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Research Article

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Haptoglobin Synthesis. II. Cellular Localization Studies *

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In previous communications (1, 2) evidence has been presented demonstrating the *in vivo* synthesis of haptoglobin by the canine liver. The results suggested, but did not prove, that the liver was the sole organ of synthesis. The purpose of the present investigation is to obtain information about the cellular sites of haptoglobin synthesis by means of specific immunofluorescence.

Immunofluorescent localization does not distinguish between sites of synthesis and sites of clearance or storage. In an attempt to distinguish between them, we have studied canine tissues obtained at different moments during the increase in concentration of haptoglobin in the plasma that follows the onset of acute inflammation. The moments chosen were the resting state, the time when the rate of haptoglobin synthesis was maximal (2), and the time when its concentration in the plasma was maximal but its synthesis diminished. We expected that the number of cells concerned with haptoglobin synthesis or the intensity of their specific fluorescence, or both, would parallel the rate of haptoglobin synthesis at the sites of production, whereas the number of cells concerned with clearance or storage of haptoglobin and the intensity of their specific fluorescence would parallel the concentration in the plasma.

Methods

Antihaptoglobin antiserum. Haptoglobin was prepared from hyperhaptoglobinemic dog plasma as described previously (1, 2). The preparation used for immunization gave a single band in vertical starch gel electrophoresis (3) at a protein concentration of about 2 g per 100 ml. The mobility of the entire band was reduced when hemoglobin in excess of the binding capacity was added before electrophoresis. Antisera were produced in rabbits by injecting 10 mg haptoglobin in complete Freund's adjuvant as a primary with booster injections at 2- to 4-week intervals. The antisera were harvested 7 to 8 days after the last booster injection. These antisera gave a single arc in immunoelectrophoresis (4) when tested against purified dog haptoglobin but five or six arcs when tested against whole dog plasma. To render these antisera specific only for haptoglobin, we serially absorbed them with anhaptoglobinemic dog plasma. The latter was obtained from dogs given large amounts of a human red cell hemolysate intravenously and intraperitoneally over the course of several days (5). These absorbed antihaptoglobin sera (aR anti-H) gave only a specific antihaptoglobin arc in immunoelectrophoresis when tested against hyperhaptoglobinemic dog plasma (Figure 1) and contained 600 to 800 μg N per ml of specific antibody (6). These antisera reacted as strongly with haptoglobin-hemoglobin complexes as with haptoglobin. When tested by Ouchterlony's method of double diffusion in agar gel (7) with different concentrations of purified haptoglobin, alternating with hyperhaptoglobinemic whole dog plasma, only a single precipitin line (of identity) formed between the wells (Figure 2). This was considered strong evidence for the purity of the haptoglobin-antihaptoglobin system.

Fluorescent horse antirabbit immune globulins. The globulin fraction precipitated by 40% saturated ammonium sulfate from horse antiserum to rabbit globulin was conjugated with fluorescein isothiocyanate and purified on DEAE cellulose columns (8). These conjugated chromatographed antibodies are referred to as F-HAR. F-HAR gave four arcs in immunoelectrophoresis when tested against either whole rabbit serum or the globulin precipitated from whole rabbit serum by 40% saturated ammonium sulfate. The major arc was identified as IgG with minor IgA and IgM arcs. The fourth arc was strong and most likely represented one of the complement components, since the horse antiserum was made against rabbit immune complexes.

Tissue sections. Healthy mongrel dogs weighing between 16 and 22 kg were made hyperhaptoglobinemic by the injection of 0.5 ml per kg of turpentine in multiple subcutaneous sites. Pieces of liver, spleen, kidney, heart, lung, and small bowel were obtained under anesthesia or immediately post-mortem. Two dogs were studied with-

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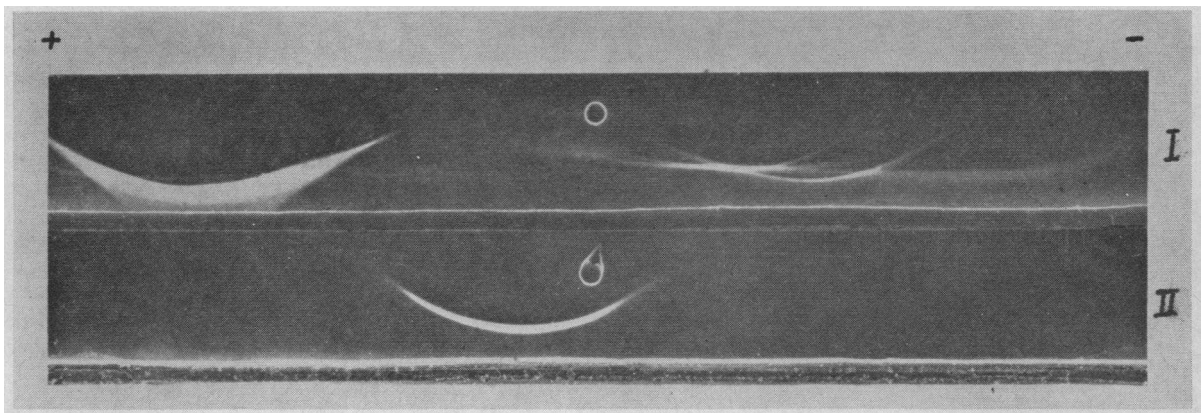

