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

**Siow-Ming Lee, Martin Harris, John Rennison, Alan McGown, Michael Bromley, Rhoderick H. Elder, Joseph A. Rafferty, Derek Crowther and Geoffrey P. Margison**

**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.

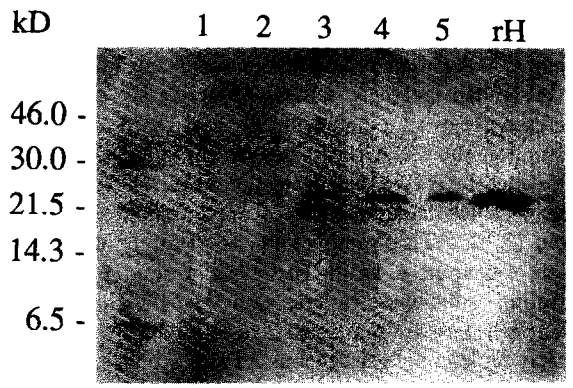


**Fig. 1. Western blot of crude sonicates of ovarian tumours with increasing ATase activity (lanes 1–5) and recombinant human ATase (rH) probed with anti-human ATase serum. The positions of the molecular weight markers (kD) are shown. See text for experimental details.**

*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).

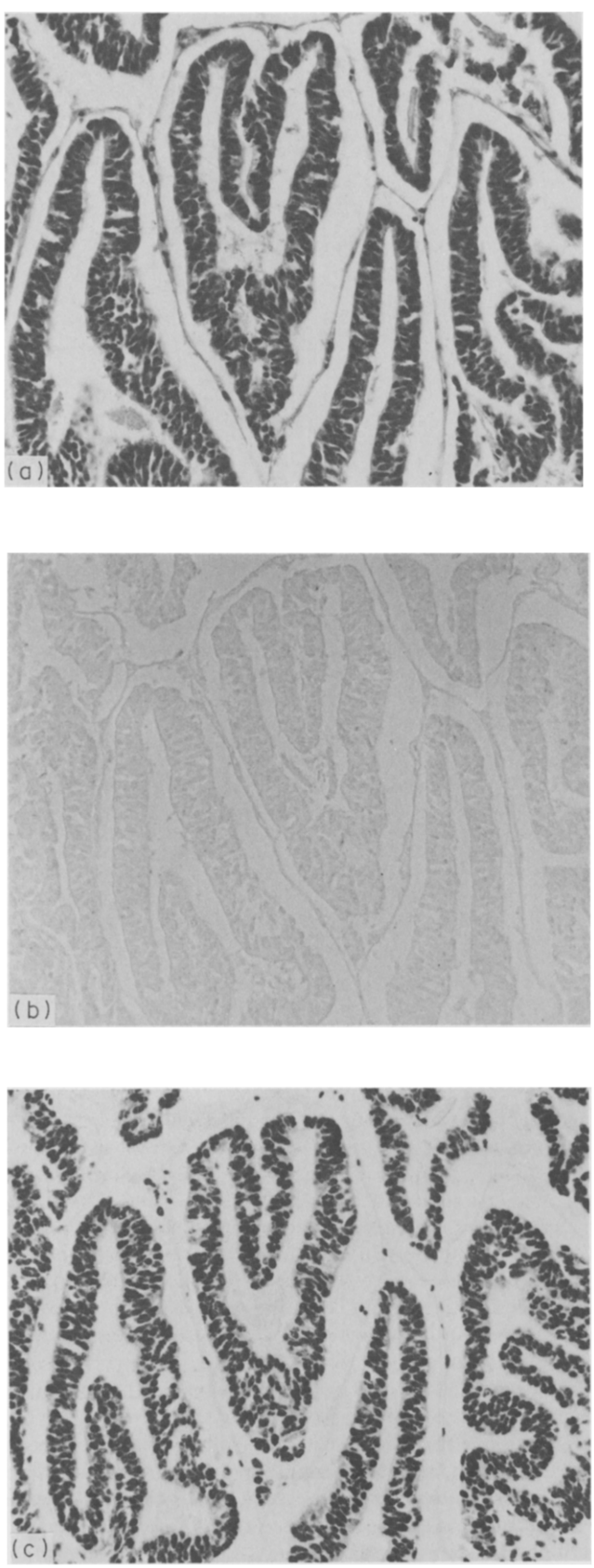


