AN IMMUNOHISTOCHEMICAL EXAMINATION OF GRANULATION TISSUE WITH GLOMERULAR AND LUNG ANTISERUMS

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A wide variety of connective tissue fibers, including lymphoid reticulin, sarcolemma, neurilemma, and most epithelial basement membranes, may be demonstrated by the so-called silver impregnation reticulin stains. Cruickshank and Hill,[1,2] using the fluorescent antibody technique of Coons and Kaplan,[3] showed that these structures apparently shared a common antigen (or antigens) as demonstrated by *in vitro* examination of rat tissues with antibody conjugates prepared against rat glomeruli and lung. This antigen, however, was not found in mature collagen. During fibrillogenesis, the immature fibers that first appear can also be demonstrated by the methods of silver salt impregnation, and hence are referred to usually as reticulin fibers; as they mature to collagen, this staining capacity is lost.

The present investigation was designed to ascertain whether the immature fibers of granulation tissue might be so constituted as to be antigenically similar to those which were found to react specifically with the anti-glomerular and anti-lung conjugates.

MATERIAL AND METHODS

Preparation of Antigens and Antiserums

Renal glomeruli from adult Wistar rats were isolated according to the method of Krakower and Greenspon.[4] The isolated glomeruli were subjected to sonic vibration for 20 minutes in a Raytheon 10 kc. oscillator and the washed fibrillar material suspended in isotonic saline to a concentration of 100 mg. per ml.

Lung tissue from the same animals was finely minced in a Waring blender, kept cool in an ice mixture, and the minced tissues subjected to sonic vibration. After being washed with isotonic saline several times, the centrifuged deposit was suspended in saline to a concentration of 350 mg. per ml.

The antigens were emulsified with 2 parts of kidney or lung suspension and 3 parts of incomplete Freund adjuvant (Difco Laboratories, Detroit, Michigan). Four interscapular subcutaneous injections of either the lung or the kidney emulsified antigen were given to 2.5 kg. rabbits at weekly intervals. Altogether 200 mg. of glomerular antigen or 700 mg. of lung antigen were injected into any given rabbit. The animals were bled 4 weeks after the last injection.

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The methods used for extracting the globulins from the antiserums, conjugating them with fluorescein isocyanate, preparing tissue sections, staining them and photographing the tissue reactions have been described in detail in a previous paper.[6]

Tissues Examined

A number of rat tissues (kidney, heart, skeletal muscle, lung, brain, peripheral nerves, liver, spleen, lymph nodes, skin, thyroid, pancreas, adrenal and ovary) were examined *in vitro* with the conjugated antiserums.

Granulation tissue was produced in rats about subcutaneously implanted gelfoam pledgets, as previously described.[6] These animals were sacrificed 1, 2, 3, 4, 7 and 9 days later. Frozen sections were prepared in a cryostat from the zones of granulation tissue and examined immunohistochemically with conjugates of both the glomerular and lung antiserums. After photographic records were made, the sections were washed in buffer, and restained with hematoxylin and eosin. Each frozen block of tissue from which the above sections had been obtained was then removed from the cryostat, fixed in formol saline, and paraffin sections were stained by Gomori's reticulin and silver methenamine methods. This allowed a comparison of the same granulation tissue area by these various stains. It was found that poor staining results were obtained with the silver impregnation methods if serial frozen sections were used.

In Vivo Experiment

Other rats received intravenous injections, 48 hours after the pledget had been implanted, with 1.5 ml. of lung or glomerular antiserum (the same antiserums used for preparing the conjugates described above). These rats were sacrificed 24 hours after the intravenous injection (granulation tissue, 72 hours' duration), and an attempt was made to trace the distribution of the injected antiserums by treating a variety of tissue sections, including the zones of granulation tissue, with anti-rabbit globulin prepared in sheep and conjugated with fluorescein (Sylvana Chemical Company, Orange, New Jersey).

Controls for Immunohistochemical Specificity

In Vitro Experiments. Serial cryostat sections were treated as follows: (1) Stained with conjugated antiserum. (2) Conjugated normal rabbit globulin. (3) Conjugated antiserum previously absorbed with the tissue extract (antigen) used in preparation of the antiserum. (4) Coons's slide blocking technique.[7] (5) Unstained section.

