Immunohistochemical demonstration of leucocyte elastase in human tissues

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SUMMARY A polyclonal antiserum, produced in sheep and reactive against purified human leucocyte elastase, has been applied to paraffin sections from a range of human tissues by means of the indirect immunoperoxidase method. Striking and consistent results have been obtained. Normal or inflammatory granulocytes were intensely positive, the reaction being "blocked" by pure elastase. Activity was not seen in other sites, including histiocytic reticulum and lymphoid cells, although some weaker reaction was present in gastric and ileal lining epithelium. Strong reactivity was also seen in the cells of acute and chronic myeloid leukaemia and in extramedullary haemopoiesis. The advantages of this technique are compared with those for muramidase (lysozyme), α_1 -antitrypsin, and naphthol-AS-D-chloroacetate esterase.

Mature myeloid cells are generally readily identified in conventional paraffin sections of human tissues. More primitive varieties, however, may be less readily recognised and their distinction from, for example, primitive lymphoid or monocytic cells may be difficult.

The naphthol-AS-D-chloroacetate esterase (NCAE) azocoupling technique shows fully or nearly mature granulocytes in sections of paraffin embedded material,¹ but the reaction is not always reliable and is highly dependent on fixation. The immunohistochemical techniques for muramidase (lysozyme)^{2,3} and α_1 -antitrypsin⁴ are unsatisfactory as a means of demonstrating granulocytes, since histiocytic cells also contain these molecules.

Accordingly, in a series of human tissues we have investigated the value of immunohistochemical localisation of the enzyme leucocyte elastase (EC 3.4.21.11), which is a serine protease. This has been extracted from malignant granulocytes. Several specimens of acute and chronic myeloid leukaemia have been included to test the usefulness of elastase staining as a means of showing these neoplasms.

Material and methods

PREPARATION OF ANTISERUM

Purified leucocyte elastase was supplied by Dr AJ Barrett, Strangeways Laboratories, Cambridge. The enzyme was isolated from cells obtained by

leucophoresis of patients with chronic myeloid leukaemia.⁵ The purified enzyme was injected intramuscularly into a sheep as a suspension in Freund's complete adjuvant. Three injections were given at intervals until a desired titre of antibodies to elastase was detected in the sheep serum. A desired titre was that resulting in a strong precipitation line on immunoelectrophoresis. The sheep was bled and the immunoglobulin fraction of the serum was isolated by ion exchange chromatography on DE 52 cellulose (Whatman Ltd); the whole sheep serum was applied to the column, which was eluted with 0.03 M phosphate buffer, pH 7.2. The non-adherent, IgG rich fraction was collected and the specificity of the antiserum was verified by immunoelectrophoresis.

TISSUES EXAMINED

Thirty five specimens of normal human tissues, obtained as surgical or fresh postmortem excisions, were studied (Table 1). These comprised: brain (2), skin (3), myocardium (1), oesophagus (1), stomach (2), ileum (2), colon (2), liver (2), spleen (2), skeletal muscle (2), bone marrow trephine (3), palatine tonsil (2), thyroid (2), adrenal (1), kidney (4), pancreas (1), and lung (3). In addition, 15 specimens showing inflammatory or other non-malignant changes were studied. These were: acute appendicitis (2), ulcerative colitis (2), pilonidal sinus (1), pulmonary tuberculosis (2), and inflammatory nasal polyps (2), together with three lymph nodes showing reactive hyperplasia and one exhibiting

Table 1 Elastase activity in human tissues

Tissue	Elastase activity
Brain (matrix and cells)	-
Skin (dermis and epidermis)	-
Myocardium	-
Oesophagus (wall and epithelium)	-
Liver (all constituent cells)	-
Skeletal muscle	-
Palatine tonsil (all cells)	-
Thyroid	-
Adrenal	-
Kidney (all cells)	-
Pancreas	-
Lung	-
Stomach (epithelial cells)	+
Ileum (epithelial cells)	+
Colon (epithelial cells)	-
Bone marrow	
Granulocyte series cells	+++
Other cells	-
Bone (cells and matrix)	-
Acute appendicitis (granulocytes)	+++
Ulcerative colitis (granulocytes)	+++
Pilonidal sinus (granulocytes)	+++
Tuberculosis (histiocytes; giant cells)	-
Nasal polyps (eosinophil polymorphs)	+
"Reactive" lymph nodes (lymphoid cells, histiocytes, sinus lining cells)	-
Extramedullary haemopoiesis	
Granulocyte series	+++
Other cells	-

+ = positive; +++ = intensely positive; - = negative.

caseous tuberculosis. The final two specimens in this group were lymph node and a liver affected by extramedullary haemopoiesis (Table 1).

