LIGHT-MICROSCOPIC AND IMMUNOPATHOLOGIC OBSERVATIONS ON CADMIUM CHLORIDE-INDUCED INJURY IN MATURE RAT TESTIS

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It is now well established that a single subcutaneous injection of cadmium chloride produces damage in the rat and mouse testis and proximal portion of the head of the epididymis (PHE).[1-9] This progresses to a state of irreversible atrophy and fibrosis of the organ in the course of a few weeks. It is also known that such damage in time evokes a castration effect.[1] Although the changes start in the interstitial tissue, they ultimately involve the tubules, so that spermatogenesis ceases and sterility results. The early changes are characterized by capillary congestion, thrombosis, and interstitial edema followed by necrosis and fibrosis.[1-9] The exact mechanism by which cadmium chloride produces these changes is not clear. It has been suggested that primary damage takes place in the endothelium of interstitial capillaries in testis,[1] the internal spermatic artery, the pampiniform plexus, and their branches.[4] The changes that follow are secondary to this effect.[1-9] Recent observations [10] indicate that an increased capillary permeability and alterations of blood flow also contribute to the damage.

Some studies suggest that the action of cadmium is directed toward spermiogenic epithelium and involves the inhibition of certain enzymes. This view is supported by the fact that a simultaneous administration of zinc,[1,3,4] selenium,[11,12] or thiol compounds [13] protects the testis from the damaging effects of cadmium. Such evidence supports the enzyme-inhibition concept, but the conclusion that spermiogenic epithelium is the primary target is open to question since it has been shown by autoradiography that the concentration of ^{109}Cd is lower in seminiferous tubules than in the interstitium of the testis.[14] This indicates that the relation between concentration and damage is unclear. Furthermore, in the same study [14] it was shown that such organs as kidney and liver accumulate much higher concentrations of cadmium than the testes though no damaging effects are seen on gross and microscopic examina-

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tion. Gunn, Gould, and Anderson[4] have suggested that organ-capillary specificity based on biochemical properties of vessels in such tissues as testes and PHE may account for the damaging effects by cadmium chloride. This may explain the escape of other organs, lacking such specificity, from the deleterious effect of cadmium.

The following study was based on the premise that the primary site of action of cadmium chloride damage was related to an increase of permeability in the vessels of the testis and PHE. The permeability changes were studied by 2 different methods. The first was aimed at the localization of carbon black particles in the cadmium-damaged vessels of the testes and PHE. The second was to determine the distribution and localization of endogenous serum albumin and gamma globulin in cadmium-damaged vessels of the testes, using fluorescein-labeled antibodies to rat serum albumin (RtSA) and the rat serum gamma globulin (RtGG).

Materials and Methods

Experiment 1

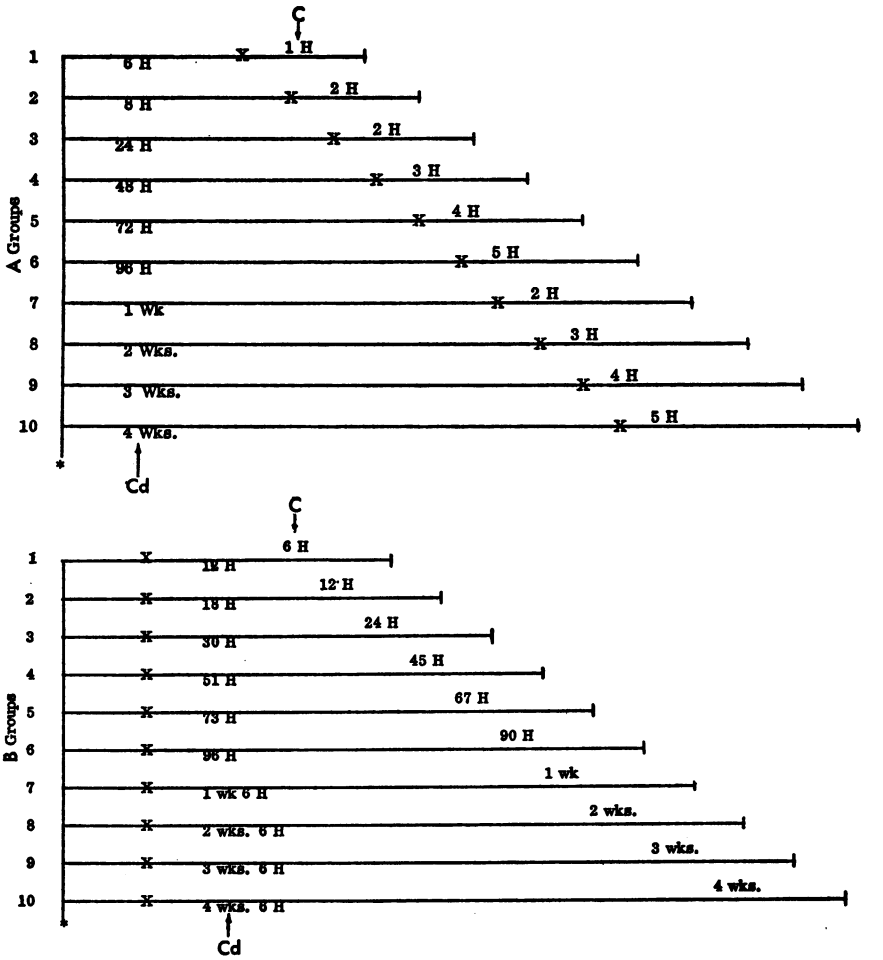
Forty mature male rats of the Holtzman strain, weighing 250–300 gm. were divided into 20 groups, each consisting of 1 experimental and 1 control animal. The rats were kept in individual cages and were fed routine diet (rat chow) and tap water ad libitum. The experimental animals were given a single subcutaneous dose of 0.02 mM/kg. body weight of cadmium chloride in normal saline into the right flank. The controls were given injections of normal saline.

