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COLLAGENASE ACTIVITY IN RHEUMATOID NODULES

Ultrastructural in vivo and in vitro Studies

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Abstract. Three typical lesions of subcutaneous rheumatoid nodules were studied with the electron microscope for evidence of collagen degeneration. Admixed with predominantly amorphous and fine filamentous substances, split or frayed collagen fibers with a granular appearance were found. Elastic fibers were not severely affected. The segment-long-spacing type of tropocollagen crystallites aggregated out of the incubation mixture of rheumatoid nodule homogenate and purified tropocollagen showed fragmentation of the molecule, most commonly producing TC^{A}_{3G} and TC^{B}_{20}. It is postulated that the initial cleavage of the collagen molecule is at β^{2}_{2}, cleaving the molecule into TC^{A}_{75} and TC^{B}_{25} and the second system of non-specific proteases contributes to the further degradation of the molecule. As an alternative, involvement of multiple collagenases, each having its own specific site of attack on the collagen molecule, is also considered.

In the light microscope literature, the pinkish, amorphous substance occupying the center of the nodule has been described as fibrinoid necrosis, necrobiosis, etc. (11, 15, 16). In a group of diseases, Pinkus & Mehregan (16) emphasized the primary nature of the damage involving the focal area of dermal connective tissue. They considered the granulomatous infiltration surrounding the degeneration as a secondary reaction. Such affections are: rheumatoid and rheumatic nodules, granuloma annulare, necrobiosis lipoidica and juxta-articular nodes in elderly syphilitics. In these conditions, not only are the fibrillar characteristics of the collagenous tissue lost, but also increased quantities of mucinous substances are found in the active lesions. The primary mechanisms leading to such collagen degeneration and an increase of PAS- and Alcian blue-positive mucopolysaccharides (11), however, have remained unknown.

During the course of ultrastructural studies of subcutaneous rheumatoid nodules, we observed a severe collagen degeneration toward the center of each nodule. Further investigation with physiochemical methods demonstrated that specific collagenase is present in active form in the lesion. In this report, ultrastructural evidence of collagenolysis in the lesions of three selected cases will be described.

MATERIALS AND METHODS

Case 1

J. F. is a 66-year-old white male with a history of rheumatoid arthritis of 25 years' duration. The onset of his disease was characterized by inflammation, pain and swelling of the joints of his hands and feet, and the disease steadily progressed to eventually involve most of the joints of his body. Physical examination revealed a severely disabled, elderly male suffering from generalized weakness, joint pain, stiffness and deformities of the majority of his peripheral joints. There were severe rheumatoid arthritic deformities of both hands with marked ulnar deviation of the fingers, thickening of the metacarpophalangeal and proximal interphalangeal joints, and muscular atrophy. Large nodules were present in both olecranon bursae, on the forearms, buttocks, and heels. Flexion deformities of the knees, in addition to crepitation on motion, were pronounced.

Laboratory data. Pertinent laboratory data included a weakly positive fluorescent antinuclear antibody test, a positive rheumatoid factor titer of 1:320, repeatedly negative LE cell tests, a moderately positive (3+) C-reactive protein, a negative VDRL test and a serum electrophoresis with decreased albumin and elevation of the gamma globulin component. Routine blood chemistries were all essentially within normal limits. A complete blood

count was normal with the exception of a mild, persistent eosinophilia of 5–11%.

Roentgenograms. The feet showed subluxation of the metacarpophalangeal joints, osteoporotic changes, and cystic lesions of several of the phalanges. Generalized osteoporosis was present in the hands, as was soft tissue swelling and narrowing of the joint spaces. A few of the phalanges of the hands showed cystic areas.

Treatment. Treatment in the past included such medications as oral prednisone, acetylsalicylic acid, propoxyphene HCl and gold sodium thiomalate injections. At the time of this examination, the patient was being treated with 600 mg of buffered aspirin five to six times a day.

Rheumatoid nodules from both forearms were surgically excised under 1% xylocaine anesthesia. They were promptly cleansed of all blood, serum and excess subcutaneous tissues.

Case 2

J. O. W. is a 50-year-old white male farmer with documented rheumatoid arthritis since 1964. On physical examination, there was swelling and tenderness of the proximal interphalangeal (PIP) and metacarpophalangeal joints of several fingers and the left knee. The left shoulder was tender to palpation. Many PIP joints of the toes were protruding and several of these overlapped. In the region of the left olecranon process there were several nodules which by previous biopsy had been diagnosed as rheumatoid nodules. Another similar nodule was present on the right Achilles tendon.

