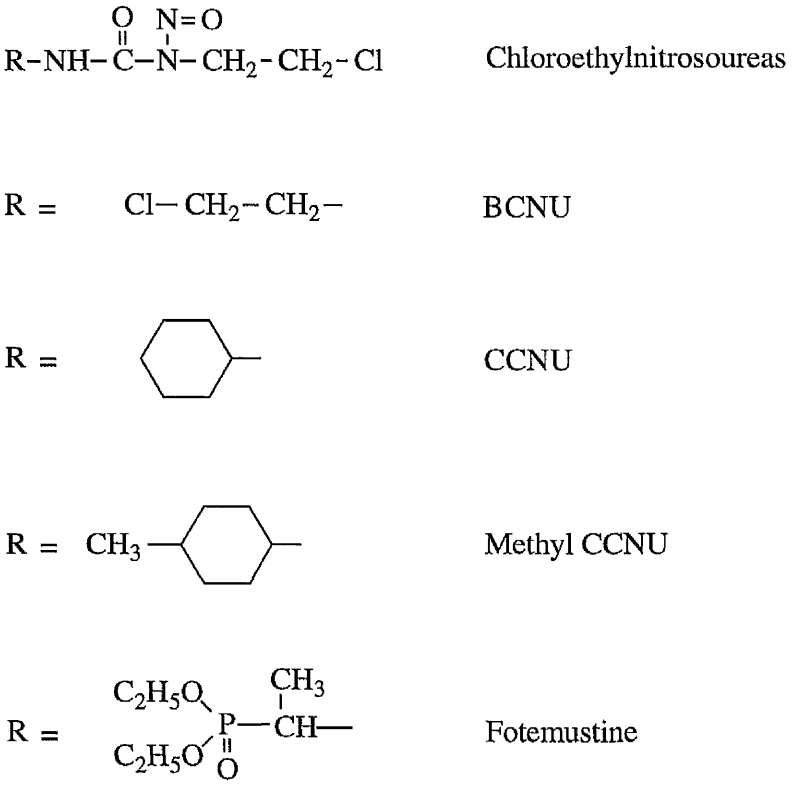


Figure 1.10. Structures of substituted chloroethylnitrosoureas.

The CNUs have shown antitumour activity against experimental leukaemia and solid tumours and clinical activity for a broad spectrum of human malignancies, including acute lymphocytic leukaemia, lymphomas, melanoma, gliomas, multiple myeloma and gastrointestinal neoplasms has been established (Mitchell and Schein 1992b; Reed 1988). Unfortunately, myelodysplastic syndromes and acute myeloid leukaemia have been reported following treatment with CNUs both as single agents and when combined with other antitumour agents either with or without irradiation (Michels et al. 1985).

Under physiological conditions, the CNUs decompose spontaneously (Colvin et al. 1976; Montgomery et al. 1975). The chemical half-lives of individual compounds in phosphate-buffered saline (pH 7.4) vary from 5 minutes for BCNU to as long as 2 hours for CCNU (Mitchell and Schein 1986). As mentioned earlier, in the process of degradation, a number of alkylating species are generated, of which the chloroethyl carbonium ions are considered the most important for antitumour activity (see Section 1.1.2). Organic isocyanate moieties are also generated and are responsible for carbamoylating intracellular proteins but no correlation has been seen between the carbamoylating activity and antitumour activity (Mitchell and Schein 1986; Montgomery and Johnson 1990; Colvin and Chabner 1990).

The main limitation of the CNUs in the clinic is related to the problem of delayed and cumulative bone marrow toxicity. Unfortunately, attempts to synthesise new nitrosoureas to overcome this have met with limited success (Montgomery and Johnston 1990; McCormick and McElhinney 1990). Chlorozotocin, synthesized by replacing the methyl group of streptozotocin with a chloroethyl group and 2-2(chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea (GANU) are less marrow suppresive than BCNU (Anderson et al. 1975) but they are much less active against a spectrum of solid tumours (Johnston et al. 1975). The chloroethyl analogue of temozolomide, mitozolomide (8-carbamoyl-3-chloroethyl-imidazole [5,1-d]-1,2,3,5-tetrazin-4(3H)-one) mentioned earlier although showing similar experimental anti-tumour activity was found to be associated with low clinical activty and severe and unpredictable myelosuppresion and therefore was withdrawn from clinical development (Blackledge et al. 1989; Harding et al. 1989; Newlands et al. 1990). More recent efforts have been focussed on the incorporation into the basic nitrosourea structure of N'-substituents derived from amino acid (Zeller et al. 1982), amino acids esters (Montgomery and Johnston 1990), amino acid amides (Suami et al. 1982; Rodriguez et al. 1984), aminocarbohydrate-amino acid derivatives (Suami et al. 1981) and hormones (Betsch et al. 1989). Among the amino acid congeners, fotemustine (diethyl-1-[3-(2-chloroethyl)-3-nitrosoureido]-ethylphosphonate, S 100036) (Fig. 10) containing a

phosphonoalanine carrier group grafted to the CNU radical was synthesized and found to possess activity against a range of solid tumour cells (Fischel et al. 1990). Similar to other nitrosoureas, delayed and cumulative myelosuppression was the main side effect (Khayat et al. 1987). In a recent phase II study of fotemustine in 153 evaluable patients with malignant melanoma, an overall response rate of 24% was achieved (Jacquillat et al. 1990).

Nitrosourea-resistant tumour cell lines and tumour xenografts can be rendered sensitive to the CNUs by pretreatment with methylating agents or O^6-AlkG as the free base (see Section 1.2.2). In one report, it was found that pretreatment of a variety of resistant human tumour cells with streptozotocin sensitized them to BCNU but as the interval between streptozotocin and BCNU increased, the amount of enhanced cytotoxicity produced was reduced and this was related to *de novo* synthesis of ATase (Pieper et al. 1991b). It is therefore not unreasonable to suggest that the timing of such combination schedules is important if improved response rates are to be achieved in a clinical situation.

1.5 Other ATase Modulating Agents

A number of antitumour agents for which there is no evidence for the production of O^6-AlkG lesions in DNA has been reported to cause ATase depletion. Pretreatment with cyclophosphamide is able to increase the amount of O^6-MeG in rodent liver DNA following a chasing dose of NMU and this was attributed this to some as yet unidentified O^6-alkylation product of guanine in DNA which is repaired by ATase and results in ATase depletion (Kleihues and Margison 1976a; Meer et al. 1989). Cisplatin (Wang and Setlow 1989), adriamycin (Link and Tempel 1991), bleomycin (Link and Tempel 1991) and suramin (Link and Tempel 1991) were also reported to inactivate ATase. However cisplatin-induced ATase-depletion was not seen in two other studies (Meer et al. 1989; Link and Tempel 1991).

Significant increases in ATase activity have been found in rat liver and in rat hepatoma cells following treatment with N-nitroso compounds, other DNA damaging agents, interferon and treatments causing increased cell proliferation. (Laval 1990; 1991; Bertini et al. 1990; Pegg 1990; Chan et al. 1992). Since interferon is regularly combined with DTIC in the treatment of malignant melanoma (Guillou et al. 1989; Mulder et al. 1990), the interferon-induced ATase expression may antagonise the cytotoxic effects of DTIC if similar change is seen in tumour tissue. Induction of ATase in rodent and human cells following exposure to ionizing radiation has also been reported (Margison et al. 1985; Pegg 1990; Chan et al. 1992; Wilson et al. 1993). This may have adverse effects on the response to O^6-alkylating anti-tumour agents if these are given after radiation. Clearly, a better knowledge of the effects of the available anticancer agents and radiation on ATase activity is warranted in order to combine treatments on a more rational basis.

1.6 Aims of the work in this thesis

The majority of evidence from work with tumour cell lines and xenograft models indicates that alkylation at the O^6-position of guanine in DNA is the principal cytotoxic lesion produced by the antitumour methylating and chloroethylating agents and that resistance to this damage stems principally from the activity of the DNA repair protein, ATase. The majority of human tumours examined so far express ATase activity but since ATase-proficient tumours cells can be rendered sensitive to these agents by treatments that reduce the ATase levels, one approach to overcome tumour resistance in clinical situation would be to deplete the endogenous ATase with methylating agents prior to administering CNUs.

In contrast to experimental systems, there is paucity of information on the effects of antitumour agents on ATase levels in human tissues. It is not known whether or not ATase is depleted and if so the rate and extent of this and the subsequent regeneration of ATase activity or whether it is related to dosage of methylating or chloroethylating

agents used, or treatment cycles. In the event of depletion, identifying the time to reach the ATase nadir and the extent of ATase depletion with different doses of methylating agents may have important therapeutic implications since a nitrosourea administered at the nadir of ATase would be anticipated to have greater cytotoxic effect: an enhanced cytotoxic effect was seen in a number of tumour cells and xenografts when nitrosourea was administered following reduction of the ATase activity. If tumour cells depleted of ATase activity are able to regenerate ATase rapidly, the cytotoxic effects of a subsequent administration of nitrosourea may not be enhanced. Residual ATase activity might be indicative of potentially resistant cells in the tumour population and conversely, complete ablation of ATase may predict cytotoxicity. In addition, it is not known whether the ATase activity of human normal or tumour tissue can be induced following treatment with alkylating agents, although such effects have been reported in rat tissues and cultured cells (Pegg 1990). The balance between the inactivation of ATase during repair of the toxic O^6-AlkG adducts and its subsequent regeneration are parameters which may predict individual tumour response to the anti-tumour O^6-alkylating agents.

In an attempt to address these questions, the first part of the thesis sets out to examine the kinetics of ATase depletion following treatment of patients with alkylating agents including DTIC, CB10-277, temozolomide, fotemustine, BCNU and cyclophosphamide. Peripheral blood lymphocytes have been used to monitor changes in ATase levels as (a) they are targets of alkylation damage (Gupta et al. 1988) and are able to repair the O^6-AlkG lesions introduced by these agents (Harris et al. 1982; Gerson 1989), (b) it is a major difficulty to obtain serial post-treatment tumour biopsies from patients on technical and ethical grounds and (c) there is some evidence to indicate a relationship between ATase levels in lymphocytes and other human tissues of the same individual (Kyrtopoulos et al 1990) although, in an attempt to examine whether a relationship exists between changes in ATase levels in tumour tissues and those in peripheral lymphocytes, sequential melanoma biopsies were taken from some patients. The present study also investigates the kinetics of O^6-MeG formation and loss in

leukocyte DNA to explore possible relationships with changes in ATase levels in peripheral lymphocytes following DTIC administration. Having established that nadir ATase activity occured at approximately 4 h after DTIC administration, the existing sequential DTIC and fotemustine schedule was altered to DTIC given at time 0 followed by fotemustine 4 h later. This was in an attempt to increase the response rate in patients with metastatic melanoma and the clinical and toxic effects of this combination chemotherapy are assessed.

Although ATase levels in extracts of many tumour types have been measured, a disadvantage of such analysis is that it represents the average ATase activity of all cells in the tumour biopsy including the normal supporting stroma and blood cells and also it takes no account of differences in the intercellular expression of ATase protein. The next part of the thesis examined the ability of a rabbit polyclonal antiserum raised against ATase to detect the protein in human tumour sections and determine its distribution in a series of human tumours including melanoma, Hodgkin's and ovarian tumours. The specificity of the antibody was initially tested by western blots analysis using crude extracts of some of the above tumours. To examine the possibility that resistance to the O^6-alkylating drugs which frequently occurs in ovarian cancer (in contrast to Hodgkin's disease) may be related to ATase expression, a comparative study of ATase expression between tumour biopsies of a series of patients with Hodgkin's disease and ovarian cancer was performed using the ATase anti-serum. This immunohistochemistry (IHC) procedure was also used to examine the extent to which cellular ATase expression is heterogeneous in tumour tissues since heterogeneous chemosensitivities of subpopulations of tumour cells to BCNU have been described (Yung et al. 1982; Shapiro et al. 1990). In the latter case, it is not unreasonable to speculate that this may be related to heterogeneity of cellular ATase expression within the tumour. Hopefully, information obtained from such studies may allow the identification of resistant tumours and hence the design of individualised treatment protocols, including resistance modifiers where necessary, to be of maximum therapeutic benefit to the patients.

2.0 Drugs and methods of administration

DTIC was purchased from Bayer (UK) Ltd and was supplied as a white or ivory-coloured, preservative-free powder in amber glass vials of 100 mg or 200 mg of the citrate salt. The drug was reconstituted with sterile distilled water before diluting into 250 ml of 0.9% NaCl in an infusion bag. It was protected from UV light during i.v. infusion by wrapping the bag with black PVC sheet.

CB10-277 was supplied by the Developmental Therapeutics Programme, National Cancer Institute, Bethesda, MD, USA as a lyophilised, pyrogen and preservative-free powder in 1000 mg vials . Each patient's dose was calculated to give the total dose, then half was reconstituted in sterile distilled water (50 mg/ml) and placed in 1 litre 0.9% NaCl and infused over 12 h. The other half was reconstituted and infused i.v. over the next 12 h in exactly the same way. The infusion was split into two 12 h batches because the drug contained no bacteriostatic agent.

Temozolomide was supplied by the Department of Pharmaceutical Sciences, Aston University, Birmingham. For the clinical study, temozolomide was formulated at Strathclyde University in hard gelatin capsules containing 20, 50 or 100 mg. Each patient's dose was calculated to give the total dose and split as equally as possible for the treatment to be given orally once per day over 5 days.

Fotemustine was supplied by Servier International Research Institute, France in vials of 200 mg of freeze dried product. The drug was dissolved in the 4 ml ethanol solvent provided and then reconstituted with 10 ml water before diluting it into 250 ml 5% dextrose. The drug was given as a 30 min i.v. infusion: it was protected from UV light during i.v. infusion by wrapping the bag with black PVC sheet.

BCNU was purchased from Bristol-Myers Squibb Pharmaceuticals Ltd and was as a white freeze-dried flaky powder supplied in 30 ml vials containing 100 mg. The drug was dissolved in the 3 ml ethanol solvent provided before diluting it further with 27 ml water. This was then added to 500 ml 5% dextrose before administering it over 2 h by i.v. infusion with electrocardiographic monitoring. It was protected from UV light during i.v. infusion by wrapping the bag with black PVC sheet.

Cyclophosphamide was purchased from Farmitalia Carlo Erba Ltd. and was supplied as a sterile, white powder in vials containing 100 mg, 200 mg, 500 mg or 1000 mg cyclophosphamide. The vials also contain sodium chloride to render the solution isotonic when the contents of a vial were reconstituted with sterile, distilled water (5 ml per 100 mg of anhydrous cyclophosphamide). After vigorous shaking, the contents were diluted into 1 litre 0.9% NaCl for i.v. infusion over 1 h.

For *in vitro* ATase inhibition experiments, DTIC, CB10-277, temozolomide, BCNU, fotemustine and cyclophosphamide were obtained from suppliers described above; procarbazine from Cambridge Laboratories; mitozolomide from May & Baker; acrolein, glutathione, MMS and NMU from Sigma Chemical Ltd. Phosphoramide mustard was kindly provided by Dr A McGown and recombinant human ATase supplied by Dr RH Elder from the Institute.

2.1 Patients and ethical consideration

All patients undergoing treatment with the alkylating anti-tumour agents described above were treated as in-patients in Christie Hospital except for those treated with temozolomide when they were admitted for one overnight stay only and subsequently followed up on the day-ward for daily blood samplings. The entry criteria for phase II trials for patients treated with combined DTIC/fotemustine, CB10-277 and temozolomide were: histological documentation of metastatic melanoma, measurable metastasis, performance status 2 or less [World Health Organization (WHO) scale] and

life expectancy greater than 2 months, adequate haematologic, hepatic and renal function, no severe medical disorders, no previous or current malignancies at other sites and no prior chemotherapy for patients treated with temozolomide and CB10-277. All patients entered into the phase II studies were informed about the background and knowledge of the drug under study and that with CB10-277, temozolomide or fotemustine, the drugs were rather new and the exact degree of activity unknown. Patients entered into the experimental studies were interviewed by one doctor (SML) and it was emphasised that the blood samplings and/or tumour and bone marrow biopsies were required for the sole purpose of research and were not part of routine clinical supervision. Financial inducements to take part in the study or payments to cover expenses were not offered. Patients were allowed to refuse the treatment under study, regular blood samplings or tumour biopsies either before or at any time during the study. All patients gave informed consent prior to the study and approval was obtained from the Ethical Committee of the South Manchester Health Authority for both the clinical and experimental studies described in this thesis.

Patients were cannulated with a green venflon (Viggo-Spectramed) in each arm; one for the drug infusion and the other for regular blood samplings for the ATase and/or O^6-MeG study. Peripheral blood aspirated through a 3-way tap attached to the venflon, was placed in a 20 ml universal container containing 0.5% EDTA and stored in+4°C before the isolation of peripheral blood mononuclear cells. Most patients received i.v. ondansetron (Glaxo Laboratories Ltd) during the study in order to suppress the nausea and vomiting induced by the cytotoxic chemotherapy. For the bone marrow study, approximately 4 ml bone marrow were aspirated from the posterior iliac crest and stored in 0.5% EDTA. Tumour skin biopsies were performed under local anasthetic using 2% xylocaine (Astra Pharmaceuticals Ltd) before removal of the metastatic nodules. The surrounding fat and skin were removed from the tumour nodules before transfer into a 20 ml universal container for immediate storage at -20°C. The phase II studies with CB10-277 and temozolamide were conducted under the auspices of Cancer Research Campaign and the phase II study with sequential DTIC/fotemustine was

supported by Servier international Research Institute. All patients entered into the phase II trials for CB10-277 and temozolomide were registered with the CRC Data Centre. Details of age, stage, histological type, treatment received and response data were retrieved from the case notes.

2.2 Clinical response

Tumour response and toxicity assessment used the WHO (1979) criteria. Complete response was defined as the disappearance of all known disease for at least four weeks; partial response was defined as a reduction in the sum of the products of the largest perpendicular diameters of each lesion by at least 50% for at least four weeks; stable disease was defined as a decrease of less than 50% in total tumour size, or an increase of less than 25% in the size of one or more lesions; progressive disease was defined as a 25% or more increase in the size of at least one lesion or the appearance of a new lesion. Early death was defined as death during the first four weeks and toxic death was defined as any death to which drug toxicity is thought to have a major contribution. Toxicity was recorded and analysed using the WHO grading system.

2.3 Isolation of peripheral blood mononuclear and bone marrow cells

Peripheral blood mononuclear cells (referred to in this thesis as lymphocytes) and bone marrow cells were isolated from peripheral blood and bone marrow aspirates by density centrifugation using Ficoll-Paque (Pharmacia Biotech Ltd; Bøyum 1968). Ten ml of Ficoll-Paque were added to a 20 ml universal container. Following this, 10 ml of blood [or 4 ml bone marrow mixed with 6 ml phosphate buffered saline (PBS; 8.5 g NaCl, 1.28 g Na_2HPO_4, 0.16 g $NaH_2PO_4.2H_2O$ per litre of H_20, pH 7.4] were carefully layered on top of the Ficoll-Paque. The samples were then centrifuged at 400 x g for 20 min at 20°C. Taking care not to disturb the lymphocyte layer at the interface, the upper layer was then removed using a Pasteur pipette and discarded into a beaker containing 2% Virkon. Using a clean plastic pipette, the lymphocyte layer (or bone marrow

mononuclear cell layer) was transferred to a clean 20 ml universal container and PBS added to make up to a total volume of 20 ml. The mixture was centrifuged at 60-100 x g to obtain a pellet. The supernatant was removed and discarded and the lymphocyte pellets were stored at -20°C prior to ATase extraction and assay.

2.4 ATase extraction

Lymphocyte pellets or biopsied tumour tissues (100-200 mg) which had been minced with a pair of scissor in 1.5 ml Eppendorf tubes were disrupted by sonication (10 sec at 10 μm peak to peak distance followed by cooling in ice and then resonication for 10 seconds at 18 μm) in 1 ml of buffer I (50 mM Tris-HCl, 1 mM EDTA, 3 mM dithiothreitol, pH 8.3). Phenymethysulphonyfluoride (8.7 mg/ml in absolute ethanol) was added to a final concentration of 87 μg/ml immediately after the second sonication to inhibit proteases. Sonicates were centrifuged at 16,000 rpm in a microcentrifuge for 10 min at 4°C to remove cell debris and the supernatants were transferred to fresh tubes in ice prior to assay for ATase activity, DNA and protein content and, in some cases, subjected to western analysis.

2.5 ATase assay

The ATase substrate, [^{3}H]-NMU methylated calf thymus DNA (provided by Dr GP Margison) was made to a working concentration of 100 μg/ml in buffer I, 100 μl of this (containing approximately 100 fmoles [3H]-O^6-MeG) were incubated at 37°C for 2 h with varying amounts of cell extract (for each sample, at least three different volumes were used varying from 25-300 μl) made up to total volume of 500 μl with 1 mg/ml of bovine serum albumin (BSA) in buffer I. After incubation, BSA (100 μl of a 10 mg/ml solution in buffer 1) and perchloric acid (200 μl of a 4 M solution) were added in rapid succession. A further 2 ml of 1 M perchloric acid were added and the mixture was heated at 75°C for 40 min to hydrolyze the DNA. Protein was recovered by centrifugation at 3000 rpm for 10 min at room temperature and the pellet was washed

once with 4 ml of 1 M perchloric acid before being resuspended in 300 ul of 0.01 M NaOH and dissolved in 3 ml of aqueous scintillation fluid (Ecoscint A; Mensura Tech Ltd). All samples were mixed thoroughly with the scintillant before being counted for three 5 min cycles in an Rackbeta (LKB) liquid scintillation counter. Counting efficiency was approximately 28%. The cpm were plotted against the volume of the extract used for each sample. Specific activity measurements were based on a minimum of 3 points on the linear part of the curve. ATase activity was expressed as fmoles [3H]-methyl transferred to protein per mg of total protein or to DNA per μg of total DNA in the extract.

2.6 Protein concentration measurement

The protein concentration estimation was based on the colour change of Coomassie brilliant blue G250 (Bradford 1976). Bio-rad protein reagent (BioRad Laboratories) was diluted 1:5 in distilled water and filtered through Whatman number 1 filter paper. A protein standard curve was set up using BSA at concentrations ranging from 0.1 mg/ml to 1 mg/ml. Two ml of diluted Bio-rad reagent were added to 40 μl of standard (in duplicate) or appropriately diluted tissue extracts (in triplicate) and absorbance at 595 nm determined. The concentration of protein in the tissue extracts was determined by extrapolation from the standard curve.

2.7 DNA concentration measurement by Hoechst Fluorescence

Standards (ranging from 0 to 5 μg of calf thymus DNA) or suitably diluted samples were pipetted in duplicate into 4 ml polystyrene tubes. Two ml of standard saline citrate (SSC; 0.15M NaCl, 15 mM Na citrate, pH 7.0) and one ml of reagent [1/100 dilution of a stock soultion of 1.5×10^{-4} M Hoechst 33258 (bisbenzamide; Sigma Chemical Ltd)] were added to the tube and fluorescence (excitation 360 nm, emission 450 nm) determined with a Shimadzu RF-5000 spectrofluorimeter. The quantity of DNA in the samples was determined by extrapolation from the standard curve.

2.8 DNA isolation and O^6-methyldeoxyguanosine analysis

DNA isolation and O^6-MeG analyses were performed by Mr John Davies in the department as described in detail elsewhere (Wild et al. 1983; Hall et al. 1991; Lee et al. 1994). Briefly, DNA was prepared from whole blood using a modified phenol extraction procedure and the isolated DNA was digested enzymatically to nucleosides before ion-exchange chromatography using Aminex A7 resin (BioRad Ltd). The four major deoxynucleosides were separated from O^6-MeG by this procedure and were quantified by peak area integration. The putative O^6-MeG containing fraction was analysed by radioimmunoassay using a monoclonal antibody to O^6-MeG (Wild et al. 1983). The results are expressed relative to the amount of parent deoxyguanosine (dG), in the DNA sample. The lower limit of detection using these small amounts of DNA was ~0.4 μmoles O^6-MedG/mole dG.

2.9 Western analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Bio-rad Mini Protean II apparatus. A 16% acrylamide gel was prepared by mixing 4.3 ml Protogel stock solution (30% w/v acrylamide, 0.8% w/v bisacrylamide; Mensura Technology Ltd), 2 ml lower Tris (1.5 M Tris-HCl, pH 8.8, 0.4% SDS), 1.7 ml H_2O, 80 μl 10% w/v ammonium persulphate and 8 μl TEMED (Bio-rad Laboratories). The acrylamide solution was pipetted between the two glass plates of the vertical gel slab apparatus. The liquid gel was overlayered carefully with butanol to ensure a level interface between the stacking and separating gels. The 5% stacking acrylamide gel was prepared by mixing 1 ml Protogel stock solution, 1.5 ml upper Tris (0.5 M Tris-HCl, pH 6.8, 0.4% SDS), 3.5 ml H_2O, 80 μl 10% ammonium persulphate amd 10 μl TEMED. After removal of the butanol from the polymerised resolving gel, the stacking gel solution was pipetted onto the resolving gel and a well-forming comb inserted. Any air bubbles trapped under the comb were removed. After polymerisation, the comb was removed and the gel slots washed thoroughly with SDS-PAGE running buffer (50 mM Tris-HCl, 192 mM glycine, 0.1% SDS).

The gel was mounted in the electrophoresis apparatus containing SDS-running buffer. Samples (30 μg), denatured pre-stained molecular weight markers (from 14.3 to 46 kDa; GIBCO, BRL) and recombinant human ATase (provided by Dr RH Elder) were prepared by heating to 100°C in SDS-PAGE loading buffer (300 mM Tris-HCl, 20% glycerol, 10% SDS, 0.25 M DTT, 0.05% bromophenol blue; prepared as 5x stock) for 10 min and then pipetted into the well slots. Electrophoresis was at 200V until the bromophenol blue reached the bottom of the gel.

The gel was removed from the apparatus, placed against a nitrocellulose membrane and assembled in a sandwich between two pieces of Whatman 3MM filter paper and porous mats. The sandwich was placed in an electrotransfer tank containing western blot transfer buffer (6.05 g Tris base, 28.8 g glycine, 400 ml methanol to 2 litres of water) with the nitrocellulose membrane on the anode side and electro-transfer was carried out at 100V for 1 h. The nitrocellulose filter was removed from the sandwich, air dried and stored between two pieces of Whatman filter paper while the residual gel was stained with Coomassie Blue R250 in 20% and destained in 5% acetic acid in order to ensure that transfer had occurred.

After soaking briefly in Tris-buffered saline containing Tween (TBST; 20 mM Tris-HCl, 150 mM sodium chloride, 0.05% v/v Tween-20, pH 7.5), the nitrocellulose membrane was incubated in 5% non-fat milk (Marvel) in TBST at room temperature for 30 min to block non-specific antibody binding sites. The membrane was then incubated with the primary ATase antibody (1:1000 dilution, 4th bleed; provided by Dr C-Y Fan) in blocking buffer for 2 h in a sealed plastic bag. Excess antibody was removed by washing three times (10 min each time) in 50 ml TBST with agitation, before incubation with the secondary antibody for 1 h at room temperature in a sealed plastic bag. The secondary antibody routinely used was goat:anti-rabbit conjugated with alkaline phosphatase (Dako Ltd) at a dilution of 1:1000. Following washing as before, the nitrocellulose was immersed in alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 10 min prior to addition of the staining

solution. The staining solution was prepared by adding 44 µl of nitro blue tetrazolium at 75 mg/ml and 33 µl of bromochloroindolyl phosphate at 50 mg/ml per 10ml of alkaline phosphatase buffer (reagent kit obtained from GIBCO, BRL). The membrane was incubated with staining solution for approximately 5-10 min until the colour was clearly visible. The reaction was stopped by rinsing with 100 ml of water containing EDTA and the membrane allowed to dry between 3MM filter paper and stored in the dark. The intensity of band staining on western blots was determined following video image capture using an Ultra Violet Products densitometer coupled with a gel analysis program.

2.10 Immunohistochemistry

Mounted sections obtained from paraffin-embedded blocks were de-waxed in xylene, washed in absolute ethanol twice, treated with 3% H_2O_2 in absolute methanol for 30 min, at room temperature, incubated with normal swine serum (10% dilution; Dako Ltd) for 30 min and exposed overnight at 4°C to the anti-human ATase (4th bleed) or preimmune serum both diluted 1:1000 in Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM sodium chloride, pH 7.5). The preimmune serum was obtained by prebleeding the the rabbit before hyperimmunization with the human ATase protein and this served as negative IHC control. The sections were then incubated with swine anti-rabbit antibody (SAR) (diluted 1:400 in TBS) for 45 min at room temperature, washed in TBS and incubated with rabbit peroxidase anti-peroxidase complex (PAP) (diluted 1:200 in TBS) for 45 min at room temperature. After washing in TBS the sections were again incubated with SAR and PAP for 15 min each. Stain development was by single-step silver intensification of the nickel-complexed diaminobenzidine (Ni-DAB; 0.5 ml of 1% $NiCl_2.6H_2O$ in 5 ml of 0.5mg/ml DAB) reaction product of peroxidase as described by Przepiorka et al (1986). Briefly, the slides were exposed to Ni-DAB for 5 min followed by 0.01% H_2O_2 in Ni-DAB for another 5 min at room temperature. After washing the slides with distilled water, the sections were incubated with silver reagent for 5 min. Silver reagent was prepared by mixing in the following order; 400 µl

distilled water, 200 µl of 0.1M NH_4NO_3, 200 µl of 0.047M $AgNO_3$, 180 µl 0.12M dodeca-tungstosilicic acid (Fisons Ltd), 15 µl 36% formalin and 1 ml 0.47 M Na_2CO_3. The slides were then washed in distilled water, fixed in 2% sodium thiosulphate for 2 min, washed in water, dehydrated and mounted. Fine black granules were seen at the sites of DAB polymerization. All tissues studied were also subjected to haematoxylin and eosin staining for histolopathological examination.

Sections of normal human liver tissue sections which had been shown to express the highest ATase activity (see Section 1.2.1) were used initially to assess whether the rabbit anti-human raised against human recombinant ATase were able to detect the human ATase at the cellular level using an IHC method. Liver sections of human ATase cDNA transgenic mice expressing human ATase (provided by Dr CY Fan, 1991) were used as a positive control as the polyclonal ATase anti-serum had been shown to be capable of detecting the human ATase in IHC (Fan, 1991). Intense nuclear staining was seen in both the normal human and trangenic liver sections whether using the standard DAB peroxidase-antiperoxidase or avidin-biotin-peroxidase method; the optimal primary antibody concentration found to be associated with the least background staining was 1/1000 dilution in TBS. The ATase finding in normal human livers has been described in Lee et al. (1992a). However when these methods were tried in a variety of tumour tissues, poor signals with high background staining were seen in the tested sections compared to the control sections, despite attempts at adjusting the dilution of primary or secondary antibodies or the length of incubation with the antibodies and DAB. Modifications using more sensitive staining methods were assessed and silver intensification of Ni-DAB as described by Przepiorka et al (1986) was found to be the most sensitive: the optimal working concentration of the primary ATase antiserum used was 1:1000 dilution. For the control sections, preimmune serum was used initially but this was subsequently changed to ATase pre-adsorbed antiserum (provided by Dr RH Elder) as this resulted in more consistent negative controls. This pre-adsorbed serum was obtained by affinity purification of ATase antiserum in a column containing pure recombinant human ATase (see Lee et al. 1992a).

2.11 Statistical analysis

The principal method of statistical analysis for pre-treatment and post-treatment ATase activities was analysis of variance, with repeated measures analysis of variance used for analysing the changes over time and 'survival' analysis for estimating the time to the ATase nadir. The computer software package BMDP was used for most analyses.

For the clinical study reported in chapter 5, the balance of prognostic variables was evaluated by the Chi square test. A comparison of haematological toxicity and response rate was made using the Chi square test and survivals were displayed using the Kaplan-Meier survival plots and were compared by the log-rank test.

Statistical analysis was carried out with the help of the staff of the Department of Statistics, Christie Hospital particularly the assistance provided by Mr M Dougal.

Chapter 3 *In vivo* and *in vitro* Effects of DTIC and Fotemustine on O^6-Alkylguanine-DNA-Alkyltransferase

3.0 Introduction

In the treatment of metastatic melanoma, DTIC is considered the single most effective chemotherapeutic agent available achieving an overall response rate of 20% (Comis 1976; Balch et al. 1989). Fotemustine alone was recently found to produce a reponse rate of 24% in a phase II study involving 153 patients with metastatic melanoma (Jacquillat et al. 1990). Resistance to both these agents is thought to involve the same DNA repair protein ATase, which repairs the cytotoxic O^6-AlkG lesions introduced in tumour DNA and in the case of fotemustine, this subsequently prevents the formation of cytotoxic interstrand DNA cross-links. In view of the results obtained with DTIC and fotemustine alone and experimental data suggesting that DTIC can deplete rodent ATase (Meer et al. 1989), a phase II trial of the sequential administration of DTIC with fotemustine was formulated to treat patients with metastatic melanoma in order to exploit a potential *in vivo* DTIC ATase depleting effect: fotemustine administered 3 h later would thus be anticipated to be associated with increased DNA cross-links.

In order to test this hypothesis, changes in ATase levels in the peripheral blood lymphocytes of patients with metastatic melanoma treated with sequential DTIC and fotemustine were assessed. This was carried out in order not only to determine the rate of ATase depletion and regeneration following different doses of DTIC and treatment cycles but also to evaluate interindividual variations in basal ATase activity and whether this influences depletion and regeneration rates. As discussed in Chapter 1 and above, identifying the time to reach the ATase nadir and the extent of ATase depletion with different doses of DTIC may have important therapeutic implications since a nitrosourea administered at the nadir of ATase activity would be expected to have greater cytotoxic effect.

3.1 Materials and Methods

Twenty-three patients with metastatic melanoma treated with sequential DTIC and fotemustine chemotherapy were included in the study. The characteristics of the patients studied are shown in Table 1.1. Patients received DTIC at 400, 500 or 800 mg/m^2 by 10 min infusion followed by fotemustine 100 mg/m^2, administered 4 h later as 30 min infusion. In the patients receiving 500 mg/m^2 DTIC, fotemustine was initially administered at 3 h after DTIC but this was subsequently changed to 4 h when it was apparent that nadir ATase occurred at approximately 4 h after DTIC administration. Treatment was repeated every 28 days and the number of cycles given depended on the individual patients response. Blood samples were collected from 30 treatment cycles just before chemotherapy and at 1, 2, 3, 4, 5, 6 and 18 h after DTIC infusion. Fourteen sets of samples were also collected from another group of 8 patients with metastatic melanoma treated with single agent fotemustine (100 mg/m^2) given by i.v. infusion over 30 min.

In order to assess the ability of DTIC, fotemustine and BCNU to inhibit pure recombinant human ATase *in vitro*, varying concentrations of these agents were incubated with 70 fmoles of ATase and 10 μg calf thymus DNA for 2 h at 37°C in buffer I. Residual ATase activity was then measured by standard assay as described in Chapter 2.

Table 3.1 Patient characteristics

Pts/ Cycle[b]	Age/ Sex	Metastatic Sites	DTIC dose[a] (mg/m^2)	ATase (fm/mg) Initial	Nadir
IP/1	66/F	nodes/parotid	400	241	137
IP/2		gland	"	138	90
IP/3			"	189	77
FE/1	61/M	lung/CNS	400	278	184
FE/2			"	168	61
KR/1	68/F	lung/ovaries	400	258	123
JG/1	54/M	skin/liver	400	400	196
MB/1	53/F	node/bone/soft tissue	400	309	163
ML/1	49/M	liver	400	191	122
MK/1	40/M	nodes/soft tissue	400	133	55
RR/1	35/M	adrenal/pleural	400	135	67
PH/1	41/M	soft tissue/nodes	400	232	140
RL/1	52/F	nodes/skin/lung	400	120	114
FM/1	41/M	liver/CNS[c]	500	135	15
FM/3			"	128	24
WB/4	69/M	nodes/bone/lung	500	175	34
PT/1	40/m	lung/soft tissue/ CNS	500	330	125
CF/6	46/M	lung/spleen	500	67	25
EHJ/3	41/M	liver/CNS	500	295	111
EHJ/1			800	107	26
EHJ/2			800	82	23
JB/4	58/M	lung/liver	800	178	102
RB/2	56/M	liver/soft tissue	800	183	52
RB/3			"	236	37
SW/2	40/M	nodes/lung	800	330	22
GS/1	67/M	skin	800	317	85
BP/1	50/M	GI[d]/soft tissue	800	250	93
DW/1	68/F	skin/bone/s oft tissue	800	211	99
DD/1	63/F	lung/CNS	800	220	0
FD/2	43/F	skin/node/CNS	800	82	0

[a] DTIC dosage received; [b] Treatment cycle;

[c] CNS, central nervous system; [d] GI, gastrointestinal.

3.2 Results

3.2.1 *In vivo* effects of increasing dosage of DTIC on lymphocyte ATase

A five-fold difference was seen between the pretreatment ATase activity in patients treated with sequential DTIC and fotemustine (Table 3.1). In the 3 groups of patients receiving 800, 500 and 400 mg/m^2 DTIC, the mean lymphocyte pretreatment ATase levels and their standard errors before the start of the 1st cycle were 230 ± 16, 233 ± 44 and 221 ± 18 fm/mg protein respectively (Fig. 3.1); there was no statistically significant difference in these values (P=0.94) and therefore subsequent effects on ATase levels were not influenced by preselection bias in the dosage groups. The data used for analysis of changes in ATase levels were measurements taken on cycle 1 because it was found that treatment cycle has a confounding effect on the statistical analysis (see Section 3.2.2 below).

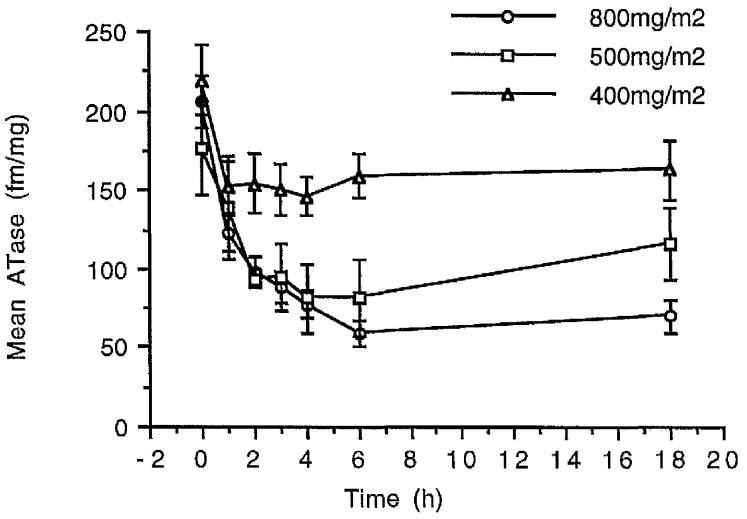


Figure 3.1. Lymphocyte ATase activity before and after first treatment with 400, 500 or 800 mg/m^2 DTIC. Fotemustine was administered 4 h after DTIC. Values shown are the patient means ± S.E.

Despite a wide interindividual variations in ATase depletion and regeneration rates, a progressive depletion of ATase activity was seen following i.v. DTIC administration (Fig. 3.1). The changes across time were very highly significant, but more interestingly so was the interaction between time and dose (P<0.0001). This shows that ATase activity changes differently over time for different doses and in general, maximum

depletion appears to occur earlier with patients receiving 400 mg/m^2 DTIC (median 2-4 h) than patients patients receiving 500 mg/m^2 or 800 mg/m^2 DTIC (median 4-6 h) (see Fig. 3.1). However, the least extensive ATase depletion was generally seen in patients receiving 400 mg/m^2 DTIC and a proportionately larger extent of ATase depletion was seen when the DTIC dose was increased from 400 to 500 mg/m^2 than from 500 to 800 mg/m^2 DTIC. The mean nadir ATase level expressed as a percentage of pre-treatment ATase for 400 mg/m^2, 500 mg/m^2 and 800 mg/m^2 was 56.3%; 26.4% and 23.9% for all treatment cycles (Fig. 3.2).

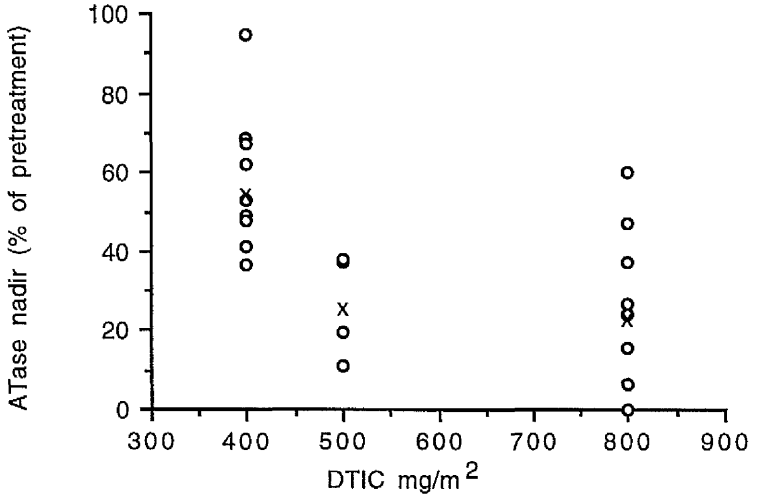


Figure 3.2. Relationship between DTIC dosage and nadir ATase activity expressed as % of the pretreatment level for all treatment cycles. The mean values are shown as x.

In two patients receiving 800 mg/m^2 DTIC, complete ATase depletion was seen and in one patient, this occurred within 1 h and no detectable ATase activity was seen over the next 18 h (FD/2 & DD/1; see Table 1.1 and Fig. 3.3).

Overall, there appeared to be no significant increase in ATase activity by 18 h even though some post-nadir increases were seen (see Fig. 3.1). In most cases this was slight, ranging from 6% to 30% of the pretreatment levels. However, in two patients receiving 400 mg/m^2 and one receiving 500 mg/m^2 recovery (FM/1, see Fig. 3.3) was very extensive and rapid, attaining close to pretreatment levels: in the former patients this was associated with an earlier ATase nadir suggesting that in some cases the lower

DTIC dosage may affect ATase recovery rates although this requires assessment using a much larger sample number.

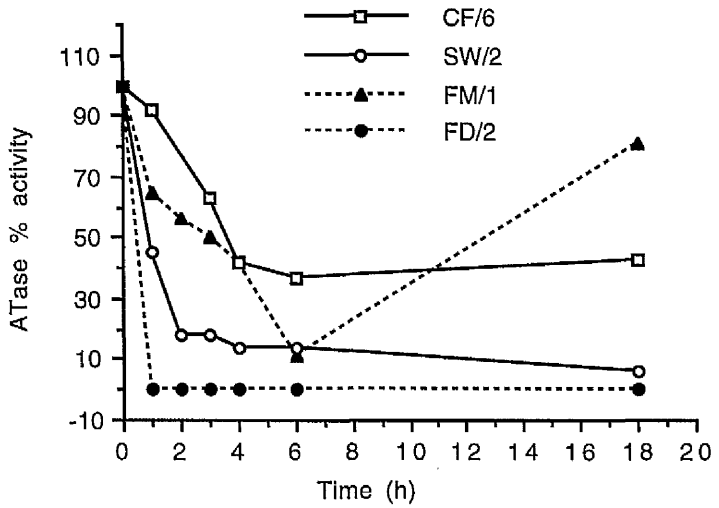


Figure 3.3. Graphs showing changes in lymphocyte ATase activity expressed as percentage of pretreatment level in 2 typical cases (CF/6 & SW/2) and 2 atypical cases (FM/1 & FD/2) following DTIC/fotemustine chemotherapy. Complete ATase depletion occurred in patient FD whereas ATase recovery was seen in patient FM.

3.2.2 *In vivo* effects of different treatment cycles on lymphocyte ATase

The mean pretreatment ATase activity seemed to be higher in treatment cycle 1 (220 ± 25 fm/mg protein) than in treatment cycles 2 to 6 (171 ± 31 fm/mg protein) (Fig. 3.4). When the study was initiated, no effect of treatment cycle on ATase activities was anticipated and as a results samples were not taken from the same patient on each treatment cycles; some patients had samples taken on different treatment cycles and some had samples taken on more than one cycle. In the interim analysis, it emerged that there was considerable difference between treatment cycle 1 and the others, but not between cycle 2 and cycles 3-6 and in the analysis presented here, the study was split into two groups: cycle 1 and cycles 2-6 and no patient appeared more than once in the analyses.

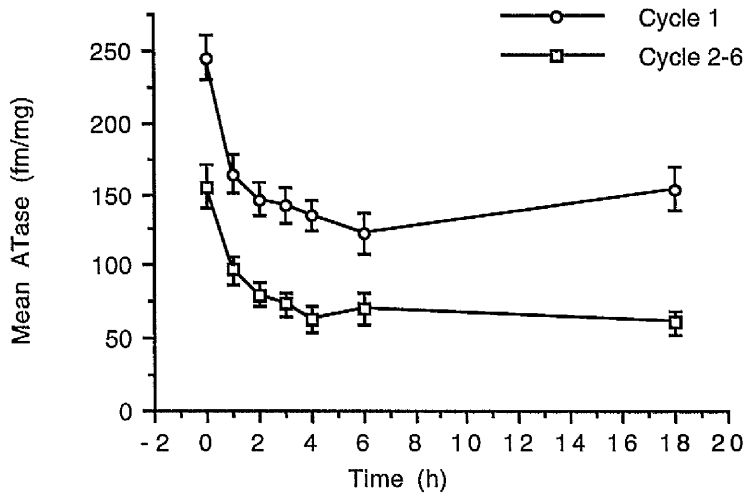


Figure 3.4. Lymphocyte ATase activity before and after chemotherapy with different treatment cycles of DTIC. Values shown are means ± S.E.

The overall difference in ATase levels between the two cycle groups was significant ($P<0.025$) but the magnitude of the difference greatly depended on the number of hours after chemotherapy (group versus time interaction $P<0.0001$). This shows that the effect of cycle changes over the sequence of measurements. The mean nadir ATase levels were 41.7% of the pretreatment level for cycle 1 and 29.8% for cycles 2 to 6 and there was no significant difference between the cycles in the time to nadir using a log rank test ($P=0.285$). The analysis and the percentages listed above were based on 23 cases (see Fig 3.4).

Overall, the extent of depletion of ATase following DTIC was directly proportional to the initial ATase level and this data is presented and discussed in Chapter 7.

3.2.3 *in vivo* effects of fotemustine on lymphocyte ATase

The overall mean lymphocyte pretreatment ATase level for patients receiving fotemustine alone was 227 ± 72 fm/mg protein which is not statistically different from the groups above receiving sequential DTIC/fotemustine. The mean pre-treatment ATase level for patients receiving fotemustine alone was 242 ± 30 fm/mg protein for cycle 1 and 176 ± 21 fm/mg protein for cycles 2 and 3 combined. All patients were

given three cycles of treatment and most patients were studied on more than one cycle, so the patients which were used in the analysis could act as their own controls.

In contrast to DTIC, there was no statistically significant change in lymphocyte ATase activity at 3-4 h or at 16-18 h after fotemustine compared to the pretreatment values (P>0.9). In addition, no significant difference was seen between ATase levels when different treatment cycles were compared (P>0.9) (see Fig. 3.5).

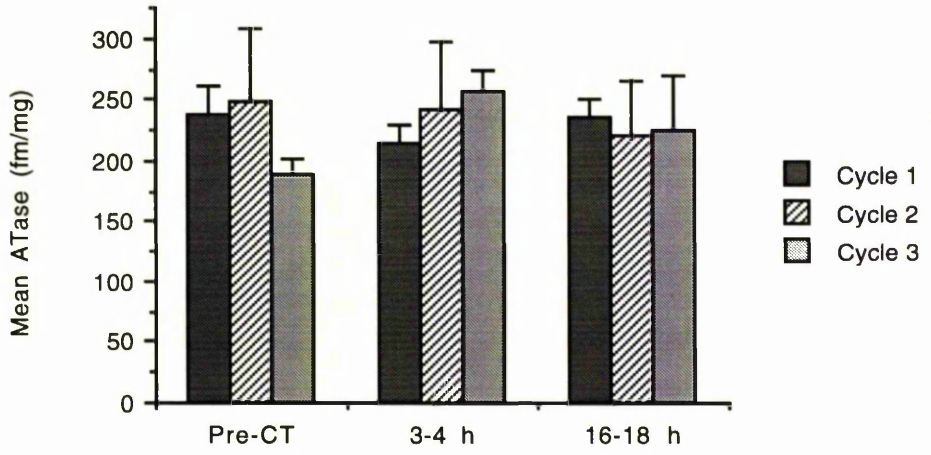


Figure 3.5. Mean lymphocyte ATase activity before, 3-4 h, 16-18 h after 100mg/m^2 fotemustine with treatment cycles 1, 2 and 3. Pre-CT= pretreatment ATase levels.

3.2.4 *In vitro* effects of DTIC, fotemustine and BCNU on recombinant ATase

No depletion of activity was seen when recombinant human ATase was incubated with up to 1 mM DTIC, whereas with BCNU and fotemustine, nearly identical dose-dependent depletion was seen, 50% depletion occurred at 10 μM and 15 μM concentrations respectively (Fig. 3.6). This finding is consistent with the spontaneous hydrolysis of BCNU and fotemustine to an alkylating species capable of alkylating DNA whereas DTIC requires the presence of cytochrome P_{450} system for metabolic activation to generate a reactive alkylating species. Slight ATase depletion was seen

with higher DTIC concentration (> 1mM) but this may be a direct effect on the ATase or to possibly contamination of DTIC with a reactive species.

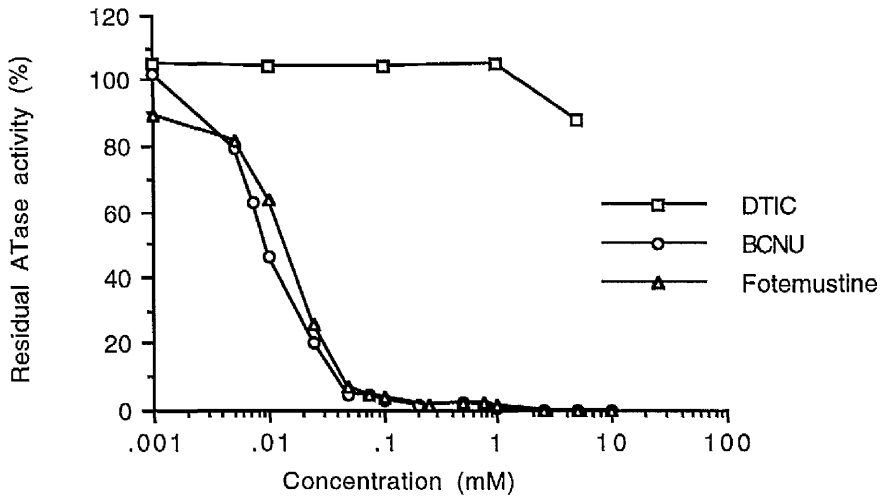


Figure 3.6. *In vitro* effects of DTIC, BCNU and fotemustine on pure recombinant human ATase.

3.3 Discussion

The present study demonstrated a progressive DTIC-induced depletion of ATase activity in human peripheral lymphocytes with time after administration. This indicates that DTIC is metabolised to the monomethyl metabolite, MTIC and that this is produced in sufficient amounts to react with DNA in peripheral blood lymphocytes to generate O^6-MeG. This is stoichiometrically repaired by lymphocyte ATase causing an apparent decrease of ATase activity with a mean nadir activity occurring at approximately 4 h in most patients. Despite a wide inter-individual variations in pretreatment ATase activity, the nadir of ATase activity generally occurred later in patients receiving 800 mg/m^2 (4-6 h) than in patients receiving 400 mg/m^2 DTIC (2-4 h). In addition patients receiving 400 mg/m^2 DTIC had the least extent of ATase depletion with a mean ATase nadir of 56.3% of pretreatment level after the first treatment cycle whereas patients receiving 500 and 800 mg/m^2 DTIC had a lower mean nadir lymphocyte ATase activity of 26.4% and 23.9% respectively. This suggests that the pharmacokinetics of DTIC is dose-dependent. Indeed, it has been shown that high-dose DTIC (850-1980 mg/m^2) follows

nonlinear pharmacokinetics with saturation occurring in the metabolism and also a slower distribution and disposition rate when compared to lower dose DTIC (Loo et al. 1968; Skibba et al. 1969; Breithhaupt et al. 1982; Buesa and Urrechaga 1991). The later nadir in ATase activity seen with 800 mg/m^2 DTIC in contrast to 400 mg/m^2 is therefore likely to be related to the more protracted production of alkylating metabolites.

Whilst ATase recovery by 18 h was generally not substantial and evident in less than half of the patients, in two patients given 400 mg/m^2, the ATase nadir was around 2 h and activity recovered rapidly attaining close to pretreatment levels by 18 h whereas complete ablation of ATase activity was seen in 2 patients given 800 mg/m^2. These results highlight the interindividual differences in the continued availability of methylating metabolites and/or in the *de novo* synthesis rates for ATase. In view of the possible saturable pharmacokinetics with high dose DTIC, it would be clearly interesting to administer DTIC by continous infusion or pulsed low doses in order to assess whether or not a complete ablation of ATase activity could be achieved using lymphocytes as a target.

Despite wide interindividual variations in pretreatment levels and post-treatment DTIC-induced lymphocyte ATase depletion, the data suggested that subsequent treatment cycles were associated with a more extensive reduction in ATase activity compared to the first treatment cycle. This indicates that a more extensive DNA methylation may have occurred with later treatment cycles. The cycle effect might be a consequence of the initial doses of DTIC or fotemustine increasing the capacity for metabolic activation of subsequent doses of DTIC. Alternately, a reduction in pre-treatment ATase levels might be the result of a drug-mediated selection of lymphocytes expressing lower levels of ATase, although how this might occur is not clear at present. A similar finding was reported in some patients treated with procarbazine (Sagher et al. 1989). This effect would be anticipated to cause greater haematological toxicity with later treatment cycles if similar changes occur in the bone marrow cells. In addition, the likelihood of patients developing a second malignancy would be anticipated to increase if they receive more

protracted treatments rather than the standard six cycle chemotherapy treatments given in many clinical situations.

Although the possibility that a synergistic effect on ATase depletion might have occurred in patients given DTIC and fotemustine cannot be excluded, no statistically significant change in lymphocyte ATase activity occurred in patients treated with 100 mg/m^2 fotemustine alone even though an *in vitro* dose-dependent inactivation of human recombinant ATase was seen. This suggests that insufficient concentrations of fotemustine were available to react with lymphocyte DNA (with the treatment dosage used) to produce a detectable lowering of ATase activity. Whether administering a higher dosage of fotemustine will be able to achieve ATase depletion will require further study. A similar finding was reported for human lymphocytes treated with low dose BCNU (40-200 mg/m^2) although a statistically significant reduction in lymphocyte ATase activity was seen after high dose BCNU (350 mg/m^2; Gerson 1989) and in patients receiving combined cyclophosphamide/BCNU (600 mg/m^2) treatments as reported in Chapter 8 (Section 8.2.1). Similar to fotemustine, an *in vitro* dose-dependent inactivation of ATase was seen following incubation of BCNU, human recombinant ATase and calf thymus DNA.

Experimental models have repeatedly shown that depletion of ATase (approximately 60-90% depletion) can sensitize tumour cells to CNUs, resulting in a 2- to 12- fold reduction in the 50% lethal dose of these compounds. Greater extents of sensitisation were seen in tumour cells expressing high ATase activity than cells with low levels of activity (Dolan et al. 1985a; Dolan et al. 1991; Gerson et al. 1985; Gibson et al. 1986a; Zlotogorski and Erickson 1984). If ATase is the principal mechanism of tumour cell resistance to methylating and chloroethylating agents and if the results obtained with peripheral lymphocytes can be extrapolated to tumour cells, the findings would support the use of sequential DTIC followed by fotemustine treatment. This would be in preference to a schedule where DTIC and fotemustine are administered concurrently or in which fotemustine is given before DTIC in order to improve the therapeutic

response. The present studies suggest that 4 to 6 h post DTIC administration would be the optimal time to administer further CNUs as this would be anticipated to lead to increased formation of the cytotoxic DNA cross-links before regeneration of ATase by *de novo* synthesis could occur. It would also be interesting to explore if repetitive administration of DTIC or a related agent every 4-6 h would further deplete ATase in lymphocytes and whether this would be associated with increased antitumour activity. Theoretically, a greater cytotoxic effects would be anticipated if complete ATase ablation could be achieved in tumour tissues before the administration of an O^6-alkylating agent.

Whilst such extrapolations to tumour tissues should be considered with appropriate caution, they should also be assessed in relation to the available relevant experimental and clinical data. Human tissues and tumours differ greatly in ATase levels (D'Incalci et al. 1988) but there is some indication that relationships between tissues may exist (Kyrtopoulos et al. 1990). In addition studies in rats have shown that DNA methylation occurred to a broadly similar level in all tissues including lymphocytes following administration of methylating agents, even those requiring metabolic activation (Kleihues et al. 1976b; Degan et al. 1988; Fong et al. 1990). As discussed earlier, in melanoma patients, DTIC alone (including high dosage) regularly produces a response rate of 20% (Cowan and Bergsagel 1971; Comis 1976; Pritchard et al. 1980; Balch et al. 1989); fotemustine alone produces a 24% response rate (Jacquillat et al. 1990). Hovever as reported in Chapter 5 in patients treated with sequential DTIC and fotemustine, an overall response rate of 30% was achieved and the response rate appears to increase with increasing dose of DTIC (see Section 5.2.2). These results provide circumstantial evidence in support of the use of lymphocyte ATase levels as a monitor for those in tumour tissues. Further support is provided by the extent of the toxic effects of the treatment as shown in the findings reported in Chapter 5; there was a statistically significant dosage-dependent development of severe haematological toxicity ($P<0.01$) in the 3 groups of patients analysed (see Section 5.2.3). It is tempting to attribute this to greater DNA alkylation with higher dosage DTIC in contrast to lower

DTIC dosage. Bone marrow has one of the lowest ATase levels of the human tissues examined so far (see Chapter 6 and Gerson et al. 1985) and this in conjunction with the possibility that ATase depletion might increase with treatment cycle, may account for its greater sensitivity to the toxic effects of DTIC.

In summary, the present work shows that DTIC can inactivate lymphocyte ATase activity and that this was dosage and cycle dependent. It also suggests that lymphocytes might be useful as a surrogate marker to monitor changes in tumour ATase activity following DTIC treatment and hence the feasibility of extending similar studies into treatments involving other O^6-alkylating anti-tumour drugs. Whether a correlative effect does occur in tumour tissues will require further investigation. Finally, the results indicate that it would be interesting to explore whether a more extensive ATase depletion can be achieved with more frequent, smaller doses of DTIC or continuous infusion of DTIC in view of its potential improved clinical effects when combined with a nitrosourea.

Chapter 4 Formation and Loss of O^6-Methyldeoxyguanosine in Human Leukocyte DNA following DTIC/Fotemustine

4.0 Introduction

In Chapter 3, a progressive depletion of ATase was seen in human peripheral lymphocytes following administration of DTIC; the rate and extent of ATase depletion was dose and cycle-dependent with the mean nadir ATase activity occurring 4 to 6 h after DTIC. In addition, large inter-patient variations were encountered both in the extent and the rate of ATase depletion. These findings can be interpreted on the basis of the metabolism of DTIC to a methylating agent capable of reacting with DNA to form O^6-MeG and the subsequent repair of this adduct leading to ATase depletion.

In the present study, the generation and loss of O^6-MeG, measured as O^6-methyldeoxyguanosine (O^6-MedG) following metabolic activation of DTIC was determined in total blood leukocyte DNA. The relationships with changes in lymphocyte ATase levels and whether O^6-MedG levels correlate with clinical responses were also examined.

The presence of persistent O^6-MedG after treaments with methylating antitumour drugs may serve as an indicator firstly of ATase deficiency and secondly of the potential toxic and carcinogenic insult received, particularly in view of the association between O^6-MeG persistence or accumulation and carcinogenesis in experimental models. Sagher et al (1988) for example reported that patients who developed a chemotherapy-related acute myeloid leukaemia (AML) following treatment for Hodgkin's disease, had lower levels of ATase compared to *de novo* AML patients or normal control subjects and they postulated that unrepaired O^6-MedG in the former group of patients may be an aetiological factor in the development of therapy-related AML. DTIC and procarbazine form part of a variety of combination chemotherapy regimes (such as MOPP, ChlVPP, MVPP, ABVD), particularly in the treatment of Hodgkin's disease which has

consistently produced high remission and cure rates (Hellman et al. 1989). However, a consequence of the treatment is the development of AML at a rate ranging from 0.5 to 2.0 percent per year for the first 10 years, with a cumulative incidence of 3 to 10 percent (Urba and Longo. 1992). This study therefore also examines the possibility of residual unrepaired O^6-MedG in patients returning for subsequent chemotherapy.

4.1 Materials and Methods

Details of the patients studied including five patients from Chapter 3 are shown in Table 4.1. All patients were diagnosed as having advanced malignant melanoma and treatment consists of 400 mg/m^2 DTIC given as 10 min infusion followed 4 h later by 100 mg/m^2 fotemustine given as 30 min infusion as described in Chapter 3. Patient FE (with brain metastasis) received an additional treatment on day 8. Blood samples were collected just before therapy and at 1, 2, 3, 4, 5, 6 and 18 h after DTIC administration from 8 patients in the first treatment cycle. A further 5 sets of blood samples were collected from 4 patients who returned for subsequent treatments. Total blood leukocytes DNA and peripheral blood lymphocytes were isolated from 2 x 10 ml blood samples by phenol extraction or by centrifugation on Ficoll gradients of whole blood respectively, as described in Chapter 2. An indication of the total exposure to O^6-MedG was obtained for each patient by integration of the area under the O^6-MedG concentration-time curve (AUC).

Table 4.1 Patient characteristics

Pts/ Cycle[b]	Age/ Sex	Metastatic Sites	Response[a]	ATase (fm/mg) Initial	Nadir	O^6-MedG Peak[c](μmol/mol dG)	AUC[d](μmol.h)
IP/1	66/F	nodes/parotid gland	CR	241	137	14.3 (3h)[e]	28.5
IP/2				138	90	24.7 (3h)	74.7
IP/3				189	77	7.6 (5h)	57.7
KR/1	68/F	lung/ovaries	PR	258	123	12.8 (3h)	27.3
KR/2				242	81	1.1 (5h)	10.9
FE/1	61/M	lung/brain	NE	278	184	7.7 (4h)	48.2
FE/2				168	61	8.3 (2h)	110.3
GB/1	53/M	skin/nodes/lung	PD	217	72	5.7 (5h)	62.2
GB/2				191	118	5.6 (3h)	76
JG/1	54/M	skin/liver	PD	400	196	1.0 (3h)	12.5
MB/1	53/F	skin/nodes/ bone/soft tissue	PD	309	163	2.0 (3h)	24.9
ML/1	73/M	skin/lung/liver/ spleen	PD	-	-	5.6 (3h)	68
MH/1	40/M	nodes/soft tissue	PD	263	55	0.7 (3h)	2.0

[a] CR= complete response; PR= partial response; PD= progressive disease; NE= not evaluable

[b] Treatment cycle

[c] Peak amount of O^6-MedG formed in leukocyte DNA (μmole/mole dG)

[d] AUC from 0 to 18h except sample KR1 which was integrated from 0-4h.

[e] Time to reach peak O^6-MedG in leukocyte DNA (h).

4.2 Results

4.2.1 O^6-methydeoxyguanosine formation in leukocyte DNA

O^6-MedG could be detected in total blood leukocyte DNA shortly after the first DTIC treatment with the peak O^6-MedG formation occuring at approximately 3 h after drug administration in 6 patients and at 4 and 5 h in the remaining 2 patients, respectively. There was a 20 fold difference in the maximum O^6-MedG levels, ranging from 0.7 to 14.3 μmoles O^6-MedG/ mole dG (mean 6.41 ± 5.53 μmoles O^6-MedG/mole dG). The kinetics of DNA methylation were broadly similar in most cases; a post-treatment peak in O^6-MedG formation occurred at 3-5 h and was followed by a decline in adduct level (Fig. 4.1). However in some cases (e.g. FE) there was a trend for O^6-MedG to rise again at 18 h (Fig. 4.1).

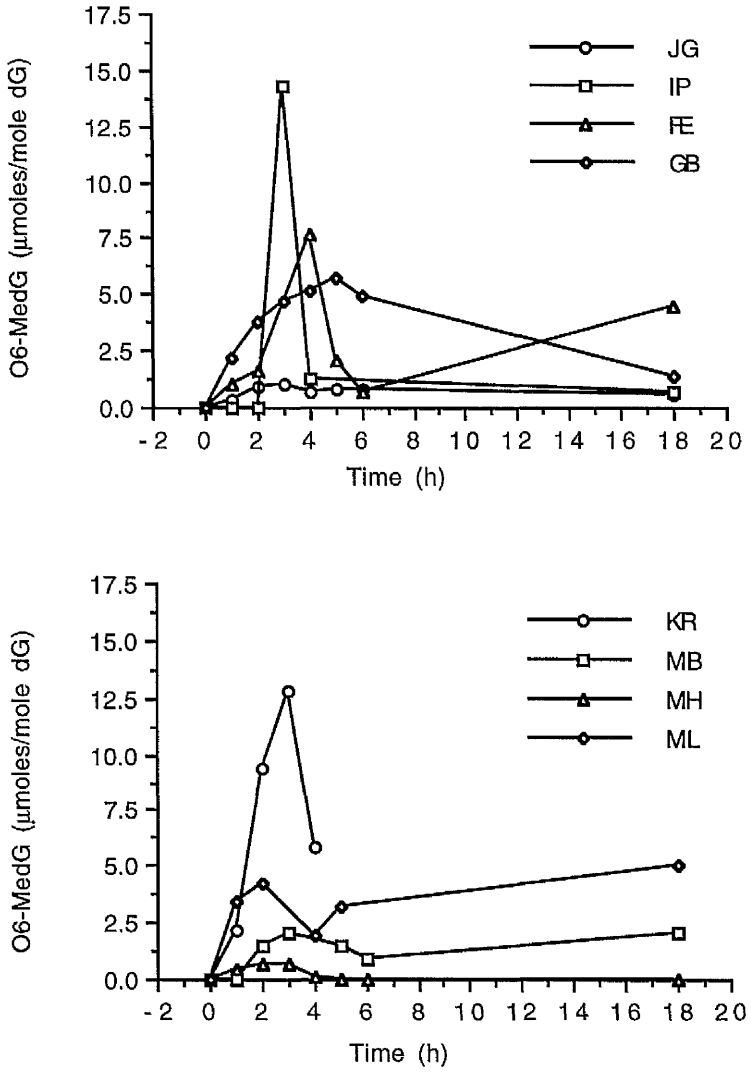


Figure 4.1. O^6-MedG levels in total peripheral leukocyte DNA at first treatment cycle. Patients IP and KR showed clinical response. In all other cases the disease progressed.

In the second treatment cycle, a broadly similar pattern of DNA methylation was observed in 4 patients with the peak of O^6-MedG formation varying from 1.1 to 24.7 µmoles/mole dG (mean 9.5 ± 8.9 µmoles O^6-MedG/mole dG, see Fig. 2). Of these four patients, two (FE and GB) achieved peak adduct levels similar to those observed in cycle one, the third patient (KR) showed an approximately 11-fold reduction and in the fourth case (IP), the peak of O^6-MedG level in cycles 2 and 3 was increased and decreased, respectively, relative to that observed for cycle 1.

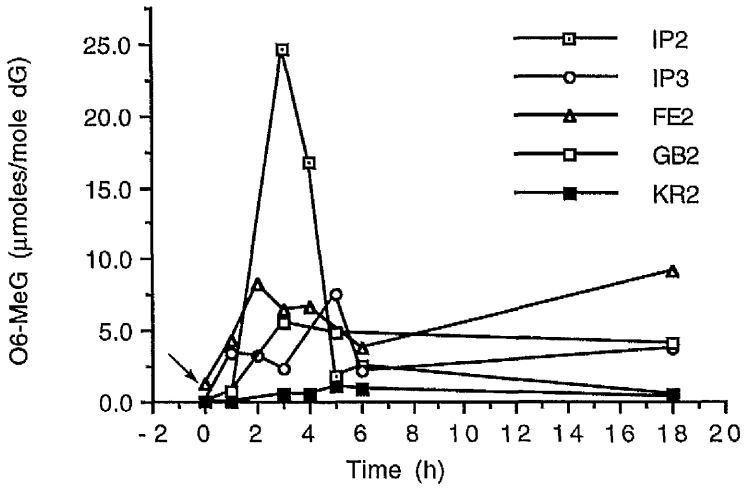


Figure 4.2. O^6-MedG levels in total peripheral leukocyte DNA at various times during subsequent treatment cycles. Number after patient's initial refers to treatment cycle.

In all but one case (MH), O^6-MedG persisted in the DNA at detectable levels for at least 18 h. One individual (FE; with brain metastasis) returned on day 8 for a second DTIC treatment and O^6-MedG was detected in leukocyte DNA at this time at a level of 1.3 µmoles/dG (Fig. 4.2, see arrow). There was no occupational history or recent cigarette consumption in this patient to suggest that the residual O^6-MedG was due to enviromental exposure other than the administered drug. For the remaining cases in which treatment was repeated every 28 days, residual O^6-MedG from previous exposure to DTIC was below the limit of detection (~ 0.4 µmoles O^6-MedG/mole dG).

The combined measurements of O^6-MedG exposure determined as AUCs are shown in Table 4.1. Again, a considerable inter-individual variation was apparent with 34-fold and 10-fold differences between the highest and lowest values for treatment cycles 1 and 2, respectively. In general, leukocyte DNA from patients returning for subsequent courses of chemotherapy (IP2, IP3, GB2, FE2) tended to be more extensively methylated (in terms of O^6-MedG AUC) than in patients receiving the first DTIC treatment (Table 4.1) and this is in accord with the data obtained from the previous study which showed a more extensive ATase depletion in peripheral lymphocytes of patients returning for subsequent treatment cycles (Section 3.2.2; Fig. 3.4).