Ten groups (1-10 A) of rats were left undisturbed for periods ranging from 6 hr. to 4 weeks following administration of cadmium chloride. Prior to sacrifice a suspension of carbon particles was injected into the jugular vein in a single dose (0.1 ml./100 gm. body weight) and allowed to diffuse for intervals of 1–5 hr. (Text-fig. 1). The carbon was especially prepared for experimental use and supplied by Guenther-Wagner-Pelikan-Werke, Hanover, Germany (Batch C11/1431 (a)). This preparation contains 100 mg./ml. of carbon with an average particle size of 200 Å, is stabilized with 4.5% fish glue, and contains 1.3% phenol as a preservative. It was filtered through Whatman No. 1 filter paper before use. The animals were killed by decapitation. The testes and epididymides were cut in half through the midsagittal plane, trimmed, and fixed, along with sections from the accessory sex organs, kidneys, heart, lungs, adrenals, and intestines, in acetic-formol alcohol (Lillie's fixative) for 72 hr. Sections were cut at 6 μ and stained with hematoxylin and eosin and/or metanil yellow (counterstain only).

In the remaining 10 groups (1–10 B) a single subcutaneous dose of cadmium chloride (0.02 mM/kg. body weight) was injected into the flank of the experimental rats, and saline was similarly injected into the control animals. After 6 hr. carbon black suspension (prepared as described above) was injected into all animals. Thereafter, all the animals in the above 10 groups were left undisturbed for a total period ranging from 12 hr. to 4 weeks 6 hr., and then sacrificed in groups as shown in Text-fig. 1. The tissues were collected and examined as in the first set of animals.

Experiment 2

Cadmium chloride damage was initiated in 10 groups of mature rats with 2 experimental rats in each group. One control rat in each group was injected with normal



TEXT-FIG. 1. Periods of cadmium (Cd) action and carbon (C) diffusion in Groups 1–10 A (top) following cadmium (*) and carbon (X) injection at varying intervals before sacrifice (cut-off points of lines). In Groups 1–10 B (bottom) the carbon injection was made 6 hr. after cadmium injection in all instances.

saline. A total of 30 mature rats was studied at 6, 8, 24, 48, and 72 hr. and at 1-, 2-, 3-, 4-, and 5-week intervals after injection of cadmium chloride by immunofluorescent protein tracing techniques.

Rat serum albumin (RtSA) was prepared from pooled rat serum by electrophoretic separation on a Spinco Model C/P apparatus. The resultant RtSA fractions from several electrophoretic runs were pooled and purified by precipitating the remaining traces of alpha and beta globulins with 2.5 M ammonium sulphate. Antiserum to RtSA was prepared by immunizing rabbits with the antigen incorporated in Freund incomplete adjuvant. Subcutaneous injections were given (15 mg. of protein per animal per week) for 3 weeks, when a booster dose of the antigen was given. Thereafter, weekly bleedings were done and monthly injections given through 2 or 3 additional cycles. These antiserums showed a single precipitation line when tested by 2-dimensional agar diffusion and agar immunoelectrophoresis against rat serum.

Rabbit antiserum to rat serum globulin (RtGG) was prepared by giving injections

in a manner similar to that detailed above for anti-RtSA. The serum globulin was precipitated several times with 1.0 M sodium sulphate. The antiserums were tested for precipitins by tube (antigen) dilution and tested for serum specificity with agar immunoelectrophoresis. The serums with separated RtSA from pooled rat serum showed a moderately heavy arc in the slow beta globulin zone and 1 heavy and 1 light arc in the gamma globulin zone. Commercial rabbit antiserums to RtGG also were used. These antiserums usually gave only 1 heavy arc and an occasional light arc in the gamma globulin zone when tested as above. The staining results with conjugates of both types of antiglobulin reagents were similar.

