Alpha-1-antitrypsin in human macrophages

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SUMMARY Preliminary studies have suggested that alpha-1-antitrypsin (A1AT) is a useful immunohistochemical marker of histiocytes (monocytes/macrophages) and malignant tumours derived from them. To confirm the reliability of this marker a wide variety of benign and malignant lymphoreticular cells and tissues have been stained by the immunoperoxidase technique for A1AT and positive staining was found to be confined to histiocytes. Immunodiffusion, isotope labelling, and isoelectric focusing studies performed on cell lysates confirmed that the positive staining shown by monocytes and malignant histiocytes is due to the presence of A1AT identical with serum A1AT and that this material is synthesised by these cells rather than taken up from their environment. Positive immunoperoxidase staining for A1AT is thus a reliable marker of lymphoreticular neoplasms of true histiocytic origin.

The development of immunologically defined markers of cell lineage has been largely responsible for recent advances in the understanding and classification of lymphoreticular malignancies. Thus, both in cell suspensions and, more importantly, in histological sections, lymphocyte subsets can now be confidently identified. Identification of macrophages (histiocytes) poses special problems however. Suspensions of macrophages are difficult to prepare from tissue and there is no consistent immunological marker for cells of the monocyte/macrophage series that can be applied to histological sections. Lysozyme can be identified immunologically and, while it is a good marker of benign reactive macrophages in tissue sections, it has, contrary to early reports,¹ proved to be a poor marker of malignant macrophages.² Consistent positive immunoperoxidase staining for alpha-1-antitrypsin (A1AT) in Kupffer cells was noted by one of us (GHMS) in the course of screening a large number of formalin-fixed paraffin embedded liver biopsies for A1AT deficiency. The possibility that this might be a good immunological marker of macrophages was explored by staining a variety of tissues for A1AT including lung, spleen, and lymph node; in each case tissue macrophages stained positively. Antiserum to A1AT was therefore added into a "panel" of antisera used to characterise malignant lymphomas³ in the hope that positive staining for A1AT would distinguish true histiocytic (that is, monocyte/macrophage derived) tumours from those of lymphoid origin. Positive staining of malignant

cells was found to be restricted to histiocytic tumours and has been particularly useful in characterising a group of intestinal lymphomas as a variant of malignant histiocytosis.⁴ This series of rather empirical observations raises several important questions. Firstly, are the routine specificity controls that were used in the immunohistochemical procedure sufficient to ensure that the substance demonstrated in macrophages is truly A1AT? Secondly, assuming that the material present is A1AT, does its presence in macrophages reflect synthesis by the cells or uptake from their environment? Thirdly, how reliable is A1AT staining as a marker of the histiocytic origin of a malignant lymphoma, and in particular do T-cells, which may resemble macrophages histochemically and immunologically,⁵ ever stain positively for A1AT? We have attempted to answer these questions by extracting material from benign and malignant macrophages and showing that it is identical with serum A1AT and, with the use of radiolabelling techniques, showing that the cells synthesise this material. Finally, we have stained a wide variety of benign and malignant lymphoreticular cells and tissues to show that positive staining for A1AT is confined to macrophages and tumours derived from them.

Material and methods

PREPARATION OF BLOOD CELLS

Peripheral blood mononuclear cells were separated from heparinised venous blood by the method described by Payne *et al.*⁶ Briefly, freshly drawn blood

was mixed with an equal volume of calcium and magnesium-free Hanks' balanced salt solution (CMF-HBSS) and layered over ficoll/trisil.⁷ Mononuclear cells were removed from the interface after centrifugation at 400 g for 30 min, washed three times in CMF-HBSS and taken into medium RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (SRPMI). All tissue culture reagents were obtained from Gibco Biocult UK Ltd (Washington Road, Paisley, Scotland).