Of the eight patients studied, only two (IP and KR) responded to therapy and, although O^6-MedG AUC for both patients was close to the mean value, their leukocyte DNA contained the highest peak levels of O^6-MedG after treatment cycle 1 (Table 4.1). Patient FE died from aspiration pneumonia shortly after cycle 2 and was not evaluable.

4.2.2 Relationship between lymphocyte ATase depletion and leukocyte O^6-methyldeoxyguanosine formation in peripheral blood

The lymphocyte ATase activity nadir occurred at approximately 3-4 h (Chapter 3, Section 3.2.1) and this coincided with the peak of DNA methylation in the leukocytes (Figs. 4.1 & 4.2). Using Spearman correlation coefficients, an inverse correlation was seen between the pretreatment lymphocyte ATase level and the amount of O^6-MedG formed in total leukocyte DNA when expressed either as peak level (R= -0.59, P=0.04) or as the leukocyte O^6-MedG AUC (R= -0.72, P=0.008; see Fig. 4.3). The extent of lymphocyte ATase depletion (i.e. pretreatment minus nadir level) was similarly related to peak O^6-MedG level (R= -0.75, P=0.005) and the AUC (R=-0.73, P=0.007; see Fig. 4.4). However, there was no evidence of a relationship between the nadir ATase levels and the extent of O^6-MedG formed in DNA (P>0.3; Fig. 4.5).

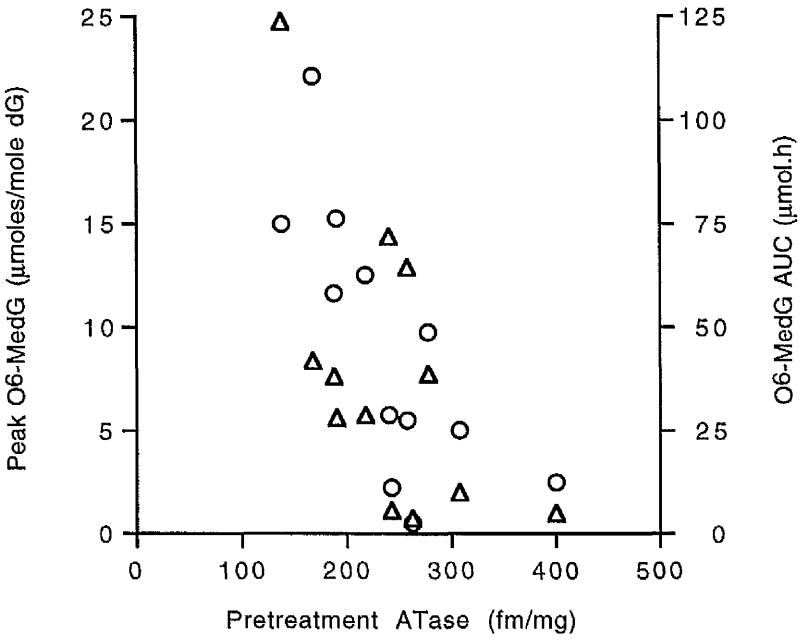


Figure 4.3. Relationship between the peak O^6-MedG level (Δ) or the O^6-MedG AUC (O) and pretreatment ATase activity.

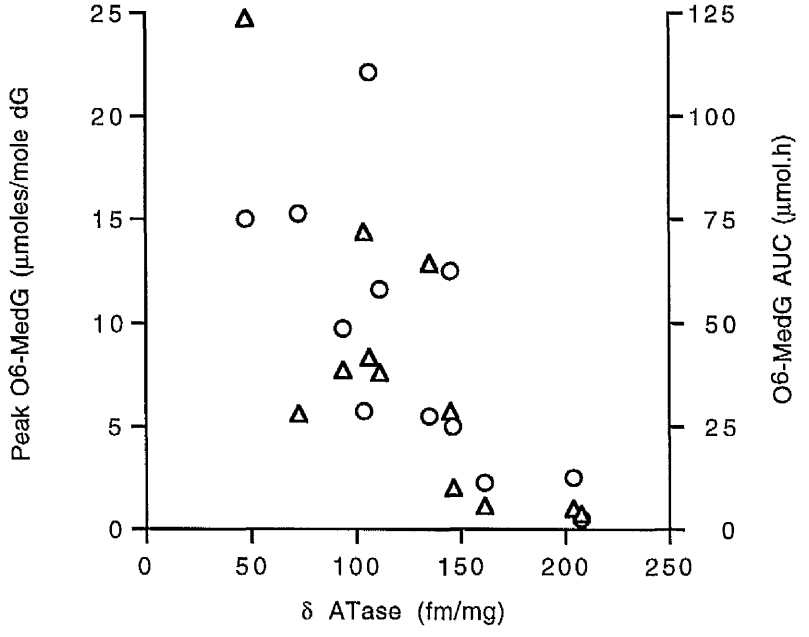


Figure 4.4. Relationship between the peak O^6-MedG level (Δ) or the O^6-MedG AUC (O) with the extent of ATase depletion (δ ATase = pretreatment minus nadir ATase level).

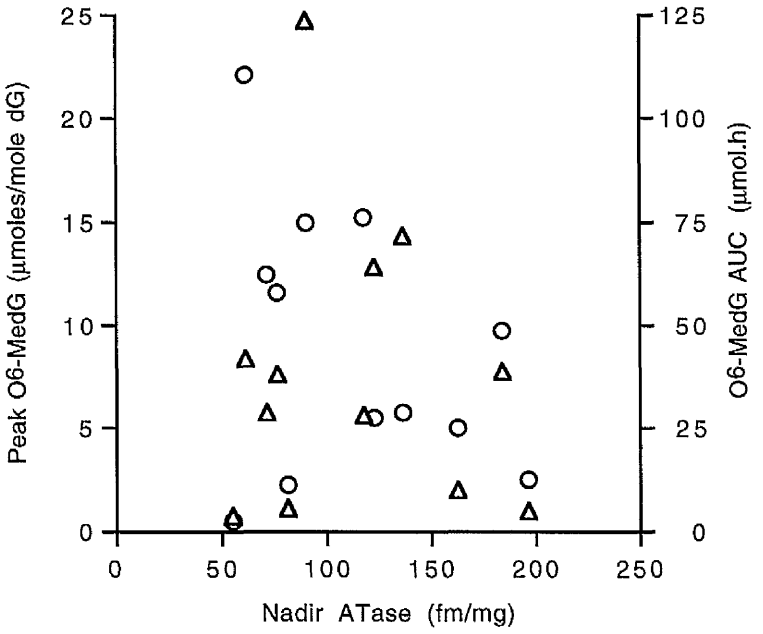


Figure 4.5. Relationship between the peak O^6-MedG level (Δ) or the O^6-MedG AUC (O) and nadir lymphocyte ATase activity.

4.3 Discussion

The present study demonstrates the presence of O^6-MedG in the DNA of peripheral leukocytes from patients receiving combined DTIC/fotemustine therapy and hence indicates the ability of these patients to activate DTIC in amounts sufficient to react with leukocyte DNA.

There was a wide variation in the amount of O^6-MedG formed with an approximately 20 fold inter-patient variation in the maximum levels of O^6-MedG determined in the 8 patients following treatment cycles 1, 2 and 3. Similar large inter-individual variation was also seen with the O^6-MedG AUC which gives a combined measure of the formation and persistence of O^6-MedG. A 2-3 fold inter-individual variation was seen when 7-MeG levels were measured in leukocyte DNA of patients receiving dacarbazine (225 mg/m^2) (van Delft et al. 1992). Amongst the factors that might contribute to these differences are capacities for DTIC activation, capacities for the cellular uptake of DTIC and/or MTIC, intracellular concentrations of glutathione or other methylating agent-

scavenging compounds, capacities for the ATase dependent or independent repair of O^6-MedG in DNA, or combinations of all of these. Some of these differences are predictable from clinical investigations on drug metabolism (Idle et al. 1983) and from studies of carcinogen metabolism in experimental animals (Nebert et al. 1979). Maximum DNA methylation was achieved at approximately 3-5 h after DTIC administration but in some cases, no definite peak of methylation occurred. Interestingly, DNA single strand breaks occurring in peripheral blood lymphocytes are also maximal approximately 5 h after DTIC administration (Walles and Ringborg, 1991).

In some patients, an increase in O^6-MedG level was seen at 18 h, and while this may be accounted for to some extent by experimental variation, the reasons for the larger increases seen in patient FE are unclear but may be related to the different turnover rates of white blood cell subpopulations with different levels of DNA methylation (Mustonen and Hemminke 1992). Some increases in 7-MeG levels at 24 h have also been observed in leukocyte DNA from patients treated with DTIC (van Delft et al. 1992).

In general, patients returning for subsequent DTIC treatments were found to show an increased O^6-MedG AUC with respect to cycle 1. These values indicating increased exposure to O^6-MedG are in accord with the observations in the previous Chapter which demonstrated that subsequent treatment cycles were associated with a more extensive reduction of ATase activity compared to the first treatment cycle.

It is tempting to speculate that the wide variations in O^6-MedG formed in leukocyte DNA which indicate differences in bioavailabity of intracellular MTIC could be amonst the factors that predict clinical response. Two of the eight patients studied here responded to treatment and in both cases the peak but not the AUC of O^6-MedG formation in leukocytes was higher than that seen in the other five patients in which disease progressed. This may reflect the need to reach a minimum threshold O^6-MedG level before cell killing can occur. Clearly, whether differences in the metabolism of

DTIC and the formation of O^6-MedG explain the wide variation in tumour response needs to be extended to a larger clinical study in order to establish the true significance of this observation. The extent to which the present results can be extrapolated to clinical response will also depend on whether or not O^6-MedG levels in peripheral leukocyte DNA correlate with those of target tumour DNA but as pointed out earlier there are some indications from experimental data that relationships between tissues exist: studies in rats have shown that DNA methylation occurred in a broadly similar levels in several different tissues including lymphocyte DNA following administration of methylating agents, even those agents requiring metabolic activation (Kleihues et al. 1976b; Degan et al. 1988; Fong et al. 1990).

In the present study, the kinetics of lymphocyte ATase depletion and leukocyte DNA O^6-MedG accumulation with time and the nadir ATase activity coinciding with peak O^6-MedG formation suggest concomitant effects in the two population of cells following DTIC treatment. Correlations were seen between the amount of O^6-MedG formed in leukocyte DNA (expressed either as peak level or AUC) with the pretreatment lymphocyte ATase activity or with the extent of ATase depletion (see Figs. 4.3 and 4.4). Although these relationships are based on a relatively few patients, a similar correlation was seen between the accumulation of O^6-MedG and ATase levels in a study of 7 patients treated with the related drug, procarbazine (Souliotis et al. 1990) and in analyses of bladder mucosa from individuals putatively exposed to enviromental alkylating agents (Badawi et al. in press). These data suggest that patients with high initial levels of ATase are therefore able to repair a greater proportion of the O^6-MedG resulting in less O^6-MedG accumulation, whilst adducts persist more extensively in the leukocyte DNA of individuals with low pretreatment ATase levels. This study underlines the importance of ATase and its relationship to O^6-MedG accumulation. Since wide differences in ATase levels have been reported in melanoma tissues (Maynard et al. 1989 and see Section 9.2.1), this would be anticipated to result in significant differences in the accumulation of the toxic O^6-MedG in the target tumour DNA and hence the tumour response.

The extent of O^6-MedG repair achieved in the eight patients following chemotherapy was highly variable. In one patient (FE, see Table 4.1) who returned 8 days later for DTIC, O^6-MedG was still detectable prior to receiving this additional chemotherapy. As discussed in Chapter 1, the persistence of O^6-MedG in DNA if unrepaired could result in G→A mutations and therefore the potential for carcinogenesis. Since myeloid precursors have low ATase activity (Gerson et al. 1985 and see Chapter 6, Section 6.2.4), it is not unreasonable to speculate that stem cells would suffer more extensive mutagenic damage and are therefore that this is the basis of their increased risk of second malignancy.

In conclusion, the results reported show that it is possible to detect and to measure O^6-MedG in total blood leukocytes. The data also support the earlier suggestion that nadir lymphocyte ATase depletion observed approximately 3-4 h after 400 mg/m^2 DTIC is a consequence of DNA methylation, which is also maximal at this time. The wide individual variations in the extent of DNA methylation if reflected in tumour tissues may result not only from differences in DNA repair capacity but also differences in the capacity for metabolic activation, uptake and detoxification of DTIC. The use of such DNA adduct measurement may therefore provide us with better information on cellular penetration as well as metabolic activation, distribution and clearance of DTIC and related methylating anti-tumour agents, which, in combination with a knowledge of the ATase activity might permit the design of individualised treatment protocols with potentially improved therapeutic benefit.

Chapter 5 Clinical Effects of Sequential Administration of DTIC and Fotemustine in Advanced Malignant Melanoma

5.0 Introduction

As indicated in Chapter 3, the most active single agent for the treatment of metastatic melanoma is DTIC which produces an overall response rate of 20% (Comis 1976; Balch et al. 1989). After DTIC, the nitrosoureas are considered the second most effective agents and produce an approximately 15 % response rate (Comis 1976; Balch et al. 1989). Doses of DTIC used have ranged from 2 mg/kg for 10 days to 1450 mg/m^2 as a single bolus every 4 to 6 weeks (Cowan and Bergsagel 1971; Comis 1976; Mastrangelo et al. 1982). The infusion of DTIC over 24 h has also been explored (Thatcher et al. 1985). The most frequently used DTIC schedule consists of 250 mg/m^2 daily intravenously for 5 consecutive days with treatment being repeated every 3-4 weeks (Mastrangelo et al. 1982; Geeraerts and Nathanson 1986). Most DTIC-containing combination chemotherapy regimens have added little to the response rate and survival duration and frequently have resulted in significant increases in haematological toxicity (Mastrangelo et al. 1982; Geeraerts and Nathanson 1986; McClay and Mastrangelo 1988).

As indicated earlier, the regimen of sequential DTIC and fotemustine treatment was formulated based on the preclinical evidence that sensitization of cells resistant to nitrosoureas can be achieved by pretreatment with a methylating agent. In Chapters 3 and 4, clinical evidence of ATase depletion in peripheral blood lymphoctes was established: the ATase nadir occurred at approximately 4 h and this was concomitant with the of peak O^6-MedG formation after DTIC administration. Having established that maximum ATase depletion occurred at 4 h after DTIC, the timing of fotemustine administration was changed to 4 h in order to maximise the potential for more cross-link formation induced by fotemustine. As indicated in Chapter 1, ATase repairs the O^6-chloroethylguanine induced by fotemustine and in the process, prevents the

formation of the cytotoxic DNA interstrand cross-links. In an effort to achieve greater ATase depletion and hence increase the response rate further, the dosage of DTIC was escalated to 800 mg/m^2 from an initial dose of 500 mg/m^2. The present study describes the clinical findings of using three different doses of DTIC (400, 500, 800 mg/m^2) with fotemustine (100 mg/m^2) administered 4 h after DTIC.

5.1 Materials and Methods

Sixty patients with widely metastatic malignant melanoma were entered into the study protocol as defined in Chapter 2, Section 2.1 and included all the patients studied in Chapters 3 and 4.

The median age was 55 years (range, 17-75 years), and there were 28 males and 32 females. The median time from primary surgery to first metastasis was 3 years (range 0 to 12 years). The number of patients with metastatic sites were: 5 patients with non-visceral sites, 21 patients with visceral sites and 34 patients with both visceral and non-visceral sites. Twelve patients had prior chemotherapy and 13 patients had localised radiotherapy, but other metastatic sites were available for evaluation in the study.

Patients received DTIC at 400, 500 or 800 mg/m^2 (i.v. infusion over 10 min) followed by fotemustine at 100 mg/m^2 (over 30 min) 4 h after DTIC and treatment was repeated every 28 days. A total of 169 cycles of chemotherapy were administered; 75, 57 and 37 treatment cycles in the 400 500 and 800 mg/m^2 DTIC groups respectively.

Tumour response and toxicity assessment used the WHO (1979) criteria as defined in Chapter 2, Section 2.2.

Lung function tests were also performed in 11 patients following the development of an adult respiratory distress type syndrome in one patient. The tests were performed by the Lung Function Unit at Wythenshawe Hospital, Manchester. Routine spirometry was

performed using a Gould Pulmonet III Spirometer (Cardiokinetics, Salford, UK). Total lung capacity was measured by body plethysmography (Eric Jaeger (UK) Ltd). Carbon monoxide transfer factor was measured by the single breath method (PK Morgan Ltd, Chatham, UK).

5.2 Results

5.2.1 Comparability of different DTIC dosage groups

As shown in Table 5.1, the three treatment groups were well balanced with no statistical differences in pretreatment characteristic in terms of distributions of age, sex (P=0.96), performance status (P=0.09), number of metastatic organ sites involved (P=0.37), prior radiotherapy (P=0.66) or chemotherapy (P=0.12) and number of treatment cycles given (P=0.84).

Table 5.1 Patient characteristics

	DTIC Dosage (mg/m^2)		
	400	500	800
Patients (number)	25	20	15
Sex (M/F)	13/12	11/9	4/11
KP (≥70/<70)	22/3	17/3	11/4
Age (≥40 yrs/<40 yrs)	21/4	16/4	12/3
Previous CT (no/yes)	23/2	15/5	10/5
Previous RT (no/yes)	21/4	15/5	11/4
No of metastatic sites			
1	6	5	1
2	12	4	6
3	4	5	5
≥4	3	5	3
No of CT courses given			
1	3	3	4
2	8	6	4
3	6	7	3
≥4	8	4	4

KP = Karnofsky performance scale.
CT = chemotherapy.
RT = radiotherapy.

5.2.2 Responses

In the 60 patients studied, 18 patients showed partial or complete responses giving an overall rate of 30%. However when based on the different treatment groups, the mean response rates (partial plus complete) were 24%, 30% and 40% in patients receiving 400, 500 and 800 mg/m^2 DTIC respectively (Table 5.2). Despite this linear DTIC dosage-dependent clinical response rate, there was no stastically significant difference in response with different DTIC dosage levels (P=0.54, chi-squared test). Two complete responders were seen in patients receiving 400 mg/m^2 and one in patients receiving 500 mg/m^2. Four patients had stable disease and fifteen patients had a partial

Table 5.2 Comparison of response rates in the treatment groups

Response	DTIC Dosage (mg/m^2) 400	500	800	total
Progression	18 (68%)[a]	13 (65%)	7 (47%)	38 (63%)
Stable	1 (4%)	1 (5%)	2 (13%)	4 (7%)
Partial response	4 (16%)	5 (25%)	6 (40%)	15 (25%)
Complete response	2 (8%)	1 (5%)	0	3 (5%)
Total number of patients	25	20	15	60

[a] Figures in brackets denote % based on total patient number in each treatment group.

response: the median duration of response was 5 months (range, 1-9 months). The responses of the metastatic sites are shown in Table 5.3.

Table 5.3 Metastatic sites and response with different DTIC doses

	DTIC Dosage (mg/m^2) 400	500	800	Total
Non-visceral sites only	1 (1)[a]	2 (1)	2 (2)	5 (4)
Visceral sites only	11 (3)	6 (1)	4 (1)	21 (7)
Both	13 (2)	12 (4)	9 (4)	34 (5)
Total number of patients	25	20	15	60

[a] Figures in bracket denotes number of patients responding.

5.2.3 Haematological Toxicity

Table 5.4 shows the haematological toxic effects seen with different DTIC dosage. Severe anaemia (WHO ≥ grade 3), neutropenia (WHO ≥ grade 3) and

thrombocytopenia (WHO ≥ grade 3) occured more often with higher dosage DTIC and this was highly statistically significant between the treatment groups. Anaemia was seen more often in the later treatment cycles (after cycle 2) than early treatment cycles.

Table 5.4 Numbers of patients showing haematological toxicity in the various treatment groups

		DTIC Dosage (mg/m^2)			
		400	500	800	
Toxicity	WHO grade				*P*-value[a]
Anaemia	2	5 (20%)[b]	2 (10%)	5 (33%)	
	≥3	1 (4%)	4 (20%)	5 (33%)	<0.05
Leucopenia	2	1 (4%)	4 (20%)	3 (20%)	
	≥3	1 (4%)	2 (10%)	6 (40%)	0.0064
Platelets	2	2 (8%)	2 (10%)	1 (7%)	
	≥3	0 (0%)	4 (20%)	6 (40%)	0.0002
Total number of patients		25	20	15	

[a] Statistical analysis for haematological toxicity between the 3 treatment groups.
[b] Figures in brackets denotes % based on total patient number in each treatment group.

5.2.4 Pulmonary Toxicity

One paient with disseminated lymphadenopathy responding to chemotherapy died from an acute respiratory distress type syndrome. This patient received 500 mg/m^2 DTIC and 100 mg/m^2 fotemustine. The history was of ten days dry cough and increasing breathlessness. The chest x-ray (CXR, Fig. 5.1) showed a bilateral alveolar shadowing and an echocardiogram demonstrated a normal left ventricular function with no evidence of pericardial effusion. Bronchial alveolar-lavage produced a fluid containing inflamatory cells. Despite high dose steroid and septrin, the patient's condition

continued to deteriorate and death occured 10 days after presentation. The post-mortem showed features of those of adult respiratory distress syndrome with interstitial fibrosis.

Following this case, the treatment protocol was amended and the DTIC dose was reduced to 400 mg/m^2 with fotemustine maintained at 100 mg/m^2 as indicated earlier. A full lung function test was undertaken in a further 11 patients before and after chemotherapy. Table 5.5 showed the physiological results of patients studied. Data was expressed as percentage of pretreatment results. As shown in the table, significant reduction of vital capacity (VC), total lung volume (TLV), residual volume (RV), total lung carbon monoxide transfer (DLCO) and transfer coefficient (KCO) occured following chemotherapy. No relationship was seen between the extent of pulmonary damage and treatment cycles (P=0.7, one-sample t-test). One patient (LP/4, table 5.5) presented with an acute onset of breathlessness and investigations revealed restrictive spirometry and small lung volumes associated with reduced DLCO (54.5% of prechemotherapy value) and KCO (44% of prechemotherapy value). CXR showed a patchy upper lobe shadowing that was more marked in the right lung (Fig. 5.2). His condition improved with high dose steroid and clinically he was still in partial remission when reviewed in the last follow-up.

Table 5.5 Results of respiratory function test (% of pretreatment value)

Patient[a]/ Cycle[b]	FEV1	VC	TLV	RV	DLCO	KCO
MB/2	95.3	93.2	102.9	112	91.3	97
MS/3	102.7	102.6	98.2	106.8	75.3	77.6
SS/3	101.5	93.7	94.8	78.3	79.2	66
IP/3	100	87.5	80.5	70.6	84.3	94.8
LP/4	100	88.5	69.8	51.6	54.5	44
PH/4	94.7	99	100	100	73.7	78.8
HG/4	91	90.8	82	86	74.4	56.5
RL/4	76.5	89.6	73	58.9	100	85.7
CW/4	60.3	59	65.1	83.2	50.5	88.4
LD/5	100	100	93.8	94.9	70.1	89.1
WL/6	105	89.7	98.2	100	69.5	80
P value[c]	0.249	0.004	0.010	0.035	0.0002	0.0035

[a] All patients received 400 mg/m^2 DTIC and 100 mg/m^2 fotemustine.

[b] Treatment cycle.

[c] *P* value based on one-sample t-test with a pretreatment value of 100%.

FEV1= Forced expiratory volume in one second; VC= Vital capacity; TLC= Total lung capacity; RV= Residual volume; DLCO= total lung carbon monoxide transfer corrected for haemoglobin; KCO= transfer coefficient.

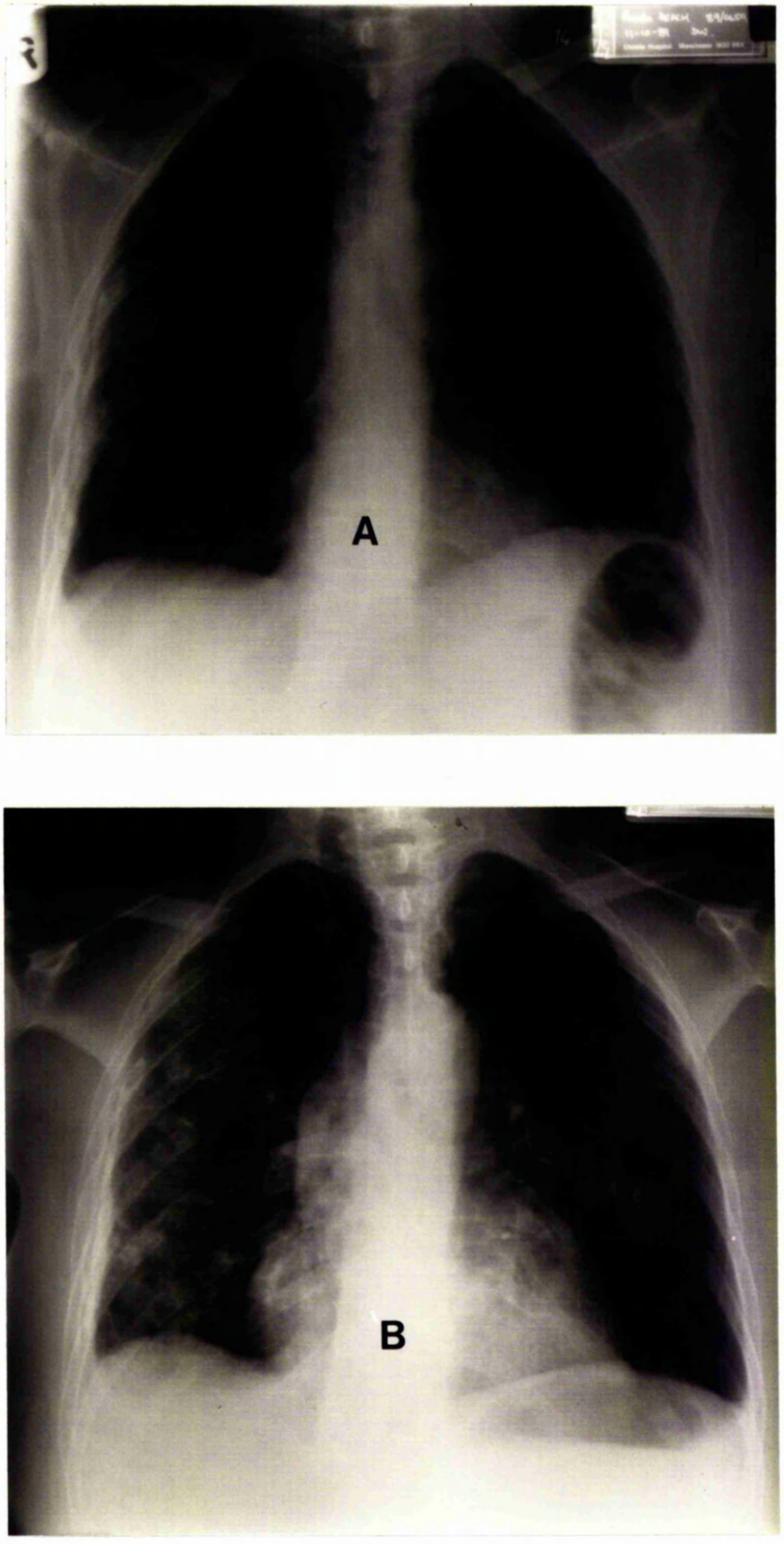


Figure 5.1. CXR obtained (A) before and (B) 3 months after chemotherapy. Pleural calcification on the right (A and B) was due to old tuberculous empyema. A extensive bilateral alveolar shadowing was seen after chemotherapy (B).

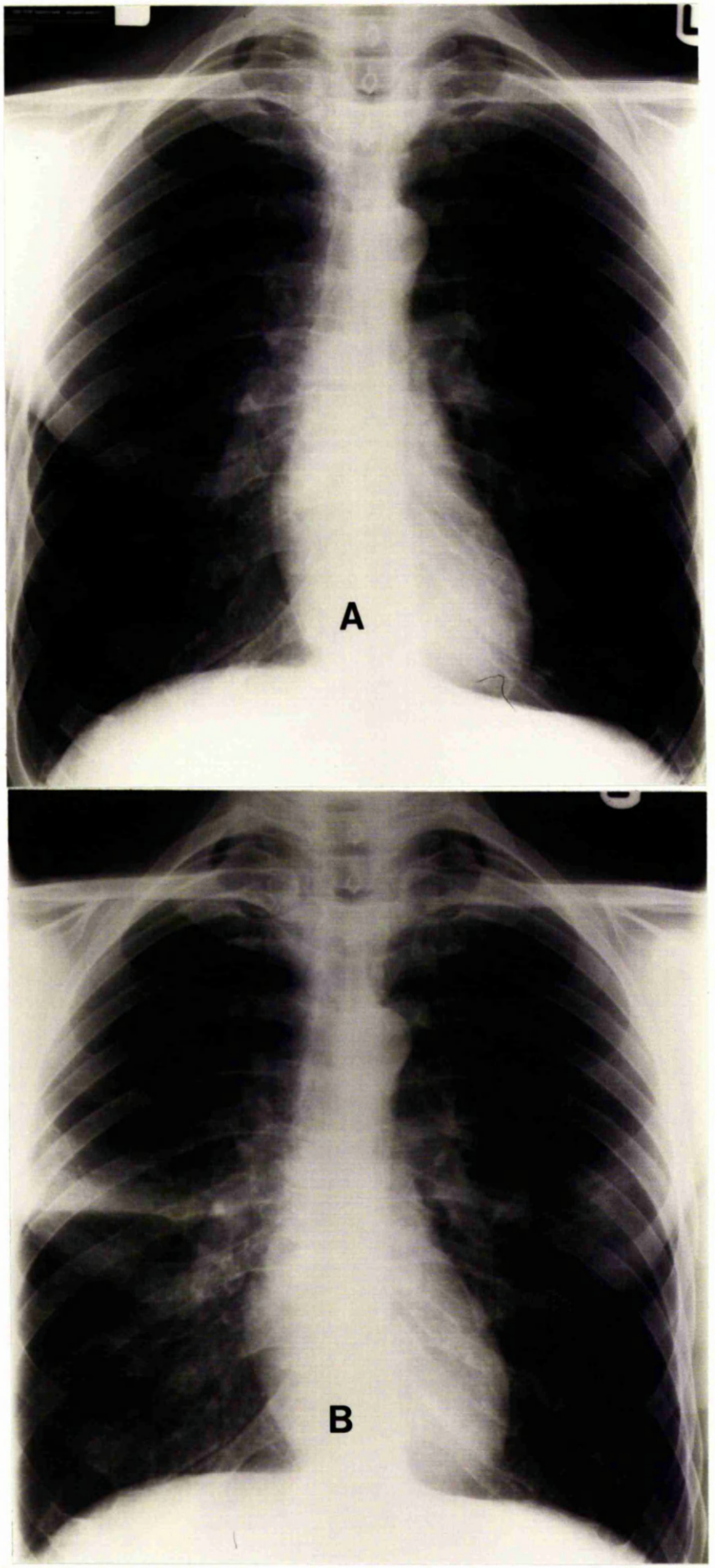


Figure 5.2. CXR obtained (A) before and (B) 4 months after chemotherapy. Patchy upper lobe shadowing more marked on the right hand side can be discerned after chemotherapy.

5.2.5 Other toxicity

Nausea and vomiting (≥ WHO 3) occured despite metoclopramide in 14 patients, elevated transaminases in 10 patients, elevated alkaline phosphatases in 12 patients and elevated bilirubin in 5 patients but these were not statistically difference between the 3 treatment groups. Five infective episodes were noted in two patients receiving 500 mg/m^2 DTIC and in 3 patients receiving 800 mg/m^2 DTIC.

5.2.6 Survival

The overall median survival was 3.6 months (range, 1-15 months). Within the treatment subgroups, median survivals were 6.3 months, 2.75 months and 3.6 months in patients receiving 400, 500 and 800 mg/m^2 DTIC respectively. However, no statistically significant difference in survival was seen between the different DTIC treatment doses (P=0.67, log-rank test; see Fig. 5.3A). Nine patients are alive at 5 to 26 months (median 10 months); three patients with no tumour and five patients with stable disease. There was a statistically significance difference (P< 0.0001 log-rank chi-square test) between survival for responders (median survival, 9 months; including patients with stable disease) compared to patients with progressive disease (median survival, 2.9 months) (Fig. 5.3B)

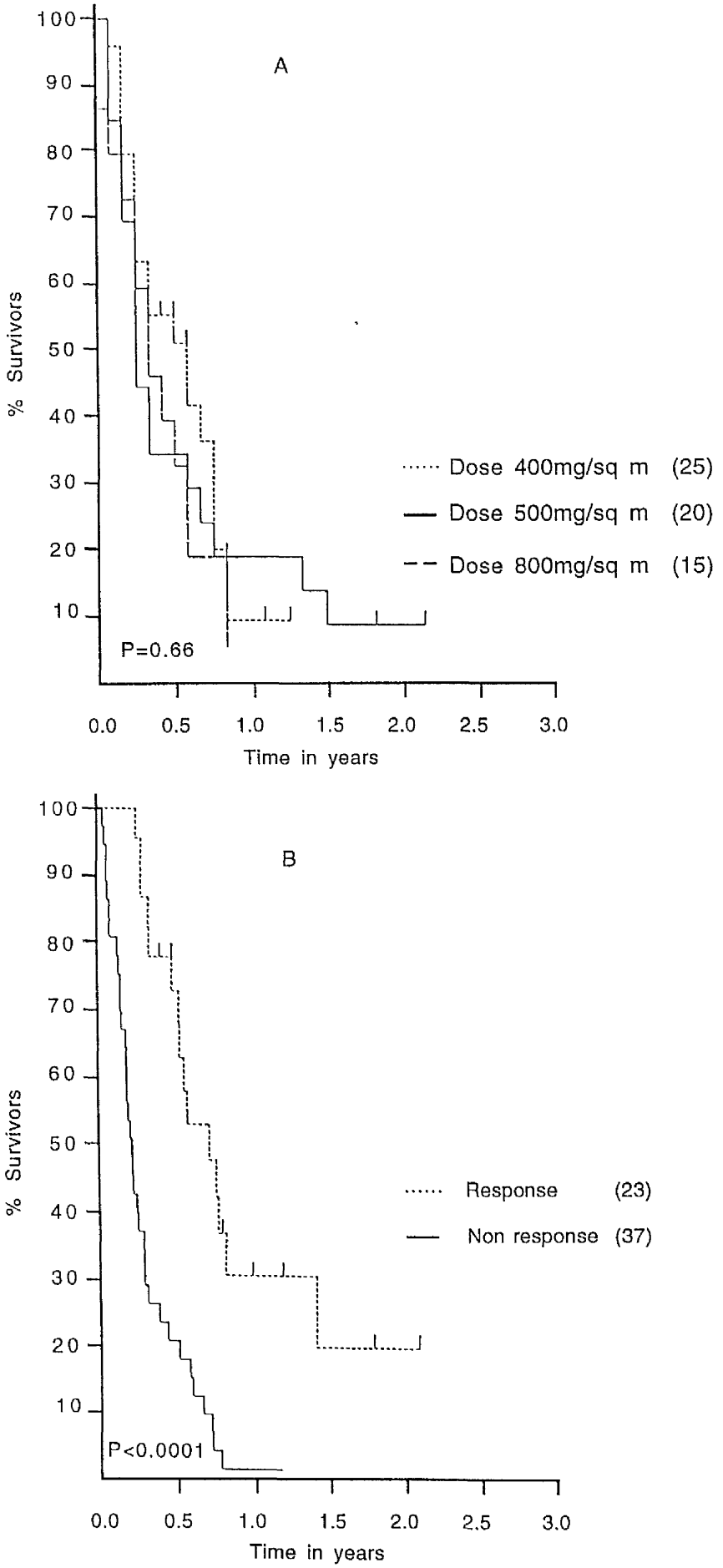


Figure 5.3. Survival curves showing (A) survival to different dosages of DTIC prior to fotemustine given at 100 mg/m^2 and (B) survival and response to chemotherapy.

5.3 Discussion

Eighteen of the 60 patients studied here responded with an overall response rate of 30%. The response rates were 24%, 30% and 40% in patients receiving 400, 500 and 800 mg/m^2 and although there appeared to be a trend towards a higher response rate with increasing dosage of DTIC this was not statistically significant and this may be due to the small number of patients entered into each treatment group. In support of this suggestion is a compatible finding by another group using a similar schedule and they reported an overall response rate of 33% in 24 patients treated with 500 mg/m^2 DTIC and 100 mg/m^2 fotemustine (Aamdal et al. 1992). In addition, in a multi-center study, the reported response rate achieved for patients treated with 800 mg/m^2 DTIC and 100 mg/m^2 fotemustine was 41% (Gerard et al. 1993).

The majority of studies in which DTIC has been given by single i.v. bolus or daily injections over 5 days, have produced an overall response rate of about 20% (Comis 1976; Mastrangelo et al. 1982; Geeraerts and Nathanson 1986; Balch et al. 1989). Single doses of DTIC of 850 mg/m^2 (Samson et al. 1978) and 800 mg/m^2 with dactinomycin (Hochster et al. 1985) or 250 mg/m^2 daily for 5 days with dactinomycin (Robidoux et al. 1982) gave response rates of 23%, 22% and 15% respectively. Fotemustine alone gave a response rates of about 24% (Jacquillat et al. 1990). Therefore the overall response rate of 30% achieved with the current study would indicate the increased effectiveness of a combination of DTIC and fotemustine in comparison with either agent used alone. In addition, based on an alternative regimen in which fotemustine was given one hour prior to DTIC, no clinical activity was seen and this suggests that the increased response rate observed with the present schedule may be due to DTIC-mediated depletion of ATase (Aamdal et al. 1990).

Unfortunately because of the development of unexpected adult respiratory distress syndrome in some of the patients treated with 500 and 800 mg/m^2 DTIC (Gerard et al. 1993), these treatment groups were withdrawn from clinical study and the true significance of the dosage-dependent response cannot be tested. Nevertheless, if the

trend towards a higher response rate observed with 500 and 800 mg/m^2 DTIC can be established with larger numbers of patients, it is not unreasonable to suggest that it may be related to the increasing extent of ATase depletion achieved in tumour tissue assuming that a depletion is similar to that occuring in lymphocytes. Since residual lymphocyte ATase activity was detected in many of the patients treated, it is possible that further improvements in response rate might be achieved if more extensive, or preferentially complete ATase suppression could be obtained prior to fotemustine administration. As indicated before, whether this can be achieved with pulsed DTIC treatment every 4 h or continous DTIC infusion followed by fotemustine or another nitrosourea will require further clinical study.

An interesting finding was the statistically significant relationship seen between the development of severe haematological toxicity and the dosage of DTIC administered. In a study of 46 patients treated with 850 mg/m^2 DTIC given as single i.v. bolus, thrombocytopenia ($\leq 100 \times 10^9$/liter) and leucopenia ($\leq 1.0 \times 10^9$/liter) was uncommon and developed in only 4% and 2% respectively of the treatment courses (Pritchard et al. 1980). In contrast, in the present study, severe thrombocytopenia ($< 75 \times 10^9$/liter) and leucopenia ($\leq 1.0 \times 10^9$/liter) occured in 47% and 60% of the patients receiving sequential 800 mg/m^2 DTIC and 100 mg/m^2 fotemustine (see Table 5.4). A more extensive marrow toxicty was seen in the schedule using 800 mg/m^2 of DTIC in comparison to the other two treatment groups and one possible reason for this might be that a more extensive ATase depletion of the already low levels of ATase in the marrow occurred (see section 6.2.4; Gerson et al. 1985) resulting in an increased sensitivity to fotemustine or subsequent doses of DTIC. In this context, ATase-deficient and, normally, hypersensitive murine haematopoietic stem cells transfected with and expressing bacterial ATase genes become highly resistant to the toxic effects of methylating and chloroethylating agents (Jelinek et al. 1988; Dumenco et al. 1989) strongly suggesting that low endogenous ATase expression is the basis of their sensitivity and hence that ATase depletion would result in further sensitization. Furthermore, there is some experimental evidence to indicate that ATase depletion of

nitrosourea-resistant melanoma cells with O^6-MeG not only sensitizes the tumour cells but also increases the toxic effects in the bone marrow cells following pretreatment with O^6-MeG (Dempke et al. 1987).

Another interesting finding was the occurence of pulmonary toxicity. Two patients presented with an acute shortness of breath; one died and post-mortem revealed features of adult respiratory distress syndrome with interstitial fibrosis. The second patient responded to high dose steroid; investigations showed a small lung volume with a significantly reduced carbon monoxide transfer factor. Follow-up studies in another 10 patients showed a significant sub-clinical deterioration in lung function following chemotherapy (Table 5.5). The clinical, radiological and histological features of symptomatic lung fibrosis have been previously been described with BCNU and other nitrosoureas (Bailey et al. 1978; Durant et al. 1979; Aronin et al. 1980; Selker et al. 1980; Weiss et al. 1981). A correlation is seen when the cumulative dosage of BCNU $\geq$ 1200 mg/m^2 (Weiss et al. 1981). However, the two cases of interstitial pneumonitis in our study received a cumulative dosage of $\leq$ 400 mg/m^2 of fotemustine suggesting that the synergy between DTIC and fotemustine (as used in the schedule here) may be responsible for the acute pulmonary event, possibly related to greater cytotoxicity in normal lung cells following depletion of the endogenous ATase. A recent phase II study of fotemustine alone in 153 patients with disseminated melanoma was not associated with any pulmonary toxicity and a similar finding was reported in another 38 patients with gliomas treated with fotemustine alone (Jacquillat et al. 1990; Frenay et al. 1991). Lung tissue has a relatively low ATase activity in comparison with other tissues (Grafstrom, 1984; Gerson et al 1986) and as a result they may be more sensitive the cytotoxic effects of DNA alkylation. This may be a particular problem in those patients whose lung tissue has low ATase activity or in which ATase depletion by DTIC has been more effective. Unfortunately none of these patients particularly the two cases with interstitial pneumonitis were monitored for lymphocyte ATase activity and it is impossible to say whether there might be a correlation between lung toxicity and ATase level in peripheral blood lymphocytes.

In conclusion, sequential DTIC and fotemustine appeared to be more effective than DTIC or fotemustine alone in patients with metastatic melanoma. There was an apparent trend towards increased response rate with higher dosage DTIC but if this is confirmed in a larger group of patients it has been achieved whilst eliciting significantly increased haematological and pulmonary toxicity. The median survival time remains short in these patients with advanced disease, but further investigations using a different schedule of DTIC combined with a nitrosoureas to overcome ATase-mediated drug resistance could be worthwhile, particularly if an increased response is achieved in the absence of any increased toxicity as a consequence of general ATase depletion in tissues. Whether the marrow toxicity can be reduced with the help of haemopoietic growth factors would require further exploration. The subclinical pulmonary damage observed indicates that it is of considerable importance to monitor these patients to prevent the possibility of acute and/or long term lung damage. Nevertheless, the present study provides a framework for other studies attempting to modulate ATase-mediated drug resistance in tumour tissues using ATase depleting agents such as a methylating agent or O^6-benzylguanine before administering a nitrosourea: any associated toxicity will need careful monitoring.

Chapter 6 *In vivo* and *in vitro* Effects of CB10-277 on O^6-Alkylguanine-DNA-Alkyltransferase

6.0 Introduction

Although DTIC is the single most active agent available for the treatment of metastatic melanoma, its clinical activity is disappointing with complete remissions infrequently seen (see Chapter 5). In addition, the duration of response and median survival remains short at 3 to 5 months and the chemotherapy for metastatic melanoma in visceral sites, liver, bone and brain is discouraging (Comis 1976; Balch et al. 1989). This is in contrast to the findings in experimental systems where DTIC has been reported to have good anti-tumour activity, particularly with murine models (Venditti 1976). Previous studies have shown a marked species-dependent activation of DTIC: the peak level of the active monomethyl metabolite, MTIC found in the plasma of mice was 10 times higher than in man or rat and this has been proposed as one possible reason for the poor clinical activity of DTIC in man (Rutty et al. 1983). As reported in Chapter 4, a wide variation in the extent of O^6-MeG formation was seen in patients treated with DTIC with clinical responses occurring in those individuals achieving the highest DNA methylation levels.

As mentioned in Chapter 1, CB10-277 (Fig. 3.6) has been shown to have marked activity against experimental murine tumours (Colombo and D'Incalci 1984) and melanoma xenografts (Foster et al. 1990) and is equally effective when compared to DTIC in inhibiting the growth of a variety of rodent tumour models including melanoma xenografts (Foster et al. 1993a). Like other dialkyltriazenes, CB10-277 requires activation by oxidative N-demethylation and the overall production of the putative active monomethyl metabolite in rats was 15-fold greater than that of DTIC, suggesting that species-dependent activation is less likely to be a problem (Rutty et al. 1986). Thus, due to its structural similarities to DTIC, its superior *in vitro* stability and solubility and its possibly improved metabolic activation, CB10-277 was selected for

clinical evaluation and a CRC phase II trial in metastatic melanoma has recently been completed.

In the initial phase I study which used a short infusion of CB10-277, it was found that the dose limiting toxicity was nausea and vomiting associated with a MTD of 6000 g/m^2. In addition, the AUC of the monomethyl metabolite at the MTD was found to be less than predicted, compared to that observed with LD10 dose in mice. The 24 h continuous infusion was chosen for the phase II study because it was anticipated that more drug would be delivered to achieve an increase in monomethyl metabolite formation despite a decrease in peak plasma values of CB10-277. The dose limiting nausea and vomiting was thought to be due to high peak values of CB10-277 and it was hoped that by changing the schedule to a 24 h continuous infusion, this would ameliorate these debilitating effects (Foster et al. 1993a; 1993b).

The present study examines the extent and the kinetics of ATase depletion and regeneration in peripheral blood lymphocytes from 9 patients and tumour biopsy material from 2 patients with metastatic melanoma treated with 24 h continous infusion of CB10-277 in a phase II study.

As part of this study, ATase levels were also measured in peripheral lymphocytes and the corresponding bone marrows obtained from 12 patients with a variety of other malignancies prior to treatment in order to examine the relationship between the ATase levels in these two tissues in the same individual.

6.1 Materials and Methods

The clinical characteristics of the 9 patients studied are shown in Table 6.1. All patients had metastatic melanoma. Patients received CB10-277 (12 g/m^2) in a 24 h i.v. infusion and treatment was repeated every 28 days. Serial blood samples were collected at various times prior to (0 h) and at 2, 4, 6, 8, 10, 16, 20, 24 h during the infusion and

for 24 h (28, 32, 40, 48 h) after completion of the first cycle of CB10-277. Serial blood samples were also taken in 2 patients who returned for subsequent treatment, whereby the CB10-277 dose was halved (6 g/m^2) because of haematological toxicity. Tumour biopsies were performed on 2 patients with adjacent metastatic subcutaneous nodules before and immediately after CB10-277.

This study also examines the ability of CB10-277, streptozotocin and NMU to inhibit pure recombinant human ATase *in vitro* by incubating varying concentrations of these agents with 70 fmoles of ATase and 10 μg calf thymus DNA for 2 h at 37°C in buffer I. Residual ATase activity was then measured as described in Chapter 2.

In order to examine the relationship between ATase levels in peripheral blood lymphocytes and bone marrow, blood samples and corresponding bone marrows were obtained from the 12 patients with a variety of malignancies (Table 6.2) admitted for routine staging investigations.

Table 6.1 Patient characteristics

Patients/Fig.	Age/Sex	Metastatic Sites	Response	ATase (fm/mg) Initial	Nadir
CM/6.1a	37/F	Lung/Nodes	PR	15	bd[c]
JR/6.1b	54/M	Lung/Brain	MR	45	bd[c]
GM/6.1c	20/F	Lung/Soft tissue	PD	250	43
FF/6.1d	50/M	Lung/Nodes	PD	206	24
MU/6.1e	66/F	Lung/Nodes	PD	163	6
MC/6.1f	66/F	Liver	PD	86	15
JH/6.1g	31/F	Lung/Nodes	MR	158	36
ED/6.1h	65/M	Liver	PD	262	80
JK/6.1i	53/F	Brain	PD	141	63

Note: PR; partial response; MR, mixed response; PD, progressive disease.
bd[c]: below detection

6.2 Results

6.2.1 *In vivo* effects of CB10-277 on lymphocyte ATase

Pretreatment lymphocyte ATase levels ranged from 15 to 262 fm/mg protein with a median level of 158 fm/mg total protein (Table 6.1). In all nine patients, progressive depletion of ATase activity was seen during the 24 h continous infusion of CB10-277. In two patients (CM & JR; Table 6.1) whose pretreatment ATase values were 15 and 45 fm/mg protein, suppression of ATase was to below detectable levels at 2 and 16 h respectively (Figs. 6.1a & b). In the remaining seven patients (Figs. 6.1c-i), the median residual ATase activity was 17% of the pretreatment value at the end of CB10-277 infusion. No recovery of ATase activity was seen in six patients for up to 24 h after completion of CB10-277 treatment (Figs. 6.1a-f) but significant recovery to 50%, 100% and 102% of pretreatment levels occurred in the other three (Figs. 6.1g-i). In two patients (CM & JR; Table 6.1), serial ATase assays were repeated for subsequent therapy, for which the CB10-277 dose was halved to 6 g/m^2 because of the

development of haematological toxicity. In comparison with treatment cycle 1 (no residual activity), a significantly less extensive ATase depletion was seen at the end of the CB10-277 infusion, with a residual ATase activity of 73% and 85% of the pretreatment levels, respectively (Fig. 6.2).

A linear relationship was seen between the pretreatment ATase level and the extent of ATase depletion and this data is presented and discussed in Chapter 7.

A 3 to 4 fold increase in the pretreatment lymphocyte ATase activity was seen in three patients (CM, MC & JK; Table 6.1) who returned after 4 weeks for subsequent chemotherapy (Fig. 6.3). No increase was seen in the other 6 patients. One of these subjects (MC) died of progressive disease, and another (JK) also had documented progressive disease and was subsequently treated with radiotherapy.

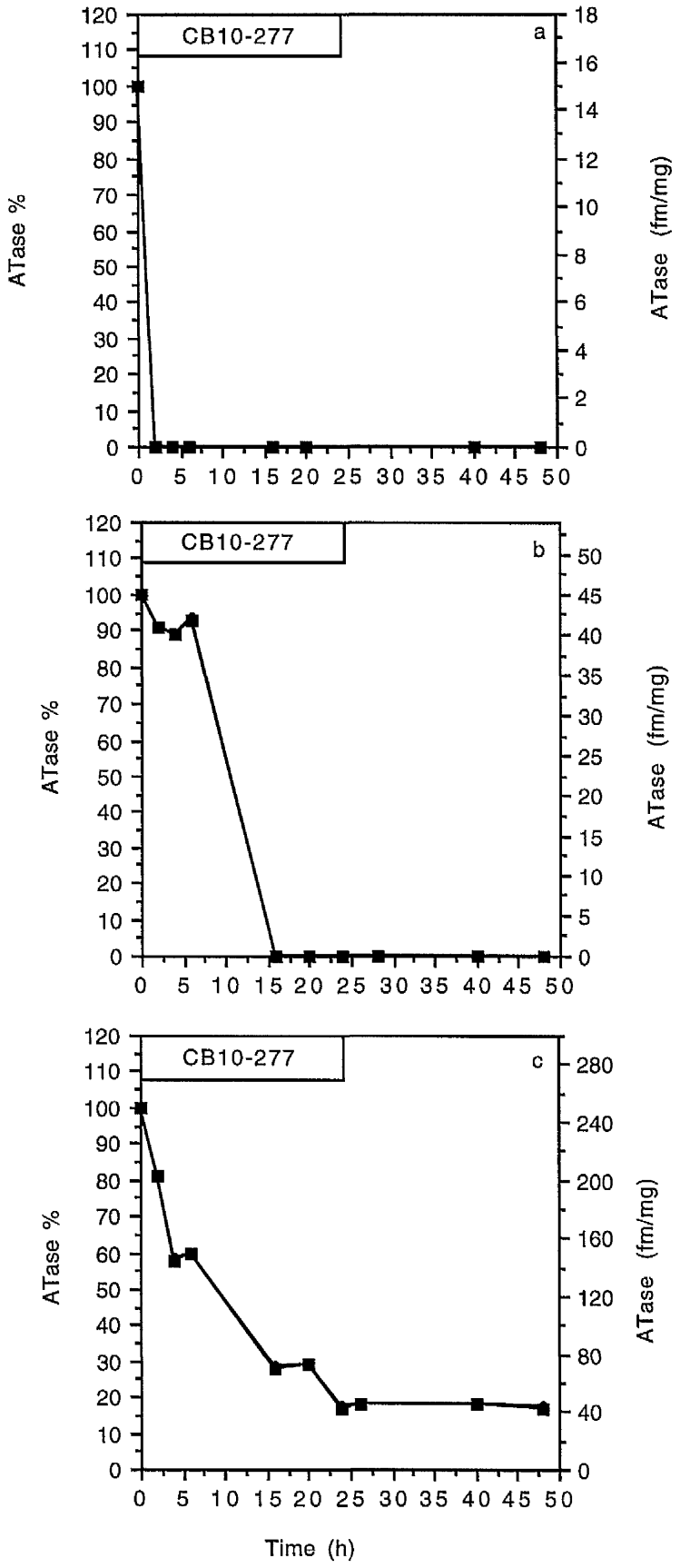


Figure 6.1 a, b & c. Lymphocyte ATase activity of individual patients expressed as % of pretreatment activity and actual levels (fm/mg protein) at various times during and after CB10-277 infusion (12 g/m^2).

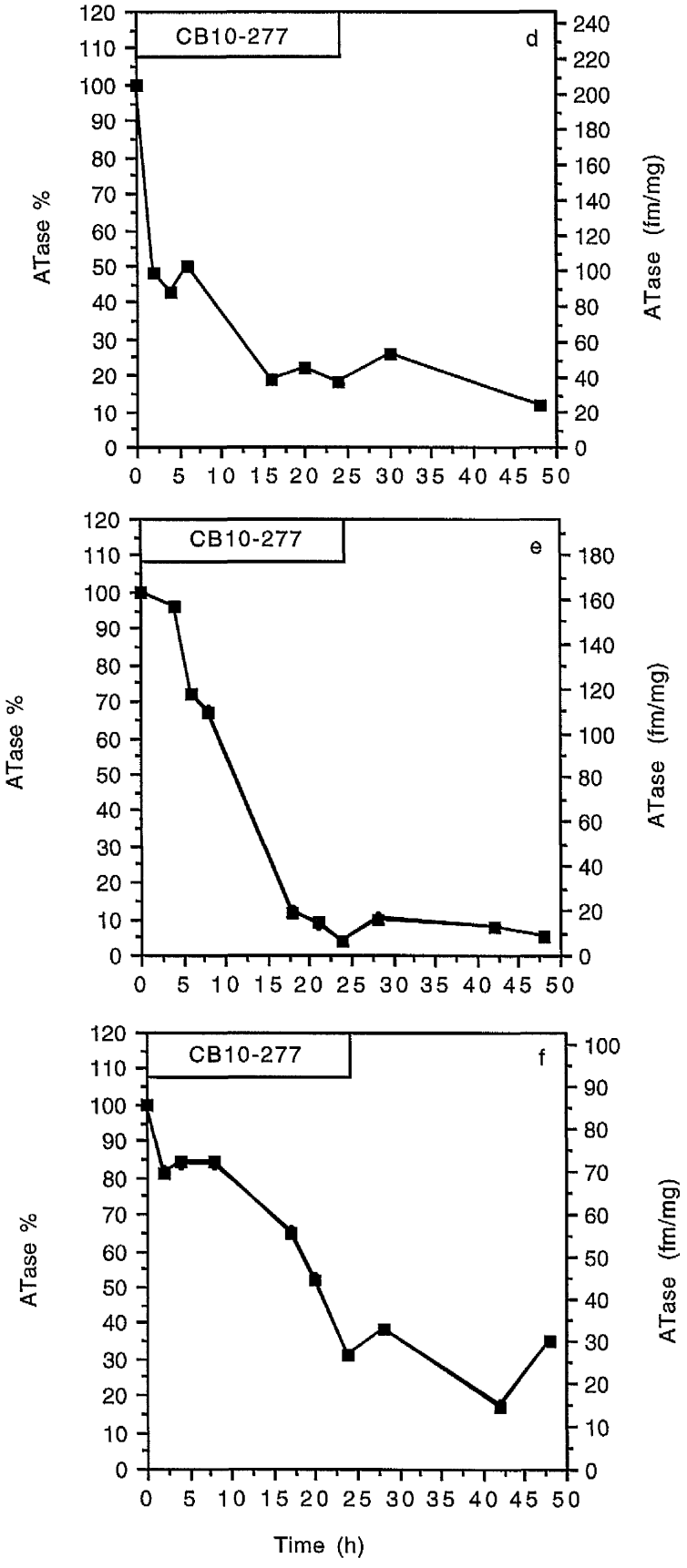


Figure 6.1 d, e & f. Continued.

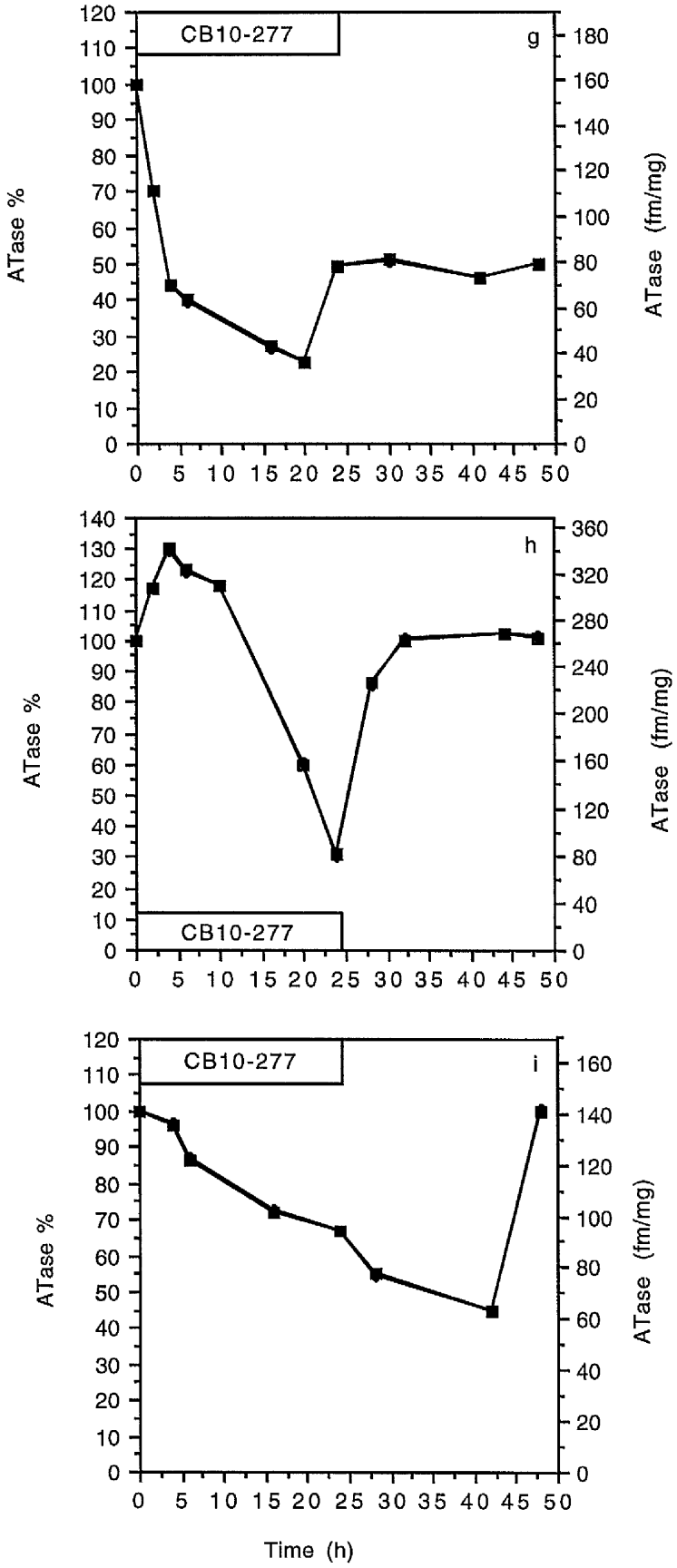


Figure 6.1 g, h & i. Continued.

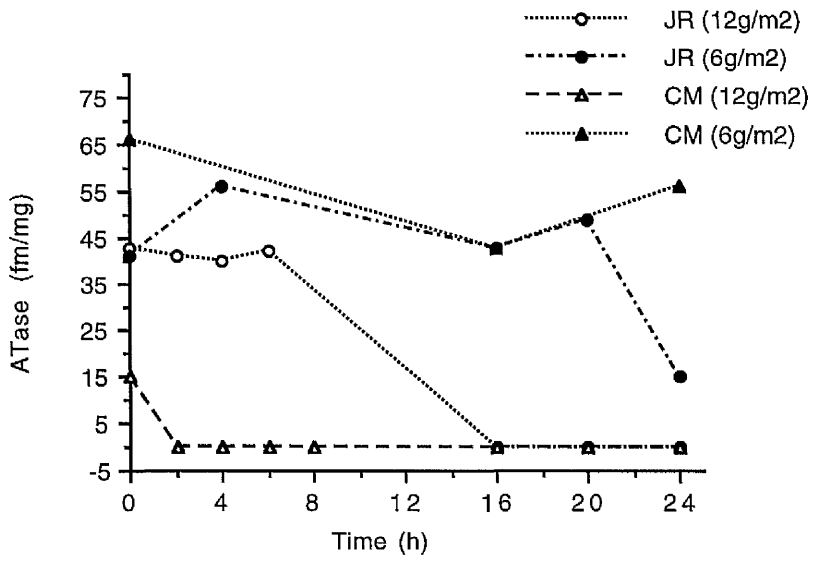


Figure 6.2. Comparison of kinetics of depletion of lymphocyte ATase (fm/mg protein) with different cycles in the 2 patients (CM & JR) who received $12g/m^2$ and $6gm/m^2$ of CB10-277 during consecutive treatment cycles.

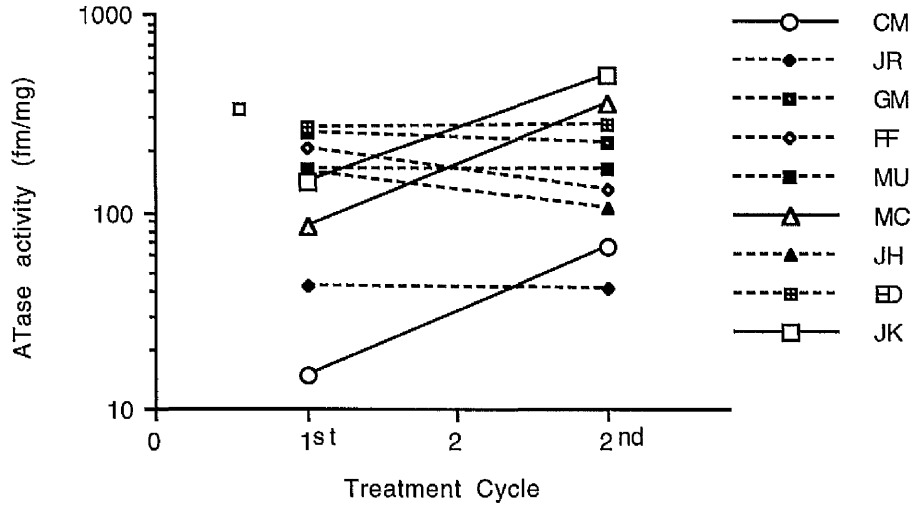


Figure 6.3. Pretreatment lymphocyte ATase activity (fm/mg protein) for individual patients during the first and second cycle of CB10-277 treatment.

6.2.2 *In vivo* effects of CB10-277 on melanoma ATase

In two melanoma biopsy samples, pretreatment ATase levels were 65 and 117 fm/mg protein, respectively. These values were within the range of the ATase activity reported in an earlier study (Maynard et al. 1989). Following CB10-277 treatment, residual ATase activity amounted to 7 and 9 fm/mg protein, respectively, in two further biopsies taken at 24 h after the start of treatment. The tumour ATase activity appeared to fall in parallel with lymphocyte ATase levels during CB10-277 treatment (Fig. 6.4). Analysis of additional paired biopsy samples before and after treatment with the other O^6-alkylating agents (DTIC & temozolomide) are presented in Chapter 9 (Section 9.2.2).

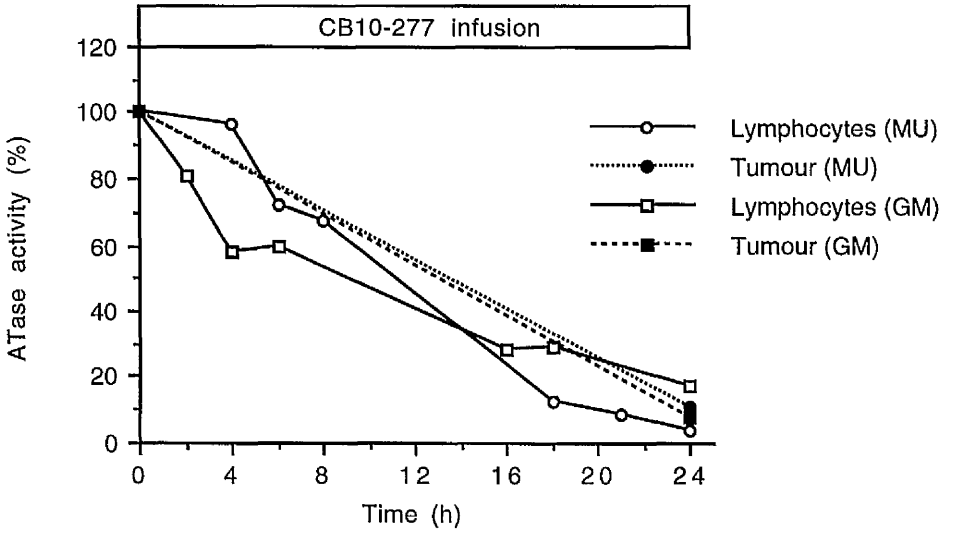


Figure 6.4. ATase activity expressed as percentage of pretreatment level in lymphocytes and biopsied melanoma tissues of 2 patients (CM; GM) following first cycle treatment.

6.2.3 *In vitro* effects of CB10-277, MNU and streptozotocin on recombinant ATase

In order to eliminate the possibilty that ATase inactivation might be due to spontaneous activation of CB10-277 to generate a species capable of alkylating DNA or a direct effect of the drug on this protein, CB10-277 was incubated with recombinant human ATase in the presence of 10 μg calf thymus DNA for 2 h at 37°C *in vitro*. The extent of ATase depletion was compared with that produced by streptozotocin and NMU, both of which agents are capable of undergoing spontaneous hydrolysis to generate a

methylating species. Dose-dependent depletion was seen with streptozotocin and NMU, 50% depletion occurred at 2 μM and 1.5 μM respectively. As would be anticipated, no ATase depletion was seen when recombinant ATase was incubated with CB10-277 up to a concentration of 1 mM (Fig. 6.5) and this is consistent with the requirement for metabolism in the liver to generate a reactive species. Slight ATase depletion was seen with higher concentration of CB10-277, but this may be due to a direct effect on the ATase or possibly to contamination of CB10-277 with a reactive species.

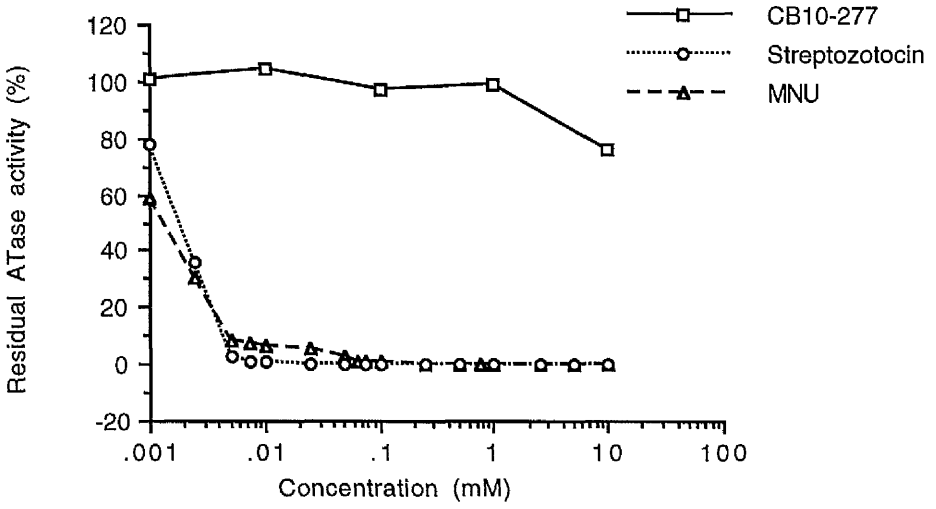


Figure 6.5. *In vitro* effects of CB10-277, NMU and streptozotocin on pure recombinant human ATase.

6.2.4 Bone marrow ATase activity

ATase activity in lymphocytes and bone marrow cells were expressed per unit DNA content (rather than per protein content) for a more comparable measurement between two different tissues (Gerson et al. 1986; Pegg 1990). The clinical characteristics of the 12 patients studied are shown in Table 6.2. The mean lymphocyte ATase activity (5.8 ± 2.2 fm/μg DNA) was approximately four fold higher than that of bone marrow (1.6 ± 0.9 fm/μg DNA; P=0.0001, paired t test; Table 6.2) but as shown in Fig. 6.6, there was a positive correlation between the bone marrow ATase activity and the corresponding lymphocyte ATase activity (R=0.67, P<0.02). This is in comparison to

other studies which showed that the mean lymphocyte ATase (210 ± 121 fm/mg protein) was 1.5 times higher than that of myeloid precursors (mean 140 ± 86 fm/mg protein) (Gerson et al. 1985).

