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The distribution of muramidase (lysozyme) in human tissues

D. Y. MASON AND C. R. TAYLOR

From the Department of Pathology, Gibson Laboratories, Radcliffe Infirmary, Oxford

SYNOPSIS The distribution of muramidase (lysozyme) in normal and pathological human tissues has been studied, using an immunohistological technique. The enzyme was demonstrated in a variety of healthy tissues, including serous salivary acinar cells, lactating mammary tissue, Paneth cells, renal tubular cells, myeloid cells (including eosinophils), and histiocytic cells.

In pathological tissues the most striking positivity was encountered in reactive histiocytic cells in granulomatous conditions such as tuberculosis and Crohn's disease.

The findings of this study are related to previous reports of the distribution of human and animal muramidase and the implications of patterns of muramidase staining in pathological histiocytes are briefly discussed.

In 1922 Alexander Fleming reported on a 'remarkable bacteriolytic' element found in tissues and secretions, and coined the name 'lysozyme' for this activity. It was subsequently established that lysozyme acts through the dissolution of N-acetylglucosaminyl-N-acetylmuramic acid linkages in the cell walls of susceptible microorganisms, and the synonym 'muramidase' is now widely used. The enzyme is present in easily detectable amounts in human milk, tears, saliva, and serum. Moderately elevated serum levels have been reported in cases of tuberculosis, sarcoidosis, and myeloproliferative diseases (Finch, Lamphere, and Jablon, 1964; Osserman and Lawlor, 1966; Catovsky, Galton, and Griffin, 1971) while relatively enormous increases in serum and urine values are found in cases of leukaemia in which the malignant clone shows monocytic differentiation (Osserman and Lawlor, 1966). Serum muramidase in normal subjects and in patients with myeloproliferative disorders is thought to represent enzyme liberated from dying polymorphonuclear leucocytes (Fink and Finch, 1968), while the elevated levels in granulomatous diseases and in leukaemia appear to reflect liberation from, respectively, tissue histiocytes and from myelomonocytic cells (Catovsky *et al.*, 1971). Muramidase in tears, saliva, and other secretions probably represents synthesis by glandular cells.

Attempts to localize the cellular origins of human muramidase have been hampered by the lack of a

simple and accurate histological technique for detecting the enzyme. Methods hitherto described have either exploited the saccharolytic properties of the enzyme, using *Micrococcus lysodeikticus* or chitin as a substrate, or have relied on the use of labelled antibodies. Neither of these approaches has allowed full examination of muramidase distribution in human tissues (table I). The former methods suffer from the disadvantage that the muramidase must still be enzymatically active when the tissue is tested, ie, tissue must be fresh and unfixed. Immunological techniques, on the other hand, whilst not requiring the preservation of enzyme activity, are also considered incompatible with formalin fixation. Thus Asamer, Schmalzl, and Braunsteiner (1971) used alcohol-fixed blood and bone marrow smears; while Erlandsen and Parson (1973) and Sternberger, Hardy, Cuculis, and Meyer (1970a) examined respectively glutaraldehyde- and osmium-fixed tissues. Immunofluorescent techniques have the additional disadvantage of poor tissue and cellular morphology, particularly in comparison with the excellent histological results which can be achieved by immunoperoxidase methods (Taylor and Burns, 1974; Burns, Hambridge, and Taylor, 1974; Taylor and Mason, 1974).

The present report describes the use of immunohistological techniques for specifically staining human muramidase. These methods utilize peroxidase-conjugated antibodies, or immune complexes of peroxidase with rabbit antibody to peroxidase (PAP), and are applicable to all formalin-fixed,

Author	Technique	Site
Briggs <i>et al</i> (1966)	Lysis of micrococcus	Myeloid cells and monocytes
Ghoos and Vantrappen (1970)	Hydrolysis of chitin	Neutrophils
Sternberger <i>et al</i> (1970b)	Electron immunocytochemistry	Leukaemic monocytes and normal and leukaemic granulocytes
Asamer <i>et al</i> (1971)	Immunofluorescence	Leukaemic myeloid and monocytic cells
Syren and Raeste (1971)	Lysis of micrococcus	Neutrophils and monocytes
Scholnik and Kass (1973)	Hydrolysis of chitin	Neutrophils and leukaemic blast cells
Catovsky and Galton (1973)	Lysis of micrococcus	Myeloid leukaemic blast cells
Erlandsen and Parson (1973)	Immunoperoxidase	Paneth cells in small intestine

