EXPERIMENTAL CUTANEOUS CALCINOSIS

AMEMIYA, Naomiki

(Citation)
The Kobe journal of the medical sciences,15(1):1-15

(Issue Date)
1969-03

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/0100489051

Kobe J. Med. Sci. 15, 1-15, March 1969

EXPERIMENTAL CUTANEOUS CALCINOSIS

Naomiki AMEMIYA and
Shigeharu SANO

Department of Dermatology
Kobe University School of Medicine

Indexing Words
calciphylaxis; dihydrotachysterol; mucopolysaccharides; phosphatase; autoradiography

Naomiki AMEMIYA and Shigeharu SANO. *Experimental Cutaneous Calcinosis.* Kobe J. Med. Sci. 15, 1-15, March 1969 —Experimental cutaneous calcinosis induced in the rat by topical calciphylaxis (Selye) was investigated histologically, histochemically and autoradiographically. Calcification in rat skin sensitized by oral administration of dihydrotachysterol occurred within 1 to 3 days after subcutaneous injection of ferric chloride. In the earlier stages of calcinosis, the presence of hyaluronic acid was confirmed histochemically and high activities of alkaline phosphatase, acid phosphatase and ATPase were also observed. In the latter stages of calcinosis, chondroitin sulfate was found to be increased.

INTRODUCTION

Much have been reported on the development of calcification in these decades. Weidmann[17] described that calcification was initiated primarily from the crystal seed in the organic matrix of tissue, and that consequently it progressed from the above nucleus. As the seed forming agent, various substances such as collagen fibers,[6] the phosphorylated matrix,[4] sulfated mucopolysaccharides[7,14] have been suspected to play a role in nucleation of the crystal seed. Selye[18] reported that systemic calcinosis (calciphylaxis) was able to be induced experimentally by the topical treatment (challenger) in animals sensitized with the hypercalcemic agent (sensitizer) such as dihydrotachysterol (DHT), vitamin D, and parathyroid hormone etc.

In this paper the experimental calcinosis produced in rat skin by Selye's method was studied histologically, histochemically and autoradiographycally.

MATERIALS AND METHODS

The animals used were female Sprague-Dawley rats weighing approximately 90 to 100 g.

The experimental cutaneous calcinosis was made by the method of Selye[18] as follows: Fourty five rats were given orally 1 mg of DTH per 100 g body weihgt as a sensitizer. The DHT used was 0.2% solution in soybean oil. After 24 hours, 0.2 ml of 0.05% ferric chloride aqueous solution was injected subcutaneously as an elicitor in mid line of the back.

The animals were killed by decapitation five each at the 5th, the 18th, the 24th,

Received for publication July 1, 1968
Authors' names in Japanese: 雨宮直幹, 佐野栄春

the 32nd hour, and on the 2nd, the 3rd, the 5th, the 7th and the 11th day after ferric chloride injection. Each injected area of the skin was removed on those periods, and utilized for the following examinations. The tissue specimens were fixed in 10% formalin containing 1% cetylpyridinium chloride for 24 hours, and after parafin-embedding and sectioning, they were examined by hematoxylin-eosin stain, van Gieson's stain, Weigert's elastic tissue stain, periodic acid-Schiff (PAS) reaction, alcian blue stain, toluidin blue stain at pH 2.5 and 4.1 (with and without bovine testicular hyaluronidase digestion), prussian blue stain, and von Kóssa stain. Besides the three normal rats, 27 rats treated with only ferric chloride injection were examined as the control three each every above-mentioned period.

For enzyme-histochemical studies, besides the normal skin, DHT-ferric chloride treated skin and only ferric chloride treated skin were examined on the activities of alkaline phosphatase, acid phosphatase and ATPase by the methods of Barka and Anderson,[1] Kagawa,[9] and Wachstein and Meisel,[16] respectively, on the 1st, the 2nd, the 3rd, the 5th, the 7th and the 11th day after ferric chloride injection. The tissue specimens that were immediately frozen after removal and sectioned by $-20°C$ cryostat at 12 micron in thickness were used for these histochemical investigations.

Eighteen rats treated with DHT and ferric chloride were divided into six groups of three rats each, and used for autoradiographic studies. $Na_2{}^{35}SO_4$ was used as a radio-active tracer. The radiosulfate of 100 μc per 100 g of the body weight was injected intraperitoneally once a day for three days before decapitation, and at 24 hours after the third injection they were sacrificed on the 1st, the 2nd, the 3rd, the 5th, the 7th and the 11th day after ferric chloride injection. Control animals which consisted of 12 rats treated with ferric chloride only and the three normal ones were also given the radiosulfate in the same way. The former control animals were examined two each every period as mentioned above. Kodak NTB_2 autoradiographic emulsion was used for autoradiographs by dipping method. After exposure for 40 days at 4 to 6°C, the microscopic slides were developed, and stained with hematoxylin-eosin.