The fluorescent staining procedure was carried out in accordance with the methods of Coons and Kaplan.[15] In this study only the testes from experimental and control animals were examined. The testicular tissue was quick-frozen immediately after collection from control and experimental animals and then transferred on dry ice. Frozen sections were cut in a cryostat, mounted on clean glass slides, and air-dried for 1 hr. Sections were then rinsed for 5–10 min. in several changes of phosphate-buffered saline pH 7.2 (PBS) and dried and fixed in 95% ethanol. Fluorescein conjugates of anti-gamma globulin and antialbumin were applied directly to tissue sections for 1–2 hr. The sections were then rinsed for 10 min. in PBS, briefly dried, and cover-slipped with buffered glycerol. Examination was done by a Zeiss fluorescent microscope equipped with an Osram HBO-200 lamp and a 35-mm. camera. Controls included tissue sections treated in 3 ways: (1) reacted with fluorescein-labeled antiserum from which gamma globulin had been precipitated by 2 M ammonium sulfate; (2) reacted with conjugated antiserum of unrelated specificity (anti-human gamma globulin); and, (3) exposed to antiserums that had been absorbed with RtGG or albumin.

Results

Observations on Cadmium-Injured Vessels Using Carbon Black Particles

Following injection of carbon black into the jugular vein, the skin and visible mucous membranes of both experimental and control rats developed a grayish hue. On gross examination, the testis and PHE of the control animals were found to be a normal light-pink color. In experimental animals sacrificed at 6, 8, and 24 hr. after the injection of cadmium, these organs were red, congested, and edematous and somewhat increased in size and weight as compared with the controls. At 72 and 96 hr. they appeared a dull-yellow color and were decreased in size. From 1 to 4 weeks the muddy appearance was more pronounced and they were shrunken and firm in consistency.

Microscopic examination of the hematoxylin and eosin-stained sections of the testes and PHE of the control animals showed normal, well-formed interstitial and tubular elements, and no carbon labeling or leakage was seen at any stage (Fig. 1). At 6 and 8 hr. after the injection of cadmium, the experimental animals showed changes limited to the interstitium, characterized by edema, few neutrophils, and marked congestion and dilatation of capillaries and venules. A few foci of red blood cells were seen in the interstitium. In these groups, after a 1- or 2-hr. interval for carbon diffusion had been allowed, focal plugging of the lumens of capillaries and venules and localization of carbon particles at the endo-

thelial lining were present (Fig. 2). No leakage of carbon was seen in the interstitium or seminiferous tubules. At 24, 48, 72, and 96 hr. the interstitial damage had progressed and was characterized by pronounced hemorrhage, marked dilatation, congestion, and thrombosis of vessels of all sizes. The carbon, when allowed to diffuse for 2–5 hr., localized freely in the interstitium and throughout the vessel walls (Fig. 3 and 4). This was confirmed by counterstaining the sections with metanil yellow, which clearly showed the carbon particles against a yellow background of other elements. In the sections from animals sacrificed at 48 and 96 hr., some carbon was present in an occasional tubule (Fig. 4).

From 1 to 4 weeks after injection of cadmium there was increased tubular damage characterized by necrosis. Despite the fact that carbon diffusion was allowed to continue up to 5 hr., no labeling or leakage of carbon was detected; instead, only hazy outlines of smudged and necrotic large-caliber vessels were seen (Fig. 5). At 3 and 4 weeks the interstitium was replaced by compact and vascularized fibrous tissue, but no carbon labeling or leakage was noted. None of the other organs showed evidence of carbon leakage or localization at any stage in either the experimental or control animals. Beginning at 1 week after cadmium administration, grossly atrophic changes were seen in the prostate and seminal vesicles of experimental animals, and by 4 weeks these organs decreased to half the size of their counterparts in the controls.

In the other 10 groups the gross changes in the experimental animals were similar to those described above, except that from 24 hr. to 4 weeks plus 6 hr. after cadmium injection the testes and PHE appeared to have a blackish color. These tissues in control rats were normal in appearance. In experimental animals the histologic sections showed an accumulation of carbon in lumens and walls of capillaries and medium-sized blood vessels up to 24 hr. after its injection. Thereafter, up to 1 week the carbon was seen as focal aggregates in the interstitium (Fig. 6). At 1 and 2 weeks some carbon had been phagocytosed by macrophages, while at 3 and 4 weeks the aggregates of carbon, though present, were being removed by markedly increased numbers of macrophages (Fig. 7). A few damaged blood vessels still contained moderate amounts of carbon, but little was seen in the seminiferous tubules of any experimental animal examined after 1 week.

Localization of Albumin and Gamma Globulin in Cadmium-Injured Testes by Immunofluorescence

In the testes of control animals no RtSA or RtGG was seen in the vessel walls, interstitium, or tubules at any stage throughout the experiment (Fig. 8 and 9).

At 6 hr. after the injection of cadmium the capillaries and venules in the interstitium of the experimental animals exhibited fluorescence to anti-RtSA and anti-RtGG (Fig. 10). Fluorescent antibodies rarely could be localized in a seminiferous tubule. At 8 hr. localization of anti-RtSA and anti-RtGG was seen in vessels of larger caliber (Fig. 11). At this time, although some albumin was detected focally in the interstitium, gamma globulin was seen in significant amounts (Fig. 12). However, most of the tubules were negative. At 24, 48, and 72 hr. both albumin and gamma globulin had diffused broadly into the walls of vessels of all sizes, and fluorescence was intense (Fig. 13). At 24 and 48 hr. both markers, particularly the gamma globulin, were also observed in the interstitium, with a concentration peak at 48 hr. (Fig. 14). At 72 hr., although the vessels and interstitium remained stained for both proteins, RtGG appeared diffusely in the tubular elements (Fig. 15).