Table I Reported distribution of human muramidase (lysozyme)

paraffin-embedded tissues, even after prolonged storage. Preliminary studies by these methods have provided information on the distribution of muramidase in normal tissues and have revealed findings of particular interest in some disease states.

Materials

TISSUES

Paraffin-embedded blocks of several different tissues were selected from the surgical histology files at the Radcliffe Infirmary. Tissues had been routinely processed on the laboratory Histokinettes by the recommended schedule, using industrial methylated spirit and chloroform following adequate formalin fixation.

BLOOD SMEARS

Buffy coat smears were prepared from fresh EDTA samples of normal human blood, air dried, and fixed for five minutes in absolute ethanol.

ANTIMURAMIDASE ANTISERA

Two antisera were used. The first was prepared by immunizing a rabbit with human muramidase isolated from the urine of a chronic monocytic leukaemic patient. After batch absorption of the urine with CM-60 Sephadex, and repeated washing of the Sephadex with 0.05 M Tris HCl buffer, pH 7.0, muramidase was eluted with 2 M Tris HCl buffer, pH 7.0. The resulting preparation of muramidase (MX 1) was dialysed extensively against distilled water, and freeze dried. Cellulose acetate and SDS-acrylamide gel electrophoresis showed a single homogeneous protein band. However, on Ouchterlony testing of concentrated MX 1, weak reactions were obtained with anti-IgG, anti-kappa, and anti-lambda antisera, indicating the presence of small amounts of polyclonal IgG. This contaminant was removed by column filtration through Sephadex G-75 giving a preparation (MX 2), which produced no reactions on Ouchterlony testing with anti-IgG, anti-kappa, or anti-lambda antisera.

A rabbit was immunized intramuscularly with

MX 1 in complete Freund's adjuvant on days 1 and 8 followed by a booster injection of MX 2 on day 83, with bleeding on days 92 and 97. The resulting pooled antiserum was tested by immunoelectrophoresis and found to contain a potent antibody to human muramidase, together with activity against human immunoglobulin. The latter component was removed by precipitation with purified human IgG, and an ammonium sulphate immunoglobulin fraction of the antiserum was prepared. Before use the antibody was absorbed twice with glutaraldehyde insolubilized normal human serum.

The second rabbit antimuramidase antiserum, together with all other antisera used in the study, was obtained from Dakopatts A/S (UK agents, Mercia Diagnostics, Sandown Road, Watford, England). The PAP (peroxidase-anti-peroxidase complexes) was prepared by Dr Agnette Ingild of Dakopatts A/S according to Sternberger's original method (Sternberger *et al*, 1970).

Methods

Serial sections were cut at 5 μ , dewaxed with xylol and processed to absolute alcohol (ethanol). Blood

Stage No.	Procedure
1	5 μ paraffin section—xylol—absolute ethanol
2	Block endogenous peroxidase with methanol containing 0.3% hydrogen peroxide: 30 minutes
3	Normal swine serum 1/20:10 minutes ³
4	Rabbit antimuramidase 1/10:30 minutes
5	Swine antirabbit serum IgG conjugated with horseradish peroxidase 1/20:30 minutes
6	Diamino-benzidine (D.A.B. ³) reaction, counterstain with haematoxylin, dehydrate, mount in DPX

Table II Peroxidase conjugate method¹

¹All reactions carried out in Tris buffer (pH 7.6), washes after stages 2, 3, 4 and 5 in Tris saline (a 1/10 dilution of Tris buffer with normal saline).

²Stage 3 is optional and is designed to reduce unwanted 'background' staining.