Laboratory data. Pertinent data includes the following: rheumatoid factor of 1 : 1280, total protein of 7.1 grams per cent with a slight decrease of albumin and an increase of all globulin fractions, a mildly positive C-reactive protein (2+), corrected sedimentation rate of 26 mm per minute, with negative LE cell and fluorescent antinuclear antibody tests. Blood chemistries were all unremarkable.

Roentgenograms. The hands and feet showed narrowing of the joint spaces with irregularities of the distal heads of the metacarpal and metatarsal bones.

Treatment. Presently, therapy includes aspirin 600 mg four times a day plus 50–75 mg of gold sodium thiomalate intramuscularly every 4 to 6 weeks. Intermittently, 25 mg of indomethacin have been taken orally three times a day for periods of 5 to 10 days.

Case 3

C. B. is a 50-year-old Caucasian male with a 10-year history of rheumatoid arthritis. This arthritic condition began as painful swelling and erythema of the fingers, wrists, elbows, ankles and knees with rapid progression to a semi-invalid state. At time of examination, his physical findings consisted of painful swelling of the carpal, metacarpophalangeal and proximal interphalangeal joints of both hands with associated limitation of motion. There was ulnar deviation of all fingers. The knee and ankle joints showed moderate periarticular swelling and tenderness with a decreased range of motion. Over the olecranon processes and dorsa of both hands there were tender, firm nodules.

Laboratory data. Pertinent laboratory data included a rheumatoid factor titer of 1 : 160, C-reactive protein 4+ and a negative LE cell test. Electrolytes and blood chemistries were all within normal limits.

Roentgenograms. X-rays of the hands, feet and ankles showed diffuse osteoporosis and joint space narrowing. These were interpreted as consistent with rheumatoid arthritis. The patient underwent a synovectomy of the left dorsal wrist. At the time of surgery, two subcutaneous nodules from the left elbow were excised under 1% xylocaine anesthesia.

Subcutaneous rheumatoid nodules obtained from these patients were divided into two parts. One piece was fixed immediately in 10% formalin and the other half in 5% glutaraldehyde buffered with 1/10 M cacodylate to pH 7.4. The formalin-fixed tissue was processed routinely and stained either with hematoxylin and eosin or with Verhoeff's elastic fiber stain. Glutaraldehyde-fixed tissue was also routinely processed and was embedded in Araldite. Thin sections, 400–600 Å, were cut in a Porter Blum MT-2 ultramicrotome, placed on 200-mesh grids and stained with lead citrate and uranyl acetate. Stained sections were observed in an Hitachi HU-12 electron microscope. The details of electron microscopic specimen preparation have been published elsewhere (4).

Preparation of segment-long-spacing crystallites from reaction mixture

Supernatants of three rheumatoid nodules were pooled and used for this study. The maximum point of the collagenolytic process was first detected by viscometry. Then, after 24 hours incubation and the confirmation of 50% viscosity loss, the reaction mixture was acidified to pH 3.8 with acetic acid and EDTA was added to a final concentration of 0.001 M to stop the enzyme activity. The acidified solution was filtered and the filtrate was centrifuged at 25 000 rpm., dialysed against 0.05% acetic acid at 4° and then lyophilized. A small amount of the lyophilized material (1–2 mg) was dissolved in 0.05 M acetic acid for 24 hours. Aliquots of this solution were kept at 15° for 100–500 hours. This incubation resulted in a better yield of high quality SLS with clearer banding pattern, both intact and fragmented, probably due to proper formation of a tropocollagen type helix (9). To each of these solutions 1% ATP was slowly added with constant stirring until the final concentration of 0.4% was reached. The solution became slightly turbid in a few seconds. If the turbidity was pronounced, the concentration of the lyophilized reaction product was reduced since SLS specimens from such solution would be overlapped and lumped together and, therefore, accurate observations on isolated single molecules would be difficult. The solution showing an ideal turbidity was kept in the cold for 60 minutes and then dropped with pipette onto Formvar-coated 200-mesh copper grids. The specimen on the grid was air dried and stained with 1% aqueous phosphotungstic acid (pH 3.7) for 5 minutes and double stained with 1% aqueous uranyl acetate for 3 hours. Stained specimens were observed in Hitachi HU-11C and HU-12 electron microscopes at accelerating voltages of 100 and 125 kV. For the control, acid-extracted tropocollagen of calf skin alone (4) was converted to SLS by the same method.