The reaction was considered to be specific if only Slide 1 showed positive fluorescence when examined by ultraviolet microscopy. Control (3) invariably caused complete inhibition of fluorescence, while the Coons technique (4) caused either complete or markedly diminished fluorescence.

In Vivo Experiments. Coons's slide blocking technique only was used.

RESULTS

In Vitro Experiments

Examination of the various rat tissues, mentioned above, with the fluorescein conjugates of both rat glomerular and lung antiserums, revealed a specific fluorescence with a wide variety of connective tissue fibrils such as epithelial basement membranes, capillaries, sarcolemma, neurilemma and lymphoid reticulin (Figs. 1 and 2). Identical results were observed in each tissue when examined with either of the conjugates. These findings directly confirm the original observations of Cruickshank and Hill,[1,2] who believed that they indicated the presence

of a common antigen (or antigens) in the reacting tissues. It should be noted that we did not observe any specific fluorescence in renal epithelium. This had been noted by Cruickshank and Hill when they used "whole kidney" antiserum but not with glomerular antiserum.

When the zones of granulation tissue about the pledgets were examined with the same antiserum conjugates, one invariably could observe specific fluorescence in newly formed capillaries from the time of their first appearance (Figs. 4 and 6), and it continued to be present in the maturing vessels up to the longest period of observation, namely, 9 days.

Examination of the same capillaries when the slide was restained by hematoxylin and eosin showed that the reacting vessels were plump and cellular (Figs. 5 and 7), and comparison with adjacent sections from the same block stained for reticulin and by the silver methenamine method gave one the impression that more than the capillary basement membranes was staining; it appeared that the reacting antigen also was present in or on the endothelial cytoplasm (Figs. 6 and 7).

In no instance was any specific fluorescence observed in the many fine immature reticulin fibrils that were prominent in neighboring sections stained by Gomori's reticulin method (Figs. 8 and 9). These fibers only showed a nonspecific autofluorescence which became somewhat more prominent as they matured.

In Vivo Experiments

The glomerular antiserum that was injected intravenously proved to be biologically active as judged by the quantitative production of proteinuria. Examination of kidney sections with the fluorescent anti-rabbit globulin showed a brilliant specific fluorescence limited to the glomerular basement membrane, indicating that the injected antiserum had united with an antigen in this location (Fig. 3). On the other hand, when granulation tissue about the pledgets was examined in a similar manner, no fluorescence was observed in either the capillaries or immature fibrils; nor could we demonstrate any specific fluorescence in a variety of other tissues that had shown a positive reaction in the *in vitro* experiment, e.g., various connective tissue fibrils and capillaries in skeletal muscle, heart, spleen, intestine, liver, adrenal, ovary, etc.

Although the lung antiserum conjugate gave identical *in vitro* fluorescent staining reactions to those observed with the antiglomerular conjugate, the lung antiserum did not produce significant proteinuria when it was injected intravenously. Examination of the kidneys from these animals with the anti-rabbit globulin conjugate, again showed specific fluorescence confined to the glomerular basement membrane. However, the degree of fluorescence was not nearly so brilliant as that observed

with the injected antiglomerular serum. These results probably indicate that the latter was a higher titer antiserum than the former. Again, no specific fluorescence was observed in the granulation tissue or in the other tissues examined.

Discussion

The observations reported above directly confirmed the original work of Cruickshank and Hill [1,2] who showed that various naturally occurring connective tissue fibrils apparently shared a common antigen (or antigens) that reacted with both anti-glomerular and anti-lung fluorescein conjugates. These various fibrillar elements, with the exception of the renal glomerular basement membranes, are argyrophilic and hence have been classified as reticulin.

The present investigation has shown that the anti-glomerular and anti-lung conjugates that had reacted with the reticulin in a wide variety of organs, failed to react with the early argyrophilic fibers in areas of fibrillogenesis. This would indicate that the latter fibers were antigenically dissimilar to the other naturally occurring reticulins or, less likely, the concentration of antigen in them was too low to be observed by this method. This observation agrees with the concept of Robb-Smith [8] who has recently summarized the work from his and other laboratories.[9,10] This has done much to clarify the confusion that envelops the term "reticulin." It is his contention that while basement membrane reticulin and the immature argyrophilic collagen fibers (usually referred to as reticulin) share certain common features, there are also distinct differences between them. Thus, the former is an extremely stable glycolipoprotein which is not a precursor of mature collagen, whereas the latter are readily soluble in sodium chloride and citrate buffer and during fibrillogenesis appear to be incorporated into and replaced by the collagen fibers. The observations reported in the present investigation would indicate that they differ antigenically as well.