T-ENRICHMENT OF LYMPHOCYTES

Enrichment of T-lymphocytes was carried out by a modification of the method of Wahl *et al.*⁸ Mononuclear cells at 2×10^6 /ml in RPMI 1640 were rosetted with 1% sheep red blood cells (SRBC) in the presence of 2% fetal bovine serum. After incubating at 37°C for 5 min and further incubation on ice overnight or for 4 h the cells were gently re-suspended. Rosetted and unrosetted lymphocytes were separated by centrifugation through ficoll/trisil; erythrocytes were lysed with distilled water before washing.

PREPARATION OF BLOOD MONOCYTES

To prepare cell suspensions enriched in blood monocytes a maximum of 5×10^7 washed whole mononuclear cells were taken into 2 ml of isotonic Percol (Pharmacia UK Ltd, Hounslow, Middlesex) adjusted

to a density of 1.050 with $10 \times$ CMF HBSS and 1 M Hepes buffer, pH 7.0. The cells were then layered over a discontinuous gradient of Percol of density 1.060 or 1.070 and centrifuged at 400 g for 30 min at room temperature. The top density fraction of this gradient after centrifugation consisted almost entirely of α -naphthyl-acetate esterase positive monocytes (Fig. 1). This fraction was used after washing for immunohistochemical and immunodiffusion studies. For isotope incorporation the monocyte fraction was further purified by adherence to tissue culture grade plastic surfaces.

MITOGEN STIMULATION

Blood mononuclear cells or T-lymphocytes were cultured for three days at a cell concentration of 2×10^6 /ml in the presence of Phytohaemagglutinin, (PHA, 1/50, Gibco); pokeweed mitogen (PWM; 1/100, Gibco); or concanavalin A (Con-A; 10 μ g/ml, Miles Laboratories Ltd, Slough, England). After culture cells were washed once in SRPMI and prepared for immunocytochemical examination.

IMMUNODIFFUSION STUDIES

Approximately 2×10^8 blood monocytes or cells from a human histiocytic lymphoma cell line, U937,⁹ were frozen and thawed three times in non-ionic detergent, 0.1% NP40 (BDH, Poole, England), centrifuged at 2000 g for 20 min and concentrated twentyfold.

Fig. 1 Cytocentrifuge preparation of peripheral blood monocytes prepared using a Percol gradient. This representative field demonstrates the homogeneity of the monocyte population. Wright-Giemsa $\times 1000$.

Concentrated lysate was tested by immunodiffusion in gels of 0.6% agarose containing 4% polyethylene glycol 6000 against antibody to A1AT and lysozyme. Culture medium containing fetal calf serum supplement was unreactive to this system.

ISOTOPE INCORPORATION

Purified blood monocytes or U937 cells were cultured at a concentration of 2×10^6 cells/ml for 48 h in leucine-free medium containing 10% dialysed fetal calf serum and ^3H -leucine to a final concentration of 25 $\mu\text{Ci/ml}$ (TRK170; Radiochemical Centre, Amersham, England). After culture cells were lysed as above and dialysed against a large volume of phosphate-buffered saline (PBS). Isoelectric focusing¹⁰ (IEF) of the lysate was carried out for 45 min on a Pharmacia FBE300 flatbed apparatus in gels of LKB Agarose-EF (0.3 g in 27 ml distilled water) with 3.6 g sorbitol and 2 ml ampholines, pH 3.5-10. Focused material was immunofixed into the gel with a 1/5 dilution of the antiserum to A1AT, the gels were then washed in PBS and either stained in CBB R250 in acetic acid:methanol:water (1:4:4) or sliced, dissolved in NCS (Amersham Corporation, Arlington Heights, Illinois, USA) and β -counted in scintillant cocktail-T (Hopkin and Williams, Chadwell Heath, Essex).

IMMUNOHISTOCHEMISTRY

Malignant lymphomas

In addition to 185 cases of malignant lymphoma previously described³ sections of formalin-fixed paraffin-embedded lymph node tissue from six cases of T-cell lymphoma were stained for A1AT. These included two cases of Sézary's syndrome, three large cell lymphomas shown to be of T-cell origin by E rosetting performed on cell suspensions and one case of nodal involvement by T-cell chronic lymphocytic leukaemia (CLL).

