

## ELASTASE DIGESTION OF THE AMYLOID-LIKE SUBSTANCE SURROUNDING MAMMARY CANCER AND BASAL CELL CARCINOMA OF THE SKIN

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**Abstract.** Ordinary amyloid (primary and secondary amyloidosis, amyloid tumours of the skin and of the lung, lichen amyloidosus) is not affected by elastase in its staining properties. Amyloid which is adsorbed to the elastic fibres i.e. "elastic amyloid" surrounding certain tumours loses the ability to stain with Congo red, elastica-dyestuffs and fluorochromes, after treatment with elastase. The case of fibroepithelioma premalignum Pinkus with deposition of amyloid occupies a special position on its own.

In previous investigations (18, 19, 20) a particularly fibrillar or finely granular, often amorphous material has been described surrounding nests of some malignant tumours, e.g. fibrous mammary cancers, basocellular carcinomas of the skin, tumours of the rectum, of the parotid gland, and of the urinary bladder. This substance, formerly termed fibrinoid, displayed some properties similar to those of amyloid. It was stained by Congo red, and even responsiveness to methyl-violet, iodine green and Lugol's solution was established. However, we also found differences from "classic" amyloid: it was not birefringent after staining with Congo red, and it took up elastica stains, whereas ordinary amyloid never reacts to them.

Due to differences between the material described and ordinary amyloid, the term "elastic amyloid" was suggested.

The purpose of this study was to establish whether the enzyme elastase would change the behaviour of the amyloid-like substance in cases of mammary cancer and of basal cell cancer of the skin.

Little is known about the pathological processes in elastic tissue. Most enzymes have no effect here. In 1890 Ewald (8) published a paper on the enzymatic dissolution of elastic fibres by crude ex-

tracts of the pancreas. In 1904 Eijkman (7) discovered an elastolytic enzyme in culture filtrates of *P. pyocyanea* and other microbes. Balo & Banga (1, 2, 9) described in 1949 an elastolytic factor in preparations from pancreas, giving it the distinctive name elastase. Their elastase was not entirely homogenous, and contained also a mucolytic enzyme. The introduction of elastase by these authors opened the possibility of research work in this field. In preparations kindly submitted by Mrs Banga we found dissolution of elastic fibres in paraffin slices. Similar results are described by Hassler & Herbertsson (12). However, they find certain difference in the type of digestion as far as man and animal, the age of patient and so on are concerned. In all these investigations, bacterial elastase and pancreas extracts had the same effect.

### MATERIAL AND METHODS

The material was the same as in our previous investigations (20). Ten specimens of non-irradiated mammary cancer—all showing the presence of elastic amyloid—were collected from surgical material, plus fifteen specimens of basal cell carcinoma, excised for diagnostic purposes. Controls were carried out on organs of patients with primary and secondary amyloidosis, amyloid tumours of the lung and of the skin, cases of lichen amyloidosus and excisions of the skin with senile elastosis.

All specimens were stained with haematoxylin-eosin, van Gieson's stain, elastica stain (resorcin-fuchsin + polychrome methylene blue), Congo red, PAS reaction, Alcian blue, and Mallory's stain as modified by Ladewig. The following methods were used in addition: fluorescence microscopy, using acridine orange and thioflavin S respectively, as fluorochromes, further investigation in polarized light preceded by Congo red staining (14) and fluorescence of the same slices. We used an elastase preparation from hog pancreas, supplied by the Sigma Chemical Company, St. Louis, USA, in a 0.5% veronal buffer solution of pH 8.4. The deparaffinized slices were