At 1 week, interstitial fluorescence decreased significantly but tubular fluorescence was increased for both markers (Fig. 16 and 17). Some vessels in the interstitium were fluorescent to a moderate degree. At 2 weeks the tubules showed even greater intensity of fluorescent staining for albumin and gamma globulin, while portions of the interstitium showed staining similar to that seen in the 1-week group.

At 3 and 4 weeks fluorescence to either conjugate persisted in the tubules, whereas the interstitium was negative. At this time the newly formed vascularized fibrous tissue, seen histologically in the interstitium, was also found to be unstained for specific fluorescence (Fig. 18) to either protein.

At 5 weeks, the necrotic tubular elements exhibited focally decreased fluorescence, the other elements being the same as at 3 and 4 weeks.

Discussion

The foregoing results indicate that cadmium-induced testicular damage first affects the vascular system of this organ. It thus confirms and extends past studies [1,4,5,10,16] that have supported this concept. In agreement with recent evidence,[10] vascular and extravascular localization of carbon particles and of both the endogenous proteins used in the present study indicates that increased vascular permeability does take place in this experimental model. The damage seems to start simultaneously in the capillaries and venules and progresses to involve the larger-caliber vessels. The leakage of both endogenous proteins and carbon particles from intravascular to extravascular compartments is probably affected by alterations in filtration pressure and increased porosity of the endothelium. This view also seems to be supported by the histological findings of edema, capillary dilatation, stasis, and hemorrhage in the early stages.

Recently, Mancini *et al.*[17] have shown significant vascular and extravascular localization of fluorescent material in the testes of normal rats of various age groups following intravenous injection of fluorescein-labeled plasma fractions or whole rat serum. Our staining results with control animals seem to be at variance with their conclusions that the testicular vasculature is highly permeable to plasma proteins. In our studies, although the animals treated with cadmium showed vascular and extravascular localization of rat serum albumin and gamma globulin, the controls showed no significant localization of either proteins in these tissues. This variation may be due to the different techniques employed, in that the previous workers[17] injected exogenous labeled plasma proteins and whole rat serum, while in our studies the albumin and gamma globulin detected by fluorescent-antibody methods were endogenous.

At this stage, on the basis of our observations, we can only conclude that a primary damage in rat testis following cadmium chloride injection starts in the capillaries and venules and progressively involves the entire vasculature of the testis. This leads to ischemic necrosis, thus secondarily affecting the seminiferous tubules.

Summary

The site of action of cadmium chloride damage in the testes was studied in mature male rats. This was done by injecting carbon particles and by tracing endogenous serum albumin (RtSA) and gamma globulin (RtGG) with fluorescein-labeled antibodies.

In experimental animals after cadmium action when intravenous carbon black was administered and retained up to 1–5 hr., it was found that diffusion of this material through the walls of blood vessels in testes and PHE occurred only during the first week. When cadmium administration after 6 hr. was followed by a single intravenous injection of carbon, this material diffused into the interstitium of testes and PHE where it aggregated and was progressively phagocytosed by macrophages in the next 4 weeks of the experiment.

Vascular damage following the injection of cadmium was further substantiated utilizing fluorescent protein tracing techniques in testes. Serum albumin and gamma globulin appeared in the walls of capillaries and venules as early as 6–8 hr. after cadmium chloride injection, while after 24 hr. vessels of all calibers showed localization of both proteins. Gamma globulin was detected in the interstitium at 8 hr., while both proteins were found in this location after 24 hr. With progressive necrosis of the tubules, both tracers localized in the seminiferous tubules. As interstitial fibrosis progressed for up to 5 weeks, localization of serum albumin and gamma globulin was found to decrease in the interstitium, but the

tubules retained significant amounts of these proteins. Testes from control animals failed to show carbon, serum albumin, or gamma globulin in the vessels, interstitium, or tubules throughout the experiment.

These observations indicate that cadmium chloride damage involves the entire vasculature of the testis and is characterized by increased permeability to injected carbon particles and to endogenous plasma proteins. Furthermore, whereas carbon accumulated extensively in the interstitium, serum albumin and gamma globulin localized and persisted in the seminiferous tubules.