Fig. 2. Well differentiated serous adenocarcinoma of the ovary: (a) haematoxylin and eosin staining; (b) immunostaining with pre-immune serum; (c) immunostaining with anti-human ATase anti-serum showing strong, uniform staining of tumour cell nuclei. Note that fibroblasts in the connective tissue septae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

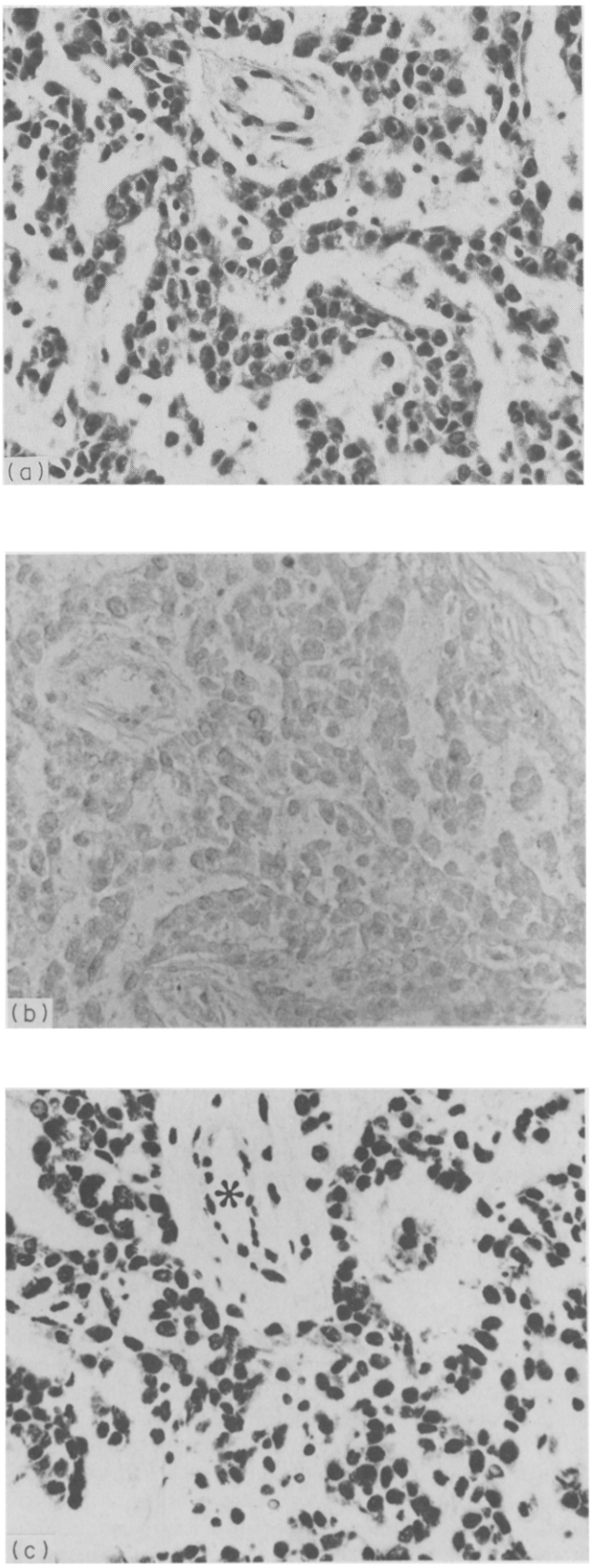


Fig. 3. Poorly differentiated serous adenocarcinoma of the ovary: (a) haematoxylin and eosin staining; (b) immunostaining with pre-immune serum; (c) immunostaining with anti-human ATase anti-serum showing similar strong, uniform staining of tumour cell nuclei; the endothelium of the blood vessel near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