Capillaries reacted specifically with both the anti-glomerular and anti-lung conjugates in all tissues examined, including the early zones of granulation tissue. The apparent thickness of the fluorescing immature and cellular capillaries in the latter, and the presence of nuclear shadows in the zones of fluorescence suggest that the reacting antibody was united to an antigenic substance related to the endothelial cytoplasm; the reaction did not appear to be limited to the capillary basement membrane *per se*. The investigation, as performed, does not allow one to specify whether this capillary antigen is identical to that in the basement membrane reticulins, or whether it was visualized by union with

some other antibody present in the conjugate that had been prepared against the relatively crude antigenic tissue extracts.

In this connection it should be noted that Pressman and colleagues[11,12] have reported that antibodies produced against kidney, lung, and liver are probably directed against antigens contained in their vascular beds and, to some extent, these antibodies are organ specific. Certainly the antibody conjugates in the present investigation were shown to react with capillaries wherever they existed.

When the glomerular and lung antiserums were injected intravenously, the only site of union with antigen that could be demonstrated was in the glomerular basement membranes. No union was observed in the other tissue sites where an *in vitro* reaction was readily demonstrated. The dissimilarity of the observations made *in vivo* and in particular the avidity of the glomeruli for injected anti-organ serums are vexing problems that remain to be solved. A discussion of these is not within the scope of this paper, but some aspects have been covered recently by Krakower and Greenspon,[13] Seegal,[14] and Hiramoto, Jurandowski, Bernecky and Pressman.[15]

Summary

Fluorescein antibody conjugates, prepared against rat glomeruli and lungs, reacted *in vitro* with a wide variety of naturally occurring reticulins but failed to react with the young argyrophilic fibers (reticulin) in areas of granulation tissue. This would indicate a lack of antigenic relationship between these groups of argyrophilic fibers which also differ in some respects chemically. The same conjugates reacted specifically with capillaries in many organs and with newly formed capillaries in the areas of granulation tissue. The reacting antigen seemed to be associated with the cytoplasm of the young capillary endothelial cells rather than with the basement membranes *per se*. When the same unconjugated antiserums were injected intravenously, the only demonstrable site of localization was in the glomerular basement membranes.

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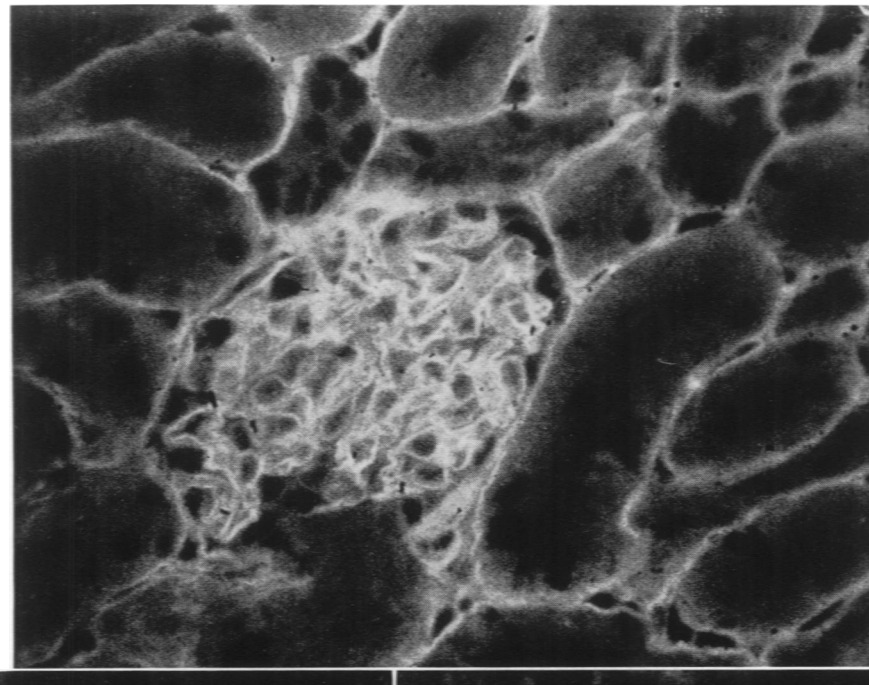
LEGENDS FOR FIGURES