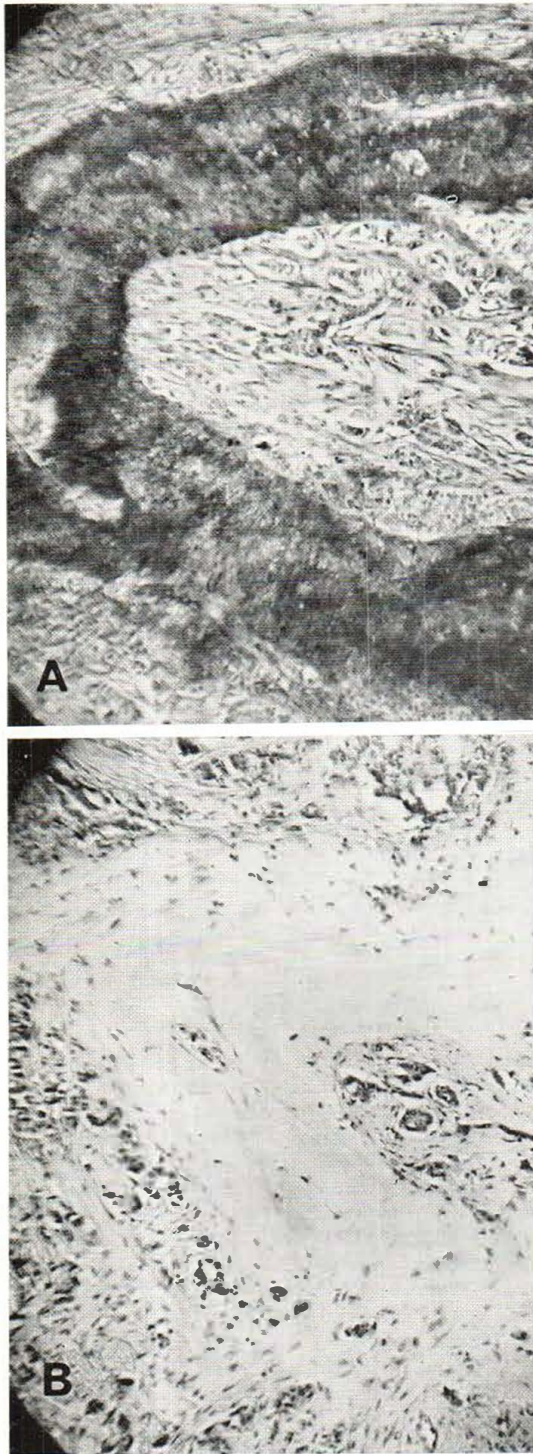


Fig. 1. (A) Cancer mammae. Tumour cell nests with elastic amyloid mass. Elastic stain.  $\times 430$ . (B) The same specimen after elastase digestion + elastic stain. No staining observed in the amyloid masses. Elastic stain.  $\times 430$ .

incubated for 3 hours at  $37^{\circ}\text{C}$  in the substrate solution. After incubation and staining by the methods mentioned above the digested samples were compared with the non-elastase-treated samples.

## RESULTS

### I. Mammary carcinoma

**Haematoxylin-eosin preparations.** The peculiar material called elastic amyloid, situated around the cancer nests, took up red stain to a significantly higher degree than did the other tissues. van Gieson's stain showed a bright yellow colour in this area. After elastase digestion, the colour was partly lost, turning either pale red or pale yellow. As already described (20) the substance was strongly stained by elastica dyestuffs, yet after treatment with elastase, absolutely no staining was noticeable. **Amyloid staining:** In these investigations only Bennholds Congo red was used. The amyloid conglomerates were stained intense brownish red without any birefringence. Elastase caused the Congo red staining to disappear completely, and still no birefringence was observed. In the fluorescence microscope a red fluorescence appeared in the Congo-red-positive areas, but disappeared after elastase-treatment. Pathological elastic fibres at the edges and in the immediate vicinity of the cancer centres, showing positive Congo-reaction, were stained dark red-violet by Alcian blue, and orange red by Laddwig's dyestuff. These stains too were negatively affected by elastase. **Fluorochrome treatment:** With acridine orange, a golden-green-yellow fluorescence was noticed, which disappeared after elastase digestion. Staining with thioflavin S, as suggested by Schwartz (21), causes a silvery blue-white fluorescence of the pathological elastic fibres and a brownish compound to the homogenous amyloid conglomerates. After elastase treatment the silver-blue fluorescence disappears, while a brown matter without fluorescence remains.

### II. Basal cell carcinoma

In these cases the pathological substance showed staining properties identical to those of the precursors of experimentally produced amyloid (4), e.g. the earliest stages of the development of elastic amyloid. In many cases we observed in the tumour cell groups small, intercellular, rather eosinophilic, homogeneous, or minutely fibrillous, limited areas which were stained light yellow by