Cytological preparations

These consisted of cytocentrifuge slides and paraffin sections of pellets of formalin-fixed cells prepared as previously described.³ Cells prepared in this way and stained for A1AT included peripheral blood monocytes, U937 cells, cells from a case of human T-cell CLL, three human T-cell lymphoma cell lines (Molt 4, HSB-2 CCRF-CEM) and T-enriched peripheral blood lymphocytes transformed by culture with PHA, PWM, and Con-A.

Immunohistochemical staining

Staining for A1AT was carried out on all tissues, cell pellets and cytocentrifuge preparations by means of the PAP immunoperoxidase technique. Sections

of paraffin-embedded tissue and cell pellets were first treated with 0.1% trypsin as previously described.¹¹ This step was omitted from cytocentrifuge preparations. Staining then followed the standard PAP procedure. Antiserum to A1AT was obtained from two sources (Behringwerke AG, Germany, and Dako-immunoglobulins AS, Denmark) which gave lines of complete identity when compared against normal human serum or A1AT standard (Sigma) by immunodiffusion and both antisera were used at a dilution of 1/1000. Controls were stained with antiserum previously absorbed with partially purified A1AT (Sigma).

Results

These are summarised in the Table.

IMMUNOHISTOCHEMISTRY

The results of staining 185 cases of malignant lymphoma for A1AT have been previously described³ and will be briefly summarised. With the exception of some Reed-Sternberg cells, positive staining for A1AT was found only in those tumours judged on morphological, histochemical or immunological grounds to be of true histiocytic derivation. Most of these were examples of malignant histiocytosis of the intestine.¹² The positive staining appeared in the malignant cells as cytoplasmic granules tucked into the nucleus in the Golgi zone. There was considerable variability in the amount of material present however. In some cases there was an abundance of cytoplasmic granules often extending into the cytoplasm as a whole (Fig. 2) while in others a scattering of cytoplasmic granules sometimes forming a peri-

A1AT in lymphoreticular cells and tissue

		Stain for A1AT
Benign	Peripheral blood monocytes	+
	Tissue macrophages	+
	liver	+
	spleen	+
	lymph node	+
	lung	+
	B-cells	—
	reactive follicle centres	—
	transformed with PWM	—
	Transformed T-cells (PWM, PHA, CON-A)	—
Malignant	U937	+
	Histiocytes	+
	histiocytic lymphoma	+
	MHI	+
	T-cell CLL	—
	T-cell lymphoma	—
	T-cell lymphoma cell lines	—
	B-cell lymphomas	—

*Shown to synthesise alpha-1-antitrypsin (A1AT).

Fig. 2 (a) Section of intestinal lymphoma from a case of MHI showing numerous large bizarre cells with abundant cytoplasm. Haematoxylin and eosin $\times 400$. (b) stain for A1AT showing granules tucked into the nucleus which, in some cells, extend into the cytoplasm as a whole. Immunoperoxidase $\times 400$.

Fig. 3 (a) A case of MHI composed of smaller more monomorphic histiocytes. Haematoxylin and eosin $\times 400$. (b) stain for A1AT showing positively staining material concentrated close to the nucleus and a scattering of granules elsewhere in the cytoplasm. Immunoperoxidase $\times 400$.

nuclear ring was observed (Fig. 3). Occasionally the amount of positively staining material was very small indeed and the oil-immersion ($\times 1000$) objective was required to delineate single cytoplasmic granules (Fig. 4). In carrying out this study benign tissue adjacent to malignant lymphoma was frequently stained. In this tissue which included lymph node, spleen, liver, and lung, reactive macrophages stained for A1AT. No staining was observed in other lymphoreticular cells.