Table 6.2 Patient characteristics

Patient	Age/Sex	Disease	ATase (fm/μg DNA)	
			Lymphocyte	Bone marrow
MR	23/M	HD	5.1	1.6
AT	66/M	HD	10.0	2.8
SH	71/M	NHL	8.1	3.1
JD	52/M	NHL	2.7	1.1
MO	68/F	NHL	4.7	0.3
JG	38/M	NHL	6.6	2.3
AG	51/M	SCLC	3.7	1.4
HW	37/F	SCLC	3.4	1.1
PFS	56/M	SCLC	6.5	0.8
BH	52/M	SCLC	4.2	0.1
FH	22/F	Sarcoma	5.7	2.4
SW	45/F	CUP	8.2	1.6

NHL, Non-Hodgkin's lymphoma; HD, Hodgkin's disease; SCLC, small cell lung cancer; CUP, carcinoma of unknown primary.

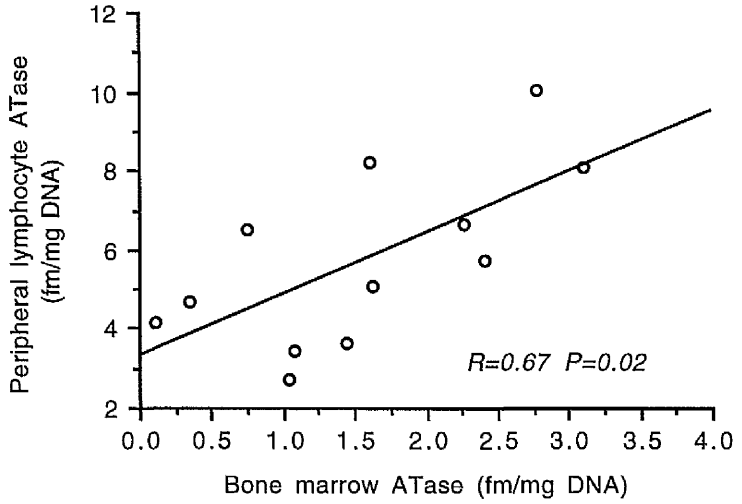


Figure 6.6. Relationship between ATase levels expressed as fm/μg DNA in peripheral blood lymphocytes and bone marrow.

6.2.5 Clinical effects of CB10-277

Haematological toxicity developed in two patients (CM & JR; table 6.1) with the lowest pretreatment lymphocyte ATase levels (15 and 45 fm/mg protein). Both leucopenia (2/3 grade 4, 1/3 grade 4, WHO scale) and thrombocytopenia (2/3 grade 4, WHO scale) occurred during 3 evaluable courses. Following a reduction of the CB10-277 dose by 50%, no further haematological toxicity was documented and this was associated with less extensive lymphocyte ATase depletion (Fig. 6.2). In the remaining seven patients whose pretreatment ATase levels exceeded 50 fm/mg protein (Table 6.1), no evidence of haematological toxicity was seen.

The response data for CB10-277 when given as a 24 h infusion are shown in Table 6.1. One partial response (CM) was observed lasting for 18 weeks. Two mixed responders were seen, in one patient (JK) partial response occured in the lung but progressed in the brain whereas the case (JH) achieved a partial response in the lung metastasis but relapsed in the axillary nodes when she returned for cycle 6 treatment. Two of these cases occurred in patients with low lymphocyte pretreatment ATase levels (CM and JK). No response was seen in the other six patients treated with CB10-277. In the phase 1 CB10-277 study, 4 responses were seen out of 11 evaluable patients

when CB10-277 was given as a short infusion and one response out of 8 evaluable patients when CB10-277 was given as a 24 h continuous infusion in patients with metastatic melanoma (Newell et al.1990; Foster et al. 1993a; 1993b).

6.3 Discussion

The present studies demonstrate that CB10-277 can cause inactivation of ATase in human peripheral lymphocytes. This indicates that like DTIC, CB10-277 is metabolised to a methylating agent in amounts sufficient to react with peripheral blood lymphocytes, generating O^6-MeG. The O^6-MeG lesion is repaired by lymphocyte ATase, causing an apparent depletion of ATase activity in lymphocyte extracts. Similar to the findings reported for patients treated with DTIC given as a single bolus dose (see Chapter 3), patients vary in their pretreatment lymphocyte ATase activity and also the rate of depletion and recovery of lymphocyte ATase activity following CB10-277 given as a 24 h infusion. The pretreatment values were not statistically significant from the pretreatment ATase activity of patients treated with sequential DTIC and fotemustine as reported in Chapter 3. Complete suppression of lymphocyte ATase activity was seen in two patients with low pretreatment ATase levels activity. Progressive depletion of lymphocyte ATase activity was seen in the remaining seven patients during CB10-277 infusion. These variations presumably reflect a combination of individual differences in CB10-277 metabolism and ATase gene transcription and translation rates.

It was also found that low pretreatment lymphocyte ATase activity (< 50 fm/mg protein) was associated with the development of grade 4 haematological toxicity in two patients (15 and 45 fm/mg protein) whose ATase activity fell to undetectable levels at 2 h and 16 h after the start of CB10-277 therapy. In the remaining seven patients whose pretreatment ATase levels exceeded 50 fm/mg protein, no evidence of haematological toxicity was seen. A major problem in cancer chemotherapy is the prediction of significant and sometimes life-threatening haematological toxicity. The present finding suggests that screening for pretreatment ATase activity in peripheral lymphocytes may

be one way of predicting and, hence, preventing such toxicity when CB10-277 or related drugs are used. Indeed, a reasonable correlation between ATase levels in peripheral lymphocytes and the corresponding bone marrow was observed here and other work supports this finding (Gerson et al. 1985). When expressed per unit DNA, bone marrow contained approximately 4 times less ATase activity in comparison to that of peripheral lymphocytes and it is not unreasonable to suggest that this may be the reason that the bone marrow is relatively more susceptible to the acute toxic and long term effects of alkylating agents compared with other tissues.

In the present report, no further haematological toxicity developed when the dosage of CB10-277 was halved in the above mentioned two patients, and this finding was also associated with reduced lymphocyte ATase depletion. It is noteworthy that in the phase I CB10-277 study, haematological toxicity developed following 4 of 8 courses involving a dose of 12 g/m^2 given as 24 h continuous infusion whereas no haematological toxicity was observed when patients received a lower dose of CB10-277 (4.7-8.0 g/m^2) in 31 evaluable courses (Newell et al. 1990: Foster et al. 1993b), and this observation was associated with a lower AUC for the monomethyl metabolites (Newell et al. 1990; Foster et al 1993b). These findings together with the lymphocyte ATase depletion data here make it tempting to speculate that less DNA alkylation may have occured in the bone marrow after treatment with the lower dose. Therefore, dose reduction following the identification of patients with low lymphocyte ATase levels which presumably reflect low ATase levels in bone marrow progenitor cells may be a strategy for the prevention of haematological toxicity following chemotherapy. No haematological toxicity was seen in the remaining seven patients receiving CB10-277 (12 g/m^2) whose pretreatment lymphocyte ATase levels exceeded 50 fm/mg protein, despite the observation of substantial ATase lymphocyte depletion at 24 and 48 h following CB10-277. These speculations are based on small patient numbers and clearly a more extensive clinical study is now needed to explore the relationship between pretreatment lymphocyte ATase activity, bone marrow ATase activity and any

associated haematological toxicity in patients treated with CB10-277 and related chemotherapeutic agents.

An extensive depletion of ATase activity was observed in biopsied tumour tissue following chemotherapy and the extent of this was similar to that seen in the peripheral lymphocytes of the same patients. This would suggest that the peripheral lymphocyte depletion corresponds to that of tumour depletion but despite generally extensive lymphocyte depletion, the general response of the tumour in patients with melanoma to treatment with CB10-277 was minimal with one partial response and two mixed response. This suggests that sufficient ATase may have remained or effective regeneration of ATase may have occurred such that the toxic O^6-MeG DNA lesions could be repaired, thereby reducing the effectiveness of the chemotherapeutic agent. It might also be that non-representative samples or nodules with particularly low levels of ATase were taken for ATase assay or that ATase levels are not homogeneous throughout the melanoma nodules and this was further explored in Chapter 9. Neverthelesss, since CB10-277 is capable of depleting tumour ATase activity, one possible approach to overcome drug resistance might be to use a nitrosourea at the end of CB10-277 infusion, when ATase nadir activity occurs as this would be predicted to increase cytotoxicity.

It was found that a small number patients (three) had an increase in pretreatment lymphocyte ATase level when they returned for subsequent CB10-277 therapy. One possible explaination is that expression of ATase activity in peripheral lymphocytes may be heterogeneous: if alkylation-mediated killing of ATase deficient lymphocytes occurs after CB10-277 treatment, the ATase-proficient lymphocytes will survive and continue to divide, the result being a net increase in ATase specific activity. If this cycle-dependent increase in ATase activity is reflected in the target tumour, it might indicate that tumour resistance will increase with the number of treatment schedules, a highly undesirable effect and one that should be monitored as closely as possible in future studies.

In conclusion, the findings here in two patients suggest that ATase depletion in peripheral lymphocytes may correlate with that in tumour tissue following CB10-277 administration and this needs to be extended to many more patients, and to related drugs and other tumours. In addition, the data shows a correlation between ATase levels of the bone marrow and corresponding peripheral lymphocytes; therefore screening patients for lymphocyte ATase activity may allow the identification of patients with increased risk of developing severe bone marrow toxicity thereby permitting the design of individualised treatment protocols to prevent this.

Chapter 7 *In vivo* and *in vitro* Effects of temozolomide on O^6-Alkylguanine-DNA-Alkyltransferase

7.0 Introduction

Temozolomide was recently selected for phase I/II clinical trials and has shown promising anti-tumour activity against high grade gliomas, melanoma and mycosis fungoides (Newlands et al., 1992; O'Reilly et al., 1993). This compound has potential for improved clinical value in comparison with DTIC or CB10-277 because it rapidly undergoes spontaneous chemical decomposition to generate the cytotoxic monomethyltriazene, MTIC (Stevens et al. 1987; Tsang et al. 1991), thus, bypassing the species and patient differences in metabolic activation that may exist in the formation of monomethyl metabolite from DTIC or CB10-277. As pointed out previously, one possible reason for the relatively poor clinical activity of DTIC in man may be the inefficient metabolic activation relative to experimental animal models (Rutty 1983). Temozolomide exhibits marked schedule dependency and has little activity when given as a single dose in experimental models or clinically in contrast to daily treatment. Because of this, the recommended dose for phase II trials is a 5 day schedule (Newlands et al., 1992).

In the present study, changes in lymphocyte ATase levels in 5 patients with metastatic melanoma were measured at various times during 24 h after the first dose of temozolomide. In 8 patients, the kinetics of ATase depletion in peripheral blood lymphocytes of patients treated with 5 consecutive daily doses were also examined.

7.1 Materials and Methods

The clinical characteristics of the 8 patients studied are shown in Table 7.1. For the first treatment cycle, temozolomide was administered orally at 150 mg/m^2 daily for 5 consecutive days. For subsequent treatment, patients received oral temozolomide (200

mg/m^2) daily on five consecutive days and this was repeated every 28 days. Serial blood samples were collected at 0, 1, 2, 3, 4, 6 and 24 h in 5 patients and at 48, 72, 96 and 120 h in 3 of these and an additional 5 patients receiving daily temozolomide (150 mg/m^2 daily from days 1 to 5).

In order to assess the ability of temozolomide to inhibit the ATase, varying concentrations of temozolomide, mitozolomide, procarbazine and methyl methanesulfonate (MMS) were incubated with 70 fmoles of pure recombinant human ATase for 2 h at 37°C. Residual ATase activity was then measured by incubation with excess [^{3}H]-methylated-DNA substrate as described in Chapter 2.

Table 7.1 Patient characteristics

Patients	Age/Sex	Metastatic sites	Response	ATase (fmol/mg) Initial	*Nadir
JP	40/F	nodes, liver, lung	PR	140	nm
MS	45/M	liver, lung	PD	434	nm
YA	26/F	nodes, soft tissues	SD	286	123
MC	39/F	lung, liver, bone	NE[a]	459	107
MF	68/F	skin, nodes	PR	197	54
IC	58/F	lung, liver, nodes	PD	300	105
AW	75/M	lung, nodes, liver	SD	593	152
KH	54/M	lung, nodes, liver	PR	243	35
GA	66/M	skin	NE[b]	69	14
MA	59/M	skin, nodes	SD	257	135

* ATase nadir during daily temozolomide administration (see Fig 7.2); nm = not measurable
PR = partial response; NC = no change; PD = progressive disease; NE = not evaluable.
[a] not evaluable because of rapidly deteriorating medical condition after cycle 1; [b] not evaluable because disease site was previously irradiated.

7.2 Results

7.2.1 *In vivo* effects of temozolomide on lymphocyte ATase

There was a wide range of pre-treatment lymphocyte ATase levels ranging from 69 to 593 fmol/mg protein with a mean value of 275 ± 182 fmol/mg protein. Depletion of lymphocyte ATase was seen within 4 h of the first oral dose of temozolomide and the median nadir was 52.9% with values ranging from 44.4% to 71.0% of pre-treatment levels in the five patients studied (Fig. 7.1). Using repeated measurement analysis and Duncan's multiple range test, nadir ATase appears to occur between 2 and 6 h after chemotherapy. Taking each individual as their own control, recovery of lymphocyte ATase activity greater than 20% was seen by 24 h in 3 of the 5 patients (See Fig. 7.1).

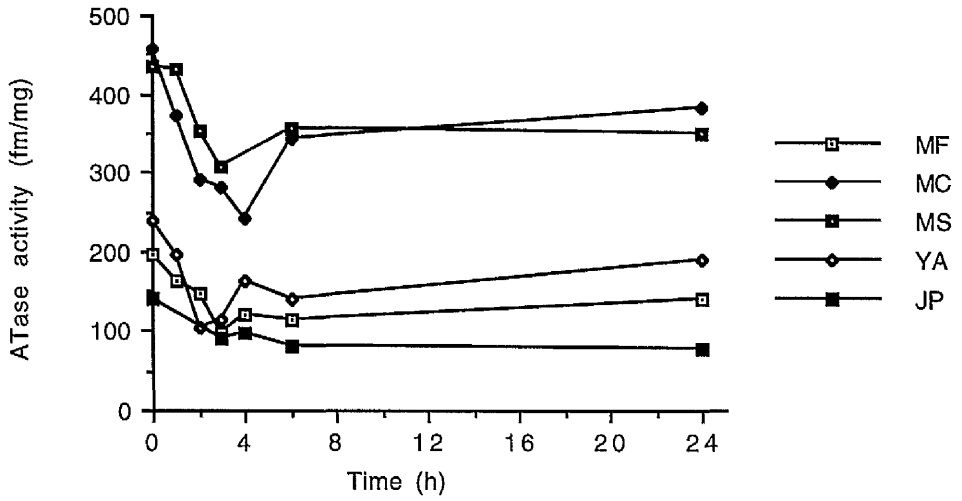


Figure 7.1. ATase activity (fm/mg) in peripheral lymphocytes of patients up to 24h after the first temozolomide dose.

During the daily administration schedule, a cumulative and progressive depletion of ATase was observed in eight patients as shown in Fig. 7.2. The consensus data for ATase activity expressed as mean % of the pretreatment value are shown in Fig. 7.3. In the two patients monitored on day 7, 48 h after the last temozolomide dose, ATase levels had recovered to 42.7% and 48.3% of the pretreatment levels; the nadirs in these patients being 25.6% and 35.0% of the pretreatment levels, respectively. Using

repeated measurement analysis and Duncan's range test, the nadir ATase activity appears to occur between days 4 and 6.

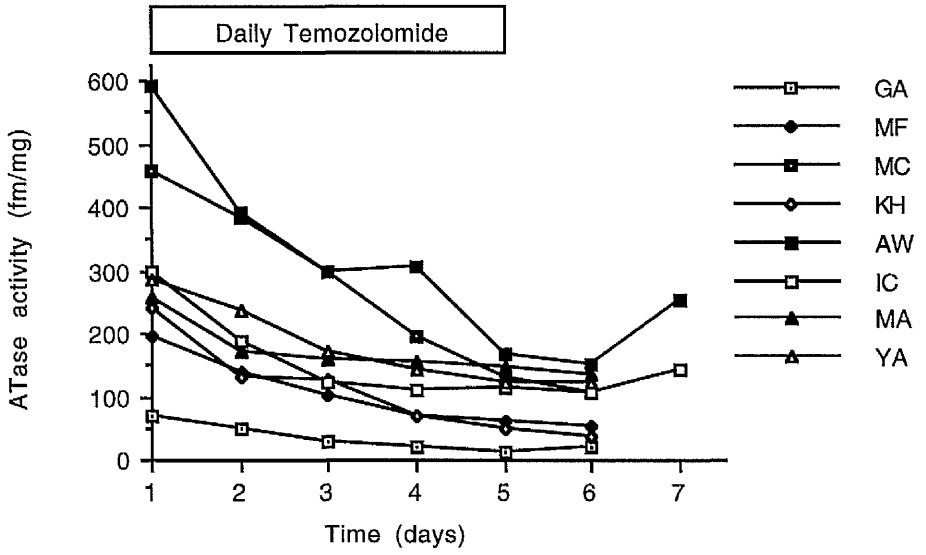


Figure 7.2. ATase activity (fm/mg) in peripheral lymphocytes of patients receiving daily temozolomide (150mg/m^2, Days 1-5).

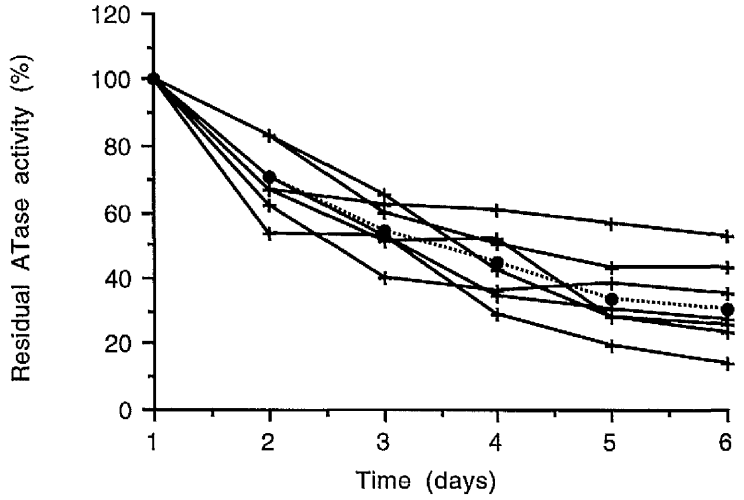


Figure 7.3. ATase activity expressed as % of pretreatment value plotted as the mean (•) and as individual patient values (+).

There was a linear relationship between the pretreatment ATase level and the extent of ATase depletion (pretreatment minus nadir ATase level) with a correlation coefficient of 0.97 (Fig. 7.4). The corresponding data from DTIC (Chapter 3, Section 3.2.1) and CB10-277 (Chapter 6, Section 6.2.1) is also presented in these cases (see Fig. 7.4) and the correlation coefficients were 0.77 and 0.96 respectively.

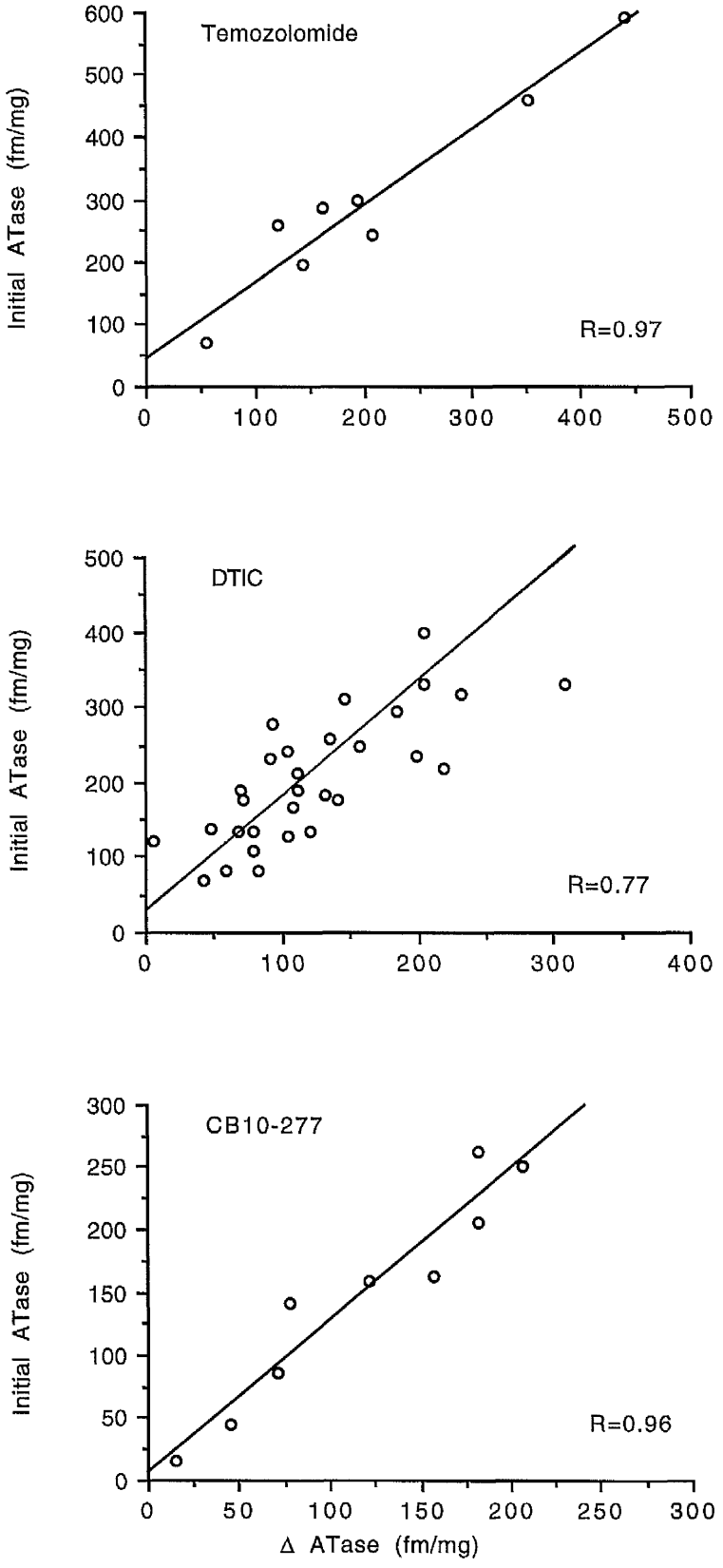


Figure 7.4. Relationship between the extent of ATase depletion and pretreatment ATase levels.

7.2.2 *In vitro* effects of temozolomide, procarbazine, mitozolomide and methyl methanesulfonate on recombinant ATase

The effect of temozolomide on pure recombinant human ATase was assessed by incubating varying amounts of the drugs with a fixed amount of ATase in the presence of 10 μg calf thymus DNA for 2 h at 37°C. The level of inactivation was compared with mitozolomide (a chloroethyl analogue of temozolomide discussed in Chapter 1), procabazine and methyl methanesulfonate (MMS, a methylating agent that does not result in appreciable levels of O^6-MeG in DNA) using identical incubating conditions. The dose-response curve of ATase inactivation by temozolomide is shown in Fig. 7.5; 50% depletion of ATase (ID_{50}) occurred at 1.5 μM temozolomide. In contrast, the ID_{50} for mitozolomide was 420 μM, approximately 300 fold higher concentration compared to temozolomide. Of the various O^6-alkylating agents capable of inactivating recombinant ATase, NMU and temozolomide are the most potent compounds (I_{50}=1.5 μM) followed by streptozotocin (I_{50}=2.0 μM; see Chapter 6, Section 6.2.3), BCNU (I_{50}=9.5 μM; see Chapter 3, Section 3.2.4), fotemustine (I_{50}=15 μM; see Chapter 3, Section 3.2.4) and mitozolomide (I_{50}=420 μM).

No significant inactivation was seen when ATase was incubated with procarbazine up to a concentration of 1 mM (Fig. 7.5) consistent with the requirement of metabolism of procarbazine in the liver to generate a reactive species capable of alkylating DNA. ATase depletion was observed with higher concentrations of procarbazine and this effect could be due to either contamination with a reactive methylating species (e.g. a 1% contamination with a direct acting agent) that directly alkylates DNA or the ATase protein resulting in ATase inactivation. However the possibility of a slow spontaneous decomposition to a methylating species during the incubation cannot be eliminated. ATase inactivation was also seen on preincubation with the direct acting agent MMS and the calculated ID_{50} was 800 μM (Fig. 7.5).

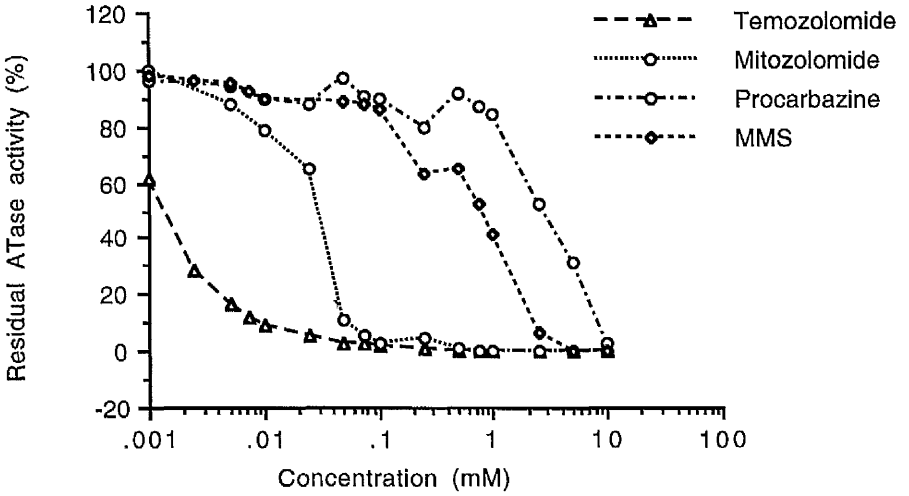


Figure 7.5. *In vitro* effects of temozolomide, mitozolomide, procarbazine and MMS on pure recombinant human ATase.

7.3 Discussion

The present study demonstrates that temozolomide is effective in depleting ATase activity in peripheral blood lymphocytes and the nadir ATase activity occurred around 2-6 h after treatment. When compared to DTIC treated patients, the extent of depletion following first oral dose of temozolomide appeared to be equivalent to ATase levels of patients receiving 400 mg/m^2 DTIC but not with patients receiving 500 or 800 mg/m^2 DTIC. The observed mean nadir ATase activity following 400 mg/m^2 DTIC was 56% versus 27% and 25% for 500 and 800 mg/m^2 DTIC respectively (see Chapter 3, Section 3.2.1). In addition the mean ATase at 24 h was appreciably higher than that seen for CB10-277 (Chapter 6, Section 6.2.1). The post nadir recovery of lymphocyte ATase activity was variable but in none of the 5 patients studied was a return to pretreatment levels observed. This residual deficit in ATase was generally increased during the repeated daily administration of temozolomide; the median ATase activities at days 2, 3, 4, 5 and 6 were 66.3%, 52.5%, 39.5%, 30.5% and 28.9% of the pretreatment values respectively. There was little inter-patient variation in the % decrease in ATase activity during the schedule, despite wide variations in pretreatment ATase levels.

There was correlation between the extent of ATase depletion (pretreatment minus nadir level) and the pretreatment ATase level (R=0.97). If the extent of ATase depletion is the result of a stoichiometric inactivation due to the repair of temozolomide-mediated methylation of DNA, the calculated amount of ATase inactivated due to repair of O^6-MeG would be expected to be relatively constant assuming drug distribution, drug uptake and *de novo* ATase resynthesis rates were consistent between patients. However, there was a marked inter-individual variation in the extent of ATase inactivation. Similar findings were seen on the results using DTIC (Chapter 3) and CB10-277 (Chapter 6).

Although ATase depletion by temozolomide would be expected to occur *via* methylation of DNA in peripheral blood lymphocytes as was seen in the *in vitro* experiments, the possibility that this non-stoichiometric depletion of ATase was due to a direct effect of temozolomide on the ATase itself cannot be dismissed. Previous *in vitro* studies have shown that inactivation of partially purified human ATase from CEM cells can occur by direct alkylation of the protein following incubation with a variety of alkylating agents including MNU, streptozotocin, BCNU, chlorozotocin, CCNU and MeCCNU (Brent 1986). In addition, it was found that MMS, a methylating agent that does not produce appreciable amounts of O^6-MeG in DNA was associated with inactivation of recombinant ATase both here and in a similar study reported elsewhere (Brent 1986). This provides additional support to the idea that the non-stoichiometric inactivation seen in the clinical situations with lymphocyte ATase may be partly due to direct alkylation of the protein.

It has been found that the anti-tumour activity of temozolomide against a number of murine tumours was very schedule-dependent (Stevens et al. 1987) and a similar finding was reported with 51 patients treated with temozolomide (Newlands et al. 1992). Thus, clinical activity has been observed for patients with malignant melanoma, astrocytomas and mycosis fungoides when temozolomide was given daily for 5 days compared to single dose administration in a phase I study (Newlands et al. 1992;

O'Reilly et al. 1993). In addition, clinical responses were also seen in 3 of the 8 patients with metastatic melanoma treated here with temozolomide (see Table 7.1). It is not unreasonable to suggest that the greater effectiveness of the daily treatment is related to the more extensive depletion of ATase, assuming that a similar effect occurs in the tumour cells. As reported in Chapter 9, ATase depletion was seen in one post-treatment tumour biopsied at 17 h after the first oral dose of temozolomide and similar ATase depletion was seen in tumour material following CB10-277 and DTIC treatments (see Chapter 9, Section 9.2.2).

If tumour sensitization is a consequence of ATase depletion then it could be that the response to treatment would be improved if the temozolomide were to be given every 2-6 h, corresponding to the ATase nadir determined after a single dose, rather than every 24 h, when recovery of ATase activity can occur. Indeed, in the treatment of melanoma with DTIC/fotemustine combinations as reported in Chapter 5, the schedule of fotemustine 4 h after DTIC was designed to exploit the anticipated nadir of ATase activity produced by DTIC and produces better response rates than the individual agents given alone. A similar dose schedule of temozolomide for 5 doses and followed 2-6 h later by a chloroethylating agent also seems worthy of consideration.

In conclusion, the present study shows that temozolomide can inactivate lymphocyte ATase activity and a progressive depletion of ATase was seen with 5 daily doses schedule suggesting that the schedule-dependent anti-tumour activity of temozolomide seen in experimental models and clinically may be related to a cumulative depletion of ATase. It would be interesting to explore whether a more extensive ATase depletion can be achieved with more frequent doses of temozolomide (every 4 h) or even with continuous infusion of temozolomide in view of its potential improved clinical effects when combined with CNUs.

Chapter 8 *In vivo* and *in vitro* Effects of Cyclophosphamide and its Metabolites on O^6-Alkylguanine-DNA-Alkyltransferase

8.0 Introduction

Autologous Bone Marrow Rescue (ABMR) and peripheral blood progenitor cell transplantation following ablative chemotherapy are being increasingly adopted for patients with high risk advanced Hodgkin's disease, who fail to obtain complete remission following primary induction chemotherapy, relapse within one year of completing chemotherapy or who are in second or subsequent relapse after receiving two or more standard chemotherapy regimens (Armitage et al. 1989; Pettengell et al. 1993). 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 (Bierman et al. 1988; Carella et al. 1988; Ahmed et al. 1989; Gribben et al. 1989). This indicates that inadequate chemotherapy is the prime reason for failure.

The most commonly used preparative treatment regimen used for ABMR in Hodgkin's disease involves 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).

Based on the observations of progressive depletion of ATase activity in human peripheral blood lymphocytes of patients with malignant melanoma treated with DTIC and CB10-277 (Chapters 3 and 6), it was considered worthwhile to examine lymphocyte ATase levels following BCNU prior to ABMR since BCNU sensitivity is known to involve ATase. The preparative variant in use was cyclophosphamide (1.5 g/m^2 on days 1 to 4) followed by BCNU (600 mg/m^2 on day 5). However an

unexpected depletion of ATase activity was seen in peripheral lymphocytes of one patient following only cyclophosphamide treatment. This observation was pursued 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).

8.1 Materials and Methods

The clinical characteristics of the 19 patients studied are outlined in Table 8.1. All the patients with Hodgkin's disease had failed front line chemotherapy [including MOPP, ChlVPP or HYBRID (Vinblastine, procarbazine, prednisolone, chlorambucil, vincristine, etoposide & 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 8.1) 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 6 fractionated doses of total body irradiation (200 cGy twice daily) to a total dose of 1200 cGy from day 4 to 6 prior to allogeneic or autograft marrow transplantation. Patients with AML had previously received combination chemotherapy comprising cytosine arabinoside, daunorubicin and thioguanine and patients with ALL 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 CML 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 at 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 at 3, 6, 15, 22, 29, 50, 64, 70, 88, 94, 112, 120 h after chemotherapy.

In order to assess the ability of cyclophosphamide, acrolein and phosphoramide mustard to inhibit ATase *in vitro*, varying concentrations of these agents were incubated with 70 fmoles of pure recombinant human ATase for 2 h at 37°C in buffer I without dithiothreitol and DNA. Residual ATase activity was then measured by incubation with excess substrate DNA as described in Chapter 2. The effect of glutathione on the inhibition of ATase by acrolein was monitored by incubating various amounts of glutathione for 2 h with 500 μM acrolein, a concentration that caused a 95% depletion in ATase activity *in vitro*. Following this, 70 fmoles of recombinant ATase was added and residual ATase activity assayed after a 2 h incubation at 37°C.

Table 8.1 Patient characteristics

Patients/Fig.	Age/Sex	Disease	Treatment	ATase Activity (fm/mg) Initial	Nadir
RD/8.1a	21/M	HD IVB	Cyclo/BCNU	162	12
JC/8.1b	39/F	HD IVB	Cyclo/BCNU	183	34
MR/8.1c	50/F	HD IVB	Cyclo/BCNU	130	25
SH/8.1d	20/M	HD IVB	Cyclo/BCNU	148	47
SJ/8.1e	34/M	HD IVB	Cyclo/BCNU	163	53
MS/8.1f	23/M	HD IVB	Cyclo/BCNU	170	55
ST/8.1g	17/F	HD IVB	Cyclo/BCNU	33	bd[a]
AW/8.2a	26/M	AML 1°CR	Cyclo/TBI	107	19
GD/8.2b	45/F	AML 1°CR	Cyclo/TBI	168	10
LW/8.2c	36/F	AML 1°CR	Cyclo/TBI	101	13
DH/8.2d	43/M	AML 1°CR	Cyclo/TBI	405	138
SA/8.2e	44/F	ALL 1°CR	Cyclo/TBI	461	95
MC/8.2f	18/F	ALL 1°CR	Cyclo/TBI	148	18
HR/8.2g	30/F	ALL 1°CR	Cyclo/TBI	103	19
TC/8.2h	21/F	ALL 3°CR	Cyclo/TBI	168	37
SK/8.2i	28/F	CML 1°CP	Cyclo/TBI	160	23
LB/8.2j	38/M	CML 1°CP	Cyclo/TBI	102	45
CN/8.2k	38/F	CML 1°CP	Cyclo/TBI	200	18
RB/8.2l	54/M	NHL 1°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; IVB: stage IVB disease; CR: complete remission; 1°: first; 3°: third; PR: partial remission; 1°CP: first chronic phase; bd[a]= below detection

8.2 Results

8.2.1 *In vivo* effects of sequential cyclophosphamide and BCNU on lymphocyte ATase

Pretreatment ATase levels in the seven previously treated Hodgkin's patients ranged from 33 to 183 fm/mg total protein and the mean value (141.3 ± 50.6) appears to be lower than that observed for previously untreated melanoma patients prior to receiving DTIC, CB10-277 or temozolomide (see Chapters 3, 6 and 7). In all seven patients,

depletion of ATase activity was seen following cyclophosphamide administration. Wide variations were noted in the rates and extents of ATase depletion between patients (see Figs. 8.1a to 8.1g). In three patients the first cyclophosphamide treatment caused depletion to 52%, 64% and 67% of the pretreatment levels (Figs. 8.1a, 8.1d & 8.1g) whilst in two other patients the first treatment caused only a 10% loss in activity (Figs 8.1b & 8.1e). Following four cyclophosphamide treatments the median ATase nadir was 32% (range 0% to 57%) of pretreatment levels. In one patient (Fig. 8.1g), no ATase activity was detectable after the fourth cyclophosphamide treatment. In two patients there was evidence of partial regeneration of ATase during the cyclophosphamide treatments (Figs. 8.1d & 8.1e). ATase activities continued to decrease after BCNU administration to a median of 19% (range 0% to 32%) of pretreatment levels.

Although in some cases, the loss of ATase was small after administration of BCNU, overall there was a substantial post-BCNU decrease in ATase and this was highly statistically significant: maximal loss occurred between the second and fourth dose of cyclophosphamide (P=0.0013) and after BCNU administration (P=0.0018). A consensus summary of the data from all seven patients is shown in Fig 8.3A.

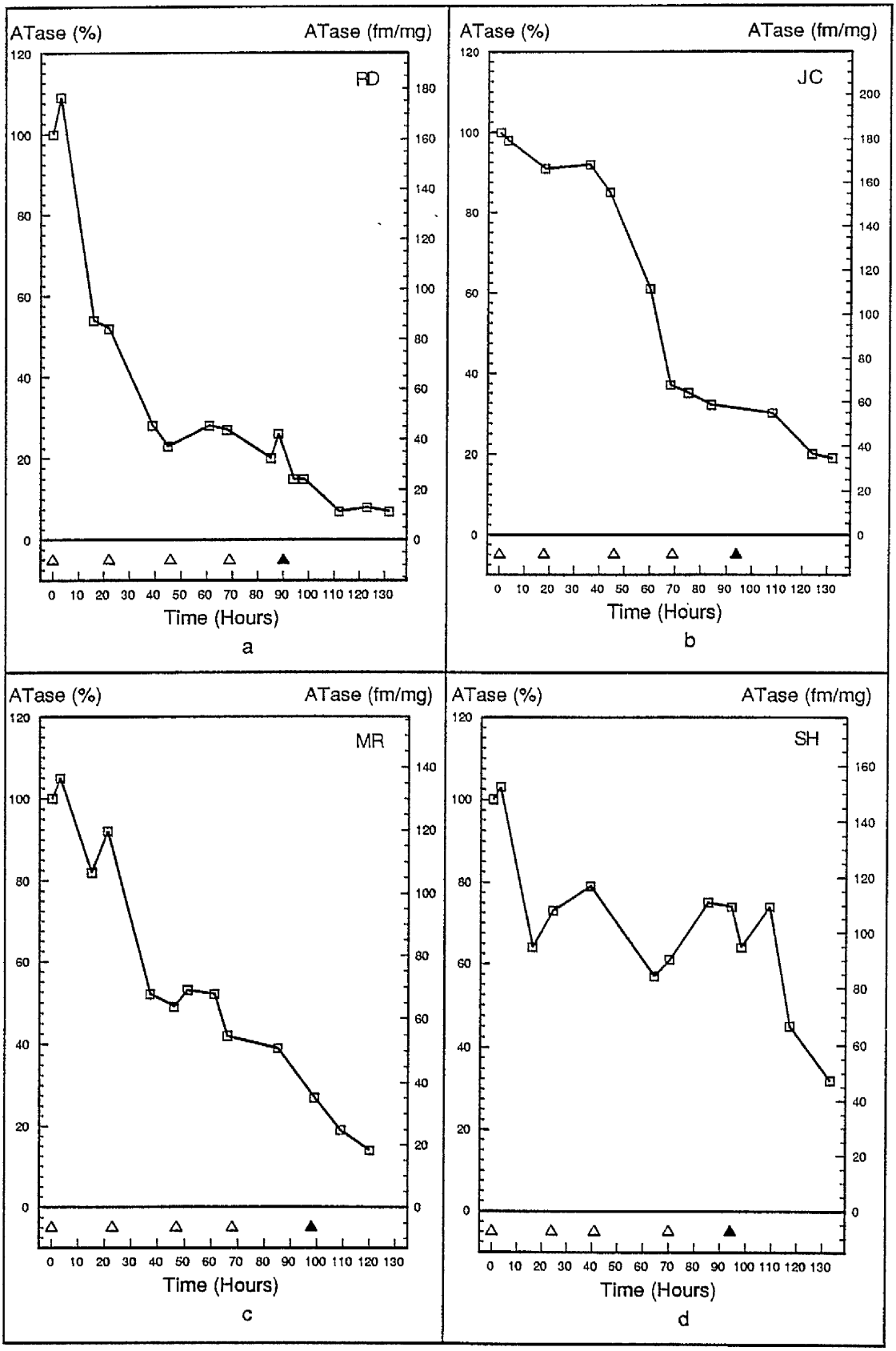


Figure 8.1 a-d. ATase activity in peripheral lymphocytes of Hodgkin's patients sampled at various times after high dose cyclophosphamide (Δ) given on day 1, 2, 3 and 4 and BCNU (▲) given on day 5. Results are expressed as % of pretreatment activity and actual levels (fm/mg protein).

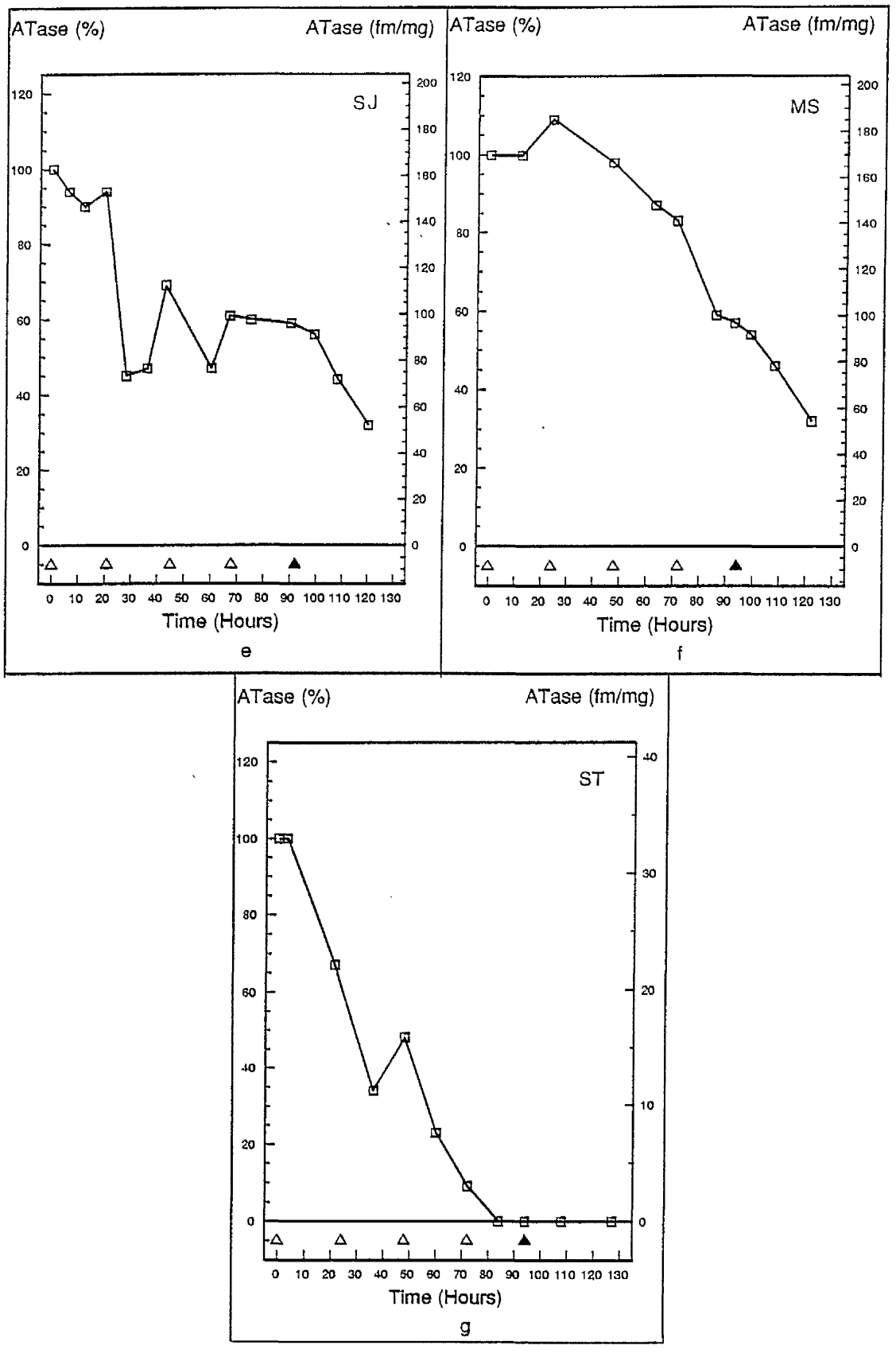


Figure 8.1 e-g. Continued.

8.2.2 *In vivo* effects of cyclophosphamide and total body irradiation on lymphocyte ATase

The pretreatment ATase levels in the twelve patients monitored ranged from 101 to 462 fm/mg total protein. As above, the extents and rates of depletion were variable with a median post-cyclophosphamide nadir of 35% (range 12% to 78%) of pretreatment levels. In seven patients there was a transient increase in ATase activity after the first dose of cyclophosphamide (Figs 8.2e-h, 8.2j, 8.2k-l) followed by depletion to nadirs from 80% to 10% of pre-cyclophosphamide levels. In two patients significant partial recovery of ATase was seen during the cyclophosphamide treatments and prior to TBI (Figs. 8.2i & 8.2l). Generally, relatively small changes in ATase activity were seen during the TBI treatments. However, in three patients, TBI itself appeared to extensively suppress ATase activity (Figs. 8.2h, 8.2k & 8.2l) but in two of these (Figs 8.2h & 8.2k) an initial marked suppression was followed by an equally extensive recovery.

Statistical analysis again showed that the effect of cyclophosphamide on ATase was cumulative, particularly between the first dose of cyclophosphamide and the second day of TBI ($P<0.0001$). A consensus summary of the data from all 12 patients is shown in Fig. 8.3B. Although the mean pretreatment ATase activity was higher and the ATase reduction rate appears faster than in the Hodgkin's group, this was not statistically significant ($P>0.05$).

Similar to the observations seen with DTIC, CB10-277 and temozolomide, a linear relationship was obtained between the pretreatment ATase level and the extent of ATase depletion in the 19 patients receiving cyclophosphamide preparative treatment (pretreatment minus nadir ATase level) with a correlation coefficient of 0.97 (Fig. 8.4).

There was progressive reduction in WBC counts over the 5-7 days of cyclophosphamide/BCNU and cyclophosphamide/TBI: the mean pretreatment leucocyte count was 7.04 x 10^9/l and mean post-cyclophosphamide leucocyte count was 3.18 x 10^9/l.

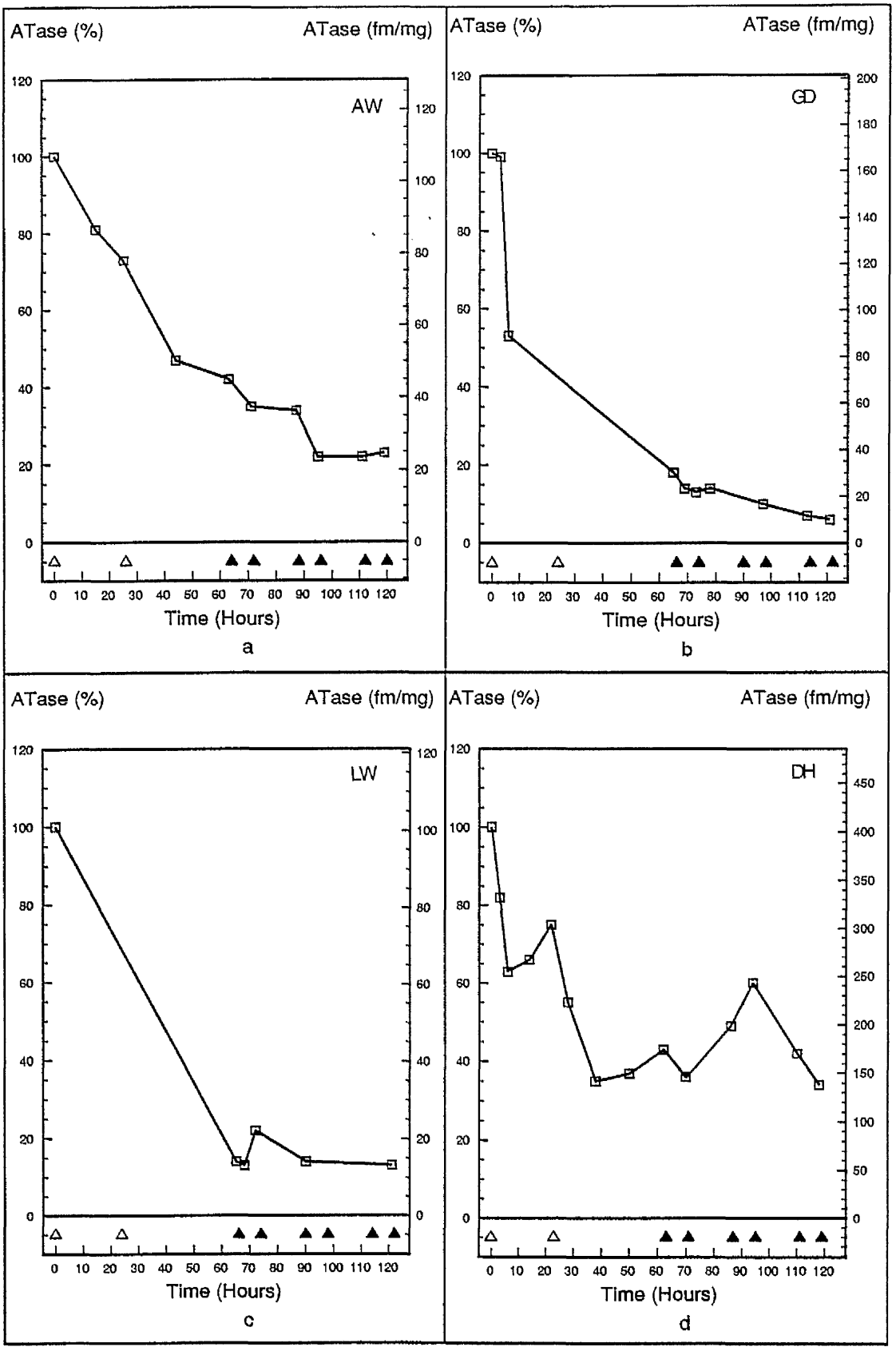


Figure 8.2 a-d. ATase activity in peripheral lymphocytes of patients sampled at various times after cyclophosphamide (Δ) given on days 1 and 2 and TBI (▲) given on days 4, 5, and 6. Results are expressed as % of pretreatment activity and actual levels (fm/mg protein).

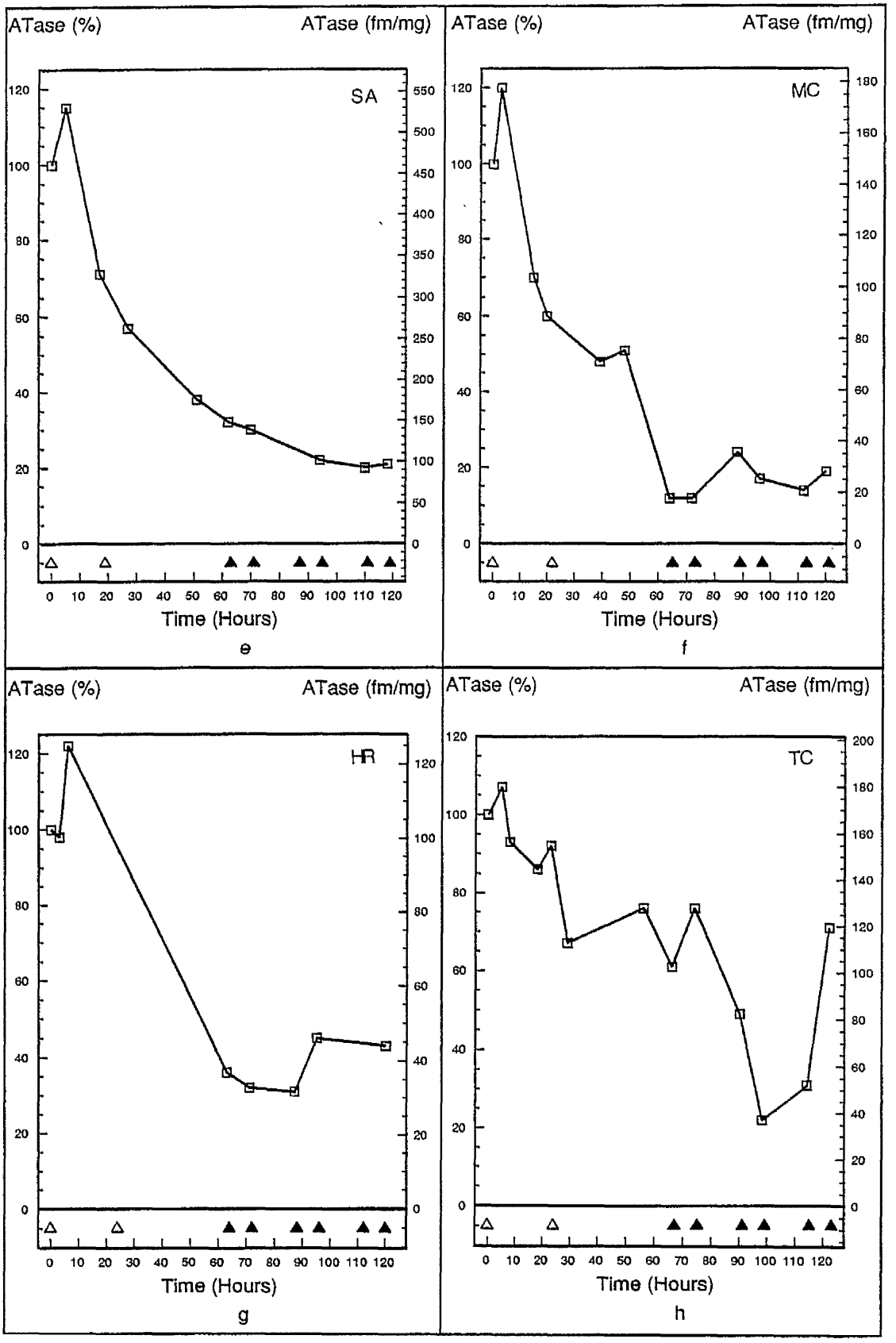


Figure 8.2 e-h. Continued.

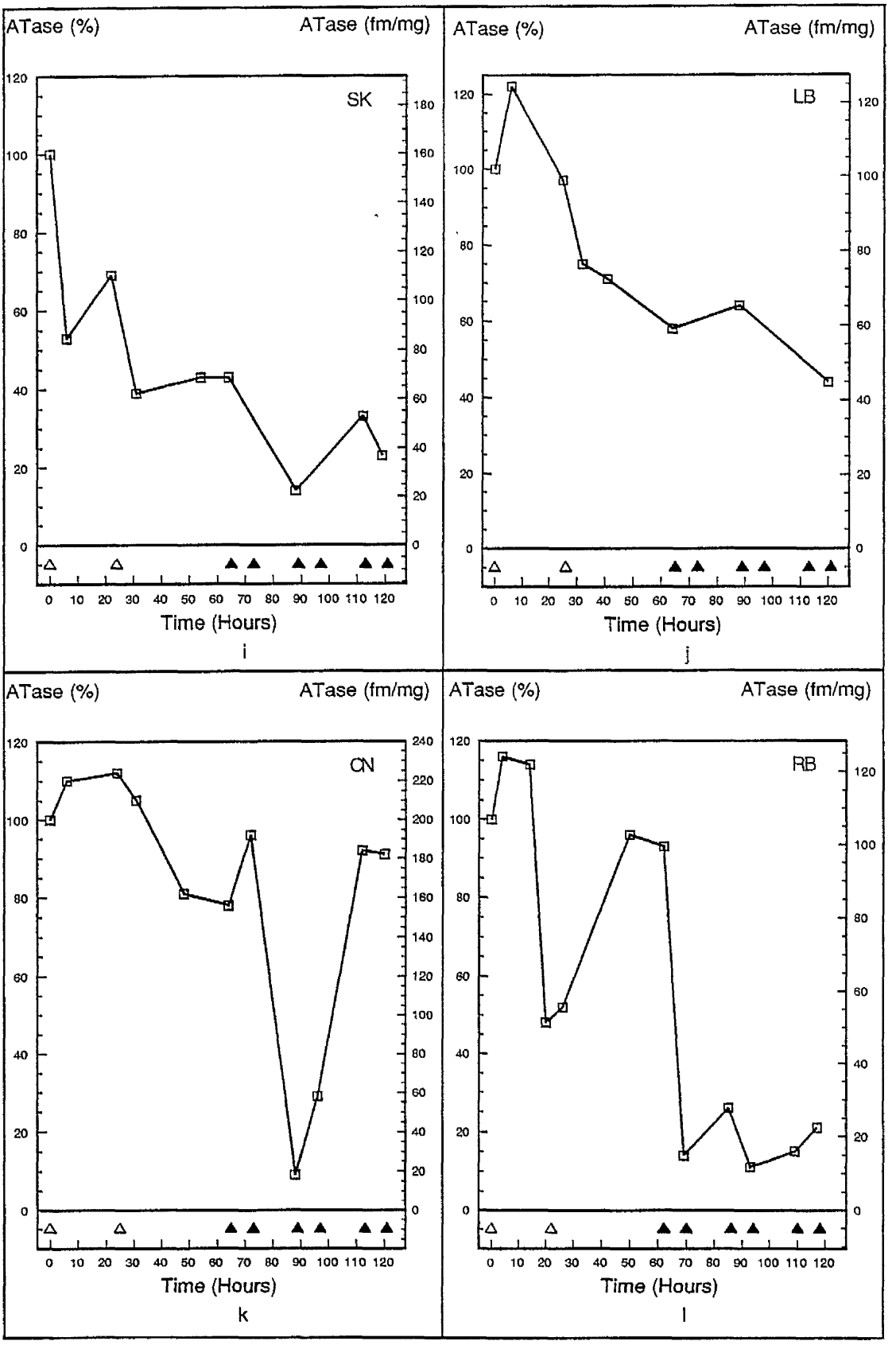


Figure 8.2 i-l. Continued.

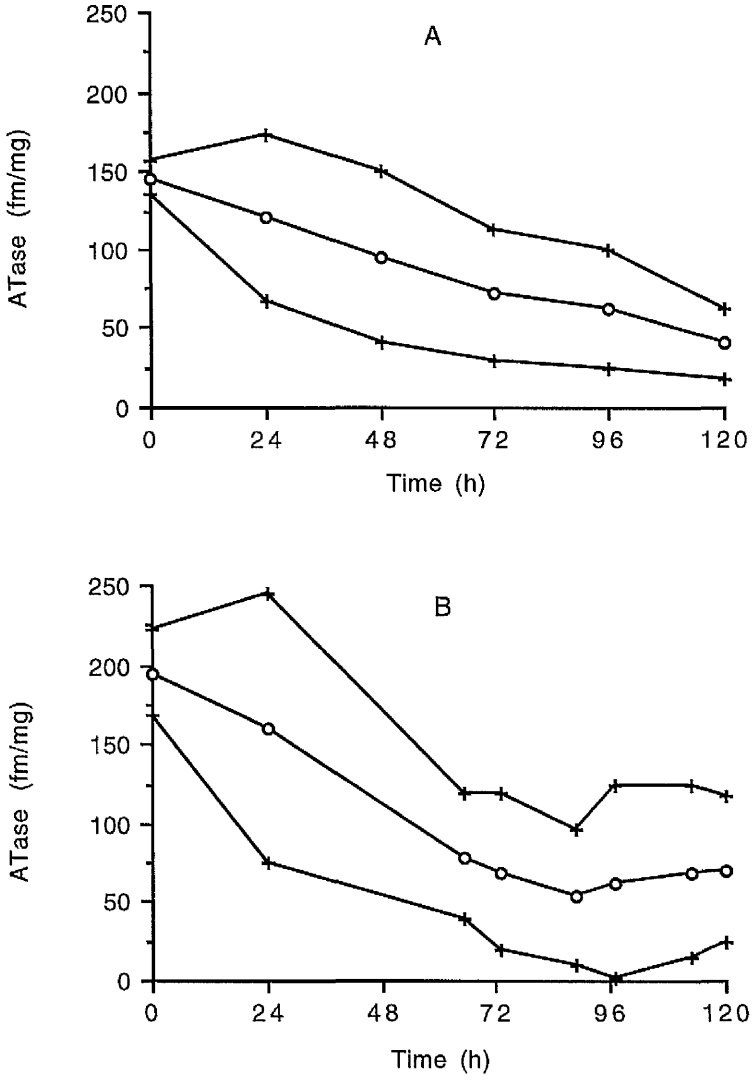


Figure 8.3. Consensus data for ATase specific activity in extracts of peripheral lymphocytes of A; patients treated with cyclophosphamide and BCNU (see Fig 8.1) and B; patients treated with cyclophosphamide and TBI (see Fig 8.2). Figures show mean values (o) and upper and lower 95% confidence intervals (+).

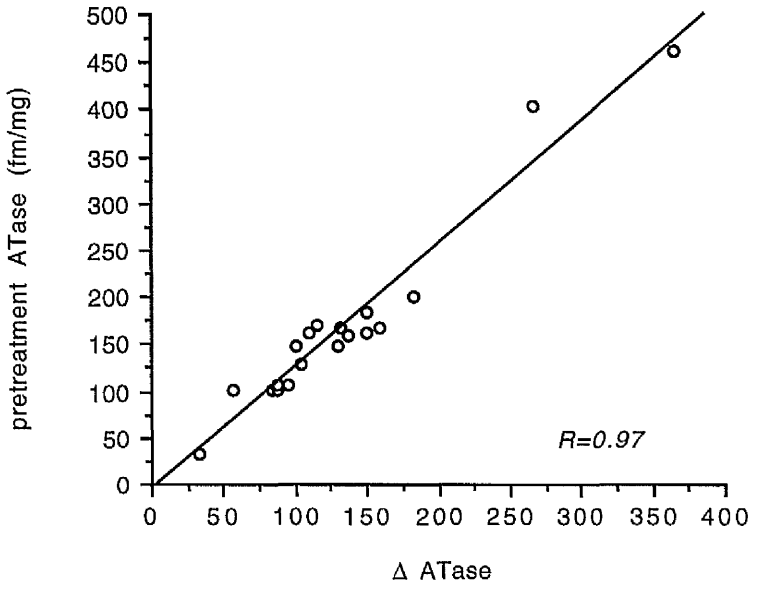


Figure 8.4. Relationship between the extent of ATase depletion and pretreatment ATase levels.

8.2.3 *In vitro* effects of cyclophosphamide and its metabolites on recombinant ATase

The direct effects of BCNU, cyclophosphamide, acrolein and phosphoramide mustard on pure recombinant human ATase was assessed by incubating the drugs with a fixed amount of ATase for 2 h at 37°C *in vitro*. Fig. 8.5 shows the dose-response curves for ATase inhibition 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 only 100 μM caused 90% inactivation. ATase depletion was also seen with phosphoramide mustard (>1 mM) but this was with a concentration far in excess of peak plasma concentration measured in patients receiving high dose cyclophosphamide (Jardine et al. 1978; Sladek et al. 1984; Colvin and Chabner 1990).

Increasing concentrations of glutathione were also incubated with a concentration of acrolein (500 μM) that caused 95% depletion of ATase activity in the competition assay

above. As shown in Fig. 8.6, 1 mM glutathione was able to completely prevent acrolein-induced depletion of ATase.

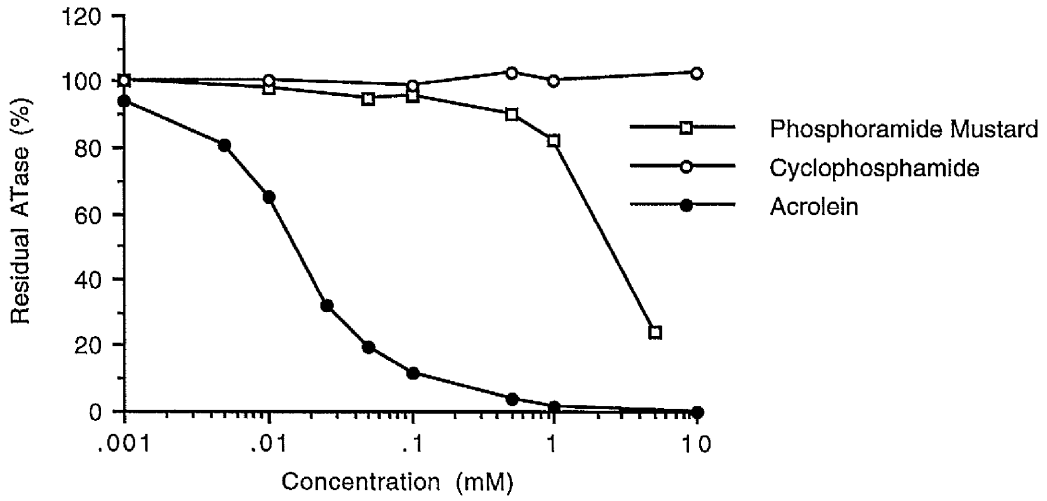


Figure 8.5. Effect of pre-incubation of pure recombinant human ATase with increasing concentrations of cyclophosphamide, phosphoramide mustard or acrolein.

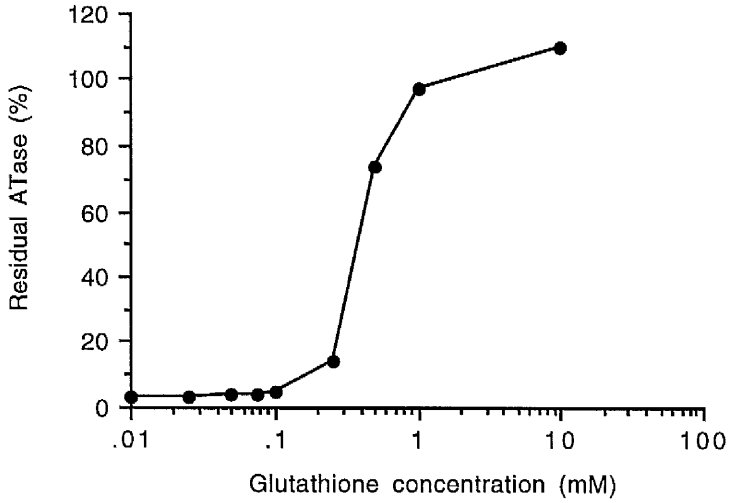


Figure 8.6. Effect on its ability to deplete the activity of pure recombinant human ATase of preincubation of acrolein with increasing concentrations of glutathione.

8.3 Discussion

The present study showed extensive depletion of ATase activity in peripheral blood lymphocytes of nineteen patients receiving cyclophosphamide preparative treatment prior to bone marrow rescue. 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, 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%). The pretreatment ATase levels in these previously treated Hodgkin's patients appears to be lower than that observed for previously untreated melanoma patients prior to receiving DTIC, CB10-277 or temozolomide (see Chapters 3, 6 & 7) and similar finding was reported by Sagher et al (1988) in 14 Hodgkin's patients previously treated with MOPP and/or ABVD. A similar picture emerged in the other group of twelve patients treated with cyclophosphamide/TBI, with a median post-cyclophosphamide nadir of 39% (range 12 to 78%).

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 experimental variation or intra-individual variation, although 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. 1989). It is possible that selection of high level ATase-expressing cells may have occurred as a consequence of cytolysis of a lymphocyte population(s) with relatively low ATase specific activity. Whilst the relative contribution of T and B lymphocytes to the overall ATase measurements was not assessed in this study, previous reports have shown ATase specific activities of 190 and 140 fm/mg protein respectively (Gerson et al. 1985). However, the overall changes observed are unlikely to be attributable to T or B specific effects since the proportion of

T and B cells is similar in Hodgkin's, non-Hodgkin's lymphoma and controls (Hermann et al. 1983).

Perhaps a more likely explanation for the loss of ATase activity is that there is a direct depleting effect on the ATase itself. Adducts at the O^6-position of guanine have not yet been identified in DNA after administration of cyclophosphamide or its metabolites but there are two reports (Kleihues and Margison 1976a; Meer et al. 1989) which showed that cyclophosphamide is able to increase the amount of O^6-MeG in DNA following a chasing dose of methylating agent in rodent liver and both groups attributed this to some as yet unidentified O^6-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: cyclophosphamide is activated by the hepatic mixed-function oxidases, to 4-hydroxycyclophosphamide, the 'transport' form which enters cells and eventually decomposes intracellularly to phosphoramide mustard, the ultimate cross-linking metabolite of cyclophosphamide, and acrolein (see Fig. 8.7; Brock 1989; Sladek 1987). In support of the ATase depleting role of the cyclophosphamide metabolite, acrolein, it was shown that 100 μM acrolein is able to deplete ATase activity by 90% when it was incubated *in vitro* with pure recombinant human ATase. This may be the result of the affinity of acrolein for sulfhydryl groups including, possibly, the alkyl-accepting cysteine residue of the ATase protein (Pegg 1990). In addition, a direct relationship was seen between the pretreatment ATase level and the extent of ATase depletion (R=0.97) in the 19 treated patients indicating that the ATase inactivation is more likely to be due to direct action of acrolein on the protein rather than *via* a stoichiometric mechanism as was discussed previously with the anti-tumour methylating compounds (see Chapter 7). The peak concentration of phosphoramide mustard achieved in the serum following high dose cyclophosphamide (60-75 mg/kg) was 50-100 μM (Jardine et al. 1978; Juma et al. 1979; Colvin and Chabner 1990)

indicating that the concentration of intracellular acrolein that depletes recombinant human ATase *in vitro* is potentially attainable *in vivo.*

The variation in ATase depletion seen in the nineteen patients studied 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 (Draeger et al. 1976; McGown and Fox 1986; Chresta 1990; Lee 1991a). It has previously been demonstrated that the amount of the ultimate active metabolites formed intracellularly is dependent on the intracellular glutathione concentration (Lee et al. 1991b). Cyclophosphamide has also been shown to be able to deplete serum glutathione (Carmichael et al. 1986). The study here shows that glutathione can inhibit acrolein-induced depletion of ATase, supporting the hypothesis that in the case of lymphocytes, intracellular glutathione levels may determine the extent of ATase depletion.