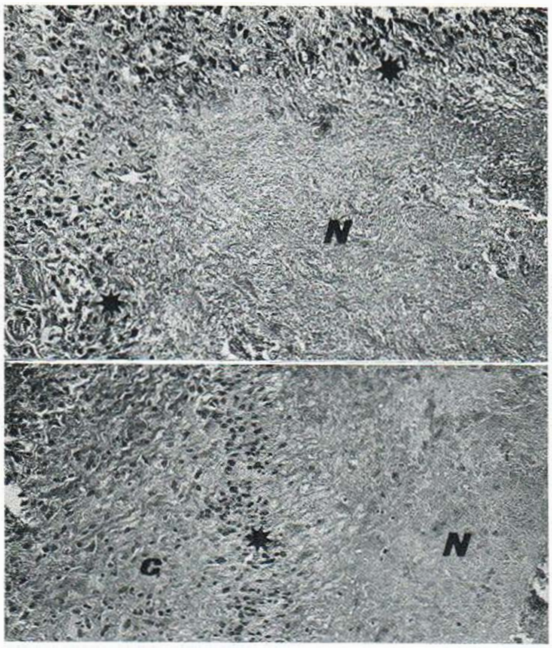

Fig. 1. Homogeneous or granular masses of necrotic material (*N*) are surrounded by histiocytic and fibrocytic infiltrations (*) in palisade along the periphery. Across this band of infiltration, a fibrillar pattern of relatively normal collagen (*c*) is seen. Notice that the centers of the necrotic masses are relatively acellular. Cases 1 and 2. × 100.

RESULTS

Light microscopy

In all three specimens, hematoxylin and eosin stained sections showed typical histopathologic pictures of rheumatoid nodule: Pink-stained homogeneous or granular masses were individually surrounded by band-like infiltrations of histiocytes and lymphocytes (Fig. 1). Verhoeff stain revealed some intact elastic fibers in most of the nodules.

Electron microscopy

Cellular elements. The center of each nodule was relatively acellular except for debris. Lipid-containing histiocytes were observed frequently (Fig. 2). A number of fibroblasts were present at the periphery of the necrotic mass. Many of them appeared to be very active in protein synthesis; they showed arrays of rough-surfaced endoplasmic reticulum that were dilated and filled with medium-electron-dense material (Fig. 3). Some fibroblasts contained bundles of microtubules (200–250 Å) and in one case they protruded into the nucleus (Fig. 3). Other cell types encountered in the peripheral cellular infiltration were histiocytes, lymphocytes and mast cells. Typical plasma cells were rarely encountered. Histiocytes, including occasional giant cells, comprised the majority of the cellular infiltration. These histiocytes were often connected to each other by desmosome-like special junctions (Fig. 4). They also produced hemidesmosome-like dense cytoplasmic plaques in apposition to basal lamina-like extracellular substance (Fig. 4).

Fibrous components. In the vicinity of active fibroblasts in the periphery, an abundance of microfilaments (100–120 Å) (Fig. 3) was seen, admixed with young collagen fibrils. The diameters of young collagen fibrils were greatly variable (Fig. 3). It was presumed that these fibroblasts represent a part of the repair processes which usually lead to fibrous organization of old nodules. Toward the center of the nodules, collagen was largely replaced by amorphous or fine, filamentous materials (Figs. 2, 5). Very fine filaments measured less than 20 Å (Fig. 5). Some of these fine filaments might be fibrin, except that no characteristic banding pattern (18) was observed. On the other hand, splitting or fraying of collagen fibrils into fine filaments was evident in many areas (Fig. 5). Also observed was granular material of moderate electron density. Possible derivation of these granular substances from damaged collagen fibrils was suggested in Fig. 5. Microfilaments admixed with collagen or forming independent masses increased in many areas (Fig. 3). A fair number of elastic fibers persisted in the lesion. Fine structural characteristics of these fibers were, however, not quite typical; protofilaments and larger fibrils were not incorporated into the matrix as in the normal elastic fibers of the skin (5, 6), yet they were visible in large numbers (Fig. 6). Some of these abnormal elastic fibers were encircled by histiocytic cells.

