

IMMUNOCYTOCHEMICAL STUDY OF GAMMA GLOBULIN IN LIVER IN HEPATITIS AND POSTNECROTIC CIRRHOSIS*

By SEYMOUR COHEN, M.D., GOROKU OHTA, M.D., EDWARD J.
SINGER, Ph.D., AND HANS POPPER, M.D.

(From the Department of Pathology, The Mount Sinai Hospital, New York)

PLATES 21 TO 24

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Spleen, lymph nodes, and bone marrow are considered sites of gamma globulin (G.G.) formation (1) while the normal liver is not supposed to form it (2-4). In active stages of cirrhosis, the serum level of G.G. is elevated and particularly so in the postnecrotic variety (5-8). This also holds true for experimentally produced coarse nodular cirrhosis (9). In both human and experimental cirrhosis, the cytoplasmic basophilia of mesenchymal cells in lymph node, spleen, and liver is conspicuous as a result of increased ribonucleoprotein (10, 11). The latter is believed to be a cytochemical indication of protein formation (12, 13). One of the proteins formed by such mesenchymal cells may be G.G. since a parallelism between increased mesenchymal cytoplasmic basophilia in these organs and the serum level of G.G. has been demonstrated (9, 10). The Coons and Kaplan fluorescent antibody technique (14) provides a means to demonstrate directly the presence of G.G. in cells. Therefore, livers, spleens, and lymph nodes of patients with hepatic diseases and other conditions were investigated with fluorescence and light microscopic techniques.

Materials and Methods

Livers and, in some instances, spleens and lymph nodes (mainly peripancreatic) from patients who had died of various hepatic diseases were studied. The same organs from patients without primary hepatic disease served as controls (Table I). Of the latter group, two patients with carcinoma and liver metastases, one with acute leukemia and one with a generalized disease of probable mumps virus etiology, had a G.G. above 2.8 gm. per 100 ml. serum. The remainder of the cases had normal livers, passive congestion of the liver, leukemia, carcinoma without liver metastases or renal disease. One case each of disseminated lupus erythematosus, scleroderma and reticulum cell sarcoma was included. Of the two cases of biliary cirrhosis, one was a liver biopsy.

Blocks of tissue were fixed in 10 per cent neutral buffered formalin for hematoxylin-eosin and periodic acid-Schiff (PAS) stains after diastase treatment and in Carnoy's solution

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followed by absolute alcohol for methyl green-pyronine stain. The latter stain was carried out before and after treatment with 0.1 per cent aqueous solution of ribonuclease for 16 hours. Other blocks were snap-frozen in a dry ice-isopentane mixture at about -70°C . and stored at -30°C . until cut; sections, 6μ in thickness, were prepared in a cryostat, dried in vacuo at 4°C ., and fixed in dehydrated acetone for 10 minutes.

Rabbits were immunized with alum-treated human G.G. by the method of Kabat and

TABLE I
Gamma Globulin Fluorescence in Liver, Spleen, and Lymph Node in Hepatic and Non-Hepatic Diseases

	Liver			Spleen		Lymph node	
	No. of cases	Gamma globulin		No. of cases	Gamma globulin in red pulp	No. of cases	Gamma globulin
		Fibrous tracts	Kupffer cells				
Postnecrotic cirrhosis	9	+ to +++	+ to ++	2	+ and ++	2	+++
Viral hepatitis							
Acute	4	++ to ++++	0 to ++++	3	+ to +++	2	++ and +++
Subacute	2	++ and +++	++	1	+	1	++
With hypogammaglobulinemia..	1	0	0				
Hepatitis—non-viral	4	+ to +++	+ to +++				
Diffuse septal (portal) cirrhosis . . .	3	+	+	3	+ to ++	2	+++
Biliary cirrhosis	2	0	0	1	++	1	++++
Cardiac fibrosis	3	0 to +	0	2	+		
Metastatic carcinoma to liver . . .	3	+	0 to +	1	+		
No primary liver disease (serum gamma globulin not elevated)	20	0 to +	0 to +	13	0 to +++	3	0 to ++
No primary liver disease (serum gamma globulin elevated)	4	0 to ++	0 to ±	3	+++	1	++

