Induction of Intravascular Coagulation and Focal Tissue Necrosis in Rats by Administration of Anti-Connective Tissue Antibodies

David H. Katz, M.D.,* Emil R. Unanue, M.D.,† and Frank J. Dixon, M.D.

ADMINISTRATION of heterologous antibodies to connective tissue elements of kidney and other organs readily induces glomerulonephritis (for complete review, see Unanue and Dixon [1]). However, there have been few reports of pathologic lesions induced by heterologous antibodies in other organs despite the clear fixation of antibody molecules to them. The intravenous injection of anti-tissue antibodies has led, in only occasional instances, to pulmonary hemorrhages.[1] Recently, during the course of a study of nephritogenic properties of antiserums to connective tissue elements of rat kidney, spleen, heart, and skeletal muscle,[2] we have observed lesions principally in the form of focal hemorrhagic necrosis in a multi-organ distribution. These lesions developed acutely and resolved without permanent sequelae, which probably explains their not being reported in the past. The present article is a description of these lesions and an attempt to determine their probable pathogenesis.

These experiments demonstrate that: (1) all anti-connective tissue antiserums contain antibodies capable of fixing specifically to the vascular and connective tissue framework of all organs; (2) depending on the amount of antibody which fixes, acute, transitory lesions are induced mainly in the liver, intestines, and reticuloendothelial organs, characterized histopathologically by focal hemorrhages, with necrosis in many instances; (3) the mechanisms responsible for tissue damage following the specific fixation of antibody involve focal, rather than diffuse, in-

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From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif. (Publication No. 297).

Supported by Grant AI 07007 from the U. S. Public Health Service, Atomic Energy Commission Contract AT(04-3)-410, and USPHS Training Grant 5T1 GM683.

Accepted for publication Aug. 6, 1968.

Address for reprint requests: Department of Experimental Pathology, Scripps Clinic and Research Foundation, 476 Prospect St., La Jolla, Calif. 92037.

* Present address; Department of Medicine, The Johns Hopkins University, Baltimore, Md.

† Fellow of the Helen Hay Whitney Foundation.

travascular coagulation, and the lesions are prevented by the administration of anticoagulants to the animals.

Materials and Methods

Animals

All rats used in these experiments were Sprague-Dawley males weighing 140-180 gm. Rabbits used for immunization were 4- to 6-lb. male albinos.

Tissue Antigens

Methods used to prepare connective-tissue-rich antigens from rat kidneys, hearts, spleens, and abdominal muscles have been described previously.[2] Rat liver antigens were obtained by treating whole organ homogenates in a similar manner and then separating by centrifugation (600 *g*; 30 min.) the saline-washed sediment into two fractions: The heavy, coarse connective tissue comprising the bottom layer of the sediment was designated Fraction A (FxA); the lighter, more homogeneous material in the clearly demarcated upper layer of the sediment was designated Fraction B (FxB). No attempt was made to characterize these crude antigenic preparations.

Antiserums

Anti-rat kidney, heart, spleen, skeletal muscle, and liver FxA and B serums were obtained from rabbits immunized with the respective antigen in complete Freund's adjuvant as previously reported.[2] The antiserums were decomplemented by heating at 56° C. for 1 hr. and absorbed with rat erythrocytes until hemagglutinins were no longer detectable. The γG-globulins (GG) of the antiserums were obtained by precipitation at 50% saturated ammonium sulfate, followed by ion-exchange chromatography.[3] The eluate contained only GG when tested by micro-immunoelectrophoresis [4] using a goat anti-rabbit serum (Colorado Serum Company, Denver, Lot No. 1). The GG preparations were stored frozen until used; immediately prior to use in in-vivo experiments they were ultracentrifuged at 81,000 *g* for 1 hr. in order to remove aggregated protein. Protein concentration was determined by the Folin-Ciocalteu method.[5]

Pathologic Examination and Immunohistochemical Studies

Tissue specimens obtained at necropsy were studied for gross and histologic abnormalities. Routine histologic stains were employed for microscopic examination of tissue sections.

Fluorescent antibody studies were carried out on sections of rat tissue by conventional procedures,[6] using the following fluorescent antiserums: rabbit anti-rat GG, rat β_{1C}-globulin, rat fibrinogen, rat serum albumin, and guinea pig β_{1C}-globulin plus sheep anti-rabbit GG. The antiserums were prepared and characterized as previously described.[2] In-vitro tissue complement (C′) fixation was determined by exposing tissue sections to fresh guinea pig serum (diluted 1:30) and then staining them with fluorescent anti-guinea pig B_{1C}-globulin.[7]

Hematologic determinations were performed on blood specimens using standard techniques.

Tissue Fixation of the Antibodies (Experiment I)

Anatomic localization of each heterologous antibody in normal rat organs was determined by using the indirect (sandwich) fluorescent antibody technique.[6]

Tissue sections were flooded with 1.0 mg. of each anti-tissue GG for 45 min., washed several times with 0.15 M NaCl, and then exposed to fluoresceinated sheep anti-rabbit GG for an additional 45 min. Specific localization of the GG was determined by fluorescent microscopic examination. A control consisted of applying to tissue sections GG from normal rabbit serum and then fluoresceinated sheep anti-rabbit GG.