Irrespective of the mechanism, the observations on peripheral lymphocytes may have important general implications in combination chemotherapy if similar changes in ATase occur in the tumour and indeed, if the extents of ATase depletion achieved are sufficient to sensitize the tumour cells to killing by BCNU. It is not unreasonable to suggest that the ATase-depleting action of cyclophosphamide would best be exploited by employing sequential cyclophosphamide and BCNU (as here) rather than a regimen in which cyclophosphamide and BCNU are administered concurrently or in which BCNU is given before cyclophosphamide. Moreover intracellularly released acrolein has been shown to deplete cellular glutathione (Gurtoo et al. 1981; Lee 1991b) and the latter is able to decrease the cytotoxic and DNA cross-linking activity of BCNU (Ali-Osman et al. 1989). Therefore, if used as in the schedule here, the glutathione-depleting property of acrolein may further sensitize tumour cells to BCNU. In support of this suggestion it is interesting to note that in one series of 54 patients with advanced Hodgkin's disease undergoing ABMR (Reece et al. 1991), where the conditioning

schedule involved administering BCNU after cyclophosphamide together with etoposide, the complete response rate and disease-free survival rate were 80% and 55% respectively. In contrast, most other series using similar preparative drugs, but different schedules, the complete response rate averaged about 45% and only approximately 10% to 30% achieved disease-free survival (Jagannath et al. 1986; Bierman et al. 1988; Carella et al. 1988; Ahmed et al. 1989).

In conclusion, cyclophosphamide is capable of depleting cellular ATase and this is probably mediated via the release of intracellular acrolein, a cyclophosphamide metabolite. This property could be exploited in designing future combination chemotherapy schedules. This method of depleting cellular ATase may be an alternative to the proposed use of agents such as O^6-benzylguanine since cyclophosphamide-induced depletion is accomplished by an agent with proven antitumour activity.

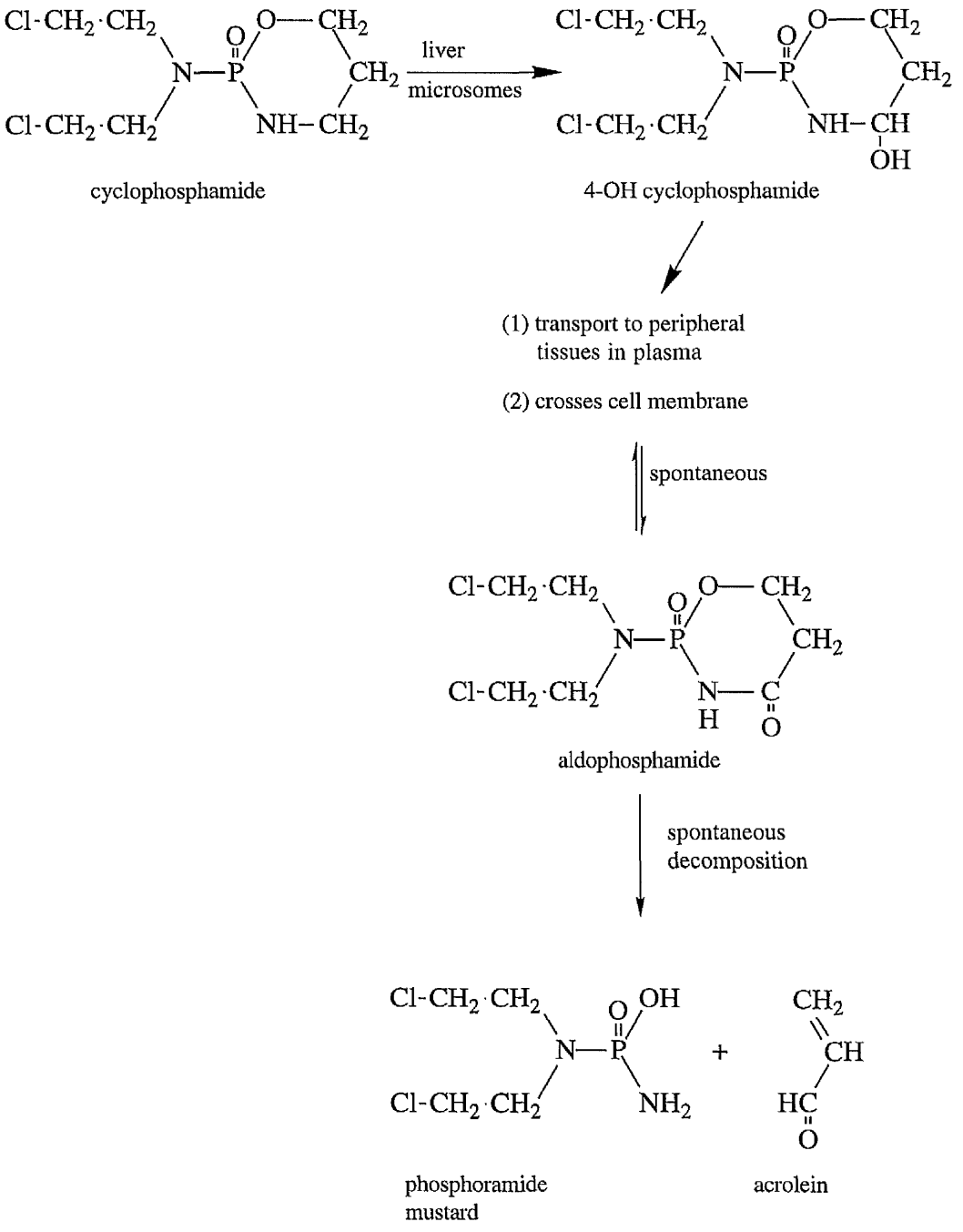


Figure 8.7. Metabolism of cyclophosphamide.
4-OH cyclophosphamide = 4-hydroxycyclophosphamide.

Chapter 9 Activity and Cellular Distribution of O^6-Alkylguanine-DNA-Alkyltransferase in Melanoma Tissues

9.0 Introduction

Melanoma tumours frequently exhibit a high degree of intrinsic and acquired drug resistance (Comis 1976, Balch et al. 1989). Since drug resistance to the most commonly used agents DTIC and the CNUs involves ATase, it is not unreasonable to suggest that high ATase activity is present in those melanoma tumours resistant to treatment with O^6-alkylating agents. Indeed in one reported study with malignant melanoma, ATase levels in melanoma biopsies varied considerably, ranging from 35 to 650 fmol/mg protein, values that differ sufficiently to explain sensitivity and resistance (Maynard et al 1989).

In an attempt to assess the possible role of ATase in the response of melanomas to O^6-alkylating treatments, ATase activity was measured in a number of melanoma biopsies before treatment in order to determine whether the levels correlated with clinical response. In nine of these patients, ATase activity was also measured in biopsies taken after treatment with O^6-alkylating agents and these include the two cases reported in Chapter 6.

Although ATase assay methods are highly sensitive, the results obtained in tissue extracts provide only an average of all cells including the normal stroma and blood cells in the tumour biopsies and do not take into account the possible cellular heterogeneity of ATase expression. This is clearly an important question in chemotherapeutic effectiveness since small numbers of cells with high levels of ATase could not only give the impression of low overall ATase level in tissue homogenates but also be the resistant cells that eventually result in tumour relapse and recurrence. The present work therefore also examined the ability of an anti-ATase antibody to detect the ATase protein in a series of melanomas and attempts were also made to correlate the staining intensity to ATase activity and clinical response. The specificity of the antiserum was initially tested using western blot analysis of crude cell-free extracts of melanoma materials.

9.1 Materials and Methods

A total of 30 melanoma tumour samples were biopsied from 25 patients under local or general anaesthesia after informed consent was obtained. In most cases biopsies were obtained from skin metastases but in four cases, they were obtained during surgery for lymph node metastasis and of these latter cases, two or more different parts of the metastasis were obtained from three patients. One sample was obtained from a tru-cut needle biopsy of a liver metastasis (see Table 9.1). In eight of these patients, tumour biopsies were also performed after chemotherapy for disseminated melanoma (see Table 9.1 for clinical details) and in one of these patientsan additional serial biopsy was performed when he returned for the 6th cycle of chemotherapy. The apparently normal tissue surrounding the tumour was removed and the tumours were frozen at -20°C until assayed for ATase activities as described in Section 2.5. In some cases, tissue extracts were subjected to western blot analysis.

Sections of seventeen melanoma biopsies obtained were examined immunohistochemically for staining intensity using an anti-ATase antibody: they were obtained from skin biopsies of patients with metastatic melanoma referred to the Department of Medical Oncology for treatment and included 14 of the 25 patients reported above (see Table 9.1 for clinical details). The melanomas were fixed in formal saline and wax embedded as described in Chapter 2, Section 2.10. The results of immunostaining were recorded on a 0 to 3+ scale in which 0 means absent staining, 1+ light staining, 2+ moderate staining and 3+ intense staining intensity.

Table 9.1 Patient characteristics

Pt/Age/ sex	Biopsied Site	Metastatic Sites	Treatment	Response	ATase (fm/mg)	Staining Intensity*
DW/80/M	skin	skin	None	PD	429 421	NA
SM/21/F	axilla node	node	Surgery, DXT	CR	70 45	1+
JH/79/M	skin	skin	Surgery	NE[a]	126	NA
JA/47/M	skin	skin, brain	steroid	PD	268	NA
DG/86/F	skin	skin, node	DXT	PD	476	2+
GS/43/M	axilla node	node, lung	Vindesine 8mg	PD	26 32 8	NA
DB/47/F	skin	skin, node, brain, liver	DTIC 400mg/m^2 Fote 100mg/m^2	NE[b]	29	NA
RL/52/F	skin	skin, node, lung	DTIC 400mg/m^2 Fote 100mg/m^2	PR	84	2+
HG/56/F	skin	skin, node	DTIC 400mg/m^2 Fote 100mg/m^2	PR	142	1+
AD/48/M	skin	skin, lung	DTIC 500mg/m2 Fote 100 mg/m2	PD	140	1+
GB/53/M	skin	skin, node, liver	DTIC 400mg/m^2 Fote 100mg/m^2	PD	223	2+
DJ/38/F	groin node	node, liver	DTIC 400mg/m^2 Fote 100mg/m^2	PD	268	NA
RB/56/M	liver	liver	DTIC 800mg/m^2 Fote 100mg/m^2	PD	228	2+
EB/45/F	skin	skin, liver	DTIC 400mg Vindesine 5mg	PD	90	NA
MW/52/F	skin	skin, node, lung	DTIC 600mg/m^2 Interferon	PD	813	NA
PT/40/M	skin	skin, nodes	DTIC 500mg/m^2 D1-D5 Melphalan 10mg/m^2	PR	21	NA
LW/61/M	skin	skin, lung	DTIC 500mg/m^2 D1-D5 Melphalan 10mg/m^2	PR	43	NA
FM/59/M	skin	skin, liver	DTIC 500mg/m^2 D1-D5 Melphalan 10mg/m^2	PD	158	NA
PC/41/F	skin	skin, node	DTIC 500mg/m^2 D1-D5 Melphalan 10mg/m^2	PD	202	NA
GT/40/F	skin	skin, bone, soft tissue	Fote 100mg/m^2	PD	392 345	3+

Table 9.1 continued

Pt/Age/ sex	Biopsied Site	Metastatic Sites	Treatment	Response	ATase (fm/mg)	Staining Intensity
MU/66/F	skin	skin, node,	CB10-277 12g/m^2	PD	65	2+
GM/20/F	skin	skin, lung,	CB10-277 12g/m^2	PD	135	2+
SR/59/M	skin	skin, node,	Temozolomide 150mg/m^2 D1-D5	PD	108	1+
JO/43/M	groin node	node, soft tissue	Temozolomide 150mg/m^2 D1-D5	PD	211 202	2+
IC/58/F	skin	skin, lung, liver	Temozolomide 150mg/m^2 D1-D5	PD	307	2+
WL/75/M	skin	skin, node, spleen	DTIC 500mg/m^2	PD	NA	3+
JG/54/M	skin	skin, liver	DTIC 400mg/m^2 Fote 100mg/m^2	PD	NA	2+
MB/53/F	skin	skin, node, soft tissue	DTIC 400mg/m^2 Fote 100mg/m^2	PD	NA	3+
ED/65/M	skin	skin, liver	CB10-277 12g/m^2	PD	NA	2+

* ATase immunostaining were recorded on a 0 to 3+ scale in which 0 means absent staining, 1+ light staining, 2+ moderate staining and 3+ intense staining intensity.
NA not assessed; NE not evaluable.
[a] Died from myocardial infarction; [b] Lost on follow-up and subsequently died in local hospital.

9.2 Results

9.2.1 ATase activity in melanoma tissues

In the 25 patients studied, a considerable variation of ATase levels was observed in crude extracts of the pretreatment melanoma biopsies and this ranged from 8 to 813 fm/mg protein with a mean activity of 200 ± 178 fm/mg protein which was not significantly different from the mean ATase activity observed in peripheral lymphocytes of patients treated with combined DTIC/fotemustine (see Chapter 3) or CB10-277 (see Chapter 6). Most of the tumour biopsies were performed on individuals with skin metastases but in four cases, the biopsies were obtained from lymph nodes and in one case, from the liver. The mean ATase activities in skin and lymph node metastases were 197 ± 176 and 139 ± 117 fm/mg protein respectively (see Table 9.1).

In five patients (DW, SM, GS, GT & JO; Table 9.1), two or more metastases were obtained from each individual and whilst the interlesion ATase activity appears varied at low ATase levels (SM & GS), at higher levels, levels in the biopsies were closely similar.

It has been suggested that comparisons of ATase activity using protein rather than DNA content do not provide the optimum basis for comparison of repair capacities between different cell and tissue types as each cell may vary in size and protein content (Gerson et al. 1986; Pegg 1990). In order to examine this issue, the ATase levels of each biopsy was expressed as activity per μg DNA and per mg protein. In 26 tumours examined, the two parameters were highly correlated (Fig. 9.1; R=0.93, P<0.001), indicating that either method of expression of the results was representative of the sample. This suggests that in the case of metastatic melanoma, the size of tumour cells or cellularity of the biopsied tumours is not an important determinant of 'absolute' ATase activity and expressing ATase activity per mg protein is sufficiently accurate for comparing ATase activity between tumour samples.

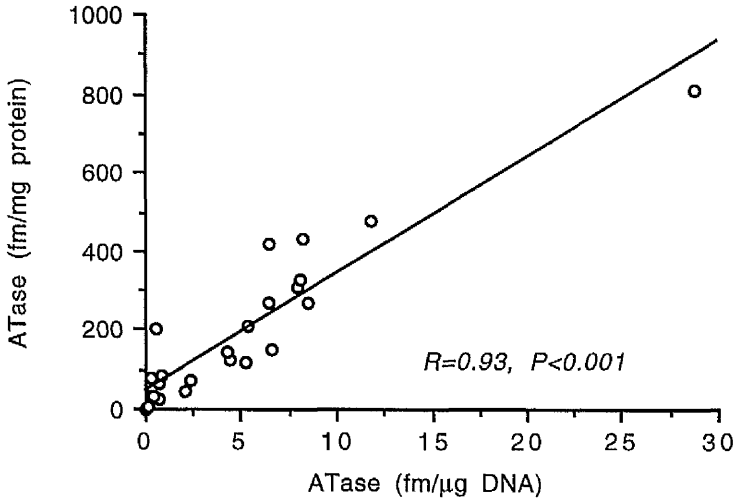


Figure 9.1. Relationship between ATase activity expressed as fm/mg protein and fm/μg DNA.

9.2.2 Relationship between tumour ATase activity and clinical response

In this series of pretreatment tumour biopsies, 19 patients were treated with single or combination treatments involving O^6-alkylating agents; 13 patients received DTIC-based combination treatments, 3 patients received temozolomide, 2 patients received CB10-277 and 1 patient received fotemustine (see Table 9.1). Four responses were seen in these cases (RL, HG, PT, LW; Table 9.1) and in these, the tumor ATase activity ranged from 21 to 142 fm/mg protein with a mean level of 73 ± 53 fm/mg protein. In the patients with progressive disease the ATase levels ranged from 65 to 813 fm/mg protein with a mean level of 237 ± 186 fm/mg protein. While 12 of the 14 patients with progresive disease had tumour ATase activity greater than 100 fm/mg protein, only one of the four responding patients had activity exceeding this level. Statistical analysis showed that the ATase activity in the non-responders was significantly greater than that of the responding patients (P=0.02; Fig. 9.2). It is interesting to note that in one patient (GS) treated with vindesine, a chemotherapeutic agent recognised not to give rise to O^6-alkylation and in whom the tumour biopsy showed a low level of ATase activity, disease progression was observed.

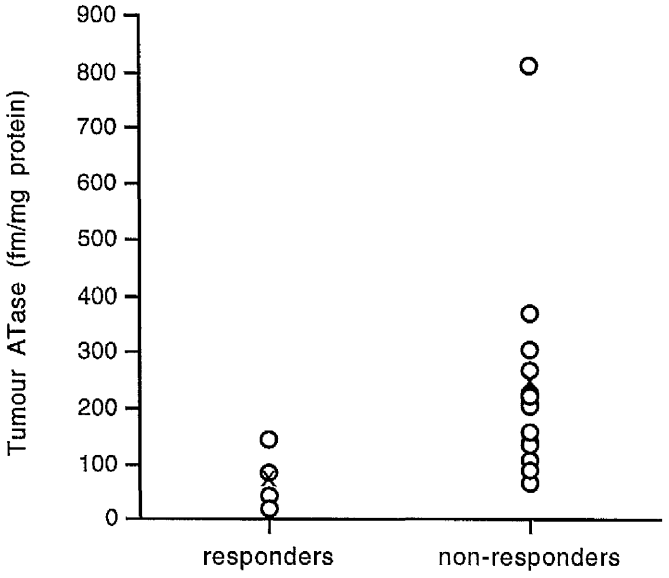


Figure 9.2. ATase activity in pretreatment biopsies of responders and non-responders treated with O^6-alkylating agents. The mean values are shown as x.

In order to assess how effectively the O^6-alkylating agents had delivered cytotoxic DNA damage to the tumour, post-treatment tumour biopsies were performed in eight of these patients during or immediately after O^6-alkylating agent treatments. The characteristics of these patients and the treatments given are shown in Table 9.2. One of these patients (PT) also had another biopsy performed when he returned for the 6th cycle of treatment. Wide individual variation in extent of ATase depletion was seen in the tumour biopsies following chemotherapy. The mean ATase activity before and after treatment were 176 ± 247 fm/mg protein and 74 ± 103 fm/mg protein respectively and this difference was found to be statistically significant (P=0.008). Four of these patients also had simultaneous blood samples taken during the biopsies and in all but one of these (RL) there appeared to be a relationship between the changes in the ATase activities in the tumour and lymphocyte samples before and after chemotherapy (see Table 9.2).

Table 9.2 Patient characteristics and chemotherapy received

Patients	Age/ Sex	Chemotherapy	Response	Tumour ATase (fm/mg) Pre	Post	Lymphocyte ATase (fm/mg) Pre	Post
PC	41/F	DTIC 500mg/m^2 D1-D5* Melphalan 10mg/m^2	PD	202	81[a]	nm	nm
PT	40/M	DTIC 500mg/m^2 D1-D5* Melphalan 10mg/m^2	PR	21	9[a]	nm	nm
PT6≠	40/M	DTIC 800mg D1-D3*		18	6[a]	nm	nm
MW	52/F	DTIC 600mg/m^2 Interferon 10mU	PD	813	266[b]	nm	nm
SR	59/M	Temozolomide 150mg/m^2 D1-D5	PD	108	77[c]	nm	nm
RL	52/F	DTIC 400mg/m^2 Fote 100mg/m^2	PR	84	32[d]	150	114
GB	53/M	DTIC 400mg/m^2 Fote 100mg/m^2	PD	223	210[d]	217	194
MU	66/F	CB10-277 12gm/m^2	PD	65	7[e]	163	6
GM	20/F	CB10-277 12gm/m^2	PD	135	9[e]	250	43

* = D1-D5 represents days 1 to 5 and D1-D3 represents days 1 to 3

[a] = Biopsy performed on Day 2; [b] = Biopsy performed 14 h after DTIC; [c] = Biopsy performed 17 h after first oral dose of temozolomide; [d] = Biopsy performed 4 hrs after DTIC; [e] = Biopsy performed immediately after completion of CB10-277 infusion (see Section 6.2.2).

≠ PT had repeat sequential tumour biopsies at cycle 6 treatment.

nm not measured.

9.2.3 Western blotting

Crude extracts from 5 melanoma biopsies obtained above with a variety of ATase levels ranging from 108 to 476 fm/mg were used for western blotting and this revealed essentially a single staining band at 22 kDa, corresponding to the size of the pure recombinant human ATase (Fig. 9.3). There was a relationship between the ATase activity of melanoma extracts and the band intensity seen in western blots when quantitated by densitometric scanning (Table 9.3; R=0.99). This indicates that the anti-serum would be highly specific, sensitive and potentially quantitative when used as a diagnostic immunohistochemical tool to evaluate ATase activity in tumour sections.

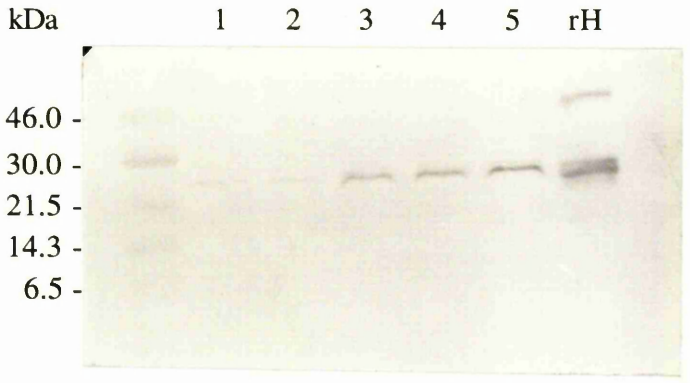


Figure 9.3. Western blot of crude sonicates of melanoma biopsies with increasing ATase activity (lanes 1-5) and recombinant human ATase (rH), probed with anti-human ATase serum. The positions of the molecular weight markers (kDa) are shown.

Table 9.3 Correlation in melanoma extracts between ATase activity and staining intensity of western blots

Patient[a]	ATase activity (fm/mg ± s.d.)	Scan intensity[b]
DG	476 ± 13.5	1.0
GT	345 ± 11.2	0.80
IC	307 ± 6.7	0.68
JH	126 ± 3.5	0.25
SR	108 ± 2.3	0.29

[a] = See Table 9.1 for patient characteristics.
[b] = Quantified by densitometric scanning and standardised to 1.0 based on DG.
s.d. = standard deviation.

9.2.4 Immunohistochemistry

With the preimmune serum, very faint staining was present in both the cytoplasm and nucleus of the melanoma sections (Plates 9.1C-9.5C). Also, in sections incubated with preadsorbed ATase antiserum, very little staining was seen, i.e. a picture indistinguishable from that obtained with pre-immune serum. The corresponding haematoxylin and eosin staining is shown in Plates 9.1A-9.5A.

With the immune serum, marked staining was seen in all the sections examined (Plates 9.1B-9.5B). Staining was seen as fine black granules mainly confined to the nucleus. In contrast to normal human liver or transgenic liver sections where ATase staining was much more homogeneous, intercellular variations in the intensity of the staining could be discerned in some of the melanoma sections, and in some sections this was very marked ranging from cells completely devoid of staining to cells with very intense staining (Plates 9.1, 9.3, 9.4 & 9.5). In some sections, virtually all the tumour cells stained homogeneously (Plate 9.2). Staining could also be discerned in the surrounding keratinocytes, endothelial cells, fibroblasts and smooth muscle cells and this was predominantly nuclear (Plate 9.2). Moreover, there was also regional heterogeneity of

ATase expression within the tumour tissue in some of the sections examined, some areas being almost devoid of staining and others being heavily stained (Plate 9.1).

Where information was available for ATase activity, staining intensity in tumour cells and clinical response to treatment involving O^6-alkylating agents, there appeared to be some relationship between these three parameters as depicted in Table 9.1. However the relationship may be complicated by variations in staining intensity between tumour cells, stromal cells and epidermal keratinocytes, regional variations of staining intensity and the tumour cellularity relative to stromal cells of the biopsied tumours. Some sections had sparsely populated islands of intensely staining tumour cells relative to the surrounding stroma cells whereas other sections showed very cellular tumour cells with few surrounding stroma cells. For examples, while the detemined ATase activity was 140 fm/mg protein in the biopsy obtained from patient HG, the tumour section showed very intense staining in epidermal keratinocytes with very light staining seen in the tumour cells and this may account for the partial response seen here. Conversely, intense staining was seen in some sections with relatively low ATase activity (patients RL, MU). The possibility that this reflects high levels of inactive ATase protein as reported by Karran et al. (1992) is unlikely in view of the correlation between westerns and assays. In general, apart from these exceptions, there is evidence of a relationship between the determined ATase activity, staining intensity and clinical response (see Table 9.1).

9.3 Discussion

Considerable variation in ATase activity was detected in the crude extracts of the pretreatment melanoma samples examined and this ranged from 8 to 813 fm/mg protein with a mean activity of 200 ± 178 fm/mg protein not significantly different from mean ATase activity observed in peripheral lymphocytes of patients treated with combined DTIC (Chapter 3) or CB10-277 (Chapter 6). In those patients who received treatment with the O^6-alkylating agents, there appeared to be a relationship between tumour ATase activity and clinical response. While 12 of the 14 patients with progresive

disease had tumour ATase activity greater than 140, only one of the four responding patients had activity exceeding this level. The mean ATase activity for responders and non-responders were 73 and 250 fm/mg protein respectively. If this observation is confirmed with a larger clinical study, the findings indicate that in the case with metastatic melanoma, tumour ATase activity may be an important determinant in predicting clinical response even though the comparison is complicated by the fact that many patients were treated with different O^6-alkylating agents which were either used alone or as combination.

The present study also demonstrates that the O^6-alkylating agents can lead to depletion of ATase activity in melanoma tissues confirming the earlier finding reported in Chapter 6 and suggesting that sufficient DNA methylation occurs in the tumour cells to inactivate ATase as a result of repair of the O^6-MeG generated in the tumour DNA. ATase depletion has also been reported with colonic tumours obtained by serial CT guided biopsy following treatment with streptozotocin (Wilson et al. 1992). The range of ATase activities in the melanoma skin metastases appears to fall within that seen with peripheral lymphocytes (Chapter 3) and in four patients, where paired tumour skin biopsies and blood samples were assayed, ATase depletion was seen in both tissues following chemotherapy (see Table 9.2). If this is confirmed with much larger patient numbers, it would suggest that DNA methylation occurs to a broadly similar extent in both tissue types. As discussed earlier, studies in the rat have shown a similar level of DNA methylation occurring in many tissues, including peripheral lymphocytes following administration of methylating agents, even many of those requiring metabolic activation (Kleihues et al. 1976b; Degan et al. 1988; Fong et al. 1990).

However, a disadvantage of measuring the ATase activity of the whole melanoma tissues as described above is that the result is a tissue average measurement and it takes no account of differences of ATase expression at the level of individual cells. In an attempt to address this issue, a rabbit anti-human ATase antiserum was used to examine a series of melanoma sections.

On western blots, the anti-serum was able to detect the ATase protein essentially as a single band in the crude extracts of a number of melanoma samples. The protein detected had an apparent molecular weight of 22 kDa, indistinguishable from that of recombinant human ATase protein. As reported elsewhere (Lee et al. 1992a), the antiserum also reacts with extracts of normal human liver and over-expressing Raji cells but not with extracts of non-expressing TK6 cells. These factors together with the correlation seen in western blots between the ATase levels and staining intensity indicate that these antibodies are highly specific.

The antiserum readily detected endogenous expression of the human ATase protein in human liver and transgenic liver sections as reported in Chapter 2 and elsewhere (Lee et al. 1992a). *In situ* detection of ATase protein was also seen in the melanoma sections examined. The staining appeared to be located predominantly over the nucleus although some cytoplasmic staining was detected in some samples. In some of the melanomas, there were marked intercellular differences in the staining of the melanoma cells suggesting cellular heterogeneity of ATase expression.

The major significance of the finding of heterogeneity of cellular ATase expression is that if ATase is the principal mechanism of resistance to the toxic effects of O^6-alkylating agents (D'Incalci et al. 1988; Margison and O'Connor 1990; Pegg 1990), it might be predicted that tumour tissue with very high proportion of ATase-expressing cells would be intrinsically resistant to DTIC and related agents, whilst tumour tissue with the majority of cells expressing low ATase activity but containing some ATase-expressing cells might initially respond but eventually acquire drug-resistance because of re-emergence of the latter cells. This selection of minor populations of pre-existing tumour cells expressing high levels of ATase was recently demonstrated in the regrowth of ATase-deficient xenograft tumours after a prolonged growth delay following treatment with BCNU (Phillips et al. 1994). A selective outgrowth of the resistant ATase-positive tumour cells may also explain the phenomenon seen in clinics

in many of the patients with metastatic melanoma who relapse after responding initially to DTIC or CNU treatments (Clark 1976).

Regional heterogeneity of ATase expression was also seen in some of the sections examined and this may explain some of the heterogeneous responses seen in tumour cell lines established from a single tumour when treated with anti-tumour agents (Yung et al. 1982). For example, studies of glioma cell lines established from tumour material demonstrated considerable heterogeneity in sensitivities to nitrosoureas (Rosenblum et al. 1983; Thomas et al. 1989) and clones from biopsies of different areas within the tumour displayed differing chemosensitivities (Yung et al. 1982; Shapiro et al. 1984). Moreover, it was found that most of the glioma cell lines established from post treatment tumour material were found to be BCNU-resistant, karyotypically and cytogenetically homogeneous and were identical to a minor subpopulation of cells obtained in the pre-treatment tumour sample (Shapiro & Shapiro 1992). As seen with the tumour xenograft models described earlier, this may be due to selection of the surviving ATase-expressing tumour cells.

Heterogeneity of cellular expression of ATase in tumour tissues may also explain the considerable number of tumour cell lines which were found to lack ATase activity (termed Mer^- or Mex^-) in contrast to the incidence reported for human tumours including those of malignant melanoma as seen here and elsewhere (Maynard et al. 1989). It is tempting to speculate that the origin of this ATase-deficient phenotype seen in 20-30% of tumour cell lines (Babich and Day 1989; Pegg 1990) is due to the selection of ATase-deficient cells from heterogeneous population because culture conditions favour the growth of this subpopulation. The upregulation of ATase expression seen following selection of ATase-deficient cell lines by treatment with methylating or chloroethylating agents *in vitro* (Morton et al. 1988, 1992) is also likely to due to selective growth advantage of the ATase-proficient cells whilst the majority of the ATase-deficient population is killed.

Where staining intensity, ATase activity and clinical response data were available, there appeared to be some relationship between these three parameters as depicted in Table 9.1. It must be emphasized that this correlation was based on very few patients and in which different O^6-alkylating drugs alone or in combination were administered. This interpretation may also be complicated by the potential presence of immunoreactive but inactive ATase protein, the presence of significant ATase expression in stromal cells relative to tumour cells or the presence of marked variation in regional distribution of ATase expressing cells as described earlier in the results section. Nevertheless, if this general correlation is confirmed in a large clinical study, this IHC method would provide us with a useful clinical tool in predicting individuals who are likely to response to O^6-alkylating agents. In addition, where tumour biopsies reveal high ATase activity, it may be possible to deplete the endogenous ATase activity with agents such as O^6-benzylguanine prior to treatment with O^6-alkylating drugs. An advantage of such IHC method is that not only it allows a cellular evaluation of tumour ATase expression but it is also able to take into consideration the cellularity of the tumour sections, variations in staining intensity between tumour and non-tumorous cells, regional variations in staining intensity and inter-cellular variations of ATase staining all of which theoretically can given rise to artificially high or low ATase activity as determined by an *in vitro* assay.

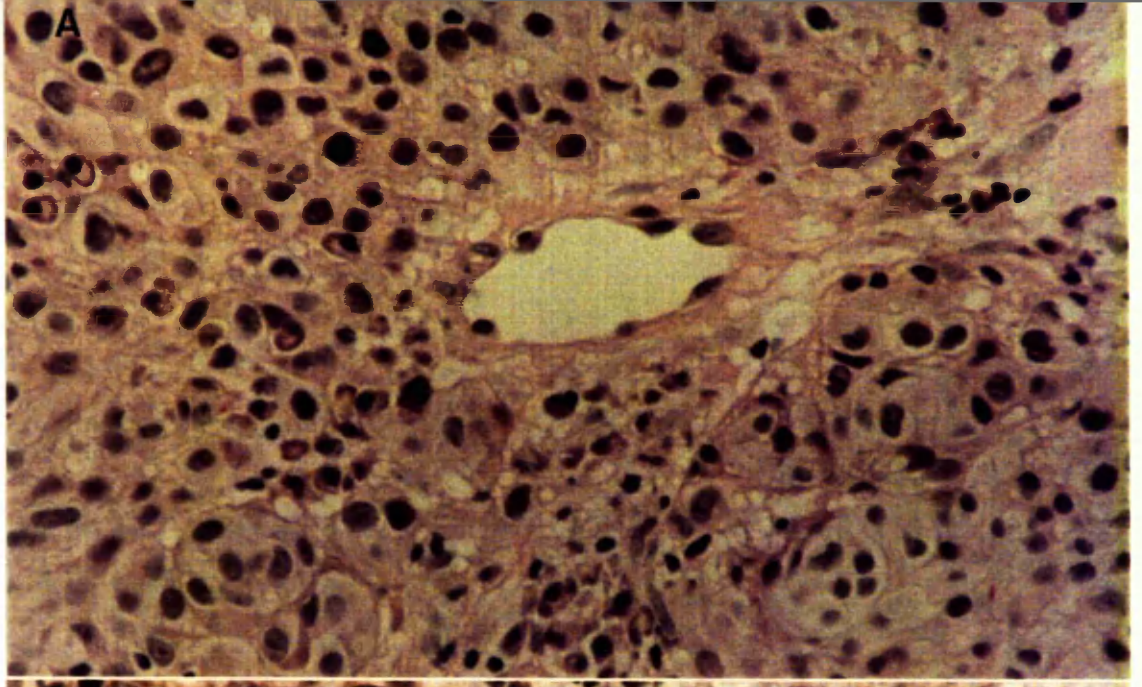
Since the findings reported here and published elsewhere (Lee et al., 1992a,b), another report examining a series of rhabdomyosarcoma xenografts recently demonstrated a good relationship between the ATase staining intensity using a similar IHC method and the ATase activity (Brent et al. 1993). The authors also demonstrated the absence of staining in ATase-deficient xenografts. In addition, other studies were able to show a good quantitative relation between the amount of immunoreactive ATase protein and ATase activity (Ostrowski et al. 1991; Pegg et al. 1991b; Zhukovskaya et al. 1992). In another study, human ATase-expressing cells grown as monolayers displayed nuclear specific staining whereas ATase-deficient cells stained very faintly (Ayi et al. 1992). More recently *in situ* hybridization of ATase mRNA with an antisense

oligodeoxynucleotide probe has been used to detect ATase expression in normal human liver and foetal kidney sections (Wani et al. 1992, 1993).

With the availabilty of the anti-human ATase antibody and related methods, it is now feasible to examine in a larger clinical studies whether or not there is a correlation between ATase levels in melanoma or other tumour extracts, the number and intensity of positively staining cells, response to O^6-alkylating agents and the frequency of intrinsic and acquired drug resistance.

Plates 9.1 to 9.5

Sections of human malignant melanoma: A, haematoxylin and eosin; B, immunostaining with primary anti-ATase antibody showing mainly heterogenous nuclear staining. Intercellular variation in intensity of the staining can be discerned, ranging from cells completely devoid of staining to cells with very intense staining. Cytoplasmic staining is present in some tumour cells in Plate 9.1B. Regional heterogeneity of ATase expression within the tumour tissue can also be seen in Plate 9.1B. In addition, Plate 9.2B shows strong cytoplasmic and nuclear staining of epidermal keratinocytes. The immunostaining with pre-immune serum shows only faint nuclear and cytoplasmic staining (C). Magnifications x 430 (Plate 9.1), x 550 (Plates 9.2 & 9.4) and x 680 (Plates 9.3 & 9.5).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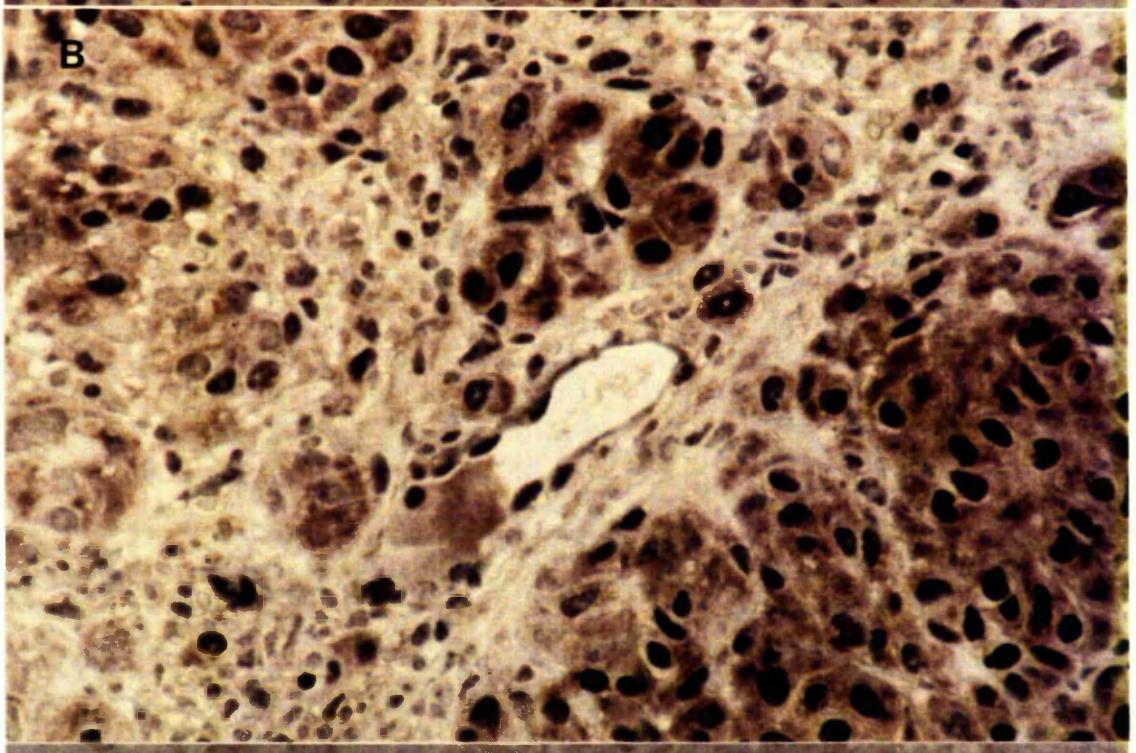
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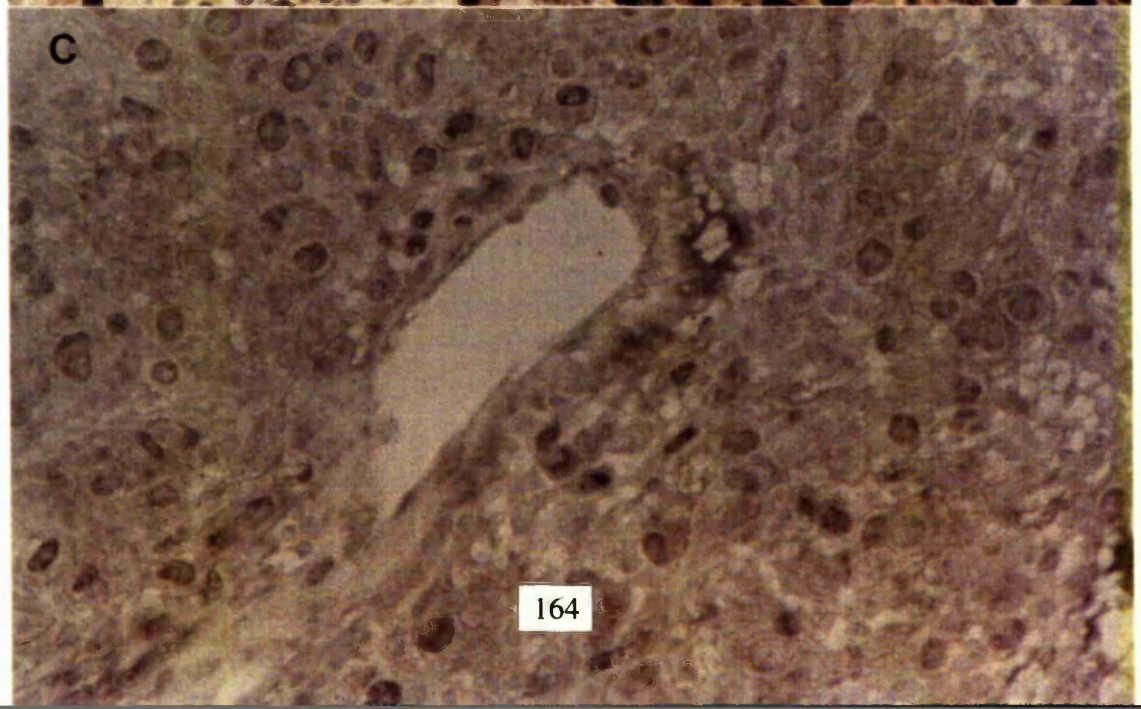
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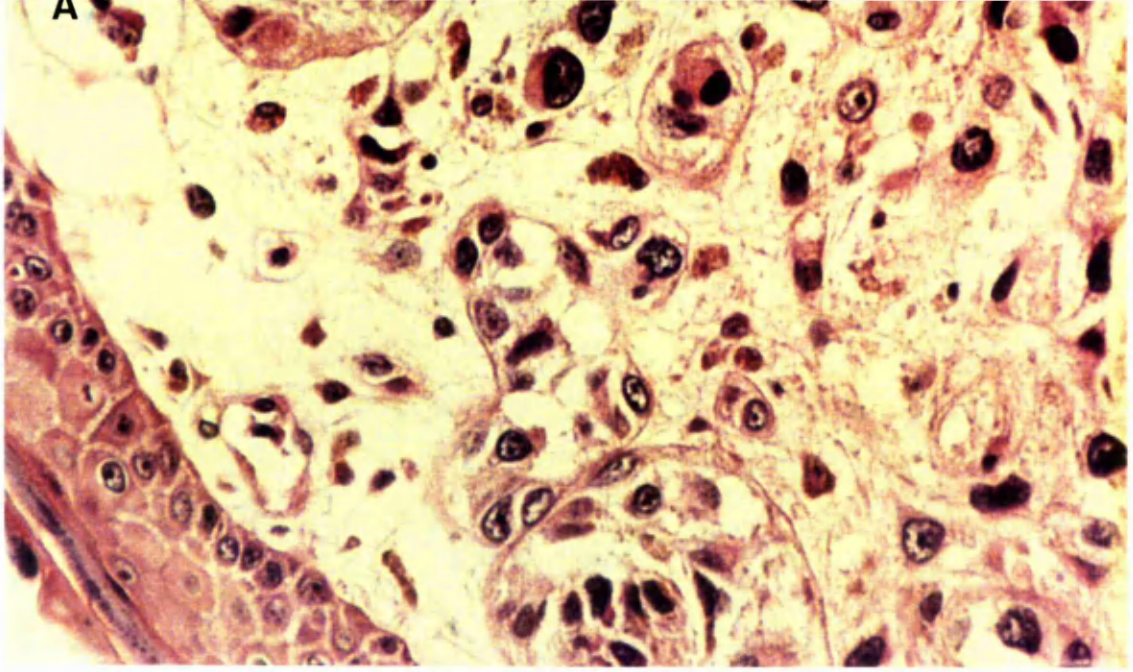
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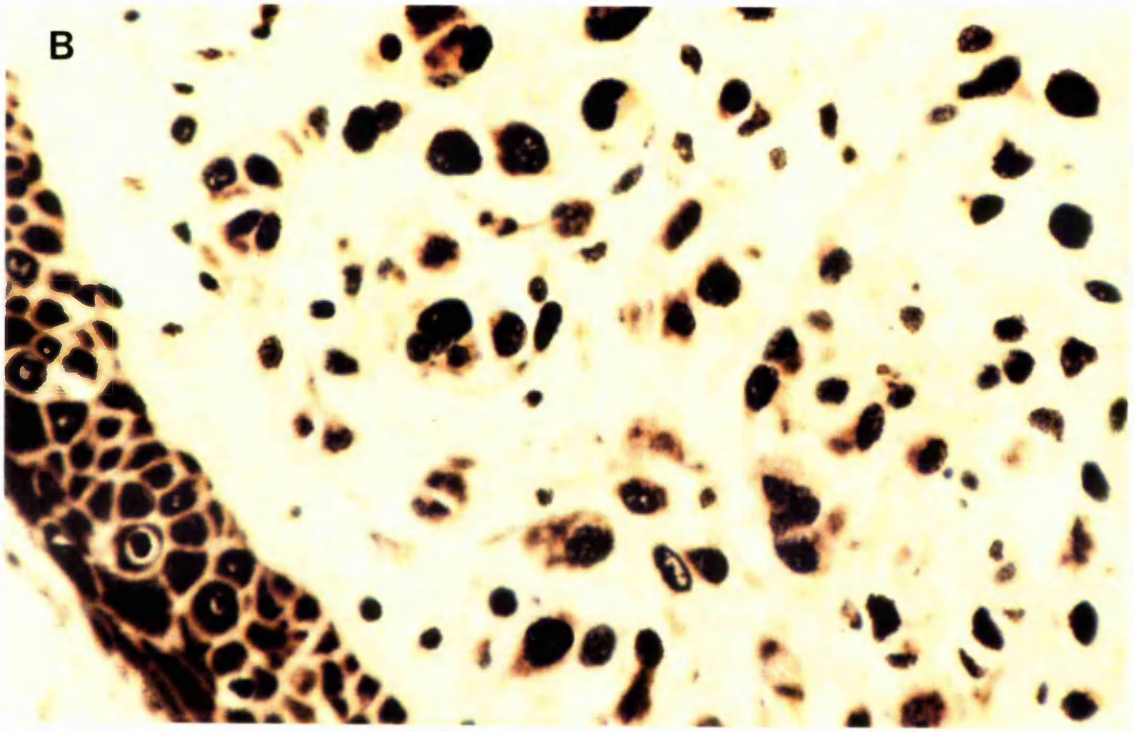
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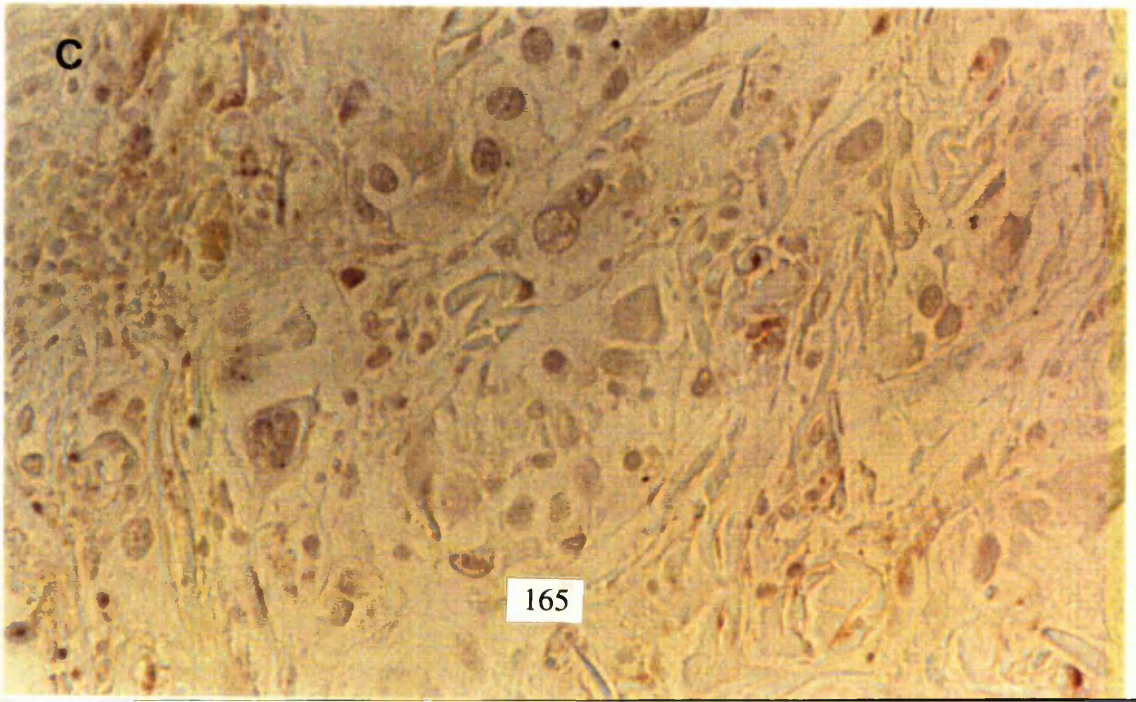
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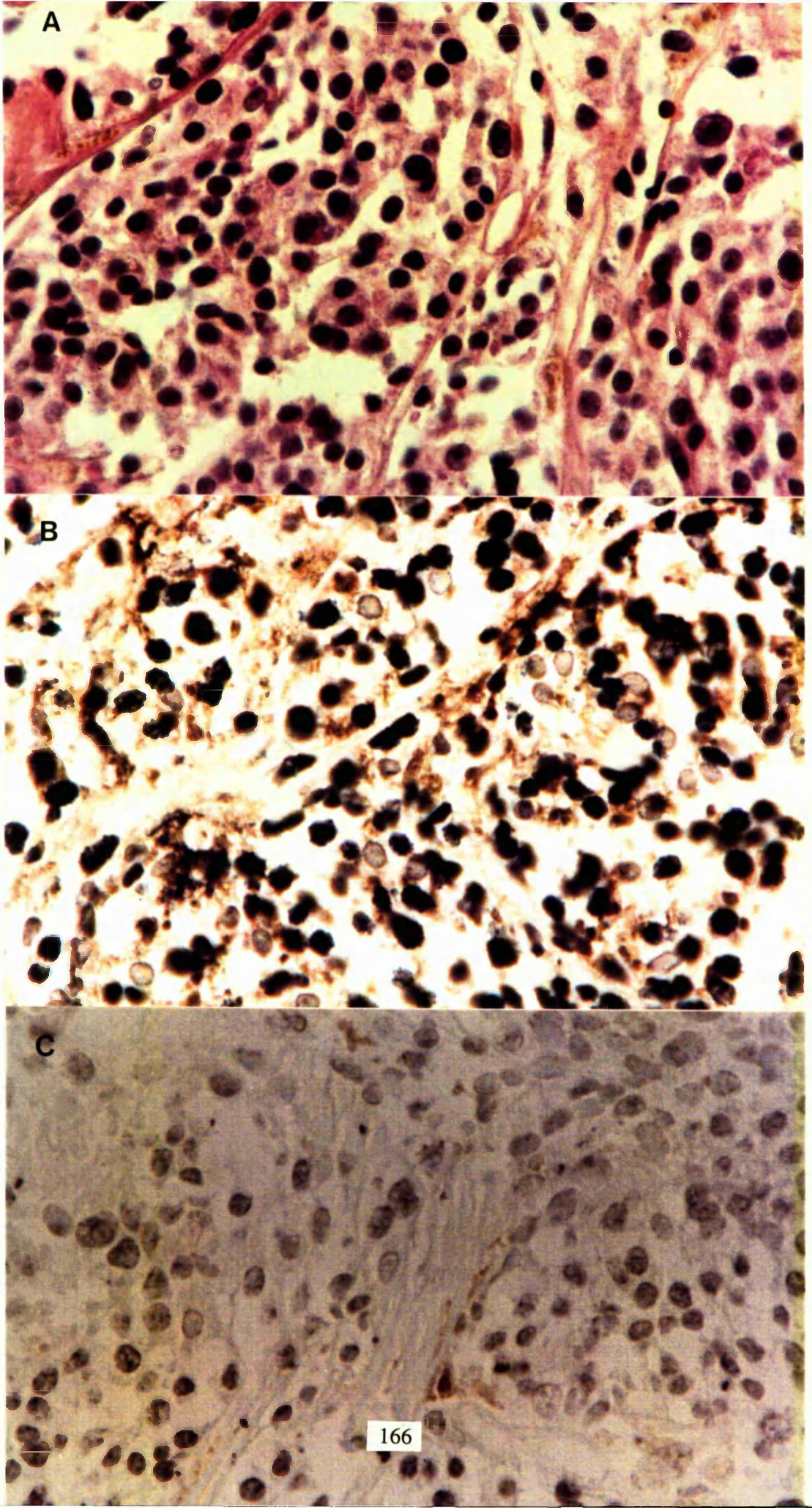
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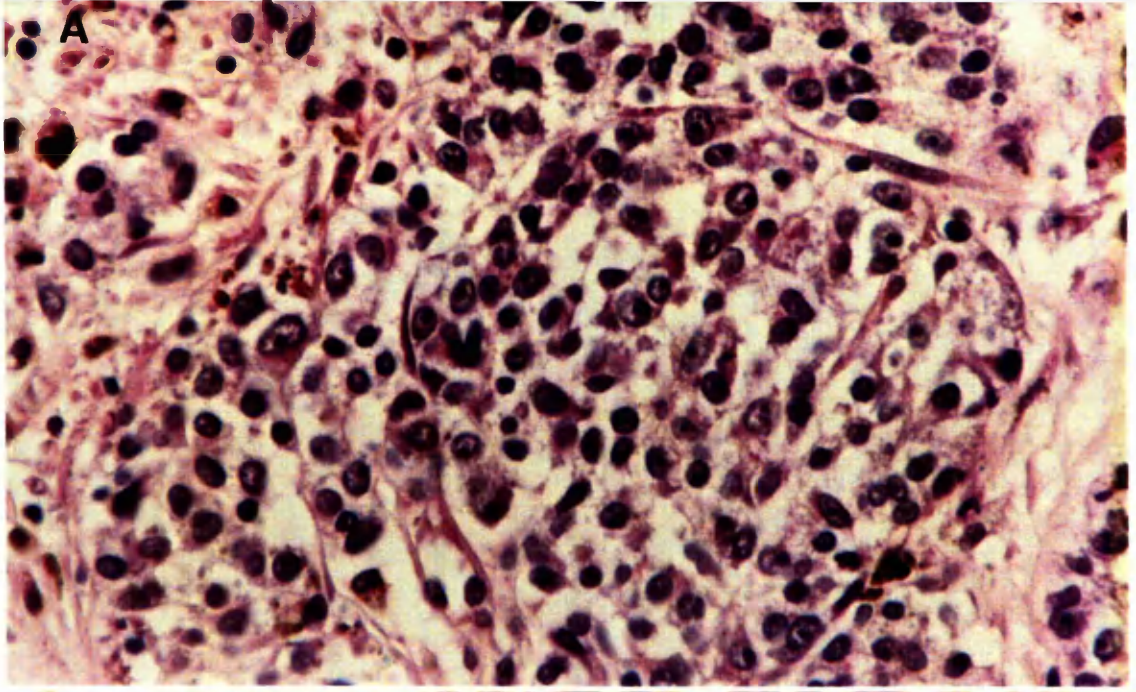
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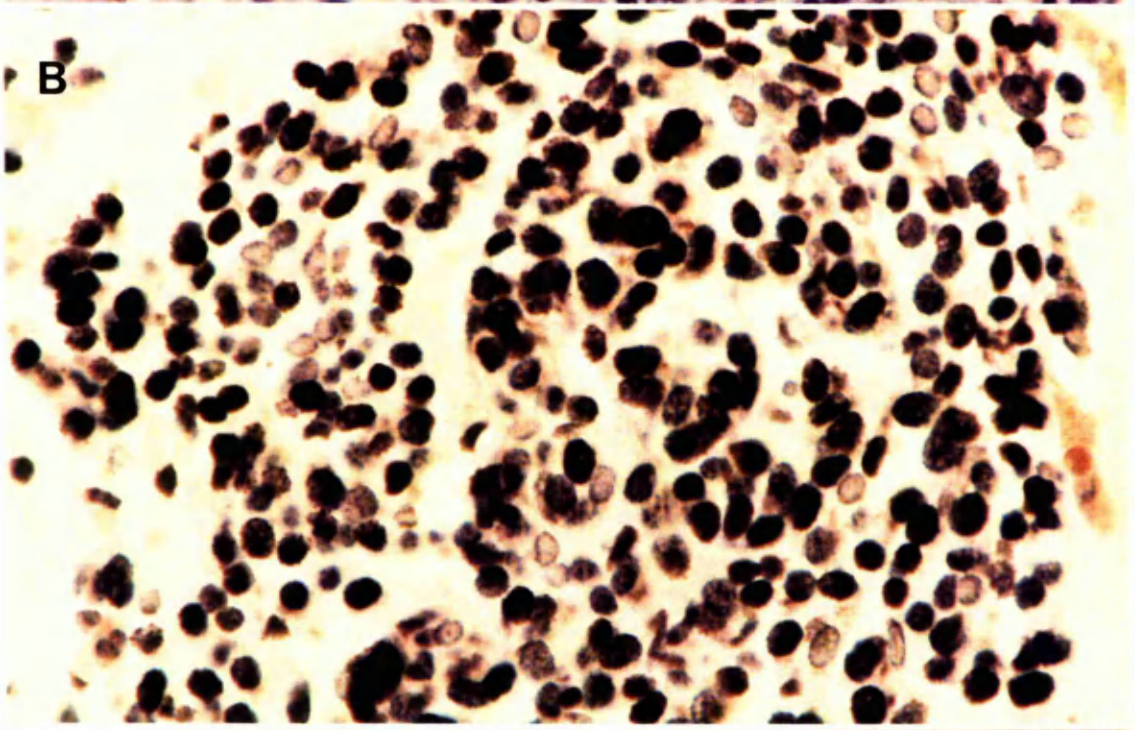
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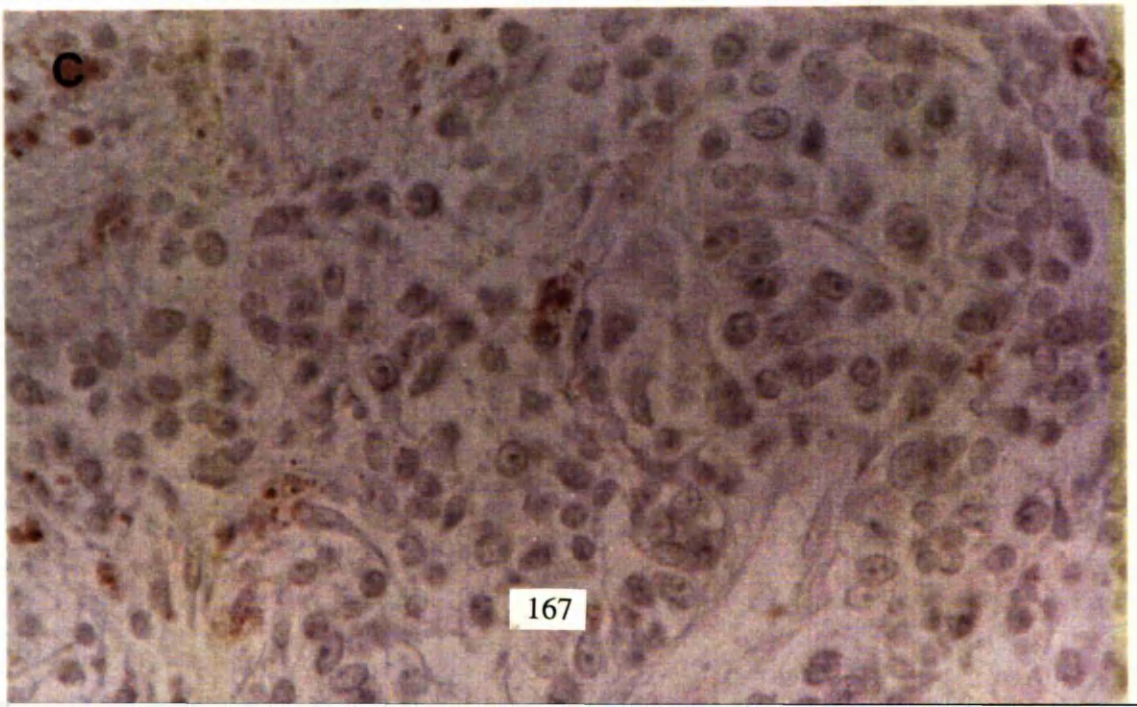
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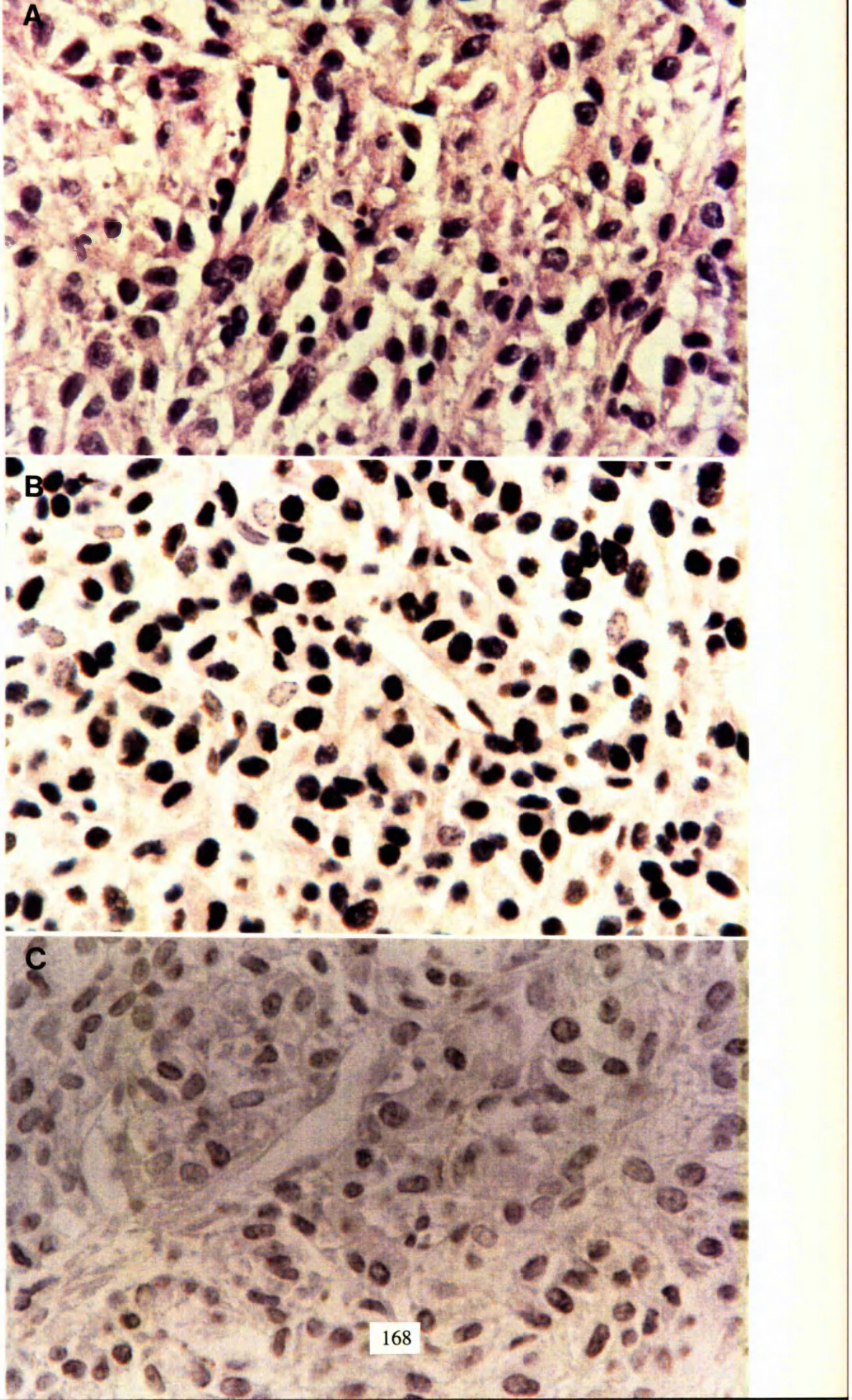
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Chapter 10 Activity and Cellular Distribution of O^6-Alkylguanine-DNA-Alkyltransferase in Ovarian and Hodgkin's Tumours

10.0 Introduction

In advanced ovarian cancer, the response rate following treatments with CNUs, DTIC and procarbazine is low in comparison with the mustard-type alkylating agents. Published reports of more than 1,000 patients treated with either melphalan, chlorambucil, thiotepa or cyclophosphamide have produced objective response rates of 35%-65% compared to less than 6% response with CNUs (Young et al.1974). This is in contrast to Hodgkin's disease where single agent therapy with the O^6-alkylating drugs BCNU, CCNU, procarbazine or DTIC achieves a response rate of 50-70% (DeVita and Hellman 1982).

Since the cellular level of ATase is a major factor in the resistance of tumour cells to the O^6-alkylating drugs (Chapter 1; Section 1.2.2), it is not unreasonable to speculate that one possible reason for the increased sensitivity of Hodgkin's tumour in contrast to ovarian cancer may be related to the cellular expression of ATase. In an attempt to address this issue, a series of ovarian and Hodgkin's tumours was examined immunohistochemically for ATase staining using the previously described ATase antiserum. The study also examined the relationship between ATase activity and western blot staining in ovarian tumours.

10.1 Materials and Methods

Fresh surgical material collected from hospitals across the North West of England was fixed in formal saline overnight and wax embedded. Diagnostic histopathology was performed on sections prepared by standard techniques from paraffin-embedded material. In addition, ten ovarian tumours were obtained at staging and second look laparotomies and samples were snap-frozen in liquid nitrogen and stored at -70°C prior

to ATase assay: 5 samples were also used for western analysis. The characteristics of the ovarian tumours studied are shown in Table 10.1. Patients received 6 cycles of intensive combination chemotherapy comprising of carboplatin/cyclophosphamide alternating with ifosphamide and adriamycin. Post-treatment samples were obtained from patients with residual or relapsed disease. The 3 samples of Hodgkin's disease were all of the nodular sclerosis type (Table 10.1).

Table 10.1 Characteristics of ovarian and Hodgkin's tumours examined

Patient	Age	Treatment sample[a]	Histology[b]	Diff[c]	Stage[d]	ATase (fm/mg)
1**	69	pre	serous	mod	3	422
2**	68	pre	mucinous	mod	3	118
3**	52	pre	serous	mod	3	20
4*	59	pre	endometroid	mod	3	850
5*	64	pre	serous	mod	3	785
6*	69	pre	mucinous	mod	3	39
7	72	pre	endometroid	well	3	nm
8	28	pre	serous	mod	3	nm
9	65	pre	undifferentiated	poor	1	nm
10	64	pre	endometroid	poor	3	nm
11	31	pre	mucinous	mod	3	nm
12	40	pre	serous	mod	3	nm
13	53	pre	serous	poor	3	nm
14**	65	post	mucinous	poor	3	366
15**	59	post	serous	poor	3	91
16	38	post	undifferentiated	poor	3	nm
17*	42	post	endometroid	poor	3	51
18*	73	post	serous	mod	3	89
19	22	pre	NS	-	2A	nm
20	44	pre	NS	-	3A	nm
21	43	pre	NS	-	3A	nm

[a] = prechemotherapy or second-look (postchemotherapy) sample.

[b] = histological types of epithelial ovarian cancer. NS=nodular sclerosing Hodgkin's disease.

[c] = histological differentiation; mod=moderate.

[d] = FIGO staging for ovarian cancer or Ann Arbor staging for Hodgkin's disease.

* = extracts assayed for ATase activity and subjected to western analysis (**).

nm = not measured.

10.2 Results.

10.2.1 ATase activity in ovarian tissues

There was a considerable variation in ATase activity among the ovarian tumours ranging from 25 to 850 fm/mg protein with a mean activity of 287 ± 314 fm/mg. As seen with melanoma biopsies, a good correlation was seen between ATase activity expressed either as fm/mg protein or fm/μg DNA measured on the same tumour tissue (R=0.91, $P<0.001$) (Fig. 40) indicating that cellularity or size of tumour cells is not an important factor in determining ATase activity in ovarian tumours.

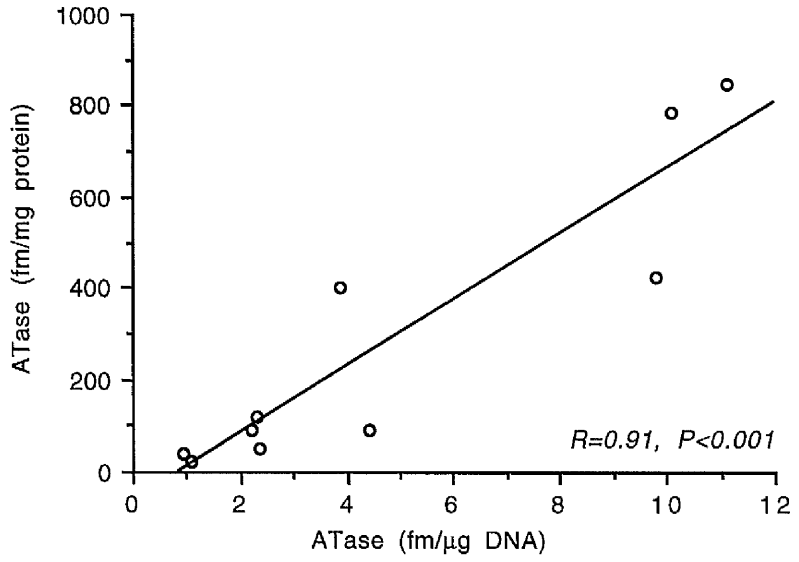


Figure 10.1. Relationship between ATase activity expressed as fm/mg protein and fm/μg DNA.