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[*Illustrations follow*]

Legends for Figures

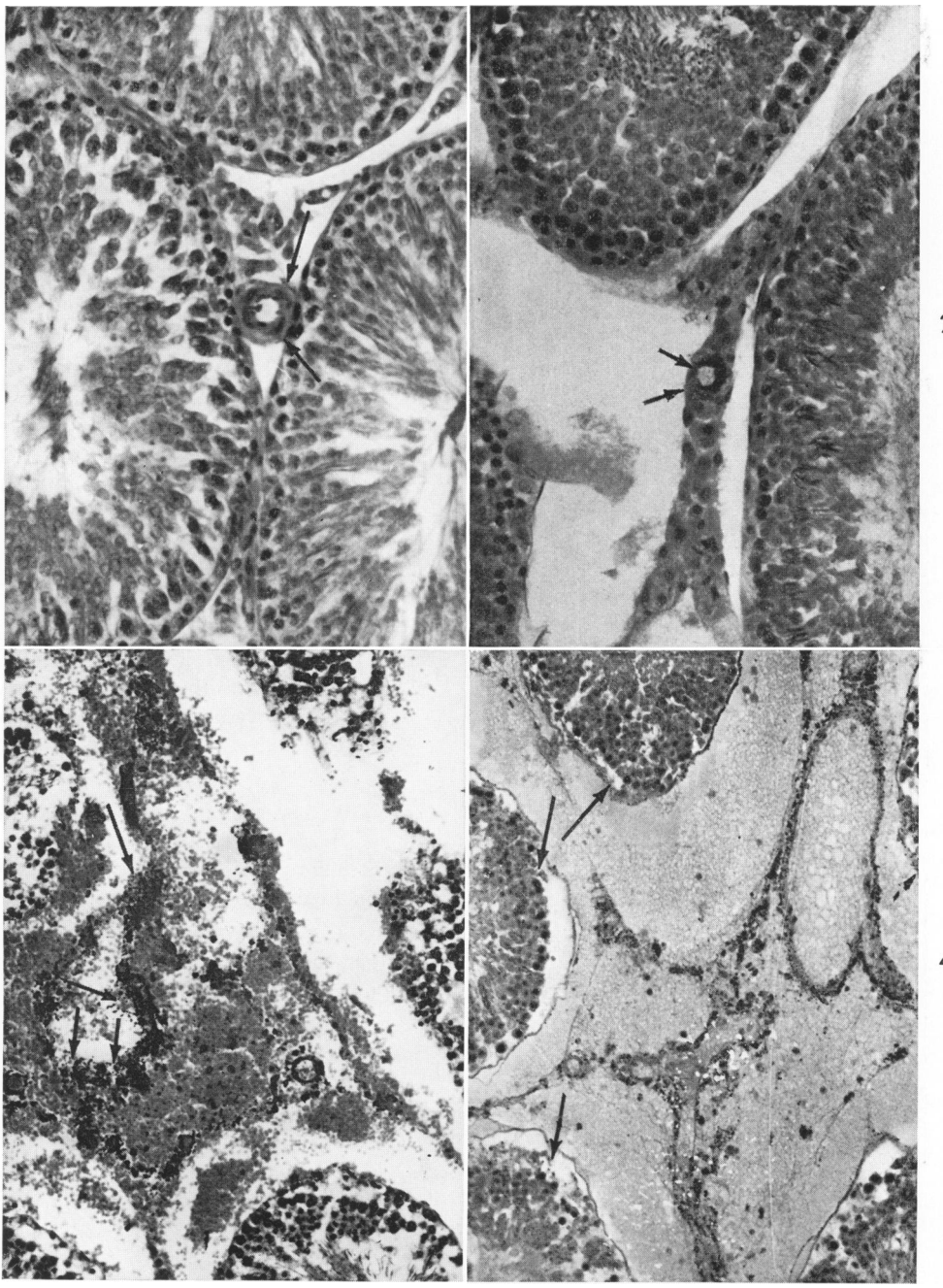
All photomicrographs show sections of rat testis. Sections illustrated in Fig. 1–7 were stained with hematoxylin and eosin; the remainder are as indicated.

Fig. 1. At 6 hr. after injection of saline and 1 hr. after injection of carbon, tubular and interstitial elements are normal. No localization or labeling of carbon appears in vessel (arrow) or any other element. × 240.

Fig. 2. At 6 hr. after injection of cadmium and 1 hr. after injection of carbon, capillary (arrow) shows circumferential localization of carbon particles. No carbon is seen in other elements. × 240.

Fig. 3. At 24 hr. after injection of cadmium and 2 hr. after injection of carbon, there are freely localized carbon particles in interstitium (arrows), apart from localization in walls of some vessels. × 220.

Fig. 4. At 96 hr. after injection of cadmium and 5 hr. after injection of carbon, there are localization of carbon in vessels and interstitial elements (center) and focal carbon particles in tubules (arrows). × 120.



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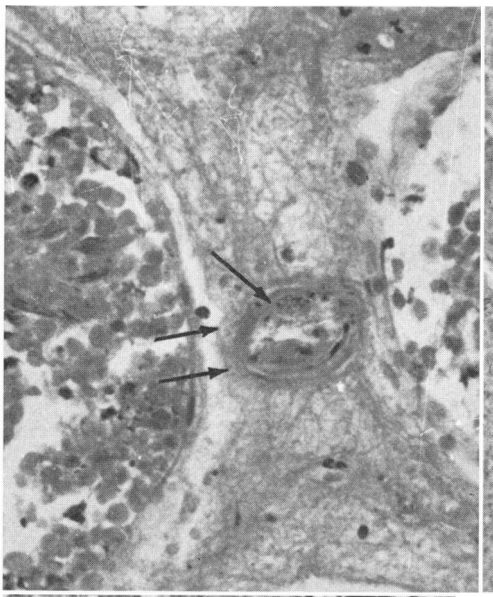