Quantitative determinations of antibodies capable of fixing in vivo to the kidneys, liver, and spleen were made with ^{131}I-labeled GG.[8] A modification [2] of the technique described by Pressman and Keighley [9] was used. In the case of rats receiving ^{131}I-labeled anti-liver FxA or FxB GG, they also received ^{125}I-labeled normal rabbit GG (Pentex, Inc., Kankakee, Ill., Lot 45P), and their organs obtained at the time of sacrifice were analyzed for ^{131}I and ^{125}I in order to provide a measure of the radioactivity of nonantibody GG remaining in the organs as well as of antibody GG. All estimations of localizing antibodies were made on tissues obtained 24 hr. after injection of the labeled GG.

Pathogenic Properties of the Antibodies (Experiment II)

Intravenous Administration of GG into Normal, Untreated Rats. Variable amounts of each anti-tissue GG (ranging from 2.5 to 80 mg.), containing, from data of the previous experiment, known amounts of tissue-fixing antibodies, were administered intravenously to rats (usually 3 rats per group). Complete necropsies were performed on representative rats sacrificed 2.5–24 hr. after injection. Tissues were either formalin-fixed for histologic examination or quick frozen in liquid nitrogen for immunohistochemical analysis.

Anti-spleen GG contained trace quantities of antibodies capable of reacting with normal constituents of rat plasma as determined by the Ouchterlony method of gel-diffusion analysis. All other anti-tissue GG appeared to be free of any similar contaminants. In order to eliminate any effect of such antibodies, all anti-tissue GG were injected after absorption with normal rat plasma. Each GG was incubated with lyophilized normal rat plasma (the equivalent of 1.0 ml. of plasma was added to every 30.0 mg. GG) at 37° C. for 3 hr. and then allowed to stand at 4° C. for an additional 12 hr. Protein aggregates were removed by ultracentrifugation (81,000 *g* for 1 hr.) and the absorbed GG retested for antibodies to rat plasma proteins by gel-diffusion analysis. The absorbed anti-spleen GG did not form precipitin lines with normal rat plasma. Groups of rats were given injections of 30-mg. doses of the absorbed GG and sacrificed 24 hr. later. Tissues from all animals were studied in the same manner as described in the preceding experiment.

To determine whether the lesions induced with anti-spleen and anti-liver FxA and FxB depended on the presence of tissue-fixing antibodies, each GG was tested after absorption with an excessive quantity (30.0 mg. sediment per 1.0 mg. GG) of its homologous tissue sediment. Quantities of each absorbed GG which, prior to absorption, were known to exert severe effects, were administered intravenously to groups of rats. The animals were sacrificed the next day and studied as in preceding experiments.

Intravenous Administration of GG into Heparin Pretreated Rats. The histopathologic features of the tissue lesions observed in the animals of Experiment II strongly suggested that intravascular coagulation might play a major role in the development of the lesions. To study this possibility, an experiment was designed in the following way: three groups of rats (3 rats per group) were given 200 units of heparin sodium (The Upjohn Co., Kalamazoo, Mich., No. 7971) intravenously. Immediately after, groups were injected with 30 mg. anti-liver FxA, and 30 mg. anti-liver FxB GG, the doses being those known to cause severe hepatic lesions.

A third group received a comparable volume of 0.15 M NaCl and served as controls. All rats were sacrificed 12 hr. later and the previously described immunopathologic tissue studies were performed.

Intravenous Administration of GG into Pre-immunized Rats. This study was made to determine if the autologous antibody response to heterologous anti-tissue GG would assume a pathogenic role in the perpetuation of the initial acute insult in other organs besides the kidney. Eight rats were immunized with 2.0 mg. of normal rabbit GG in Freund's incomplete adjuvant in the footpads and subcutaneously. Three days later, the immunized rats and a group of 8 nonimmunized rats were given 30 mg. of anti-spleen GG intravenously. Two immunized and 2 nonimmunized rats were sacrificed for detailed tissue studies at 1, 4, 7, and 15 days after injection of GG.

Results

Tissue Fixation of the Antibodies (Experiment I)

All the anti-connective tissue GG fixed to the same anatomical sites in kidney, liver, spleen, and muscle, which were as follows:

Kidney. Glomerular capillary walls, tubular basement membranes and interstitial capillaries

Liver. Endothelial walls of arteries and veins, and sinusoids, portal tracts, and interlobular connective tissue

Spleen. Reticulum, capillaries, and sinusoids

Muscle. Sarcolemma, capillaries, and perimysium

There were subtle differences among the GG in the amount of fluorescence at some tissue sites which probably reflected quantitative differences in the heterogeneous antibody population of each GG.

Table 1 shows that all the GG contained antibodies capable of fixing, in vivo, to kidney, spleen, and liver.

Table 1. Pathologic Effects of γG-Globulins in Normal Rats

γG-globulins	Total amt. GG injected (mg.)	Specific organ-fixing antibodies injected (μg.)*			Extrarenal focal tissue necrosis
		Liver	Kidney	Spleen	
Anti-liver FxA	50	1470	220	90	Severe
Anti-spleen	40	1440	200	188	Severe
Anti-liver FxB	50	1050	125	40	Severe
Anti-kidney	30	1080	900	231	Mild
Anti-heart	80	432	248	32	None
Anti-muscle	40	296	104	64	None

The data are from one representative pool of each anti-connective tissue GG.

