

## Technical reports

# A sensitive fluorescent assay for measuring the cysteine protease activity of Der p 1, a major allergen from the dust mite *Dermatophagoides pteronyssinus*

O Schulz, H F Sewell, F Shakib

### Abstract

**The potent allergenicity of Der p 1, a major allergen of the house dust mite *Dermatophagoides pteronyssinus*, is thought to be related to its cysteine protease activity. Therefore, there is considerable interest in developing a sensitive assay for measuring Der p 1 activity to screen for specific inhibitors. This study demonstrates for the first time that the activity of Der p 1 can be measured conveniently in a continuous rate assay with the fluorogenic substrate Boc-Gln-Ala-Arg-AMC ( $K_m = 280 \mu\text{M}$  and  $k_{cat}/K_m = 4.6 \times 10^3/\text{M/s}$ ).**

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Keywords: cysteine proteases; Der p 1; IgE

IgE antibody mediated hypersensitivity to the house dust mite *Dermatophagoides pteronyssinus* is a major cause of allergic diseases, such as asthma.<sup>1</sup> More than 80% of individuals who are sensitive to *D pteronyssinus* produce IgE antibodies to Der p 1, one of several mite allergens with enzymatic activity.<sup>2</sup> Der p 1 is a 25 kDa cysteine protease showing considerable sequence similarity with papain.<sup>3</sup> The expression pattern of Der p 1 in the mite gut<sup>4</sup> suggests a digestive function and it is, indeed, found in high concentration in the faecal pellets, which can become easily airborne and inhaled.<sup>5</sup>

The potent immunogenicity of Der p 1 (EC 3.4.22.-) is thought to be related to its proteolytic activity,<sup>6</sup> which has been shown in vitro to compromise functions of the innate and adaptive immune systems. For instance, we have shown that Der p 1 cleaves the serine protease inhibitor  $\alpha_1$ -antitrypsin,<sup>7</sup> whose function is to protect the airway mucosa against proteolytic damage. Der p 1 has also been reported to increase the permeability of the bronchial mucosa to macromolecules,<sup>8</sup> thus facilitating the passage of itself and other allergens across the mucosa, and consequently leading to allergic sensitisation. Furthermore, Der p 1 has been shown to induce IgE independent release of histamine and interleukin 4 (IL-4) from mast cells,<sup>9</sup> and disrupts the negative IgE regulatory mechanism mediated by membrane

CD23 (low affinity receptor for IgE) on B cells.<sup>10-11</sup> Given its deleterious clinical effects, there is considerable interest in developing a sensitive method for measuring Der p 1 activity, particularly to screen for specific inhibitors. Previous assays to measure Der p 1 activity have been tedious and of low sensitivity, such as those that use protein or peptide<sup>12-13</sup> substrates. This prompted us to investigate the enzymatic activity of Der p 1 using a range of fluorogenic peptide substrates. Here, we demonstrate that the activity of Der p 1 can be measured conveniently in a continuous rate assay using Boc-Gln-Ala-Arg-AMC (AMC, 7-amino-4-methylcoumarin; Boc, N-tert-butoxy-carbonyl), and that Der p 1 has a more restricted substrate specificity than most other cysteine proteases.

### Materials and methods

#### ENZYME SUBSTRATES

Boc-Gln-Ala-Arg-AMC, Boc-Gln-Gly-Arg-AMC, Boc-Gln-Arg-Arg-AMC, Succinyl-Leu-Leu-Val-Tyr-AMC, Boc-Val-Pro-Arg-AMC, and Cbz-Phe-Arg-AMC (Cbz, N-carboxybenzoxy) were obtained from the Sigma Chemical Company (Poole, Dorset, UK). Succinyl-Ala-Ala-Ala-AMC, Arg-AMC, Cbz-Arg-AMC, Cbz-Lys-AMC, and Cbz-Gly-Gly-Arg-AMC were obtained from Bachem Limited (Saffron Walden, Essex, UK).

#### PURIFICATION OF Der p 1

Der p 1 was purified from either lyophilised house dust mite culture supernatant (SmithKline Beecham Pharmaceuticals, Worthing, Sussex, UK) or from faecal pellets (Pharmacia Allergon AB; Pharmacia, Angelholm, Sweden) by affinity chromatography using monoclonal anti-Der p 1 antibody (4C1; Indoor Biotechnologies, Clwyd, UK) as described elsewhere.<sup>14</sup> The affinity purified Der p 1 was then passed through a soybean trypsin inhibitor agarose column to remove traces of serine protease activity.<sup>15</sup> The protein concentration was determined using a bicinchoninic acid (BCA) microtitre plate assay (Pierce and Warriner Limited, Chester, Cheshire, UK) and confirmed spectrophotometrically using the