FIG. 1. IMMUNOELECTROPHORESIS OF HYPERHAPTOGLOBINEMIC DOG PLASMA. Two separate slides are placed in apposition for comparison. Hyperhaptoglobinemic dog plasma was placed in both center wells to undergo electrophoresis for 3 hours. The troughs were filled with rabbit antiwhole dog plasma (slide I) and absorbed rabbit antidog haptoglobin (aR anti-H) (slide II). Only one arc develops with the latter antiserum.

out turpentine injection, two 65 hours after turpentine injection when haptoglobin synthesis is approximately maximal, and two 125 hours after injection when synthesis is diminished but the plasma haptoglobin concentration is maximal (9). The tissue pieces were immediately placed in 95% ethanol at 4° C and fixed for immunofluorescent studies by the method of Sainte-Marie (10). Other fixation procedures using acetone, acid acetone, acid-alcohol, and so on were tried but were found to be less satisfactory than cold ethanol. Once in the paraffin stage, tissues kept well for at least 6 months. Sections 4 μ in thickness were deparaffinized with xylene

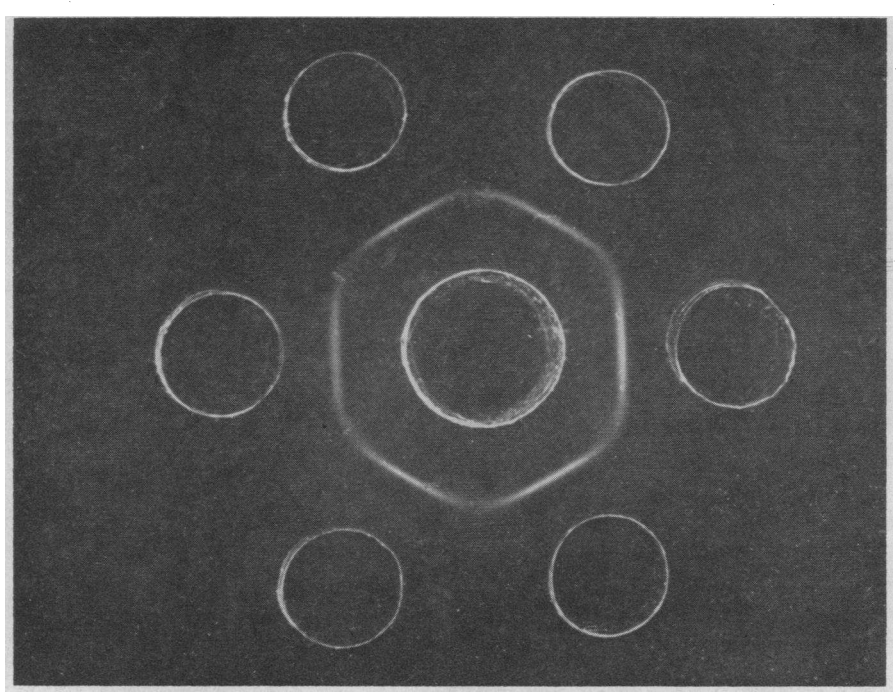

FIG. 2. DOUBLE DIFFUSION IN AGAR GEL OF HYPERHAPTOGLOBINEMIC DOG PLASMA, PURIFIED DOG HAPTOGLOBIN, AND ABSORBED RABBIT ANTIDOG HAPTOGLOBIN (aR ANTI-H). The undiluted antiserum is in the center well, and the purified haptoglobin (2 mg per ml) alternates with hyperhaptoglobinemic plasma (1:16 dilution in isotonic saline) in the six peripheral wells. The slide was allowed to incubate at room temperature in a moist chamber for 48 hours. See text for description of results.