* The amount of organ-fixing antibodies in each GG was determined from experiments in which rats were given injections of 500 μg. of ^{131}I-labeled GG (specific activity of 20 to 40 μc./mg. of GG). The radioactivity in the tissue was measured 24 hr. later. All values of organ-fixing antibodies have been corrected for the presence of non-antibody GG.[2]

Pathogenic Properties of the Antibodies (Experiment II)

Intravenous Administration of GG into Normal, Untreated Rats. The maximum dose of each GG tested, the corresponding quantities of

organ-fixing antibodies, and the estimate of the degree of pathologic effects are listed in Table 1. Characteristic histologic and immunohistochemical findings of acute nephrotoxic serum nephritis were seen in rats that received injections of sufficient quantities of anti-kidney, spleen, heart, and liver FxA GG but not in rats given injections of anti-liver FxB or anti-muscle GG. These findings were the subject of an earlier report [2] and will not be further elaborated upon here. It should be noted that with anti-liver FxA and anti-spleen GG the doses of GG which induced nephritis also induced extrarenal lesions. However, because of its higher specificity for the kidney, this was not true for anti-kidney GG. Nephritis was induced with doses of anti-kidney GG (approximately 5 mg.) which did not cause extrarenal lesions.

The extrarenal pathologic changes induced by sufficient doses of certain GG were characterized by focal tissue necrosis in several organs. The lesions were invariably present in liver, intestines, lymph nodes, thymus, and spleen. Less constantly, focal hemorrhages were found in the lungs, adrenal cortex, pancreas, gonads, and skeletal muscle. The incidence and severity of lesions, particularly in the liver, correlated with the quantity of antibodies in each GG (Table 1).

The clinical and pathologic effects of the anti-spleen and both anti-liver GG, when given in high doses, were indistinguishable. Clinical manifestations were evident soon after injection, and the most severe reactions were characterized by progressive dyspnea, prostration, and eventual death, usually in 2–6 hr. Gross abnormalities seen in the animals that died or were sacrificed in the first 2.5–10 hr. consisted of congestion of the abdominal viscera, and scattered petechial hemorrhages on the serosal surface of the intestines and in Peyer's patches, mesenteric lymph nodes, thymus, and lungs. After 10 hr., tiny light-yellow foci were prominently visible on the surface and throughout the parenchyma of the congested liver.

Microscopically, the following abnormalities were observed:

LIVER. Within 2.5 hr. focal hemorrhages were present primarily in the midzonal areas and occasionally in the centrilobular regions. In animals sacrificed at later times, the hemorrhage became more extensive, neutrophil migration into damaged areas could be seen, and changes indicating early coagulation necrosis were evident. After 4–6 hr. small, spherical hyaline masses could be seen occluding the sinusoids and within the cytoplasm of Kupffer cells in the vicinity of the lesions (Fig. 1). By 24 hr. the foci of necrotic parenchyma were more sharply demarcated as cellular changes had progressed, the inflammatory elements consisting of a mixture of neutrophils and macrophages. Hepatic parenchymal cells near the margins of the necrotic areas showed early

changes of degeneration—i.e., cloudy swelling (Fig. 2 and 3). It is important to note that these acute lesions appeared to be reversible in most animals. A full description of their resolution is given below in *Intravenous Administration of GG in Pre-immunized Rats.*

INTESTINES. Focal hemorrhages were randomly present along the base of the glandular epithelium, and within the submucosa, muscularis, and adventitia of the wall. Small hyaline masses were evident in the lumens of small vessels in the affected areas, and inflammatory cells surrounded the hemorrhages.

LYMPHATIC ORGANS. Prominent hemorrhagic foci were present in the mesenteric lymph nodes, Peyer's patches, and thymus as early as 2.5 hr. after injection. The hemorrhages were located in perifollicular and medullary areas. The spleen showed focal areas of congestion in the red pulp. Small vessels in the vicinity of affected areas in all of these organs were occluded with small hyaline plugs similar to the ones seen in other organs.

OTHER ORGANS. Other organs such as lungs, adrenals, pancreas, gonads, and skeletal muscle had occasional small areas of hemorrhages. Bilateral renal cortical necrosis or occlusion of glomerular capillaries with hyaline material were not observed in any of the rats.

Immunofluorescence studies of the liver demonstrated specific fixation of rabbit GG to the walls of sinusoids, intralobular reticulum, and large portal vessels (Fig. 4). Heterologous GG was also present in the reticulum and walls of sinusoids and vessels in lymph nodes, spleen, and thymus, and to vessel walls in the intestine. No specific fixation of either autologous GG or β_{1C}-globulin could be demonstrated in similar sites of these organs. In liver, however, there was in-vitro C′ fixation to sites occupied by the heterologous GG.

When tissue sections were exposed to fluoresceinated anti-rat fibrinogen, many small spherical bodies showing intensely positive fluorescence were observed. The size and location of these bodies indicated that they were the same spherical hyaline masses seen under the light microscope in the sinusoids and Kupffer cells of the liver and occluding small vessels in other organs. The masses also stained positively, although less intensely, for rabbit GG, rat GG, and albumin. Rat fibrinogen also lined the walls of sinusoids and small vessels of the liver.

Rats given injections of anti-spleen and anti-liver FxA and FxB GG absorbed with plasma had the same clinical symptoms and pathologic changes as those seen in rats that had received injections of the unabsorbed GG. Immunohistochemical findings were likewise unaffected.