³D.A.B. 3.3' Diamino-benzidine tetrahydrochloride (BDH Chemicals, Poole, England) 6 mg in 10 ml of Tris buffer containing freshly added hydrogen peroxide 0.01%.

Stage No.	Procedure
1	5 μ paraffin section—xylol—absolute ethanol
2	Block endogenous peroxidase with methanol containing 0.3% hydrogen peroxide 30 minutes
3	Normal swine serum 1/20:10 minutes ²
4	Rabbit antimuramidase 1/40:30 minutes
5	Swine antirabbit serum protein 1/20:30 minutes
6	PAP (peroxidase anti-peroxidase complexes) 1/20:30 minutes
7	Diamino-benzidine (DAB ³) reaction, counterstain with haematoxylin, dehydrate, mount in DPX

Table III The PAP (peroxidase-anti-peroxidase immune complex) method¹

¹All reactions in Tris buffer (pH 7.6), all washes after stages 2, 3, 4, 5, and 6 in Tris saline.

²Stage 3 optional.

³DAB 3 3' diamino-benzidine tetrahydrochloride (BDH Chemicals, Poole, England) 6 mg in 10 ml of Tris buffer containing freshly added hydrogen peroxide 0.01%.

smears were allowed to dry after ethanol fixation and subsequently treated in the same way as tissue sections. All slides were transferred to absolute methanol containing 0.3% hydrogen peroxide in order to block the endogenous peroxidase activity (of granulocytes and red cells) which interferes with the interpretation of results (see Burns *et al.*, 1974). The subsequent method is summarized in tables II and III.

All tissues were initially examined by the 'peroxidase conjugate' method (table II and Taylor and Burns, 1974). Those tissues in which results were equivocal were subsequently re-examined by the more sensitive PAP method (table III and Taylor, 1974). In this procedure the tissue-bound rabbit antiserum to muramidase (stage 4, table III) is linked to the rabbit antiserum against horseradish peroxidase in the PAP complex (stage 6, table III) by the intermediate addition of a swine antiserum to rabbit serum protein (stage 5, table III). Background staining was minimal by this method due to the intrinsic purity of the PAP, and the higher dilution factor of the antimuramidase antiserum.

Troublesome background staining in the peroxidase conjugate method may be reduced by pretreatment of sections with normal swine serum. This reduces non-specific absorption of antisera and avoids cross reaction with the swine anti-immunoglobulin antisera used in stage 5. In order to facilitate comparisons between the different methods all sections were subjected to 'pretreatment' with normal swine serum (stage 3, tables II and III).

In addition, all sections showing positive staining with our antimuramidase antiserum, together with the majority of the 'negative' sections, were checked by the identical procedure using Dakopatts antimuramidase. In every case results were in agreement.

Controls

Serial sections of selected cases were subjected to the procedures presented in tables II and III, with the omission of each stage in turn. Also in every case examined by anti-muramidase, a parallel section was subjected to the identical procedure, except that rabbit anti-IgM antiserum was substituted for the rabbit anti-muramidase. There was no evidence of specific staining in any of these control preparations, other than occasional IgM-containing plasma cells.

	Tissue	Muramidase Activity	
Normal Tissue	Digestive system	Stomach Small bowel Colon Liver Pancreas	Negative Paneth cells positive ¹ Negative Kupffer cells positive ¹ Negative
	Endocrine	Adrenal Thyroid	Negative Negative
Genitourinary	Testis Ovary Prostate Kidney	Negative Negative Negative Proximal tubular cells positive ¹	
	Uterine cervix	Negative, including endo-cervical glands	
Exocrine	Endometrium Lacrimal glands Salivary glands Bronchial glands Sebaceous glands Sweat glands	Negative Positive ¹ Serous elements positive ¹ Serous elements positive ¹ Negative Negative	
	Mammary tissue	Non-lactating Lactating	Negative Positive ¹
Musculoskeletal	Striated muscle Smooth muscle Myocardium Cartilage	Negative Negative Negative Positive ¹	
	Lymphatic and haematological tissue	Lymph nodes Spleen Peripheral blood	Positive histiocytes ¹ Positive histiocytes ¹ Positive polymorphs and monocytes
Respiratory system		Bone marrow Lung	Positive myeloid cells Alveolar lining cells and iron or carbon containing macrophages, negative
	Integument	Bronchial epithelium Skin	Mucus secreting cells negative Sweat and sebaceous glands negative
Pathological Tissue		Reactive	Granulation tissue Fat necrosis Tuberculosis, sarcoidosis, and Crohn's disease
	Lipid-containing phagocytes	Xanthoma Gaucher's disease	Positive giant cells ¹ Positive histiocytes ¹
Miscellaneous		Histiocytoma Giant cell tumour of tendon sheath Eosinophilic granuloma	Negative Negative Positive eosinophils and histiocytes