FIG. 1. Fluorescent microphotograph of a rat kidney stained *in vitro* with rat anti-lung fluorescein conjugate. Glomerular and tubular basement membranes as well as intertubular capillaries show specific fluorescence. Identical results were obtained with rat anti-glomerular conjugates. × 500.

FIG. 2. Rat pancreas similarly stained. The alveolar basement membranes show specific fluorescence as do the capillaries which are shown well in the islet of Langerhans, upper left. × 500.

FIG. 3. Kidney in a rat given rat-glomerular antiserum intravenously. The antiserum was prepared in a rabbit, and the kidney was stained with fluorescent anti-rabbit globulin. The *in vivo* glomerular antiserum has only united with the glomerular basement membrane. Compare with Figure 1. × 750.



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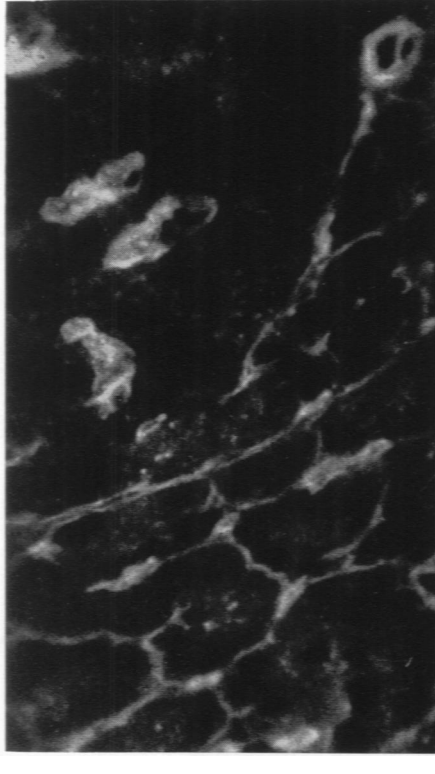

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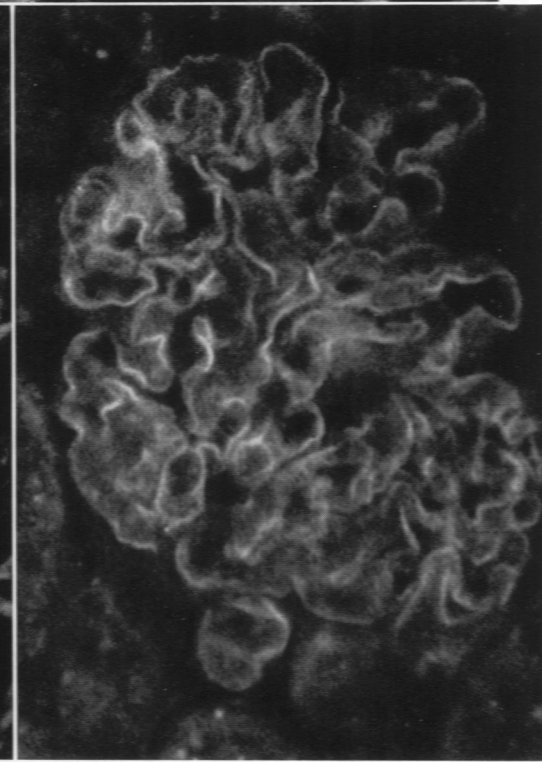