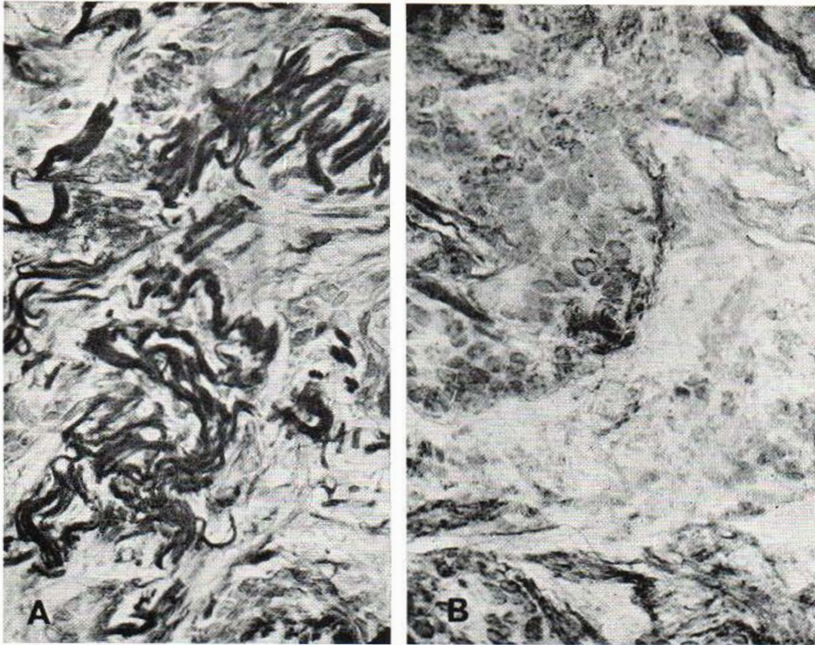


Fig. 2. (A) Basal cell cancer. The proliferating pathological elastic fibres are stained dark purplish-blue with Alcian blue.  $\times 315$ . (B) The same specimen after elastase digestion + Alcian blue staining.  $\times 315$ .

van Gieson's dyestuff and showed a positive PAS-reaction. With toluidine-staining, a metachromasia could be observed in these deposits and also between the tumour cell nests. After digestion with elastase, the staining by eosin and by van Gieson's dyestuff became a little paler, whereas the red colour of the PAS-reaction grew more pronounced. The toluidine metachromasia disappeared. These foci were stained sky blue by Alcian blue but were resistant to elastica dyestuffs and to Congo red. No change was seen after elastase treatment. Towards the periphery of the tumour nests, this material is eliminated and it is adsorbed to the elastic fibres. At the same time, the hitherto positive PAS-staining capacity, as well as the toluidine-metachromasia, is gradually lost. However, the material will accept elastica- and Congo red staining, exactly as we have described previously in connection with mammary cancer. The pathological elastic fibres display a certain exuberant growth, accepting a red or orange yellow stain with Ladewig's method and a dark purple colour with Alcian blue. After elastase treatment the staining ability will disappear with elastica dyestuffs, Congo red, Alcian blue and Ladewig's staining. The same observations have been made for the small condensed areas between the tumour cell nests. No birefringence after Congo red staining ever occurred, but the red Congo fluorescence

was clearly visible in the fluorescence microscope. This fluorescence also disappears after elastase treatment as well as the fluorescence after acridine orange, and the blue-white fluorescence after thioflavin S.

### III. Control material

Senile elastosis is often encountered in areas around basal cell carcinoma sites. It stains greyish red with haematoxylin-eosin and shows intense elastica staining. It often assumes a light brownish colour with Congo red. The remaining methods cause a staining rather paler than in the surroundings. No birefringence or fluorescence occurs. After elastase treatment the elastica staining will disappear, and the other stains will become even less distinct.

In cases of secondary amyloidosis (liver, spleen, lymph node) and one case of primary amyloidosis (heart, liver, lymph node), all these organs were stained by Congo red, showing birefringence and an intense red fluorescence after staining. Elastase did not change these phenomena. Elastin staining and other stains had no positive effect. A small amyloid tumour of the lung behaved quite similarly, as also did lichen amyloidosus and the amyloid tumours of the skin. All these control cases displayed an intense fluorescence after treatment with acridine orange and thio-

Table I. Results of the described histological and histochemical investigations on elastic- and common amyloid

Methods	Basal cell cancer				Mammary cancer		Common amyloid		Pinkus tumor	
	Not adsorbed to elastica		Adsorbed to elastica		Before elastase	After elastase	Before elastase	After elastase	Before elastase	After elastase
	Before elastase	After elastase	Before elastase	After elastase						
Eosin	++	+	++	+	+++	+	+++	+++	+++	+++
v. Gieson	++	+	+++	+	+++	+	+++	+++	+++	+++
Congo red	-	-	+++	-	+++	-	+++	+++	+++	+++
Congo fluorescence	-	-	+++	-	+++	-	+++	+++	+++	+++
Birefringence	-	-	-	-	-	-	+++	+++	-	-
Elastica	-	-	+++	-	+++	-	-	-	-	-
PAS reaction	++	++	-	-	+++	-	-	-	++	+++
Alcianblue	++	-	dark-blue	-	<sup>a</sup>	-	-	-	-	-
Ladewig	-	-	red	-	<sup>a</sup>	-	-	-	red	blue
Acridinorange	-	-	++	-	++	-	++	++	++	++
Thioflavin S	-	-	++	-	++	-	++	++	++	++

<sup>a</sup> Only the pathological elastic fibres.

flavine S, which after elastase treatment intensified, rather than disappearing.