Absorption of anti-spleen and anti-liver FxA and FxB GG with ex-

cess quantities of their homologous antigens completely abolished the in-vivo effects observed after administration of the same dose of unabsorbed GG. Previous experiments with ^{131}I-labeled anti-spleen GG had demonstrated that absorption with the homologous antigen effectively removed 95% or more of the antibodies capable of fixing to kidney, spleen, and liver (unpublished data). Therefore, the removal of tissue-fixing antibodies from the GG parallels the abrogation of its pathologic properties.

Hematologic studies consisting of total and differential white blood cell counts (WBC) were made in rats before, and 2.5 and 24 hr. after the administration of anti-spleen GG. Within 2.5 hr. the total WBC became elevated (average 27,600 WBC/cu. mm.) with increase in the relative number of neutrophils. In the peripheral blood film, the erythrocytes and WBC appeared normal in structure, and the platelets were moderately decreased in number. After 24 hr. the total WBC was still elevated, but the ratio of neutrophils to lymphocytes was nearly normal in proportions, thus indicating that an absolute lymphocytosis had occurred. The peripheral blood film was unchanged except for an increase in the quantity of circulating platelets.

Intravenous Administration of GG into Heparin-pretreated Rats. Heparin was effective in preventing the clinical and pathologic manifestations in rats given anti-liver FxA and FxB GG injections. No gross or histologic abnormalities could be found in the extrarenal organs of any of these animals. Immunofluorescent studies of the livers, on the other hand, demonstrated rabbit GG in a distribution pattern identical to that observed in untreated rats. Rat fibrinogen was also localized in the walls of sinusoids and vessels. The striking difference between the untreated and heparin-treated rats was the complete absence, in the latter group, of the microscopic fibrin-containing hyaline masses, seen in most organs of the former group.

The control rats given injections of 200 units heparin alone had no untoward reactions to this dose of anticoagulant, and their tissues were entirely normal in all parameters.

Intravenous Administration of GG in Pre-immunized Rats. There was no difference between the pathologic changes observed in the immunized and nonimmunized rats. Both groups of rats manifested the acute lesions shortly after receiving the anti-spleen GG. The gross microscopic and immunohistochemical findings at 24 hr. were exactly as described in the results of GG admininistration to normal, untreated rats. After 48–72 hr., all rats appeared clinically normal, and by the fourth day, no gross abnormalities could be detected at necropsy.

Microscopically, after 4 days the necrotic areas in the liver were less prominent than those observed after 24 hr., as were the accompanying mononuclear cell infiltrates. The spherical hyaline masses were no longer present in the sinusoids and Kupffer cells, but the walls of some hepatic arteries had a smudgy, thickened appearance, and small clusters of plasma cells and lymphocytes had begun to accumulate in the portal tracts. The parenchymal cell structure was essentially normal by the fifteenth day. There was, however, no apparent improvement in the smudgy thickening of the vessel walls, and the cell clusters in the portal tracts had increased somewhat in size; some connective tissue fibers surrounding the parenchymal lobules were also noted (Fig. 5–8).

Immunofluorescent studies revealed that autologous GG—probably anti-rabbit GG—was fixed specifically only to the livers of immunized rats by the fourth day. The pattern of localization was identical to that of the heterologous GG which persisted in these sites, though in apparently decreasing amounts, throughout the course of the experiment. The intensity of positive fluorescence for rat GG increased with time, and by Day 15, there was positive staining for both heterologous and autologous GG in the walls of sinusoids and vessels, in intralobular reticulum, and in portal tracts, including the newly formed connective tissue projections (Fig. 7 and 8). Autologous GG was never detected in the livers of nonimmunized rats, but the heterologous GG was localized in all of the sites mentioned above. Autologous β_{1C}-globulin could not be demonstrated in the livers of rats from either group.

Discussion

The present study demonstrates that certain anti-connective tissue serums, when administered intravenously, can induce lesions in other organs besides the kidneys. The characteristic extrarenal lesion, focal hemorrhagic necrosis, occurs most constantly in the liver, intestines, and lymphatic organs. The development of the lesions is related quantitatively to the specific fixation of antibodies to connective tissue elements. This point is proved by the following observations: (1) Immunofluorescent studies demonstrate specific fixation of antibodies to sinusoids, vessels, and reticulum of liver, and to comparable sites in other organs. (2) Development of the lesions can be prevented by absorbing the anti-tissue GG with its homologous antigen prior to in-vivo administration. (3) In the case of the liver, lesions are induced only by those antiserums which contain a critical amount of fixing antibodies.

The multi-organ distribution of the lesions, the early development of thrombi, the resulting coagulative necrosis, and the ability to prevent all

of these changes with heparin strongly suggest that following the specific fixation of antibodies there is a process consisting of intravascular coagulation, vascular disturbances, and ischemia playing a major role in the pathogenesis of the lesions. The initiation of local intravascular coagulation by the in-vivo fixation of antibody has been reported before and may be explained in several ways. If fixation of antibody molecules causes endothelial injury, then any damaged site will serve as a nidus for the formation of thrombi. Also, it has been clearly demonstrated that interaction of antibody and antigen molecules can directly activate the clotting mechanism,[10] the effect being mediated through platelet agglutination, which can be inhibited by heparin.[11]

A difference between the lesions obtained with anti-tissue antiserums and those observed in the generalized Shwartzman reaction is that the latter phenomenon is usually "disseminated" throughout the vascular system so that thromboses and their sequelae can be found in most tissues examined. In the present study, none of the rats developed myocardial lesions or deposits of fibrinoid material in the renal glomerular capillaries, changes usually seen as a result of the Shwartzman reaction in rats.[12] Participation of complement may not be required for development of the lesions, as suggested by the failure to demonstrate its presence on the antigenic sites occupied by antibody molecules and also by the observation that similar lesions can be induced with duck anti-kidney, a non-C′-fixing antibody (unpublished data).