Table IV Distribution of muramidase in human tissues

¹See text

Incubation of the anti-muramidase antisera with a solution of purified muramidase completely abolished specific activity (see results).

Results

Morphological detail in all sections examined was excellent, equivalent to any orthodox haematoxylin- and -eosin paraffin section. Positive brown staining for muramidase clearly contrasted with the blue haematoxylin counterstain.

The results of testing a variety of human tissues for muramidase activity are given in table IV. At least two examples of each tissue have been examined.

All sections contained a variable number of positive polymorph leucocytes (both eosinophil and neutrophil), and these cells provided a useful positive control, demonstrating in each case that the technique was working satisfactorily. Tissues in

which only granulocytes and the occasional scattered mononuclear cells (? monocytes) stained for muramidase were classified in table IV as negative.

TISSUES CONTAINING MURAMIDASE

Small bowel

Positivity was confined to Paneth cells, all of which appeared to contain the enzyme. Activity was predominantly localized in the apex of the cells, where it outlined the membranes of the secretion granules.

Liver

Some Kupffer cells gave positive reactions, but an undefined proportion were clearly negative. There was no discernible pattern to the distribution of positive and negative cells.

Kidney

Proximal tubular cells were positive (fig 1). Tubular

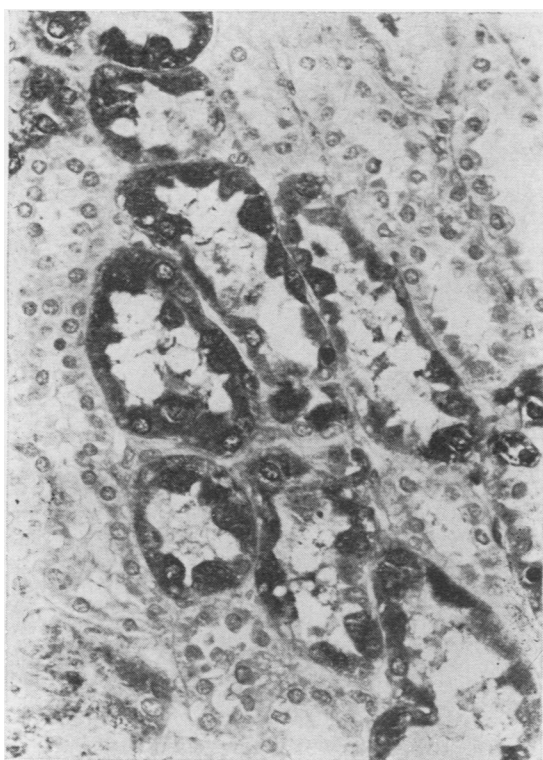


Fig 1

Fig 1 *Kidney. Epithelial cells of proximal tubule clearly positive (black) distal tubule negative. Cyan blue filter $\times 750$.*