A case of fibroepithelioma premalignum Pinkus showing amyloid deposits in the stroma stood in a category of its own. These conglomerates were stained by Congo red, showed an intense red fluorescence, but no birefringence, however. Elastase had no effect. Elastica dyestuffs were not taken up. The PAS-reaction became strongly positive and even more intense after elastase treatment. With Ladewig's staining, the material assumed an intensely red colour, changing to vivid blue after elastase. Fluorochromes affected the amyloid deposits in the same way as normal amyloid. The results are summarized in Table I.

In short, we observed the development in this tumour of an amyloid-like substance consisting of a peculiar mixture of neutral and acid mucopolysaccharides and paraproteins and not entirely corresponding either to elastic or classic amyloid. The substance is rather similar to the amyloid precursors of Christensen (4). A marked simultaneous staining by PAS and by Congo red occurs, and the birefringence has not yet developed. Furthermore, the result of the Ladewig staining does not agree with the arbitrarily introduced amyloid groups.

We may assume the existence of a primary development of amyloid precursors in tumours, from which elastic as well as ordinary amyloid

may develop within the course of a maturation process which has not so far been described in greater detail.

## DISCUSSION

Thus, deposits of a material similar to amyloid are first observed in the cell groups of basal cell carcinoma. This is stained by PAS and Alcian blue, but is resistant to elastica dyestuffs and to Congo red. Elastase does not alter these reactions. The material, consisting of proteins and mucopolysaccharides (MPS), is then partially expelled in the vicinity and adsorbed to elastic fibres. This complex, instantly losing its PAS-activity, is stained by Congo red and elastica dyestuffs. It is stained in a pathological way by Alcian blue and Ladewig's dyestuffs and displays fluorescence with fluorochromes. It is not birefringent but it is red-fluorescent after Congo red staining and sensitive to elastase. We have termed it elastic amyloid (18).

After elastase digestion the MPSs are removed by the elastomucinase component and after liberation of the carbohydrate-containing substance, the degenerated elastica is easily dissolved. All kinds of amyloid and elastica stainings as well as fluorescence will disappear.

In cases of mammary cancer, the maturation of the described substance proceeds, the pathological elastic fibres forming a thick layer around the

cancer cell groups. They split like aged elastic tissue and form a granular, subsequently amorphous conglomerate. In this case too, elastase will prevent amyloid staining, elastica staining and the fluorescence of the fibres.

The control cases of primary and secondary amyloidosis, amyloid tumours of the skin and of the lung, containing true amyloid, showed birefringence after Congo red staining, and elastica staining was always negative. Neither the different kinds of staining nor the strong fluorescence were affected by elastase treatment.

The adsorption of the precursors to elastic fibres as established in these investigations is not acknowledged by Missmahl (15), who discovered the adsorption of the amyloid to collagenous and to reticular fibres. Other authors, such as Sary (17), accept such an adsorption, however, believing that the MPSs will adsorb to the surface of the elastica and will split from the elastine molecules, due to the elastase and elastomucoproteinase content of the pancreas extracts (11). The adsorption of the amyloid to elastica has, furthermore, been described by Schwartz (21) in the aorta of old people and animals and in elastica-rich large blood-vessels, and also by Glaus (10) and Dahme (5). We have also had the same experience. Divry (6) has described the tendency of the amyloid to form deposits at the different fibres and membranes of the tissue. Kozlowsky et al. (13) have found a metachromatic material in basal cell cancer, closely attached to the elastic fibres.

The effect of elastase on the different types of amyloid may be explained as being due to removal of the mucopolysaccharide-containing "cement" substance from the elastica which will itself be dissolved (3, 16, 17, 22). Our own investigations show good agreement with this explanation. The material developing from the precursors as "classic" amyloid, is not attached to elastic fibres, but to collagenous and reticular ones. For this reason the material is not affected by elastase. The regions containing pure MPS, which are visible in some areas, also remain unaffected by elastase.

These observations give us reason to believe that it is possible to detect an important difference between the normal and the elastic amyloid in the vicinity of different tumours, in addition to the histochemical distinction. This difference implies that because of its construction the elastic amy-

loid after elastase digestion loses its ability to be stained by Congo red, elastica, MPS dyestuffs and fluorochromes, whereas normal amyloid remains unaffected. Intermediate stages between the precursors and the different types of amyloid, e.g. the case of fibroepithelioma Pinkus with amyloid, require further investigation.

In conclusion, we would like to emphasize that routine stainings of amyloid obviously do not suffice to study these complicated substances. Other, different, simultaneous histochemical investigations are strongly advocated.

#### ACKNOWLEDGEMENT

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