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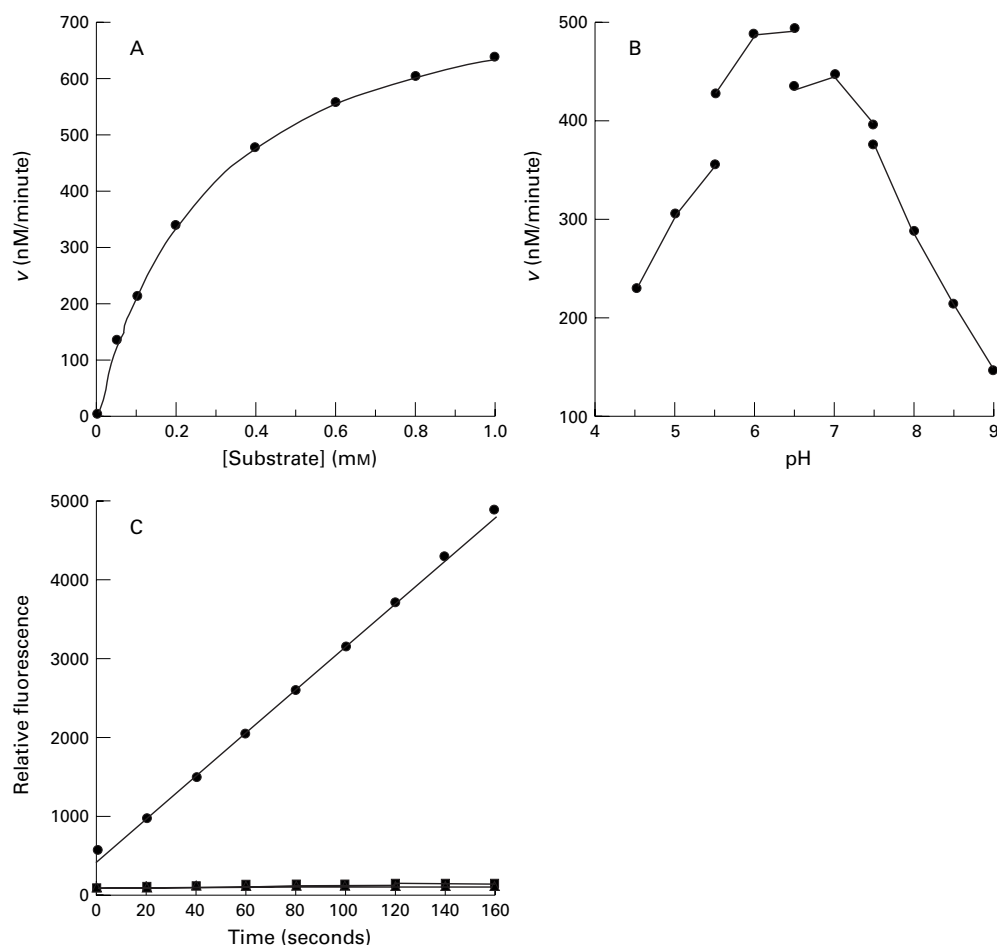


Figure 1 (A) Substrate kinetics for the hydrolysis of Boc-Gln-Ala-Arg-AMC by Der p 1. Data were fitted to the Michaelis-Menten equation and the following kinetic constants were deduced:  $K_m = 280 \mu\text{M}$ ,  $k_{cat} = 1.3/\text{s}$  (based on enzyme concentration) and  $k_{cat}/K_m = 4.6 \times 10^3/\text{M}/\text{s}$ . (B) The pH activity profile of Der p 1. Buffers, each containing 1 mM EDTA and 1 mM dithiothreitol (DTT), were 50 mM sodium acetate (pH 4.5–5.5), 50 mM sodium citrate (pH 5.5–6.5), 50 mM sodium phosphate (pH 6.5–7.5), and 50 mM Tris (pH 7.5–9.0). (C) Progression curves for the hydrolysis of 280  $\mu\text{M}$  of Boc-Gln-Ala-Arg-AMC (circles), Boc-Gln-Gly-Arg-AMC (squares), and Boc-Gln-Arg-Arg-AMC (triangles).

empirical absorption coefficient value for Der p 1 of  $E^{1\%}_{1\text{cm}}$  (280 nm) = 16.4.<sup>16</sup> The purity of the preparation was assessed by silver stained separation dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (15% gel) and its identity confirmed by N-terminal sequencing on an automatic amino acid sequencer (Applied Biosystems, Foster City, California, USA). The sequence obtained (TNACSINGNA) matched the published sequence of Der p 1.<sup>3</sup>

#### ENZYME ASSAY

Continuous rate assays were conducted in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 mM dithiothreitol (DTT) at 25°C in a total volume of 1 ml. Hydrolysis of AMC substrates was monitored using a Hitachi F-2000 fluorescence spectrophotometer (Hitachi Limited, Tokyo, Japan) with  $\lambda_{ex} = 380 \text{ nm}$  and  $\lambda_{em} = 460 \text{ nm}$  and results were related to an AMC standard curve (0–1000 nM). The assays were started by adding DTT activated Der p 1 to a final concentration of 10 nM. Kinetic constants for the substrates were obtained using the Michaelis-Menten equation:

$$y = V \cdot x / (K_m + x)$$

#### Results and discussion

We tested the proteolytic activity of Der p 1 against eleven fluorogenic peptide substrates. Only Boc-Gln-Ala-Arg-AMC, for which the kinetic data and pH profile are shown (fig 1A and B), was sensitive to hydrolysis by Der p 1. This indicates that Der p 1 has a more restricted substrate specificity than most other cysteine proteases, which explains previous difficulties in finding suitable substrates. The reactivity of Der p 1 with Boc-Gln-Ala-Arg-AMC was most notably influenced by the residue in the P<sub>2</sub> position, because Der p 1 did not react with two other similar peptides, namely Boc-Gln-Gly-Arg-AMC and Boc-Gln-Arg-Arg-AMC (fig 1C).