Mayer (15). They were bled from the heart and the G.G. isolated from their pooled sera by precipitation in a dialysis sac immersed in 18 per cent Na_2SO_4 at 37°C . (16). The precipitated G.G. was washed with 18 per cent Na_2SO_4 , dissolved in physiological saline, and the sulfate removed by dialysis against normal saline at 4°C . Fluorescein isothiocyanate was prepared and conjugated with the purified rabbit immune G.G. (17, 18); conjugate solutions were twice adsorbed with acetone-extracted pork liver powder before use (19). The fixed frozen sections were washed 3 times for 5 minutes each in pH 7.0 phosphate buffered saline. They were then treated for 30 minutes with fluoresceinated rabbit serum G.G. containing anti-human G.G., washed 3 times for 5 minutes each in pH 7.0 phosphate buffered saline and covered with 1 to 2 drops of phosphate buffered glycerol as a mounting medium. Immunologic specificity was controlled by treating one section with unconjugated anti-human G.G. and another section with normal rabbit serum G.G. for 30 minutes; each of these sections was washed 3 times for

5 minutes each in pH 7.0 phosphate buffered saline and then treated with the fluoresceinated sera as the test sections. A Zeiss fluorescence microscope with a BG 12 filter between the 200 watt mercury vapor light source and the objective and an OG 5 secondary filter was used. Observations were recorded in terms of the maximum number of G.G.-containing cells in each of at least three high power fields; + represents 2 to 3 such cells, ++ 4 to 6 such cells, +++ 7 to 9 such cells, and ++++ 10 or more. Some of the sections which had been exposed to fluorescent antibody were then counterstained with hematoxylin and eosin or with methyl green-pyronine to confirm the localization of the G.G. and some were examined under the phase microscope.

TABLE II
Correlation of Gamma Globulin Fluorescence with Other Histologic Features in Postnecrotic Cirrhosis and Hepatitis

	Autopsy No.	Gamma globulin		Blurring of border of nodules	Extent of liver cell necrosis	Amount of inflammatory cells	Degree of cholestasis	Scavenger cells in parenchyma	Amount of plasma cells	Parenchymal collapse	Fatty metamorphosis	Regeneration
		in fibrous septa	in Kupffer cells									
Postnecrotic cirrhosis	17,880	+++	++	+	+++	+++	0		+	+++	0	++
	18,070	+++	+	++	+	++	0		++	0	+	++
	18,140	++	+	+	+	+	++		+	+	+	++
	18,180	+++	++	+	+++	+++	++		+	++	0	++
	18,290	+	++	++	+	++	+		0	0	0	++
	18,315	++	+	+	+	++	+		+	+	0	+++
	18,391	++	+	+	+	+++	++		±	++	+	++
	18,375	+++	++	+	++	+	0		++	+	+	+++
18,432	+++	++	+	+++	+++	++		+	++	0	++	
Acute viral hepatitis	64	++	+++		++++	++		++	0	+++		0
	18,098	++++	+++		++++	++		+	++	++++		0
	18,236	++++	++++		++	++		+	+	++		++
	18,401	++	0		++++	+		+	0	++		+
Subacute viral hepatitis	17,891	++	++		0	++		++	0	+		++
	18,559	+++	++		+	+		+	±	++		++