Electron microscopy of segment-long-spacing (SLS) tropocollagen crystallites

Segment-long-spacing (SLS) tropocollagen crystallites aggregated from the incubation mixture were mainly of two types. One had a length of about 56% of the full molecule of tropocollagen from the A end ($-NH_2$ terminal) (Fig. 7 left) and the other had about 20% of the length of the full

Fig. 2. Collagen is largely replaced by fine filamentous (*) and amorphous substances. Some areas appear granular. One histiocyte contains lipid globules (*L*). *H*, portions of histiocytes. Case 3. × 10 600.

molecule from the B end (–COOH terminal) (Fig. 7 middle). However, none of the complementary pieces such as 44% B-end fragments nor 80% A-end fragments were discovered. A-A dimer of the 56% molecules was commonly observed (Fig. 7 middle). B-end fragments which were further degraded and shorter than 20% in length were common. The last-cited possibility was actually observed in the homogenate of primary cutaneous melanoma (19): 75% fragments from the A-end were found in hybridization with 56% fragments (Fig. 7 left). However, the complementary 25%

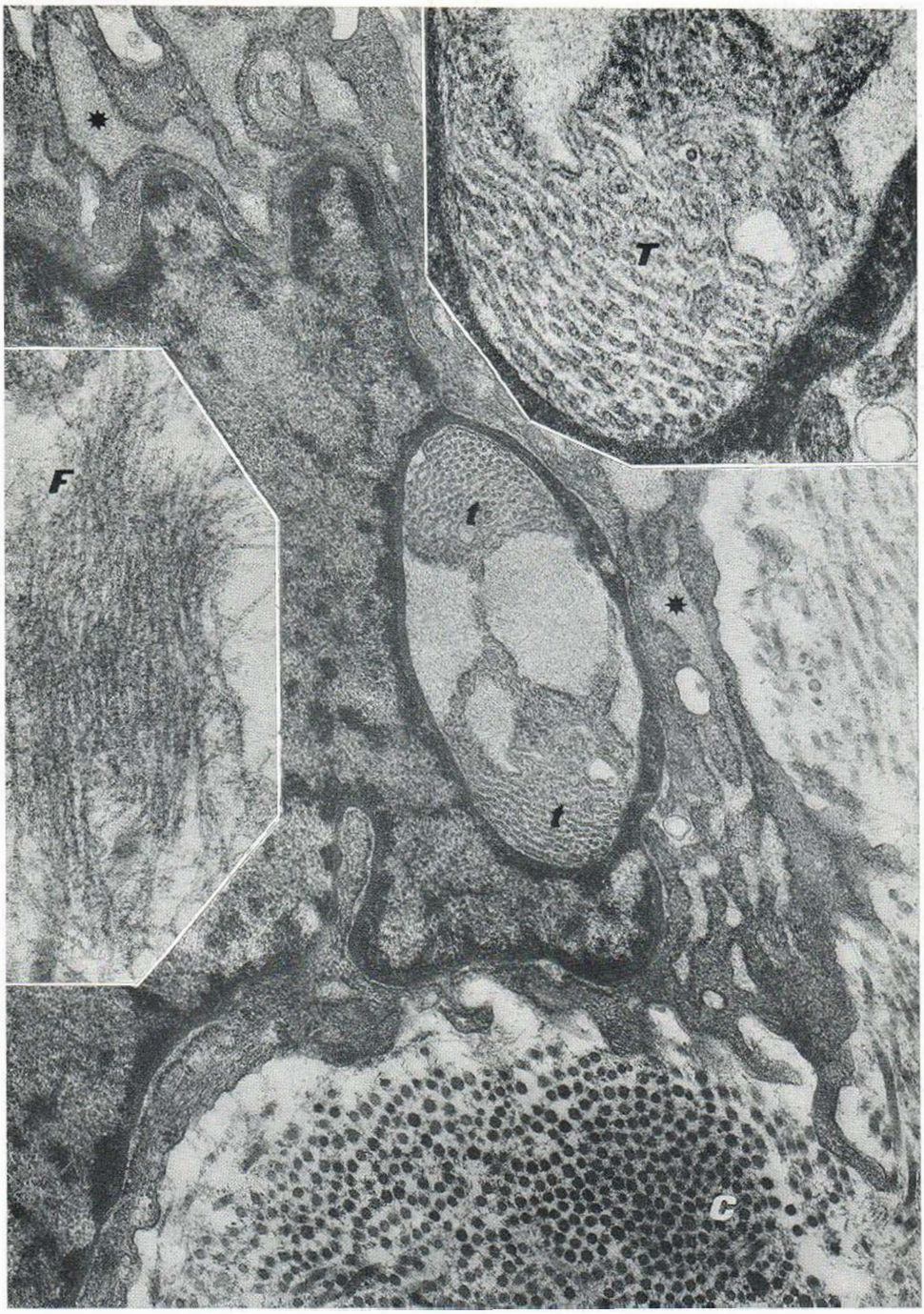

Fig. 3. Fibroblast found at the periphery of a necrotic mass contains a peculiar bundle of microtubules (*t*) (200–250 Å) protruding into the nucleus. Compared at a high magnification (*T*) with microfilaments (*F*) (100–120 Å), which are also increased extracellularly