It has been reported recently that Cbz-Phe-Arg-AMC is a suitable substrate for Der p 1.<sup>17</sup> However, we believe that the antibody affinity purified Der p 1 used by those authors might have been contaminated with mite serine proteases because, in our hands, hydrolysis of this substrate can be demonstrated with antibody affinity purified Der p 1, but not with a Der p 1 preparation that has been purified additionally using immobilised soybean trypsin inhibitor. The availability of a Der p 1 substrate suitable for high throughput screening should

facilitate attempts to find specific inhibitors for further characterisation of the biological activities of this ubiquitous cysteine protease.

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## Upregulation of ATM in sclerosing adenosis of the breast

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### Abstract

**The gene mutated in ataxia telangiectasia (ATM) has an established tumour suppressor role in breast cancer. ATM appears to be expressed in most normal cells, including breast epithelium, where it has been postulated to have a nuclear role in cell cycle regulation following DNA damage. However, ATM is not upregulated after DNA damage. In this study, we demonstrate an absence of immunohistologically detectable levels of ATM in the normally quiescent myoepithelial cells that line normal breast ducts. This contrasts dramatically with the significant expression of ATM in the proliferative myoepithelium of sclerosing adenosis (n = 7). This upregulation of ATM suggests that ATM expression is coupled to the proliferative status of the myoepithelium. Our results also indicate that there are factors other than ATM gene mutations that can dramatically influence ATM expression in the breast and that these factors should be considered for their possible implications in carcinogenesis.**

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Keywords: breast; ATM upregulation; carcinogenesis

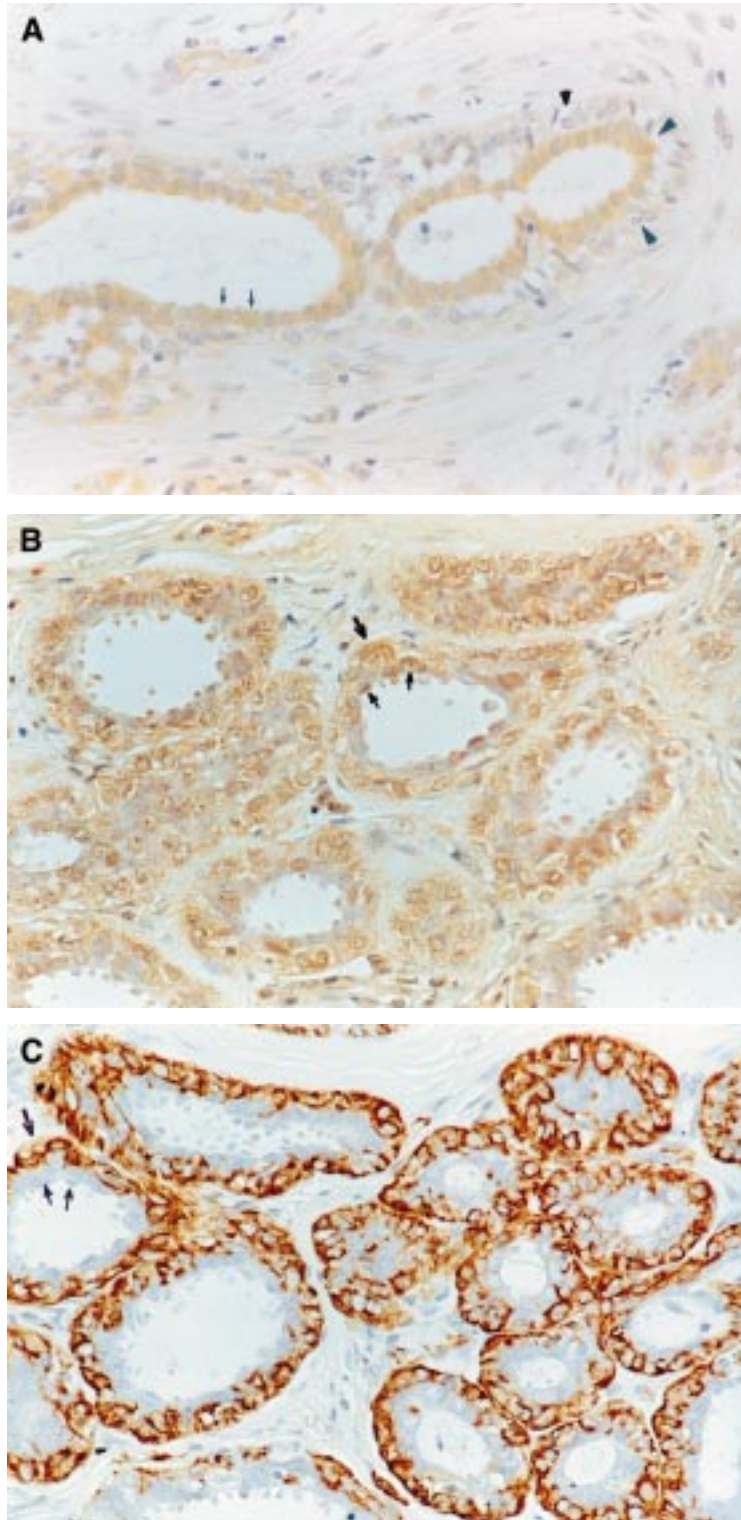
One of the many anomalous features associated with mutations in the gene mutated in ataxia telangiectasia (ATM) that usually result in the absence of ATM expression is an increased risk of developing breast cancer.<sup>1–4</sup> One function of ATM is in the control of cellular proliferation, where it acts upstream in the p53 signal transduction pathway and leads to the induction of G1/S arrest.<sup>5</sup>