RESULTS

1) Histological and autoradiographic findings:

a) The normal skin.

Concerning uptake of radiosulfate, the radioactivity of ^{35}S was hardly seen in the epidermis. In the papillary layer and the cytoplasm of fibroblasts of the upper dermis, a considerable number of radioactive particles were recognized (Fig. 1), and also recongnized more in the mast cells (Fig. 2), the dermal hair papillae and the outer hair root sheath. However, the radioactive particles were scarcely found in the other sites of the dermis.

b) The skin treated with DHT and ferric chloride.

Five hours after ferric chloride injection:

The dermis was found to be edematous. Inflammatory cell infiltrations were

observed in the lower dermis, the muscular layer (pannuculus carnosus muscle), and the subcutaneous loose connective tissue which mainly consisted of neutrophiles and lymphocytes with a few eosinophiles, fibroblasts, and histiocytes (Fig. 3). The mast cell count was found to be decreased in this inflammatory region. In the lower dermis, dilated blood vessels and the extravasation of erythrocytes were seen. The subcutaneous loose connective tissue was found to be slightly positive with alcian blue stain, and labile for hyaluronidase digestion. Iron was demonstrated in the lower dermis and the subcutaneous tissue with prussian blue stain. The mast cells, the degenerated muscle fibers, and blood vessel walls were PAS positive. Von Kossa positive substances could not be detected.

Eighteen hours after ferric chloride injection :

Inflammatory infiltrate was found to be more remarkable. Swelling and basophilic degeneration of the collagen fibers were observed in the lower dermis. Few mast cell was seen in the inflammatory area.

Twenty four hours after ferric chloride injection :

In the lower dermis, the number of neutrophiles was slightly decreased, while a few number of the mast cells appeared at the perivascular area in the peripheral part of the inflammatory area. In most specimens any calcified area was not recognized. However, only one specimen showed von Kóssa positive granules and small patches of calcification along connective tissue fibers in the inflammatory region of the subcutaneous tissue (Fig. 4). This area weakly showed to be positive with alcian blue stain. The loose connective tissue adjacent to this calcified area was more positive with alcian blue stain. The radiosulfate particles were demonstrated in the mast cells and some dermal fibroblasts, while any particles were demonstrated neither in the inflammatory area nor in the calcified area of this stage.

Two days after ferric chloride injection :

More patchy calcified areas were observed in the lower dermis and the region between the muscle bundles and the subcutaneous loose connective tissue in most specimens. The calcified area was positive with alcian blue stain, and labile for hyaluronidase digestion.

Three to five days after ferric chloride injection :

Calcification is more intense (Fig. 5). In these areas a number of fibroblasts and histiocytes were seen and occasionally foreign body giant cells appeared among the calcified, coarse and wavy fibers. The calcified area was not positive with Weigert's elastic tissue stain. Reactions to PAS, alcian blue stain, and metachromasia with toluidin blue at pH 4.1 were found. The ground substances of the non-calcified connective tissue adjacent to the calcified region were observed to be more positively reactive with alcian blue stain. With prussian blue stain, iron was demonstrated in both the calcified area and the adjacent non-calcified area, and also the cytoplasm of fibroblasts in these areas. At this stage, around the calcified areas neutrophiles and lymphocytes were decreased in number, while fibroblasts and histiocytes were

predominant. Though the mast cells were not seen in the calcified area, they increased in the dermis adjacent to the calcified area. Radiosulfate particles were demonstrated in the calcified area a little. However, fibroblasts, the mast cells, and the connective tissue ground substances adjacent to the calcified area revealed to be moderate in ^{35}S-uptake.

Seven days after ferric chloride injection:

Calcified areas were positive with alcian blue stain, PAS reaction, and slightly positive with prussian blue stain. Although the ^{35}S particles were demonstrable in the calcified area, it is noted that more uptake of radiosulfate was seen in the mast cells appeared around the calcified area.

Eleven days after ferric chloride injection:

Some of the calcified areas were intensely positive with alcian blue, resistant to hyaluronidase digestion and showed toluidine blue metachromasia at pH 2.5 (Fig. 6). More ^{35}S-uptake was seen in the calcified area (Fig. 7).

c) The skin treated with ferric chloride only.