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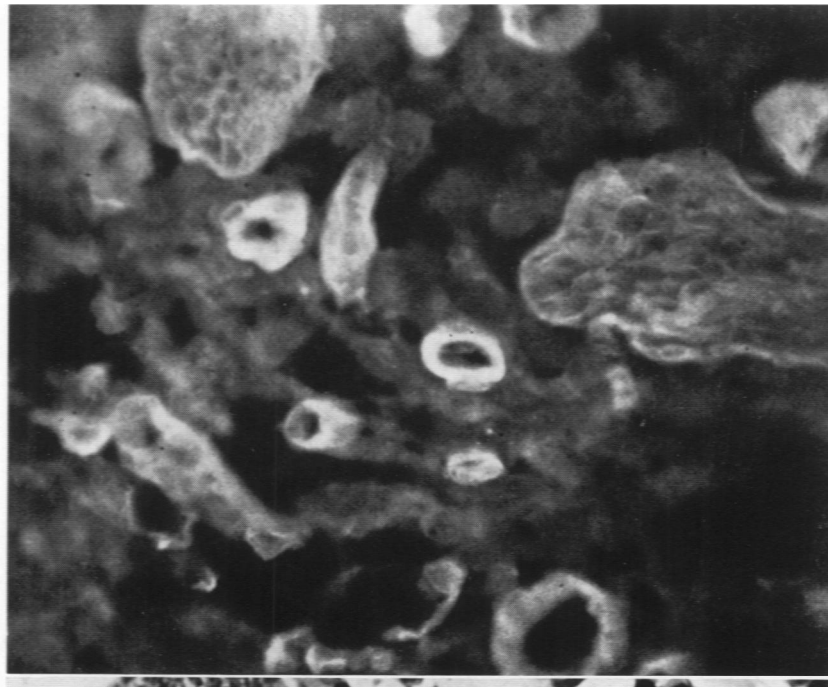

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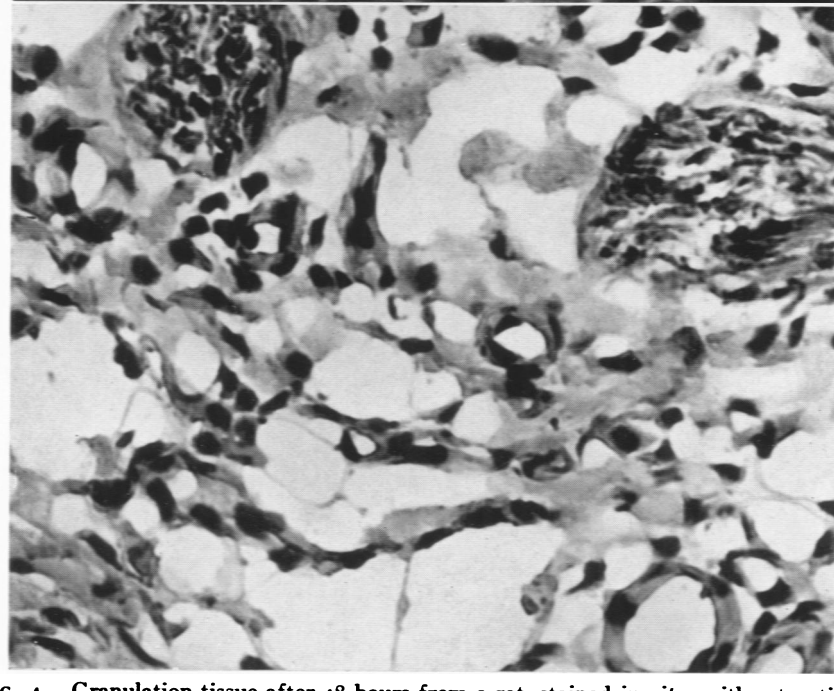