A second group, consisting of 15 specimens infiltrated by diagnostically established leukaemias, was studied. This comprised spleen, liver, or bone marrow specimens containing acute lymphoblastic leukaemia (2), chronic lymphocytic leukaemia, acute myeloid leukaemia (3), and chronic myeloid leukaemia (6) (Table 2).

Thus 65 specimens from the same number of patients were submitted to staining for leucocyte elastase.

FIXATION AND STAINING

All specimens, other than bone marrow trephines, were fixed in 10% formol-saline. The trephine specimens were fixed in 5% acetic acid in 10% formol-saline.⁶ After fixation, all tissues were proces-

sed to paraffin wax, sectioned at 3 μ m, and then stained for elastase activity, having been taken to Tris buffer at pH 7.8. Endogenous peroxidase activity was blocked by 0.1% hydrochloric acid in methanol for 30 min. The indirect peroxidase labelled second antibody routine was utilised. Sheep antihuman elastase was applied, at a titre of 1/100, for 45 min. After a thorough wash in Tris buffer, pH 7.8, peroxidase conjugated rabbit antisheep immunoglobulins were applied, at a concentration of 1/50. After a further wash in Tris buffer, pH 7.8, the peroxidase was shown in the usual way, by means of the 3, 3'-diaminobenzidine reaction. Sections were counterstained in Mayer's haemalum, dehydrated, cleared, and mounted in synthetic medium.

The usual controls were performed: the optimum titre was sought in the first stage, the first antiserum was replaced by normal sheep serum and both of the two stages, respectively, were omitted. The reaction was completely "blocked" by pure antigen at a concentration of 2 mg/ml and the 3, 3' diaminobenzidine reaction applied direct to the sections was negative. (Trypsinisation, before immunostaining, was unnecessary, resulting in no improvement in staining response.)

Results

The results of immunostaining for elastase are summarised in Tables 1 and 2. Certain generalisations may be made.

NORMAL TISSUES

In all but a limited number of tissues, positivity was seen only in polymorphonuclear leucocytes. In these cells, staining was uniformly intense and highly granular. The only other site in which activity was detected was in the basal part of the lining cells of the stomach and ileum, where weaker but finely granular staining was seen (Fig. 1). No other epithelia were positive. Connective tissue elements, including smooth and striated muscle, fibroblasts, cartilage (cells and matrix), and bone (cells and matrix) were uniformly negative, as were histiocytes and endothelial cells. In lymphoid tissues (for example, tonsils and gut associated lymphoid tissue), histiocytic reticulum cells, fibroblasts, and all lymphoid cells themselves were negative. Squamous and glandular epithelia (apart from the gastric and ileal linings) were uniformly negative. In bone marrow, only granulocyte series cells were positive (Fig. 2).

TISSUES AFFECTED BY BENIGN CONDITIONS

The specimens selected showed uniformly intense granular staining of polymorphonuclear neutrophils

Table 2 Elastase activity in leukaemic specimens

Type of leukaemia	Elastase activity of malignant cells
Acute lymphoblastic	-
Chronic lymphocytic	-
Acute myeloid	+++
Chronic myeloid	+++

+++ = intensely positive; - = negative.

Fig. 1 *Ileal lining epithelium. Fine, moderate staining for elastase activity is seen in a supranuclear position in the epithelial cells. Peroxidase-antiperoxidase technique for elastase; haemalum counterstain $\times 270$.*

and, less strongly, eosinophils (Fig. 3) in acute inflammatory areas. In granulomatous lesions, histiocytes, epithelioid cells, and multinucleate giant cells were negative. In extramedullary haemopoiesis, the granulocytic component stained intensely (Fig. 4); the other cellular moieties were negative. Reactive lymph nodes possessed no elastase activity in lymphoid cells, histiocytic reticulum cells, or sinuses (Fig. 5); some positive structures, probably corresponding to mast cells, were seen within and beneath the marginal sinus.