To investigate the relation between ATM and mammary cellular proliferation, as distinct from carcinogenesis, we investigated the immunohistological expression of ATM in sclerosing adenosis of otherwise normal individuals. Sclerosing adenosis is a proliferative disease of the breast characterised by extensive fibrosis, an increase in the number of glandular elements, and proliferation of myoepithelial cells.<sup>6,7</sup>

### Materials and methods

The study group consisted of seven female patients with sclerosing adenosis of the breast. Histologically normal breast tissue samples (n = 7) with ductal epithelium and acini were obtained from areas of the breast located away from the primary lesion. Specimens were routinely fixed in formalin, embedded in paraffin wax, and stained with haematoxylin and

eosin. Representative tissue sections were cut at 4 µm, mounted, and air dried. Two adjacent sections were used, one for immunostaining and the other as a negative control. After



**Figure 1** (A) Strong ATM immunoreactivity in the inner epithelial cells of a normal breast duct (small arrows). The outer myoepithelial cells show no ATM immunoreactivity (large arrows) and form a clear background. Surrounding connective tissues display an intermediate level of ATM expression. Immunoperoxidase, CT-1;  $\times 200$  magnification. (B) Strong ATM immunoreactivity in sclerosing adenosis in the outer myoepithelial layer (large arrow) and the inner epithelial layer (small arrows). CT-1;  $\times 400$  magnification. (C) Strong smooth muscle actin (SMA) immunoreactivity in sclerosing adenosis in the outer myoepithelial layer (large arrow) and lack of immunostaining in the inner epithelial cells (small arrows). Immunoperoxidase, SMA;  $\times 400$  magnification.

**Table 1** Immunostaining of the myoepithelium in sclerosing adenosis of the breast

Patient	Nuclear staining		Cytoplasmic staining	
	Intensity	Number of cells (%)	Intensity	Number of cells (%)
1	3+	10	3+	80
2	0	—	2+	60
3	2+	70	2+	30
4	1+	10	2+	40
5	2+	40	2+	80
6	1+	30	0	—
7	2+	50	0	—

Intensity of staining: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining.

blocking of endogenous peroxidase, washing in Tris/HCl saline, pH 7.6, and blocking of non-specific binding of secondary antibody with skimmed milk solution, tissue sections were incubated overnight and stained with the ATM monoclonal antibody CT-1 (1/10 dilution) (a gift from Geoff Birrell, Queensland Institute of Medical Research, Queensland, Australia). CT-1 is a mouse monoclonal antibody raised against a synthetic 16 amino acid ATM fragment from the extreme carboxyl terminus of the ATM protein; its specificity has been confirmed and it is comparable to that of polyclonal ATM4BA.<sup>8</sup> We used an antibody directed against smooth muscle actin (SMA, Sigma clone 1A4 (St Louis, USA); 1/800 dilution for one hour) to stain selectively for myoepithelial cells. The primary antibodies were detected with a streptavidin–biotin–peroxidase system (DAKO, Carpinteria, USA). In sections used as negative controls, the primary antibodies were substituted with skimmed milk solution. The sections were counterstained with Harris's haematoxylin. Distinct brown nuclear or cytoplasmic staining was classified as positive ATM expression. Immunoreactivity was evaluated in a semiquantitative manner based on the intensity of staining and proportion of immunoreactive cells. The intensity of ATM immunostaining (table 1) was graded as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining. Table 1 shows the proportion of myoepithelial cells that stained positively for ATM.

## Results

The ATM protein was not detected in the myoepithelial cells of histologically normal breast tissue sections (fig 1A). In contrast, ATM expression was evident in myoepithelial cells in all of the sclerosing adenosis cases tested ( $n = 7$ ) (fig 1B and table 1). ATM was also detected in the epithelium and to a lesser extent in the surrounding connective tissue (fig 1A and B). An antibody directed against SMA confirmed the myoepithelial nature of the cells in the outer layer of the ducts in sclerosing adenosis lesions (fig 1C).