Since none of the 185 cases had been characterised as a T-cell lymphoma, six cases of proven T-cell lymphoma were stained for A1AT as part of the present study; all stained negatively, although accompanying benign macrophages showed positive staining (Fig. 5). Negative staining was also found in cell pellets and cytocentrifuge preparations from the case of T-cell CLL, the human T-cell lymphoma cell lines and T-enriched human lymphocytes transformed with PWM, PHA, and Con-A. Freshly isolated peripheral blood monocytes and U937 cells stained positively for A1AT in both cell pellets and cytocentrifuge preparations. In the monocytes positive staining was found in approximately 50% of the cells and was usually confined to an area close to the nucleus (Fig. 6). In U937 cells most of the cells stained positively but with variable intensity, the finely granular staining being unevenly distributed throughout the cytoplasm (Fig. 7). In all cases positive staining could be abolished by prior absorption of the antiserum with partially purified A1AT.

Fig. 4 (a) Histiocytic lymphoma of spleen composed of indistinctive immature lymphoreticular cells. Haematoxylin and eosin $\times 1000$. (b) stain for A1AT; high magnification is required to distinguish isolated granules of A1AT situated close to the nucleus (arrows). Immunoperoxidase $\times 1000$.

IMMUNODIFFUSION

A single precipitin line was obtained when lysates of U937 and peripheral blood monocytes were precipitated against the antiserum to A1AT and lines of identity were formed with serum A1AT and partially purified A1AT (Fig. 8).

ISOELECTRIC FOCUSING

Isoelectric focusing of leucine-labelled lysates of U937 cells or purified blood monocytes produced labelled proteins which focused at a pH equivalent to that observed with A1AT standards and which were fixed in the gel with antibody specific to A1AT (Fig. 9).

Discussion

Positive immunohistochemical staining with specific antiserum need not necessarily be due to the antigen to which the antiserum was raised. Non-specific cross-reactions may occur and even abolition of positive staining by absorption of antiserum with specific antigen, generally accepted as the best control for specificity of immunohistochemical staining, is not entirely reliable since, particularly with high molecular weight antigens it is difficult to exclude cross-reactions due to a few shared antigenic deter-

Fig. 5 (a) Lymph node from a case of T-cell CLL showing malignant infiltrate on left and residual containing macrophages on right. Haematoxylin and eosin $\times 320$. (b) stain for A1AT showing positive staining in sinusoidal macrophages while tumour infiltrate is negative. Immunoperoxidase $\times 320$.

Fig. 6 Cytocentrifuge preparation of peripheral blood monocytes stained for A1AT. Approximately half of the cells stain positively with coarse granules tucked into the nuclear indentation. Immunoperoxidase $\times 1000$.

Fig. 7 Cytocentrifuge preparation of malignant histiocytes (U937) stained for A1AT. There is variable finely granular staining of the cytoplasm. Immunoperoxidase $\times 1000$.

Fig. 8 Immunodiffusion plate prepared over 24 h at 4°C. Antibody to alpha-1-antitrypsin is present in the centre well. The precipitin line shows complete identity between the antigen extracts present. STD: alpha-1-antitrypsin standard (Sigma); SER: human serum; MO: Percoll purified blood monocytes; U937: extracted U937 histiocytic lymphoma cells.

minants. Strong evidence that our antisera are indeed recognising A1AT in macrophages is obtained from the immunodiffusion studies which showed single precipitin lines of identity, without spur formation, between lysates of positively staining cells (benign monocytes, and cultured malignant histiocytes (U937)), human serum and partially purified A1AT. Confirmation that the antiserum is recognising newly synthesised A1AT in these cell preparations is obtained from the isoelectric focusing studies of ^3H -labelled lysates of short-term cell cultures. In dialysed preparations of both blood monocytes and U937 cells ^3H -leucine was incorporated into material with the immunological and focusing characteristics of A1AT.