LEUKAEMIC SPECIMENS

In all specimens of myeloid leukaemia, whether acute or chronic, there was intense granular staining of the majority of malignant cells for elastase. This was seen in liver, spleen, and bone marrow (Figs. 6 and 7) and in a case of "chloroma." In contrast, the specimens of acute lymphoblastic and chronic lymphocytic leukaemia were uniformly negative.

Fig. 2 *Bone marrow. Numerous positive cells, which were of granulocytic series. Peroxidase-antiperoxidase technique for elastase; haemalum counterstain $\times 310$.*

Fig. 3 *Acute appendicitis. A heavy neutrophil polymorphonuclear leucocyte infiltrate is present in the mucosa, submucosa, and wall; the constituent cells are intensely positive with the elastase technique. Two lymphoid follicles are negative. Peroxidase-antiperoxidase technique for elastase; haemalum counterstain $\times 120$.*

Fig. 4 Lymph node. The interfollicular area is infiltrated by extramedullary haemopoietic tissue. The granulocyte series component cells are intensely positive for elastase. Peroxidase-antiperoxidase technique for elastase; haemalum counterstain $\times 140$.

CONTROLS

All negative controls gave appropriately negative staining results. Elastase staining was totally "blocked" by preadsorption of the antiserum with purified elastase.

Discussion

A not infrequent problem in diagnostic histopathology is that of recognising myeloid series cells or of distinguishing them from lymphoid cells. This can be

Fig. 5 Follicle from "reactive" lymph node. Elastase activity is absent from lymphoid and histiocytic cells. Peroxidase-antiperoxidase technique for elastase; haemalum counterstain $\times 520$.

Fig. 6 Liver, infiltrated by cells of chronic myeloid leukaemia, which show positive staining for elastase. Peroxidase-antiperoxidase technique for elastase; haemalum counterstain $\times 250$.

especially difficult in paraffin sections, where the finer cytological details afforded by smear preparations are not always available. The present study was undertaken in an attempt to clarify this problem by the use of an immunohistochemical technique for leucocyte elastase.

There are several methods for showing cells of the granulocyte series, but these may be difficult to perform or may be rather non-specific. For example, the NCAE reaction can be used on smears⁷ or on paraffin sections¹ to show granulocytic cells. On paraffin sections, however, the success of the reaction depends critically on good fixation and the results may be disappointing. In addition, NCAE activity may be seen in cells of other lineages, including occasional benign and malignant monocytes, megakaryocytes, and some erythroid cells in Di Guglielmo's disease.⁷ Furthermore, myeloblasts are not always positive.⁷

An alternative approach lies in the application of immunohistochemical methods to show antigenic substances present in cells of the granulocyte series. Both muramidase^{2,3} and α_1 -antitrypsin⁴ are present in such cells but they also occur in histiocytes and some monocytes, as may, variably, peroxidase.^{8,9} Conversely, the cysteine protease cathepsin B may be used to differentiate histiocytic cells from those of the granulocytic series: it occurs in lymphoid tissue only in the histiocytic cells and in sinus lining cells.^{10,11}

The present findings indicate that leucocyte elastase is a useful "marker" of benign and malignant granulocytes. In the benign group neutrophils, eosinophils, and (probably) mast cells are all stained

Fig. 7 Spleen, infiltrated by cells of acute myeloid leukaemia, which are intensely positively stained. Peroxidase-antiperoxidase technique for elastase; haemalum counterstain $\times 550$.

but neutrophil polymorphonuclear leucocytes are especially active. The intense positivity encountered in acute and chronic myeloid leukaemias and the absence of staining in acute lymphoblastic and chronic lymphocytic leukaemias, together with a lack of staining in histiocytes, suggest that one potentially useful diagnostic role of leucocyte elastase staining would be that of differentiating leukaemias of granulocyte type from those of lymphoid origin. The technique seems to offer considerable advantages in relation to the other means of showing granulocytes, as described above.

It is of interest that neutrophil type elastase has been shown in extracts from U937 cells, which are monocyte like.¹² Of course, this may represent a different elastase from that shown in the current study or may be a finding related to the different sensitivities of the techniques applied. Certainly, macrophage type cells were uniformly negative with our technique. The finding of leucocyte elastase activity in some gut lining cells is also of interest and may relate functionally to luminal digestive activity. In the diagnosis of leukaemia it is, of course, of little importance.

We now intend to carry out a prospective study of leucocyte elastase activity in bone marrow smears and trephines with a view to distinguishing between myeloid and lymphoid leukaemias.

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