Twenty four hours after the injection:

In the lower dermis and the subcutaneous tissue, inflammatory infiltrates which mainly consisted of neutrophiles, lymphocytes and eosinophiles, with a few fibroblasts were observed.

Two to three days after the injection:

Inflammatory infiltrates were still observed. Slightly positive reaction was shown in some areas of the subcutaneous loose connective tissue with alcian blue stain. The radioactive particles were not demonstrable in the inflammatory area. No calcified area was demonstrated.

Five to seven days after the injection:

The specimens showed a decrease in number of the inflammatory cells, while the count of fibroblast increased in the lower dermis and the subcutaneous tissue. Iron was demonstrated among the interfibrillar spaces of the subcutaneous connective tissue, and in the fibroblasts. The radioactive particles of ^{35}S were observed in the mast cells, some fibroblasts and the dermal hair papillae, but not in the inflammatory area. Calcification was not seen.

Seven to eleven days after the injection:

Calcification was not demonstrated. Any specific uptake of ^{35}S was not found.

2) Enzyme histochemical findings:

a) The normal skin.

Alkaline phosphatase activity was observed in the capillary endothelium, the dermal hair papillae, the outer root sheath of hair follicle, and the cytoplasm of dermal fibroblasts.

Acid phosphatase activity was sightly observed in the basal layer of the epidermis, and along the connective tissue fibers of the dermis. It was also revealed in some dermal hair papillae and the outer hair root sheath moderately.

ATPase activity was observed in the outer hair root sheath, blood vessel walls in the middle and the lower dermis, and slightly in the papillary layer of the dermis.

b) The skin treated with DHT and ferric chloride.

Twenty four hours after ferric chloride injection:

Intense alkaline phosphatase and acid phosphatase activities were demonstrated in most of the neutrophiles and the fibroblasts (Fig. 8). Along the connective tissue fibers, moderate activities of these enzymes were also recognized. ATPase activity was not observed in the inflammatory cells, but in the connective tissue fibers in the inflammatory area.

Two days after ferric chloride injection:

Strong alkaline phosphatase activity was observed in the earlier calcified area and its adjacent area of the lower dermis and the subcutaneous tissue (Fig. 9). Many neutrophiles and fibroblasts located in these areas showed intense alkaline phosphatase activity. Acid phosphatase and ATPase activities were demonstrated in the calcified area. However, stronger activities were observed in the peripheral part of the calcified area.

Three days after ferric chloride injection:

Although strong activities of these three enzymes were found in the peripheral part of the calcified area, scarcely any activities were found in the central area.

Five to seven days after ferric chloride injection:

The similar findings were observed on these enzyme activities as compared with the specimen taken on the 3rd day after the injection (Fig. 10). It seemed, however, that the activities were found to be more decreased as time goes on.

c) The skin treated with ferric chloride only.

Histochemical studies on the specimens which had been passed 24 hours after the injection showed the similar results, compared with those treated with DHT and ferric chloride. Specimens taken on the 2nd, or the 3rd day showed no calcified area. And the cell infiltrations mainly consisted of neutropiles and fibroblasts as described before. Alkaline phosphatase positive cells decreased gradually at the later stages. ATPase activity was not found in these cells.

Table 1 summarizes the results of the above findings.

DISCUSSION

The importance of acid mucopolysaccharides (AMPS), especially its action as a matrix substance for the initial crystal formation, in the general area of calcifica-

Table 1. AMPS, enzyme activities and ^{35}S uptake in normal skin and calcifical area.

Calcification		——							
Alcian blue		Mast cells and hair papillae		±	±	+	+	+	+
Toluidine blue metachromasia	pH 4.1	Mast cells and hair papillae		−	−	+	+	+	+
	pH 2.5			−	−	−	−	±	+
PAS		Mast cells and blood vessel walls		−	±	+	+	+	+
^{35}S uptake		Mast cells, hair papillae and fibroblasts		−	−	−	− peripheral fibroblast (+)	±	+
ATPase		Hair follicles and blood vessel walls		+	++	− peripheral zone (+)	− peripheral zone (+)	− peripheral zone (+)	−
Alkaline phosphatase		Hair follicles and capillaries		+ Inflammatory cell (++)	++	− peripheral zone (+)	− peripheral zone (+)	− peripheral zone (+)	−
Acid phosphatase		Hair follicles and dermis		+ Inflammatory cell (+)	++	− peripheral zone (+)	− peripheral zone (+)	− peripheral zone (+)	−
		Normal skin	DHT → $FeCl_3$ →	1 day	2 days	3 days	5 days	7 days	11 days

tion has been of current interest. It was reported by Johnson et al[7,8] that AMPS were demonstrated histochemically in the calcified area of various diseases and experimental cutaneous calcinosis.