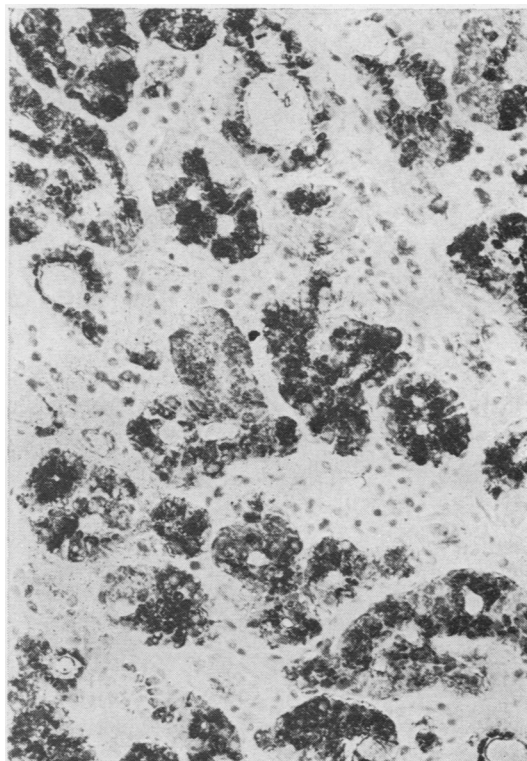


Fig 2

Fig 2 *Lacrimal glands. Positive (black) staining for muramidase in the majority of the glandular epithelial cells. Cyan blue filter $\times 750$.*

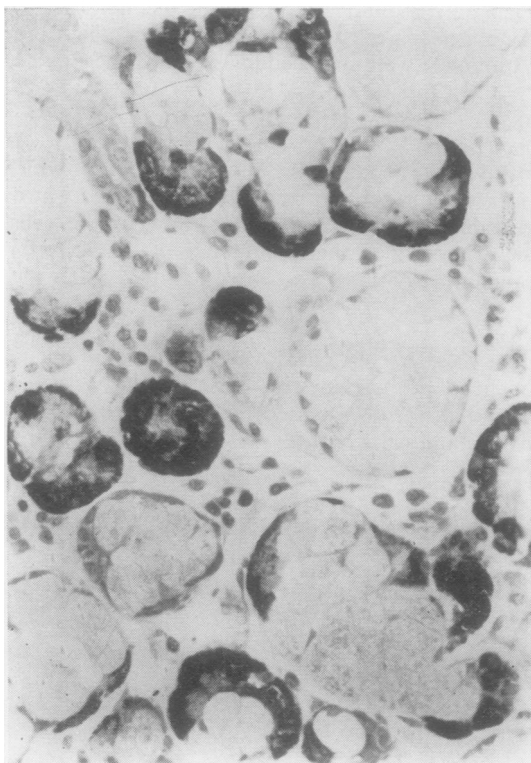


Fig 3

Fig 3 *Mixed type salivary gland. Serous 'caps' or 'demilunes' clearly positive (black), mucus-secreting element negative. Cyan blue filter $\times 750$.*

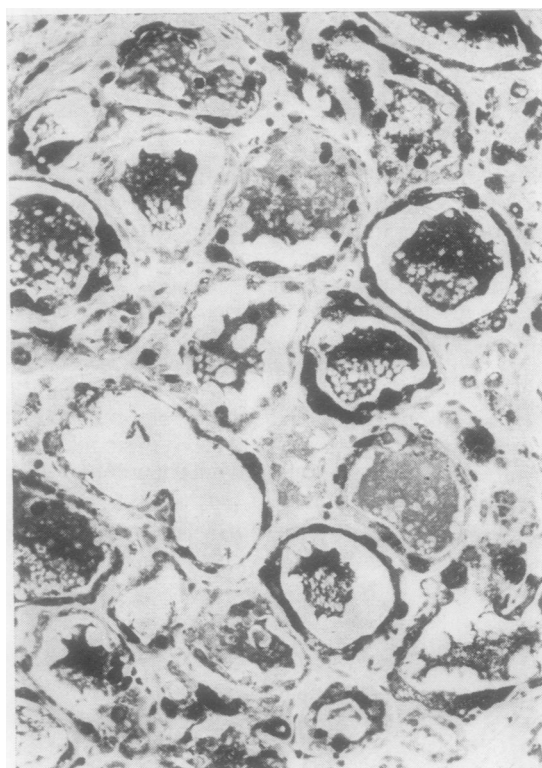


Fig 4

Fig 4 *Lactating breast. Positive (black) muramidase-containing cells line the secretory acini. Secretions themselves show diffuse positive staining. Cyan blue filter $\times 750$.*

cells in the upper part of the descending limb of Henle's loop were less strongly positive, and the reaction disappeared as the loop progressed into the medulla.