The liver is the organ in which the relationship between fixation of antibodies and lesions is best studied. Similar lesions, presumably caused by the presence of immune complexes in the hepatic circulation, have been previously reported by many investigators.[13-17] However, in the present studies any possible role of circulating complexes is eliminated. Absorption of antiserums with normal rat plasma has no appreciable effect on the development of lesions by either anti-spleen or anti-liver antiserums. The small, spherical hyaline masses seen occluding the sinusoids and small vessels of liver (and other organs) clearly represent thrombi and not immune complexes. However, we cannot rule out the possibility that these spherical bodies in some areas may represent emboli rather than in-situ thrombosis. It is striking that despite the extensive necrosis, there is, in most rats, complete recovery of the lobular architecture. Later, however, some extension of portal connective tissue into the hepatic lobules and infiltration of plasma cells, lymphocytes, and fibroblasts into portal tracts and septums is observed. Any effect of the autologous antibody response in the development of a chronic liver lesion is not seen during the 2-week period of observation. However,

autologous GG, fixed to the same anatomic sites occupied by the heterologous antibody, can be demonstrated by immunofluorescent studies as early as the fourth day after injection into pre-immunized rats. Thus, the substratum for a continuous immunologic injury is present, and it is not unlikely that, as in the kidney, a chronic lesion may develop after a longer period of time.

Conclusion

Extrarenal lesions were consistently induced by the injection of a variety of anti-connective tissue antibodies. Once injected into the circulation, the heterologous antibodies became fixed primarily to the connective tissue framework of liver, intestines, and reticuloendothelial organs, and, if present in sufficient amounts, led to local intravascular coagulation in the vicinity of these sites. The resulting vascular disturbances and occlusion of sinusoids and small vessels apparently compromised blood supply, leading to the development of ischemic necrosis. The lesions could be prevented by either removal of the antibodies by prior absorption with the homologous tissue antigen or by administering an anticoagulant simultaneously with the antiserum.

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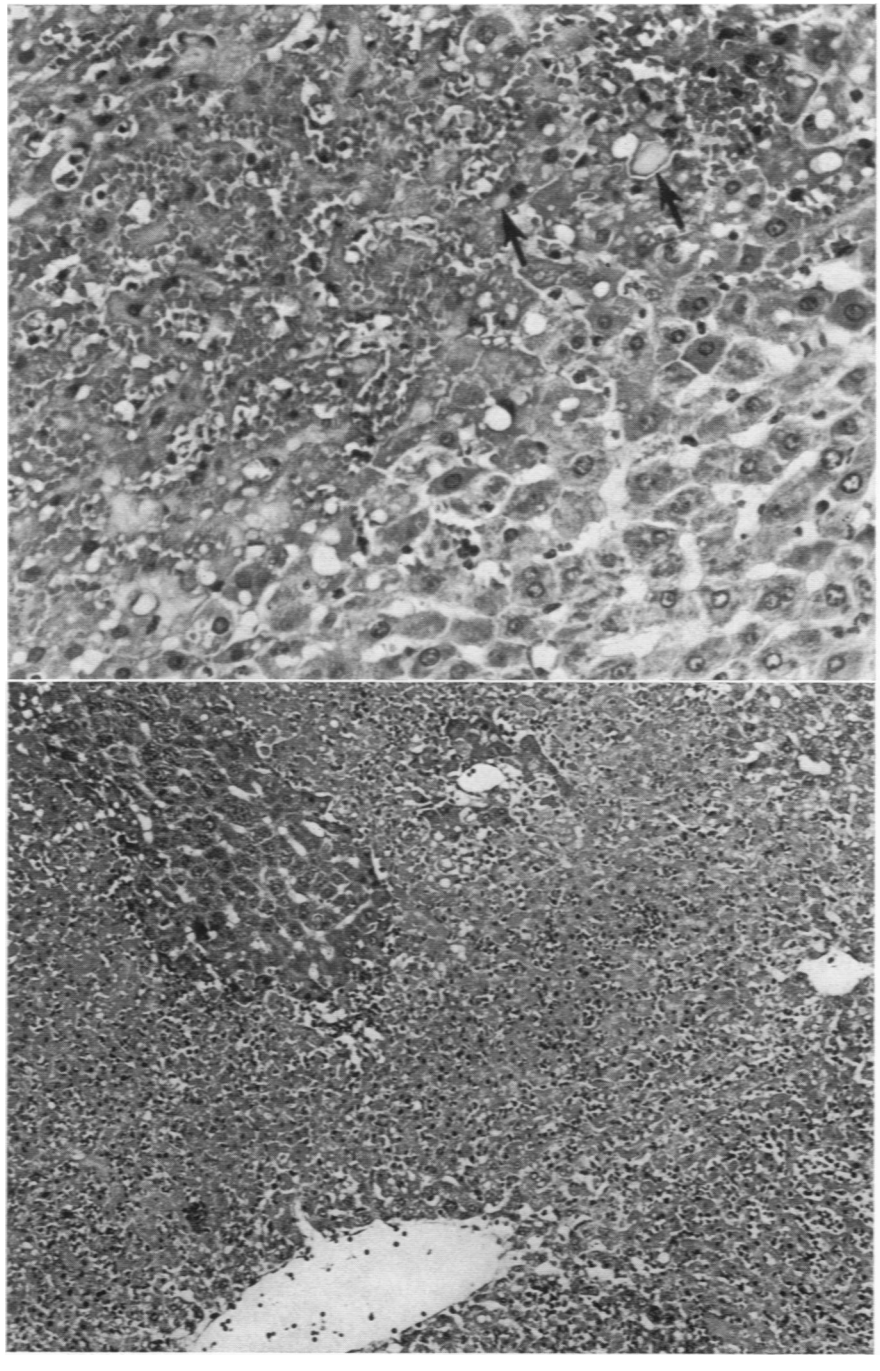
[*Illustrations follow*]