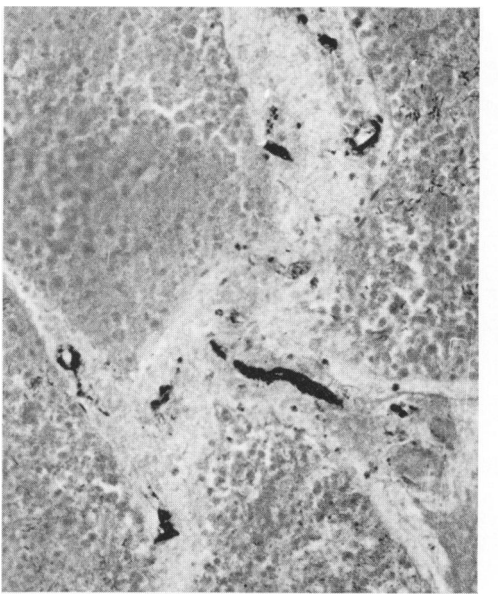

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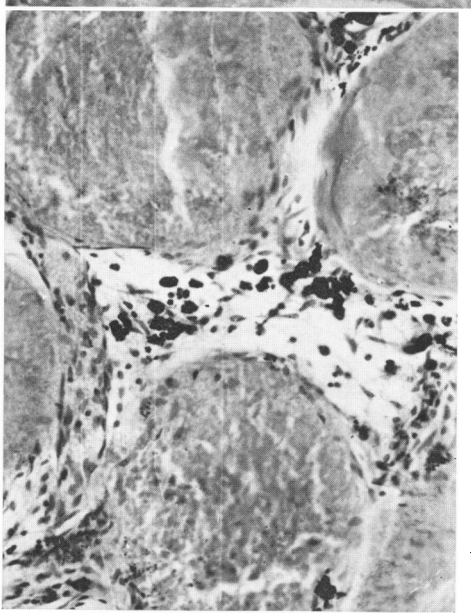


FIG. 5. At 2 weeks after injection of cadmium and 3 hr. after injection of carbon, blood vessel (arrows) appears smudged and necrotic but carbon-free. × 240.

FIG. 6. At 6 hr. after injection of cadmium and 1 week after injection of carbon, there are prominent aggregates of carbon in damaged interstitium. × 200.

FIG. 7. At 6 hr. after injection of cadmium and 4 weeks after injection of carbon, there is increase in number of macrophages with phagocytosed carbon. Necrotic tubules do not contain carbon. × 150.