Lacrimal glands

The majority of the glandular acini were strongly positive (fig 2). A few acini, and the ducts of the gland, contained no demonstrable muramidase.

Salivary glands

Mucous gland acini were consistently negative. In the accessory mixed-type glands underlying the buccal mucosa, 'caps' of serous cells were clearly positive (fig 3). The parotid gland, although an almost pure serous gland, contained no activity.

Bronchial glands

An appearance very similar to that of mixed salivary

glands was seen, with positive serous 'caps' to many mucous acini.

Lactating breast

Many of the flattened epithelial cells lining dilated secretory acini were strongly positive (fig 4), as were the secretions within the acini. In all of the acini negative cells could be found lying adjacent to morphologically identical muramidase-containing cells.

Cartilage

Faint diffuse granular staining of cartilage was observed, together with strong staining in lacunae.

Lymphatic tissue

The appearances varied greatly according to the degree of reactivity of the lymphoreticular tissue. In quiescent lymph nodes the small germinal centres

were entirely negative. In addition, there was no evidence of positive reaction in sinus lining cells or in paracortical or sinus histiocytes.

In follicular hyperplasia a proportion of tingible body macrophages showed distinct positivity, while in other cases morphological 'dendritic reticular cells' in the peripheral parts of the follicles appeared positive. In sinus histiocytosis an increasing proportion of sinus histiocytes showed positive staining of marked intensity, and small aggregations of histiocytes in paracortical areas showed similar reactions. These features were observed in 'dermatopathic lymphadenitis' and in lesser degrees of non-specific histiocytosis.

The most marked positive reactions, however, were seen in lymph nodes containing tuberculous or Kveim-positive sarcoid lesions. An essentially identical pattern of numerous strongly positive epithelioid histiocytes and giant cells was seen in both these conditions (fig 5). However it was always possible, even in the most active granulomata, to see negative cells of similar morphology.

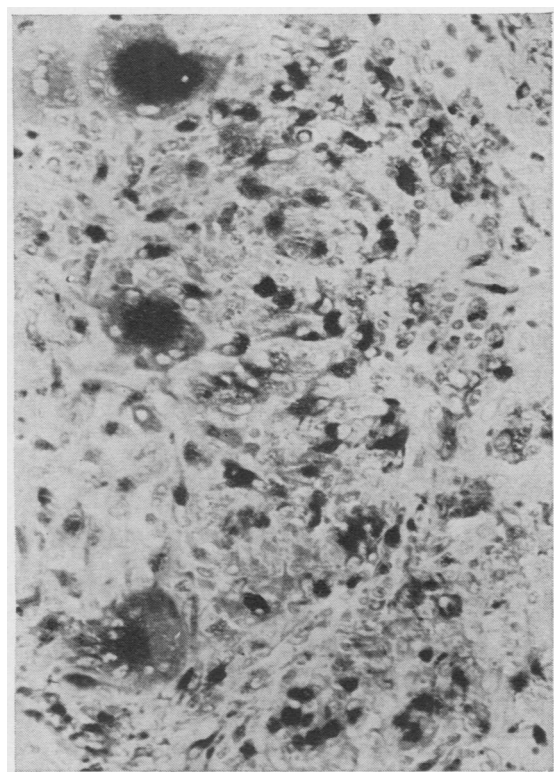


Fig 5 *Sarcoidosis. Epithelioid granuloma with positive (black) and negative histiocytes and three strongly positive giant cells. Cyan blue filter $\times 750$.*

In areas of healthy lymphoid tissue adjacent to granulomata there were numerous strongly positive smaller histiocytes, possibly migrating towards the diseased regions.

Granulomata in samples of bowel affected by Crohn's disease were also found to contain numerous strongly positive histiocytic cells.