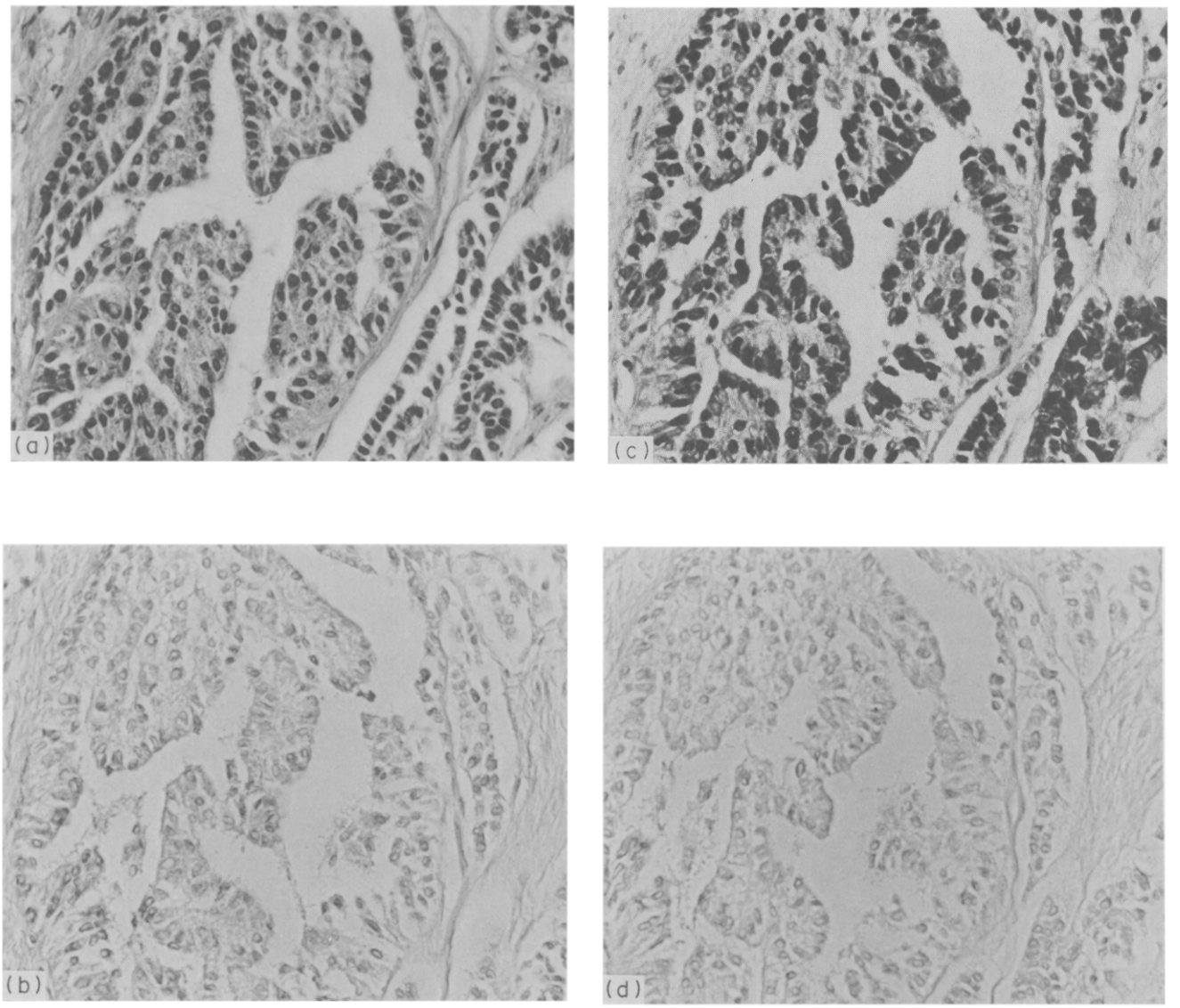


**Fig. 4. Moderately differentiated serous adenocarcinoma of the ovary: (a) haematoxylin and eosin staining; (b) immunostaining with pre-immune serum; (c) immunostaining with anti-human ATase antiserum showing strong, uniform staining of tumour cell nuclei; (d) immunostaining with ATase antiserum affinity-purified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