RESULTS

In postnecrotic cirrhosis (Fig. 1), G.G. fluorescence was observed in the cytoplasm of cells lining the sinusoids of the cirrhotic nodules and in many cells of the fibrous septa (Fig. 2) (Table II). The cytoplasm of all these cells was abundantly stained while the usually eccentric nucleus was not stained (Fig. 3 A). Under the phase contrast microscope, most of the G.G.-containing cells located in the sinusoidal wall appeared to have processes connecting with neighboring littoral cells (Fig. 3 B). Under the light microscope, in sections stained with hematoxylin and eosin after treatment with fluoresceinated sera, the majority of the G.G.-containing cells, and similar cells in paraffin sections, had most characteristics of Kupffer cells. In contrast to typical Kupffer cells, their nuclei appeared denser and sometimes exhibited cart-wheel arrangement of the chromatin (Fig. 4). Their cytoplasm frequently showed a

diffuse PAS reaction in contrast to the usual granular reaction of Kupffer cells. It stained deeply red with pyronine before but not after treatment with ribonuclease. Occasionally the cells with G.G. fluorescence contained cytoplasmic inclusions which gave a strong spontaneous orange-brown fluorescence. This was also visible in sections not treated with fluoresceinated sera and apparently was given by lipofuscin. Few G.G.-containing cells were not attached to the sinusoidal wall, were round, devoid of inclusions, and appeared under the light microscope as plasma cells in the sinusoids. The G.G.-containing cells in the septa were similar under the phase or light microscope to the G.G.-containing Kupffer cells. The autofluorescent inclusions of these portal cells were more conspicuous. Plasma cells in vessels or interstitial tissue were very sparse.

In fatal viral hepatitis (Fig. 5), many more cells with G.G. fluorescence were noted attached to the sinusoidal wall and lying free either in the capillary lumen or in tissue spaces which appeared enlarged because of the disappearance of necrotic liver cells (Fig. 6) (Table II). The sinusoidal cells (Figs. 7 A and 7 B) which contained G.G. and similar cells in paraffin sections, when stained with hematoxylin and eosin, exhibited under the light microscope a large, heavily basophilic cytoplasmic rim. It was strongly PAS-positive. The free cells were round, their nuclei were frequently eccentric and sometimes exhibited cartwheel distribution of chromatin. Most of them were smaller than plasma cells to which they showed resemblance. Both attached and free G.G.-containing cells only occasionally had PAS-positive granules which were found abundantly in the other Kupffer cells and in the macrophages in blood and tissue spaces. These granules apparently represented material engulfed by phagocytosis, much of which had the orange-brown autofluorescence of lipofuscin. In the portal tracts similar G.G.-containing cells were found, here intermixed with a large number of lymphocytes or primitive reticulum cells, segmented leucocytes and histiocytes.

In one case of fatal viral hepatitis, G.G. was found only in the fibrous tracts while the collapsed lobular parenchyma was almost devoid of cells. In two cases of subacute hepatitis with considerable regeneration, much G.G. was found in fibrous tracts and parenchyma but in another case with a similar histologic picture in the presence of acquired hypogammaglobulinemia, no G.G.-containing cells at all were found. Four cases of acute hepatitis without histological indications of viral etiology (one associated with myeloid metaplasia) also had a considerable number of cells with G.G. fluorescence.

In both hepatitis and postnecrotic cirrhosis, the G.G. fluorescence of the Kupffer cells showed some correlation to the amount of inflammatory cells present while the G.G. fluorescence in the fibrous areas which include both the portal tracts and the connective tissue septa in cirrhosis showed only questionable correlation (Table II). The G.G. fluorescence in both sites was related to the degree of liver cell necrosis and collapse of parenchyma but not at all to the amount of plasma cells present or to fatty metamorphosis or cholestasis.

In three cases of diffuse septal or portal cirrhosis, very few Kupffer cells or R.E. cells in fibrous tracts exhibited G.G. fluorescence while in the two examined cases of biliary cirrhosis, they were entirely free. In only one of the cases of hepatic fibrosis associated with chronic passive congestion, few cells with G.G. fluorescence were found in areas with beginning transition to cirrhosis. In the presence of metastatic

carcinoma, G.G.-containing cells were regularly found in the portal tracts and none in the parenchyma. In the cases without primary hepatic disease, as a rule no G.G.-containing cells were found. In two out of twenty cases, few fluorescent portal R.E. cells or Kupffer cells were noted. The negative group included four patients with total serum globulin above 3.5 gm. per 100 cc. serum, G.G. partition not being available. Of the four cases with established serum G.G. elevation, one had a significant number of G.G.-containing cells in the portal tract, one few, and one very few, while only one had sparse fluorescent Kupffer cells. Hepatic granulomas, one associated with mumps and the other with Hodgkin's disease, had plasma cells which contained G.G.