Peripheral blood

In alcohol-fixed buffy coat smears intense cytoplasmic staining was apparent in all but a very few neutrophils. Monocytes stained less strongly, and in a proportion no enzyme was detectable.

Bone marrow

Sections of bone marrow revealed large numbers of muramidase-positive cells. The majority of these were morphological neutrophils, or eosinophils, or recognizable segmented precursors. A proportion of 'mononuclear' cells were also positive, but it is not yet clear whether these represent more primitive granulocytic cells or monocytoid cells.

Granulation tissue

In granulation tissue from the base of a peptic ulcer the majority of cells (including histiocytes and fibroblasts) were negative. In areas where acute inflammatory cells were present, a few histiocytes showed granular positivity.

Fat necrosis

A minority of the giant cells found in association with cholesterol clefts were faintly positive.

Lipid-containing phagocytes

A proportion of Gaucher's histiocytes, and a few giant cells in one of the two xanthomata examined stained weakly and diffusely for muramidase. In the latter instance the positive reaction for muramidase was associated with other features of chronic irritation, possibly the result of minor trauma.

Eosinophilic granuloma

The eosinophil polymorphs which are a striking feature of this condition showed strong granular positivity. In addition scattered foamy histiocytes contained moderate amounts of the enzyme. The majority of other histiocytic cells, which tended to be smaller, were negative or very weakly positive.

SPECIFICITY OF MURAMIDASE STAIN

The antimuramidase antiserum was diluted 1/20 in a 1.0 mg/ml solution of purified muramidase (MX 2), and applied to several sections in which muramidase-positive cells appeared to be numerous. In each case the positive staining was completely abolished,

confirming the specificity of the reaction for muramidase.

Discussion

The present investigation confirms most of the previously reported work on the cellular distribution of human muramidase, in that normal myeloid and monocytic cells contained the enzyme. A single discrepancy lies in the fact that we found strong muramidase activity in mature eosinophils in tissue sections but not in blood films. This possibly reflects differences in the methods of processing for sections and smears. Three other reports have suggested that there is little or no enzyme in eosinophils in smears (Scholnik and Kass, 1973; Briggs, Perillie, and Finch, 1966; Syren and Raeste, 1971).

Studies of human muramidase (quoted in table I) have been supplemented by investigations of muramidase activity in animal tissues, and the present survey may be considered in relation to this work. Because of the species antigenic specificity of muramidase, we have not been able to apply the immunoperoxidase technique to tissue of non-human origin.

RENAL TUBULAR CELLS

The enzyme has been demonstrated in rat and mouse proximal tubular cells (Glynn and Parkmann, 1964; Klockars and Osserman, 1974), a finding which was confirmed for man in the present study. The kidney is known to excrete muramidase readily into the glomerular filtrate, whence it is reabsorbed and catabolized by proximal tubular cells (Balazs and Roepeke, 1966).

LACRIMAL GLAND

Fleming, in his original paper on muramidase, described powerful activity in human tears, and our present findings demonstrate that the majority of the secretory cells of the lacrimal gland are capable of muramidase synthesis. Scholnik and Kass (1973) had previously found muramidase activity in rabbit lacrimal glands. However, Klockars and Osserman (1974) discovered an apparent species difference in that rat lacrimal tissue was devoid of this enzyme.

CARTILAGE

Muramidase has been detected in chick and human rat cartilage (Kuettner, Eisenstein, Soble, and Arsenis, 1971; Greenwald and Sajdera, 1971; Greenwald, Josephson, Diamond, and Tang, 1972). In all these studies muramidase was found to be extracellular. Kuettner *et al* (1971), using an immunofluorescent technique, showed localization around chondrocyte lacunae, an appearance similar to the

pattern which we encountered in human cartilage. It is therefore possible that cartilage muramidase is not synthesized by the chondrocytes themselves, but taken up instead from tissue fluids. This concept is supported by the reports of Greenwald *et al* (1972) and of Eisenstein, Arsenis, and Kuettner (1970) that exogenous muramidase, because of its strongly cationic charge, will bind to cartilage.