In this study, alcian blue stain positive substances were detected in the adjacent connective tissue of the inflammatory region of the treated area. In the earlier stages of calcification, the above substances were recognized more positively in the peripheral sites than in the calcified area. They were supposed to be a hyaluronic acid mainly, because of being easily digested by hyaluronidase and negative to toluidin blue metachromasia at pH 2.5. The more calcification developed, the more positively the calcified area was stained with alcian blue, and resistant to hyaluronidase digestion, showing toluidin blue metachromasia at pH 2.5. A number of radiosulfate particles were seen in these areas. It can be assumed by these results that there exists a large amount of sulfated mucopolysaccharides, probably chondroitin sulfate in the highly calcified area.

It is well known that the ^{35}S-labelled sulfate is incorporated into sulfuric ester group of the sulfated mucopolysaccharides such as chondroitin sulfate[2,10]. Dziewiatkowski et al.[5] observed that the injected ^{35}S-labelled sulfate was mostly incorporated into chondroitin sulfate in the cartilage. Weidmann[17] showed autoradiographically that radiosulfate particles were accumulated in the hypertrophic zone of the epiphyseal part of the cartilage. These findings support the observations that chondroitin sulfate was formed in and around the calcified area. The author's results that chondroitin sulfate seemed to be little accumulated in the earlier stages of the experimental calcification and that it appeared to be increased in the highly calcified stages, may lead to a conclusion that it behaves for the secondary reaction of the calcification mechanism.

Since Robinson and Rosenheim[12] reported on the role of alkaline phosphatase in calcification, especially concerning its catalyzing action on synthesis of phosphoric ester, the existence of this enzyme would indicate the crystal formation in the development of calcification. It was described by Selye[13] that a hypophysectomy was one of the most effective prevention of the topical calciphylaxis. Solomon et al.[15] reported that the hypophysectomized and DHT-sensitized animals showed a calciphylactic response in the subcutaneously injected area of alkaline phosphatase. The present investigations indicate that the activities of alkaline phosphatase, acid phosphatase, and ATPase are strong in adjacent calcifing areas or in itself, and that it was not found in the central portion of highly calcified area.

In the heterotropic bone formation following the epithelial transplantation of the urinary baldder, marked activities of alkaline and acid phosphatases were demonstrated in the peripheral part of the formed bone by Kagawa[9]. Bona et al[3] found that high levels of alkaline phosphatase, acid phosphatase and ATPase activities were shown in the chondrocytes of the young human cartilages. Mori et al[11] reported that acid phosphatase activity was presented strikingly in the development of the teeth in the area of calcification, while slight activity was observed in the insufficiently calcified zone. They also reported that the acid phosphatase was directly related to mineralization, and that the alkaline phosphatase was connected with cell differentiation, matrix formation and other metabolisms of the tissue. It is well known that

ATPase is concerned with the formation of glucose-6-phosphate and glucose-1-phosphate which may take part in synthesis of AMPS. In these respects, it is assumed that these enzymes play a certain role in the mechanism of calcification. According to the present study, it would be suggested that alkaline phosphatase, acid phosphatase, and ATPase play an important role in calcification, and that the role of these enzymes in the development of calcification bears certain relations to the formation of some organic matrix, such as AMPS or AMPS-protein complexes, probably one of the components of which is hyarulonic acid.

SUMMARY

The experimental cutaneous calcinosis in the rat induced by topical calciphylaxis (Selye) was studied by means of histological, histochemical and autoradiographic techniques.

1) The calcified changes in the rat skin sensitized with the oral administration of dihydrotachysterol occurred within 1 to 3 days after the subcutaneous injection of ferric chloride.

2) In the earlier stages of calcinosis, the presence of acid mucopolysaccharide (AMPS) was demonstrated, the properties of which suggested that it was hyaluronic acid. The higher activities of alkaline phosphatase, acid phosphatase and ATPase were also observed. Chondroitin sulfate was found to be increased in the later stages of calcinosis.

3) Particularly hyaluronic acid was found to be important in the mechanism of calcinosis among AMPS, while chondroitin sulfate appeared secondarily in the stages of higher calcinosis.

4) The roles of alkaline phosphatase, acid phosphatase and ATPase were also discussed in the development of calcifing mechanism.