In almost all spleen and lymph nodes examined, G.G.-containing cells were encountered which were, in general, in the red pulp of the spleen and in the medullary cords and sinuses of lymph nodes (Table I). Many of them were plasma cells. In both organs some of the G.G.-containing cells and similar cells in paraffin-embedded tissue also contained PAS-positive granules whereas others contained fluorescent lipofuscin as well as PAS-positive granules. The same was true of some of the G.G.-containing cells which were littoral reticuloendothelial cells. In the cases of cirrhosis examined, these cells were in distinct clusters. In only two cases without primary liver disease, G.G.-containing cells were not demonstrated in the spleen. Patients with elevated serum G.G. levels tended to have high amounts of G.G. in spleen and lymph nodes. This held true for all types of cirrhosis, including diffuse septal and biliary.

DISCUSSION

The elevation of serum G.G. in liver diseases and particularly in postnecrotic cirrhosis has attracted interest because of the possibility that at least part of the excess serum G.G. is antibody to liver tissue. This in turn raised the possibility that such an antibody to liver tissue is a factor in either initiation or more probably in progression or chronicity of liver disease (20). Antibodies against liver tissue in serum have been repeatedly demonstrated (21, 22) and their presence has recently been emphasized in so called lupoid hepatitis (23, 24).

In agreement with perfusion studies (2) in which it was shown that G.G. is not formed by the liver, the immunocytochemical technique indicates absence of G.G.-containing cells from normal liver. Few such cells are seen in livers altered by non-specific chronic changes such as passive congestion or metastatic carcinoma. This is in contrast to the large number of mesenchymal cells with G.G. fluorescence in postnecrotic cirrhosis and even more so in acute hepatitis. Various observations indicate that the G.G. is formed by these cells and is not present as a result of phagocytosis. In hypergammaglobulinemia of non-hepatic' etiology, in which spleen and lymph nodes are rich in G.G., few cells in the liver contain it. Moreover, the cells in question, as a rule, show little lipofuscin and PAS-positive material derived from phagocytosis (25). Their prominent cytoplasmic ribonucleoprotein suggests considerable protein

formation and protein formed by such cells would presumably be G.G. (26). The absence of G.G. in biliary cirrhosis is of special interest because of the high amount of complement-fixing anti-liver antibodies reported in this disease (27).

Spleen and lymph nodes almost always contain a considerable number of G.G.-containing cells and in biliary and diffuse septal cirrhosis they are as rich in such cells as in postnecrotic cirrhosis and hepatitis. This fact is in accordance with the view that these organs are the main site of G.G. production in liver disease (9).

The classification of the cells in the liver which seem to produce G.G. is problematic. Only a few are plasma cells by cytological criteria. Some are free, round cells which do not have all the characteristics of plasma cells and sometimes, particularly in viral hepatitis, contain material apparently engulfed by phagocytosis. Most of them are littoral cells attached to the wall of the sinusoids; others lie in the portal tracts. All seem to be reticuloendothelial cells with some modifications, such as abundant basophilic cytoplasm, diffuse strong PAS reaction, and occasionally eccentric nucleus. These observations are best reconciled with the hypothesis that the G.G.-containing cells in the liver are reticuloendothelial cells exhibiting varying degree of transition to plasma cells, some of them still retaining phagocytic activity. Formation of plasma cells from reticuloendothelial cells in sites other than the liver appears to be accepted (1). The transformation of reticuloendothelial cells into plasma cells as a consequence of immunoallergic shock *in vitro* has recently been demonstrated by microcinematography (28). A rounding of Kupffer cells with an increased basophilia, an eccentric nucleus and a loss of the capacity to phagocytose carmine has been described in the rabbit in response to stimulation with bacterial antigens (29).