Nuclear and cytoplasmic immunoreactivity was detectable in the myoepithelium in six of seven and four of seven patients with sclerosing adenosis, respectively (table 1). Two cases (two of six) demonstrated positive nuclear staining only and one case (one of five) displayed solely cytoplasmic staining.

## Discussion

The dramatic increase in the numbers of ducts in the breast in sclerosing adenosis makes this an ideal model to study myoepithelial cell proliferation.<sup>7</sup>

In our study, we report the immunohistological detection of ATM in the myoepithelium of all sclerosing adenosis cases tested together with its presence in the nucleus in most of these cases. In contrast, ATM was absent from normal quiescent myoepithelial cells in normal breast tissue (fig 1A).

Previous immunohistochemical studies have suggested that ATM might be expressed constitutively in all cell types and that it is not upregulated in response to DNA damage.<sup>9</sup> Our results indicate that ATM is not expressed constitutively in the myoepithelial cells of normal breast tissue (fig 1A). The well defined ATM immunostaining seen in the myoepithelial cells of sclerosing adenosis (fig 1B) presented a dramatic contrast to the absence of ATM immunostaining in myoepithelial cells of normal breast tissue (fig 1A). This dramatic increase can best be explained by the upregulation of ATM expression at either the transcriptional or translational levels. This upregulation of ATM in the proliferative myoepithelium of sclerosing adenosis suggests that ATM expression has been “switched on” in response to molecular changes that permit or initiate cell proliferation. This would be consistent with the established role for ATM as a cell cycle regulator.<sup>10</sup> Coupling of ATM expression and proliferation is, however, not ubiquitous—for example, we have identified high levels of ATM expression in the non-dividing Purkinje cells of the cerebellum (data not shown).

In response to DNA damage, ATM can act upstream of the p53 tumour suppressor in a signal transduction pathway leading to cell cycle regulation, including G1/S arrest and apoptosis.<sup>5</sup> Mutation or loss of the ATM gene predisposes ataxia telangiectasia sufferers to many different types of cancer including breast cancer.<sup>3,4</sup> Our study indicates that ATM expression can be regulated dramatically within the breast, suggesting that the cellular

expression of ATM per se is not of primary importance in the predisposition of patients with ataxia telangiectasia to cancer. However, the uncoupling of ATM expression and cell proliferation is more likely to be fundamental in ATM related carcinogenesis. Our results also indicate that there are factors other than ATM gene mutations that can dramatically influence ATM expression in the breast and that these factors should also be considered for their possible implications in carcinogenesis.

In conclusion, we have described ATM upregulation in the proliferative myoepithelium of sclerosing adenosis of the breast, discounting the notion of consistent and constitutive nuclear expression of ATM.<sup>9</sup> These results remain consistent with the roles of ATM in cell cycle regulation during cell proliferation and recognition/repair of DNA damage,<sup>5,10</sup> and provide impetus for studies into the upstream regulation of ATM expression.

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# Immunohistochemical retrieval of the principal HIV antigens p24, gp41, and gp120 in formalin fixed tissue: an investigation using HIV infected lymphoblasts and postmortem brain tissue from AIDS cases

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## Abstract

**This paper describes the use of an autoclaving procedure followed by immunocytochemistry to enhance the detection of the human immunodeficiency virus (HIV) antigens p24, gp41, and gp120. This procedure greatly improved the detection rate of the p24 and gp41 HIV surface antigens in formalin fixed, paraffin wax embedded, HIV positive central nervous system (CNS) tissue while restricting staining to areas of the CNS showing evidence of neuropathology. However, the technique did not improve retrieval of the gp120 antigen in either HIV positive, formalin fixed CNS tissue or HIV infected T lymphoblasts. The inclusion of the high temperature autoclave step was validated using both HIV infected lymphoblasts and pre-adsorption of the specific antibodies with the appropriate recombinant HIV proteins. Using the methodology described here, formalin fixed CNS tissue from potential or known HIV positive cases can be processed reliably and safely. To ensure the reliability of this technique, it is recommended that an assessment of both the p24 and gp41 antigens is undertaken.**

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Keywords: high temperature antigen retrieval; human immunodeficiency virus; AIDS dementia

The detection of antigens using immunohistochemistry in formalin fixed, paraffin wax embedded tissue can be problematical, particularly after the material has undergone a prolonged period of fixation. For this reason, the retrospective detection of human immunodeficiency virus (HIV) surface antigens in formalin fixed material using immunohistochemistry has been unreliable and has produced conflicting results.<sup>1-4</sup> A recent development designed to enhance the retrieval of antigens in formalin fixed tissue is the pretreatment of sections with high temperature, either using a pressure cooker (or wet autoclave)<sup>5-7</sup> or a microwave technique.<sup>8-11</sup> With both techniques, immunohistochemical staining of formalin fixed material has been enhanced while retaining antibody specificity. High temperature antigen retrieval methods have also been applied systematically to formalin fixed, post-

mortem central nervous system (CNS) tissue with encouraging results.<sup>6, 10</sup>

In our paper we describe the use of a wet, high temperature autoclave technique, which has been combined with immunoperoxidase based immunohistochemistry to evaluate the detectability of HIV-1 surface (gp120) and core (p24) antigens in formalin fixed, postmortem brain tissue.