in the vicinity, the microtubules are slightly larger (compare two inset pictures). This fibroblast shows dilated rough-surfaced endoplasmic reticulum (*). Young collagen (*C*) in the vicinity shows variation in diameter. Case 1. Main picture: × 30 000. Both insets: × 60 000.

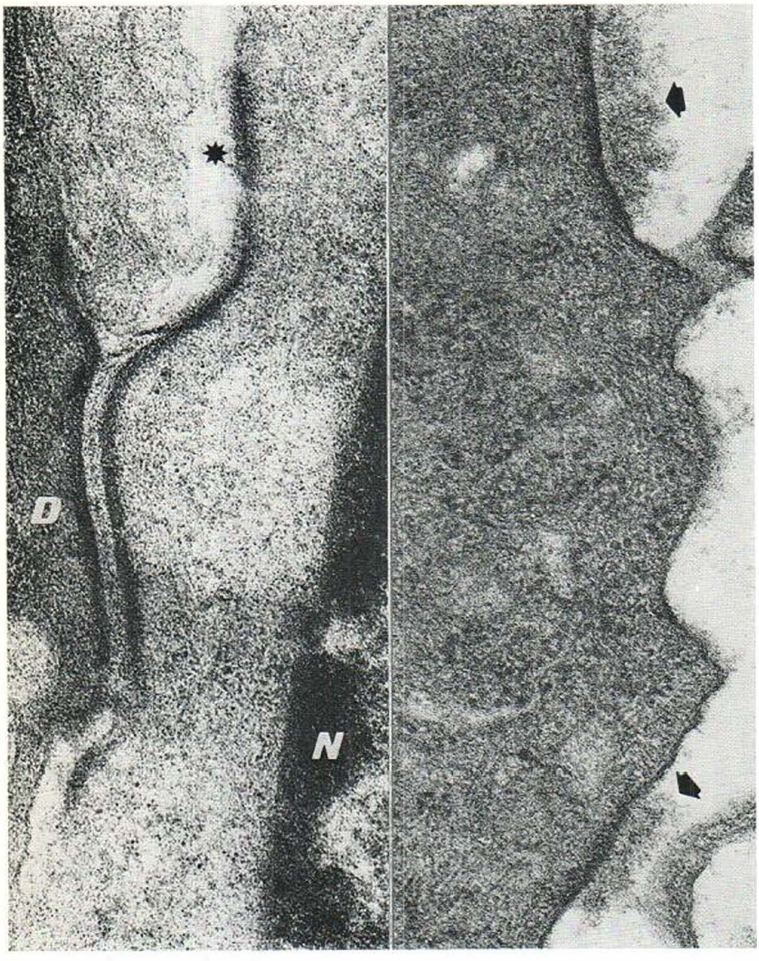

Fig. 4. (*Left*) Desmosome-like junction (*D*) between two histiocytes consists of thickening of cell periphery similar to the attachment plaque of desmosome and intercellular dense layer. Dense thickening of cell periphery extends beyond the area of apposition (*). *N*, nucleus. Case 3. × 75 000. (*Right*) Hemidesmosome-like peripheral density of a histiocyte is matched extracellularly with accumulation of dense amorphous substance (*arrows*). Case 1.

pieces were not found in the same preparation. From these observations the following three possibilities were considered: (i) Tropocollagen molecules were mainly cleaved into 56% A-piece (TC^{A}_{56}) and 44% B-piece (TC^{B}_{44}), of which only TC^{A}_{56} could be found; (ii) the molecules were mainly segmented into TC^{A}_{80} and TC^{B}_{20} pieces and only TC^{B}_{20} were recovered; or (iii) the specific collagenase severed the molecules into TC^{A}_{75} fragments as most of the animal collagenases do (2, 3, 8, 12, 20) and non-specific proteases further eroded these scission products into smaller pieces such as TC^{A}_{56} and TC^{B}_{20}.