10.2.2 Western Blotting

Crude extracts from 5 ovarian tumours with a variety of ATase levels ranging from 20 fm/mg to 450 fm/mg (Table 10.2) were used for western blotting and as previously seen with melanoma extracts, this revealed essentially a single staining band at 22kDa, corresponding to the size of the pure recombinant human ATase (Fig. 10.2). In order to exclude the possibility that some of the ATase protein in ovarian tumours may be inactive protein but IHC reactive, the relative intensity of these bands were quantitated by densitometry scanning (Table 10.2); there was a linear correlation between the ATase activity in tumour extracts and the intensity of staining on the western blots

(R=0.99, P<0.001) again indicating that this anti-peptide is very specific and should prove sensitive enough for evaluating ATase expression in ovarian tumour and probably Hodgkin's samples using the immunohistochemical method described previously in Chapter 9.

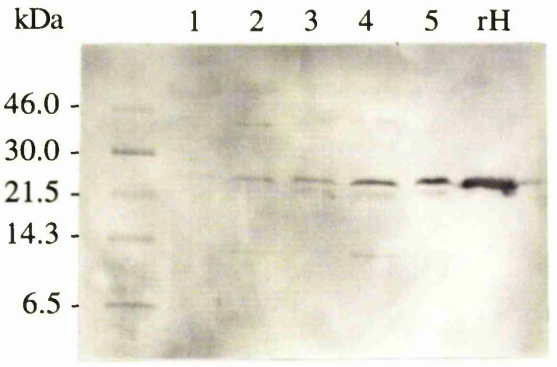


Figure 10.2. Western blot of crude sonicates of ovarian tumours with increasing ATase activity (lanes 1-5) and recombinant human ATase (rH) and probed with anti-human ATase serum. The positions of the molecular weight markers (kDa) are shown.

Table 10.2 Correlation in ovarian tumours between ATase activity and staining intensity of western blots

Ovarian Tumour[a]	ATase activity (fm/mg ± s.d.)	Scan intensity[b]
1[b]	422 ± 32.7	1.0
14	366 ± 7.0	0.76
2	118 ± 1.2	0.22
15	91 ± 2.7	0.122
3	20 ± 0.7	0.04

[a] = See Table 10.1 for tumour characteristics.

[b] = Quantified by densitometric scanning and standardised to 1.0 based on tumour no. 1.

s.d. = standard deviation.

10.2.3 Immunohistochemistry

With the preimmune serum, very faint staining was seen in both the cytoplasm and nucleus of the ovarian and Hodgkin's sections (Plates 10.1C-10.4C)

In the ovarian tumours, staining with the ATase antiserum was seen in all the 18 cases examined. Similar to the previous findings with melanoma sections, staining was seen as fine black granules mainly confined within the nucleus and present in most tumour cells (Plates 10.1B and 10.2B). Some intercellular variation in intensity of cellular staining was again observed indicating heterogeneity of cellular expression but unlike that observed with melanoma sections, little difference in regional distribution of positively staining ATase cells could be discerned. In some of the sections, where adjacent non-tumorous cells could be identified, staining was seen in the supporting stromal fibroblasts, endothelial cells and adipocytes, principally in the nuclei (Plate 10.2B). The extent to which staining was quantitatively related to ATase levels in tissue extracts was also assessed: staining intensity appeared to be less in two sections with low ATase levels (Table 1, tumour no. 3 & 6) in comparison to two sections with high ATase activity (Table 1, tumour no. 4 & 5). However, staining intensity did not appear to correlate with ATase levels in those extracts with intermediate levels. Indeed, in one of the sections with low ATase activity (tumour no. 6), haematoxylin and eosin staining revealed mainly fibrous tissue sparsely populated with tumour cells, the latter nevertheless still stained positively for ATase.

Relative to the ovarian cancers, staining in the Hodgkin's disease specimens was significantly less in intensity (Plates 10.3B and 10.4B). Reed-Sternberg cells of lacunar type were identified in all three biopsies and showed weak cytoplasmic staining and variable, weak nuclear staining. Cytoplasmic staining of similar intensity was also discerned in the surrounding 'reactive' lymphocytes (Plates 10.3B and 10.4B).

10.3 Discussion

The mean ATase activity in ovarian tumours before chemotherapy (372 ± 374 fm/mg) appears to be higher than that determined for malignant melanoma (200 ± 178 fm/mg). Similar to the findings seen in melanoma tumours, the correlation seen in western blots between ATase levels and staining intensity indicates that this ATase antiserum is highly specific and should be sensitive enough to be used as an IHC tool to evaluate ATase expression in ovarian tumours and the possibility that increased staining due to inactive ATase protein as reported by Karran et al. (1992) is unlikely to be a major problem in this particular tumour type.

The polyclonal antibodies readily detected endogeneous expression of ATase protein in the ovarian sections examined by IHC. Strongly positive staining was seen in all the 18 ovarian tumours examined. Staining was essentially confined to the nucleus and where cytoplasmic could be discerned it was very faint when compared to the nuclear staining. In contrast, in the Hodgkin's sections examined, staining was very much less intense and was mainly confined to the cytoplasm of both Reed-Sternberg cells and surrounding reactive 'lymphocytes'. These findings suggest that a possible reason for the low response rate of ovarian cancer observed in the clinics to the O^6-alkylating agents (Young et al. 1974) is a consequence of high levels of ATase expression in the tumour cells. Conversely, the increased sensitivity of Hodgkin's disease to O^6-alkylating agents may be due to low ATase expression.

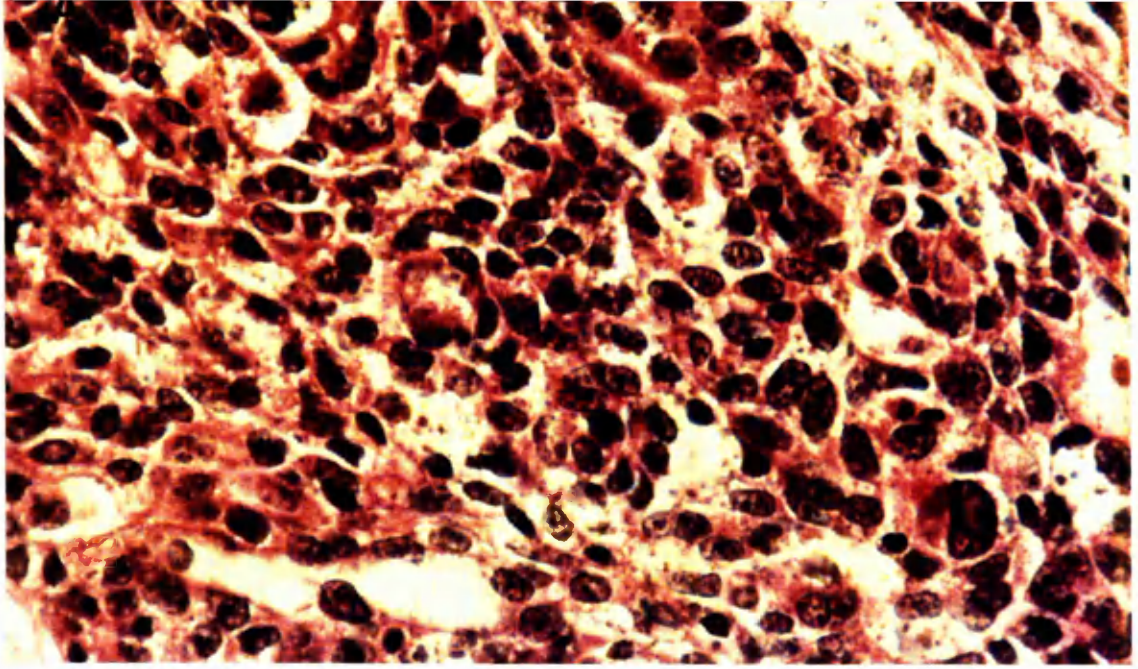
It is interesting to note that ATase protein was found to be located in the cytoplasm of many of the malignant Reed-Sternberg cells, away from its site of action in the cell nucleus and this could be an additional factor contributing to the increased sensitivity of Hodgkin's disease to O^6-alkylating agents. In support of this, immunohistochemical studies on mammalian cells (NIH-3T3) expressing a bacterial ATase gene indicated that the protein was predominantly cytoplasmic and that these cells were only slightly more resistant to BCNU than the control cells, despite a 15-fold rise in total ATase activity quantitated by an *in vitro* assay (Dumenco et al. 1989). This suggests that the

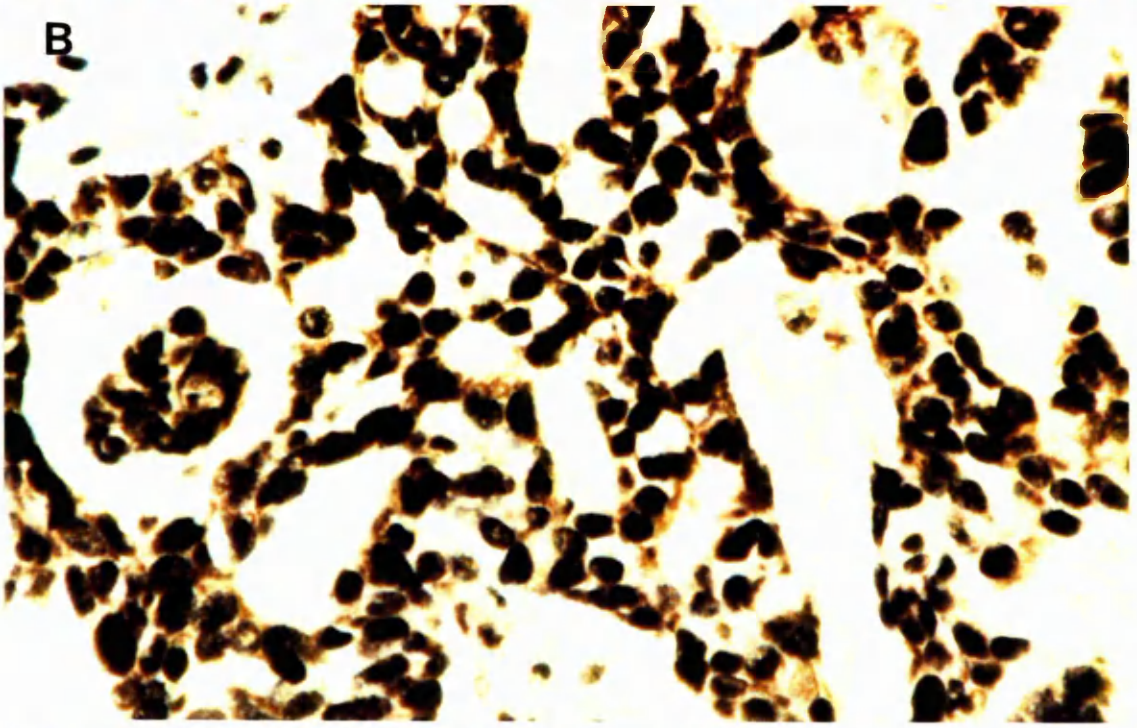
cytoplasmic protein may not be fully functional in the cell. It is possible that in Hodgkin's disease, these malignant cells may exhibit a defective cytoplasmic nuclear transport mechanism which normally transports the ATase protein synthesized in the cytoplasm into the nucleus.

The findings reported here and previously in Chapter 9 with melanoma sections establish that the anti-human ATase anti-serum might allow the individual identification of resistant tumours and hence the design of individualised treatment protocols, including resistance modifiers where necessary, to be of maximum therapeutic benefit to the patients. As mentioned above, it is therefore important to explore prospectively whether a relationship exists between ATase levels in tumours detected by quantitative immunohistochemistry, tumour response to O^6-alkylating drugs, frequency of intrinsic or acquired drug resistance and survival, particularly in Hodgkin's disease where dacarbazine, procarbazine and BCNU regularly form part of combination chemotherapy. In addition, since archival material is available, it will be worthwhile to examine previously treated Hodgkin's disease for ATase expression and to see whether or not this correlates with response to chemotherapy and patient survival.

Plates 10.1 & 10.2

Sections of poorly differentiated adenocarcinoma of ovary: A, haematoxylin and eosin; B, immunostained with polyclonal human anti-ATase antibody showing strong staining of tumour cell nuclei. Note that stromal fibroblasts also exhibit nuclear staining (arrows, Plate 10.2B). Immunostaining with pre-immune serum shows only faint nuclear and cytoplasmic staining (C). Magnifications x 550 (Plate 10.1) and x 430 (Plate 10.2).

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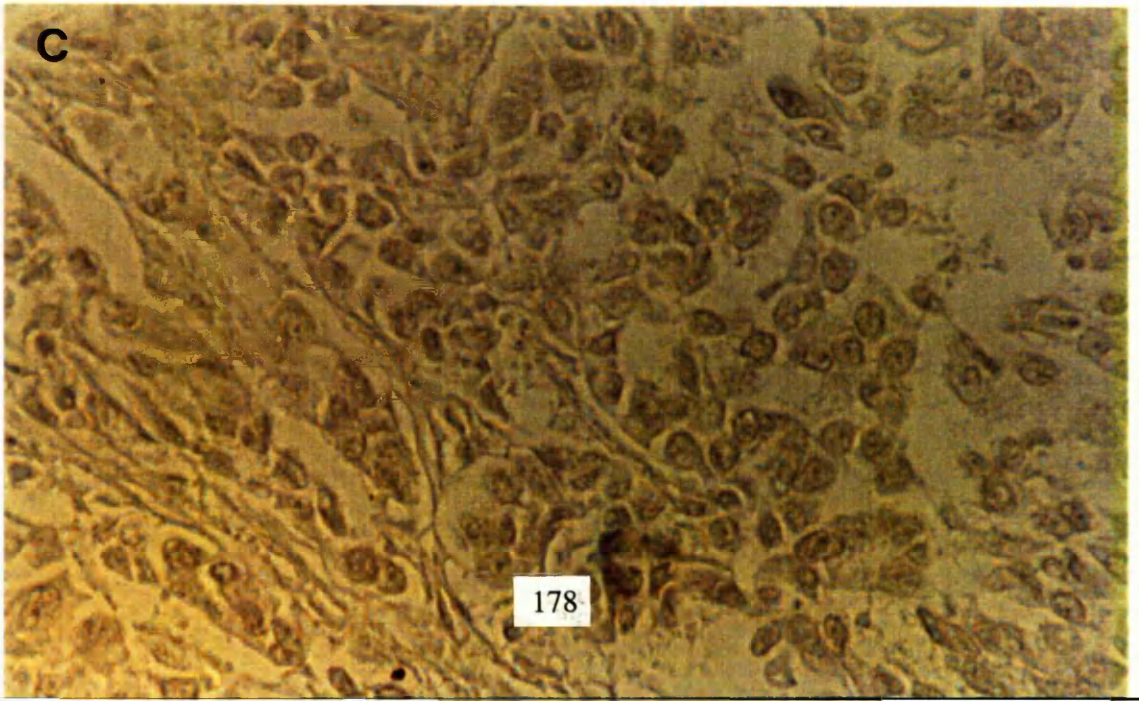
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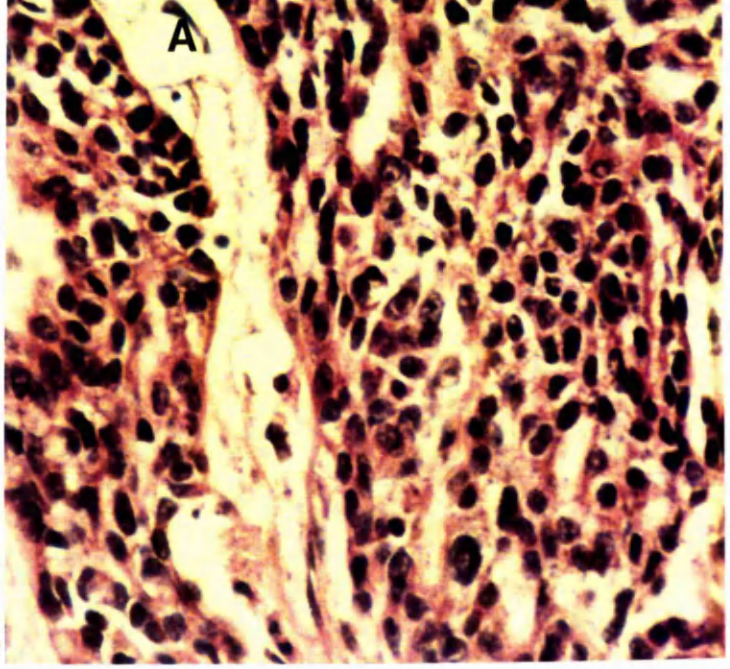
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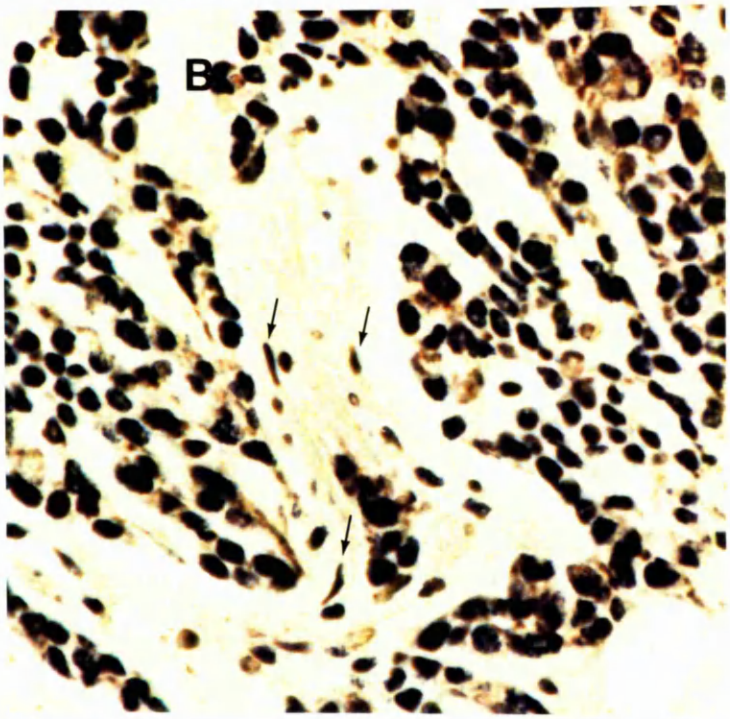
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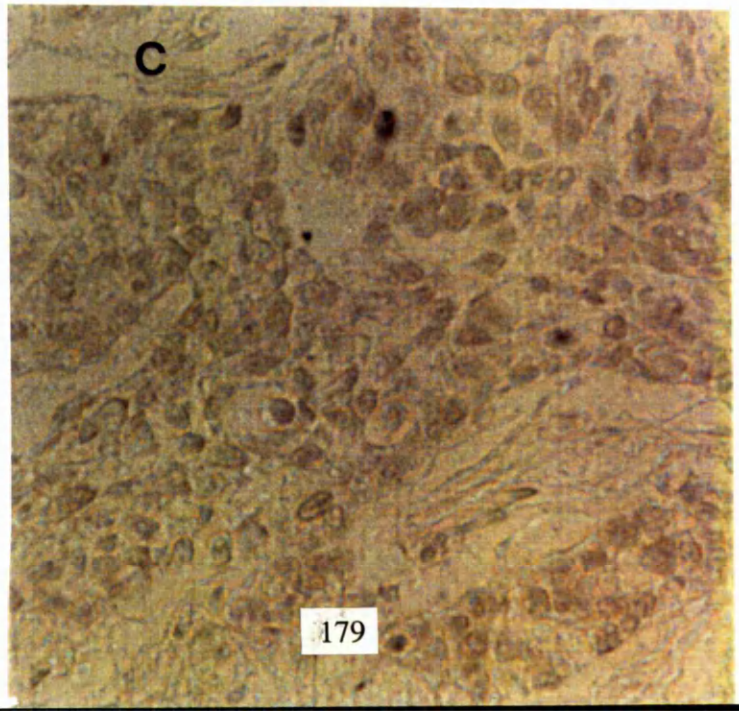
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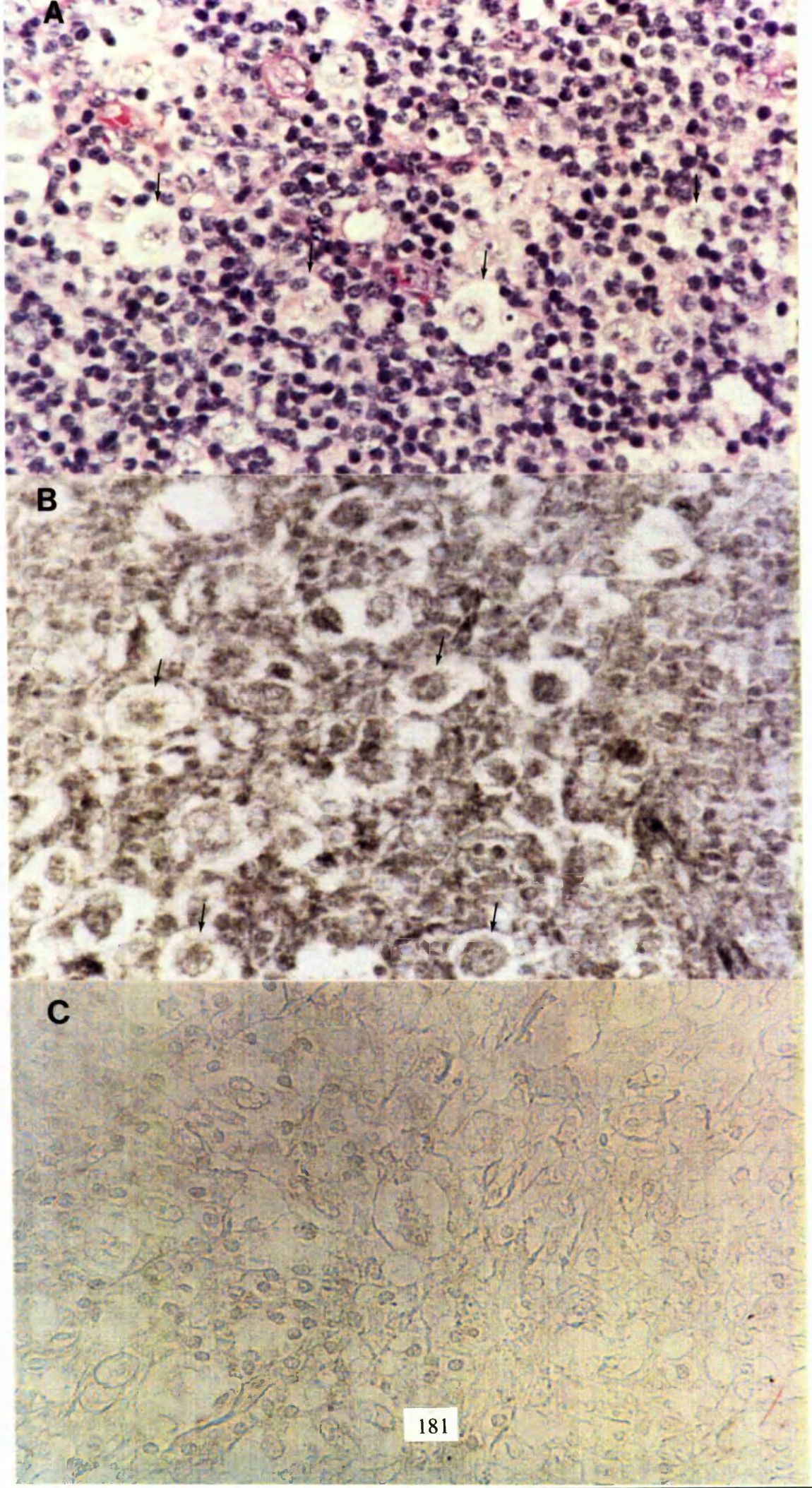
Plates 10.3 & 10.4

Sections of Nodular sclerosing Hodgkin's disease: A, haematoxylin and eosin. Lacunar-type Reed-Sternberg cells are plentiful (arrows) in a background population of small lymphocytes; B, immunostaining with polyclonal human anti-ATase antibody showing weak staining in the nuclei and cytoplasm of the lacunar Reed-Sternberg cells (arrows, Plate 10.3B) and moderate cytoplasmic staining with negative nuclei (arrows, Plate 10.4B). Weak staining can be discerned in surrounding lymphocytes; C, immunostaining with pre-immune serum showing weak non-specific staining present in the small lymphocytes (C). Magnification x 430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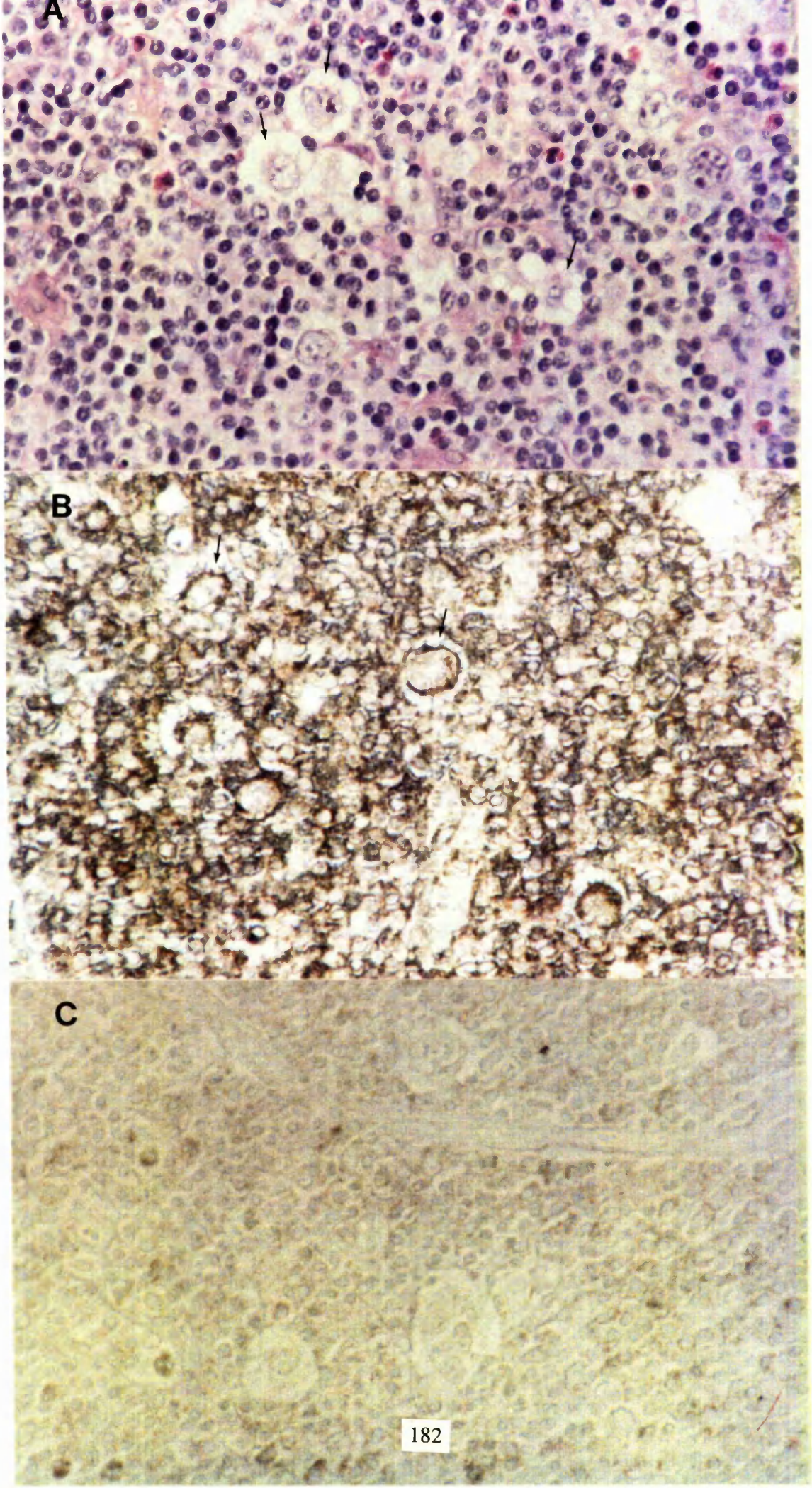
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The present studies demonstrated that the O^6-alkylating agents DTIC, CB10-277, temozolomide and BCNU were able to deplete ATase activity in peripheral blood lymphocytes of patients and a wide inter-individual variations in the depletion and regeneration was noted.

With DTIC, a dosage and cycle dependent depletion of ATase was observed with nadir ATase activity occurring at approximately 4 h. In support of this finding, a recent report using sequential administration of DTIC and TCNU in xenografts demonstrated that maximal depletion of ATase activity also occurred at 4 h after DTIC (100 mg/kg) in both tumour and normal tissues including liver, lung, gut and bone marrow and no differences in ATase depletion between tumour and normal tissues were seen (Matthew et al. 1994). Moreover, these authors observed that higher dosage DTIC (200 mg/kg) produced a similar but a more protracted ATase depletion in the tumour xenografts which was followed by ATase recovery and this supports the suggestion that high-dose DTIC may be indeed limited by a saturation in DTIC metabolic activation as was seen in the peripheral blood lymphocytes of patients receiving high dose DTIC (see Chapter 3). This report (Matthew et al. 1994) together with the findings reported in Chapter 3 suggest that it would be worthwhile to examine whether repetitive administration of DTIC every 4 h may achieve further ATase depletion and whether this might be associated with increased antitumour activity: since temozolomide was shown to cause cumulative ATase depletion in peripheral blood lymphocytes of patients receiving daily oral treatment, a more extensive ATase depletion would be likely to occur with a repetitive 4 h treatment schedule rather than a schedule employing a single bolus treatment. In addition, it would be interesting to explore whether continuous DTIC administration could also achieve more extensive ATase depletion particularly in light of the findings in patients receiving 24 h continuous infusion of CB10-277 where complete ablation of lymphocyte ATase was seen in some cases. Clearly much more

work still needs to be done in order to identify the optimal DTIC schedule capable of achieving maximal ATase depletion.

Because of bed shortages and ease of administration, there is now an increasing tendency among oncologists to treat metastatic melanoma with a single bolus DTIC instead of a daily DTIC schedule. If ATase is the principal mechanism of resistance to O^6-alkylating agents, the findings reported here and in xenograft models suggest that a single bolus DTIC schedule may be associated with potentially reduced response rate particularly when this is associated with lower doses of DTIC. It has been demonstrated in experimental models that fractionated doses of DTIC and temozolomide are more effective than a single administration and this may be related to more extensive ATase depletion (Connors et al. 1976; Stevens et al. 1987). Since the mean ATase activity determined in melanoma tissues (see Chapter 9) was not substantially different from that seen in peripheral blood lymphocytes of patients treated with combined DTIC/fotemustine or CB10-277, it is not unreasonable to postulate that a broadly similar level of ATase depletion would generally occur in both tumour and lymphocyte tissues (see Chapter 9). In this particular situation therefore, determining changes in lymphocyte ATase activity may be a useful surrogate marker for predicting likely changes in ATase activity in tumour tissues.

In the CB10-277 studies, haematological toxicity developed in two patients with the lowest peripheral lymphocyte ATase activity (<50 fm/mg protein) and since studies here (Chapter 6) and elsewhere (Gerson et al. 1985) comparing lymphocyte and bone marrow ATase activitity, showed significant correlation between the two tissues, it is tempting to speculate that this was due to more extensive ATase depletion of the already low pretreatment levels of ATase in the bone marrow. If this is confirmed in larger patient numbers, then peripheral lymphocyte ATase activity may be used as a surrogate marker to predict potential bone marrow toxicity. Unfortunately CB10-277 treatment was not associated with significant anti-tumour activity and in a recently completed CRC phase II trial, only one partial responder was seen in the 22 treated patients with

metastatic melanoma (Bleehen et al, in preparation). Together with the emergence of temozolomide as an oral monomethyl triazene prodrug with activity against melanoma, this unfortunately has lead to the demise of CB10-277 for consideration in a phase III study. However, as demonstrated in Chapter 6, CB10-277 is capable of achieving significant lymphocyte ATase depletion with nadir activity occurring at the end of CB10-277 infusion and extensive ATase depletion was also seen in tumour biopsy material. This suggests that a potential increase in anti-tumour activity may occur if CNUs were to be administered at the end of CB10-277 infusion. One particularly interesting observation was the achievement of clinical responses in two patients in whom complete depletion of lymphocyte ATase occured; one patient obtained a partial response and in the other patient, a response in the lung metastases was seen although progression in the brain metastases occurred after cycles of 3 treatment and the latter effect may have been due to the inability of CB10-277 to penetrate the blood brain barrier. Thus, it may be worthwhile resurrecting CB10-277 into clinical use, particularly when combined with a CNU, since it is possible that the poor response rate seen with CB10-277 in the phase II study may be due to chance either because of the small number of patients entered into the study or that the tumours in these patients had high levels of ATase.

A progressive depletion of ATase was observed during the five days of continous temozolomide therapy. This suggests that the schedule-dependent anti-tumour activity of temozolomide seen in experimental models and clinics may be related to a cumulative depletion of ATase. If tumour sensitization is a consequence of ATase depletion then it could be that response to treatment would be better if the temozolomide were to be given every 2-6 h, corresponding to the ATase nadir determined after a single dose, rather than every 24 h, during which time recovery of ATase activity can occur. A similar dose schedule of temozolomide for 5 doses and followed 2-6 h later by a CNU also seems worthy of consideration.

Cyclophosphamide and BCNU are used regularly in many preparative regimens for a variety of cancer diseases prior to bone marrow or peripheral stem cell transplantations. There was an unexpected depletion of lymphocyte ATase in patients treated with cyclophosphamide and the *in vitro* study with pure recombinant human ATase suggests that this may be related to the direct reaction of the cyclophosphamide metabolite, acrolein with ATase. These findings suggest that the ATase-depleting action of cyclophosphamide could best be exploited by employing sequential cyclophosphamide and BCNU rather than a schedule administering BCNU before cyclophosphamide or concurrently. In this way, ATase depletion would be accomplished by an agent with considerable anti-tumour activity so that additional synergisms may operate.

Analysis of the data on ATase depletion with DTIC, CB10-277 and temozolomide showed that ATase inactivation was non-stoichiometric varying considerably between patients. There was, however, a strong correlation between the extent of ATase depletion and the pretreatment ATase level. If the extent of ATase depletion is the result of a stoichiometric inactivation due to the repair of the drug-induced methylation of DNA, then the calculated amount of ATase inactivated due to repair of O^6-MeG would be expected to be relatively constant assuming drug dosage, distribution and uptake and *de novo* ATase resynthesis rates were consistent between patients. That the kinetics of ATase depletion with temozolomide were very similar to that observed with DTIC (Chapter 3) and CB10-277 (Chapter 6), both of which require metabolic activation in order to produce a methylating species suggests that the process of metabolic activation of the latter agents occurs very rapidly and might not be the rate-limiting step in ATase depletion.

The possibility that the non-stoichiometric inactivation of ATase was due to direct reaction of the corresponding metabolites with ATase cannot be dismissed since similar correlation between pretreatment ATase activity and extent of ATase depletion in peripheral blood lymphocytes was also seen with cyclophosphamide and this was shown to be the likely result of the direct reaction of acrolein with ATase (see Chapter

8). Furthermore, MMS, a methylating agent that does not produce appreciable amounts of O^6-MeG was also associated with the *in vitro* inactivation of recombinant human ATase (see Chapter 7) and previous *in vitro* studies have shown inactivation of partially purified human ATase by direct alkylation of the protein following incubation with a variety of alkylating agents including MNU, streptozotocin, BCNU, chlorozotocin, CCNU and MeCCNU (Brent 1986). If ATase depletion by alkylating agents *in vivo* is predominantly a direct effect and not unique to peripheral lymphocytes, one possible consequence might be that the extent of ATase inactivation would be greatest in those cells and tissues expressing the highest levels of protein. Thus in tumour cells which express high ATase levels (Chapters 9 & 10) improved therapeutic index might be achieved since sensitization to killing by alkylating agents would be more extensive than in bone marrow which generally expresses low levels of ATase (Chapter 3; Gerson et al. 1985).

In the analysis of O^6-MeG formation in leukocyte DNA of patients undergoing sequential DTIC and fotemustine treatment, large variations were seen in the amounts of O^6-MeG initially formed in and subsequently removed from leukocyte DNA and these change differently in individual patients at different treatment cycles. Correlations were seen between the amount of O^6-MedG formed in leukocyte DNA (expressed either as peak level or AUC) and the pretreatment lymphocyte ATase activity or the extent of ATase depletion (Chapter 4). Similar correlations were also seen with CB10-277 when given by 24 h infusion (Lee et al. 1993). These data indicate that patients with high initial levels of ATase are able to repair a greater proportion of the O^6-MedG resulting in less O^6-MedG accumulation, whilst adducts persist more extensively in the leukocyte DNA of individuals with low pretreatment ATase levels. Since wide differences in ATase levels have been reported in melanoma tissues (Chapter 10; Maynard et al. 1989), this would be anticipated to result in significant differences in the accumulation of the toxic O^6-MedG in the target tumour DNA and tumour response. Indeed, one complete and one partial clinical response were seen and these occurred in the two patients with the highest O^6-MedG levels (Chapter 4). For a more comparative

study, it would be worthwhile to measure also 7-MeG since the level of this adduct would not be influenced by ATase-mediated repair and reflect more closely to the absolute level of DNA methylation. This may be important as the administered dose may not be a good indicator of the absolute dose reaching the tumour particularly if metabolic activation is required. A preliminary finding measuring 7-MeG in leukocyte DNA appears to show less heterogeneity of 7-MeG formation in 3 patients receiving the same dose of DTIC (van Delft et al.1992).

Clinical studies with sequential DTIC and fotemustine have provided important insights into potential clinical problems which are likely to be encountered using any agent that depletes ATase including the use of O^6-BeG which in combination with BCNU, is expected to enter clinical studies in America. As demonstrated in Chapters 3 and 5, it appears that increased ATase depletion that was achieved by escalating DTIC dosage may result in higher response rates but at the expense of increased bone marrow toxicity. Two patients developed interstitial pneumonitis and significant subclinical pulmonary damage was also seen in some of the patients where the lung function was monitored during the course of treatment. Unfortunately none of these patients were assayed for lymphocyte ATase activity and it is not possible to determine whether there might be a correlation between pulmonary toxicity and ATase levels in peripheral blood lymphocytes. In two other phase I studies where streptozotocin was administered before BCNU in order to deplete ATase activity, thrombocytopenia was the dose-limiting toxicity and this was associated with reduced MTD of BCNU to 50% that of BCNU used alone (Micetich et al. 1992; Panella et al. 1992). The haematological toxicity observed in these patients is presumably the result of ATase depletion in the bone marrow which prior to treatment would be expected to have a low constitutive ATase activity. This, however, may be an acceptable risk if it is accompanied by a significant increase in response rate and survival. Some of the resulting haematological toxicity profile, particularly neutropenia, may be ameliorated with haemopoietic growth factors but platelet toxicity still remains a limiting factor and this may preclude any

further escalation of O^6-alkylating agent dosage even in the presence of recombinant human granulocyte-macrophage colony stimulating factor (Rampling et al. 1994).

Since ATase-deficient murine haemopoietic stem cells when transfected with a bacterial ATase gene are highly resistant to the toxic effects of methylating agents and CNUs, which strongly indicates that ATase would protect against the haematological effects of these agents (Jelinek et al. 1988; Dumenco et al. 1989), an alternative approach which might be taken to overcome the haematologic toxic effects is *via* ATase gene therapy. Human pluripotent haemopoietic stem cells obtained from autologous bone marrow or peripheral stem cell harvest could be transduced with the human ATase cDNA packaged into a retrovirus *ex vivo* and then returned to the patients during the course of bone marrow transplantation. If this achieves high levels of ATase expression, one can envisage treating patients with repetitive high dose O^6-alkylating agents which might result in the elimination of the tumour but spare the ATase transduced bone marrow precursors. Alternatively with the proposed use of O^6-BeG, it would be possible to transfect the stem cells with a bacterial ATase gene (*Ada* gene) since the bacterial ATase protein is only marginally inactivated by O^6-BeG. Preliminary work here at Manchester suggest long term transfection of the bacterial ATase gene can be achieved in murine pluripotent haemopoietic stem cells in a long term bone marrow culture (J. A. Rafferty, personal communication).

The availability of a specific anti-human ATase has opened up other areas for potential clinical research. Since IHC is a reasonably easy procedure to perform, work needs to be extended into other tumour types treated with O^6-alkylating agents particularly gliomas, Hodgkin's disease and gastrointestinal tumours. Current studies continue to examine ATase expression in melanoma biopsies and whether this correlates with response and survival of patients treated with temozolomide. In addition, it would be worthwhile to evaluate the formation and persistence of O^6-MeG and 7-MeG by immunostaining using the corresponding antibodies (Fan et al. 1989; Benthem et al. 1991) in post-treatment tumour biopsies in order to ascertain whether DNA methylation

occurred uniformly throughout the tumour cell population. Recent progress in this area indicates that these two DNA adducts could be stained for simultaneously (J.A. Bailey, personal communication). Together with concurrent staining for cellular ATase distribution, it would be possible to document the progress of therapy at the level of individual tumour target cells. Such information would be expected to increase our understanding of sensitivity or resistance to chemotherapy in individual cells and may eventually indicate a more rational approach in the use of O^6-alkylating drugs in the clinic.

The generation and use of antibodies to human ATase also means that it will be possible to assess ATase activity in normal tissues obtained for examples, from bronchosopic normal lung and bone marrow biopsies and when performed in conjunction with ATase staining in tumour tissues, it may be possible to tailor treatment individually to patients who are likely to benefit most Thus, patients with high tumour ATase but low ATase activity in normal tissues are the cases which would be anticipated to obtain the best therapeutic index to O^6-alkylating agent treatments. Alternatively, patients identified with weak/absent ATase staining in bone marrow or lung tissues could be treated treated concurrently with haemopoietic growth factor to ameliorate the anticipated bone marrow toxicity or could be offered a non-O^6-alkylating agent treatment protocol or prophylatic steroids in order to prevent potential pulmonary fibrosis induced as a result of O^6-alkylating treatments.

Another major obstacle to overcome with O^6-alkylating agent treatments is the potential adverse biological effects likely to be induced in many of the patients surviving long term particularly in Hodgkin's patients where there is a high incidence of secondary leukaemia ranging from 0.5 to 2.0 percent per year for the first 10 years, with a cumulative incidence of 3.3 to 10 percent (Urba & Longo 1992). Significant number of these patients will also develop second neoplasms (especially non-Hodgkin's lymphoma, breast, ovary and lung; Kaldor & Lasset, 1989; Hancock et al. 1993). The schedule of chemotherapy combining ATase-depleting agents (e.g. O^6-BeG) with

methylating agents or CNUs is likely to increase mutagenesis and hence carcinogenic risks but it is possible that this may eventually be prevented with ATase gene therapy as discussed earlier. Nevertheless, the findings in Chapters 3 and 4 which showed greater DNA methylation with subsequent DTIC treatment cycles indicates that caution should be exercised when treating young Hodgkin's patients with DTIC or procarbazine containing regimens (such as MOPP, MVPP, ABVD) beyond the standard six cycles because of the likelihood of increased risk of developing a second malignancy. Indeed a collaborative group comprising several cancer registries found that more than six cycles of MOPP-like chemotherapy was associated with a significantly greater risk of secondary leukaemia than fewer than six cycles (Kaldor et al. 1990).

In summary, it is anticipated that approaches such as frequent repetitive O^6-alkylating treatments followed by CNU or combining ATase-inactivators such as O^6-BeG to CNUs in attempts to modulate ATase activity in tumour tissues will increase the sensitivity of inherently resistant tumours, broaden the range of tumour types that can be treated and possibly allow the reintroduction of agents previously shown to be of marginal clinical value. Whilst the associated toxicity will need careful monitoring, this type of strategy, together with antibody based methods of assessing tumour resistance in combination with haemopoietic growth factors or even gene therapy to overcome toxicity in non-target tissues including bone marrow, could lead to individualised therapy thereby improving prospects for clinical response, reduced toxicity and increased survival.

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Appendix

Publications arising from this thesis

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O^6-Alkylguanine-DNA Alkyltransferase Depletion and Regeneration in Human Peripheral Lymphocytes following Dacarbazine and Fotemustine[1]

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ABSTRACT

O^6-Alkylguanine-DNA alkyltransferase (ATase) levels were measured in peripheral blood lymphocytes of 13 patients with advanced malignant melanoma treated with sequential dacarbazine (DTIC) and fotemustine. Wide interindividual variation in the pretreatment levels and in depletion and regeneration of ATase activity was noted. Depletion of ATase was seen within the first h after DTIC administration with values ranging from 44 to 92% of pretreatment levels. In 10 patients, progressive depletion of ATase activity occurred with nadir activity occurring at about 4 to 6 h with values ranging from 0 to 67% of pretreatment activity; at 18 h after DTIC infusion, ATase activity varied from 6 to 81%. No significant difference was seen between the rates of ATase depletion or regeneration between the two groups of patients receiving either 500 or 800 mg/m^2 of DTIC with the same dose of fotemustine (100 mg/m^2). In one patient, maximum depletion occurred within 1 h and no ATase activity was detectable over the next 18 h. In another patient, maximum depletion occurred at 2 h after DTIC followed by recovery of ATase activity to 71% at 18 h. In 2 patients who returned for subsequent cycles of chemotherapy, an increase in pretreatment ATase activity was seen. Overall, the extent of depletion of ATase following DTIC/fotemustine was directly proportional to the initial ATase level.

INTRODUCTION

There is a considerable amount of experimental evidence to indicate that the endogenous ATase[3] level may be responsible for cellular resistance following administration of certain alkylating agents. Thus, ATase-deficient cell lines are more sensitive to killing by simple methylating and chloroethylating agents than ATase-proficient cells (1, 2). ATase status correlates with the Mer phenotype which was originally defined on the basis of reactivation of methylated adenovirus 5 (3). Viral (SV40) transformation of Mer$^+$ human fibroblast cell lines into Mer$^-$ cells rendered them more sensitive to killing by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine than their untransformed parent lines (3). Additional evidence comes from *in vitro* studies which show that depletion of endogenous ATase by either prior exposure to O^6-methylguanine (4–6) or pretreatment of Mer$^+$ cells with nontoxic doses of alkylating agents (7–9) rendered the cells more sensitive to subsequent treatment with methylating or chloroethylating agents. This is thought to be a consequence of the stoichiometric autoinactivating mechanism of ATase action and the requirement for *de novo* synthesis to restore cellular ATase levels (2, 4–9). One postulated mechanism of the cytotoxic effect of cloroethylating agent is the formation of guanine-cytosine DNA interstrand cross-links which are produced in a two-step reaction from the monoadduct O^6-chloroethyl guanine (10) which itself has been shown to be a substrate for ATase. It is thought that ATase reduces the cytotoxicity of this product by removing the alkyl group from the O^6 position of guanine before interstrand cross-links can be formed (2).

Further evidence supporting a role for ATase in tumor resistance comes from *in vivo* studies showing that human tumor xenografts low in ATase activity were more sensitive to alkylating agents than xenografts with high ATase activity (11). More recently transfection of an *Escherichia coli* ATase gene into various Mer$^-$ cell lines rendered them more resistant to killing by a variety of alkylating agents (12–15).

DTIC (Fig. 1) has unquestioned activity in Hodgkin's disease, sarcomas, and melanoma. The mechanism of its antitumor activity remains unclear but there is evidence to suggest that it requires metabolic activation to a monomethyl metabolite (MTIC) and that it methylates DNA, producing among 12 other lesions, O^6-methylguanine (2). Possibly as a consequence, MTIC is more cytotoxic to Mer$^-$ than to Mer$^+$ cells (16, 17). The biological activity of a new methyltriazene, temozolomide, which spontaneously decomposes to MTIC without metabolic activation, again correlates with the Mer phenotype (18). Additional evidence indicating a cytoprotective role of ATase in melanoma comes from a recent study showing that melanoma xenografts with high ATase activity implanted into nude mice are more resistant to DTIC than xenografts with low activity (19).

Based on the evidence discussed above, sequential administration of DTIC and fotemustine was used to treat patients with metastatic melanoma in order to increase the response rate and we have recently reported this to be 34% (20). Fotemustine (Fig. 1) is a new drug containing a phosphonoalanine carrier group grafted to the nitrosourea radical and has shown good antitumor activity in several rodent tumors (21) and promising clinical efficacy (22).

However, if tumor cells which are depleted of ATase activity following DTIC (or any other methylating agent) are able to rapidly regenerate ATase, the toxic effects of subsequent nitrosoureas might not be enhanced. The rate and extent of depletion and subsequent regeneration of ATase activity in human tissues have not been explored, nor is it known whether this is related to dosage of methylating or chloroethylating agents. In some animal tissues and cells in culture ATase activity can be induced and can reach 3 times control level following treatment with alkylating agents (23).

In patients, it is a major difficulty to obtain tumor tissue; therefore we examined the ATase levels in peripheral blood lymphocytes following sequential DTIC and fotemustine. It was possible not only to determine the rate of ATase depletion and regeneration following different doses of methylating agent (in this case DTIC) but also to evaluate interindividual variations in basal ATase activity and whether this influences deple-

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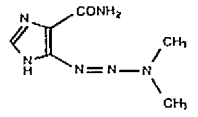
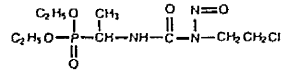
[3] The abbreviations used are: ATase, O^6-alkylguanine-DNA alkyltransferase; DTIC (dacarbazine), 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; MTIC, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide.



Dimethyltriazineimidazolecarboxamide (DTIC)



Fotemustine

Fig. 1. Structures of DTIC and fotemustine.

Table 1 *Patient's characteristics*

Name	Age (yr)	Sex	Metastatic sites
FM	41	M	Liver and CNS[a]
WB	69	M	Axillary, bone, and lung
PT	40	M	Lung, pelvic mass, and CNS
CF	46	M	Lung and spleen
EHJ	57	F	Skin, lung, and retroperitoneal
JB	58	M	Lung and liver
RB	56	M	Liver and retroperitoneal
SW	40	M	Axillary and lung
GS	67	M	Skin
BP	50	M	Small bowel and retroperitoneal
DW	68	F	Skin, bone, and mesenteric nodes
DD	63	F	Lung and CNS
FD	43	F	Skin, inguinal node and CNS

[a] CNS, central nervous system.

tion and regeneration rates, effects which may have important therapeutic implications. It also allows examination whether an "adaptive" response operates by monitoring ATase activity in lymphocytes in patients who return for subsequent cycles of chemotherapy. Such a phenomenon may explain some of the tumor resistance which is common in metastatic melanoma. The balance between the inactivation of ATase during repair of O^6-alkylguanine adducts and its subsequent regeneration are parameters which may predict individual tumor response to chemotherapy.

We therefore investigated changes in ATase activity in peripheral blood lymphocytes of patients with metastatic melanoma treated with sequential DTIC and fotemustine before and various times after chemotherapy.

MATERIALS AND METHODS

Blood Samples. Blood samples were collected from patients with metastatic melanoma treated with sequential DTIC and fotemustine. Patients received DTIC at 500 or 800 mg/m² by i.v. infusion over 10 min followed by fotemustine in a 100 mg/m² infusion over 30 min at 3 or 4 h after DTIC, respectively. Treatment was repeated every 28 days. Details of individuals studied are shown in Table 1. Blood samples were collected just before chemotherapy and at 1, 2, 3, 4, 6, and 18 h after DTIC infusion. Where possible, a final collection of blood was taken when patients returned to the clinic on days 7 and 14 after treatment. Bloods were drawn into a 20-ml universal container containing 0.5% EDTA and stored in 4°C before isolation of lymphocytes.

Isolation of Lymphocytes and ATase Extraction. Peripheral blood lymphocytes (mononuclear cell fraction) were isolated by centrifugation on Ficoll (Pharmacia, Uppsala, Sweden), washed with phosphate-buffered saline, centrifuged again into a pellet, and stored at −20°C. The pellets were sonicated (10 s at 10-μm peak to peak distance followed by cooling in ice and then resonication for 10 s at 18 μm) in 1 ml of buffer I (50 mM Tris-HCl-3 mM dithiothreitol-1 mM EDTA, pH 8.3). Phenylmethylsulfonyl fluoride (8.7 mg/ml in ethanol) was added to a final concentration of 87 μg/ml immediately after the second sonication. Sonicates were centrifuged at 16,000 rpm in a microcentrifuge for 10 min at 4°C. Supernatants were assayed for ATase activity and protein concentration (Bio-Rad protein reagent).

O^6-Alkylguanine-DNA Alkyltransferase Assay. This was carried out as described previously (24) with slight modifications. Thus, varying amounts of cell extract were incubated with [³H]methylnitrosourea-methylated calf thymus substrate DNA (specific activity, 23 Ci/mmol) at 37°C for 2 h in a total volume of 300 μl of 1 mg/ml of bovine serum albumin in buffer I. After incubation bovine serum albumin (100 μl of a 10-mg/ml solution in buffer I) and perchloric acid (100 μl of a 4 M solution) were added in rapid succession. A further 2 ml of 1 M perchloric acid were added and the mixture was heated at 75°C for 40 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 (Ecoscint A; National Diagnostics). Counting efficiency was approximately 28%. ATase specific activity was calculated from the region where activity was proportional to the amount of extract added since with higher amounts of extracts the reaction becomes substrate limiting. ATase activity is expressed as fmol methyl transferred to protein per mg of total protein in the extract.

RESULTS

Alkyltransferase Activity in Peripheral Blood Lymphocytes. A 5-fold difference was seen between pretreatment ATase activity in patients treated with sequential DTIC and fotemustine. To ensure that the 500- and 800-mg/m² patients had equivalent ranges of ATase activity before assessing depletion and regeneration rates, we compared the baseline values of the two groups of patients (see Tables 2 and 3). No significant difference was seen (unpaired t test, $P < 0.05$).

Alkyltransferase Depletion and Regeneration. Wide variations in ATase depletion and regeneration rates were noted between various individuals. ATase depletion was easily detectable at 1 h after completion of DTIC infusion with values ranging from 44 to 92% of control values. Maximum depletion seemed to

Table 2 *ATase activity in peripheral lymphocytes various times after DTIC (500 mg/m²) and fotemustine (100 mg/m²)*

		ATase activity (fmol/mg protein)							
Patient	Cycle	0 h	1 h	2 h	3 h	4 h	6 h	18 h	7 days
FM	1	135	88	76	67	56	15	110	131
WB	4	175	—[a]	88	53	34	67	91	—
PT	1	330	266	125	215	206	220	235	311
CF	6	67	62	—	42	28	25	29	67
FM	3	128	56	36	26	24	24	25	68
EHJ	3	295	—	145	125	111	130	123	—

[a] —, not determined.

Table 3 *ATase activity in peripheral lymphocytes various times after DTIC (800 mg/m²) and fotemustine (100 mg/m²)*

		ATase activity (fmol/mg protein)							
Patient	Cycle	0 h	1 h	2 h	3 h	4 h	6 h	18 h	7 days
EHJ	1	107	86	29	26	28	—[a]	—	—
JB	4	178	138	121	123	120	108	107	102
RB	2	183	125	122	95	79	57	52	105
SW	2	330	154	61	60	45	45	22	—
EHJ	2	82	69	54	38	23	23	36	90
RB	3	236	—	101	77	37	66	67	—
GS	1	317	166	142	130	109	85	181	229
BP	1	250	190	173	150	148	98	93	176
DW	1	211	160	124	120	99	109	112	150
DD	1	220	100	79	75	72	0	25	40
FD	2	82	0	0	0	0	0	0	—

[a] —, not determined.

occur at about 4 h in patients receiving 500 mg/m² DTIC and 4–6 h in patients receiving 800 mg/m² DTIC with activity ranging from 0 to 67% of controls (see Figs. 2 and 3). Comparison of the two groups of patients receiving either 500 or 800 mg/m² of DTIC at 2, 3, 4, 6, and 18 h and 7 days (unpaired *t* tests) showed no significant difference. However, taking each individual as its own control, a significant recovery greater than 20% was noted in 1 patient receiving 800 mg/m² of DTIC (Fig. 3*b*, GS1) and 3 patients receiving 500 mg/m² (Fig. 2*a*, FM1, WB4, PT1) at 18 h suggesting that in some cases the lower dosage DTIC may affect regeneration rate although this requires assessment using a much larger sample number.

In one patient, complete depletion was seen within 1 h with no detectable activity seen over the next 18 h (Fig. 3*c*, FD2) and this individual's ATase activities were not included in the analysis above.

In two patients who returned for cycle 3 chemotherapy, an increase in baseline prechemotherapy ATase level by factors of 1.4 and 3, respectively, compared with cycle 1 chemotherapy was seen.

DISCUSSION

In the present study we have shown progressive depletion and recovery of ATase activity in peripheral blood lymphocytes with time after sequential DTIC and fotemustine. Wide inter-individual variations in ATase depletion and recovery were seen with depletion measurable even after 1 h. However, in most patients the nadir activity occurred at around 4 to 6 h after DTIC treatment.

Given the constraints of study, we are unable to say whether fotemustine contributes to the ATase depletion since no data are yet available on fotemustine depletion of ATase in human lymphocytes. Other chloroethylnitrosoureas such as 1,3-bis(2-chloroethyl)-1-nitrosourea or 1-(2-chloroethyl)-1-nitroso-3-cyclohexylurea did not cause ATase depletion (25); however, activity was measured 20 h after drug exposure and our study shows that in some patients ATase recovery can occur already by 18 h. Clearly, the extent to which the present results can be extrapolated to tumor cells will depend on whether or not changes in peripheral blood lymphocyte ATase activity correlate with target tumor activity following administration of alkylating agents. Human tissues and tumors differ greatly in ATase levels (2) but there is some indication that relationships between tissues may exist (26). Another complication is that the levels of DNA adducts following alkylating agent treatment may vary between tissues depending on metabolic activation, the stability of alkylating metabolites and the blood supply, etc. However, studies in the rat have shown that DNA methylation occurred to a broadly similar level in all tissues following administration of methylating agents, even those requiring metabolic activation (27, 28). The difficulties of access to serial tumor tissue biopsies on technical and ethical grounds should also be borne in mind when such studies are considered. Nevertheless, the present findings suggest that 4- to 6 h would be the optimal time to administer further methylating or chloroethylating agents in order to maximize the effects mediated via O^6-

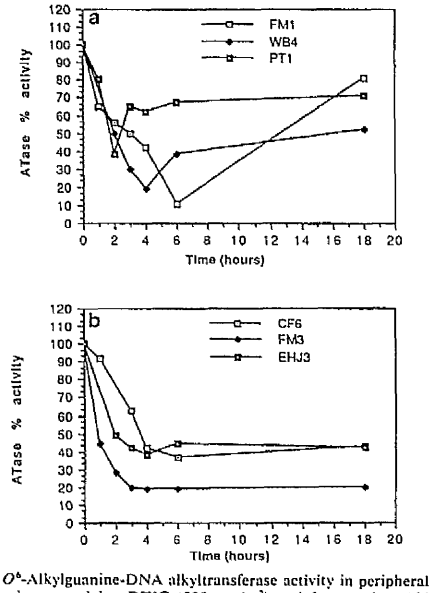


Fig. 2. O^6-Alkylguanine-DNA alkyltransferase activity in peripheral lymphocytes of patients receiving DTIC (500 mg/m²) and fotemustine (100 mg/m²) expressed as percentage of baseline prechemotherapy level. Number after patient's initial refers to treatment cycle (see Table 2). Data presented in 2 graphs (*a* and *b*) for clarity.

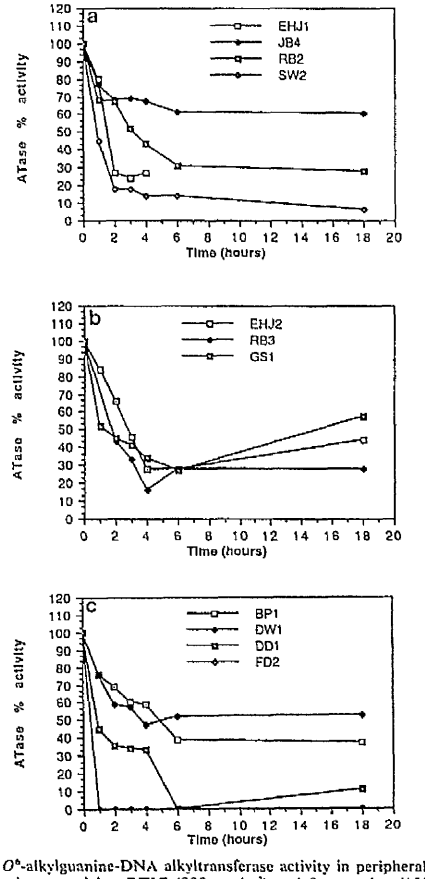


Fig. 3. O^6-alkylguanine-DNA alkyltransferase activity in peripheral lymphocytes of patients receiving DTIC (800 mg/m²) and fotemustine (100 mg/m²) expressed as percentage of baseline prechemotherapy level. Number after patient's initial refers to treatment cycle (see Table 3). Data presented in 3 graphs (*a*, *b*, and *c*) for clarity.

alkylation of guanine in DNA before regeneration of ATase by *de novo* synthesis can occur. It has been observed that fractionated doses of DTIC are more effective than a single administration (29) and the reason for this may be related to slow or incomplete recovery of ATase. Our study indicates that the ATase depletion rate was not correlated with dosage of DTIC; it may therefore alternatively be that repetitive fractionated doses of DTIC achieve accumulating ATase depletion with each dose. It would now be interesting to explore if repetitive administration of DTIC or a related agent every 4 to 6 hours would further deplete ATase and moreover whether it would have increased antitumor activity.

Very few agents produce any significant clinical activity in metastatic melanoma apart from DTIC and nitrosoureas (30). DTIC alone regularly produces a response rate of 20% (30) and it is tempting to speculate that this is related to ATase levels found in nodule samples (31); fotemustine alone produces a 24% response rate (22). However, with sequential DTIC and fotemustine, scheduled as in the present study, we recently achieved a 34% response rate (20). Since extrapolation from the lymphocyte data would have suggested the scheduling of DTIC and fotemustine actually used in this study, this adds support to the possibility that lymphocytes may indeed be an indicator of ATase activity in melanomas. Thus, if more extensive and consistent depletion of lymphocyte ATase levels can be achieved, for example by smaller, more frequent, doses of DTIC, this may further improve response rate and should be explored. An additional finding from our studies was that two patients returning for further cycles of chemotherapy showed increases in the pretreatment ATase levels in their lymphocytes. Although further confirmation is clearly required, if this effect is reflected in the target tumor, it might indicate one mechanism of tumor resistance. As with many chemotherapeutic agents, it has been shown that resistance to DTIC or MTIC develops rapidly *in vivo* (32) and *in vitro* (33) and in the latter case this arises not from decreased drug uptake but from enhanced repair of methylation damage in DNA (34). Similar effects have been observed using chloroethylating agents (24). Whether the resistance can be overcome by compensatory increases in dose or dose frequency can now be addressed using the lymphocyte as a monitoring system.

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Cyclophosphamide decreases O⁶-alkylguanine-DNA alkyltransferase activity in peripheral lymphocytes of patients undergoing bone marrow transplantation

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Summary O⁶-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^{-2}, daily) administered on days 1 to 4 and BCNU (600 mg m^{-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^{-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 or with >1 mM phosphoramide mustard. 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 kiling by BCNU is initiated by the formation of the mono-adduct, O⁶-chloroethylguanine which undergoes an intramolecular rearrangement to form O⁶, 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 O⁶-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 choroethylating 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 (Futscher *et al.*, 1989; Zlotogorski & Erickson, 1984) or O⁶-methylguanine (Dolan *et al.*, 1985; Gerson *et al.*, 1988*a*; Yarosh *et al.*, 1986) or O⁶-benzylguanine (Dolan *et al.*, 1990) rendered the cells more sensitive to subsequent treatment with chloroethylating agents. Conversely, transfer and expression of ATase genes in ATase deficient cells renders them more resistant to chloroethylating 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 fotemustine (Lee *et al.*, 1991*a*), 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).

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Materials and methods

Chemicals

Cyclophosphamide was obtained from Farmitalia Carlo Erba Ltd; acrolein and glutathione were from Sigma Chemical Co Ltd. and phosphoramide mustard was a generous gift from Dr A. McGown (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; ChlVPP: Chlorambucil, vinblastine, procarbazine and prednisolone or HYBRID: Vinblastine, procarbazine, prednisolone, chlorambucil, vincristine, etoposide and adriamycin) and/or salvage chemotherapy (including VAPEC-B: Adramycin, 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 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.

Table I Patients characteristics

Patient	Age/sex	Disease	Treatment	ATase activity (fm mg^{-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 1° CR	Cyclo/TBI	107	19
GD	45/F	AML 1° CR	Cyclo/TBI	168	10
LW	36/F	AML 1° CR	Cyclo/TBI	101	13
DH	43/M	AML 1° CR	Cyclo/TBI	405	138
SA	44/F	ALL 1° CR	Cyclo/TBI	461	95
MC	18/F	ALL 1° CR	Cyclo/TBI	148	18
HR	30/F	ALL 1° CR	Cyclo/TBI	103	19
TC	21/F	ALL 3° CR	Cyclo/TBI	168	37
SK	28/F	CML 1° CP	Cyclo/TBI	160	23
LB	38/M	CML 1° CP	Cyclo/TBI	102	45
CN	38/F	CML 1° CP	Cyclo/TBI	200	18
RB	54/M	NHL 1° 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; 1°: first; 3°: third. BD* = below detection.

O^6-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.*, 1991*a*) with slight modifications. Briefly, cells were disrupted by sonication in 1 ml of buffer I (50 mM Tris–HCl, 3 mM dithiothreitol, 1 mM EDTA, pH 8.3) and centrifuged to prepare cell extracts. Varying amounts of cell extract were incubated with ^{3}H-methylnitrosourea-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 (100 µl of a 10 mg ml^{-1} solution in buffer I) and perchloric acid (200 µl of a 4 M solution) were added in rapid succession. 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 (Ecoscint 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 fmoles 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 phosphoramide mustard to inhibit the ATase *in vitro*, varying concentrations of these agents were incubated with 70 fmoles of pure recombinant human ATase (Santibanez-Koref *et al.*, 1992 in press) for 2 h at 37°C in buffer I without dithiothreitol. Residual ATase activity was then measured by incubation with excess substrate DNA. The effect of glutathione on the inhibition of ATase by acrolein was monitored by incubating various amounts of gluthathione for 2 h with 500µM acrolein, a concentration that caused a 95% depletion in ATase activity *in vitro*. Following this, 70 fmoles 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) fm mg^{-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,

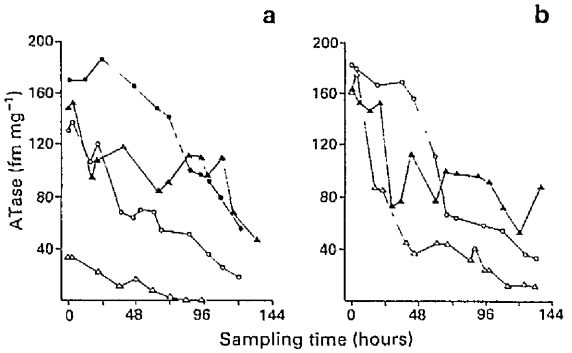

Figure 1 O^6-alkylguanine-DNA-alkyltransferase (ATase) specific activity (fmoles mg^{-1} protein) in extracts of peripheral lymphocytes of Hodgkin's patients sampled at various times after high dose cyclophosphamide given on day 1, 2, 3 and 4 and BCNU given on day 5. a, Patients ST (△), SH (▲), MR (○) and MS (●). b, Patients RD (△), SJ (▲) and JC (○).

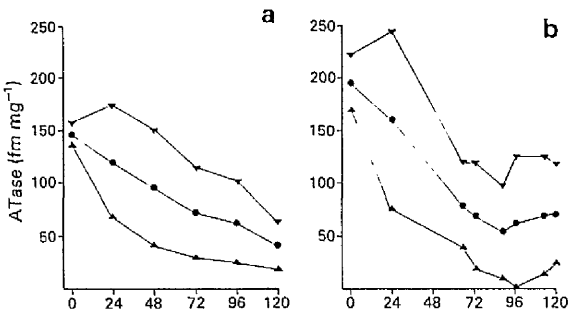

Figure 3 Consensus data for O^6-alkylguanine-DNA-alkyltransferase (ATase) specific activity (fmoles mg^{-1} protein) in extracts of peripheral lymphocytes of a, patients treated with cyclophosphamide and BCNU (see Figure 1) and b, patients treated with cyclophosphamide and TBI (see Figure 2). Figures show mean values (●) and upper (▼) and lower (▲) 95% confidence intervals.

Figure 1a), no ATase activity was detectable after the fourth cyclophosphamide treatment. In two patients there was evidence of partial regeneration of ATase during the cyclophosphamide treatments (SH and SJ, Figures 1a and 1b). ATase activities continued to decrease after BCNU administration to a median of 19% (range 0% to 32%) of pretreatment levels.

Although in some case, the loss of ATase was small after administration of BCNU; overall there was a substantial post-BCNU decrease in ATase and this was highly statistically significant: maximal loss occurred between the second and fourth dose of cyclophosphamide ($P = 0.0013$) and after BCNU administration ($P = 0.0018$). A consensus summary of the data from all seven patients is shown in Figure 3a.

Decrease in ATase in vivo *following cyclophosphamide and TBI*

Pretreatment ATase levels in the 12 patients monitored ranged from 101 to 462 (mean 186) fm mg^{-1}. As above, the extents and rates of decrease in activity were variable with a median post-cyclophosphamide nadir of 35% (range 12% to 78%) of pretreatment levels. The extent of ATase loss did not appear to be related to the pretreatment levels. In seven patients there was a transient increase in ATase activity after the first dose of cyclophosphamide (SA, MR, HR, TC, LB, RB and CN, Figures 2b and 2c) followed by reduction to nadirs from 80% to 10% of pre-cyclophosphamide levels. In two patients significant partial recovery of ATase was seen during the cyclophosphamide treatments and prior to TBI (RB and SK, Figure 2c). Generally, relatively small changes in ATase activity were seen during the TBI treatments. However, in three patients TBI itself appeared to extensively suppress ATase activity (RB, TC and CN, Figures 2b and 2c) but in two of these (TC and CN, Figures 2b and 2c) an initial marked suppression was followed by an equally extensive recovery.