The role of the G.G. found in mesenchymal cells of abnormal livers particularly in hepatitis and postnecrotic cirrhosis is a second problem. Most, if not all, G.G. seems to be antibody (30). The hepatic G.G. is apparently not related to the hepatitis virus, because of its presence in what appears to be non-viral type of hepatitis. Its occurrence seems best correlated with the degree of liver tissue destruction which is less violent in diffuse septal and biliary cirrhosis; inflammation *per se* appears less important. The presence of PAS-positive granules and lipofuscin, which are regarded as cell breakdown products (25), in some cells which contain G.G. suggests that these substances might stimulate the formation of G.G. during the transformation of R.E. cells to plasma cells. Some liver disease then might, as has been postulated (20), either be caused or be perpetuated by antibodies to liver cell breakdown products. The main sites of antibody production under these circumstances would seem to be spleen and lymph nodes. However, it is still a question for further investigation whether the increase in hepatic G.G. in some liver diseases

reflects an immunologic process at all and also what the significance is of the material engulfed by phagocytosis.

SUMMARY

Gamma globulin was demonstrated by immunocytochemical fluorescence technique in many reticuloendothelial cells of the hepatic sinusoids and of the fibrous tracts in various forms of hepatitis and in postnecrotic cirrhosis. In other liver diseases and in normal livers, even in the presence of hypergamma-globulinemia, few if any gamma globulin-containing cells were found. In contrast, spleen and lymph nodes showed no difference between postnecrotic cirrhosis or hepatitis and other types of cirrhosis or non-hepatic hypergamma-globulinemias. The gamma globulin-containing cells in the liver are on cytologic grounds considered reticuloendothelial cells showing transition to plasma cells and exhibiting little or no phagocytosis of tissue breakdown products. These cells are assumed to form rather than engulf gamma globulin. The possibility that the gamma globulin formed represents antibody to liver cell breakdown products is discussed.

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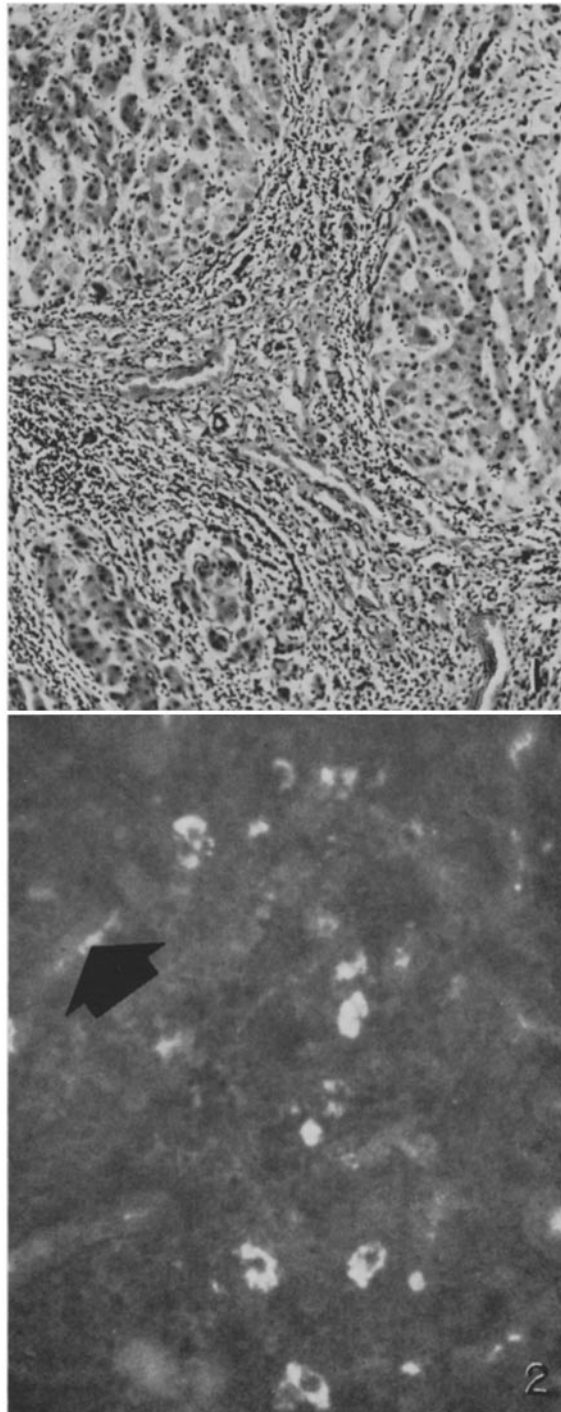
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EXPLANATION OF PLATES