DISCUSSION

In the present study, collagen fibrils in the necrobiotic center of the nodule were found to exhibit various degenerative processes such as splitting, granular changes and final disintegration into amorphous substances. It seems very likely, from our previous physiochemical studies (1) as well as the present works, that collagen degradation in the lesion was initiated by specific collagenase or multiple collagenases (see below).

There is no way at present to correlate the collagen molecule cleavage demonstrated in SLS molecules in vitro with those degenerative changes observed in the lesion in vivo. In order to define the specific ultrastructural changes caused by specific collagenase, it is necessary to purify the enzyme, experimentally inject it into animal skin and observe the changes thus produced. Since our collagenase was a crude preparation and, furthermore, there is always a possibility that a non-specific protease system may be participating in the degradative action (1) as in another system (13), we do not claim specificity

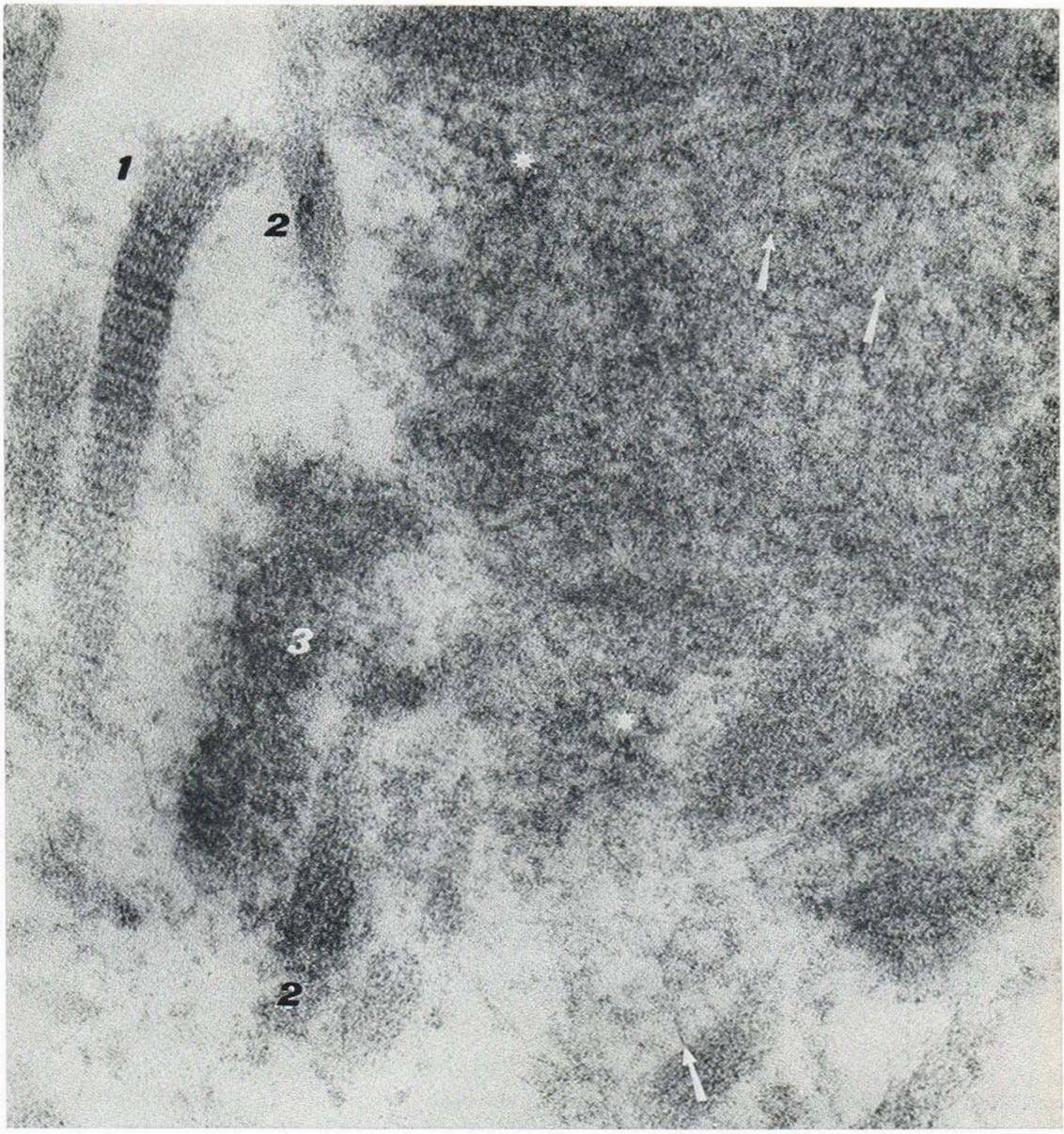

Fig. 5. Collagen degeneration in the area similar to that shown in Fig. 2 is enlarged. There is normally banded collagen with frayed end (1), slightly granulated fiber (2), swollen and markedly granular fiber (3) and what could be interpreted as further degraded materials (*). Very fine filaments (*arrows*) and amorphous substance are seen in such an area.