A1AT was first demonstrated in human alveolar macrophages by Cohen in 1973¹³ by means of an immunofluorescence technique. He noted that the intensity of fluorescence declined over 72 h and interpreted this finding as evidence that the macrophages had taken up plasma A1AT which was then gradually lost from the cells. Gupta *et al.*¹⁴ found A1AT in the pulmonary macrophages of smokers but did not discuss their observations in any depth. Initially, we were also of the opinion that macrophage A1AT reflected uptake from the plasma, perhaps in the form of complexes with proteases, but our preliminary studies on pellets of blood monocytes and U937 cells caused us to revise our views and suggest that macrophages synthesised A1AT.¹⁵ This suggestion has been confirmed by our present study and also by the report of Wilson *et al.*¹⁶ who showed isotopically-labelled A1AT in the supernatants of

Fig. 9 Isoelectric focusing of detergent lysates of peripheral blood monocytes and U937 cells. The shaded area indicates the position of alpha-1-antitrypsin standard and vertical columns show the level of β -counts, representing synthesised material, fixed in each gel slice by specific antibody to alpha-1-antitrypsin. Material present in the cell lysate which is of too great a size to leave the loading trough of the gel is indicated by the arrowed thin column. The stained patterns show immunofixed alpha-1-antitrypsin standard and below that trichloroacetic acid precipitated whole human serum run under the same conditions.

short-term cultures of the adherent (that is, monocyte) fraction of blood mononuclear cells.

Lipsky *et al.*¹⁷ showed positive staining for A1AT on the surface, but not in the cytoplasm, of lymphocytes transformed with Con-A and incubated with the antiserum in suspension. Staining of cell surface is not successful in formalin-fixed tissues so that confusion between macrophages and transformed

lymphocytes in paraffin sections is unlikely on this account. Our own studies of mitogen-transformed lymphocytes showed no positive staining either in cell pellets or in cytocentrifuge preparations which were stained on the slide. In addition to monocytes Benitez-Bibiesca & Frere-Horta¹⁸ demonstrated A1AT in granulocytes. In our study we too have noted strong and apparently specific staining in granulocytes. These cells are, however, sufficiently distinctive and unlikely to be confused with macrophages.

When interpreting A1AT staining, care must be taken not to confuse positive staining caused by non-specific uptake of the antigen from the plasma. This occurs in dead or damaged cells in common with other plasma proteins such as IgG and albumin.³ The nature of staining is, however, quite different from the granular staining of cells synthesising A1AT, being diffuse and non-granular and frequently staining the nucleus as well as the cytoplasm.

Recent advances in the understanding and classification of lymphoreticular tumours have rested heavily on the demonstration of chemical, immunological, and morphological features in malignant cells that mimic those of their benign counterparts. To an almost uncanny extent, a normal cell type has been found to account for almost every type and subtype of malignant lymphoreticular cell, and classification of lymphoreticular malignancies has followed accordingly. In normal tissues, while certain non-lymphoreticular cells such as granulocytes may contain A1AT the macrophage is the only lymphoreticular cell in which we have consistently been able to demonstrate A1AT. Before being certain, however, that the presence of A1AT is indicative of macrophage/monocyte origin, a thorough search for A1AT in other benign and malignant lymphoreticular cells is necessary. This applies particularly to T-cells which, in any case, may show certain resemblances to macrophages.⁵ We have not observed A1AT in B-cell lymphomas of any type, in benign reactive follicle centres (apart from macrophages), or in B-lymphocytes transformed with pokeweed mitogen.³ Our inability to demonstrate A1AT in T-cell lymphomas, T-cell lymphoma cell lines, T-cell CLL cells and T-cells transformed with PHA and Con-A makes it highly unlikely that A1AT positive malignant lymphomas could be of T-cell origin. At present, therefore, it would seem justifiable to classify cases in which the cells stain positively for A1AT as of true histiocytic (monocyte/macrophage) origin. Our recent report of A1AT in some Reed-Sternberg cells³ is of interest in this respect. This finding, also reported by Poppema,¹⁹ suggests a histiocytic derivation of these cells.

The role of macrophage A1AT is presumably one

of neutralisation of proteases either intracellularly or after their release. The relation of macrophage A1AT to the A1AT deficiencies needs to be explored as does the possibility of a relation to certain inflammatory diseases. At present, however, we can state with confidence that human macrophages synthesise A1AT and that the presence of A1AT in the cytoplasm of malignant lymphoreticular cells is a reliable indicator of their histiocytic (monocyte/macrophage) derivation.

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