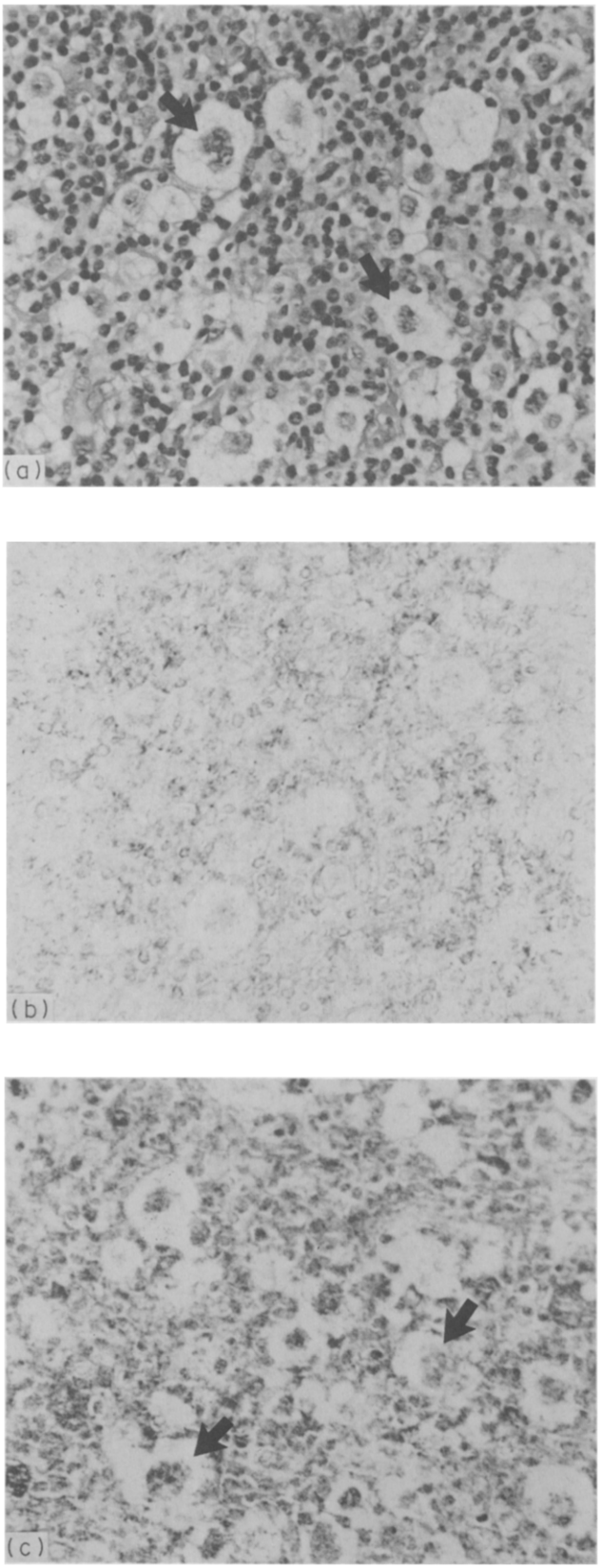


**Fig. 5. Nodular sclerosing Hodgkin's disease: (a) haematoxylin and eosin. Lacunar-type Reed–Sternberg cells are plentiful (arrows) in a background population of small lymphocytes; (b) immunostaining with pre-immune serum. Weak non-specific staining is present in the small lymphocytes; (c) immunostaining 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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