Legends for Figures

Fig. 1. Liver from rat sacrificed 5.5 hr. after receiving 30 mg. anti-spleen GG. Note area of necrosis of hepatic cells with extravasation of erythrocytes. Occasional hyaline masses *(arrows)* are seen in sinusoids. There is scanty cellular infiltration at this time. Hematoxylin and eosin. × 75.

Fig. 2. Liver from rat sacrificed 24 hr. after receiving 30 mg. anti-spleen GG. Note extensive zone of necrosis of the hepatic lobule. Hematoxylin and eosin. × 75.



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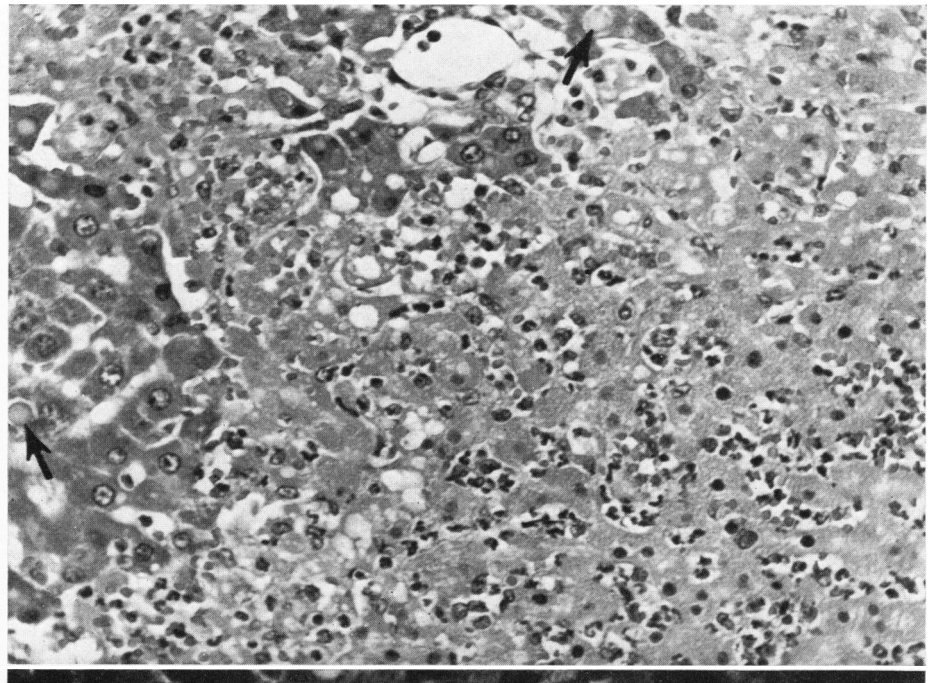