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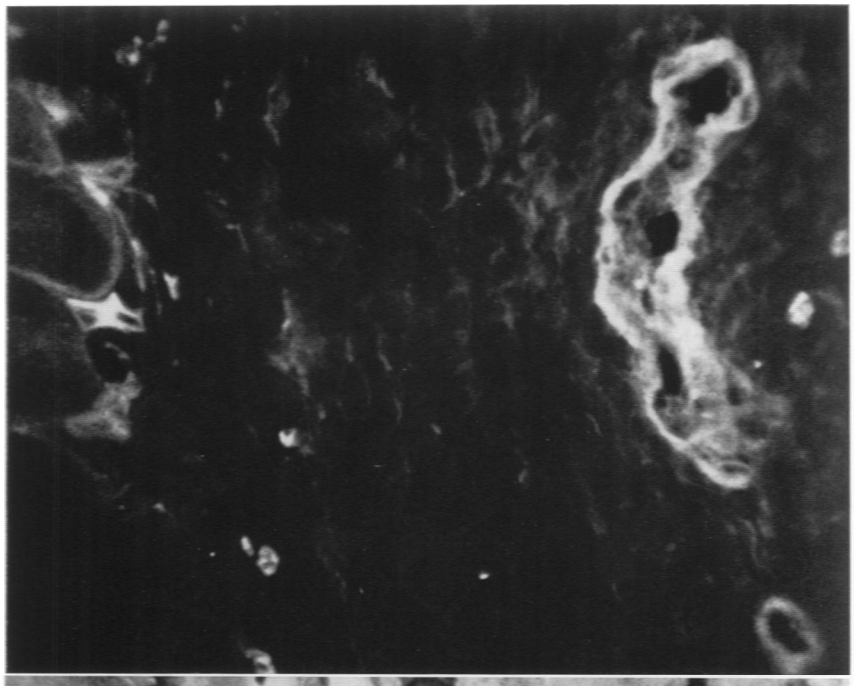
FIG. 4. Granulation tissue after 48 hours from a rat, stained *in vitro* with rat antiglomerular fluorescein conjugate. The two larger structures, in the upper left and right, are nerves in which the neurilemma shows specific fluorescence. Multiple newly formed capillaries also show a specific reaction. × 500.

FIG. 5. The same section as Figure 4 having been washed in buffer and restained with hematoxylin and eosin. By comparing this illustration with Figure 4, one can identify the immature cellular capillaries that showed the specific fluorescence. The nerves are also shown. × 500.



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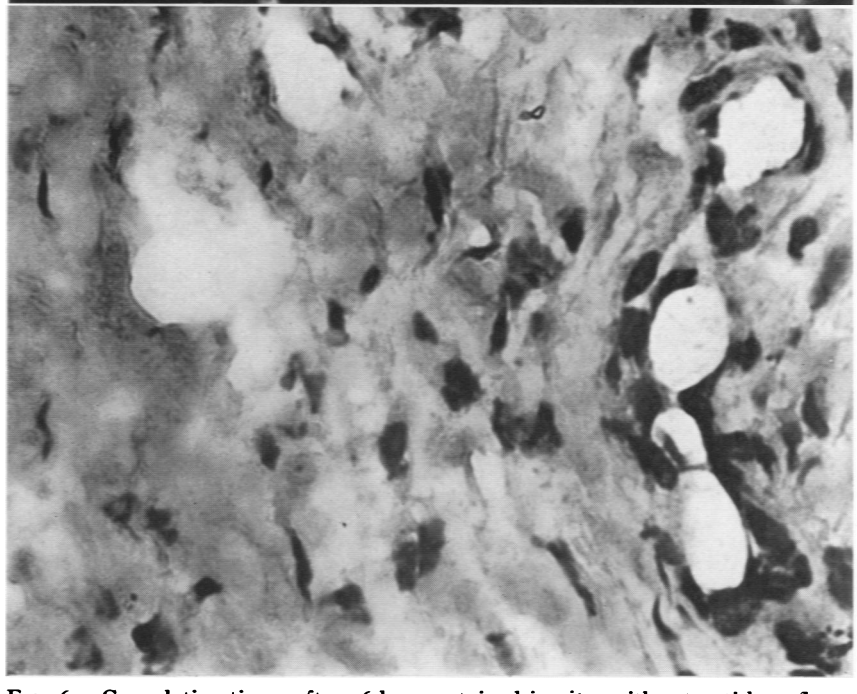

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FIG. 6. Granulation tissue after 96 hours, stained *in vitro* with rat anti-lung fluorescein conjugate. The capillary on the right shows specific fluorescence as does the sarcolemma of skeletal muscle just seen on the far left. The fibrils barely visible in the background show nonspecific autofluorescence. × 500.

FIG. 7. The same section as in Figure 6 restained with hematoxylin and eosin. Comparison enables one to identify nuclear shadows in the fluorescent capillary. These shadows are seen best on the upper half of the fluorescent microphotograph, and their presence suggests that the reacting antigen, which has combined with the fluorescent antiserum, is present in the endothelial cytoplasm. × 750.