of the alterations described above. It will suffice to say, however, that specific collagenase should have been involved initially to produce the massive collagen degradation in vivo as observed in our materials, since collagen is quite resistant to degradation by ordinary proteolytic enzymes. Enzymes such as trypsin are not regarded as collagenases since they only erode an end of the native collagen molecule rather than cleave it, although they can further degrade the molecules initially cleaved by specific collagenase (17). We therefore believe that the collagenase mechanism

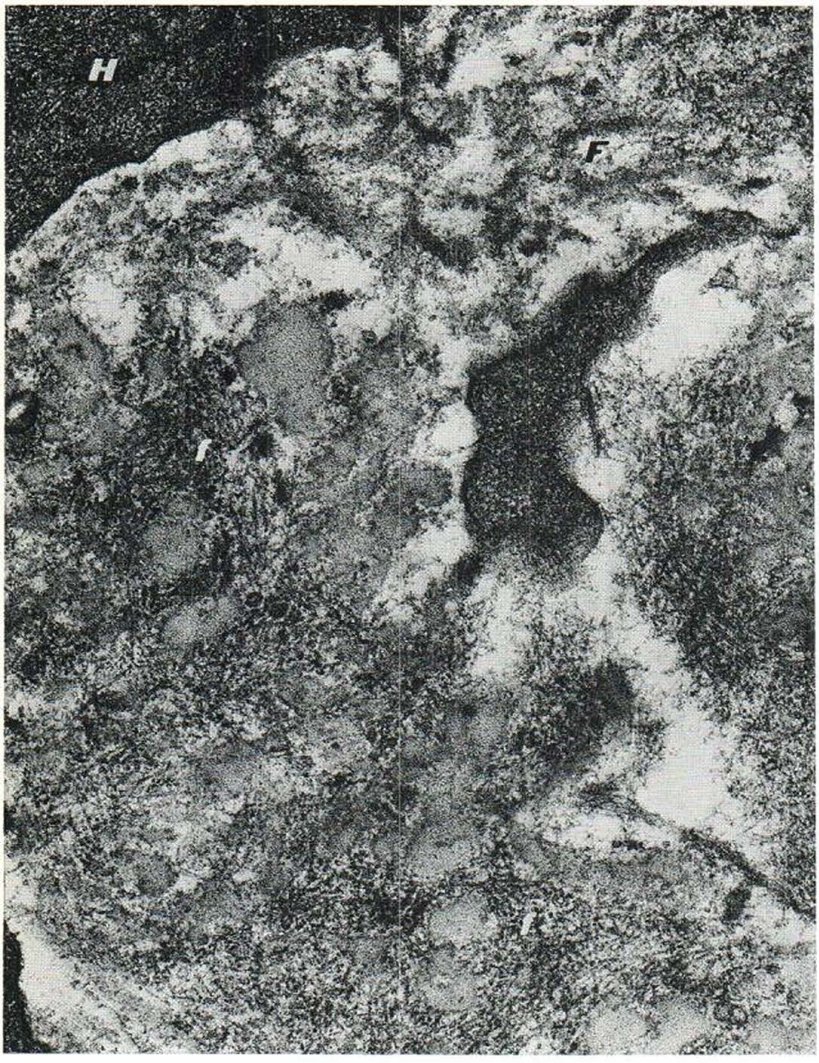

Fig. 6. A large number of protofilaments (*f*) are still visible in mature elastic fibers found in the necrotic area of a nodule. Larger fibrils (*F*) have not yet been incorporated into the fiber and are still visible. The entire mass of abnormal elastic fiber is surrounded with a histiocyte of which a part is shown (*H*). Case 1. × 38 500.

is involved at the outset of the degeneration process. Necrobiotic collagen thus formed may subsequently elicit granulomatous (foreign body) reaction and, as it subsides, fibrotic organization. Active fibroblasts and associated microfilament production as shown in Fig. 3 seem to be participating in this repair process. Desmosome-like junctions between fibroblasts and histiocytes and formation of hemidesmosomes with ground substance have been observed in several abnormal conditions in which collagen alteration or deposition of abnormal substances such as hyalin and amyloid occur (7, 8). The significance of microtubular bundles in fibroblasts is not clear.