REFERNECES

1. BARKA T. and ANDERSON P. J.
Histochemistry. 1963 p. 234
Hoeber medical division, Harper & Row publischers Inc. New York, Evanston and London.
2. BESTRÖM H.
J. Biol. Chem. 1952. 196. 477/481
On the metabolism of the sulfate group of chondroitin sulfuric acid.
3. BONA C., STANESCU V., DUMITRESCU M., CHYKA GR., and JONESCU V.
Acta Histochem. 1965. 21. 98/119
Histochemical studies on the growing human tibial cartilage.
4. CARTIER P. et PICARD J.
Bull. Soc. Chim. Biol. 1955. 37. 485/494
La minéralisation du cartilage ossifiable: I.—La minéralisation du cartilage *in vitro*.
5. DZIEWIATKOWSKI D. D., FERRANTE N. D., BRONNER F. and OKINAKA G.
J. Exptl. Med. 1957. 106. 509/524
Turnover of ^{35}S-sulfate in epiphyses and diaphyses of sackling rats.

6. FLEISCH H. and NEUMAN W. F.
Amer. J. Physiol. 1961. 200. 1296/1300
Mechanisms of calcification: role of collagen, polyphosphatases and phosphatase.
7. JOHNSON W. C., GRAHAM J. H., and HELWING E. B.
J. Invest. Derm. 1964. 42. 215/224
Histochemistry of the acid mucopolysaccharides in cutaneous calcification.
8. JOHNSON W. C., FORBES P. D., GRAHAM J. H., and GRAY H.
J. Invest. Derm. 1964. 43. 454/466
Experimental cutaneous calcinosis: a histopathologic and histochemical study.
9. KAGAWA S.
J. Histochem. Cytochem. 1965. 13. 255/264
Enzyme histochemistry of bone induction by urinary bladder epithelium.
10. MANCINI R. E., VILAR O., STEIN E., and FIORINI H.
J. Histochem. Cytochem. 1961. 9. 278/291
A histochemical and radioautographic study of the participation of fibroblasts in the production of mucopolysaccharides in connective tissue.
11. MORI M., TAKADA K. and OKAMOTO J.
Histochemie. 1962. 2. 427/434
Histochemical studies on the localization and activity of acid phosphatase in calcifying tissues.
12. ROBINSON R. and ROSENHEIM A. H.
Biochem. J. 1934. 28. 684/698
Calcification of hypertrophic cartilage *in vitro*.
13. SELYE H.
Calciphylaxis. 1962
The university of Chicago press, Chicago.
14. SOBEL A. E. and BURGER M.
Proc. soc. Exptl. Biol. Med. 1954. 87. 7/13
Investigation of the role of chondroitin sulfate in the calcifying mechanism.
15. SOLOMON R. D., NADKARNI B. B. and RICHARDSON L. R.
Arch. Path. 1966. 82. 60/64
Local calciphylaxis in dichydrotachysterol-sensitized rats.
16. WACHSTEIN M. and MEISEL E.
Amer. J. Clin. Path. 1957. 27. 13/23
Histochemistry of hepatic phosphatases at a physiologic pH.
17. WEIDMANN S. M.
"Calcification of Skeletal Tissues" 1963. p339/377
In international review of connective tissue research. vol. 1 (Ed. by Hall, D. A.) Academic press, New York and London.

LEGENDS

Fig. 1. ^{35}S uptake in upper dermis of the normal skin (10×10).
Fig. 2. ^{35}S uptake in mast cells of the normal skin (10×40).
Fig. 3. Inflammatory infiltrate in lower dermis of DHT and ferric chloride treated skin (5 hours after injection) (10×10, HE).
Fig. 4. Early calcified area of DHT and ferric chloride treated skin (24 hours after injection) (10×10, von Kóssa-HE).
Fig. 5. Highly calcified area in the subcutaneous region of DHT and ferric chloride treated

skin (5 days after injection) (10×10, von Kóssa-HE).

Fig. 6. Toluidine blue metachromasia at pH 2.5 in mast cells and highly calcified area of DHT and ferric chloride treated skin (11 days after injection) (10×10).

Fig. 7. ^{35}S uptake in highly calcified area of DHT and ferric chloride treated skin (11 days after injection) (10×40).

Fig. 8. Alkaline phosphatase activity in the inflammatory area of DHT and ferric chloride treated skin (24 hours after injection) (10×10).

Fig. 9. Alkaline phosphatase activity in the early calcified area and its peripheral zone of DHT and ferric chloride treated skin (2 days after injection) (10×10).

Fig.10. Alkaline phosphatase activity in the peripheral zone of calcified area of DHT and ferric chloride treated skin (7 days after injection) (10×10).