Statistical analysis again showed that the effect of cyclophosphamide on ATase was cumulative, particularly between the first dose of cyclophosphamide and the second day of TBI ($P < 0.0001$). A consensus summary of the data from all 12 patients is shown in Figure 3b. Although the mean pretreatment ATase activity was higher and the ATase reduction rate appears faster than in the Hodgkin's group, this was not statistically significant ($P = 0.097$ and $P = 0.89$ respectively) and there was therefore no correlation between pretreatment history and ATase loss. The apparently greater individual variation in ATase in the cyclophosphamide/TBI than the cyclophosphamide/BCNU group might be related to the fact that the former group received two and the latter four consecutive days of cyclophosphamide treatment, or to the different pretreatment history.

There was progressive reduction in WBC counts over the 5–7 days of cyclophosphamide/BCNU and cyclophospha-

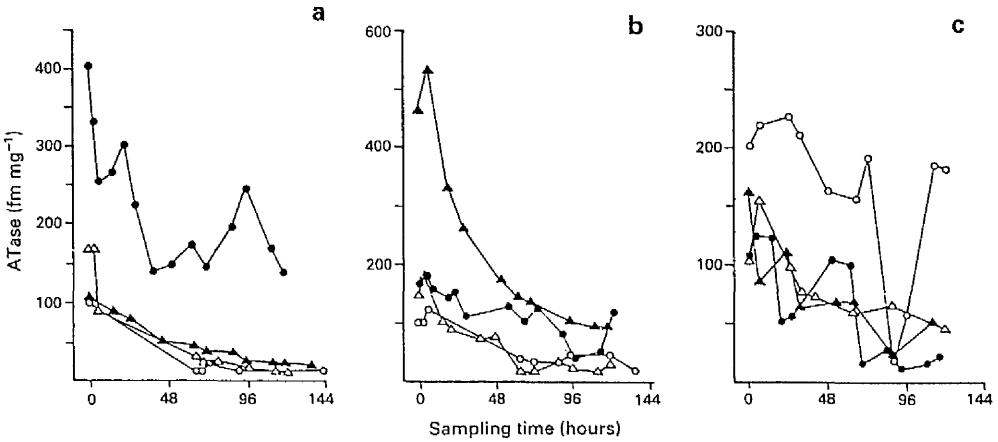

Figure 2 O^6-alkylguanine-DNA-alkyltransferase (ATase) specific activity (fmoles mg^{-1} protein) in extracts of peripheral lymphocytes of patients sampled at various times after cyclophosphamide given on day 1 and 2 and TBI given on days 4, 5 and 6. a, Patients AW (▲), GD (△), CW (○) and DH (●). b, Patients SA (▲), MR (△), HR (○) and TC (●). c, Patients SK (▲), LB (△), CN (○) and RB (●).

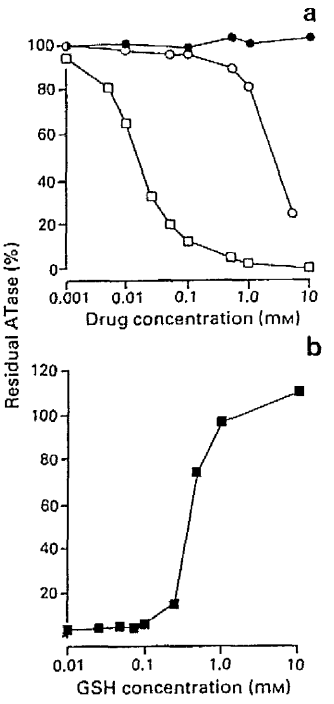

Figure 4 a, Effect of pre-incubation of pure recombinant human O^6-alkylguanine-DNA-alkyltransferase (ATase) with increasing concentrations of cyclophosphamide (●), phosphoramide mustard (○) or acrolein (□). See text for experimental details. b Effect of preincubation of acrolein with increasing concentrations of glutathione (GSH) on its ability to deplete the activity of pure recombinant human ATase.

mide/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 2 h 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 occuring 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 experimental variation or intra-individual variation. 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 ATase specific activities of 190 and 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, O^6-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; Meer *et al.*, 1989) which showed that cyclophosphamide is able to increase the amount of O^6-methylguanine in DNA following a chasing dose of methylating agent in rodent liver and both authors attributed this to some as yet unidentified O^6-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-hydroxycyclophosphamide, 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 sulfhydryl groups including, possibly, the alkyl-accepting cysteine residue of the ATase protein. The peak concentration of phosphoramide 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*. Phosphoramide 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 (Chresta *et al.*, 1990; Draeger *et al.*, 1976; Lee, 1991*b*; 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.*, 1991*c*). Cyclophosphamide has also been shown to be able to deplete serum glutathione (Carmichael *et al.*, 1986). Here we were able to show that glutathione can inhibit acrolein-induced depletion of ATase, supporting the hypothesis that in the case of lymphocytes, intracellular glutathione levels may be one of the factors that determines the extent of ATase decrease.

Irrespective of the mechanism, our observations on peripheral lymphocytes may have important general implications in combination chemotherapy if similar changes in ATase occur in the tumour and indeed, if the extents of ATase loss achieved are sufficient to sensitise the tumour cells to killing by agents such as BCNU. In cultured human tumour cells lines that express high levels of ATase, depletion of the enzyme following exposure to O^6-benzylguanine increases their sensitivity to the toxic effects of subsequent doses of chloroethylating agents, greater extents of sensitisation being produced in cells expressing higher levels of ATase: no such sensitisation occurs in equivalent ATase deficient cells (Dolan *et al.*, 1991). It might therefore be argued that enzyme depletion in ATase-expressing tumour cells would considerably enhance chemotherapeutic effectiveness without significantly increasing the toxic side effects in tissues such as bone marrow that generally express low levels of the enzyme.

It is not unreasonable to suggest that the ATase-reducing action of cyclophosphamide would best be exploited by employing sequential cyclophosphamide and BCNU (as here) rather than a regimen in which cyclophosphamide and BCNU are administered concurrently or in which BCNU is given before cyclophosphamide. Further support for such a schedule is that intracellularly released acrolein has been shown to deplete cellular glutathione (Gurtoo *et al.*, 1981; Lee, 1991*b*) and the latter is able to decrease the cytotoxic and DNA cross-linking activity of BCNU (Ali-Osman *et al.*, 1989). Therefore, if used as in the schedule described here, the glutathione-depleting property of acrolein may further sensitise tumour cells to BCNU. In support of this suggestion it is interesting to note that in one series of 54 patients with advanced Hodgkin's disease undergoing ABMR (Reece *et al.*, 1991), where the condition schedule involved administering BCNU after cyclophosphamide together with etoposide, the complete response rate and disease-free survival rate were 80% and 55% respectively. In contrast, most other series using similar preparative drugs, but different schedules, the complete response rate averaged about 45% and only approximately 10 to 30% achieved disease-free survival (Ahmed *et al.*, 1989; Bierman *et al.*, 1988; Carella *et al.*, 1988; Jagannath *et al.*, 1986).

In conclusion, cyclophosphamide is capable of reducing ATase activity in peripheral lymphocytes and one possible explanation is that this is mediated via the release of intracellular acrolein, a cyclophosphamide metabolite. This property could be exploited in designing future combination chemotherapy schedules; this method of reducing cellular ATase levels may be an alternative to the proposed use of agents such as O^6-benzylguanine since, in the case of cyclophosphamide, reduction is accomplished by an agent with proven antitumour activity.

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Immunohistological examination of the inter- and intracellular distribution of O⁶-alkylguanine DNA-alkyltransferase in human liver and melanoma

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Summary The tissue and cellular distribution of the DNA repair protein O⁶-alkylguanine-DNA-alkyltransferase (ATase) is an important question in relation to the response of tumour and normal tissues to chemotherapeutic regimes employing alkylating agents such as methyltriazenes and nitrosoureas. In order to examine this issue by immunostaining, we have raised a rabbit antiserum to apparently pure recombinant human enzyme. The antiserum is highly specific and sensitive, detecting a band at 24 kDa on western blots of crude extracts of ATase-expressing human lymphoblastoid cells, liver and melanoma. Adjacent sections of acetone or formalin fixed normal human liver and subcutaneous malignant melanoma were reacted with preimmune serum or antiserum and an immunoperoxidase detection system with silver enhancement was used to locate binding of the primary antibody to the antigen. In sections reacted with preimmune serum or with antigen-preadsorbed antiserum, only faint cytoplasmic and little or no nuclear staining was seen. In contrast, using antiserum, the reaction in positively staining cells was very intense and predominantly nuclear. In the liver, there was interindividual variation in the cellular distribution of reaction with staining present in all discernable cell types in most samples but confined to the hepatocytes and bile duct epithelial cells in others. In the melanoma sections, all discernable cell types showed mainly nuclear staining: the intensity of staining varied between tissue samples and there was evidence of a range of intermediate staining intensities with some melanoma cells showing no detectable reaction.

Some antitumour alkylating agents including the methylating agents dacarbazine (DTIC), procarbazine, temozolomide, CB10277 and streptozotocin and the chloroethylating agents chlorozotocin, BCNU and related nitrosoureas such as TCNU and fotemustine exert their effects by interaction with DNA. There is increasing evidence that one of the principal mechanisms of cellular resistance to the cytotoxic and other biological effects of these agents is related to the expression of the DNA repair enzyme O⁶-alkylguanine-DNA-alkyltransferase (ATase): cultured cells or tumour xenografts that express high levels of this enzyme either from the endogenous or a cloned, transfected gene are generally more resistant to the toxic effects of these agents than those expressing low levels (D'Incalci *et al.*, 1988; Margison & O'Connor, 1990; Pegg, 1990). There is currently considerable interest in measuring the amounts of ATase in tumour and normal biopsy material (Myrnes *et al.*, 1984; Weistler *et al.*, 1984; Maynard *et al.*, 1989; Kyrtopoulos *et al.*, 1990) and also in peripheral lymphocytes (Sagher *et al.*, 1988; Gerson *et al.*, 1988; Lee *et al.*, 1991), which have the distinct advantage of being more accessible and amenable to repeat sampling. The aim of such work is to assess whether or not there is any evidence for a similar correlation between ATase levels and the response of the tumour, or the tissues in which toxic side effects occur, to chemotherapeutic regimens that include these types of agents and also to monitor the effects of various drugs and treatment schedules on ATase activities (Gerson *et al.*, 1988; Lee *et al.*, 1991).

Although ATase assay methods are extremely sensitive, the results obtained using human tumour biopsies are always a tissue-average measurement and take no account of cellular heterogeneity in ATase expression This is clearly a very critical question in relation to chemotherapeutic effectiveness since small numbers of cells with high levels of ATase could not only give the impression of a low overall ATase level in tissue homogenates but also be the resistant cells that eventually result in tumour relapse and recurrence.

A similar question arises with respect not only to the toxic side effects of chemotherapeutic alkylating agents but also to the numerous adverse biological effects of environmental or endogenously formed alkylating agents, or their precursors (Bartsch & Montesano, 1984). In this case individual cells that express very low levels of ATase might be expected to be the most susceptible to these effects, which include mutation and malignant transformation (see Margison & O'Connor, 1990). Indeed, such a situation has been observed in an animal model system in which specific target cells for mesenchymal tumour induction in rats were shown to be damaged by an environmental alkylating agent and to lack the capacity for repair of O⁶-methylguanine, even over a period of several weeks (Fan *et al.*, 1990).

In order to address these questions, we have generated polyclonal antibodies to the human ATase and these have been used to visualise the enzyme in human normal and tumour tissue. Staining was heterogeneous and almost exclusively nuclear in normal liver and in subcutaneous malignant melanoma nodules.

Materials and methods

Antibody production

E.coli harbouring pRBShAT (see Potter *et al.*, 1991) were grown in LB medium containing carbenicillin (Sigma, 0.1 mg ml^{-1}) at 30°C to an E600 of 0.2 then at 42°C for 3 h prior to harvesting by centrifugation. Crude sonicates of these bacteria, which expressed the recombinant human protein to approximately 3% of total protein, were subjected to

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DNA cellulose affinity purification essentially as previously described (Wilkinson *et al.*, 1989). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining showed the pooled, concentrated material to be apparently homogeneous (estimated >95% pure). Samples (100 μg) of this were used to immunise prebled Half-lop rabbits: the primary injection was followed by three boosts at 4-week intervals and bleeds were taken one week after each of the boosts for preparation of serum. Dilutions for use in western blotting and immunohistology were estimated by ELISA.

Western blotting

Crude sonicates of the human lymphoblastoid cell lines RAJI and TK6 were assayed for ATase activity as described (Lee *et al.*, 1991). These and similar extracts of human tissues (see below) each containing 30 μg of total protein were subjected to SDS-PAGE and transferred to Hybond C (Amersham International PLC) membranes. After blocking with non-fat milk (5% Marvel in Tris-buffered saline (TBS)), the membranes were incubated with anti-human ATase antiserum (3rd bleed serum diluted 1:1000 in blocking buffer) and then goat anti-rabbit alkaline phosphatase (Dako Ltd., High Wycombe UK). Antibody complexes were revealed by reaction with nitro blue tetrazolium and bromochloroindolyl phosphate.

Immunohistology

Ethical committee approved human liver and melanoma tissue samples were obtained by trucut needle or surgical biopsy. Tissues were fixed in formalin or acetone and wax embedded. Sections (3 μ) were cut and mounted onto gelatin-subbed slides, dewaxed and rehydrated. The sections were treated with methanol and exposed overnight at 4°C to the anti-human ATase antiserum (3rd bleed) or preimmune serum diluted 1:1000 in PBS. As an additional control, an aliquot of the diluted immune serum was preincubated with the pure recombinant human ATase at 4°C overnight prior to use in the above procedure. The sections were then incubated with swine anti-rabbit antibody (SAR, (Dako) diluted 1:40 in PBS containing 10% normal rat serum) for 45 min at room temperature, washed in PBS and incubated with rabbit peroxidase-antiperoxidase complex (PAP, (Dako) diluted 1:400 in PBS) for 45 min at room temperature. After washing in PBS the sections were incubated twice for 15 min with SAR and PAP. For DAB development, slides were incubated for 5 min in 50 mM Tris-HCl, pH 7.5 containing 10 mM imidazole then for 5 min in the same medium containing 0.5 mg ml^{-1} DAB and 3% hydrogen peroxide. Slides were washed in water, dehydrated, mounted and photographed. For silver detection, after the second application of PAP, the sections were washed in TBS then incubated at room temperature for 5 min in TBS containing nickel-complexed DAB (0.5 mg ml^{-1} DAB in 80% TBS containing 10% aqueous ($NiCl_2 6H_2O$). The sections were then incubated in the above solution containing 10 μl of 30% hydrogen peroxide for 5 min and the reaction stopped by three 1 min washes in distilled water. This was followed by incubating in silver reagent (prepared according to Przepiorka and Myerson, 1986, by mixing 400 μl water with 200 μl 0.1 M ammonium nitrate, 200 μl 0.047 M silver nitrate, 180 μl 0.12 M dodecatungstosilic acid (Fisons), 15 μl 36% formalin and 1 ml 0.47 M sodium carbonate). The slides were given three 1 min washes in water, a 2 min wash in 2% sodium thiosulphate and a 5 min wash in running tap water, dehydrated in alcohols, cleared in xylene and mounted in XAM (BDH).

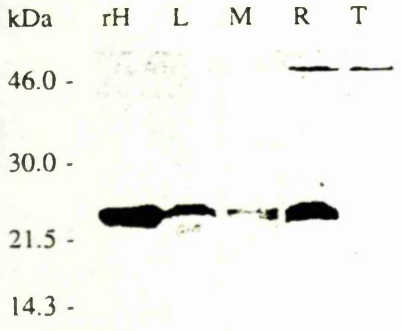


Figure 1 Western blot using anti-human rabbit antiserum: rH, pure recombinant human ATase; L, human liver extract; M, human melanoma extract; R, Raji cell extract; T, TK6 cell extract. The positions of the molecular weight marker proteins are shown. See text for details.

Results

Western blotting

The ATase specific activities in the crude sonicates of the TK6 and RAJI cells were <2 fm mg^{-1} and 400 fm mg^{-1} respectively. Western blotting revealed a heavily staining band in the RAJI but not the TK6 extracts at ca. 24 kDa, corresponding to the size of the pure recombinant human ATase and the bands seen in crude extracts of human liver and melanoma (Figure 1). An additional higher molecular weight protein was faintly detected at around 48 kDa in both of the cell extracts but not in the human tissue extracts or the recombinant protein (Figure 1).

Immunostaining

In the present report, six liver and 16 melanoma samples were assessed histopathologically for the inter and intracellular distribution and intensity of staining. Using preimmune serum, faint cytoplasmic and nuclear staining were seen such that in normal liver (Figures 2a and 3a) and melanoma (Figures 4a1 and 4b1) tissue architecture was easily discerned. In general, incubation with the ATase antiserum showed very heavy nuclear staining although more faint cytoplasmic staining was seen in some sections. The results for two liver samples are shown in Figures 2 and 3 and for two melanoma samples in Figures 4a and 4b: the corresponding haematoxylin and eosin staining is shown in Figures 2c, 3c, 4a3 and 4b3.

In the liver sections there was relatively homogeneous and intense staining of the hepatocytes and this was predominantly in the nucleus with little or no cytoplasmic stain in most of the samples. The bile duct epithelial cells presented a similar picture although cytoplasmic staining was also seen in one of the samples. In most of the samples, the portal vein endothelial cells and the Kuppfer cells were not stained. There was no apparent predominance of centrilobular or periportal staining in any of the sections.

In the melanoma sections, all cell types that could be discerned, including melanoma cells, keratinocytes, endothelial cells, fibroblasts and smooth muscle cells showed staining that was predominantly nuclear. In some cases the melanoma cell staining was heterogeneous and many of the nuclei appeared free of stain (e.g. Figure 4a2). As with the liver samples, there was interindividual variation in staining intensity.

As further confirmation of the specificity of the antiserum, liver sections serial to a sample showing marked antibody staining were incubated with antigen-preincubated antiserum then subjected to the standard protocol. The result (Figure 3c) was indistinguishable from that obtained with preimmune serum.

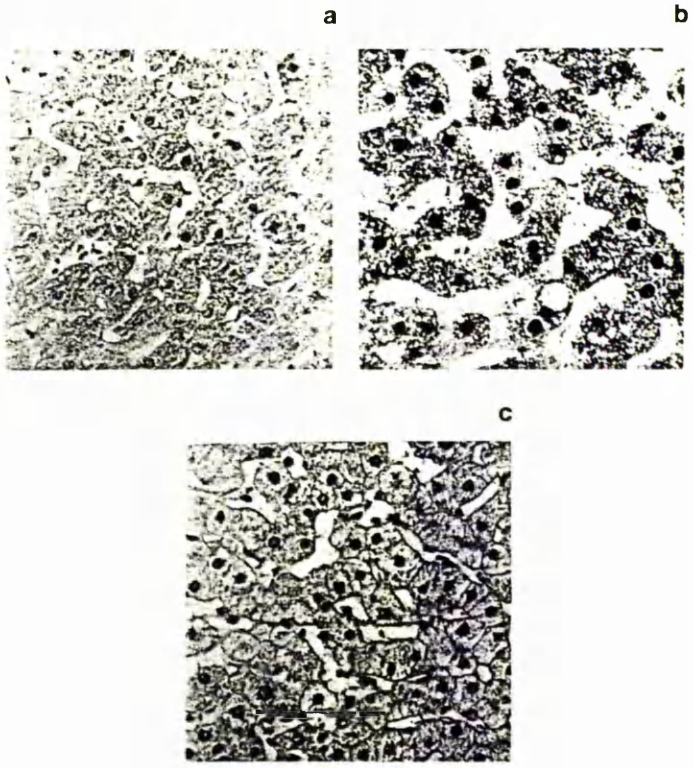


Figure 2 Staining of a normal human liver sample with: (**a**) preimmune serum, (**b**) ATase antiserum and (**c**) haematoxylin and eosin. The antiserum produces strong, uniform nuclear staining which is absent in (**a**). Magnification × 210.

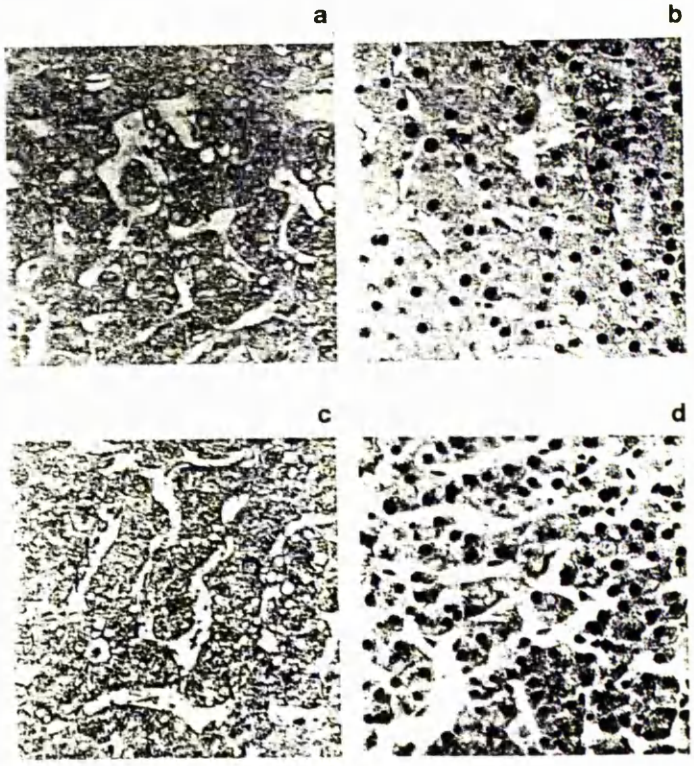
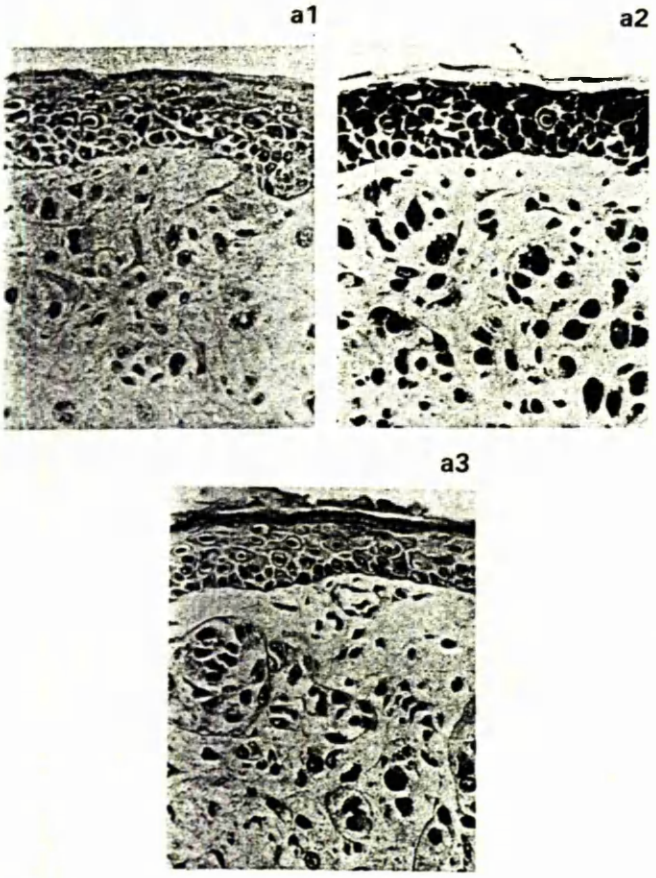


Figure 3 Staining of a normal human liver sample with: (**a**) preimmune serum, (**b**) ATase antiserum, (**c**) antigen-preadsorbed antiserum and (**d**) haematoxylin and eosin. Magnification × 230.



Discussion

The antiserum we have produced is highly specific for the human ATase detecting a strongly reacting 24 kDa band in extracts of RAJI cells that expressed high levels of ATase but not in TK6 cells that expressed almost undetectable levels of this protein. Although the human cell extracts contained a cross-reacting high molecular weight protein, this was not seen in human tissue extracts and appeared not to be present in the pure recombinant protein used as the immunogen. The antiserum has also recently been shown to inhibit the human but not rodent ATases in liquid hybridisation experiments (Santibanez-Koref *et al.*, 1992; Rafferty *et al.*, 1992).

The antiserum readily detected endogenous expression of the human ATase protein in sections of human liver and melanoma and in sections of human ATase-transgenic mice (Fan *et al.*, 1990; Fan *et al.*, in preparation). In both cases the staining appeared to be located predominantly over the nucleus although some cytoplasmic staining was detected in some samples. The specificity of the antiserum was further confirmed by preadsorption of the antiserum with the pure recombinant ATase protein after which staining was reduced to the levels seen with preimmune serum.

There are several reports in which the intracellular distribution of ATase has been addressed using subcellular fractionation procedures. The cytosolic fraction of rat liver was reported to contain 35% (Jun *et al.*, 1985), 59% (Pegg *et al.*, 1983) or 72% (Hora *et al.*, 1983) of the total ATase activity. Earlier indications were that rat liver nuclei contained 75% of the total cellular enzyme (Renard & Verly, 1980). Since the polyclonal antibodies used in the present work have not so far detected the rat ATase in liver sections we are unable to confirm these findings. In the present report, cytoplasmic staining showed considerable intercellular and interindividual variation in human liver and melanoma, but was in most cases much less intense than in the nuclei. It may be that rat and human tissues are very different in the cellular distribution of ATase but it might also be that cell fractionation procedures disturb the true location of the protein. Alternatively the antibodies may be better able to detect the human ATase when it is located in the nucleus, in chromatin or bound to DNA, rather than in the cytoplasm. Another explanation is that the processing procedure used here might effectively remove the enzyme from the cytoplasm or in some other way render it undetectable. These possibilities are being investigated.

In the liver, the hepatocytes and bile duct epithelial cells were stained and whilst this staining was apparently relatively uniform, in some of the samples there was no detectable staining of the portal vein endothelial cells or Kuppfer cells. This highly heterogeneous intercellular distribution of staining suggests that the ATase gene is not being transcribed and translated at a level that can be detected in all of the liver cells. In the rat liver, a degree of heterogeneity of repair of O^6-methylguanine is likely to occur since hepatocytes lose this lesion in a matter of hours whereas in endothelial cells and fibroblasts the lesion is retained for much longer. Indirect evidence of repair enzyme deficient cells has also been observed in rat lung, kidney cortex and glandular stomach (O'Connor *et al.*, 1990). It may be possible to confirm the heterogeneity of ATase staining by *in situ* hybridisation using riboprobes on normal tissue sections or by

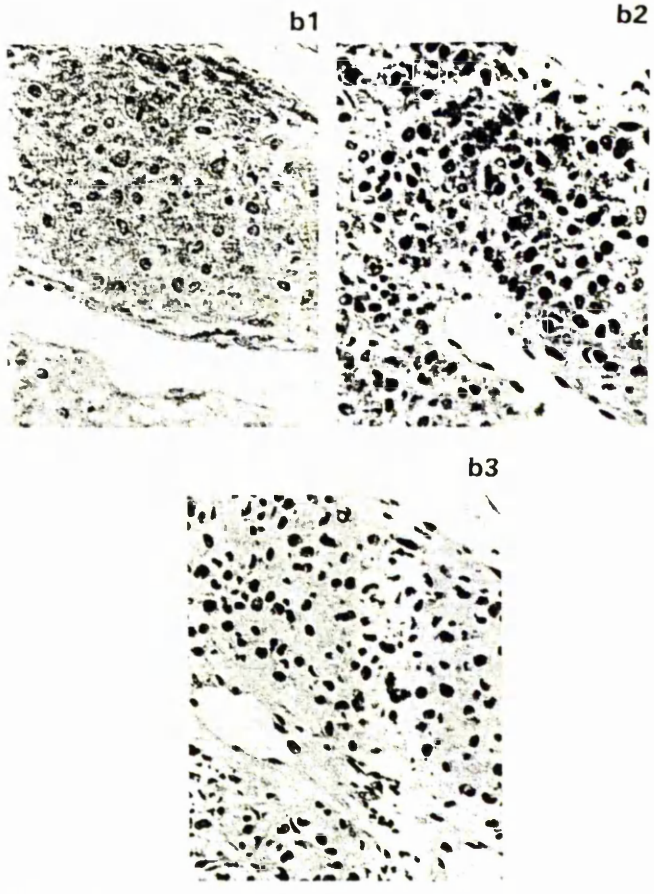


Figure 4 Staining of two human malignant melanoma samples (**a** and **b**) with: (**a1** and **b1**) preimmune serum, (**a2** and **b2**) ATase antiserum and (**a3** and **b3**) haematoxylin and eosin. Note that in (**a2**), the epidermal keratinocytes show strong cytoplasmic and nuclear staining whilst the melanoma cells show mainly nuclear staining. In (**b3**), melanoma cells show nuclear staining with variation in intensity between cells. Vascular endothelial cell nuclei are also strongly stained. Magnification (**a**) × 180 (**b**) × 200.

immunostaining using anti-O^6-methylguanine antibodies (O'Connor *et al.*, 1988) on tissue sections from patients treated with methylating antitumour agents. The significance in carcinogenesis of a heterogeneous cellular distribution of ATase remains to be established, however it is tempting to speculate that higher levels of expression might provide greater protection against the carcinogenic effect of environmental and endogenously produced alkylating agents.

In some of the melanomas, there was a marked intercellular heterogeneity in the staining of the melanoma cells. The major clinical significance of this finding is that if ATase is the principal mechanism of resistance to the toxic effects of antitumour alkylating agents (D'Incalci *et al.*, 1988; Pegg, 1990; Margison & O'Connor, 1990), it might be predicted that whilst a portion of the cells would be killed (assuming that they received a sufficiently high dose of the agent) there would be a number of resistant cells in the population. It is tempting to speculate that it would be these cells that would continue to grow and be responsible for the re-emergence of the disease, unless the numbers were reduced to below a level at which immune surveillance would be effective. Indeed it has been shown that in melanoma, resistance to DTIC or its metabolite MTIC develops rapidly *in vivo* (Clark, 1976) and *in vitro* (Parsons *et al.*, 1982) and in the latter case not by decreased uptake of the drug but *via* enhanced repair of methylation damage in DNA (Parsons *et al.*, 1982; Hayward & Parsons, 1984; Maynard *et al.*, 1988; Foster *et al.*, 1990).

An extensive study is now required in order to establish whether or not there is a correlation between ATase levels in tumour extracts, the number of ATase positively staining cells and the intensity of staining, the response of the tumour to treatment and the frequency of relapse. A large number of tumour types will also need to be examined in order, eventually, to assess whether or not alkylating agent treatment would be appropriate for any individual patient.

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In vivo depletion of O^6-alkylguanine-DNA-alkyltransferase in lymphocytes and melanoma of patients treated with CB10-277, a new DTIC analogue

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Summary. There is increasing evidence to suggest that alkylation of guanine residues in DNA at the O^6 position is the critical cytotoxic event following treatment with dacarbazine (DTIC) and related drugs and that endogenous O^6-alkylguanine-DNA alkyltransferase (ATase) gene expression may be a major factor in resistance to such agents. 1-*p*-Carboxyl-3,3-dimethylphenyltriazene (CB10-277) was recently selected for clinical evaluation as a DTIC analogue with improved solubility, stability and (possibly) metabolic activation. Serial ATase levels were measured in peripheral blood lymphocytes of nine patients and in biopsied melanoma samples of two patients undergoing treatment with 24-h continuous infusion of CB10-277 (12 g/m^2). Wide individual variations in pre-treatment levels as well as in the post-treatment depletion of lymphocyte ATase were seen. Progressive depletion of lymphocyte ATase was seen during continuous infusion of CB10-277 in all patients. Complete suppression of lymphocyte ATase activity occurred in two patients whose pre-treatment ATase levels were low. Immediately following completion of the CB10-277 infusion, the median ATase activity was 17% of pre-treatment levels (range, 0–67%). At 24 h after the end of the infusion, no recovery of lymphocyte ATase activity was observed in six patients, but significant recovery to 50%, 100% and 102% of pre-treatment activity occurred in the other three. In three patients who returned for subsequent cycles of chemotherapy at 4 weeks after the first dose, pre-treatment ATase levels showed a 3- to 4-fold increase relative to the original pre-treatment values. A significant correlation was found between the extent of ATase depletion and the initial lymphocyte ATase levels ($r = 0.725$, $P < 0.05$). Haematological toxicity developed in two patients and was associated with low pre-treatment ATase activity. Depletion of tumour ATase activity was noted in these patients, with residual activity amounting to 8% and 11% of pre-treatment levels, respectively, in the biopsied melanoma tissues. These results indicate extensive metabolism of CB10-277 to a methylating agent capable of mediating alkylation of DNA and subsequent depletion of lymphocyte and tumour ATase levels and further indicate that the effects on lymphocytes may reflect effects on the target tumour.

Abbreviations: ATase, O^6-alkylguanine-DNA alkyltransferase; CB10-277, 1-*p*-carboxyl-3,3-dimethylphenyltriazene; DTIC, dacarbazine, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; MTIC, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide

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Introduction

The effective treatment of metastatic melanoma is disappointing; dacarbazine (DTIC) remains the standard treatment and in a recent cumulative review of 1133 patients with metastatic melanoma, DTIC produced a 21% response rate [2]. Although DTIC has been reported to have good activity in murine tumour models in vivo, one possible reason for its disappointing clinical activity is that following DTIC administration, plasma levels of the active monomethyl metabolite MTIC [5-(3-methyl-1-triazeno)imidazole-4-carboxamide] are much lower in rats and humans than in mice [34]. CB10-277 (Fig. 1) has been shown to have marked activity against experimental murine tumours [10] and melanoma xenografts [16] and is significantly more effective than DTIC in inhibiting the growth of the Walker tumour in the rat [35]. Like other dialkyltriazenes, CB10-277 requires activation by oxidative *N*-demethylation, and the overall production of the putative active monomethyl metabolite in rats was 15-fold that of DTIC, suggesting that species-dependent activation is less likely to be a problem in humans [35]. Thus, due to its structural similarities to DTIC, its superior in vitro stability and solubility and its possibly improved metabolic activation, CB10-277 was selected for clinical evaluation as a DTIC analogue and is currently undergoing phase II trial in metastatic melanoma under the auspices of the

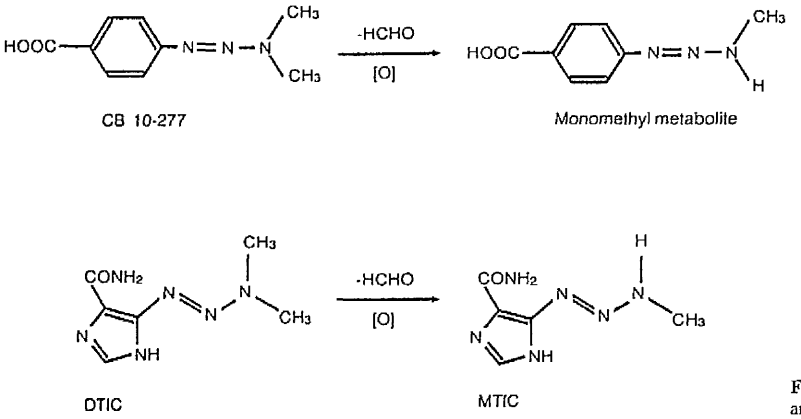

Fig. 1. Structures of CB10-277 and DTIC and their monomethyl metabolites

Cancer Research Campaign (UK) Clinical Trial Committee.

The mechanism of the antitumour activity of DTIC remains unclear, but there is evidence to suggest that the metabolite MTIC methylates DNA, producing, among 12 other lesions, O^6-methylguanine [11]. It has been shown in experimental models that resistance to DTIC and other methylating agents involves the DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase (ATase), which transfers the methyl group from the O^6 position of guanine to an internal cysteine residue in an auto-inactivating stoichiometric reaction. Thus, ATase-deficient cell lines are more sensitive to killing by these alkylating agents [8, 11, 20, 21, 27, 32]. Further evidence for the importance of ATase comes from in vitro studies in which depletion of endogenous ATase by prior exposure to either O^6-methyl- or -benzylguanine or non-toxic doses of simple methylating agents [13, 15, 18, 19, 38, 39] rendered the cells much more sensitive to subsequent treatment with methylating and chloroethylating agents. In addition, melanoma xenografts with high ATase activity are more resistant to DTIC than are xenografts with low activity [16]. The strongest evidence for the cytoprotective role of ATase comes from experiments in which ATase-deficient cultured mammalian cells that had been transfected with cloned pro- or eukaryotic ATase genes and expressed them at high levels were more resistant to the toxic effects of such alkylating agents [3, 7, 12, 14, 23, 36, 37].

Determination of the pre-treatment levels and the depletion rates of ATase in peripheral lymphocytes and tumour tissue following treatment with alkylating agents should provide therapy-relevant information; if ATase depletion is seen in tumour tissue following the administration of CB10-277 or related drugs, one can assume that DNA methylation and, hence, metabolic activation has occurred. In addition, if complete loss of ATase activity is demonstrated, an excess of the toxic O^6-methylguanine lesions and, hence, killing of cells might be expected. Residual ATase activity may indicate resistance or the presence of resistant cells in the tumour population. Indeed, in HL-60 cells the methylating agents need to inactivate ATase before cytotoxicity is observed; in contrast, the chloroethylating agents induce inactivation of ATase only after the cells have been incubated with concentrations 7–12 times higher than the cytotoxic dose [18]. Unlike the methylating agents, the toxic event following treatment with the chloroethylating nitrosoureas is the formation of DNA interstrand cross-links. Thus, ATase measurements may allow us to improve drug dosage and delivery schedules or may indicate useful combinations with the nitrosoureas in which drug resistance is also mediated by ATase.

The present study was based on our recent observations of progressive depletion of ATase activity in peripheral blood lymphocytes of patients with metastatic melanoma treated with sequential DTIC and fotemustine [24]. We examined the extent and the kinetics of ATase depletion and regeneration in peripheral blood lymphocytes of nine patients and in biopsied tumour materials of two patients with metastatic melanoma treated with 24-h continuous infusion of CB10-277.

Patients and methods

Drugs and treatment of patients. CB10-277 (sodium salt, MW 215) was supplied as a lyophilised, pyrogen- and preservative-free powder in 1.000-mg vials by the Developmental Therapeutics Programme, National Cancer Institute (Bethesda, Md., USA). All nine patients had metastatic melanoma and the clinical characteristics are shown in Table 1. Patients received CB10-277 (12 g/m^2) in a 24-h i.v. infusion and treatment was repeated every 28 days. Serial blood samples were collected at various times during the infusion and for 24 h after completion of the first course of CB10-277. Serial blood samples were also taken from two patients who returned for subsequent treatment, whereby the CB10-277 dose was halved (6 g/m^2) because of haematological toxicity. Blood samples were drawn into a 20-ml universal container containing 0.5 ml 0.5% ethylenediaminetetraacetic acid (EDTA) and were stored at 4° C before the isolation of lymphocytes. Tumour biopsies were performed on two patients with metastatic s.c. melanoma nodules before and after CB10-277 treatment: because of the possibility of tissue heterogeneity in ATase expression, biopsy specimens were taken from two adjacent metastatic nodules. Signed informed consent was obtained according to the guidelines of the South Manchester Health Authority Ethical Committee and the Royal College of Physicians, London. The phase II trial of CB10-277 was carried out under the auspices of the Cancer Research Campaign (UK) Clinical Trials Committee.

Table 1. Patients' characteristics and response

Patient/ figure	Age (years)/ sex (M/F)	Metastatic sites	Response	ATase (fmol/mg ± SD)	
				Initial	Nadir
1/Fig. 2a	37/F	Lung/nodes	PD[a]	15 ± 0.4	BD
2/Fig. 2b	54/M	Lung/brain	MR	45 ± 1.1	BD
3/Fig. 2c	20/F	Lung/soft tissue	PD	250 ± 9.5	43 ± 1.0
4/Fig. 2d	50/M	Lung/nodes	PD	206 ± 4.5	24 ± 0.8
5/Fig. 2e	66/F	Lung/nodes	PD	163 ± 0.8	6 ± 0.1
6/Fig. 2f	66/F	Liver	PD	86 ± 2.4	15 ± 0.6
7/Fig. 2g	31/F	Lung/nodes	PD[b]	158 ± 10.0	36 ± 0.3
8/Fig. 2h	65/M	Liver	PD	262 ± 0.9	80 ± 5.0
9/Fig. 2i	53/F	Brain	PD	141 ± 4.7	63 ± 2.5

MR, Mixed response; PD, progressive disease; BD, below detection levels

[a] This patient showed a partial response at chemotherapy cycle 4 but relapsed after cycle 6

[b] This patient showed a partial response at chemotherapy cycle 3 but relapsed after cycle 6

Tissue and ATase extract preparation. Peripheral blood lymphocytes (mononuclear cell fraction) were isolated by centrifugation on Ficoll (Pharmacia, Uppsala, Sweden) [5], washed with phosphate-buffered saline, centrifuged again into a pellet and stored at –20° C. Apparently normal tissue was removed from the melanoma nodules, which were then stored at –20° C. The lymphocyte pellets and melanoma nodules (200–400 mg) were sonicated (10 s at 10 μm peak distance followed by cooling in ice and then resonication for 10 s at 18 μm) in 1 ml buffer I (50 mM TRIS-HCl, 3 mM dithiothreitol, 1 mM EDTA; pH 8.3). Phenylmethysulphonyl fluoride (8.7 mg/ml in ethanol) was added to a final concentration of 87 μg/ml immediately after the second sonication. Sonicates were centrifuged at 16,000 rpm in a microcentrifuge for 10 min at 4° C and supernatants were transferred to fresh tubes in ice and assayed for ATase activity. Protein concentration was measured by the Bradford method using bovine serum albumin as the standard [6].

ATase assay. ATase assay was carried out as previously described [24], with slight modifications. Varying amounts of cell extract were incubated with [^{3}H]-methylnitrosourea-methylated calf-thymus substrate DNA (specific activity, 19 Ci/mmol) at 37° C for 2 h in a total volume of 500 μl of a 1-mg/ml solution of bovine serum albumin in buffer I. After incubation, bovine serum albumin (100 μl of a 10-mg/ml solution in buffer I) and perchloric acid (200 μl of a 4-M solution) were added in rapid succession. A further 2 ml 1 M perchloric acid was added and the mixture was heated at 75° C for 40 min. Samples were clarified by centrifugation, and the precipitates were washed with 4 ml 1 M perchloric acid before being resuspended in 300 μl 0.01 M sodium hydroxide and then dissolved in 3 ml aqueous scintillation fluid (Ecoscint 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 femtomoles of methyl transferred to protein per milligram of total protein in the extract.

Results

ATase depletion and recovery in peripheral lymphocytes

Pre-treatment lymphocyte ATase levels ranged from 15 to 262 fmol/mg protein (median, 158 fmol/mg total protein; Table 1). In all nine patients, progressive depletion of ATase activity was seen during the 24-h continuous infusion of CB10-277. In two patients whose pre-treatment ATase values were 15 and 45 fmol/mg protein, respectively, suppression of ATase was to below detectable levels at 2 and 16 h, respectively (Figs. 2a, b). In the remaining seven patients (Figs. 2c–i), progressive depletion of ATase activity occurred, the median residual ATase activity amounting to 17% of the pre-treatment value at the end of the CB10-277 infusion. No recovery of ATase activity was seen in six patients for up to 24 h after completion of the CB10-277 treatment (Figs. 2a–f) but significant recovery to 50%, 100% and 102% of pre-treatment levels occurred in the other three (Figs. 2g–i). A significant correlation was found between the extent of ATase depletion and the initial lymphocyte ATase levels ($r = 0.725$, $P < 0.05$; Fig. 3). In two individuals (patients 1 and 2, Table 1), serial ATase assays were repeated for subsequent therapy, whereby the CB10-277 dose was halved to 6 g/m^2 because of the development of haematological toxicity. In comparison with therapy cycle 1 (no residual activity), a significantly less extensive ATase depletion was seen at the end of the CB10-277 infusion, with residual ATase activity of amounting to 73% and 85% of the pre-treatment levels, respectively (Fig. 4).

A 3- to 4-fold increase in the pre-treatment lymphocyte ATase activity was seen in three individuals (patients 1, 6 and 9) who returned 4 weeks later for subsequent chemotherapy (Fig. 5). No increase was seen in the other six patients. One of these subjects (patient 6) died rapidly of progressive disease, and another (patient 9) also had documented progressive disease and was subsequently treated with radiotherapy.

ATase activity in tumour biopsies

Pre-treatment ATase levels were 65 and 117 fmol/mg protein, respectively, in two melanoma biopsy samples. These values were within the range of ATase activity detected in our earlier melanoma biopsies [28]. Following CB10-277 treatment, residual ATase activity amounted to 7 and 9 fmol/mg protein, respectively, in further biopsies. The tumour ATase activity appeared to fall in parallel with lymphocyte ATase levels during CB10-277 treatment (Fig. 6). Although it is based on data from only two patients, this is the first clinical demonstration of ATase depletion in tumour tissue following chemotherapy.

Haematological toxicity and clinical response

An interesting finding was the development of haematological toxicity in the two individuals (patients 1 and 2) with the lowest pre-treatment ATase levels (15 and 45 fmol/mg protein, respectively). Both leucopenia (2/3 grade 4, 1/3 grade 4, WHO scale) and thrombocytopenia (2/3 grade 4, WHO scale) occurred during 3 evaluable courses. Following a reduction of the CB10-277 dose by 50%, no further haematological toxicity was documented, but this observation was also associated with less extensive ATase depletion (Fig. 4). In the remaining seven patients whose pre-treatment ATase levels exceeded 50 fmol/mg protein (Figs. 2c–i), no evidence of haematological toxicity was seen. It is noteworthy that in

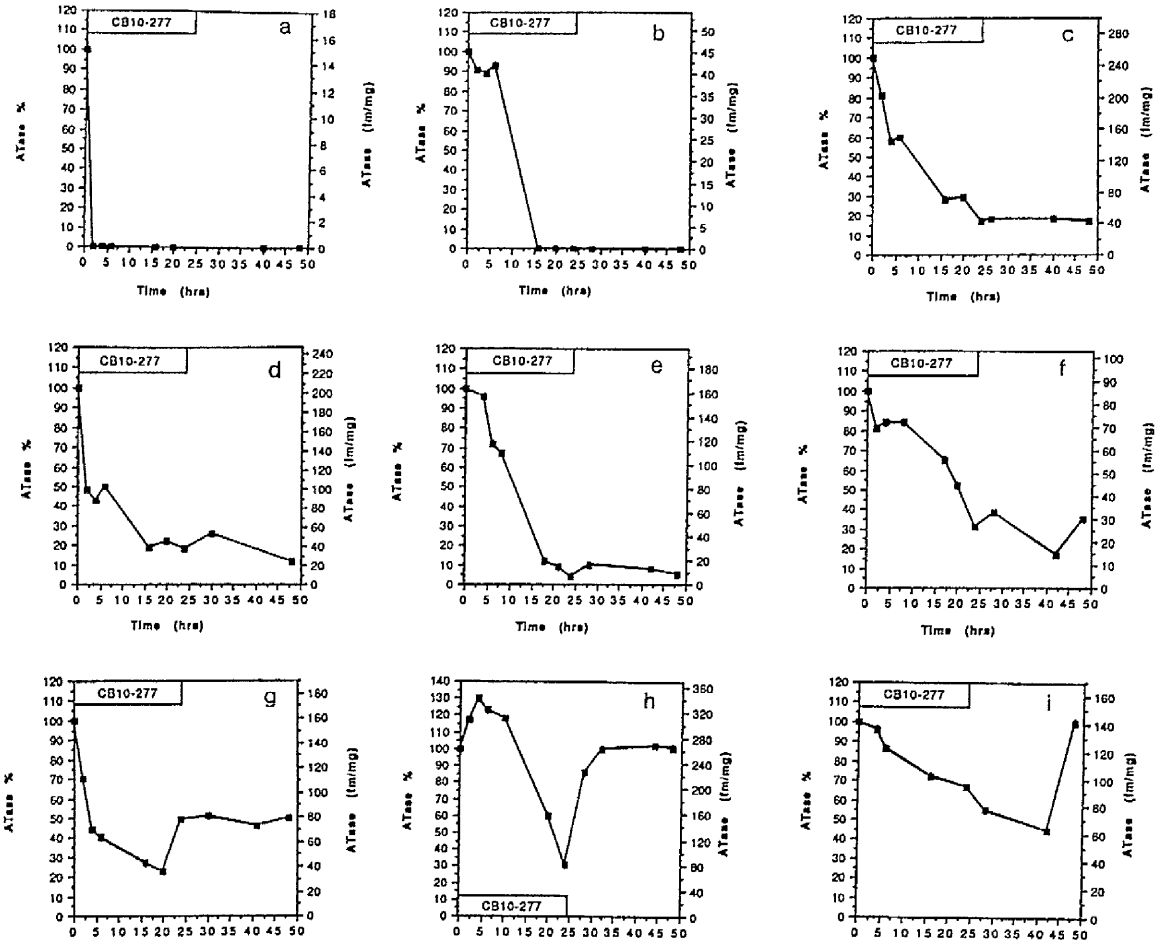

Fig. 2a–i. ATase activity expressed as a percentage of pre-treatment activity and actual levels (fm/mg protein) measured at various times during and after CB10-277 continuous infusion (*box*). Data points represent mean values for 3 estimations, which agreed within ± 10%

the phase I CB10-277 study, haematological toxicity developed following 4 of 8 courses given at a dose of 12 g/m^2 whereas no haematological toxicity was observed when patients received a lower dose of CB10-277 (4.7–8.0 g/m^2) in 31 evaluable courses [30].

The response data for CB10-277 given as a 24-h infusion are shown in Table 1. In one mixed responder, a response occurred in the lung but the disease progressed in the brain (patient 2). This result may not be surprising, as it has been demonstrated that negligible DNA alkylation occurs in the rodent brain as compared with other organs following DTIC administration [29]. There were two early responses, but the disease progressed after the last cycle of chemotherapy (patients 1 and 7). Interestingly, two responders had low pre-treatment lymphocyte ATase levels (patients 1 and 2). No response was seen in the other six patients treated with CB10-277. In the phase I CB10-277 study, 4 responses were seen in 11 evaluable patients when CB10-277 was given as a short infusion [30].

Discussion

The present studies demonstrate that CB10-277 can lead to inactivation of ATase in human peripheral lymphocytes. This indicates that like DTIC, CB10-277 is metabolised, presumably predominantly in the liver [30, 35], to a methylating agent, which is produced in sufficient amounts to react with peripheral blood lymphocytes, generating O^6-methylguanine. The O^6-methylguanine lesions repaired by lymphocyte ATase, causing an apparent depletion of

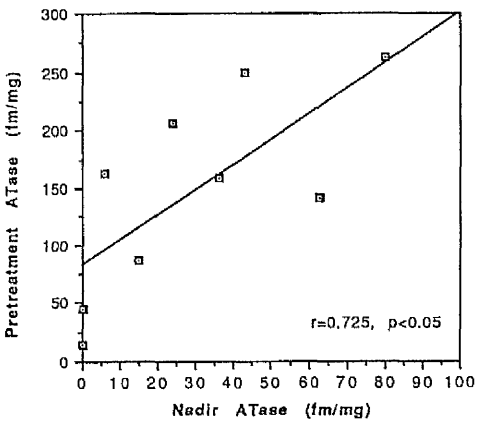

Fig. 3. Correlation between pre-treatment ATase activity and nadir ATase activity

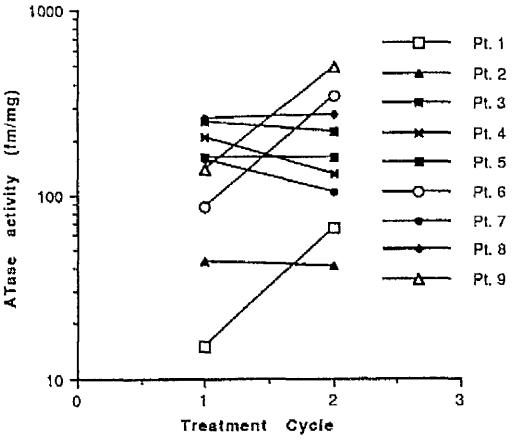

Fig. 5. Pre-treatment lymphocyte ATase activity (fm/mg protein) measured in patients (*Pt.*) 1–9 at the first and second cycles of CB10-277 treatment

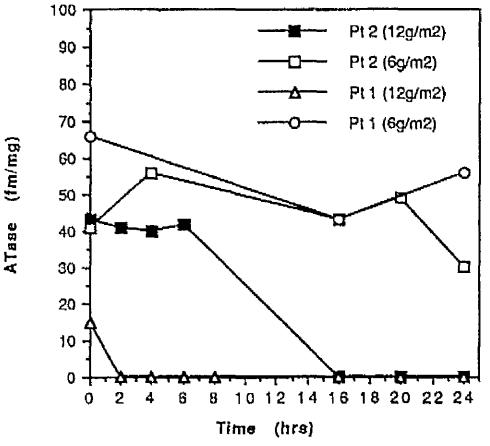

Fig. 4. Comparison of the kinetics of lymphocyte ATase (fm/mg protein) depletion during different cycles in 2 patients (*Pt.*) who received both 12 and 6 g/m² CB10-277

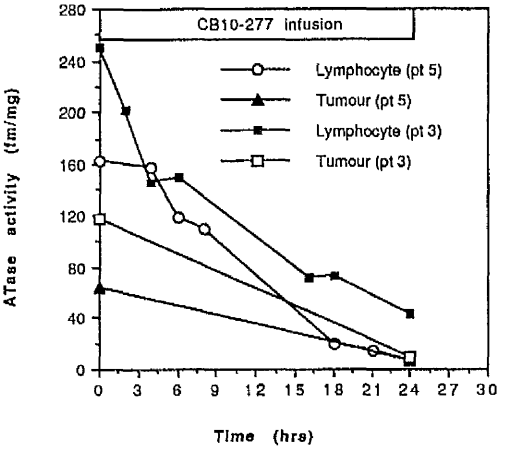

Fig. 6. ATase activity (fm/mg protein) measured in lymphocytes and biopsied melanoma tissues of 2 patients (*Pts.* 3 and 5; Table 1). Biopsies were performed before and immediately after the CB10-277 infusion

ATase activity in lymphocyte extracts. Similar to our earlier findings in patients treated with DTIC given in a single bolus dose [24] the present trial revealed variations in pre-treatment lymphocyte ATase activity as well as in the rate of depletion and recovery of lymphocyte ATase activity in patients given CB10-277 in a 24-h infusion. Complete suppression of lymphocyte ATase activity was seen in two patients whose pre-treatment ATase levels were low. Progressive depletion of lymphocyte ATase activity was seen in the remaining seven patients during CB10-277 infusion. At 24 and 48 h following the initiation of CB10-277 treatment, median lymphocyte ATase activity amounted to 17% of pre-treatment levels. Following completion of the CB10-277 infusion, no recovery of ATase activity was observed in six patients, but recovery to 50%, 100% and 102% of pre-treatment activity had occurred by 24 h in the other three patients, respectively. These variations presumably reflect a combination of individual differences in CB10-277 metabolism and in ATase gene transcription and translation rates.

We also found that low pre-treatment lymphocyte ATase activity was associated with the development of grade 4 haematological toxicity in two patients whose ATase activity fell to undetectable levels at 2 and 16 h after the start of CB10-277 therapy. A major problem in cancer chemotherapy is the prediction of significant and sometimes life-threatening haematological toxicity. Our finding suggests that screening for pre-treatment ATase activity in peripheral lymphocytes may be one way of predicting and, hence, preventing such toxicity when CB10-277 or related

drugs are used. Indeed, we have previously shown that ATase-deficient murine haematopoietic stem cells transfected with an *Escherichia coli* ATase gene are highly resistant to the toxic effects of methylating and chloroethylating agents, which strongly suggests that ATase provides protection against the haematological effects of these agents [22]. In addition, a significant correlation has been demonstrated between ATase levels in peripheral lymphocytes and those in bone marrow myeloid precursors [17]. In the present trial, no further haematological activity developed when the dose of CB10-277 was halved in the abovementioned two patients, and this finding was also associated with incomplete ATase depletion. In phase I studies of CB10-277, no evidence of haematological toxicity was encountered when lower dose of CB10-277 (<12 g/m^2) were used, and this observation was associated with a lower level of monomethyl metabolites [30]. These findings together with our ATase depletion data make it tempting to speculate that less DNA alkylation may have occurred in the bone marrow after treatment with the lower dose. Therefore, dose reduction following the identification of patients with low lymphocyte ATase levels may be a strategy for the prevention of haematological toxicity following chemotherapy. No haematological toxicity was seen in the remaining seven patients receiving CB10-277 (12 g/m^2) whose pre-treatment ATase levels exceeded 50 fmol/mg protein, despite the observation of substantial ATase depletion at 24 and 48 h following CB10-277 administration. A more extensive clinical study is now needed to explore the relationship between pre-treatment lymphocyte ATase activity and bone marrow toxicity in patients treated with CB10-277 and related chemotherapeutic agents.

A novel finding in the present study was an extensive depletion of ATase activity in biopsied tumour tissue following chemotherapy. However, despite this finding, the rate of response by patients with melanoma to treatment with CB10-277 was disappointing, with one mixed response and two early responses being obtained. This suggests that sufficient ATase may have been present or effective regeneration of ATase may have occurred such that the toxic O^6-methylguanine lesions could be effectively repaired, thereby reducing the effectiveness of the chemotherapeutic agent. It might also be that ATase levels are not homogeneous throughout the melanoma nodules [25] and that non-representative samples or nodules with particularly low levels of ATase were taken for ATase assay. In this case, the ATase-deficient tumour cells may be killed, but the resistant population could survive and continue to grow. A further disadvantage of CB10-277 is that it is a monofunctional agent and it has been shown to be 300–2,400 times less cytotoxic than an equivalent dose of a bifunctional agent [4]. Since CB10-277 is capable of depleting tumour ATase activity, one possible approach to overcome drug resistance might be to use a chloroethylnitrosourea after CB10-277, since the principal mechanism of chloroethylnitrosourea resistance involves the same DNA repair enzyme, ATase, which can remove the chloroethyl group from the O^6 position and, hence, prevent the formation of cytotoxic DNA interstrand cross-links [11, 32]. In fact, using sequential DTIC and the new chloroethylating agent fotemustine, we have recently improved the clinical response rate in metastatic melanoma [1] from 21% for single-agent treatment to up to 40%, supporting the use of such drug combination and delivery schedules.

As observed in our earlier study in patients treated with DTIC [24], in the present trial we found that a small number of patients (three) showed an increase in pre-treatment lymphocyte ATase levels when they returned for subsequent CB10-277 therapy. As is the case for many chemotherapeutic agents, resistance to DTIC and MTIC develops rapidly in vivo [9] and in vitro [31], and in the latter case this arises from enhanced repair of the cytotoxic O^6-guanine lesions [21]. This resistance may be related to the DNA cytosine methylation status of the ATase gene controlling regions in surviving lymphocytes following CB10-277 treatment, as it has recently been shown that methylation of the ATase gene is correlated with ATase expression [33]. Another possibility is that expression of ATase activity in peripheral lymphocytes may be heterogeneous [25]; if alkylation-mediated killing of ATase-deficient lymphocytes occurs after CB10-277 treatment, the ATase-proficient lymphocytes will survive and continue to divide, the result being a net increase in ATase specific activity. If this cycle-dependent increase in ATase activity is reflected in the target tumour, it might indicate that tumour resistance will increase with the number of treatment schedules, a highly undesirable effect and one that should be monitored as closely as possible in future studies. There appeared to be a decrease in pre-treatment ATase levels following one cycle of DTIC therapy, which suggests that different agents might produce different longer-term responses, although this possibility needs to be investigated in much larger groups of patients [26]. With the availability of ATase cDNA probes and ATase antibodies, it might be possible to elucidate the molecular mechanism behind the development of drug resistance in tumour tissues.

Clearly, many factors are involved in the prediction of tumour sensitivity in patients. A major clinical difficulty is to obtain serial tumour biopsies during the course of chemotherapy. One approach that would include all of the variable factors discussed above would be to use peripheral lymphocytes to monitor ATase activity so as to identify the optimal chemotherapy dose, schedule and delivery to overcome drug resistance. Our findings in two patients suggest that ATase depletion in peripheral lymphocytes may correlate with that in tumour tissue following CB10-277 administration and this needs to be extended to related drugs and other tumour types.

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Dosage and cycle effects of dacarbazine (DTIC) and fotemustine on O^6-alkylguanine-DNA alkyltransferase in human peripheral blood mononuclear cells

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Summary There is increasing experimental evidence to suggest that endogenous expression of O^6-alkylguanine-DNA-alkyltransferase (ATase) is a major factor in cellular resistance to certain chemotherapeutic agents including dacarbazine (DTIC). We have recently shown wide interindividual variation in the depletion and subsequent regeneration of ATase in peripheral blood mononuclear cells (PMCs) following DTIC and this has now been extended to ascertain whether or not depletion is related to dosage of DTIC used and repeated treatment cycles of chemotherapy. ATase levels were measured in three groups of 25 patients (pts) up to 24 h after receiving DTIC at 400 mg m^{-2}, 500 mg m^{-2} or 800 mg m^{-2}. Each group also received fotemustine (100 mg m^{-2}), 4 h after DTIC. The lowest extent of ATase depletion (highest nadir ATase) was seen in patients receiving 400 mg m^{-2}. The mean nadir ATase, expressed as a percentage of pre-treatment ATase, was respectively 56.3%, 26.4% and 23.9% for 400 mg m^{-2}, 500 mg m^{-2} and 800 mg m^{-2}. The median nadir of ATase activity for pts receiving 800 mg m^{-2} pts was at 4–6 h and for pts given lower doses it was at 2–3 h. In addition, repeated measures analysis of variance of observations before chemotherapy, then at 2, 3, 4, 6 and 18 h after chemotherapy provides some evidence that ATase was depleted to a lesser extent after cycle 1 than after subsequent cycles ($P = 0.025$). It also provides evidence that the change in ATase activity over time varied with dose and cycle. The findings can be interpreted on the basis of a dosage-dependent metabolism of DTIC to an agent capable of methylation of DNA and subsequent depletion of PMC ATase: with higher DTIC doses, the extent of ATase depletion may be limited by the pharmacokinetics of DTIC metabolism. PMC ATase was measured in another group of 8 pts at various times after receiving only fotemustine (100 mg m^{-2}) and in contrast to DTIC, no ATase depletion was seen suggesting that insufficient concentrations of fotemustine and/or its metabolites were available to react with DNA to produce a depletion of PMC ATase activity.

In the treatment of metastatic melanoma, dacarbazine [5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; DTIC] is considered the single most effective chemotherapeutic agent available (Balch *et al.*, 1989; Comis, 1976). It undergoes metabolic N-demethylation to give the cytotoxic monomethyl triazene, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC) which methylates DNA, producing among 12 other lesions, O^6-methylguanine (Meer *et al.*, 1986). There is increasing evidence to suggest that O^6-methylguanine is the principal cytotoxic event following DTIC and that O^6-alkylguanine-DNA alkyltransferase (ATase) gene expression may be a major factor in cellular resistance to such agents. ATase is able to transfer the methyl group from the O^6 position of guanine to an internal cysteine residue in an auto-inactivating stoichiometric reaction. Experimental models using ATase-deficient cell lines or xenografts show them to be more sensitive to DTIC than lines or xenografts with high activity (Catapano *et al.*, 1987; D'Incalci *et al.*, 1988; Foster *et al.*, 1990; Gibson *et al.*, 1986*a*; Hayward and Parsons, 1984; Lunn & Harris, 1988). The strongest evidence for the cytotoxic effects of O^6-alkylguanine in DNA comes from ATase cDNA transfection experiments which show that expression of prokaryotic or eukaryotic ATase cDNA in mammalian cells protects them against the toxic effects to these agents (Brennand and Margison, 1986; Jelinek *et al.*, 1988; Kataoka *et al.*, 1986; Samson *et al.*, 1986; Kaina *et al.*, 1991).

We recently showed that there was wide interindividual variation in the DTIC-mediated depletion and subsequent recovery of ATase levels in human peripheral blood cells (Lee *et al.*, 1991*a*) and this work has now been extended to explore whether or not the rate and extent of depletion and regeneration of ATase activity is related to the dosage of DTIC used or the number of treatment cycles. Identifying the time to reach the ATase nadir and the extent of ATase depletion with different DTIC dosage may have important therapeutic implications especially when DTIC is combined with the subsequent administration of a chloroethylating nitrosourea. In this case, drug resistance appears to involve the same ATase DNA repair enzymes which remove the chloroethyl lesions from the O^6-position of guanine, thereby preventing the formation of a cytotoxic DNA interstrand cross-link (see Lee *et al.*, 1991*a*). Theoretically, enhanced therapeutic effects might be obtained when the nitrosourea is administered at the nadir of ATase activity following DTIC treatment assuming that the effect in peripheral mononuclear cells reflects that of tumour tissues.

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Materials and methods

Patients and blood samples

Blood samples were collected from 30 treatment cycles of 25 pts with metastatic melanoma treated with sequential DTIC and fotemustine chemotherapy. Approval was obtained from the local ethical committee and all pts gave informed consent for the study. Pts received DTIC at fixed doses (for each pt) of 400, 500 or 800 mg m^{-2} by i.v. infusion followed by fotemustine 100 mg m^{-2}, 4 h later. Treatment was repeated every 28 days and the number of cycles given depended on the individual pts response. Blood samples were collected just before chemotherapy and at 1, 2, 3, 4, 6 and 18 h after DTIC infusion; in addition, 5 h samples were collected for the 400 mg m^{-2} patients. Blood was drawn into a 20 ml univeral container containing 0.5% EDTA and stored at 4°C before isolation of PMCs. Fourteen sets of samples from were also collected from another group of 8 pts with metastatic melanoma receiving only fotemustine (100 mg m^{-2}).

When the study was initiated, no effect of treatment cycle on ATase concentrations was anticipated. As a result samples were not taken from pts on the same cycles. Some pts had samples taken on cycle 1, other on cycle 2, etc. and some pts had samples taken on more than one cycle. In the event, the

cycle in which ATase was measured had an effect on the ATase level and as a result the dose and cycle effects are confounded. To avoid this, samples were randomly selected for each analysis so that no more than one set of observations was used from a patient. As this resulted in loss of data, we present this aspect of the study as a guide to possible future studies. The principal method of statistical analysis was analysis of variance, with repeated measures analysis of variance used for analysing the changes over time and 'survival' analysis for estimating the time to the ATase nadir. The computer software package BMDP was used for most analyses.

Isolation of mononuclear cells, ATase extraction and assay

This was carried out as described previously (Lee *et al.*, 1991*a*). Briefly, the PMCs were isolated by centrifugation on Ficoll (Pharmacia, Uppsala, Sweden) (Boyum, 1968), sonicated and the supernatants were assayed using [^{3}H]-methylated DNA containing 0.01 picomoles O^6-methylguanine per μg DNA. Activity was expressed as fmoles methyl transferred to protein per mg of protein. Measurements were in triplicate. The mean ATase values are presented ± standard error of the mean.

Results

PMC ATase activity following DTIC and fotemustine

The change in ATase activity over time was analysed using repeated measures analysis of variance, unweighted means method (Winer *et al.*, 1991*a*). The factors used were the three doses and the cycles, grouped as cycle 1 and cycles 2 to 6. The level of significance was $P \leqslant 0.05$.

Dose effects

In the three groups of pts receiving 400, 500 and 800 mg m^{-2} DTIC, the mean pretreatment PMC ATase levels and their standard errors before the start of the 1st cycle were 230 ± 16, 233 ± 44 and 211 ± 18 fm mg^{-1} respectively. A one-way analysis of variance revealed no statistically significant difference ($P = 0.936$) and therefore subsequent effects of ATase levels were not influenced by inadvertant preselection bias in the dosage groups. The analysis used all cases with measurements on cycle 1.

Following intravenous DTIC administration, progressive depletion of ATase activity was seen (Figure 1). The changes with time were, as expected from earlier studies (Lee *et al.*, 1991*a*) very highly significant, but more interestingly so was the interaction between time and dose ($P < 0.0001$). This shows that ATase activity changes differently over time for different doses. The least extensive ATase depletion was generally seen in pts receiving 400 mg m^{-2} (Figure 2). The mean nadir ATase, expressed as a percentage of pretreatment ATase, was 56.3%, 26.4% and 23.9% for 400, 500 and 800 mg m^{-2} respectively (Figure 2) and a two way analysis of variance, which included the effect of the two cycle groups, confirmed that the observed difference in the nadir between the doses of DTIC was significant at the 5% level ($P < 0.005$).

The time to the nadir of ATase activity was analysed like a survival analysis and tested with the log rank test. Cases whose lowest ATase levels were after 6 h can be considered as 'survivors' whether or not the 18 h figure is higher: it is not possible to indicate whether or when in the 12 h interval the nadir has occurred and 6 h is therefore a censored time. Pts who received 400 mg m^{-2} were pooled with pts who received 500 mg m^{-2} so that there were enough cases in each

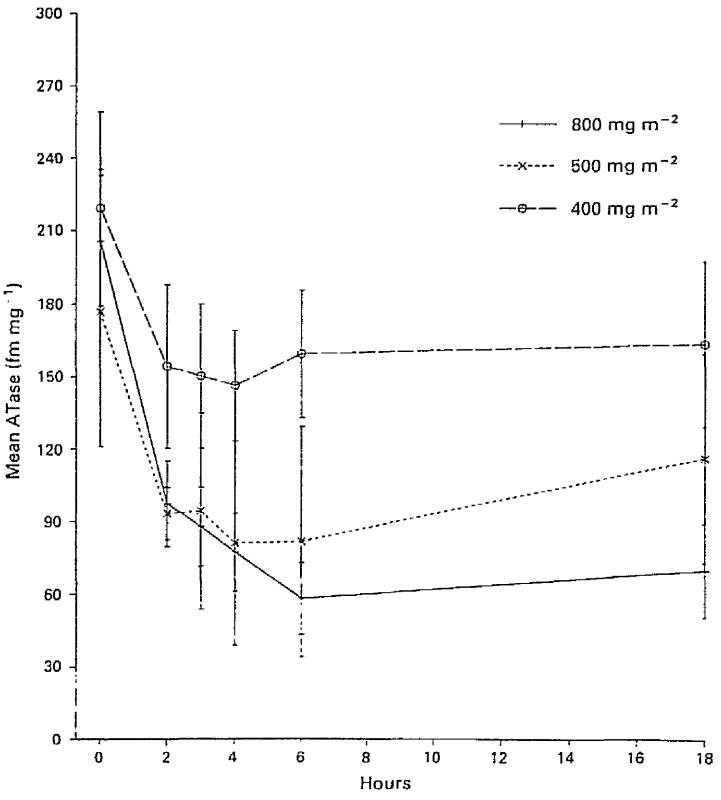

Figure 1 Lymphocyte ATase activity before and after chemotherapy with 400 (o), 500 (x) or 800 mg m^{-2} (+) DTIC. Fotemustine was administered 4 h after DTIC. Values shown are the means ± standard error. An average of >9, 4 and 10 pts were analysed for each time point in the 400, 500 and 800 mg m^{-2} groups, respectively.