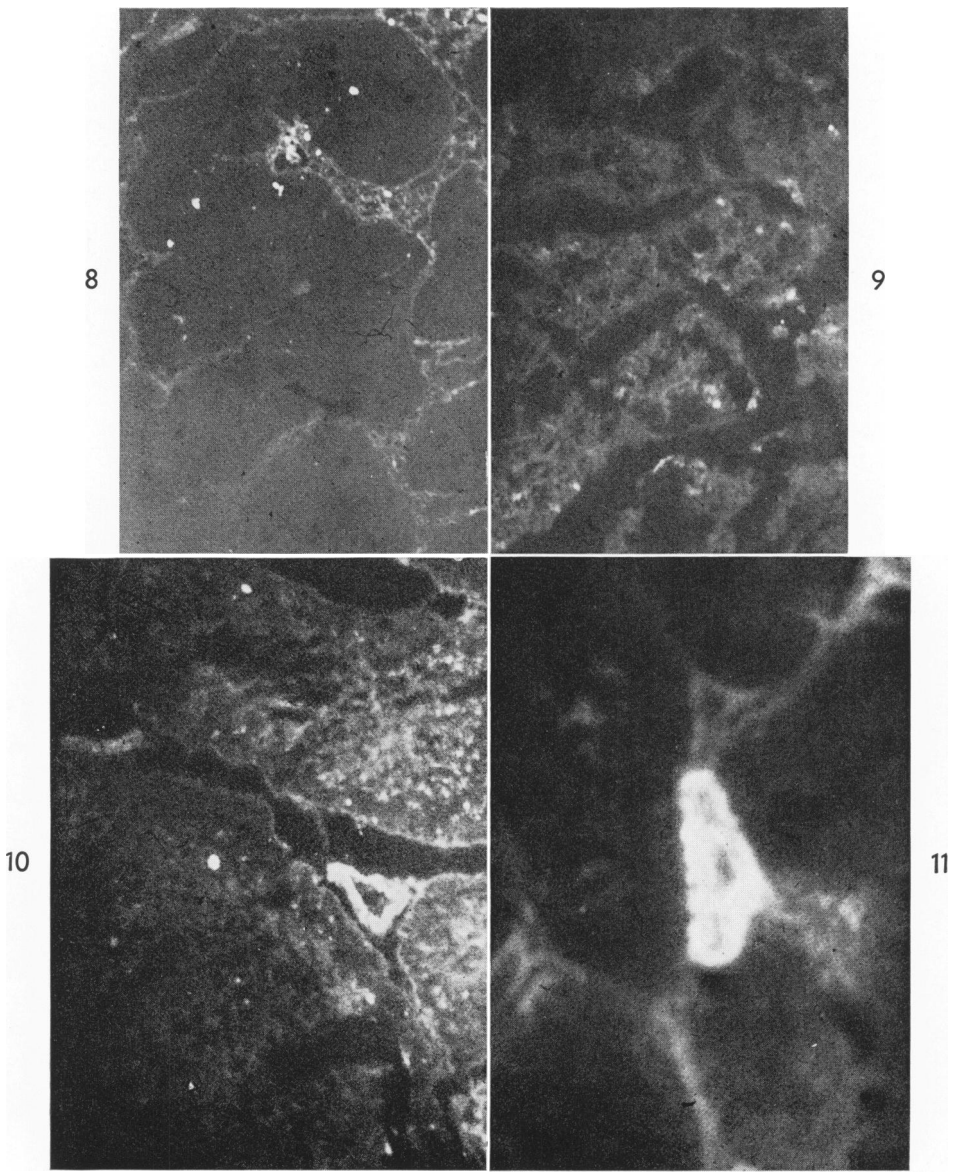


FIG. 8. Control rat 8 hr. after injection of saline. There is faint or negative staining of fluorescent anti-RtSA. × 100.

FIG. 9. Section from same rat as in Fig. 8, stained with fluorescent anti-RtGG, shows weak reactivity to the conjugate in some tubules. × 100.

FIG. 10. At 6 hr. after injection of cadmium there is focal localization of RtSA in a few tubules. At this interval capillary shows distinct localization of protein. × 125.

FIG. 11. At 8 hr. after injection of cadmium there is marked localization of RtSA in vessel wall. Tubules are unstained, while interstitium shows a little RtSA focally. × 125.

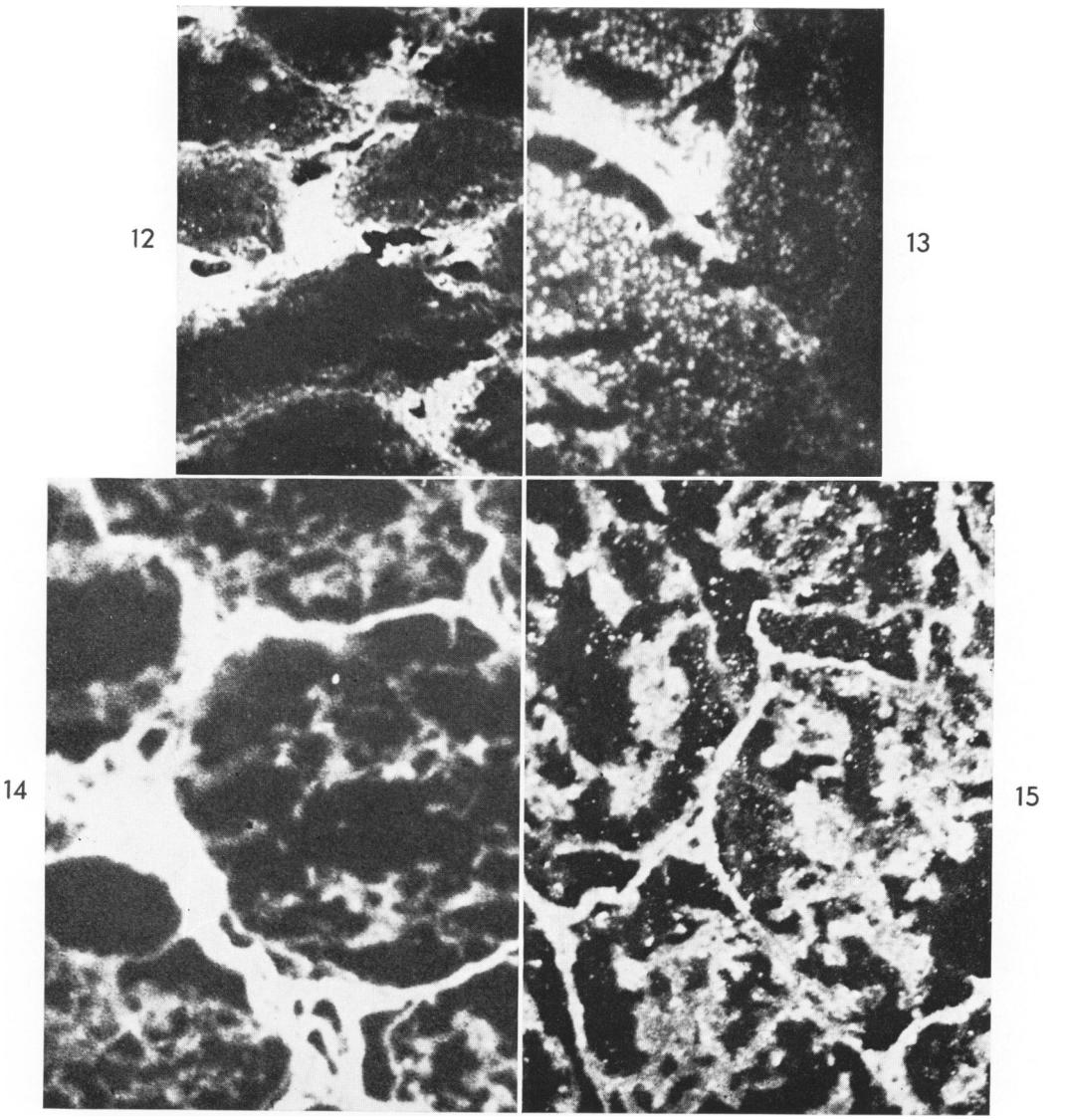
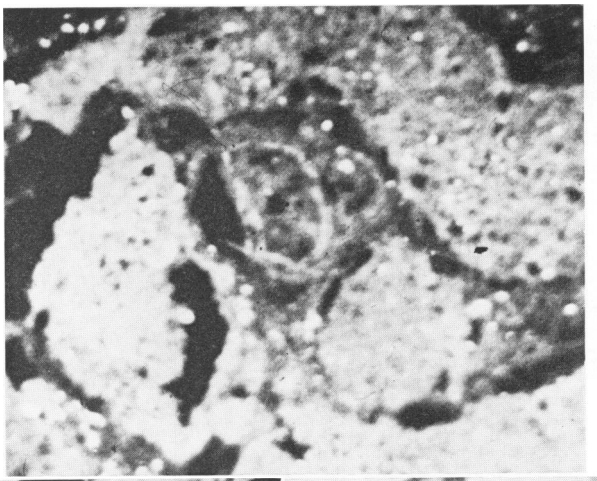