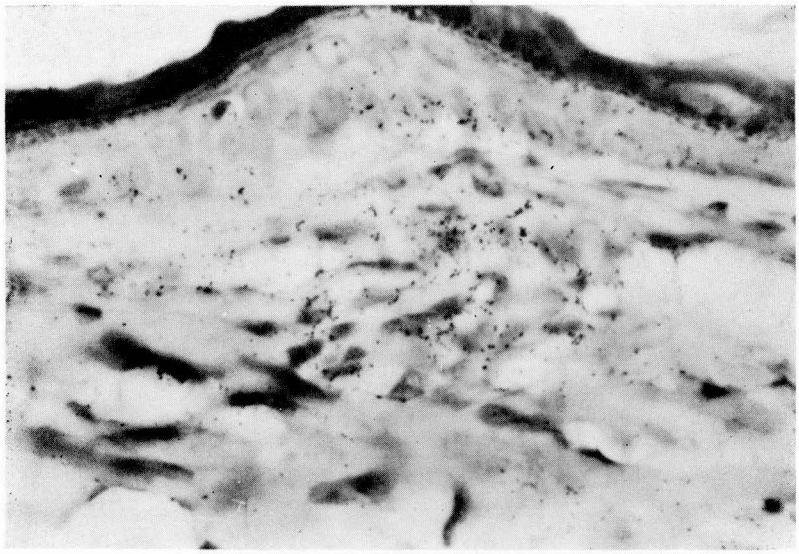

Fig. 1.

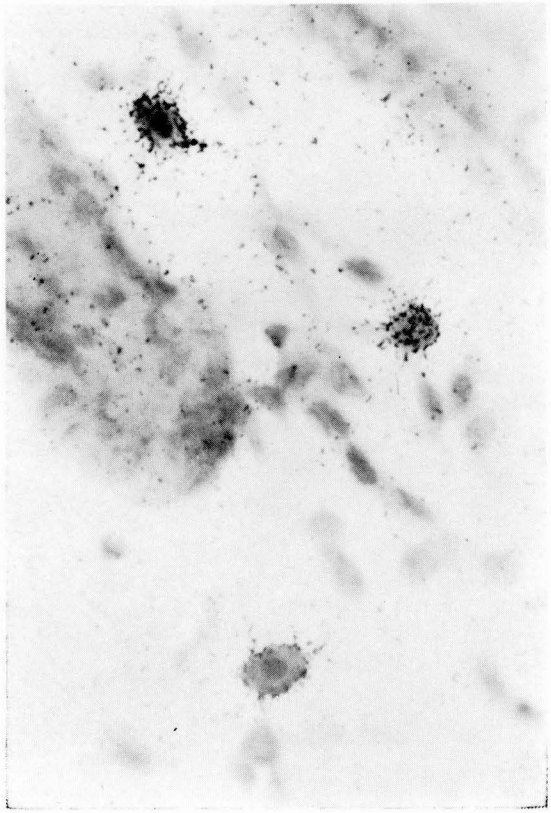

Fig. 2.

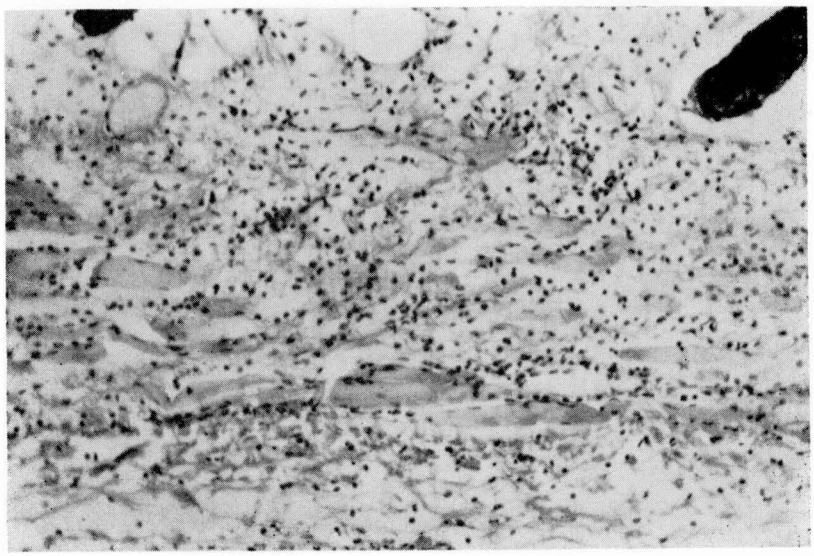

Fig. 3.