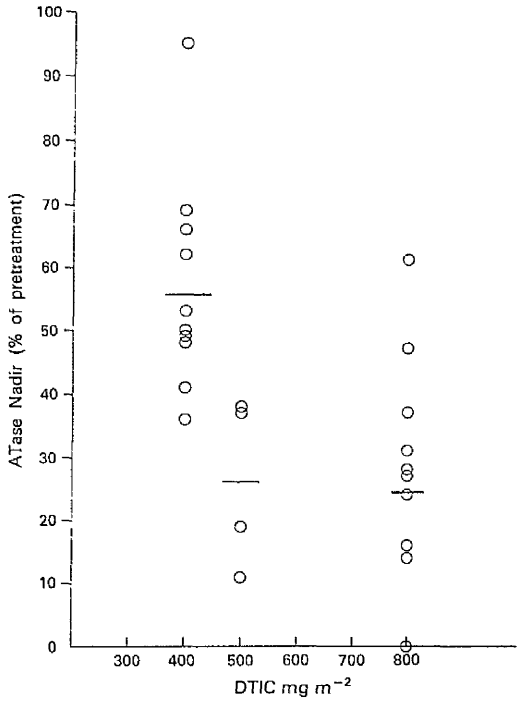

Figure 2 Relationship between DTIC dosage and nadir ATase activity expressed as % of the pretreatment level. The mean values are shown as a horizontal bar.

group for an effective analysis and seven observations were deleted so that no patient appeared more than once in the analysis. The time to nadir was significantly less for pts receiving 400 mg m^{-2} or 500 mg m^{-2} (median 2–3 h) than 800 mg m^{-2} (median 5–6 h) ($P = 0.0407$).

In less than half of the patients, there was a post nadir increase in ATase levels by 18 h after treatment. In most cases this was slight, ranging from 6% to 30% of the pretreatment levels. However, in two pts receiving 400 mg m^{-2} and one receiving 500 mg m^{-2}, recovery was very extensive and rapid, attaining close to pretreatment levels: in the former pts this was associated with an earlier ATase nadir. In order to establish whether the increase in ATase by 18 h was statistically significant, the mean ATase at 18 h was compared with the of the nadir. The nadir was taken as being between 4 and 6 h at which time the means were 99.6 fm/mg and 99.4 fm mg^{-1} respectively: the 18 h mean was 111.7 fm mg^{-1}. The difference (12.2 fm mg^{-1}) was not significant at the 5% level using the Tukey method for comparing means (Winer *et al.*, 1991*b*). Thus by 18 h the mean ATase had not recovered and in fact in some pts the 18 h value is the lowest recorded, although this does not necessarily indicate that it is the nadir.

Cycle effects

The mean pre-treatment ATase activity would seen to be higher in cycle 1 (220 ± 25 fm mg^{-1}) than in cycles 2–6 (171 ± 31 gm mg^{-1}) (Figure 3). However, the *P*-value from an analysis of variance is greater than 0.1. Hence the observed difference could be due to chance. Seven observations were deleted so that no patient appeared more than once in the analysis and the listed means were derived from the remaining cases.

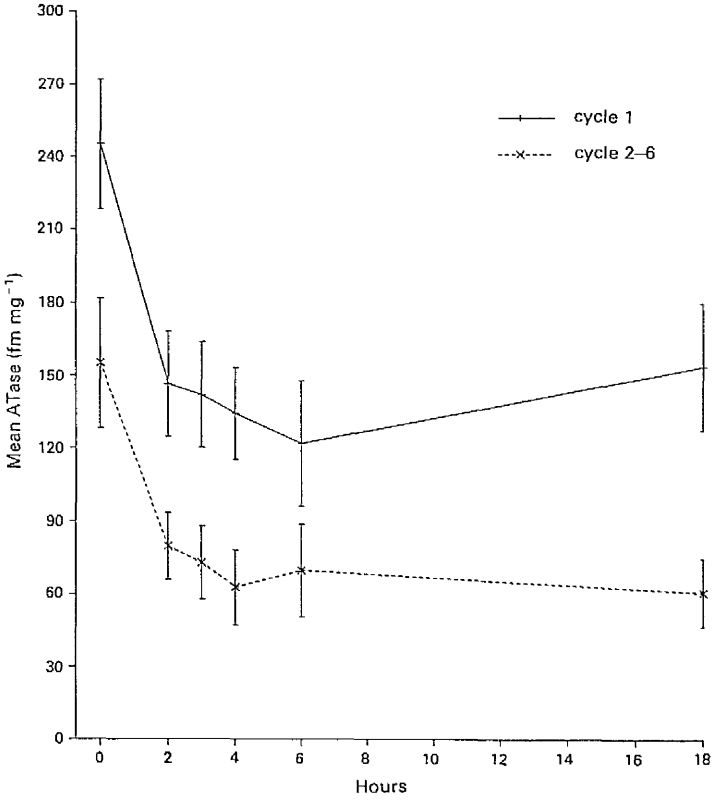

Figure 3 Lymphocyte ATase activity before and after chemotherapy with different treatment cycles of DTIC. Values shown are the means ± 95% confidence interval. There were 15 and 8 pts in the cycle 1(+) and subsequent combined cycle (x) groups, respectively.

The overall difference in ATase levels between the two cycle groups was significant ($P < 0.025$) but the magnitude of the difference greatly depended on the number of hours after CT (group versus time interaction $P < 0.0001$). This shows that the effect of cycle changes over the sequence of measurements. The mean nadir ATase levels were 41.7% of the pretreatment level for cycle 1 and 29.8% for cycles 2 to 6 but the previously mentioned 2-way analysis of variance provided inadequate evidence for an effect of cycle at the nadir ($P > 0.1$). The analysis and the percentages listed above were based on 23 cases. There was no significant difference between the cycles in the time to nadir using the log rank test ($P = 0.285$).

PMC ATase activity following fotemustine alone

The mean pre-treatment ATase level for pts receiving fotemustine only was 242 ± 30 fm mg^{-1} for cycle 1 and 176 ± 21 fm mg^{-1} for cycles 2 and 3 combined. All pts were given three cycles of treatment and most pts were studied on more than one cycle, so the pts which were used in the analysis could act as their own controls. Each patient analysed contributed one observation only to both cycle 1 and the combined cycles 2 and 3 and the means quoted above were for the cases used in the analysis. A Wilcoxon paired sample test was used to test the difference between the cycles and a P value of 0.0625 provided inadequate evidence that the observed difference was due to anything other than chance.

The ATase levels at 3–4 h or at 16–18 h after fotemustine were not significantly different from the pretreatment values ($P > 0.9$) and there was no significant difference ($P > 0.9$) between ATase levels when different treatment cycles were compared (Figure 4).

Discussion

In the present study, we were able to demonstrate DTIC-induced depletion of ATase activity in human PMCs. This is consistent with the metabolism of DTIC to the monomethyl metabolite, MTIC which is produced in sufficient amounts to react with DNA in PMCs to generate O^6-methylguanine. This is stoichiometrically repaired by ATase causing an apparent decrease of PMC ATase activity. The nadir of ATase activity generally occurred later in pts receiving 800 mg m^{-2} than in the lower dosage groups; pts receiving 400 mg m^{-2} seemed to have the lowest extent of ATase depletion (highest ATase nadir) with a mean ATase nadir of 56.3% of pretreatment level. In contrast, pts receiving 500 and 800 mg m^{-2} DTIC had a lower mean nadir PMC activity of 26.4% and 23.9%. This suggests that the pharmacokinetics of DTIC is dose-dependent. Indeed, it has been shown that high-dose DTIC (850–1980 mg m^{-2}) follows nonlinear pharmacokinetics with saturation occurring in the metabolism and also a slower distribution and disposition rate when compared to lower dose DTIC (Breithaupt *et al.*, 1982; Buesa & Urrechaga, 1991; Loo *et al.*, 1968; Skibba *et al.*, 1969). The later nadir in ATase activity seen with 800 mg m^{-2} DTIC in contrast to 400 mg m^{-2} may be related to the more protracted production of alkylating metabolites.

Whilst ATase recovery by 18 h was generally not substantial and evident in less than half of the pts, in two pts given 400 mg m^{-2}, the ATase nadir was around 2 h and activity recovered rapidly to attain close to pretreatment levels. These results further suggest interindividual differences in the continued availability of methylating metabolites and/or in the *de novo* synthesis rates for ATase. In view of the possible saturable pharmacokinetics with high dose DTIC, it would be clearly interesting to administer DTIC by continuous infusion or pulsed low doses in order to assess whether or not a complete loss of ATase activity could be achieved using PMCs as a target.

Despite wide interindividual variations in pretreatment levels, post-treatment DTIC-induced PMC ATase depletion and subsequent recovery, the data suggested that ATase depletion occurred to a lesser mean extent in treatment cycle 1 compared to subsequent treatment cycles. A similar finding was reported in some pts treated with procarbazine (Sagher *et al.*, 1989). This effect might be a consequence of the initial doses of methylating agent (or in the present case, fotemustine) increasing the capacity for metabolic activation of subsequent doses leading to increased levels of DNA methylation. If a similar increase occurred in tumour cells it might be expected that later treatment cycles might be more therapeutically effective than the first cycle.

Whilst the mean pretreatment ATase activity was apparently reduced in subsequent treatment cycles in comparison to cycle 1, in the present study this was not statistically significant and is unlikely to contribute to the differential extent of ATase depletion in later cycles. If, however, a reduction in the mean pre-treatment ATase levels is observed in a larger group of pts, it might presumably be the result of a drug-mediated selection of PMCs expressing lower levels of ATase, although how this occurs is not clear at present.

No statistically significant change in PMC ATase activity occurred in pts treated with 100 mg m^{-2} fotemustine alone although the possibility that synergistic effect on ATase depletion might have occurred in patients given DTIC prior to fotemustine cannot be excluded. This suggests that

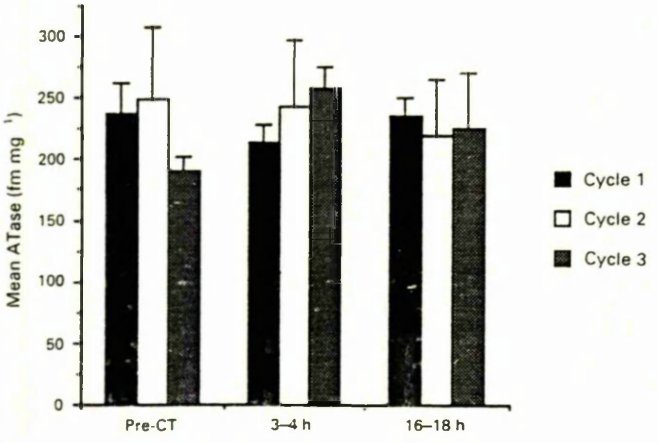

Figure 4 Mean lymphocyte ATase activity before and after chemotherapy with fotemustine alone (100 mg m^{-2}). Values shown are the mean ± standard error of the mean for treatment cycles 1 to 3.

insufficient concentrations of fotemustine or its metabolites were available to react with PMC DNA to produce a detectable lowering of ATase activity. A similar finding was reported for human PMCs treated with low dose BCNU (40–200 mg m^{-2}) although a statistically significant reduction in PMC ATase activity was seen after high dose BCNU (350 mg m^{-2}) (Gerson, 1989). Experimental models have repeatedly shown that depletion of ATase (approximately 60–90% depletion) can sensitise tumour cells to chloroethylnitrosoureas, resulting in a 2- to 12- fold reduction in the 50% lethal dose of these compounds. Greater extents of sensitisation were seen in tumour cells expressing high ATase activity than cells with low levels of activity (Dolan *et al.*, 1985; Dolan *et al.*, 1991; Gerson *et al.*, 1985; Gibson *et al.*, 1986*b*; Zlotogorski & Erickson, 1984) If ATase is the principal mechanism of tumour cell resistance to methylating and chloroethylating agents and if the results obtained with PMC can be extrapolated to tumour cells, our findings would advocate the use of sequential DTIC then fotemustine treatment (as here) rather then a schedule where DTIC and fotemustine are administered concurrently or in which fotemustine is given before DTIC.

Whilst such extrapolations should be considered with appropriate caution, they should also be assessed in relation to the available relevant clinical data. Thus in melanoma pts, DTIC alone (including high dosage) regularly produces a response rate of 20% (Balch *et al.*, 1989; Comis, 1976; Cowan & Bergsagel, 1971; Pritchard *et al.*, 1980); fotemustine alone produces a 24% response rate (Jacquillat *et al.*, 1990). However in pts treated with sequential DTIC and fotemustine, we achieved an overall response rate of 34% and there was a trend towards higher response rate with pts treated with 800 mg m^{-2} DTIC, followed by 500 mg m^{-2} and 400 mg m^{-2} DTIC respectively (Aamdal *et al.*, 1991; Lee *et al.*, 1991*b*). These results provide circumstantial evidence in support of the use of PMC ATase levels as a monitor for those in tumour tissues. Further support is provided by the extent of the toxic side effects of the treatment. Thus there was a statistically significant dosage-dependent development of severe haematological toxicity ($P<0.01$) in the three groups of pts analysed (Lee & Thatcher, unpublished data). It is tempting to attribute this to greater DNA alkylation with higher dosage DTIC: bone marrow has one of the lower ATase levels of the human tissues examined so far (Gerson *et al.*, 1986) and this, together with the possibility that ATase depletion might increase with treatment cycle, may account for its greater sensitivity to the toxic effects of DTIC. Indeed we have previously shown that ATase-deficient chloroethylnitrosourea-sensitive murine haemopoietic stem cells transfected with a bacterial ATase gene become highly resistant to the toxic effects of methylating and chloroethylating agents, strongly suggesting that ATase would protect against the haematological effects of these agents (Jelinek *et al.*, 1988); other work (Dumenco *et al.*, 1989) supports this finding.

As the bone marrow is generally the principal target organ for the toxic side effects of these agents, it may be possible to protect this tissue by transfection of human pluripotent stem cells with an ATase cDNA. These cells may be returned to the pts in the course of bone marrow transplantation. If this achieves high levels of ATase expression, one can envisage treating pts with high dose alkylating agents which, assuming a linear dose response curve, might result in the elimination of the tumour but spare the ATase transfected bone marrow precursors.

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Sequential administration of varying doses of dacarbazine and fotemustine in advanced malignant melanoma

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Summary There is increasing experimental evidence to suggest that expression of O^6-alkylguanine-DNA-alkyltransferase (ATase) is a major factor in resistance to dacarbazine (DTIC). We recently demonstrated a progressive ATase depletion in human peripheral lymphocytes with nadir levels occurring at 4–6 h after DTIC administration (Lee *et al.*, 1991). Therefore in an attempt to improve the clinical response rate of DTIC, fotemustine was administered 4 h after DTIC administration; since in the case of fotemustine, ATase removes the chloroethyl lesions from the O^6-position of guanine, thereby preventing the formation of the cytotoxic cross-links. Sixty patients with widely metastatic melanoma received DTIC at 400, 500 or 800 mg m^{-2} followed by fotemustine (100 mg m^{-1}) at 4 h after DTIC administration. Treatment was repeated every 28 days with a total of 169 cycles of chemotherapy administered; 75, 57 and 37 treatment cycles with 400, 500 and 800 mg m^{-2} DTIC groups respectively. Eighteen of the 60 patients responded (with three complete response); response rates were linearly related to dose, being 24%, 30% and 40% in patients receiving 400, 500 and 800 mg m^{-2} of DTIC respectively and the overall response rate was 30%. Median survival was 3.6 months (range, 1–15 months) with no statistically significant difference between the different DTIC treatment groups ($P = 0.67$). Nine patients are alive at 5 to 26 months (median 10 months); three patients with no tumour and five patients with stable disease. A statistically significant relationship was seen between the development of severe haematological toxicity (WHO ≥ 3) with increasing dosage of DTIC and significant subclinical pulmonary damage was seen in 11 patients where the lung function was monitored during the course of treatment. In conclusion, it appears that with this small group of patients, escalation of DTIC dosage might not significantly affect response rates but does increase haematological toxicity. The present study provides a framework for other studies in an attempt to modulate ATase-mediated drug resistance in tumour tissues but the associated toxicity will need careful monitoring.

Dimethyl-triazeno-imidazole-carboxamide (Dacarbazine, DTIC) is still considered one of the more effective chemotherapeutic agents used in the treatment of advanced disseminated melanoma and regularly produces a response rate of approximately 20% (Comis, 1976; Balch *et al.*, 1989). After DTIC, the nitrosoureas are considered the second most effective agents and produce an approximately 15% response rate (Comis, 1976; Balch *et al.*, 1989). The doses of DTIC used have ranged from 2 mg kg^{-1} for 10 days to 1450 mg m^{-2} as a single bolus every 4 to 6 weeks (Cowan & Bergsagel, 1971; Comis, 1976; Mastrangelo *et al.*, 1982). Infusion of DTIC over 24 h has also been explored (Thatcher *et al.*, 1985). The most popular DTIC schedule consists of 250 mg m^{-2} daily intravenously for 5 consecutive days, treatment being repeated every 3–4 weeks (Mastrangelo *et al.*, 1982; Geeraerts & Nathanson, 1986). Combination chemotherapy has added little to the response rate and survival duration and frequently resulted in significant increases in toxicity (Mastrangelo *et al.*, 1982; Geeraerts & Nathanson, 1986; McClay & Mastrangelo, 1988).

DTIC undergoes metabolic N-demethylation to give the cytotoxic monomethyl triazene, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC) which methylates DNA, producing among 12 other lesions, O^6-methylguanine (Meer *et al.*, 1986). There is increasing evidence to suggest that O^6-methylguanine is the principal cytotoxic event following DTIC and that O^6-alkylguanine-DNA alkyltransferase (ATase) expression may be a major factor in cellular resistance to such agents (D'Incalci *et al.*, 1988; Pegg, 1990). ATase is able to transfer the methyl group from the O^6 position of guanine to an internal cysteine residue in an auto-inactivating stoichiometric reaction. Experimental models using ATase-deficient cell lines or xenografts show them to be more sensitive to DTIC than lines or xenografts with high activity (Hayward & Parsons, 1984; Gibson *et al.*, 1986; Catapano *et al.*, 1987; D'Incalci *et al.*, 1988; Lunn & Harris, 1988; Foster *et al.*, 1990). The strongest evidence for the cytotoxic effects of O^6-alkylguanine in DNA comes from ATase cDNA transfection experiments which show that expression of prokaryotic or eukaryotic ATase cDNA in mammalian cells protects them against the toxic effects to these agents (Brennand & Margison, 1986; Kataoka *et al.*, 1986; Samson *et al.*, 1986; Kaina *et al.*, 1991).

We recently demonstrated a progressive ATase depletion in human peripheral lymphocytes with nadir ATase levels occurring at 4–6 h after DTIC administration (Lee *et al.*, 1991). Assuming that a similar depletion effect occurs in the tumour cells, an enhanced therapeutic effect might be obtained if a nitrosourea is administered at the nadir of ATase activity following DTIC treatment since in the case of nitrosoureas, ATase removes the chloroethyl lesions from the O^6-position of guanine, thereby preventing the formation of cytotoxic cross-links (D'Incalci *et al.*, 1988; Pegg, 1990). Therefore in an attempt to improve the clinical response rate of DTIC, fotemustine was administered at 4 h after DTIC administration, the time which was shown to be associated with maximal ATase depletion in the peripheral blood lymphocytes. Fotemustine is a new drug containing a phosphonoalanine carrier grafted to the nitrosourea radical and it has shown promising clinical efficacy (Jacquillat *et al.*, 1990). The present study evaluates and compares the clinical results of using three different doses of DTIC (400, 500 and 800 mg m^{-2}) with fotemustine (100 mg m^{-2}).

Materials and methods

Sixty patients with widely metastatic malignant melanoma were entered into the study protocol. The protocol required histological documentation of metastatic melanoma, measurable metastasis, Karnofsky index ≥ 50, a white blood count $\geq 4.0 \times 10^9 l^{-1}$, a platelet count $\geq 100 \times 10^9 l^{-1}$, a haemoglobin $\geq 11 g l^{-1}$ and no major disturbance of renal or hepatic biochemistry. Local ethical approval was obtained for the study.

The median age was 55 years (range, 17–75 years), and there was 28 males and 32 females. The median time from

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surgery to first metastasis was 3 years (range 0 to 12 years). Number of patients with metastatic sites were: five patients with non-visceral sites, 21 patients with visceral sites and 34 patients with both visceral and non-visceral sites. Twelve patients had prior chemotherapy and 13 patients had localised radiotherapy, but other metastatic sites were available for evaluation in the study.

Patients received DTIC at 400, 500 or 800 mg m^{-2} by i.v. infusion over 10 min followed by fotemustine (100 mg m^{-2}) over 30 min at 4 h after DTIC. Treatment was repeated every 28 days. A total of 169 cycles of chemotherapy were administered; 75, 57 and 37 treatment cycles in the 400, 500 and 800 mg m^{-2} DTIC groups respectively.

Tumour response and toxicity assessment used the World Health Organization (WHO) criteria (WHO, 1979). Complete response was defined as the disappearance of all known disease for at least 4 weeks; partial response was defined as a reduction in the sum of the products of the largest perpendicular diameters of each lesion by at least 50% for at least 4 weeks; stable disease was defined as a decrease of less than 50% in total tumour size, or an increase of less than 25% in the size of one or more lesions. Toxicity was recorded and analysed using the WHO grading system.

Lung function tests were also performed in 11 patients following the development of an adult respiratory distress type syndrome in one patient. The tests were performed at the Lung Function Unit at Wythenshaw Hospital, Manchester. Routine spirometry was performed using a Gould Pulmonet III Spirometer (cardiokinetics, Salford, UK). Total lung capacity was measured by body plethysmography (Eric Jaeger (UK) Ltd). Carbon monoxide transfer factor was measured by the single breadth method (PK Morgan Ltd, Chatham, UK).

Results

Comparability of different DTIC dosage groups

As shown in Table I, the three treatment groups were well balanced with no statistical differences (chi-squared tests) in pretreatment characteristic in terms of distributions of age, sex, performance status, number of metastatic organ sites involved, prior radiotherapy or chemotherapy and number of treatment cycles given.

Responses

In the 60 patients studied, the overall response rate was 30% with 18 patients responding to therapy: when based on the different treatment groups, the mean response rates were 24%, 30% and 40% in patients receiving 400, 500 and 800 mg m^{-2} of DTIC respectively (Table II). Despite this apparently linear DTIC dosage-dependent clinical response rate, there was no statistically significant difference in response with different DTIC dosage levels ($P = 0.29$, test for linear trend). Two complete responders were seen in patients receiving 400 mg m^{-2} and one in patients receiving 500 mg m^{-2}. Four patients had stable disease and fifteen patients had partial response. The median duration of chemotherapy response was 5 months (range, 1–9 months). The sites of response for the metastatic sites available is shown in Table III.

Haematological toxicity

Table IV shows the haematological toxic effects seen with different DTIC dosage. Severe anaemia (WHO $\geqslant$ grade 3), neutropenia (WHO $\geqslant$ grade 3) and thrombocytopenia (WHO $\geqslant$ grade 3) occurred more often with higher dosage DTIC and this was statistically significant. Anaemia was seen more often in the later treatment cycles (after cycle 2) than early treatment cycles.

Pulmonary toxicity

One patient with disseminated lymphadenopathy responding to chemotherapy died from an acute respiratory distress type syndrome. This patient received 500 mg m^{-2} DTIC and 100 mg m^{-2} fotemustine. The history was of 10 days dry cough and increasing breathlessness. The CXR showed a bilateral alveolar shadowing and echocardiogram demon-

Table I Comparison of patients characteristics

	DTIC Dosage (mg m^{-2})			
	400	*500*	*800*	*P-value*[a]
Patients (n)	25	20	15	
Sex (M/F)	13/12	11/9	4/11	$P = 0.2$
KP ($\geqslant 70/<70$)	22/3	17/3	11/4	$P = 0.09$
Age ($\geqslant 40$ yrs/<40 yrs)	21/4	16/4	12/3	$P = 0.96$
Previous CT (no/yes)	23/2	15/5	10/5	$P = 0.12$
Previous RT (no/yes)	21/4	15/5	11/4	$P = 0.66$
No of metastatic sites				$P = 0.37$
1	6	5	1	
2	12	4	6	
3	4	5	5	
$\geqslant 4$	3	5	3	
No of CT courses given				$P = 0.84$
1	3	3	4	
2	8	6	4	
3	6	7	3	
$\geqslant 4$	8	4	4	

[a] = chi-squared test. CT = chemotherapy. RT = radiotherapy.

Table II Comparison of response rates

	DTIC Dosage (mg m^{-2})			
Response	*400*	*500*	*800*	*Total*
Progression	18 (68%)	13 (65%)	7 (47%)	33
Stable	1 (4%)	1 (5%)	2 (13%)	4
Partial response	4 (16)	5 (25%)	6 (40%)	15
Complete response	2 (8%)	1 (5%)	0 (0%)	3
Patients (number)	25	20	15	60

( ) % based on total patient number in each treatment group.

Table III Metastatic sites and response with different DTIC doses

No of patients with metastatic sites[a]	*DTIC Dosage (mg m^{-2})*			
	400	*500*	*800*	*Total*
Non-visceral sites only	1 (1)	2 (1)	2 (2)	5 (4)
Visceral sites only	11 (3)	6 (1)	4 (1)	21 (7)
Both	13 (2)	12 (4)	9 (4)	34 (5)

[a] $P > 0.5$, chi-squared test. ( ) number in bracket denotes number of patients responding.

Table IV Haematological toxicity for each DTIC dose

Toxicity	*WHO grade*	*DTIC Dose (mg/m^{-2})* *400(25 pts)*	*500(20 pts)*	*800(15 pts)*	*P-value*[a]
		Number of patients			
Anaemia	2	5 (20%)	2 (10%)	5 (33%)	
	$\geqslant 3$	1 (4%)	4 (20%)	5 (33%)	<0.05
Leucopenia	2	1 (4%)	4 (20%)	3 (20%)	
	$\geqslant 3$	1 (4%)	2 (10%)	6 (40%)	<0.01
Platelets	2	2 (8%)	2 (10%)	1 (7%)	
	$\geqslant 3$	0 (0%)	4 (20%)	6 (40%)	0.0005

[a] = chi-squared test. ( ) % based on total patient number in each treatment group.

Table V Physiological results of respiratory function test

Patient	FEV1 % PreT	VC % PreT	TLV % PreT	RV % PreT	DLCO % PreT	KCO % PreT
MB[C2 a]	95.3	93.2	102.9	112	91.3	97
MS[C3]	102.7	102.6	98.2	106.8	75.3	77.6
SS[C3]	101.5	93.7	94.8	78.3	79.2	66
IP[C3]	100	87.5	80.5	70.6	84.3	94.8
LP[C4]	100	88.5	69.8	51.6	54.5	44
PH[C4]	94.7	99	100	100	73.7	78.8
HG[C4]	91	90.8	82	86	74.4	56.5
RL[C4]	76.5	89.6	73	58.9	100	85.7
CW[C4]	60.3	59	65.1	83.2	50.5	88.4
LD[C5]	100	100	93.8	94.9	70.1	89.1
WL[C6]	105	89.7	98.2	100	69.5	80
P value	0.249	0.004	0.010	0.035	0.0002	0.0035

%PreT = Per cent of prechemotherapy value. [a]All patients received 400 mg m^{-2} DTIC and 100 mg m^{-2} fotemustine and number after patient's initial refers to treatment cycle. FEV1 = Forced expiratory volume in one second; VC = Vital capacity; TLC = Total lung capacity; RV = Residual volume; DLCO = total lung carbon monoxide transfer corrected for haemoglobin; KCO = transfer coefficient. *P* value: based on one-sample *t*-test.

strated a normal left ventricular function with no evidence of pericardial effusion. Bronchial alveolar-lavage produced fluid containing inflammatory cells. Despite high dose steroid and septrin, the patient condition's continued to deteriorate and death occurred 10 days after presentation. Post-mortem showed features of those of adult respiratory distress syndrome with interstitial fibrosis.

Following this case, the treatment protocol was amended and the DTIC dosage was reduced to 400 mg m^{-2} with fotemustine maintained at 100 mg m^{-2}. A full lung function assessment was undertaken in 11 patients before and after chemotherapy. Table V shows the physiological results of patients studied. Data was expressed as percentage of pretreatment results. As shown in the table, significant reduction of vital capacity (VC), total lung volume (TLV), residual volume (RV), total lung carbon monoxide transfer (DLCO) and transfer coefficient (KCO) occurred following chemotherapy. No relationship was seen between the extent of pulmonary damage and treatment cycles ($P = 0.72$, one-sample *t*-test). One patient (LP[C4], Table V) presented with an acute onset of breathlessness and investigations revealed restrictive spirometry and small lung volumes associated with reduced total lung carbon monoxide transer (DLCO of 54.5% of prechemotherapy value) and transfer coefficient (KCO of 44% of prechemotherapy value). CXR showed patchy upper lobe shadowing that was more marked on the right hand side.

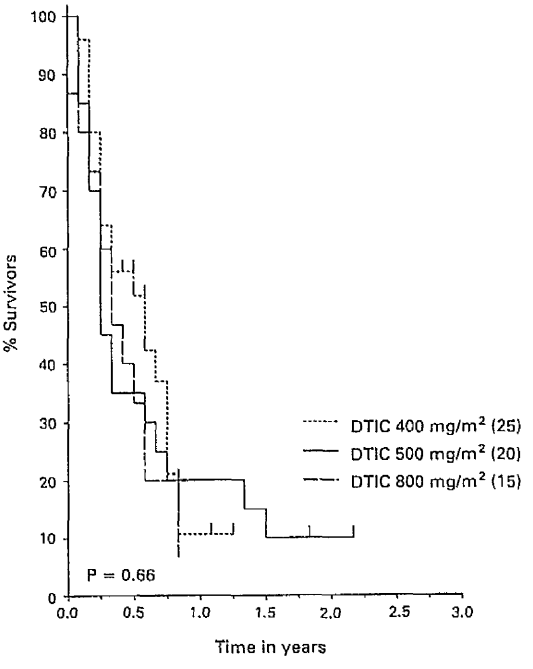

Figure 1 Survival to different dosages of DTIC with fotemustine maintained at 100 mg m^{-2}.

Other toxicity

Nausea and vomiting (≥ WHO 3) occurred in 14 patients despite metoclopramide, elevated transaminases in ten patients, elevated alkaline phosphatases in 12 patients and elevated bilirubin in five patients and these were not statistically different between the three treatment groups. Two infective episodes were noted in two patients receiving 500 mg m^{-2} DTIC and in three patients receiving 800 mg m^{-2} DTIC.

Survival

The overall median survival was 3.6 months (range, 1–15 months). Within the treatment subgroups, median survivals were 6.3 months, 2.75 months and 3.6 months in patients receiving 400, 500 and 800 mg m^{-2} DTIC respectively. However, no statistically significant difference in survival was seen between the different DTIC doses ($P = 0.67$, log-rank test; see Figure 1). Nine patients are alive at 5 to 26 months (median 10 months); three patients with no tumour and five patients with stable disease. There was a statistically significant difference ($P < 0.0001$ log-rank test) between survival for responders (median survival, 9 months; including patients with stable disease) compared to patients with progressive disease (median survival, 2.9 months).

Discussion

The current study reports an overall response rate of 30% obtained with sequential DTIC then fotemustine. Although there appeared to be a trend towards a higher response rate with increasing dosage of DTIC this was not statistically significant and may be due to the small number of patients entered to each treatment group. The majority of studies in which DTIC has been given by single i.v. bolus or daily injections over 5 days, have produced an overall response rate of about 20% (Comis 1976; Mastrangelo *et al.*, 1982; Geeraerts & Nathanson, 1986; Balch *et al.*, 1989). Single doses of DTIC of 850 mg m^{-2} (Samson *et al.*, 1978) and

800 mg m^{-2} with dactinomycin (Hochster *et al.*, 1985) or 250 mg m^{-2} daily for 5 days with dactinomycin (Robidoux *et al.*, 1982) gave response rates of 23%, 22% and 15% respectively. Fotemustine alone gave a response rate of about 24% (Jacquillat *et al.*, 1990). Therefore the overall response rate of 30% achieved with the current study would support the use of a combination of DTIC and fotemustine, although, because of the absence of a direct comparison, no conclusions about the scheduling can be reached. In one study of 18 patients treated with combined fotemustine/DTIC chemotherapy, giving fotemustine (100 mg m^{-2}) 1 h prior to DTIC not only had no clinical effect but also caused unexpected antagonism and modification of the pattern of toxicity (Aamdal *et al.*, 1990) and supports the use of our administration schedule. If the trend towards a higher response rate with higher dosage DTIC is substantiated with larger number of patients, it is not unreasonable to suggest that it may be related to the increasing extent of ATase depletion achieved in tumour tissue assuming that a depletion is similar to that occurring in lymphocytes (Lee *et al.*, 1991). However, with the dosages of DTIC used in the current study, complete suppression of lymphocyte ATase was not acheived; the mean nadir ATase activities were approximately 56%, 27% and 24% of the pretreatment activity in patients receiving 400, 500 and 800 mg m^{-2} of DTIC (Lee *et al.*, 1993). If this is reflected in the tumour cells, residual ATase activity following DTIC administration may be sufficient to repair any potentially toxic O^6-chloroethyguanine lesions induced by the subsequent administration of a chloroethylating agent. The lymphocyte ATase depletion data (Lee *et al.*, 1991; Lee *et al.*, 1993) suggest that it would be interesting to explore whether or not pulsed DTIC treatment every 4 h or continuous DTIC infusion, followed or not by fotemustine or another nitrosourea will be able to improve the response rate, since an improved clinical response might be achieved if complete tumour ATase suppression is attained prior to fotemustine administration.

An interesting finding was the statistically significant relationship seen between the development of severe haematological toxicity and the dosage of DTIC administered. In one study of 46 patients treated with 850 mg m^{-2} DTIC given as single i.v. bolus, thrombocytopenia ($\leqslant$ 100,000 ml^{-1}) and leucopenia ($\leqslant$ 1000 ml^{-1}) was uncommon and developed in only 4% and 2% of the treatment courses (Pritchard *et al.*, 1980). In contrast, in the present study this occurred in 40% and 53% of the patients receiving sequential 800 mg m^{-2} DTIC and 100 mg m^{-2} fotemustine. A more extensive marrow toxicity was seen in the schedule using 800 mg m^{-2} of DTIC and one possible explanation for this might be due to a more extensive ATase depletion of the already low levels of ATase in the marrow (Gerson *et al.*, 1985) resulting in an increased sensitivity to fotemustine or subsequent doses of DTIC. In this context, ATase-deficient murine haematopoietic stem cells transfected with and expressing bacterial ATase genes are highly resistant to the toxic effects of methylating and chloroethylating agents strongly suggesting that endogenous ATase expression would protect against the haematological effects of these agents (Jelinek *et al.*, 1988) and hence ATase depletion would result in sensitisation. Furthermore, these is some experimental evidence to indicate that ATase depletion of nitrosourea-resistant melanoma cells with O^6-methylguanine not only sensitises the tumour cells but also the normal bone marrow cells following subsequent exposure to a chloroethylating nitrosourea (Dempke *et al.*, 1987).

Another interesting finding was the occurrence of pulmonary toxicity. Two patients presented with an acute shortness of breath; one died and post-mortem revealed features of adult respiratory distress syndrome with interstitial fibrosis. The second patient responded to high dose steroid; investigations showed a small lung volume with significantly reduced carbon monoxide transfer factor. Follow-up studies in another 10 patients showed a significant sub-clinical deterioration in lung function following chemotherapy (Table V). The clinical, radiological and histological features of 'early onset' lung fibrosis have previously been described with BCNU and other nitrosoureas (Bailey *et al.*, 1978; Durant *et al.*, 1979; Aronin *et al.*, 1980; Sekler *et al.*, 1980; Weiss *et al.*, 1981); correlation is seen when cumulative dosage of BCNU $\geqslant$ 1000 mg m^{-2} (Weiss *et al.*, 1981). However, the two cases of interstitial pneumonitis in our study received a cumulative dosage of $\leqslant$ 400 mg m^{-2} of fotemustine suggesting that the synergy between DTIC and fotemustine (as used in the schedule here) may be responsible for the acute pulmonary event, possibly related to greater cytotoxicity in normal lung cells following depletion of the endogenous ATase. A recent phase II study of fotemustine alone in 153 patients with disseminated melanoma was not associated with any pulmonary toxicity and similar finding was reported in another 38 patients with gliomas treated with fotemustine alone (Jacquillat *et al.*, 1990; Frenay *et al.*, 1991). Lung tissue has a relatively low ATase activity in comparison with other tissues (Grafstrom *et al.*, 1984; Gerson *et al.*, 1986) and as a result they may be more sensitive to the cytotoxic effects of DNA alkylation. This may be a particular problem in those patients whose lung tissue has low ATase activity or in which ATase depletion by DTIC has been more effective.

In conclusion, sequential DTIC and fotemustine appears to be more effective than DTIC or fotemustine alone. There is a trend towards increased response rate with higher dosage DTIC however, if this is confirmed in a larger group of patients it has been achieved whilst eliciting significantly increased haematological and possibly pulmonary toxicity. The median survival time remains short in these patients with advanced disease, but we might speculate that further investigations using different schedules of DTIC combined with a nitrosourea to overcome ATase-mediated drug resistance could be worthwhile particularly if an increased response is achieved in the absence of increased toxicity because of general ATase depletion in both normal and tumour tissues. Whether the marrow toxicity can be reduced with the help of haemopoietic growth factors would require further exploration. The subclinical pulmonary damage observed indicates that it is of considerable importance to monitor these patients to prevent the possibility of acute and/or long term lung damage. Nevertheless, the present study provides a framework for other investigations using ATase depleting agents such as a methylating agents or O^6-benzylguanine before administering chloroethylating nitrosoureas.

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Expression of O^6-Alkylguanine-DNA-Alkyltransferase *in situ* in Ovarian and Hodgkin's Tumours

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The cellular expression of O^6-alkylguanine-DNA-alkyltransferase (ATase) may be an important factor in determining tumour sensitivity to certain alkylating agents. In a comparative study, we have examined the inter- and intracellular distribution of ATase in tumour biopsies of a series of patients with Hodgkin's disease and ovarian cancer using a rabbit antihuman ATase antiserum. The antibody recognises the ATase protein on western blots of cell-free extracts of a number of ovarian tumours with ATase activities varying from 20 to 420 fmol/mg protein as determined by *in vitro* assay and there was a linear correlation between ATase activity and the intensity of the band on western blots ($r = 0.993$). Immunohistochemical staining was seen in all of the ovarian tumours examined and was confined to the nucleus. This is in contrast to the Hodgkin's tissue, where staining was much reduced and present in both nuclei and cytoplasm. The results suggest that in ovarian tumours the general resistance to nitrosourea chemotherapy may be related to the high cellular expression of ATase protein: this is in contrast to the more chemosensitive Hodgkin's disease. This raises the possibility that it might be feasible to predict sensitivity or resistance to these alkylating agents by immunohistochemical staining of tumour or tissue specimens.
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INTRODUCTION

THE RESPONSE rate of ovarian cancer following treatments with chloroethylating nitrosoureas, dacarbazine and procarbazine is low in comparison with the mustard-type alkylating agents: published reports of more than 1000 patients treated with either melphalan, chlorambucil, thio-tepa or cyclophosphamide have produced objective response rates of 35–65% compared to less than 6% response with nitrosoureas [1]. This is in contrast to Hodgkin's disease where single-agent therapy with either 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-

cyclohexyl-1-nitrosourea (CCNU), procarbazine or dacarbazine achieves a response rate of 50–70% [2]. For these agents, which generate O^6-alkylation products as a significant portion of the total DNA damage (here termed O^6-alkylating drugs) there is increasing experimental evidence to suggest that cellular expression of the DNA repair protein, O^6-alkylguanine-DNA alkyltransferase (ATase) can protect cells against cytotoxicity by repairing O^6-alkylguanine, one of the principal toxic lesions induced [3–5]. ATase removes the alkyl group from the O^6 position of guanine to an internal cysteine residue in an auto-inactivating stoichiometric reaction [3–5]. Thus, ATase-deficient cell lines are more sensitive to killing by such agents [3–6] and depletion of endogenous ATase by pretreatment with non-toxic doses of methylating agents [7–9] or O^6-alkylguanine [8–13] in ATase-proficient cells rendered the cells more sensitive to subsequent treatment with nitrosoureas and related agents. Similarly, tumour xenografts with high ATase activity are more resistant to O^6-alkylating drugs than xenografts with low activity [14]. Possibly, the strongest evidence for the cytoprotective role of ATase comes from transfection experiments which show that expression of prokaryotic or eukaryotic ATase cDNA in mammalian cells protects them against the toxic effects of these agents [15–19].

In the present report we have examined the possibility that resistance to O^6-alkylating drugs which frequently occurs in ovarian cancer in contrast to Hodgkin's disease may be related to the cellular expression of ATase. Although ATase levels in extracts of many tumour types have been measured, a disadvantage of this is that it is a tissue average measurement and it takes no account of differences in intercellular or intracellular expression or regional distribution of ATase protein. In an attempt to address this issue, we used a rabbit anti-human ATase antiserum to examine, by immunostaining, sections of 18 ovarian tumours and three Hodgkin's disease tissues. Using the former tissues, we have also examined the relationship in tissue extracts between the ATase activity and western blot staining.

MATERIALS AND METHODS

Tumour material

Fresh surgical material collected from hospitals across the northwest of England was fixed in formal saline overnight and embedded in wax. Diagnostic histopathology was performed on sections prepared by standard techniques from paraffin-embedded material. The characteristics of the ovarian tumours studied are shown in Table 1. Patients received six cycles of intensive combination chemotherapy comprising of carboplatin/cyclophosphamide alternating with ifosphamide and doxorubicin. Post-treatment samples were obtained from patients with residual or relapsed disease. The three samples of Hodgkin's disease were all of the nodular sclerosis type (Table 1). Local ethical approval was obtained for the study.

O^6-Alkylguanine-DNA alkyltransferase assay

Ten ovarian tumours were obtained at staging and second-look laparotomies and samples were snap-frozen in liquid nitrogen and stored at −70°C. Cell-free sonicates of these were assayed for ATase activity as described previously [20] except that the total incubation volume was 500 μl and the specific activity of the [^{3}H]-*N*-nitrosomethylurea-methylated DNA substrate was 629 GBq/mmole. Activity was expressed as fmoles [^{3}H]-methyl transferred from [^{3}H]-O^6-methylguanine to protein per mg of protein under protein limiting conditions and was the mean of three estimations. Protein content of the extracts were measured by the Bradford method [21] using Bio-Rad protein assay reagent and bovine serum albumin (BSA) as standard.

Table 1. Characteristics of ovarian and Hodgkin's tumours examined

No.	Age	Treatment sample*	Histology†	Histological differentiation	Staged‡	ATase (fmol/mg)
1	69	Pre	Serous	Moderate	3	422
2	68	Pre	Mucinous	Moderate	3	118
3	52	Pre	Serous	Moderate	3	20
4§	59	Pre	Endometroid (Fig. 4)	Moderate	3	850
5§	64	Pre	Serous (Fig. 2)	Well	3	785
6§	69	Pre	Mucinous	Moderate	3	39
7	72	Pre	Endometroid	Well	3	nm
8	28	Pre	Serous	Moderate	3	nm
9	65	Pre	Undifferentiated	Poor	1	nm
10	64	Pre	Endometroid	Poor	3	nm
11	31	Pre	Mucinous	Moderate	3	nm
12	40	Pre	Serous (Fig. 3)	Poor	3	nm
13	53	Pre	Serous	Poor	3	nm
14	65	Post	Mucinous	Poor	3	366
15	59	Post	Serous	Poor	3	91
16	38	Post	Undifferentiated	Poor	3	nm
17§	42	Post	Endometroid	Poor	3	51
18§	73	Post	Serous	Moderate	3	89
19	22	Pre	NS (Fig. 5)	—	2A	nm
20	44	Pre	NS	—	3A	nm
21	43	Pre	NS	—	3A	nm

*Prechemotherapy or second-look (postchemotherapy) sample. †Histological types of epithelial ovarian cancer. NS = nodular sclerosing Hodgkin's disease. Brackets indicate where samples were subjected to immunostaining *in situ* and the corresponding Figure number. ‡FIGO staging for ovarian cancer or Ann Arbor staging for Hodgkin's disease. §Extracts assayed for ATase activity and subjected to western analysis. ¶nm = not measured.

Western blotting

Extracts of five of the above ovarian tumours containing 30 μg of total protein (but different ATase activity) and a sample of pure recombinant human ATase protein and molecular weight markers (Amersham International) were subjected to SDS–PAGE in a 0.75-mm thick 16% polyacrylamide gel, in a Bio-Rad mini-gel apparatus at 200 V for 45 min. Proteins were electroblotted onto Hybond C (Amersham International) membrane for 1 h at 100 V in a Bio-Rad mini trans-blot apparatus. The blotted membrane was blocked with non-fat milk powder [5% Marvel in tris-buffered saline (TBS)] and probed with the rabbit anti-human ATase antiserum (fourth bleed serum diluted 1:1000 in TBS) [22] and then goat anti-rabbit alkaline phosphatase (Dako Ltd, U.K.). The antibody reaction was visualised by reaction with nitro blue tetrazolium and bromochloroindolyl phosphate (GIBCO BRL, Life Technologies, U.S.A.). The

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intensity of staining on western blots were scanned using an Ultra Violet Products (UVP) densitometer and analysed by UVP gel analysis software.

Immunohistochemistry

The sections were dewaxed in xylene, washed twice in absolute ethanol, treated with 3% H_2O_2 in methanol for 30 min at room temperature, washed in TBS, incubated with normal swine serum (Dako) for 30 min and exposed overnight at 4°C to the anti-human ATase antiserum (fourth bleed) or pre-immune serum diluted 1:1000 in TBS [22]. As an additional control, sections were also incubated with ATase antiserum (1:1000 dilution) that has been affinity purified using pure recombinant human ATase. The sections were then incubated with swine anti-rabbit antibody [SAR, (Dako) diluted 1:400 in TBS] for 45 min at room temperature, washed in PBS and incubated with rabbit peroxidase–antiperoxidase complex [PAP, (Dako) diluted 1:200 in TBS] for 45 min at room temperature. After washing in TBS the sections were incubated with SAR and PAP again for 15 min. Stain development was by a single-step silver intensification of nickel-complexed DAB (3',3'-diaminobenzidine-4 HCl) peroxidation product as described by Przepiorka *et al.* [23]. Briefly, the slides were exposed to nickel-complexed diaminobenzidine (Ni-DAB) (0.5 ml of 1% $NiCl_2$-$6H_2O$ in 5 ml of 0.5 mg/ml DAB) for 5 min followed by 0.01% H_2O_2 in Ni-DAB for another 5 min at room temperature. After washing the slides with distilled water, they were incubated with silver reagent for 5 min. Silver reagent was prepared by mixing in the following order: 400 μl distilled water, 200 μl of 0.1 mol/l NH_4NO_3, 200 μl of 0.047 mol/l $AgNO_3$, 180 μl 0.12 mol/l dodecatungstosilicic acid (Fisons, U.K.), 15 μl 36% formalin and 1 ml 0.47 mol/l Na_2CO_3. Fine black deposits of silver were seen at the sites of DAB polymerisation. All the staining intensity was assessed without prior knowledge of the tumour characteristics.

RESULTS

Western blotting

Crude extracts from five ovarian tumours with a variety of ATase levels ranging from 20 to 420 fmol/mg protein (Tables 1, 2) were used for western blotting. As shown in Fig. 1, western blotting revealed essentially a single staining band at 22 kD, corresponding to the size of the pure recombinant human ATase. The relative intensities of these bands were quantitated by densitometry scanning (Table 2) and there was a linear correlation between the ATase activity in tumour extracts and the intensity of staining on the western blots ($r = 0.993$).

Table 2. Correlation in ovarian tumours between ATase activity and staining intensity of western blots

Ovarian tumour*	ATase activity (fmol/mg ± S.D.)	Staining intensity†
1	422 ± 32.7	1.0
14	366 ± 7.0	0.76
2	118 ± 1.2	0.22
15	91 ± 2.7	0.12
3	20 ± 0.7	0.04

*See Table 1 for tumour characteristics. †Quantified by densitometric scanning and standardised to 1.0 based on tumour no. 1.

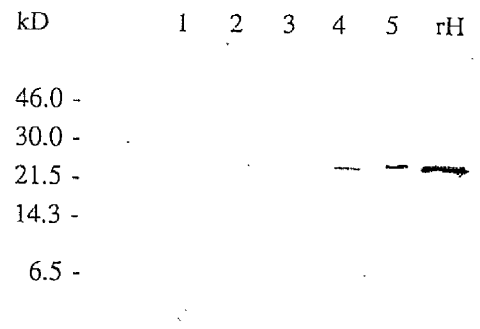


Fig. 1. Western blot of crude sonicates of ovarian tumours with increasing ATase activity (lanes 1–5) and recombinant human ATase (rH) probed with anti-human ATase serum. The positions of the molecular weight markers (kD) are shown. See text for experimental details.

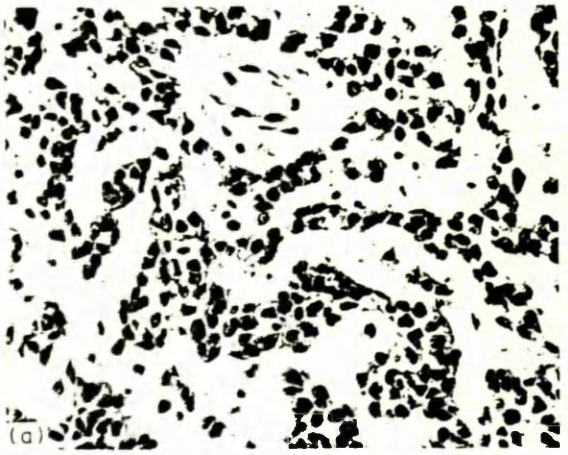
Immunohistochemical staining

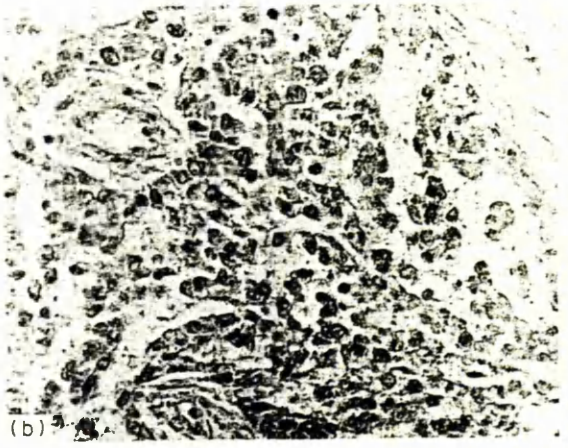
With the preimmune serum, very faint staining was seen in both the cytoplasm and nucleus of the ovarian and Hodgkin's sections (Figs 2b, 3b, 4b, 5b). In sections incubated with ATase antiserum that had been affinity purified using pure recombinant human ATase, very little nuclear and cytoplasmic staining was seen, i.e. a picture similar to that observed with the pre-immune serum (Fig. 4d) and thus further confirming the specificity of the ATase antiserum.

In the ovarian tumours, staining with the ATase antiserum was seen in all the 18 cases examined. Staining was seen as fine black granules mainly confined within the nucleus and present in virtually all the tumour cells (Figs 2c–4c). Some intercellular variation in intensity of staining was observed possibly indicating heterogeneity of cellular expression but little difference in regional distribution of positively staining ATase cells could be discerned. In some of the sections, where adjacent non-tumorous cells could be identified, staining was seen in the supporting stromal fibroblasts, endothelial cells and adipocytes, principally in the nuclei (Figs 2c, 3c). Whilst staining was considered essentially a qualitative parameter, the extent to which staining was quantitatively related to ATase levels in tissue extracts was also assessed: staining intensity appeared to be less in two sections with low ATase levels (Table 1, tumour nos 3 and 6) in comparison to two sections with high ATase activity (Table 1, tumour nos 4 and 5). However, staining intensity did not appear to correlate with ATase levels in those extracts with intermediate levels. Indeed, in one of the sections with low ATase activity (tumour no. 6), haematoxylin and eosin staining revealed mainly fibrous tissue sparsely populated with tumour cells, the latter nevertheless still stained positively for ATase. This indicates an advantage of immunohistochemical staining which takes into consideration the cellular content of ATase in contrast to the *in vitro* ATase assay which is a tissue average measurement.

Relative to the ovarian cancers, staining in the Hodgkin's disease biopsy specimens was substantially less in intensity (Fig. 5c). Reed–Sternberg cells of lacunar type were identified in all three biopsy specimens and these showed relatively weak cytoplasmic staining with variable, weak nuclear staining. Cytoplasmic staining of similar intensity was also discerned in the surrounding 'reactive' lymphocytes (Fig. 5c).

Fig. 2. Well differentiated serous adenocarcinoma of the ovary: (a) haematoxylin and eosin staining; (b) immunostaining with pre-immune serum; (c) immunostaining with anti-human ATase antiserum showing strong, uniform staining of tumour cell nuclei. Note that fibroblasts in the connective tissue septae also exhibit nuclear staining. Magnification ×215.





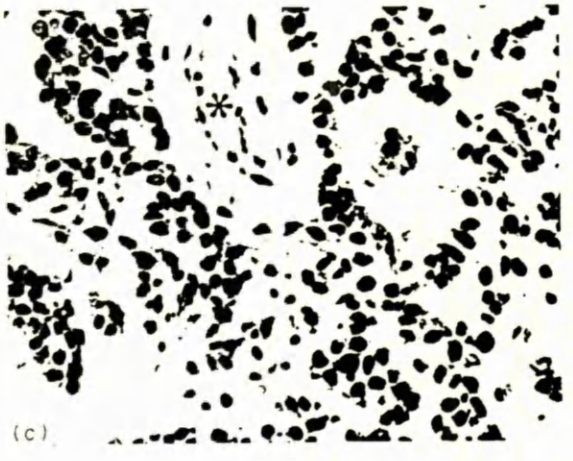


Fig. 3. Poorly differentiated serous adenocarcinoma of the ovary: (a) haematoxylin and eosin staining; (b) immunostaining with pre-immune serum; (c) immunostaining with anti-human ATase antiserum showing similar strong, uniform staining of tumour cell nuclei; the endothelium of the blood vessel near the centre of the field is similarly stained (asterisk). Magnification ×350.

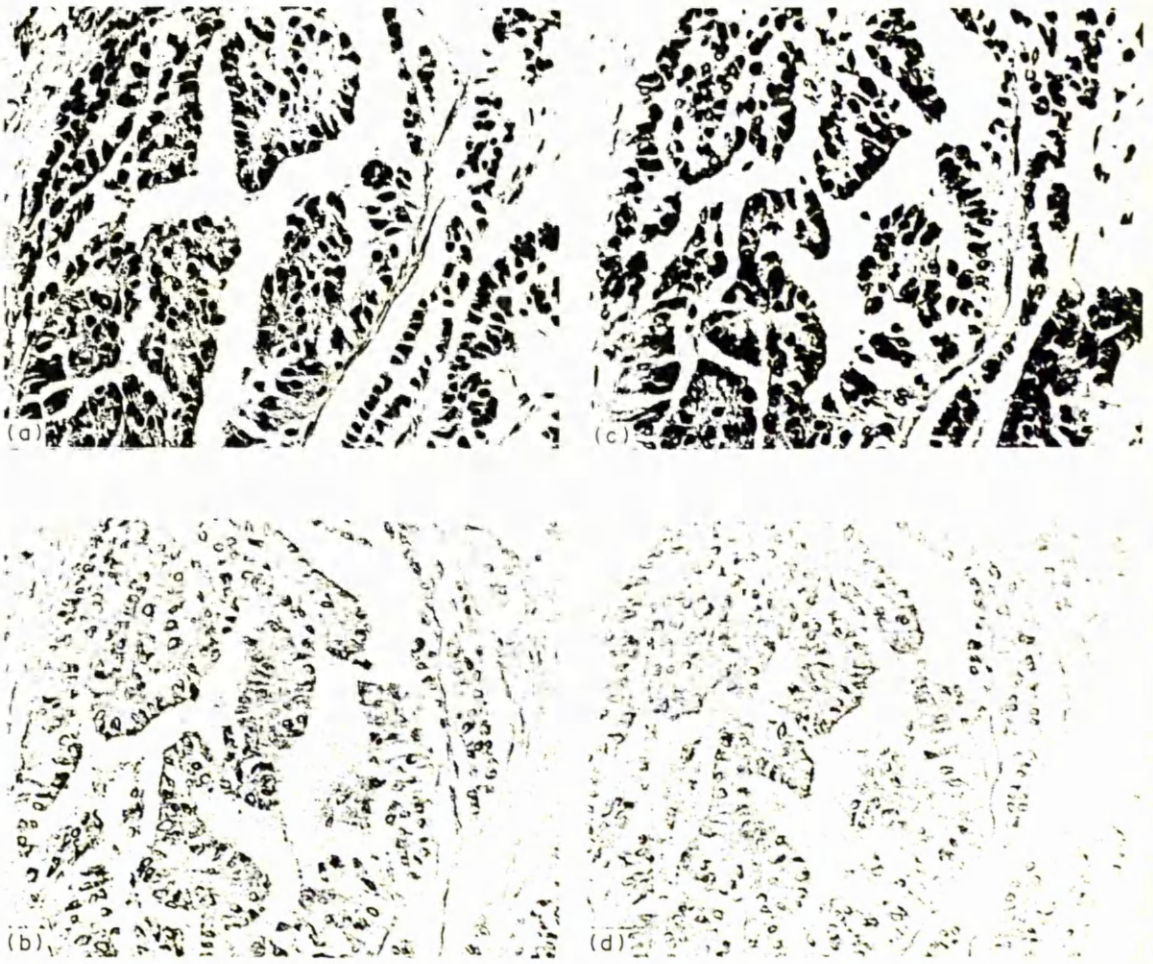


Fig. 4. Moderately differentiated serous adenocarcinoma of the ovary: (a) haematoxylin and eosin staining; (b) immunostaining with pre-immune serum; (c) immunostaining with anti-human ATase antiserum showing strong, uniform staining of tumour cell nuclei; (d) immunostaining with ATase antiserum affinity-purified with pure recombinant human ATase showing very little nuclear and cytoplasmic staining. Magnification ×345.

DISCUSSION

Previous studies with the anti-human ATase antiserum have indicated the high specificity of ATase detection. Thus, in liquid hybridisation experiments, we have shown that there is a dose-dependent inhibition of recombinant human ATase protein when it was incubated with increasing concentrations of ATase antiserum [24]. Moreover, whilst intense nuclear staining was seen in normal human liver cells with the antiserum, there was background staining with either pre-immune or pre-adsorped serum [22]. The polyclonal antibodies were able to react with ATase from crude extracts of ovarian tumours on the western blots to produce essentially a single band of an apparent molecular weight of 22 kD that was indistinguishable from that of the recombinant human ATase protein. This factor and the correlation seen in western blots between ATase levels and staining intensity further indicate that the ATase antiserum is highly specific in support of our earlier findings [22].

The polyclonal antibodies readily detected expression of ATase protein in the tumour sections examined by immunohistochemistry. The ability of the antiserum to detect the ATase protein in both the western blots and tumour sections indicates that they recognise common epitope site(s) in both the denatured and intact ATase protein. This is in contrast to other studies where the human ATase antibodies generated only recognise an exposed antigenic site following SDS–PAGE [25–27].

The level of ATase activity has been shown in many experimental models to be an important factor in the sensitivity of tumours to alkylating agents that form adducts at the O^6-position of guanine, including procarbazine, dacarbazine, temozolomide, CB10-277, streptozotocin and the chloroethylating nitrosoureas. Ovarian cancer is highly resistant to such O^6-alkylating drugs and this is in contrast to Hodgkin's disease which is sensitive. We have, therefore, examined this issue using the rabbit anti-human ATase antiserum to probe a series of ovarian and Hodgkin's tumours by immunohistochemistry. Strongly positive staining was seen in all the 18 ovarian tumours examined. Staining was essentially confined to the nucleus and where cytoplasmic staining could be discerned it was very faint when

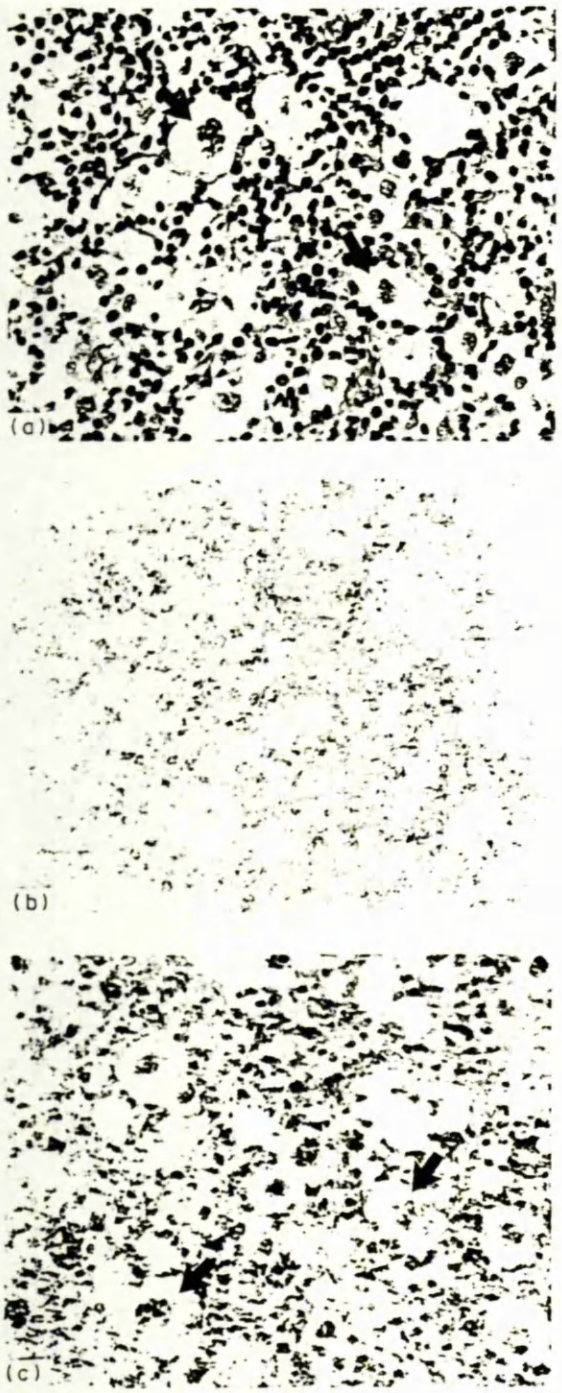


Fig. 5. Nodular sclerosing Hodgkin's disease: (a) haematoxylin and eosin. Lacunar-type Reed–Sternberg cells are plentiful (arrows) in a background population of small lymphocytes; (b) immunostaining with pre-immune serum. Weak non-specific staining is present in the small lymphocytes; (c) immunostaining with anti-human ATase antiserum showing weak staining in the nuclei and cytoplasm of the lacunar Reed–Sternberg cells (arrows) and in the small lymphocytes. Magnification ×350.

compared to the nuclear staining. Some differences were seen in the intensity of intercellular staining suggesting heterogeneous or possibly cell-specific expression of the ATase. In contrast, in the Hodgkin's sections, staining was much less intense and was mainly confined to the cytoplasm of both Reed–Sternberg cells and surrounding reactive lymphocytes. Since the ATase protein is synthesised in the cytoplasm, it is possible that in Hodgkin's disease, the Reed–Sternberg cells may contain a defective cytoplasmic nuclear transport mechanism. It is interesting to note that immunohistochemical studies on mammalian cells (NIH-3T3) expressing a bacterial ATase gene indicated that the protein was predominantly cytoplasmic and that these cells were only slightly more resistant to BCNU than the control cells, despite a 15-fold rise in total ATase activity quantitated by an *in vitro* assay [28]. This suggests that the cytoplasmic protein may not be fully functional in the cell.

These findings suggest that a possible reason for the low response rate of ovarian cancer observed in clinics in the O^6-alkylating agents [1] is a consequence of significant levels of ATase expression in the tumour cells. Conversely, the sensitivity of Hodgkin's disease to the O^6-alkylating agents may be due to low ATase expression; clinical studies in Hodgkin's disease show that single agent O^6-alkylating agents administered alone including procarbazine, dacarbazine and BCNU regularly achieve a 50–70% response rate [2] and these agents regularly form part of combination chemotherapy regimes such as MOPP (nitrogen mustard, vincristine, procarbazine, prednisolone), ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) and BVCPP (BCNU, vinblastine, cyclophosphamide, procarbazine, prednisolone) [29].

In the present report we have established that the anti-human ATase antiserum might allow the identification of resistant tumours and hence the design of individualised treatment protocols, including resistance modifiers where necessary, to be of maximum therapeutic benefit to the patients. It, therefore, appears to be important to explore prospectively whether a relationship exists between ATase levels in tumours detected by quantitative immunohistochemistry (using image analysis technology), tumour response to O^6-alkylating agents, frequency of intrinsic or acquired drug resistance and survival, particularly in Hodgkin's disease where dacarbazine, procarbazine and BCNU regularly form part of combination chemotherapy. In addition, since archival material is available, it is now feasible to examine previously treated Hodgkin's disease for ATase expression and to see whether or not this correlates with response to chemotherapy and patient survival.

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Inactivation of O^6-alkylguanine-DNA alkyltransferase in human peripheral blood mononuclear cells by temozolomide

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Summary O^6-alkylguanine-DNA-alkyltransferase (ATase) activity was measured in extracts of peripheral blood mononuclear cells (PMCs) taken from eight patients at various times during 5 days of oral treatment with temozolomide (150 mg m^{-2}, days 1-5). Pretreatment ATase levels ranged from approximately 70 to 600 fmol per mg of protein. Depletion of PMC ATase was seen within 4 h of the first dose of temozolomide and had a median nadir of 52.9% and values ranging from 44.4% to 71.0% of pretreatment levels. There was a correlation between the extent of ATase depletion (pretreatment minus nadir level) and the pretreatment ATase level ($r = 0.97$). A progressive depletion of ATase was observed during the 5 days of continuous temozolomide therapy with median ATase activities of 66.3%, 52.5%, 39.5%, 30.5% and 28.9% of the pretreatment values at days 2, 3, 4, 5 and 6 respectively. This suggests that the schedule-dependent anti-tumour activity of temozolomide seen in experimental models and clinics may be related to a cumulative depletion of ATase.

Temozolomide (CCRG 81045; M&B 39831; NSC 362856) was recently selected for clinical trials and has shown promising anti-tumour activity against high-grade gliomas, melanoma and mycosis fungoides (Newlands *et al.*, 1992; O'Reilly *et al.*, 1993). In contrast to dacarbazine (DTIC) or CB10-277, which require metabolic activation, temozolomide undergoes spontaneous chemical degradation to generate the cytotoxic monomethyl triazene, 5-(3-methyl-1-triazeno) imidazole-4-carboxamide (MTIC) (Figure 1) (Stevens *et al.*, 1987; Tsang *et al.*, 1991), which methylates DNA, generating among 12 other DNA lesions O^6-methylguanine (O^6-MeG). There is increasing experimental evidence to suggest that the anti-tumour activity of this class of drugs is linked to the alkylation of the O^6 position of guanine in DNA and that endogenous expression of O^6-alkylguanine-DNA alkyltransferase (ATase) may be a major factor in resistance to such agents (D'Incalci *et al.*, 1988; Margison & Connor, 1990; Pegg & Byers, 1992); ATase transfers the methyl group from O^6-MeG to an internal cysteine residue in an autoinactivating, stoichiometric reaction. A similar mode of drug resistance applies to the chloroethylating nitrosoureas: ATase prevents the formation of the cytotoxic interstrand cross-links which are produced in a two-step reaction from the monoadduct O^6-chloroethylguanine, which has itself been shown to be a substrate for ATase (Tong *et al.*, 1982; Gonzaga *et al.*, 1992; Baer *et al.*, 1993). The strongest evidence for the cytotoxic effects of O^6-alkylguanine in DNA comes from experiments which show that the expression of a transfected prokaryotic or eukaryotic ATase cDNA in mammalian cells protects them against the toxic effects of these agents (Brennand & Margison, 1986; Kataoka *et al.*, 1986; Samson *et al.*, 1986; Jelinek *et al.*, 1988; Kaina *et al.*, 1991).

While the majority of human tumours examined so far express ATase activity (D'Incalci *et al.*, 1988), it is possible to sensitise resistant tumour cells in culture or xenografts by pretreatment with methylating agents (Zlotogorski & Erickson, 1983, 1984; Gibson *et al.*, 1986) or the modified base O^6-benzylguanine (Dolan *et al.*, 1991), which renders them sensitive to the cytotoxic effects of subsequent treatment with methylating or chloroethylating agents. Two- to 12-fold increases in sensitivity to these agents have been observed with tumour cell lines which have high levels of ATase: these include colon (Karran & Williams, 1985; Baer *et al.*, 1993), melanoma (Dempke *et al.*, 1987), glioma (Aida *et al.*, 1987; Baer *et al.*, 1993), breast (Baer *et al.*, 1993) and leukaemic cell lines (Gerson *et al.*, 1988).