PLATE 21

FIG. 1. Active postnecrotic cirrhosis. Hematoxylin-eosin stain. $\times 63$.

FIG. 2. Postnecrotic cirrhosis. Cryostat section treated with rabbit serum gamma globulin containing anti-human gamma globulin. The cytoplasm of cells containing gamma globulin stains brightly while the nuclei show no fluorescence. The weakly fluorescent dots and streaks are bile within canaliculi (arrow). $\times 450$.

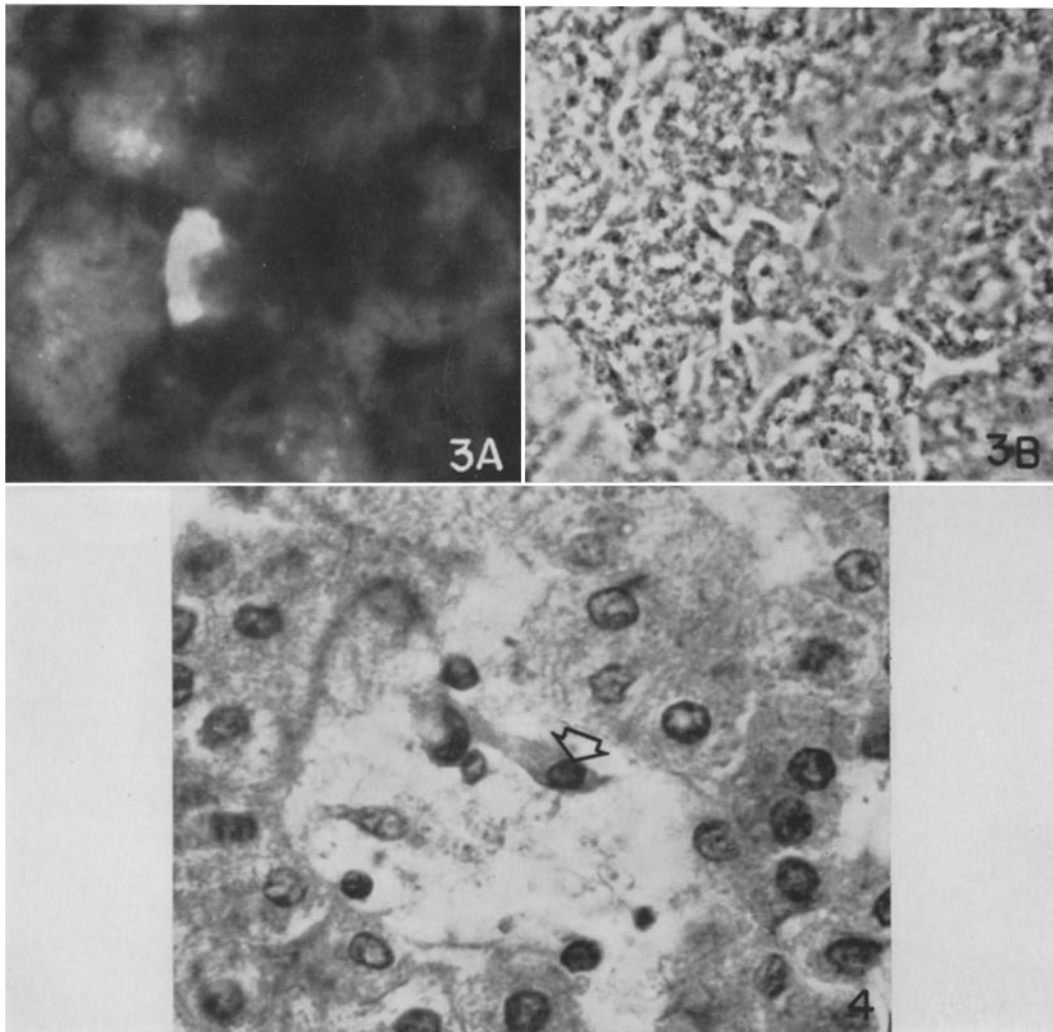


(Cohen *et al.*: Immunocytochemical study of gamma globulin)

PLATE 22