## Materials and methods

### LYMPHOCYTE CULTURES

HIV-1 infected and uninfected H9 human lymphoblasts from a T cell lymphoma were obtained from the MRC AIDS directed programme research project. The HIV infected lymphoblasts were cultured as described by Shapshak and colleagues<sup>12</sup> for three weeks in RPMI 1640 culture medium supplemented with 10% foetal calf serum and were maintained by subculture at 2-4 day intervals. The cells were harvested by centrifugation (500×g for 10 minutes) and the pellet was fixed in 10% formalin for 24 hours. The cells were then embedded in agar and 5 µm thick sections were prepared for further examination.

### CNS TISSUE

The brains from ten male patients positive for HIV (age range, 26-55 years) were removed and placed in 10% formalin for up to three weeks. The postmortem retrieval intervals were between 24 and 48 hours. Control brains were also obtained from HIV negative cases of the same age, sex, and postmortem intervals as those obtained from the HIV cases. Following fixation, the brains were cut coronally into 2 cm thick sections and from these slices small tissue blocks were prepared, representing different parts of the brain, which were processed for paraffin wax embedding. Sections (5 µm thick) were cut and mounted on to APES coated slides. The slides were dried at 37°C before transfer to a 60°C oven overnight. Individual sections from several different areas of the same brain were stained with haematoxylin and eosin. These were examined for the presence of neuropathological features commensurate with HIV infection<sup>13, 14</sup> and these blocks, together with areas of the same brain that did not show histological evidence of neuropathology and sections from the corresponding areas of the control brains, were used in this study.

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Table 1 Details of primary antibodies

Antigen	Type of antibody	Supplier	Dilution
Glial fibrillary protein (GFAP)	Mouse monoclonal	DAKO	1/100
HIV-1 p24	Mouse monoclonal	Dupont NEA 9306	1/1000–1/6000
HIV-1 gp41	Mouse monoclonal	Dupont NEA 9303	Undiluted–1/500
HIV-1 gp120	Mouse monoclonal	MRC AIDS programme	1/100–1/10 000

#### HIGH TEMPERATURE ANTIGEN RETRIEVAL PROCEDURE

A technique based on that described by Bankfalvi *et al* was used to enhance antigen retrieval.<sup>5</sup> Sections were dewaxed and any endogenous peroxidase activity was inhibited before they were immersed in 10 mM citrate buffer (pH 6) and autoclaved at 15 pounds per square inch (psi) (120°C) for 10 minutes using a portable stainless steel autoclave. They were then cooled in tap water before being transferred to phosphate buffered saline (PBS).

#### RECOMBINANT PROTEIN ADSORPTION ASSAY

The specificity of the primary HIV antibodies was confirmed by adsorption with the appropriate HIV recombinant protein as described below.

Recombinant proteins for HIV-1 p24 (core protein; molecular weight, 24 000 kDa), expressed in pGEX as a glutathione 5 transferase fusion protein, and HIV gp120 (external glycoprotein; molecular weight, 120 000 kDa), produced in a baculovirus system, were obtained from the MRC AIDS directed programme. They were diluted in PBS (pH 6) before being immobilised on hydroxy succinimidyl activated matrices, which were then used to adsorb out the appropriate antibody before immunohistochemistry. Control adsorptions were carried out using bovine serum albumin (BSA; molecular weight, 67 000 kDa) coated matrices. Insufficient recombinant HIV gp41 protein was available for use in this part of the study. This validation procedure was applied to autoclaved and non-autoclaved lymphoblast and brain sections.

#### IMMUNOCYTOCHEMISTRY

The lymphocyte and CNS sections were processed for immunohistochemistry using a standard two step indirect immunoperoxidase technique and the secondary antibody was visualised using a diaminobenzidine (DAB) procedure.<sup>15</sup> The primary antibodies used in the study are shown in table 1. All dilutions were prepared in 0.6% BSA in PBS.

#### Results

Figure 1 shows photomicrographs of the immunohistochemically stained lymphoblasts and brain sections obtained in our study.