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The kinetics of the inactivation of ATase during the repair of O^6-MeG and the subsequent resynthesis of ATase are parameters which may predict an individual patient response to treatment. We have previously found a depletion of ATase in peripheral blood mononuclear cells (PMCs) of patients receiving a single intravenous bolus of dacarbazine or 24 h continuous infusion of CB10-277 (Lee *et al.*, 1991, 1992, 1993*a*). ATase depletion was also seen in the tumour biopsies of patients receiving the latter treatment schedule (Lee *et al.*, 1992). Furthermore, using DTIC, very large inter-patient variations in the extents and rates of ATase depletion were observed (Lee *et al.*, 1991, 1993*a*). In the present study we therefore examined the kinetics of ATase depletion in PMCs of eight patients with metastatic melanoma treated with the direct-acting agent temozolomide on five consecutive days. In five patients, changes in PMC ATase levels were also measured at various times during the 24 h after the first dose of temozolomide. In contrast to daily temozolomide administration, a single intravenous bolus of this drug was not associated with any tumour xenograft response in rodent models (Stevens *et al.*, 1987) or in clinics (Newland *et al.*, 1993) and we have therefore also compared ATase levels during 24 h of a single dose of temozolomide with those after 1-5 days of treatment.

Materials and methods

Chemicals

Temozolomide was supplied by the Department of Pharmaceutical Sciences, Aston University, Birmingham, UK. Dacarbazine was obtained from Bayer UK (Newbury, UK) and CB10-277 from the National Cancer Institute (Bethesda, MD, USA).

Treatment of patients

For this clinical study, temozolomide was formulated at Strathclyde University in hard gelatin capsules containing 20, 50 or 100 mg. All patients had metastatic melanoma and the clinical characteristics are shown in Table I. For the first treatment cycle, temozolomide was administered orally at 150 mg m^{-2} daily for five consecutive days. For subsequent treatment, patients received oral temozolomide (200 mg m^{-2}) daily on 5 consecutive days and this was repeated every 28 days. Serial blood samples were collected at 0 h, 1 h, 2 h, 3 h, 4 h, 6 h and 24 h in five patients and at 48 h, 72 h, 96 h, 120 h in three of these and an additional five patients receiv-

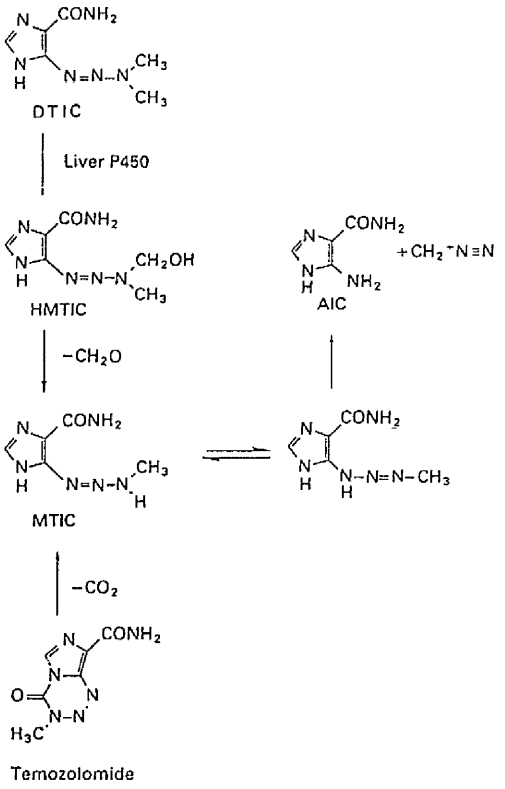

Figure 1 Metabolism of DTIC and decomposition pathway of temozolomide. Abbreviations used: AIC, 5-aminoimidazole-4-carboxamide; HMTIC, 5-(3-hydroxymethyl-3-methyl-1-triazenyl) imidazole-4-carboxamide; MTIC, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide.

ing daily temozolomide (150 mg m^{-2} daily from days 1 to 5). Blood was dispensed into 20 ml universal containers containing 0.5 ml of 0.5% EDTA and kept at 4°C before isolation of PMCs. Signed informed consent was obtained following the guidelines of the local health authority ethical committee. The phase II trial of temozolomide was carried out under the auspices of the Cancer Research Campaign (UK) Clinical Trials Committee.

Isolation of PMC, ATase extraction and assay

This was carried out as described previously (Lee *et al.*, 1991). Briefly, the PMCs were isolated by centrifugation on Ficoll (Pharmacia, Uppsala, Sweden) (Boyum, 1968), sonicated and the supernatants were assayed using 10 μg of [^{3}H]methylated DNA containing 0.1 pmol of O^6-methylguanine. ATase activity was expressed as fmol of methyl transferred to protein per mg of total protein in the extract and measurements were in triplicate.

Results

Effect of temozolomide on PMC ATase levels

In this series of patients, there was a wide range of pretreatment PMC ATase levels ranging from 69 to 593 fmol mg^{-1} protein (mean 275 ± 182 fmol mg^{-1} protein) (Table I). Depletion of PMC ATase was seen within 4 h of the first oral dose of temozolomide and the median nadir was 52.9% with values ranging from 44.4% to 71.0% of pretreatment levels in the five patients studied (Figure 2). Using repeated measurement analysis and Duncan's multiple range test, nadir ATase appears to occur between 2 and 6 h after chemotherapy. Taking each individual as their own control, recovery of PMC ATase activity greater than 20% was seen by 24 h in three of the five patients (see Figure 2).

Following 5 days' oral administration, a cumulative and progressive depletion of ATase was observed in eight patients (see Figure 3) with median ATase levels of 66.3%, 52.5%, 39.5%, 30.5% and 28.9% of pretreatment values at days 2, 3, 4, 5 and 6 respectively. In two patients on day 7, 48 h after the last temozolomide dose, ATase levels had recovered to 42.7% and 48.3% of the pretreatment levels, the nadirs in these patients being 25.6% and 35.0% of the pretreatment levels respectively. Using repeated measurement analysis and Duncan's range test, the nadir ATase activity appears to occur between days 4 and 6. There was a linear relationship between the pretreatment ATase level and the extent of ATase depletion (pretreatment minus nadir ATase level) with a correlation coefficient of 0.97 (Figure 4). The corresponding data from Lee *et al.* (1991, 1992) are also presented in Figure 4 and correlation coefficients of 0.88 and 0.96 were calculated for DTIC and CB10-277 respectively.

Discussion

In the present study, we clearly demonstrate that temozolomide is effective in depleting ATase activity in PMCs and that the nadir of activity following a single dose is around 2–6 h after treatment (Figure 2). If the ATase depeletion (pretreatment minus nadir levels) seen had been a consequence of temozolomide-mediated methylation of DNA in PMCs and the subsequent autoinactivation of ATase by the repair of O^6-MeG thus generated, it would have been predicted that, particularly with an agent not requiring metabolic activation, the actual amount of ATase inactivated in this way would be relatively constant, assuming that drug uptake and ATase resynthesis rates were consistent. However, we found that the extent of ATase inactivation varied

Table I Patient characteristics

Name	Age (years)/ sex (M/F)	Metastatic sites	ATase (fmol mg^{-1} ± s.d.) Initial	Nadir[a]
JP	40/F	Nodes, liver, lung	140 ± 6.9	NM
MS	45/M	Liver, lung	434 ± 5.7	NM
YA	26/F	Nodes, soft tissues	286 ± 6.5	123 ± 4.4
MC	39/F	Lung, liver, bone	459 ± 1.8	107 ± 7.2
MF	68/F	Skin, nodes	197 ± 12.8	54 ± 1.3
IC	58/F	Lung, liver, nodes	300 ± 4.8	105 ± 1.0
AW	75/M	Lung, nodes, liver	593 ± 17.3	152 ± 2.0
KH	54/M	Lung, nodes, liver	243 ± 16.6	35 ± 2.3
GA	66/M	Skin	69 ± 4.9	14 ± 0.6
MA	59/M	Skin, nodes	257 ± 13.5	135 ± 2.2

[a]ATase nadir during daily temozolomide administration (see Figure 3). NM, not measurable.

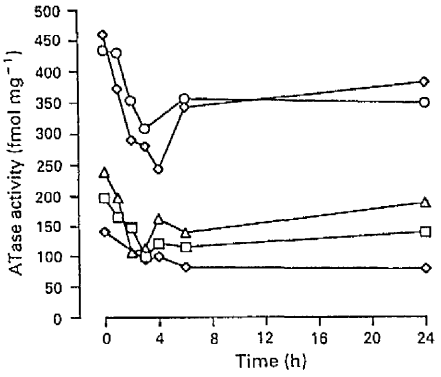

Figure 2 ATase activity (fmol mg^{-1} protein) in PMCs of five patients up to 24 h after the first temozolomide dose (150 mg m^{-2}).

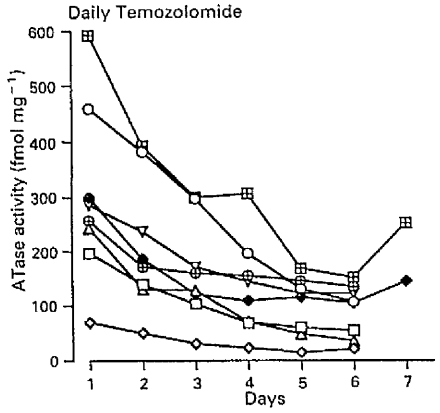

Figure 3 ATase activity (fmol mg^{-1} protein) in PMCs of eight patients receiving daily temozolomide (150 mg m^{-2}, days 1–5).

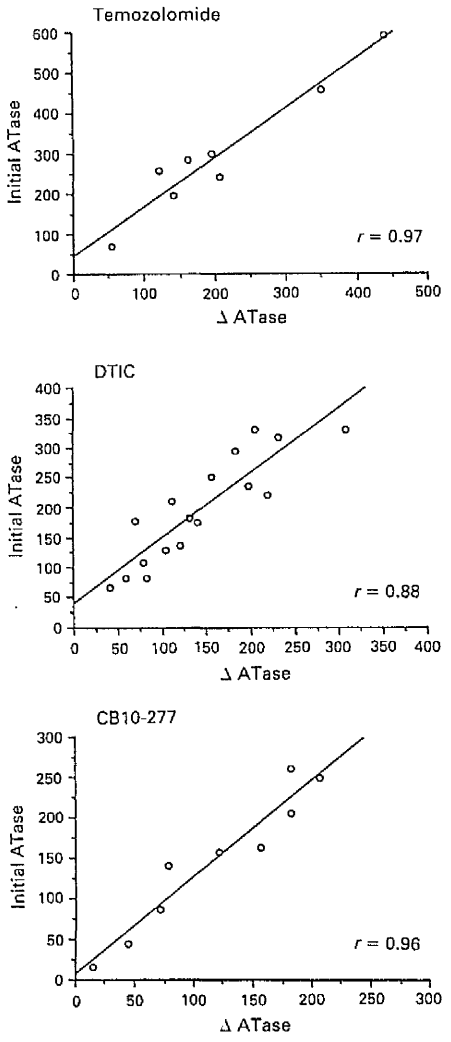

Figure 4 Relationship between the extent of ATase depletion (Δ ATase = pretreatment minus nadir ATase levels) and pretreatment ATase level in patients receiving temozolomide, DTIC or CB10-277. r = correlation coefficient.

considerably from patient to patient, but that there was a strong correlation between the extent of ATase depletion and the pretreatment ATase level.

Although ATase depeletion would be expected to occur via methylation of DNA in PMCs by temozolomide, the possibility that this non-stoichiometric depletion of ATase was due to a direct effect of temozolomide on the ATase itself cannot be dismissed. Previous studies have shown that inactivation of partially purified human ATase from CEM cells can occur *in vitro* following incubation with a variety of alkylating agents, including MNU, streptozotocin, BCNU, chlorozotocin, CCNU and MeCCNU, and, of the agents tested, methylmethanesulphonate was the most effective, producing 50% inactivation at 70 μM (Brent, 1986).

Reanalysis of earlier results using DTIC and CB10-277 (Lee *et al.*, 1991, 1992) also shows a correlation between pretreatment ATase levels and the amounts of ATase inactivated (Figure 4). Here too, the depletion may therefore be a consequence of the direct reaction of the corresponding metabolites with PMC ATase. That the kinetics of ATase depletion with temozolomide were very similar to that observed with DTIC (Lee *et al.*, 1991) and CB10-277 (Lee *et al.*, 1992), both of which require metabolic activation in order to produce a methylating species (Figure 1), suggests that the process of metabolic activation of the latter agents occurs very rapidly and might not be the rate-limiting step in ATase depletion.

If ATase depletion by alkylating agents *in vivo* is predominantly a direct effect and not unique to PMC, one possible consequence might be that the extent of ATase inactivation would be greatest in those cells and tissues expressing the highest levels of enzyme. Thus, in tumour cells which can express high ATase levels (Dolan *et al.*, 1991), sensitisation to killing by alkylating agents might be more extensive than in bone marrow, which generally expresses low levels of ATase (Gerson *et al.*, 1985). Indeed, extrapolation of the data in Figure 4 suggests that a threshold ATase level exists below which no ATase depletion occurs. For temozolomide and DTIC this value is 40–45 fmol mg^{-1} protein; for CB10-277, the value was about 10 fmol mg^{-1}, although there were fewer patients in this study.

The post-nadir recovery of PMC ATase activity was variable, but in none of the five patients studied was a return to pretreatment levels observed. This residual deficit in ATase was generally increased during the repeat daily administration of temozolomide such that, 24 h after the final dose,

ATase levels were between 14.4% and 52.5% of the pretreatment values (Figure 3). There was little inter-patient variation in the percentage decrease in ATase activity during the schedule, despite wide variations in pretreatment ATase levels, suggesting that depletion was possibly a direct effect on ATase.

It has been shown that the anti-tumour activity of temozolomide in tumour-bearing mice is schedule dependent (Stevens *et al.*, 1987), and a similar finding was reported with 51 patients treated with temozolomide (Newlands *et al.*, 1992). Thus, improved therapeutic effectiveness was noted when temozolomide was given daily for 5 days compared with single-dose administration. It does not seem unreasonable to suggest that the greater effectiveness of the daily treatment is related to the more extensive depletion of ATase, assuming that a similar effect occurs in the tumour cells. While tumour tissue has not been assessed in the present study, we have previously shown that CB10-277 is able to deplete ATase levels in both PMCs and melanoma (Lee *et al.*, 1992).

If tumour sensitisation is a consequence of ATase depletion, then it might be speculated from the present results that response to treatment would be more extensive if the temozolomide was given every 2–6 h, corresponding to the ATase nadir found here after a single dose, rather than every 24 h, when recovery of ATase activity can occur. Indeed, in the treatment of melanoma with DTIC/fotemustine combinations, the schedule of fotemustine 4 h after DTIC was designed to exploit the anticipated nadir of ATase activity produced by DTIC (Lee *et al.*, 1991) and produces better response rates than the individual agents given alone (Lee *et al.*, 1993*b*). The possibility therefore of giving a chloroethylating agent 2–6 h after the last of five doses of temozolomide given every 2–6 h also seems worthy of consideration.

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Abbreviations: ATase, O^6-alkylguanine-DNA alkyltransferase; MNU, *N*-methyl-*N*-nitrosourea; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MeCCNU, 1-*trans*-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea.

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Formation and loss of O^6-methyldeoxyguanosine in human leucocyte DNA following sequential DTIC and fotemustine chemotherapy

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Summary There is increasing evidence to indicate O^6-methyldeoxyguanosine (O^6-MedG) formation in DNA is a critical cytotoxic event following exposure to certain anti-tumour alkylating agents and that the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (ATase) can confer resistance to these agents. We recently demonstrated a wide inter-individual variation in the depletion and subsequent regeneration of ATase in human peripheral blood lymphocytes following sequential DTIC (400 mg m^{-2}) and fotemustine (100 mg m^{-2}) treatment, with the nadir ATase activity occurring approximately 4 h after DTIC administration. We have now measured the formation and loss of O^6-methyldeoxyguanosine (O^6-MedG) in the DNA of peripheral leucocytes of eight patients receiving this treatment regimen. O^6-MedG could be detected within 1 h and maximal levels occurred approximately 3–5 h after DTIC administration. Following the first treatment cycle, considerable inter-individual variation was observed in the peak O^6-MedG levels, with values ranging from 0.71 to 14.3 μmol of O^6-MedG per mol of dG (6.41 ± 5.53, mean ± s.d.). Inter- and intra-individual variation in the extent of O^6-MedG formation was also seen in patients receiving additional treatment cycles. This may be a consequence of inter-patient differences in the capacity for metabolismm of DTIC to release a methylating intermediate and could be one of the determinants of clinical response. Both the pretreatment ATase levels and the extent of ATase depletion were inversely correlated with the amount of O^6-MedG formed in leucocyte DNA when expressed either as peak levels ($r = 0.59$ and -0.75 respectively) or as the area under the concentration time-curve ($r = 0.72$ and -0.73 respectively). One complete and one partial clinical response were seen, and these occurred in the two patients with the highest O^6-MedG levels in the peripheral leucocyte DNA, although the true significance of this observation has yet to be established.

Dacarbazine (5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; DTIC) is considered the single most effective chemotherapeutic agent available for the treatment of metastatic melanoma (Comis, 1976; Balch *et al.*, 1989). It undergoes metabolic *N*-demethylation to give the monomethyl triazene, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC), which methylates cellular macromolecules including DNA. Among the 12 DNA lesions so formed, O^6-methyldeoxyguanosine (O^6-MedG) is thought to be the principal cytotoxic product (Meer *et al.*, 1986). It has been shown that resistance to MTIC and related agents involves the activity of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (ATase), which transfers the methyl group from O^6-MedG to an internal cysteine residue in an autoinactivating, stoichiometric reaction (Hayward & Parsons, 1984; Gibson *et al.*, 1986; Catapano *et al.*, 1987; D'Incalci *et al.*, 1988; Lunn & Harris, 1988; Foster *et al.*, 1990). The strongest evidence for the cytotoxic effects of O^6-alkylguanine on DNA comes from experiments which show that the expression of a transfected prokaryotic or eukaryotic ATase cDNA in mammalian cells protects them against the toxic effects of these agents (Brennand & Margison, 1986; Kataoka *et al.*, 1986; Samson *et al.*, 1986; Jelinek *et al.*, 1988; Kaina *et al.*, 1991).

We recently examined the levels of ATase in human peripheral lymphocytes following combination therapy with DTIC and the chloroethylating agent, fotemustine. ATase activity was depleted and the rate and extent of ATase depletion was patient, dosage and cycle dependent, with the nadir of ATase activity occurring 4–5 h after DTIC administration (Lee *et al.*, 1991, 1993*a*). These findings have been attributed to autoinactivation of ATase during the repair of O^6-MedG formed in lymphocyte DNA. It was also shown that fotemustine administration was not associated with ATase depletion in peripheral lymphocytes (Lee *et al.*, 1993*a*).

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The present study investigates the kinetics of formation and loss of O^6-MedG in total blood leucocyte DNA to explore possible relationships with changes in ATase levels in peripheral lymphocytes following DTIC administration. These factors may have important therapeutic implications, particularly in combination with the effects of administration of a nitrosourea for which drug resistance can also involve ATase activity (D'Incalci *et al.*, Pegg, 1990).

Materials and methods

Patients and blood samples

Details of the individuals studied are shown in Table I. Each patient received DTIC (400 mg m^{-2}) by i.v. infusion over 10 min followed 4 h later by fotemustine (100 mg m^{-2}) given as a 30 min i.v. infusion. The treatment was repeated every 4 weeks except for patient F.E. (with brain metastasis), who received an additional treatment on day 8. Blood samples (20 ml) were collected just before therapy and at 1, 2, 3, 4, 5, 6 and 18 h after DTIC administration from eight patients in the first treatment cycle. A further five sets of blood samples were collected from four patients who returned for subsequent treatments. Each sample was divided into two universal containers (10 ml each) containing 0.5 ml of 0.5% EDTA, pH 8.0. One half was stored at −20°C prior to DNA extraction and radioimmunoassay for O^6-MedG, while the second half was kept at 4°C before isolation of lymphocytes for ATase assays. Approval was given by the local ethical committee and informed consent was obtained from all patients prior to the study.

DNA isolation

Blood samples (10 ml) were thawed, combined with 10 ml of lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, 2 mM EDTA, pH 7.5) and allowed to stand at 4°C for 30 min. Nuclei were collected by centrifugation at 625 *g* for 10 min at 4°C, resuspended in 1.5 ml of 75 mM sodium chloride, 24 mM EDTA, pH 7.5, and lysed by the

Table I Patient characteristics

Patient/treatment cycle	Age/sex	Metastatic sites	Response[a]	ATase activity Pretreatment	ATase activity Nadir	O^6-MedG Peak amount[b]	O^6-MedG AUC[c]
I.P./1	66/F	Nodes/parotid gland	CR	241	137	14.3 (h3)[d]	28.5
I.P./2				138	90	24.7 (h3)	74.7
I.P./3				189	77	7.6 (h5)	57.7
K.R./1	68/F	Lung/ovaries	PR	258	123	12.8 (h3)	27.3
K.R./2				242	81	1.1 (h5)	10.9
F.E/1	61/M	Lung/brain	NE	278	184	7.7 (h4)	48.2
F.E./2				168	61	8.32 (h2)	110.3
G.B./1	53/M	Skin/nodes/lung	PD	217	72	5.7 (h5)	62.2
G.B./2				191	118	5.6 (h3)	76.0
J.G./1	54/M	Skin/liver	PD	400	196	1.0 (h3)	12.5
M.B./1	53/F	Subcutaneous/nodes/bone/ soft tissue	PD	309	163	2.0 (h3)	24.9
M.I./1	73/M	Skin/lung/liver/spleen	PD	–	–	5.6 (h3)	68.0
M.H./1	40/M	Nodes/soft tissue	PD	263	55	0.7 (h3)	2.0

[a]CR, complete response; PR, partial response; PD, progressive disease; NE, not evaluable. [b] Peak amount of O^6-MedG in total peripheral leucocyte DNA (μmol mol^{-1} dG). [c]Area under curve of O^6-MedG (μmol mol^{-1} dG) *vs* time (hours) from 0 to 18 h except for sample K.R. 1, which was integrated from 0 to 4 h. Values calculated following the trapezoid rule using Sigmaplot 5.0 graph-plotting software. [d]Time to reach peak O^6-MedG in total peripheral leucocyte DNA (hours).

addition of sodium dodecyl sulphate (SDS) to a final concentration of 1%. The lysate was then incubated overnight at 37°C in the presence of proteinase K (0.1 mg ml^{-1}). The following day, 1.5 ml of phenol (saturated with 100 mM Tris-HCl, pH 8.0) was added and after shaking at room temperature for 10 min the mixture was centrifuged at 625 *g* for 5 min. The upper, aqueous phase was re-extracted with phenol as above and residual protein was removed from the final aqueous phase by extraction with 1.5 ml of chloroform-isoamyl alcohol (25:1, v/v). DNA was precipitated by the addition of sodium acetate (10 μl of a saturated solution) and 3.75 ml of ethanol. Following sequential washing in 70% ethanol, ethanol, ethanol-ether (1:1, v/v) and ether, residual solvent was removed by evaporation in a stream of nitrogen. The yield of DNA was 0.2–1.0 mg and the product was free from RNA contamination.

O^6-*alkylguanine-DNA alkyltransferase assay*

ATase activity was measured in cell-free extracts of peripheral blood lymphocytes by monitoring the transfer of radioactive methyl groups from substrate DNA to protein. The substrate was prepared by *in vitro* methylation of calf thymus DNA using nitroso-[^{3}H]methylurea and activity is expressed as fmol of [^{3}H]methyl transferred per mg of protein in the extract (Lee *et al.*, 1991).

O^6-*Methyldeoxyguanosine analysis*

The procedure used for the determination of O^6-MedG in DNA has been described in detail elsewhere (Wild *et al.*, 1983; Hall *et al.*, 1991) and is presented here in outline only. DNA was digested enzymatically to nucleosides before ion-exchange chromatography using Aminex A7 resin (BioRad, Hemel Hempstead, UK). The four major deoxynucleosides were separated from O^6-MedG by this procedure and were quantified by peak area integration. The putative O^6-MedG containing fractions and control fractions (i.e. similar volumes of buffer from a nucleoside-free region of the column elution profile) were analysed by radioimmunoassay using a monoclonal antibody to O^6-MedG (Wild *et al.*, 1983). The results are expressed relative to the amount of dG in the DNA sample. The lower limit of detection using these small amounts of DNA was ~0.4 μmol of O^6-MedG per mol of dG.

Where sufficient sample remained, duplicate radioimmunoassay (RIA) determinations were performed, and these indicated an inter-assay variation which was generally $< \pm 20\%$. At values approaching the limit of detection, greater inter-assay variation was apparent, and this approached $\pm 35\%$ as previously reported (Badawi *et al.*, 1992). Assay variation has also been monitored by inclusion of a control sample which varied from 0.072 to 0.102 pmol of O^6-MedG (0.086 ± 0.010, mean ± s.d.) over a period of approximately 3 months.

Results

O^6-*Alkylguanine-DNA alkyltransferase activity*

The data regarding the changes in ATase activity during combination chemotherapy with DTIC and fotemustine have been reported previously as mean values of a group of patients (Lee *et al.*, 1993*a*). Here, we present the pretreatment and nadir (i.e. the minimum level reached during therapy) lymphocyte ATase activity on an individual basis for all the patients in the present study (Table I). Prior to the first cycle of chemotherapy, ATase activity among the different patients varied by a factor of approximately 2 (range: 217–400 fmol mg^{-1} protein) with a mean value of 289 ± 60 fmol mg^{-1} protein. This value fell to a mean nadir level of 133 ± 54 fmol mg^{-1} protein 4–5 h after treatment and inter-individual variation at this time increased to 3.6-fold (range: 55–196 fmol mg^{-1} protein). Following subsequent cycles of therapy, both the mean pretreatment and mean nadir levels of ATase activity (186 ± 38 and 85 ± 21 respectively) were reduced by a factor of 1.6 when compared with cycle 1.

O^6-*Methyldeoxyguanosine formation in leucocyte DNA*

O^6-MedG could be detected in total blood leucocyte DNA shortly after DTIC administration in all patients studied at cycles 1, 2 or 3 of treatment (Figure 1). The kinetics of DNA methylation was broadly similar in all cases: a post-treatment peak in O^6-MedG formation occurred at 3–5 h and was followed by a decline in adduct level (Figure 1). However, in some cases (e.g. FE1, Figure 1a) there was a tendency for O^6-MedG levels to rise again towards the end of the treatment cycle.

There was an approximately 20-fold inter-individual variation in the O^6-MedG maxima both among patients in cycle 1 (range: 0.7–14.3 μmol of O^6-MedG per mol of dG; 6.4 ± 5.5, mean ± s.d.) and in cycle 2 (range: 1.1–24.7 μmol of O^6-MedG per mol of dG; 9.4 ± 8.9, mean ± s.d.). Of the four patients who returned for subsequent courses of DTIC

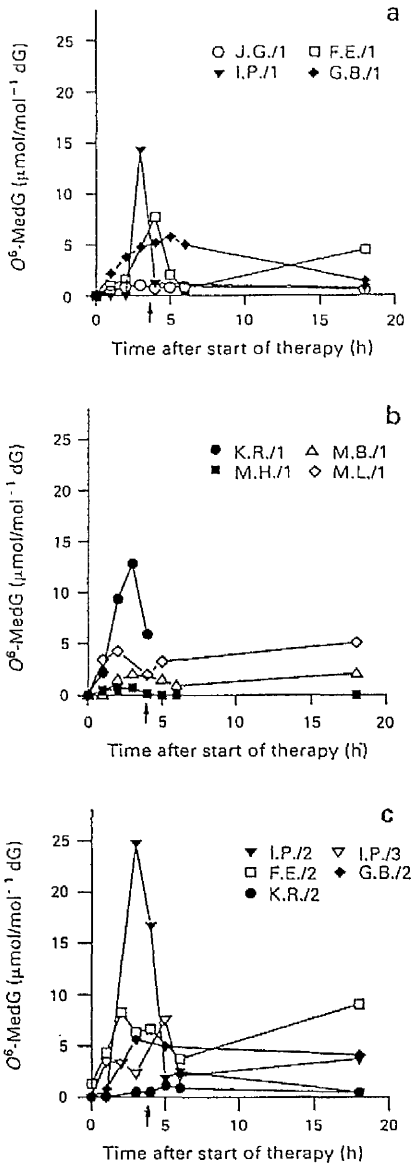

Figure 1 O^6-MedG levels in peripheral leucocyte DNA in eight patients at various times during cycle 1 (a and b) or subsequent cycles (c) of sequential DTIC (400 mg m^{-2}) fotemustine (100 mg m^{-2}) chemotherapy for metastatic melanoma. Arrows indicate the time at which fotemustine was administered.

therapy, two (F.E. and G.B.) achieved peak adduct levels similar to those observed in cycle 1, a third (K.R.) showed an approximately 11-fold reduction and in the fourth patient (I.P.) the peak O^6-MedG level in cycles 2 and 3 was increased and decreased, respectively, relative to that observed for cycle 1 (Figure 1 and Table I).

In all but one case (M.H./1), O^6-MedG persisted in the DNA for at least 18 h. One individual (F.E.; with brain metastasis) returned on day 8 for a second DTIC treatment and O^6-MedG was detected in leucocyte DNA at this time at a level of 1.3 μmol of O^6-MedG per mol of dG (Figure 1c). For the remaining cases in which repeat therapy was given at 28 day intervals, residual O^6-MedG from previous exposure to DTIC was not detected.

A combined measure of O^6-MedG formation and its persistence was obtained for each patient by integration of the area under the O^6-MedG concentration-time curve (AUC; Figure 1). The values obtained are shown in Table I. Again, considerable inter-individual variation was apparent with 34-fold and 10-fold differences between the highest and lowest values for cycles 1 and 2 respectively. In general, leucocyte DNA from patients returning for subsequent courses of chemotherapy tended to be more extensively methylated (in terms of the O^6-MedG AUC) than in patients receiving the first DTIC treatment (Table I).

Relationship between lymphocyte ATase activity and leucocyte O^6-methyldeoxyguanosine formation in peripheral blood

The nadir of ATase activity in peripheral lymphocytes occurred 4–5 h after DTIC administration, and this coincided with the peak of DNA methylation in the leucocytes. An inverse correlation was seen between the pretreatment ATase activity and the amount of O^6-MedG formed in DNA expressed as either the maxima or the AUC (Figure 2a and c respectively). The extent of lymphocyte ATase depletion (i.e. the difference between pretreatment and nadir ATase activity) was similarly related to the O^6-MedG maxima and the AUC (Figure 2b and d respectively). On the other hand, no correlation between the nadir ATase activity level and the extent of O^6-MedG formed in DNA was apparent.

Discussion

DTIC is a prodrug that requires metabolic activation to produce the methylating agent MTIC which can then react with DNA (Meer *et al.*, 1986). The present study demonstrates the presence of O^6-MedG in the DNA of peripheral leucocytes from patients receiving combined DTIC/fotemustine therapy and hence the ability of these patients to activate DTIC.

Maximum levels of O^6-MedG were observed 3–5 h after DTIC administration, and this coincided with the nadir in ATase activity in peripheral lymphocytes (Lee *et al.*, 1993*a*). Interestingly, DNA single-strand breaks occurring in peripheral blood lymphocytes are also maximal approximately 5 h after DTIC administration (Walles & Ringborg, 1991).

In treatment cycle 1 there was an approximately 20-fold inter-individual variation in the O^6-MedG maxima, which was also evident in the group of patients that went on to receive further courses of therapy (Figure 1 and Table I). The O^6-MedG AUC gives a combined measure of the formation and persistence of O^6-MedG, and these values also show large inter-individual variations, with differences of 34-fold and 10-fold for cycle 1 and subsequent cycles respectively. In some patients, an increase in O^6-MedG level was seen at 18 h, and while this effect may be accounted for to some extent by experimental variation (see Materials and methods) reasons for the larger increases seen in patient F.E. (cycles 1 and 2) are unclear. However, similar changes in 7-methyldeoxyguanosine levels have been observed in leucocyte DNA from patients treated with DTIC, and this was attributed to changes in turnover rates of white blood cell subpopulations (van Delft *et al.*, 1992).

Of the four patients returning for additional DTIC therapy, three show an increased O^6-MedG AUC with respect to cycle 1. These values (I.P. 2, I.P. 3, G.B. 2 and F.E. 2) are associated with relatively low pretreatment ATase levels and form a small cluster in Figure 2c. The effect is most pronounced in F.E., an individual with brain metastases who received a second course of therapy 8 days after the initial treatment when O^6-MedG was still detectable in leucocyte DNA (Figure 1c). Presumably, as a consequence of this, ATase activity remained depressed. Such an explanation does not apply to the remaining three instances (I.P. 2, I.P. 3 and G.B. 2) as O^6-MedG was not detected at the start of

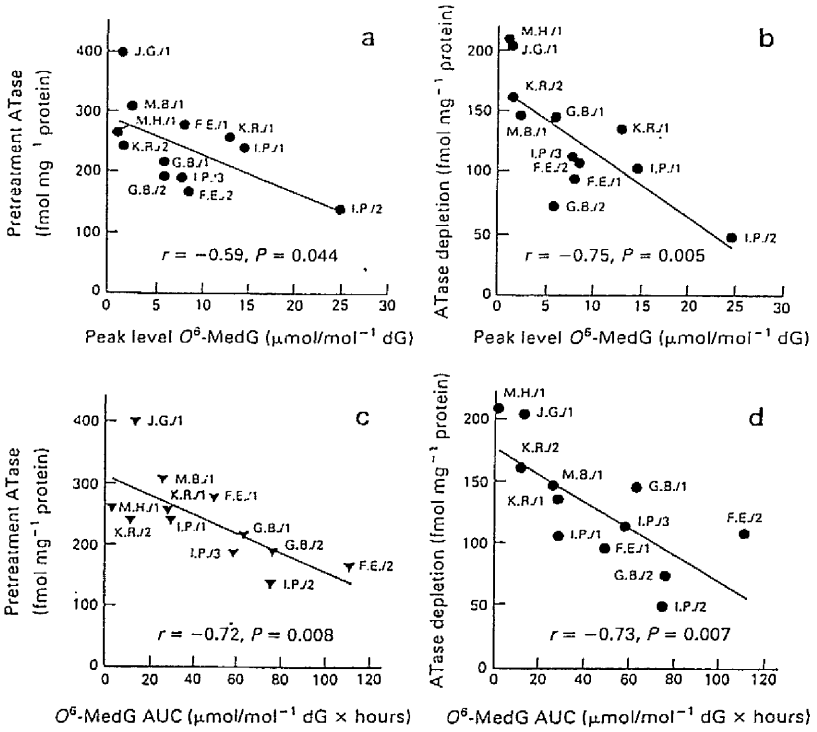

Figure 2 The relationship between the peak O^6-MedG level (a and b) or the O^6-MedG AUC (c and d) for leucocyte DNA and pretreatment ATase (a and c) or ATase depletion (b and d).

subsequent DTIC cycles, suggesting that in some cases ATase expression dies not readily recover.

Clearly, the extent to which these present results can be extrapolated to tumour cells depends on the extent to which O^6-MedG levels in leucocyte DNA correlate with those of target tumour DNA. Human tissues and tumours differ greatly in their levels of ATase (D'Incalci *et al.*, 1988), but there are indications that relationships between tissues may exist (Kyrtopoulos *et al.*, 1990): studies in rodent models have shown that DNA methylation occurrs to a broadly similar extent in most tissues following administration of methylating agents, even those requiring metabolic activation (Kleihues *et al.*, 1976; Fong *et al.*, 1990), and to a similar extent in leucocyte DNA (Degan *et al.*, 1988).

In fact, in the present study, the kinetics of lymphocyte ATase depletion and leucocyte DNA O^6-MedG accumulation does suggest concomitant effects in the two populations of cells. Following DTIC treatment, correlations were seen between the amount of O^6-MedG formed in leucocyte DNA (expressed either as the peak value or the AUC) with the pretreatment lymphocyte ATase activity (Figure 2a and c) or with the extent of ATase depletion (Figure 2b and d). Although these relationships are based on a relatively few patients, a similar trend was observed in a study of patients treated with the related drug, procarbazine (Souliotis *et al.*, 1990), and strong correlations between pretreatment ATase activity and both peak O^6-MedG levels and O^6-MedG AUC have recently been established in patients treated with 1-*p*-carboxyl-3,3-dimethylphenyltriazene (CB10-277; Lee *et al.*, 1993*b*; S.M. Lee *et al.* in preparation). A similar relationship between ATase activity and the amount of O^6-MedG in DNA was evident also in analyses of bladder mucosa from individuals exposed to environmental alkylating agents (Badawi *et al.*, 1991). The present results suggest that patients with high initial levels of ATase are therefore able to repair a greater proportion of the O^6-MedG resulting from DTIC therapy, whilst the adduct accumulates in the leucocyte DNA of individuals with low pretreatment ATase levels.

Of the eight patients studied, only two (I.P. and K.R.) responded to therapy and, although O^6-MedG AUC for both patients was close to the mean value, their leucocyte DNA contained the highest peak levels of O^6-MedG after treatment cycle 1 (Table I). This may reflect the need to reach a minimum threshold O^6-MedG level before cell killing can occur.

In conclusion, the results reported here lend support to our earlier suggestion that the nadir lymphocyte ATase depletion observed approximately 4 h after 400 mg m^{-2} DTIC is a consequence of DNA methylation, which is also maximal at this time. The wide individual variations in the extent of DNA methylation may result not only from differences in DNA repair capacity but also from differences in the capacity for metabolic activation, uptake and detoxification of DTIC. Use of DNA adduct measurement can therefore provide information on variations in cellular penetration, metabolic activation, distribution and clearance of methylating anti-tumour agents such as DTIC, which in combination with knowledge of ATase activity will permit the design of individualised treatment protocols with improved therapeutic benefit.

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Abbreviations: ATase, O^6-alkylguanine-DNA alkyltransferase; AUC, area under concentration-time curve; dG, 2'-deoxyguanosine; DTIC (dacarbazine), 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; MTIC, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide; O^6-MedG, O^6-methyl-2'-deoxyguanosine.

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Heterogeneity in the Mechanisms of Resistance to Chemotherapy Related to DNA Damage and Repair

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INTRODUCTION

The alkylating agents are a large group of biologically active chemicals, many of which are carcinogenic and some are widely used in chemotherapy. It is now generally accepted that these agents exert their toxic, mutagenic and carcinogenic effects via interaction with DNA. Their mutagenic effects have been ascribed with some certainty to the presence of the promutagenic lesions *O^6-alkylguanine and O^4-alkylthymine* in DNA and there is a considerable body of circumstantial evidence implicating these lesions as the initiating events in alkylating agent-induced carcinogenesis. The toxic effects of alkylating agents, which are important for chemotherapy, were originally associated with the lesions lying in the narrow groove of DNA (the 3-alkylpurines), but more recently the toxicity of O^6-alkylguanine in DNA has been elegantly demonstrated using techniques for the transfection of foreign DNA repair genes into repair deficient cells (Margison and O'Connor, 1990). The purpose of this article is to discuss heterogeneity in mechanisms of resistance to the action of alkylating agents as related to DNA damage and repair, within the context of chemotherapy. First it is necessary to consider the initial reactions with DNA.

REACTION WITH DNA

Alkylating agents can be conveniently divided into two groups. Those which need to be metabolised in order to generate the spontaneously reactive alkylating species (the so-called indirect agents) before they can

interact with the constituents of cells and thereby exert their biological effects and others which decompose spontaneously (i.e. the direct alkylating agents). In the former case the distribution of enzymes capable of metabolising the agent in question will determine not only the relative effectiveness of the agent, but also, those cells which will be affected.

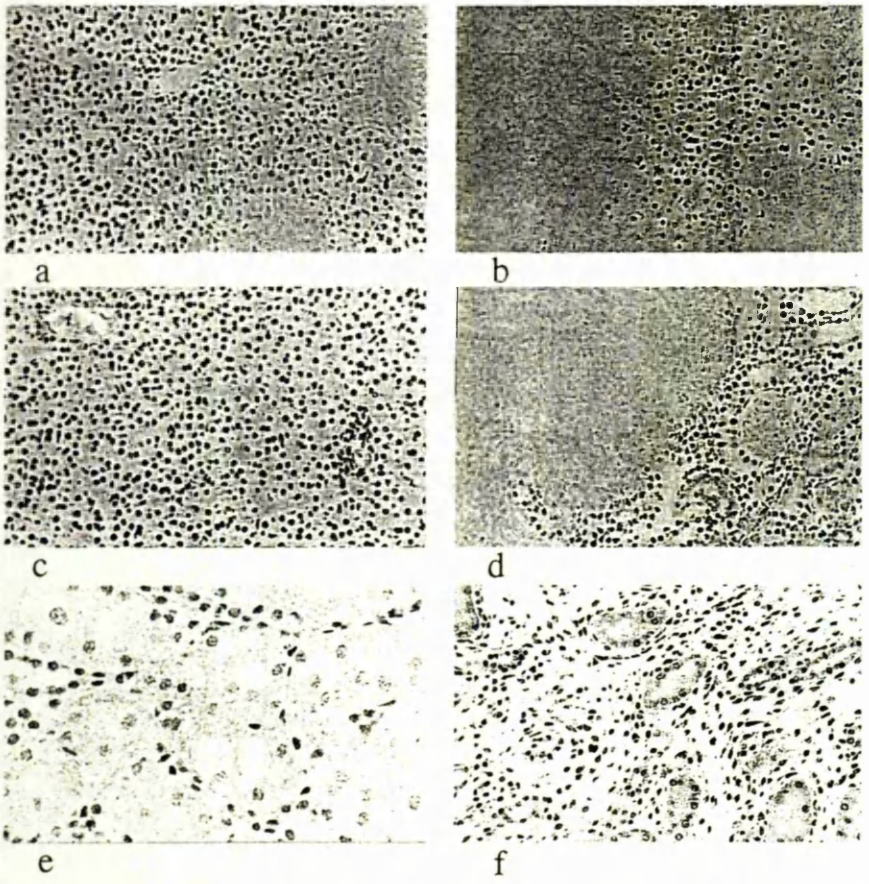

FIG. 1. Immunohistochemical staining of 35 paraffin wax sections for the presence of O^6-methyl-2'-deoxyguanosine in nuclear DNA. Liver of weanling rats maintained either on a normal diet, (a) or for 3 days on a protein deficient diet, (b) and then given NDMA (30 mg/kg) 3 hours prior to sampling; (c), the liver of a rat maintained on a normal diet and given MNU (75 mg/kg) 1 hour before sampling; (d), the kidney of an animal treated as in (b); (e), idem, after 10 weeks and (f) a mesenchymal tumour arising 20 weeks after administration of NDMA to an animal on a protein deficient diet. The conditions for staining using 3',3'-diaminobenzidine and the appropriate control procedures are described in Fan et al, (1989); Zaidi et al., (1992).

In the case of the spontaneously reactive agents, or those which although requiring metabolic activation have a long lived reactive intermediate, their reactivity will be less cell selective and depend mainly upon accessibility and

drug concentration (Margison and O'Connor, 1979; Saffhill et al., 1985). We can gain some insight into the cell-specific nature of the reactions of the indirect alkylating agents by turning briefly to an animal model. If weanling rats are given a single dose of *N-nitrosodimethylamine* (NDMA) they eventually produce mesenchymal tumours of the kidney and if they are kept for a day or two on a protein deficient diet then the tumour yield is greatly increased (Hard and Butler, 1972; Swann et al., 1980; Driver et al., 1987). Using a polyclonal antibody raised against *O^6-methyl-2'-deoxyguanosine* (0^6-MedG) (Wild et al., 1983) in an immunohisto-chemical procedure (O'Connor et al., 1988; Fan et al., 1989) we can readily determine which cells contain 0^6-MedG and hence which cells were exposed to the alkylating species derived from this agent. Figure 1 (a and c) show the localised distribution of alkylated cells in the hepatic lobule of an animal given NDMA (30 mg/kg) compared to

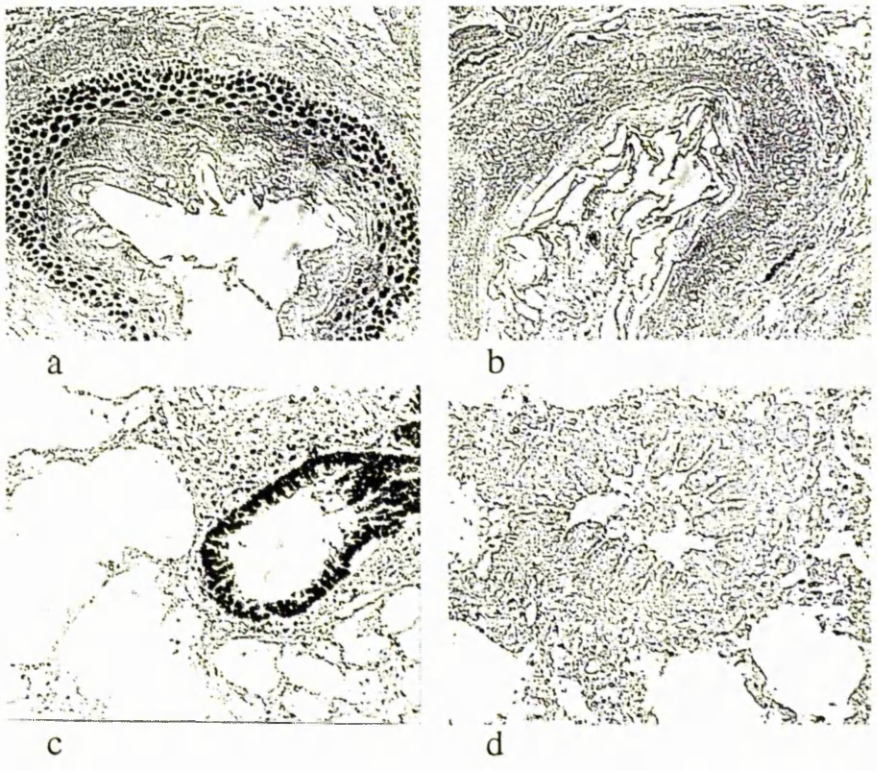

FIG. 2. *Immunohistochemical staining for* the presence of *0^6-methyl-2'-deoxyguanosine* in nuclear DNA of epithelial cells in sections of rat lung and oesophagus. Intense staining of the oesophagus (a) and bronchiolar epithelia (c) is observed after treatment with the asymmetrical nitrosamine N-nitrosomethylbenzylamine (30 mg/kg) but not with the symmetrical nitrosamine, NDMA (4 mg/kg) (b) and (d), respectively. These doses given intraperitoneally give similar levels of methylation in hepatic DNA. The conditions for staining with 3',3'-diaminobenzidine and control procedures are described in O'Connor et al., 1988 and Zaidi et al., 1992.

the non-selective staining of all cell nuclei observed in an animal given *N-methyl-N-nitrosourea* (MNU) (75 mg/kg). Sections of rat liver stained for the presence of the metabolising enzyme itself, the NDMA-demethylase (P450 II E1) (Nebert et al., 1991) after first treating the animal with NDMA to activate the enzyme, show that the distribution of this protein corresponds well with the centrilobular distribution of the alkylated cells in rats given NDMA.

However, if the same animals are maintained on a protein-free diet, then the hepatic capacity for the *metabolism of NDMA* is significantly reduced (Swann and MacClean, 1971) and there is a marked reduction in the number of alkylated cells within the liver lobule (Fig 1b; Fan et al., 1989). This reduced efficiency of the hepatic "first pass" effect permits more of the nitrosamine to circulate around the body and so to alkylate other tissues, with the corresponding observed increase in the yield of renal tumours. In the kidney we also see a marked heterogeneity of alkylation damage (Fig 1d). The medulla is not alkylated and neither are the glomeruli, but the cells of the Bowman's capsule are positively stained and so are the epithelial cells of the renal tubules and the mesenchymal cells of the intertubular regions. If we look at the kidney 10 weeks later (Fig 1e), when the animals are almost full grown, we see that just a few cells remain alkylated. These *cells* are not only metabolically proficient for the activation of the nitrosamine, but also *repair deficient* for the removal of O^6-methyl-guanine (Fan et al., 1990).

They are the presumed mesenchymal cells from which the renal tumours arise (Hard and Butler, 1972). As a population of cells which we know to be continuously, but slowly undergoing cell replacement (Fan et al., 1990), they are therefore continually at risk for the initiation of carcinogenesis. At this time, regions of mesenchymal hyperplasia are observed and 20 weeks after the initial treatment small *mesenchymal tumours* begin to appear in the same region *of the kidney* (Fig 1f; Hard and Butler, 1972; Driver et al., 1987). If, on the other hand, the rats are treated with an asymmetrical nitrosamine such as N-nitrosomethylbenzylamine then the liver is no longer the most heavily alkylated tissue and the epithelia of the bronchioles, oesophagus (Fig 2), trachea, and nasal sinuses become the principle and highly selective targets (Koenigsmann et al., 1988; O'Connor, 1989). In liver itself, differences occur in the distribution of alkylated cells within the liver lobule when treatment with NDMA is compared with that of N-nitrosomethylbenzylamine (Dai et al., 1991).

From these and other studies it is clear that the character of the alkylating agent (i.e. indirect or direct), the distribution of metabolising enzymes, physiological state and the structure of the alkylating agent itself, along with other factors which include DNA repair, DNA sequence (Hartley et al., 1988), the location of the DNA within the cell e.g mitochondria (Myers et al., 1988) and chromatin domains (Ryan et al., 1986), can all markedly effect the identity and degree to which individual cells are affected (Table 1).

Table 1. *Factors affecting the heterogeneity of DNA alkylation*

Intercellular:
Character of agent (e.g. direct or indirect acting)
Enzymes of Metabolism:-
distribution
induction
inhibition
Structure of Agent (e.g. asymmetrical nitrosamines)
DNA Repair (e.g. repair deficient cells)
Intracellular:
Location of DNA
mitochondria *vs* nucleus
within chromatin structure
DNA Sequence (e.g. effects of adjacent bases)
DNA Repair (location and effects of adjacent bases)

DNA damage and repair in human tissues

From the chemotherapist's viewpoint, information on parameters such as those discussed above would be invaluable tools in the tailoring of more effective chemotherapy. Although in principle we now have the methods to study the process of DNA damage and repair in human tissues, the problems of sensitivity and the availability of samples are still limiting factors for these investigations. One solution to this problem is through the use of xenograft models; with *normal human breast xenografts* we have been able to show, using a polyclonal antibody to O6-MedG, that the cells of the ductal epithelium and the myoepithelium have the capacity to activate NDMA (Fig 3; Zaidi et al., 1992). Such cells could be targets for the carcinogenic action of this nitrosamine which is ubiquitously present in the human environment (Bartsch and Montesano, 1984). These models, however, are only of limited use as they may fail to mimic the in vivo situation. If the mechanisms of chemotherapy using the alkylating agents are to be properly understood and rationally used, then a fuller understanding of these processes in normal and tumour tissue as they occur during chemotherapy regimes is essential.

Our earliest venture in this area of work was in response to speculations (see Yarosh, 1985; Saffhill et al., 1985) that tumour cells might be predominantly repair deficient. Assays of *O^6-alkylguanine-DNA-alkyltransferase* (ATase) activity in extracts of paired samples *of normal and tumour tissue* from the stomach and colon of patients undergoing surgery for these gastrointestinal problems showed that this was true for only 38% of the tumours (Margison

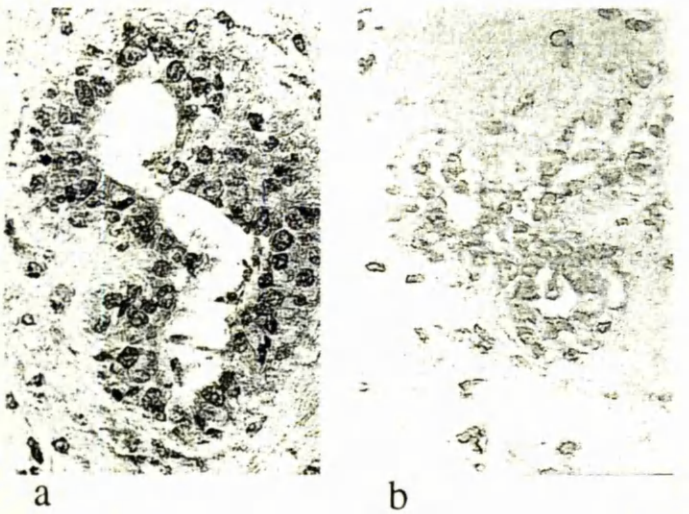

FIG. 3. Immunohistochemical demonstration of O^6-methyl-2'-deoxyguanosine in the ductal and myoepithelial nuclei of human breast tissue maintained as xenografts in Nu/Nu mice. The host animals were given NDMA (20 mg/kg), (a) or saline as a control, (b) 4 hours before sampling (Zaidi et al., 1992).

et al., 1990). Thus, in the majority of cases the tumour cells would appear to be able to rid themselves more effectively of the toxic lesion O^6-MedG than would the corresponding normal tissue. Apparently therefore, ATase activity differentials between tumour and normal tissues offer no simple advantage for exploitation and more systematic studies of DNA damage and repair are needed.

More detailed studies were undertaken in patients undergoing treatment for malignant skin tumours. *In melanoma* patients the methylating agent DTIC (Fig 4) has proved particularly useful, giving responses in ~20% of cases. It was administered by infusion over 10 minutes at 400, 500 or 800 mg/m^2 followed 4 or 5 hours later by an infusion of 100 mg/m^2 of the chloroethylating, cross-linking agent, fotemustine (Fig 4) over 30 minutes. Peripheral blood lymphocytes (PBL) or total blood leukocytes (TBL) DNA were isolated from 2x10 ml blood samples by centrifugation on Ficoll gradients or by phenol extraction of whole blood, respectively (Lee et al., 1991; Lee et al., in preparation).

In the first experiments with DNA binding, DNA was isolated from blood samples taken just before the fotemustine infusion, digested to nucleosides enzymatically and chromatographed on an Aminex A7 column to separate the O^6-MedG. After solid phase extraction on Bond Elut columns to purify and concentrate the O^6-MedG the amount was determined by *RIA* using a *monoclonal antibody specific for O^6-MedG* (Wild et al., 1983; Fig 5). Initially, in patients given 200, 400 or 800 mg DTIC/m^2 in the first cycle of treatment

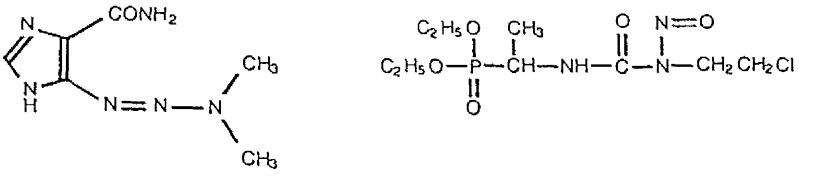

FIG. 4. Formula of (a), 5-(3,3-dimethyl-1-triazino)imidazole-4-carboxamide (*DTIC; dacarbazine*) and (b), *fotemustine*.

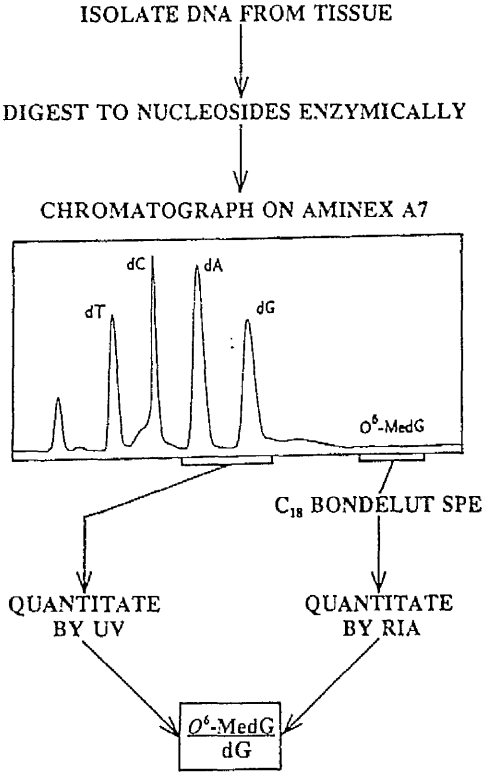

FIG. 5. Procedure for the isolation and analysis of DNA containing O^6-methyl-2'-deoxyguanosine (see Wild et al., 1983).

there was an indication of a linear dose response relationship. The amounts of O^6-MedG formed appeared to suggest a valuable guide to the extent of DNA binding, however, when the kinetics of alkylation were determined in individual patients given the first cycle of DTIC treatment this proved to be

an oversimplification. Samples taken at 1, 2, 3, 4, 5, 6 and 18-20 hours after administration of 400 mg/m^2 for the 8 patients studied so far showed curves for the formation of O^6-MedG with evidence of an early peak of methylation (see Fig 6). There was, however, a 20-fold difference between the lowest and the highest levels of peak methylation (0.71-14.33 μmoles O^6-MedG/mole dG) with a mean value of 6.4±5.53 μmoles O^6-MedG/mole dG and the DNA reaction curves differed considerably in character, from an early, high, transient peak (IP1) to a shallow but sustained level of reaction (GB1). This indicated considerable inter-patient differences in the kinetics of metabolism of the drug and presumably therefore of the availability of the drug for the alkylation of tumour DNA. It was of interest to note that the 2 patients which showed the high transient levels of O^6-MedG (KR1 and IP1; 12.7 and 14.5 μmoles O^6-MedG/mole dG respectively) were the only two which were judged to respond and the remaining six cases were classified either as having progressive disease or not evaluable (1 case).

O^6-MedG formation was also examined in patients given subsequent cycles of DTIC/fotemustine treatment and again a similar pattern of inter-individual variation was observed. One patient (IP) who returned for treatment cycles 2 and 3 achieved peak methylation levels of 24.7 and 7.57 μmoles O^6-MedG/mole dG respectively, compared to 14.33 μmoles O^6-MedG/mole dG in the first cycle. On the other hand the patient KR who returned for a second treatment cycle achieved a maximum of only 1.14 compared to 12.09 μmoles O^6-MedG/mole dG attained in the first cycle. It may be of significance that a complete response was recorded for the patient IP, compared to only a partial response for the patient KR.

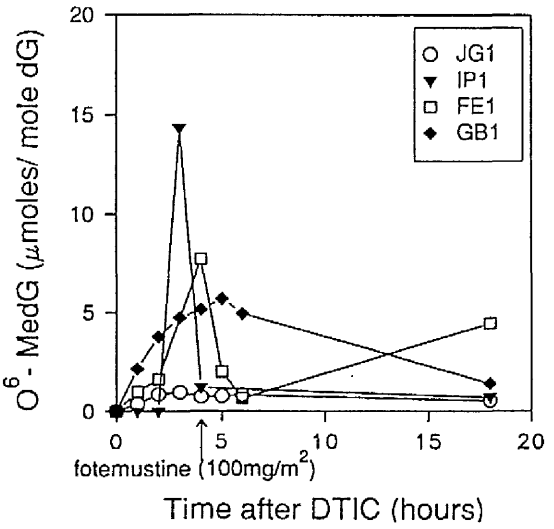

FIG. 6. *O^6-methyl-2'-deoxyguanosine in total blood leukocyte DNA* of 4 patients at various times after the first cycle of chemotherapy with DTIC and fotemustine. The curves shown are typical of those for this study.

A preliminary study has commenced using another metabolism-requiring methylating agent, *CB10-277*. In this case the infusion of 12g/m^2 took place over 24 hours and the kinetics of DNA alkylation, after an apparent 5 hour lag period, rose sharply to 20 hours and then increased slowly over the next 20 hours. In this one case a significantly higher and more sustained level of DNA alkylation was achieved than with DTIC.

Whilst these studies indicate the period for which the alkylating agent is still circulating in the blood stream and therefore available for alkylation of tissue DNA, they do not define the extent or kinetics of alkylation of DNA in the tumour itself. Some limited studies are in progress to determine (by RIA) the alkylation levels of tumour biopsy DNA but now that we have developed more sensitive procedures involving immunoaffinity columns coupled with 32P DNA-postlabelling for O^6-MedG (Cooper et al., 1991), more detailed studies can be contemplated using small tumour biopsy samples.

The activity of the DNA repair protein ATase has also been studied. The ATase activity in PBL was measured in patients entering into a treatment schedule with DTIC at 400, 500 or 800 mg/m^2 followed by fotemustine as indicated above. ATase activity was rapidly depleted in all cases by 4-6 hours but to a nadir level which varied from 44 to 99% of the pretreatment value. Typical depletion curves are shown in Figure 7. The nadir of ATase activity tended to occur earlier after 400 mg/m^2 than either 500 or 800 mg/m^2 DTIC. In some cases recovery occurred within 20 hours but in others, substantial recovery had not occurred, even by 7 days. In one case activity was depleted completely within one hour and there was no return of activity over the next 18 hours (Lee et al., in press).

When the data for a group of 25 patients treated with 400, 500 or 800 mg/m^2

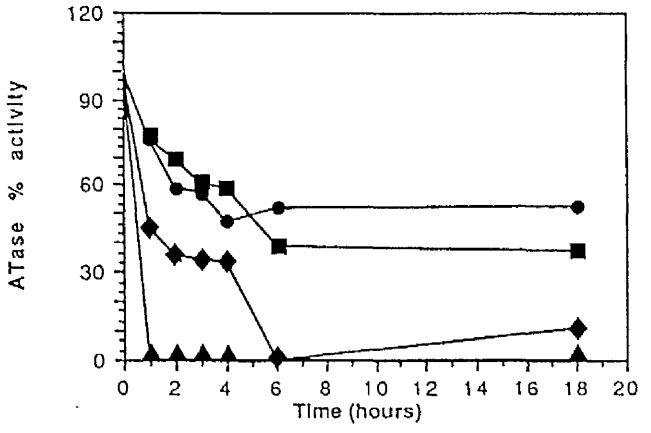

FIG. 7. *O^6-Alkylguanine-DNA-alkyltransferase activity in peripheral blood lymphocytes* of patients at various times after the first cycle of DTIC and fotemustine chemotherapy (From Lee et al., 1991).

DTIC (9, 5 and 11 patients, respectively) were averaged there was a relationship between the nadir of ATase activity and dose. This relationship is not simple, as the degree of inhibition at the highest dose was only slightly greater than that after 500 mg/m^2 (Lee et al., in press). It does, however, conform very closely to a semi-log relationship and if substantiated the effect is therefore potentially predictable.

When DTIC treatment cycles 1, 2, 3, 4 and 6 were compared there was some evidence that ATase was depleted to a lesser extent after cycle 1 than after subsequent cycles and that the change of ATase activity over time varied with the dose and cycle (Lee et al., in press). Fotemustine given alone on the other hand, despite being a chloroethylating agent (Zlotogorski and Erikson, 1984), had no significant effect upon ATase activity when pretreatment levels were compared with those obtained at 3-4 hours or 16-18 hours after 100 mg/m^2 fotemustine for each of three treatment cycles (Lee et al., in press).

In a number of patients the ATase activity in PBL and the O6-MedG content of TBL was compared. There was no indication of a relationship between O6-MedG content and the pretreatment ATase level or with the nadir ATase levels (expressed as a percentage of pre-treatment levels: $p = 0.37$) (Lee et al., in preparation).

There was, however, a striking relationship between the timing of the *nadir ATase activity* (Lee et al, 1991) and that of the *peak of DNA methylation*, both of which occurred ~3-5 hours after DTIC administration. If events in the tumour tissue mirror those taking place in the peripheral blood, then this suggests that fotemustine treatment given at 4-5 hours post DTIC administration has been timed appropriately to achieve the maximum lethal effect of DNA cross-links arising from the chloroethylation of DNA guanine by fotemustine, since crosslinking would otherwise be prevented by the activity of ATase in repairing O^6-chloroethylguanine.

Location of O^6-alkylguanine-DNA-alkyltransferase

The events observed so far tell us something about inter-individual differences, the duration of the period during which active alkylating DTIC and CB10-277 metabolites are available and the relationship between DNA methylation and ATase depletion. They do not necessarily reflect events at the level of individual cells, particularly those of the tumour cells. Methods are, however, now becoming available that will allow us to address these issues.

The availability of a cloned DNA sequence containing the human ATase (hAT) gene has enabled production of purified human ATase protein for the preparation of a rabbit antiserum (Lee et al., 1992). This reacts with extracts of normal human liver, melanoma cells, the over-expressing Raji cells and

purified human ATase, but not with extracts of the non-expressing TK6 cells and to a much lesser extent with rat ATase protein, as shown by Western blots on these proteins. This specificity was confirmed and extended by liquid inhibition experiments in which human ATase, rat ATase and mouse ATase were mixed with increasing amounts of either anti-human ATase or anti-rat ATase.

The presence of anti-human ATase specifically inhibited only human ATase, whereas anti-rat ATase inhibited rat and mouse, but not human or Chinese hamster ATase (Rafferty et al, 1992; Santibanez-Koref et al., 1992).

After the polyclonal antibody had been characterised it was used in immunohistochemical (IHC) procedures to detect human ATase in the tissues of mice that were transgenic for the human ATase cDNA and for which studies of the expression and induction of the ATase protein had been made (Fan et al., 1990; Rafferty et al., 1992). The protein was expressed in a wide variety of tissues and it was of interest to observe differences in the extent to which expression varied from cell to cell. Although traces of the human ATase protein were detected in the cytoplasm, by far the majority was concentrated within the nucleus, possibly in some way bound to chromatin DNA structures and thereby conserved against the advent of DNA damage (Fan et al, 1990).

Once validated, these IHC procedures were then used to examine a variety of human tissues. In all cases, pre-immune serum was used as a control. In normal human liver cells, a similar pattern of nuclear staining was observed (Lee et al., 1992). There was no apparent predominance of centrilobular or periportal staining but portal vein endothelial cells and Kuppfer cells were mostly unstained. There was, however, a marked intercellular heterogeneity in the staining of the nuclei in the ATase positive cells. When these liver sections were treated with primary antibody preadsorbed with human ATase as an additional control, all nuclear staining was abolished.

In the melanoma sections, all recognisable cell types including the melanoma cells, keratinocytes, vascular endothelial cells, fibroblasts and smooth muscle cells showed predominantly nuclear staining. In melanoma cells, there was a marked intercellular variation in the intensity of staining and in some melanoma cells the nucleus was free of stain. In epidermal keratinocytes, strong cytoplasmic and nuclear staining was observed. This pattern was observed for the 16 melanoma samples that we have examined (Lee et al., 1992). Similar patterns of heterogeneity have been observed in other tumours e.g. ovarian adenocarcinoma, Hodgkins and glioblastoma (Fig 8).

It is desirable now to use IHC to detect O6-MedG, 7-methyldeoxyguanosine and ATase in the nuclei of the melanoma cells post-treatment in order to verify the kinetics of DNA binding that has been established for TBL DNA and the depletion of ATase activity observed for PBL.

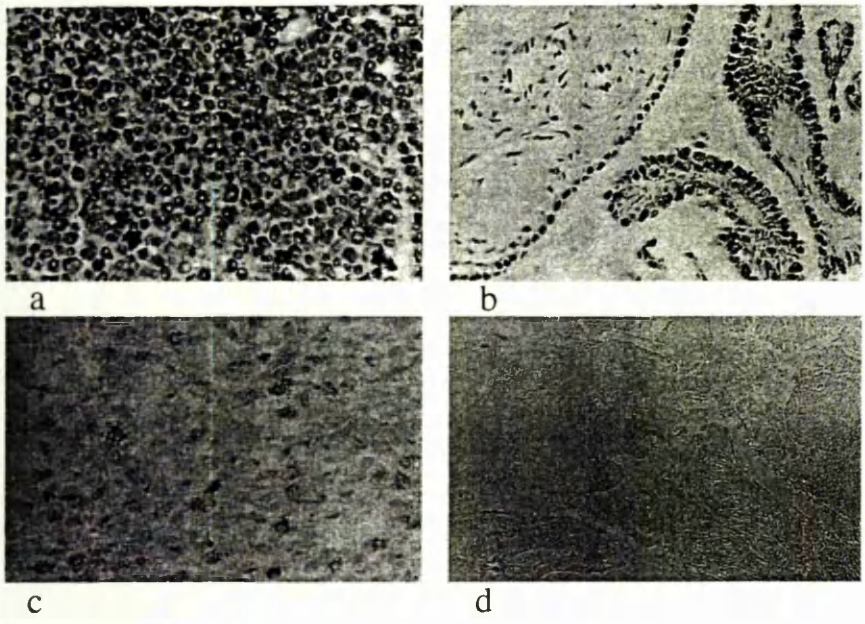

FIG. 8. Immunohistochemical demonstration of the presence of the O^6-alkylguanine-DNA-alkyltransferase in 35 paraffin wax sections of various human tumours to show the heterogeneous distribution of the protein: a) melanoma; b) an ovarian tumour of the serous cystadenocarcinoma type; (c), glioblastoma and (d), control section of the ovarian tumour. The sections were treated either with a *polyclonal anti-O^6-alkylguanine-DNA-alkyltransferase* (a, b, c) or, with pre-immune serum, (d). The conditions for staining with 3',3'-diaminoazobenzidine and appropriate control procedures are described in Lee et al, (1992).

CONCLUSIONS

Apart from the practical limitations of obtaining clinical samples, there remains the problem of sensitivity for the DNA adduct staining as well as certain technological difficulties associated with this type of staining which sometimes limit the direct transfer of this IHC technique from one species or tissue to the next. Nevertheless, we are now close to providing the clinician with a detailed account of the progress of therapy at the level of the individual tumour target cell. It is already evident that the intercellular variation which we have observed in the animal model discussed at the outset is being repeated in human tissues. We now need to know whether or not repair efficient or deficient cells are a feature of specific tumour cell types and the extent to which the DNA of these cells is damaged by the reactive intermediates of DTIC. Such information is expected to increase our understanding of tumour resistance to chemotherapy and eventually to indicate ways in which this problem may be overcome. In doing so, our un-

derstanding of dose will be refined to estimates of the biologically effective dose to the DNA of target cells and ultimately, the dose to the DNA of specific gene sequences by agents tailored for lethality on interaction at these sites. As our knowledge in this area increases, we anticipate that a more informed use of existing agents, together with the introduction of novel approaches will significantly improve the prospects for the success of cancer chemotherapy.

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