FIG. 3 A. Kupffer cell containing fluorescent gamma globulin. The eccentric nucleus fails to stain. The small, rather weakly fluorescent granules in liver cells are auto-fluorescent lipofuscin. $\times 900$. Fig. 3 B. The same Kupffer cell as in Fig. 3 A seen under the phase contrast microscope. $\times 900$.

FIG. 4. Kupffer cell (arrow) in postnecrotic cirrhosis. The cell is rounder than typical Kupffer cells, with a basophilic cytoplasm and an eccentric nucleus which is denser than usual. Hematoxylin-eosin stain. $\times 600$.

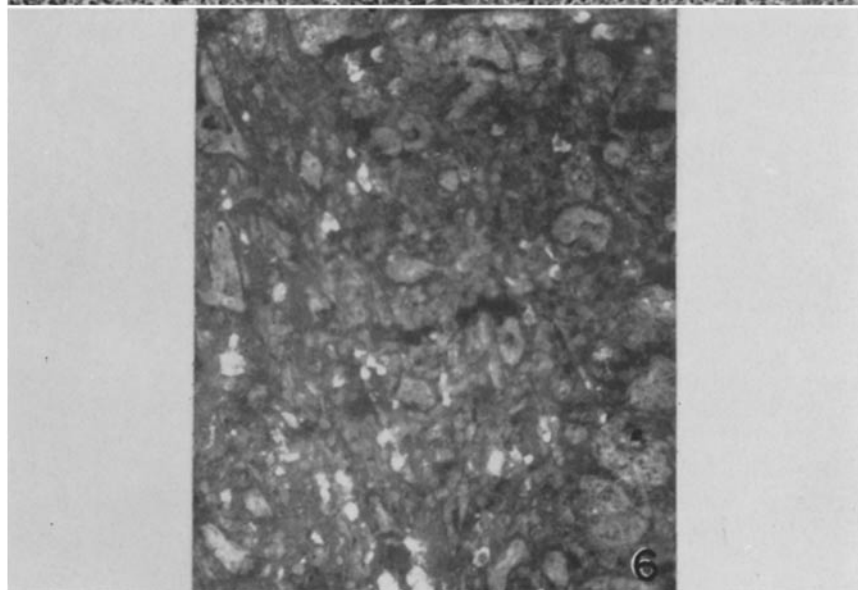
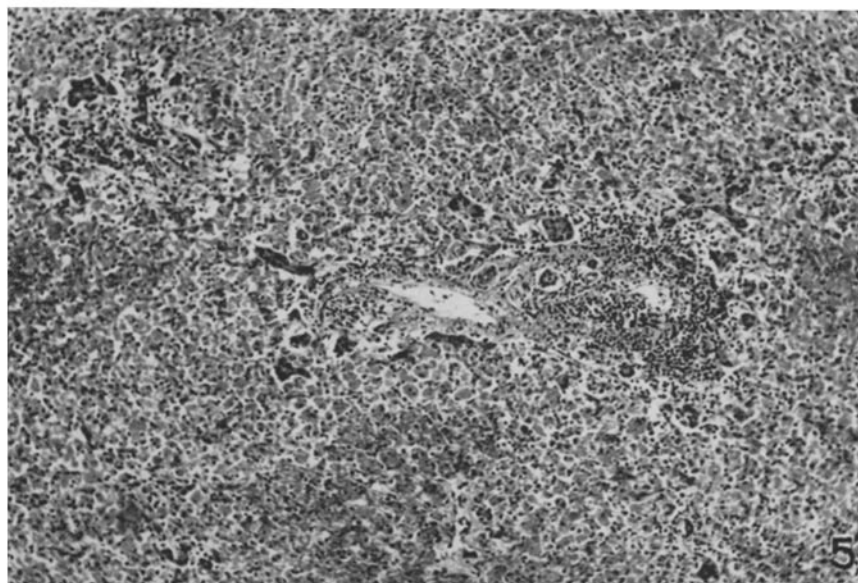