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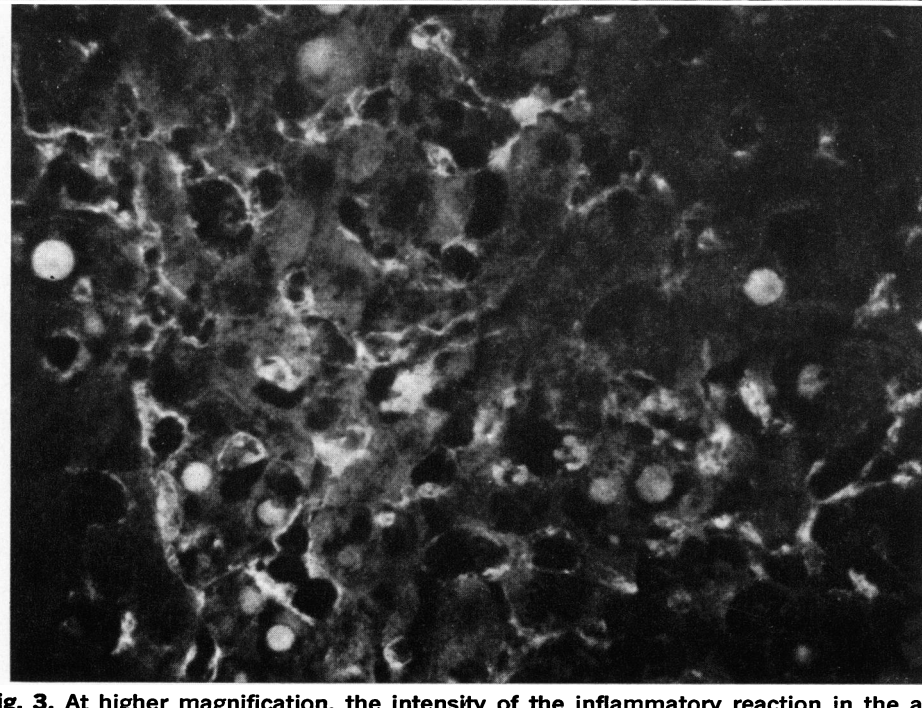
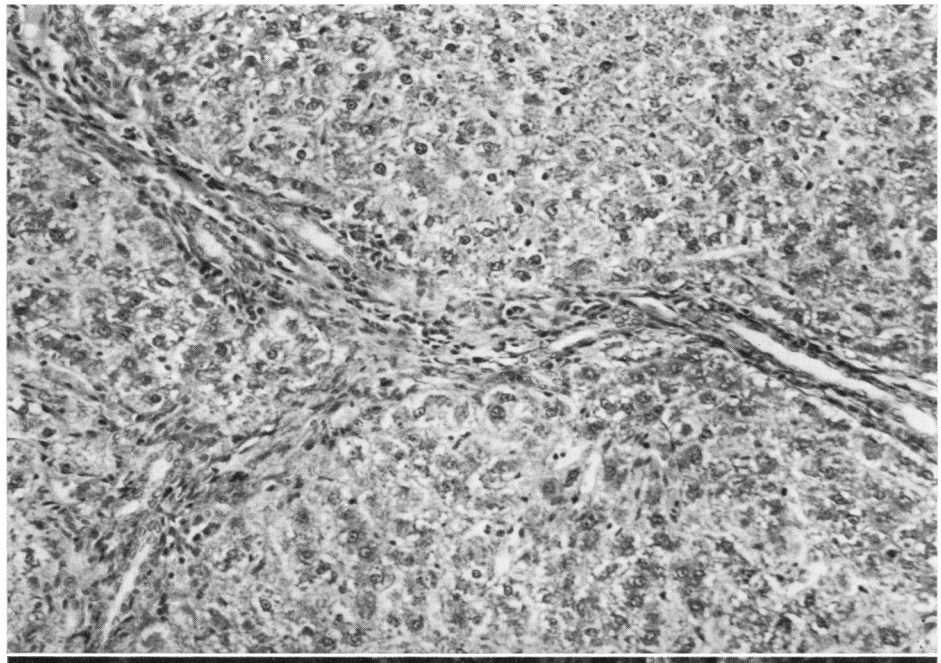


Fig. 3. At higher magnification, the intensity of the inflammatory reaction in the area of necrosis shown in Fig. 2 can be better appreciated. Characteristic thrombi are indicated by arrows. Note cloudy swelling and vacuolation in parenchymal cells adjacent to necrotic focus. Hematoxylin and eosin. × 190.

Fig. 4. Immunofluorescence study of liver from same rat as shown in Fig. 2 and 3, stained for rabbit GG, demonstrates fixation of antibody to walls of sinusoids and vessels and to reticulum. The spherical masses correspond to the thrombi observed by light microscopy and contain rat fibrinogen, GG, and albumin. × 250.



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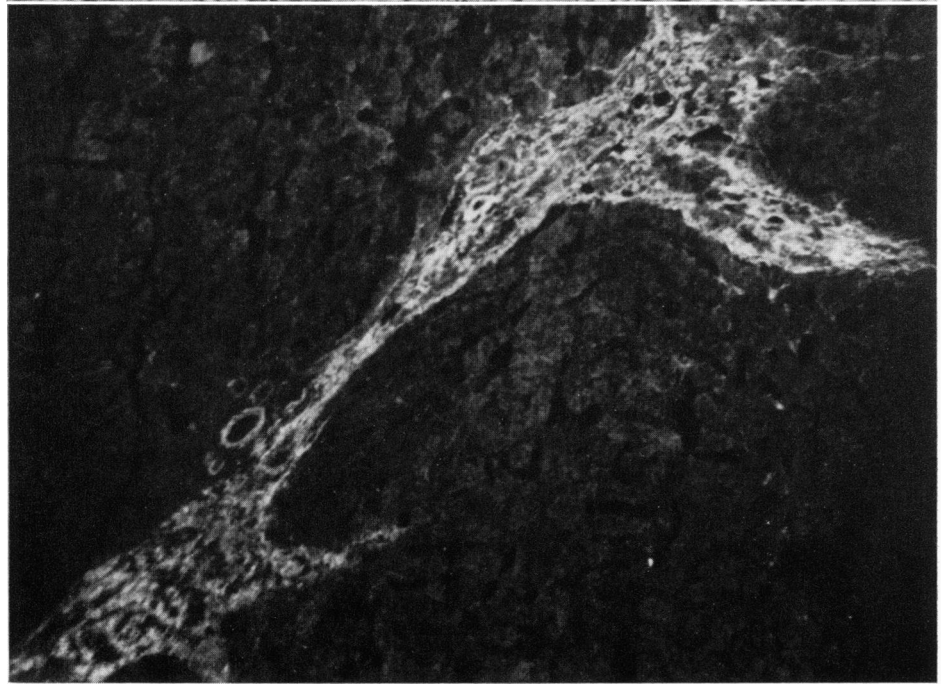