The origin of the rheumatoid nodule collagenase is not known at present. In the early lesions of this group of diseases, accumulations of histiocytes and fibroblasts are present, surrounding very small foci of "necrobiotic" collagen. It is only surmised that these cells might produce collagenase. In the subcutaneous lesions such as ours, epidermal or dermal papilla collagenase activity (2, 14) can be ruled out. The majority of the cells infiltrating the periphery of the necrosis are histiocytes and fibroblasts, thus the presence of granulocyte collagenase (12, 13) can also be ruled out. The fact that elastic fibers were more or less spared may indicate the absence of significant amounts of elastase such as may be released from the granulocytes (10). Further

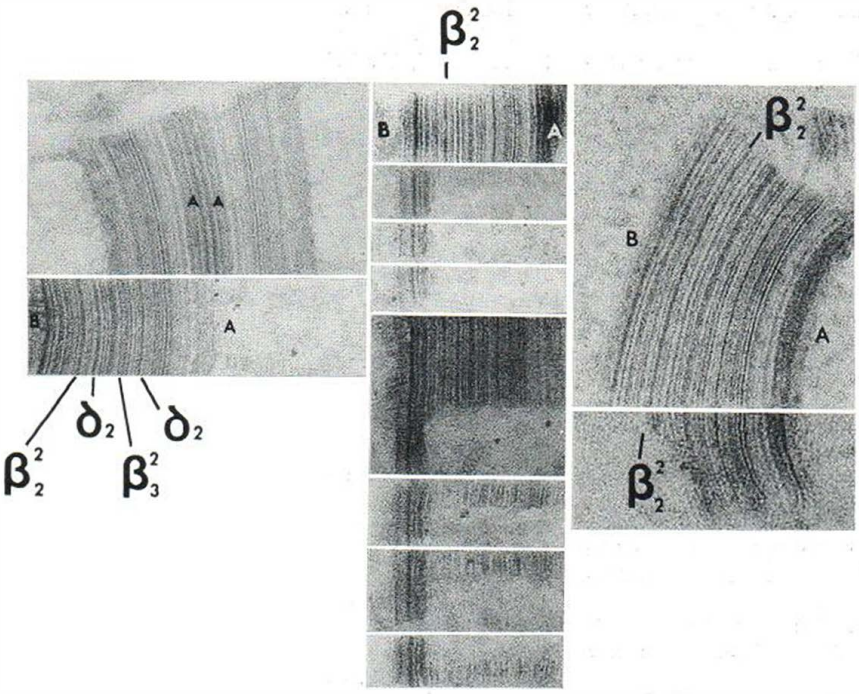

Fig. 7. (*Left*) A–A dimer of TC_{56}^{A} (*top*) is matched up with a full-length molecule (*bottom*). It is seen that the B-terminal side ends between β_3^2 and δ_2 lines, approximately 56% from A-terminal. (*Middle*) TC_{20}^{B} and hybrids of TC_{20}^{B} with full molecules are shown. If matched with a full molecule (*top*), it is seen that the A-side of the fragment terminates short of the β_2^2 line representing approximately 20% of the molecular length from the B-end. (*Right*) Hybrid of TC_{75}^{A} cleaved at β_2^2 and TC_{56}^{B} (*bottom*) was found in the incubation mixture of primary cutaneous melanoma and purified calf skin tropocollagen (9). This hybrid is matched with a full-length molecule (*top*). × 112 500.

studies are in progress to characterize this enzyme, using physiochemical and electron microscopic methods.

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Addendum

Since the completion of this study, Harris, Jr (J Clin Invest *51:* 2973–2976, 1972) has published data that a collagenase and a neutral protease could be extracted from the culture media of rheumatoid nodule. Both the collagenase and protease were active at physiological pH and were inhibited by chelating agents, SH compounds and 1 : 40 dilutions of human serum. The purified enzyme was said to cleave the collagen molecule into 75% and 25% fragments, but no electron microcscopic picture was presented.