In contrast to these findings Klockars and Osserman (1974) could demonstrate no muramidase activity in rat cartilage.

THYROID TISSUE

A further discrepancy, also possibly species related, lies in the finding by Klockars and Osserman of muramidase in rat thyroid follicles. We could not demonstrate even weak activity in human thyroid sections.

SALIVARY GLANDS, BRONCHIAL GLANDS, AND LACTATING BREAST

We could find no record that muramidase has been detected in these organs previously, although both human saliva and milk have long been known to contain the enzyme. The observation that serous elements in the parotid were negative was of interest and accords with Klockars and Osserman's observation on rat parotid tissue.

PANCREAS

We found no evidence of pancreatic muramidase activity, in contrast to the report by Speece (1964) that the enzyme is present in mouse pancreatic tissue.

INTESTINAL CELLS

Paneth cells were found to be strongly positive in the present study, in accordance with the reports of Erlandson and Parson (1971) on human tissue, by Klockars and Osserman (1974) on rat small intestine, and by Ghoos and Vantrappen (1971) on mouse jejunum. The function of Paneth cells is unknown. Klockars and Osserman (1974) cite the large number of Paneth cells in the small intestine of the Brazilian ant-bear as evidence that they were originally evolved for the secretion of chitin-dissolving muramidase. An alternative view, that they function primarily as fixed phagocytes, is supported by the work of Erlandson and Chase (1972) in which rat Paneth cells were shown to be actively phagocytic for two different species of intestinal microorganism.

Klockars and Osserman also reported that rat colonic and rectal epithelial cells contained muramidase. We initially found weak staining at intestinal epithelial surfaces by the peroxidase conjugate method. This method, however, is prone to give non-specific staining at tissue interfaces, and there

was no suggestion of positivity using the more sensitive PAP method in which non-specific staining is greatly reduced.

MACROPHAGES AND HISTIOCYTES

It was apparent in this study that many histiocytes whether in hepatic, pulmonary, or lymphatic tissues, were negative for muramidase. These negative cells included such declared phagocytes as pulmonary 'heart failure' alveolar macrophages and carbon-containing histiocytes.

Those histiocytes or macrophages which were found to contain the enzyme fell into three categories:

1 Morphologically unremarkable cells in normal tissues

Scattered histiocytic cells in all tissues were occasionally strongly positive. Kupffer cells were the most frequently positive cells in this category.

2 Storage, reactive, and giant cells

A proportion of cells such as Gaucher's cells, xanthoma cells, and giant cells in areas of fat necrosis contained muramidase. The reaction was always weak, however, and frequently at the threshold for detection of the enzyme.

3 Epithelioid histiocytes, activated histiocytes

The cells, exemplified by the histiocytes seen in sarcoid, tuberculous, and Crohn's granulomata, were frequently strongly positive.

This triple hierarchy of positive cells suggests that muramidase may be synthesized predominantly in 'reactive' histiocytes, rather than in resting unstimulated phagocytes, or in cells which have ingested relatively bland substances such as lipid carbon particles or red cells. This concept is supported by Klockars and Osserman (1974) who reported negative staining in all the rat lymph nodes which they examined with the exception of a single para-aortic node in which germinal centre hyperplasia and sinus histiocytosis were associated with the presence of many muramidase-positive histiocytes. There is further evidence for the hypothesis that muramidase is synthesized only by 'activated' histiocytes in the increased serum levels found in tuberculosis and sarcoidosis (Finch *et al*, 1964; Osserman and Lawlor, 1966) and also in the work of Cappucino, Winston, and Perri (1964) and of Glynn (1968) who demonstrated an increase in splenic muramidase in mice receiving BCG injections.

Conclusion

The technique described here may be of value in the study of the monocyte/histiocyte series in health

and in disease. Possible clinical applications include the investigation of monocytic and myelomonocytic leukaemias, particularly when the latter appears as a terminal event in cases of multiple myeloma (Andersen and Videbaek, 1970).

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D Y Mason and C R Taylor

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