*Figure 1 (A) HIV-1 infected T lymphoblasts and (B) non-infected T lymphoblasts immunostained using a primary antibody directed against p24 (antibody dilution 1/5000) after high temperature pretreatment (the position of chronically HIV-1 infected T lymphoblasts is indicated by an arrow). Chronically infected T lymphoblasts immunostained using anti-p24 (1/5000 dilution) (C) without adsorption and (D) after adsorption with p24. (E) HIV-1 infected T lymphoblasts and (F) non-infected T lymphoblasts immunostained using anti-gp41 (1/500 dilution) after high temperature pretreatment (the arrow indicates positive cytoplasmic staining). (G) HIV-1 infected T lymphoblasts and (H) non-infected T lymphoblasts immunostained using anti-gp120 (1/500 dilution) after high temperature pretreatment (the position of chronically HIV-1 infected T lymphoblasts is indicated by an arrow). Chronically infected T lymphoblasts immunostained using anti-gp120 (1/500 dilution) (I) without adsorption and (J) after adsorption with gp120. Paraffin wax embedded brain tissue sections from (K) an HIV positive case and (L) a non-HIV case immunostained with anti-gp41 (1/500 dilution) after high temperature pretreatment (the position of an immunostained multinucleated cell is indicated by an arrow). Magnification ×40 for all sections.*

#### FORMALIN FIXED HIV INFECTED LYMPHOBLASTS

Immunohistochemistry of HIV infected lymphoblasts using anti-HIV p24 or anti-HIV gp120 in the absence of high temperature treatment showed only some granular staining of the cytoplasm. No staining was seen with anti-HIV gp41. However, the addition of an autoclaving step produced a positive staining reaction with infected lymphoblasts for p24 (fig 1A), gp41 (fig 1E), and gp120 (fig 1G). No staining reaction was seen, however, when autoclaved sections of uninfected lymphoblasts were used (fig 1B, F, and H), giving a clear distinction between HIV infected and non-infected lymphoblasts. The application of high temperature treatment to these cells did not induce any false positive staining reaction when non-infected lymphoblasts were probed with any of the anti-HIV antibodies.

#### PRE-ADSORPTION STUDIES

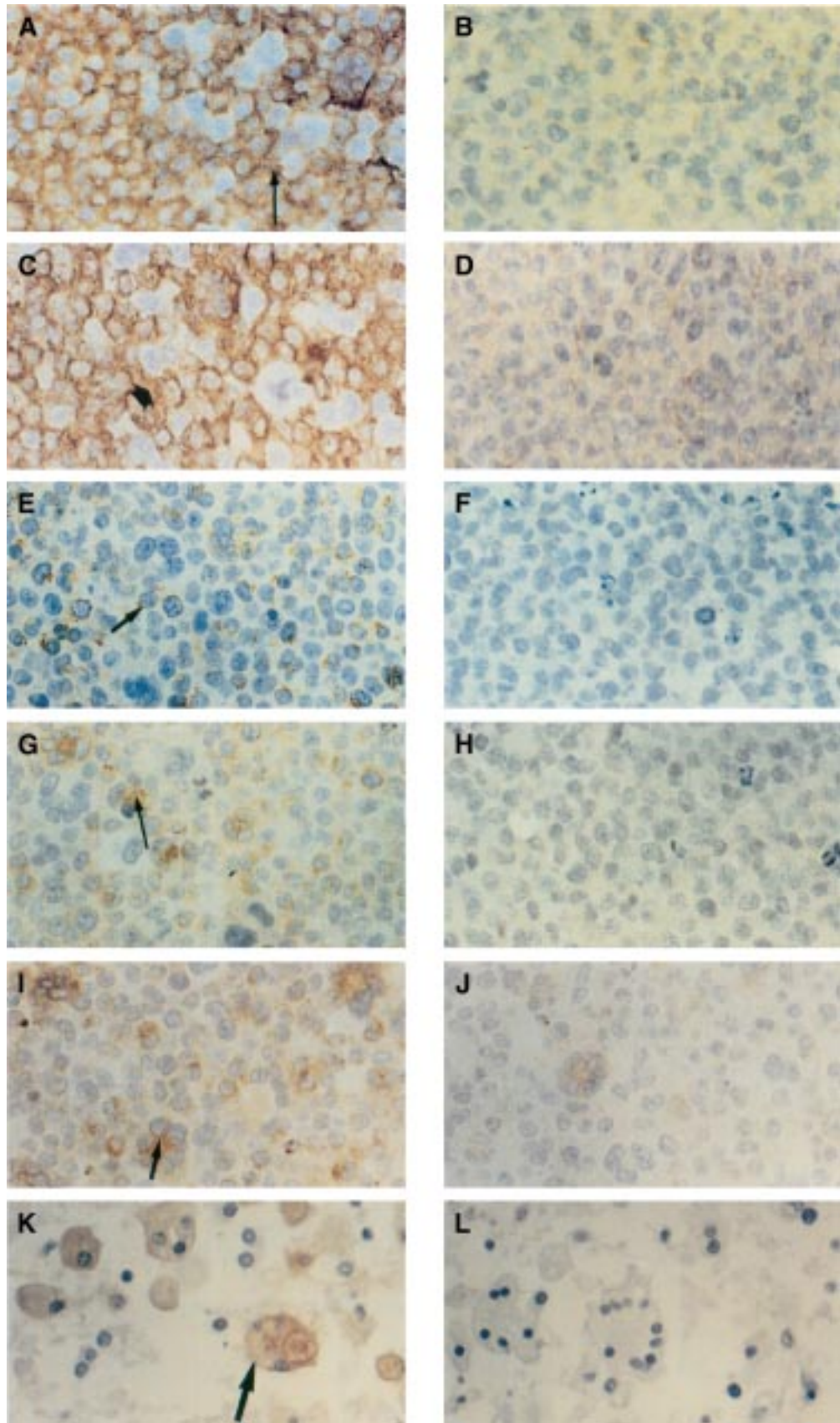
No immunoreactivity was seen when the anti-p24 antibody used in immunohistochemistry was adsorbed with immobilised recombinant protein before immunostaining (fig 1D). This was true for all the experimental material, whether it had been heat treated or not. This resulted in no immunoperoxidase staining even when autoclave pretreatment was omitted for both HIV infected and HIV positive CNS sections. The same was also true when the anti-gp120 antibody was adsorbed with its corresponding recombinant protein (fig 1J). These observations confirm the immunospecificity of these two antibodies. Pre-adsorption studies were not undertaken with the gp41 antibody because of the limited availability of the corresponding recombinant protein.