(Cohen *et al.*: Immunocytochemical study of gamma globulin)

PLATE 23

FIG. 5. Fatal acute viral hepatitis with extensive necrosis of parenchymal cells and conspicuous accumulation of mesenchymal cells. Hematoxylin-eosin stain. $\times 63$.

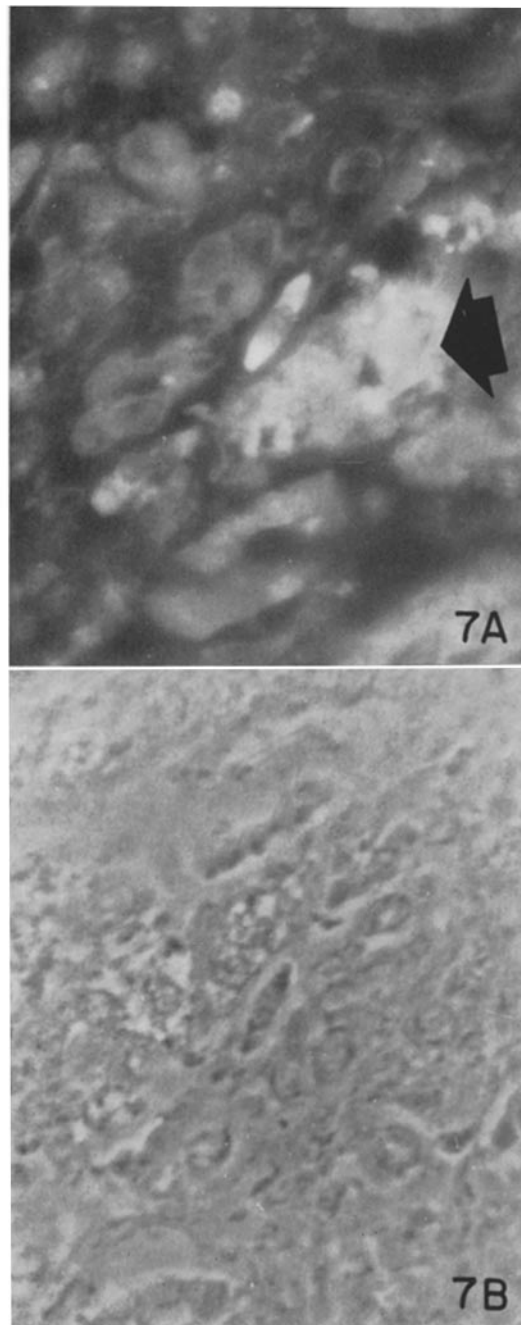
FIG. 6. Liver in acute viral hepatitis treated with anti-human gamma globulin. Many cells show bright gamma globulin fluorescence. $\times 250$.



(Cohen *et al.*: Immunocytochemical study of gamma globulin)

PLATE 24

FIG. 7 A. Kupffer cells in acute viral hepatitis showing gamma globulin fluorescence in the cytoplasm. In the neighboring liver cells are many autofluorescent lipofuscin granules (arrow). $\times 900$. FIG. 7 B. The same cells as seen in Fig. 7 A under the phase contrast microscope. Processes of the Kupffer cells are attached to neighboring littoral cells. $\times 900$.



(Cohen *et al.*: Immunocytochemical study of gamma globulin)