FIG. 12. Section from same rat as in Fig. 11 shows specific localization of RtGG in interstitial elements. Tubules are unstained. × 125.

FIG. 13. At 24 hr. after injection of cadmium there is localization of RtGG in blood vessel. Some fluorescence is also seen in tubules. × 125.

FIG. 14. At 48 hr. after injection of cadmium there is localization of RtGG in interstitium and blood vessels. Moderate amount of fluorescence is seen in tubules. × 150.

FIG. 15. At 72 hr. after injection of cadmium there is intense localization of RtGG in tubules. Interstitial elements are also heavily stained. × 150.



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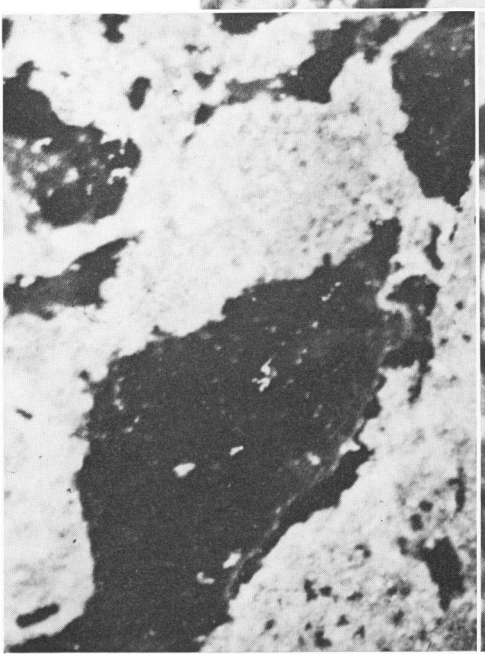

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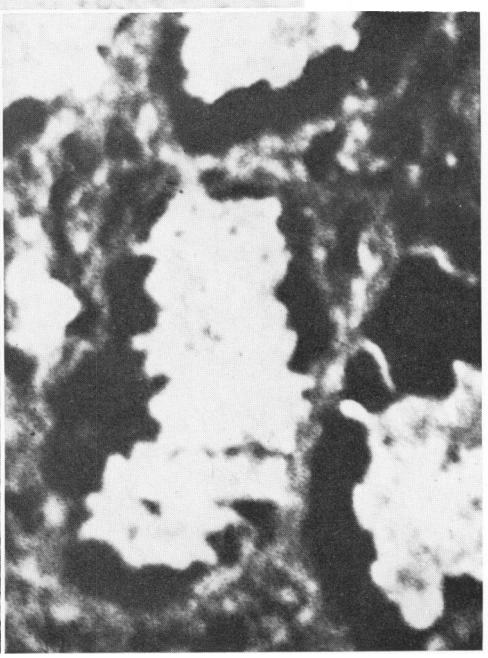

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FIG. 16. At 1 week after injection of cadmium, localization of RtSA in tubules is increased. Interstitium shows significant decrease of staining to conjugate. × 150.

FIG. 17. Section from same rat as in Fig. 16 shows similar distribution of anti-RtGG. × 150.

FIG. 18. At 4 weeks after injection of cadmium, when anti-RtGG was used, there was significant localization of conjugate in tubules. Newly formed vascularized fibrous tissue replacing interstitium is mostly unstained. × 150.