#### FORMALIN FIXED CNS TISSUE

In the absence of heat treatment, immunohistochemistry of brain tissue showing HIV associated neuropathology (as indicated in haematoxylin and eosin stained sections of the same area of brain) was uniformly negative for both multinucleated giant cells and microglia. Results obtained with the anti-p24 antibody were variable, but did produce positive staining of the Purkinje neurons in HIV positive tissue. Following heat treatment, immunohistochemistry with the anti-p24 antibody showed

immunostaining restricted to the microglial aggregates and to areas containing neuropathology (as demonstrated by the correspond-

ing haematoxylin and eosin section). The results obtained with the anti-gp41 antibody also showed positive staining limited to the



microglia and multinucleated giant cells (fig 1K). There was no significant improvement in the staining reaction obtained with the anti-gp120 antibody when the tissue sections were heat treated.

No positive staining reaction was seen in either HIV brain tissue without pathology or in sections obtained from non-HIV infected CNS tissue, irrespective of whether the sections had been heat treated or not.

### Discussion

Our data show that the use of a wet autoclaving technique applied to sections of HIV infected lymphoblasts and formalin fixed CNS tissue from HIV infected patients with proven neuropathology enhances the retrieval and specificity of immunohistochemical staining using antibodies directed at the p24 and gp41 antigens. No immunostaining was seen when either of these antibodies was used to immunostain control lymphoblasts or control brain tissue. Similarly, in HIV positive cases without evidence of CNS histopathology, neither of these antigens could be detected by immunohistochemical staining after autoclave treatment.

Therefore, the addition of the autoclave step enhances immunohistochemical detection of these HIV antigens in formalin fixed tissue. Furthermore, heat pretreatment does not impair the specificity of these reactions: the distribution of the immunostaining with p21 and gp41 was similar to that found in non-heat treated specimens and was restricted to those parts of the brain showing evidence of HIV associated pathology. In addition, this immunohistochemical staining was completely abolished after pretreatment of the antibodies with their respective recombinant antigen. Our findings are also corroborated by in situ hybridisation data showing that HIV RNA is located exclusively within the microglia and is restricted to areas of the brain that show abnormal histopathology commensurate with HIV infection.<sup>16 17</sup>

A number of previous studies using immunohistochemistry on formalin fixed tissue have failed to demonstrate reliably the presence of the p24 or gp41 antigen in CNS tissue from known HIV positive cases with neuropathology.<sup>1-5</sup> This was attributed variously to a prolonged period of fixation with formalin, the differential stability of envelope and core (p24) proteins in microglia, or the differential staining of different populations of microglia within the CNS.<sup>2 3</sup>

An alternative explanation for the variability of immunohistochemistry in formalin fixed tissue could be that a reaction between the HIV proteins and formaldehyde might result in hydroxy methyl groups that could mask the antigens.<sup>8 18</sup> The local interaction of hydroxymethyl residues with tissue is postulated to produce crosslinking between adjacent amino acids<sup>19</sup> and also to lead to the masking of potential antigenic sites by the formation of protein complexes induced by calcium ions.<sup>20</sup> Hydrolysis of these complexes requires a large amount of energy, in the form of heat, if poten-

tial antigenic sites are to be unmasked.<sup>18 21</sup> The lack of staining with antibodies directed against gp120 could indicate that the bond between gp120 and formaldehyde is not reversible by this mechanism.

Furthermore, the addition of tissue autoclaving improved the specificity of this reaction by preventing the recognition of non-HIV epitopes. As yet, we have no adequate explanation for this.

In conclusion, the use of autoclaving enhanced retrieval and improved the specificity of immunohistochemistry using antibodies directed against p21 and gp41, while not impairing tissue preservation. There was, however, no improvement in the detection of the gp120 antigen. In addition, the technique is biologically 'safe', removing the necessity to use frozen, unfixed tissue for diagnosis. When applying this technique to detect the presence of HIV antigens in formalin fixed tissue we recommend that both anti-p21 and anti-gp41 antibodies are used.

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