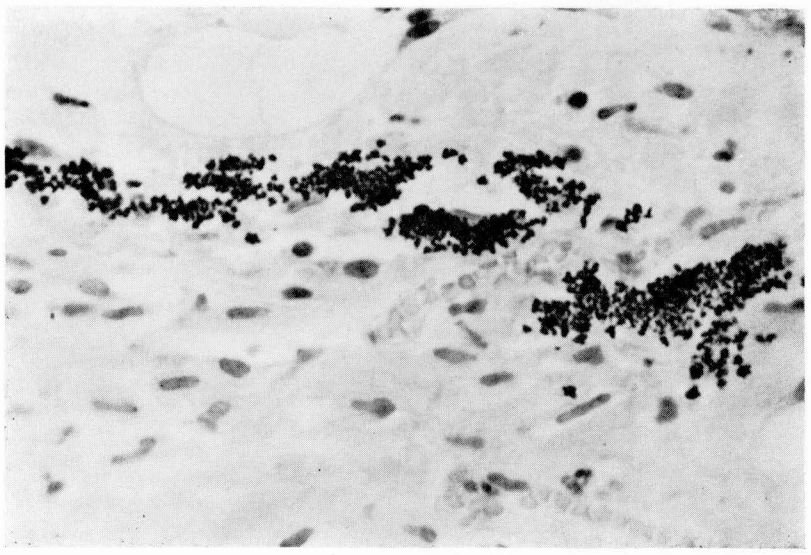

Fig. 4.

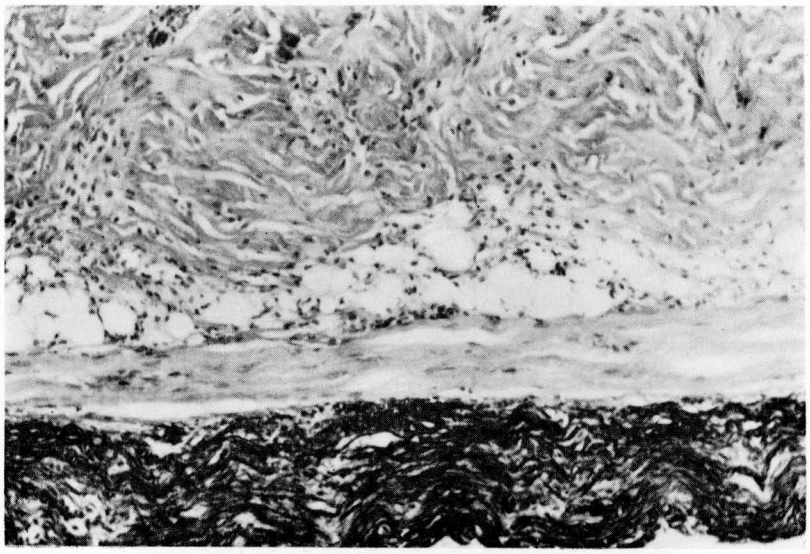

Fig. 5.

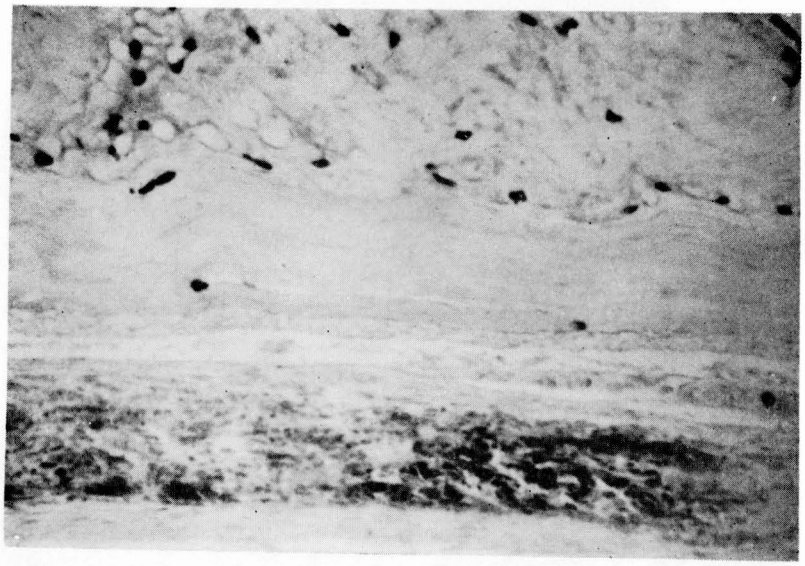

Fig. 6.