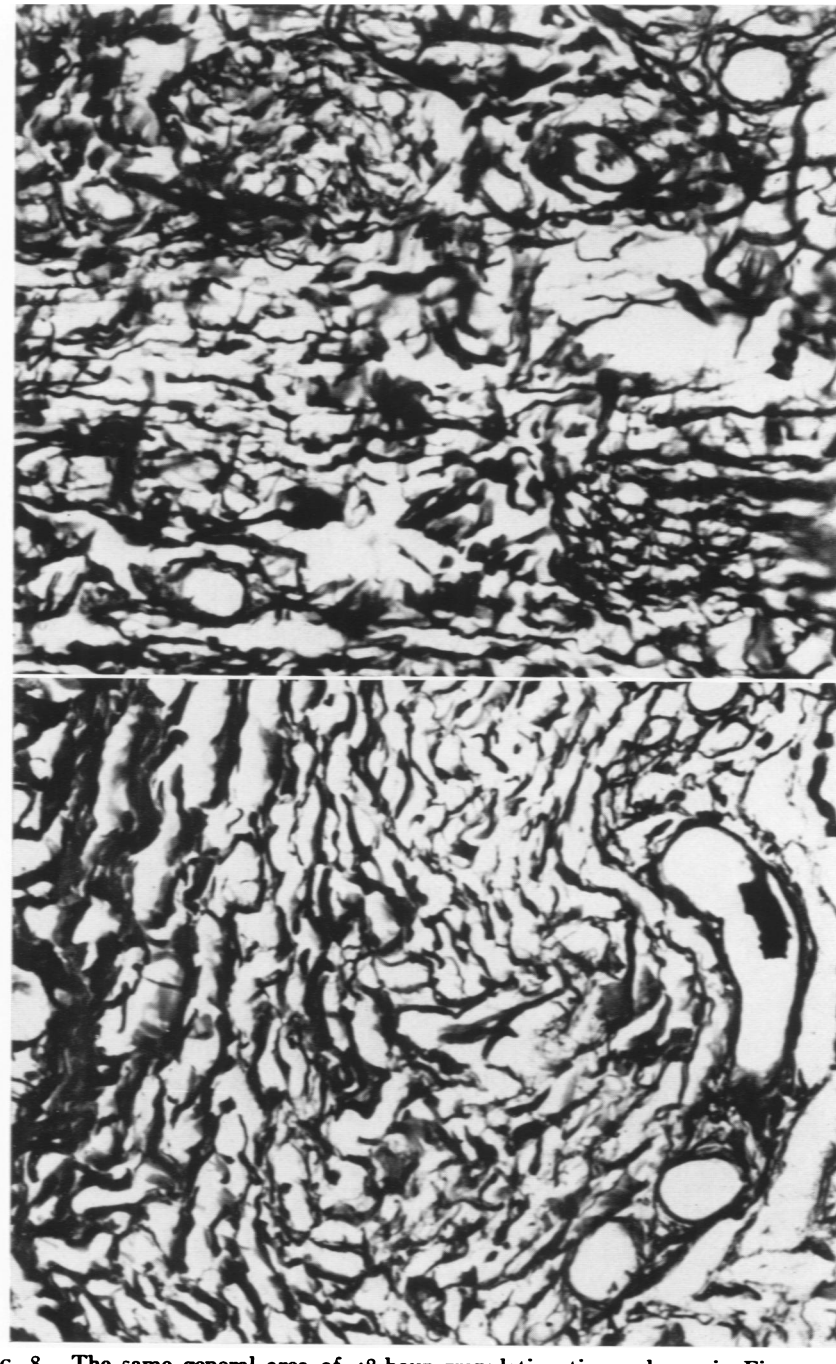

FIG. 8. The same general area of 48-hour granulation tissue shown in Figures 4 and 5. Note the large numbers of argyrophilic "reticulin" fibers which had not reacted with the fluorescent antiserums. Gomori's reticulin stain. × 650.

FIG. 9. Granulation tissue after 96 hours, again showing numerous argyrophilic fibers. Same area as that shown in Figures 6 and 7. Gomori's reticulin stain. × 650.