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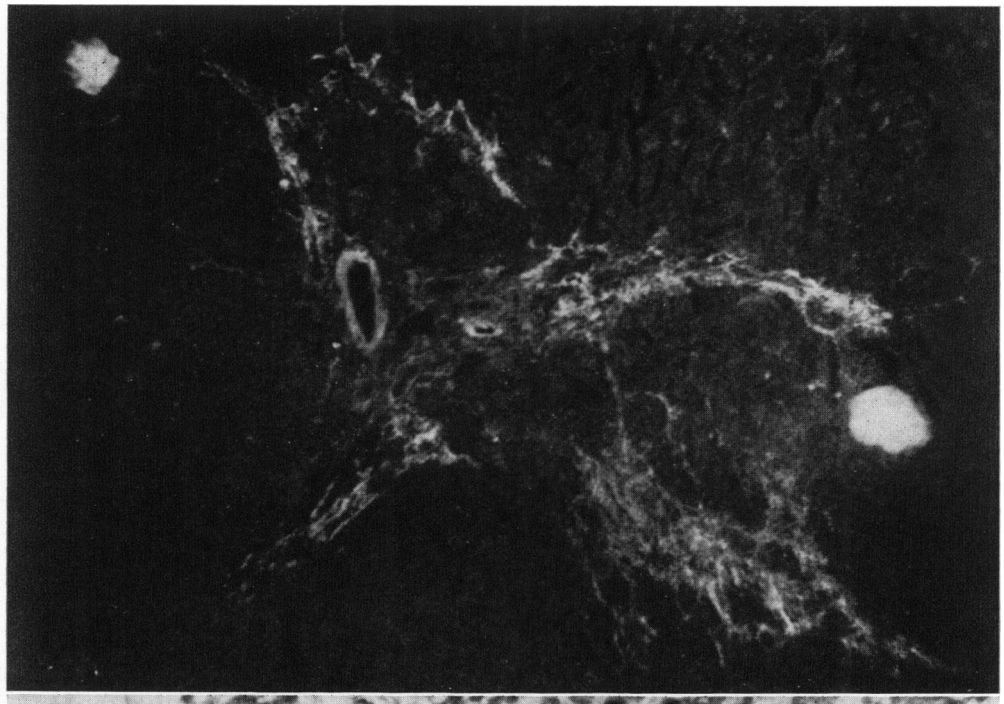
Figures 5–8 illustrate liver from a rat immunized to rabbit GG and sacrificed 15 days after receiving 30 mg. anti-spleen GG.

Fig. 5. Connective tissue extends from portal tract into parenchyma. Masson trichrome stain. × 84.

Fig. 6. Fluorescence examination of liver stained for rabbit GG reveals positive staining of connective tissue extending from portal tract and patchy distribution of antibody fixed to walls of sinusoids. × 100.



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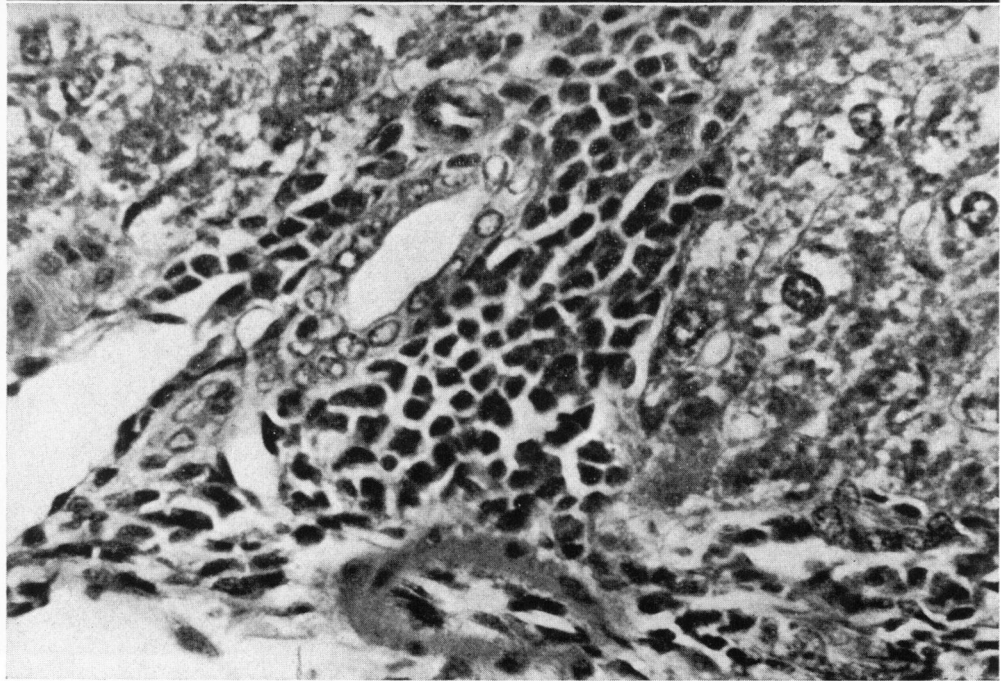

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Fig. 7. Immunofluorescence study of liver stained for rat GG demonstrates fixation of the autologous antibody to connective tissue septums, vessels, reticulum, and sinusoids. × 100.

Fig. 8. Cluster of plasma cells, lymphocytes, and a few fibroblasts in portal tract surrounding normal-appearing bile duct and portal vein. Note smudgy appearance of the wall of hepatic artery at lower center of field. × 320.