Fig. 7.

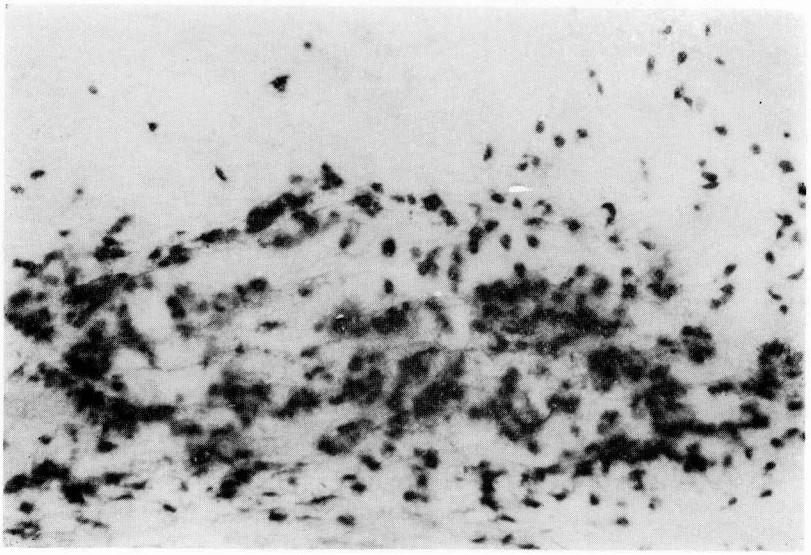

Fig. 8.

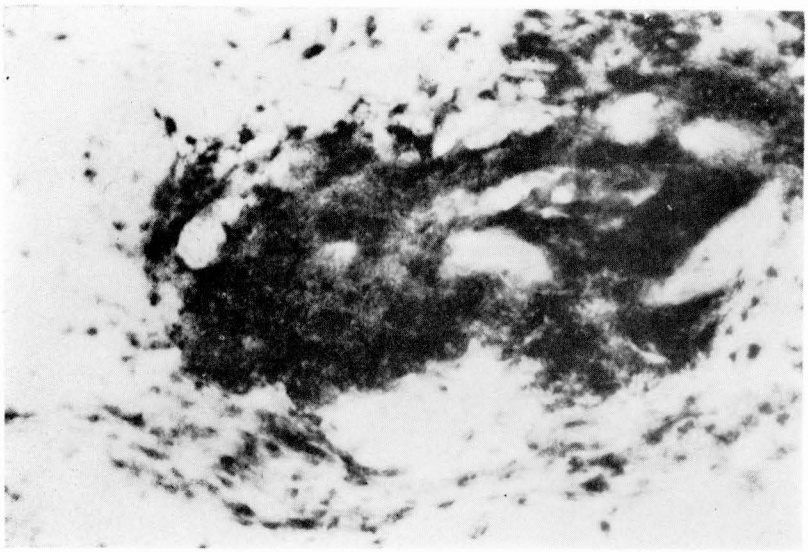

Fig. 9.

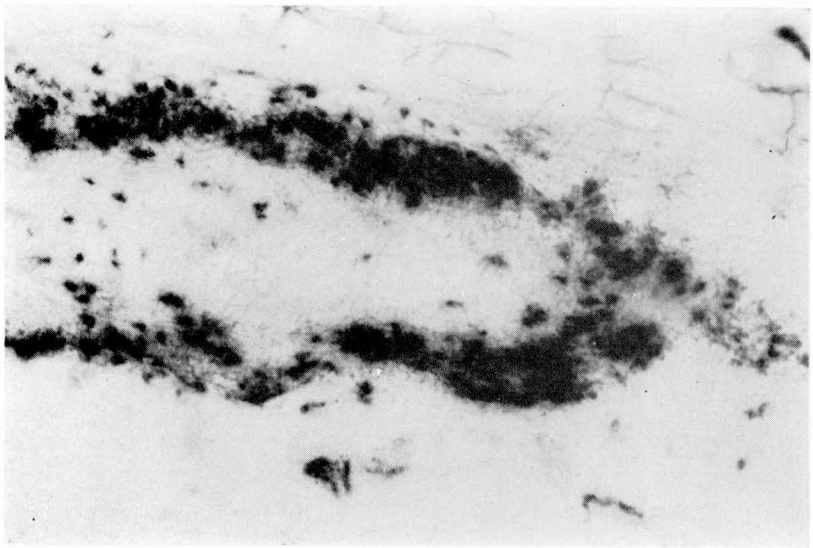

Fig. 10.