TABLE I

*A summary of the steps in immunofluorescent staining of dog tissue for haptoglobin**

Purpose	Intermediate layer	Final layer
Specific staining	aR anti-H	F-HAR
Control	Phosphate-buffered saline	F-HAR
Control	Normal rabbit serum	F-HAR
Control	Rabbit antipneumococci antiserum	F-HAR
Control	None	F-HAR
Control	aR anti-H	Fluorescent horse anti-diphtheria toxoid
Control	aR anti-H serially absorbed with haptoglobin	F-HAR

* aR anti-H = absorbed rabbit antidog haptoglobin; F-HAR = fluorescent horse antirabbit immune globulins.

at room temperature and rehydrated through successive ethanol baths (95%, 80%, 70%, 50%) and finally, 0.15 M phosphate-buffered saline, pH 7.2.

Immunofluorescence techniques. The rehydrated tissue sections in moist chambers were layered with a 1 : 4 or 1 : 5 dilution of aR anti-H for 60 minutes. After thorough washing with phosphate-buffered saline, pH 7.2, F-HAR was layered over the sections for 30 minutes to localize fixed rabbit globulin (11). The slides were then washed in phosphate-buffered saline for 20 minutes, mounted in buffered glycerol, and examined with a Zeiss fluorescent microscope. Table I summarizes the techniques and control procedures used.

Cell counting. Two tissue sections from each of two tissue blocks from each of two dogs at each of the three points in the response to inflammation were studied (a total of 24 tissue sections). All the hepatic parenchymal cells within five discrete 500- × 500-μ^2 areas of each tissue section were counted with the aid of a 10 × ocular fitted with a grid. The average number of total parenchymal cells in the 120 (500- × 500-μ^2) areas was 7,737 with a range of 7,110 to 8,225. The total number of cells examined was thus somewhat more than 900,000 and the number for each stage of inflammation a little over 300,000. Similar counting was done to obtain average values for the total number of positive fluorescent cells, binucleate cells, and fluorescent cells that were binucleate in these areas.

Haptoglobin plasma concentration. The paper electrophoretic method of Nyman (12) was used to determine haptoglobin concentration in dog plasma. The plasma haptoglobin concentration was followed in dogs at 12- to 24-hour intervals for periods up to 22 days after turpentine injection. Maximal plasma haptoglobin concentrations were observed at approximately 125 hours after turpentine stimulation. The time of maximal haptoglobin synthesis was taken to be the point of inflection (or maximal slope) of the haptoglobin concentration versus time curves. This occurred at approximately 65 hours after turpentine stimulation.

Results

Haptoglobin detected by specific immunofluorescence was present only in the liver and spleen. Specific fluorescence was found only in the cytoplasm of scattered individual or clusters of liver parenchymal and Kupffer cells and in occasional splenic cells that had the appearance of macrophages. The fluorescent staining was diffuse throughout the cytoplasm of the hepatic parenchymal cells and under high magnification appeared finely granular. The intensity of fluorescence varied from cell to cell, but always produced a sharply demarcating cellular outline in contrast with neighboring nonfluorescent cells. No nuclear fluorescent staining was observed in any of the tissues studied. The distribution of cells containing haptoglobin was random throughout the liver lobules. All sections stained as controls (Table I) for nonspecific fluorescence were completely negative.

In normal (or 0 hour) dogs, only 2.4% of the parenchymal cells stained specifically for haptoglobin (Table II). These cells appeared mostly

TABLE II

Specific haptoglobin cellular fluorescence in liver cells at discrete intervals after stimulation with turpentine

Time	$\frac{\text{Fluorescent cells}}{\text{Total cells*}} \times 100$	$\frac{\text{Binucleate fluorescent cells}}{\text{Total cells*}} \times 100$	$\frac{\text{Binucleate fluorescent cells}}{\text{Total fluorescent cells}} \times 100$	Plasma haptoglobin concentration†
hours	%	%	%	
0	2.4 ± 0.16‡	0.11 ± 0.02	4.4 ± 1.0	140
65	11.2 ± 0.50	1.03 ± 0.06	9.1 ± 0.58	315
125	9.5 ± 0.39	0.32 ± 0.06	3.6 ± 0.50	465

* Average number of total parenchymal cells in a 500- × 500-μ^2 area = 7,737 (see text).
† Expressed as mg per 100 ml hemoglobin binding capacity.
‡ Standard error of the mean.

alone or in pairs and only occasionally in clusters, as seen in Figure 3. About 4.4% of the fluorescent cells appeared to be binucleate. Few Kupffer cells in the normal dogs contained haptoglobin, and the intensity of staining was only 1 to 2 + on a 1 to 4 + scale.

At 65 hours after turpentine injection, when the rate of haptoglobin synthesis was maximal, we

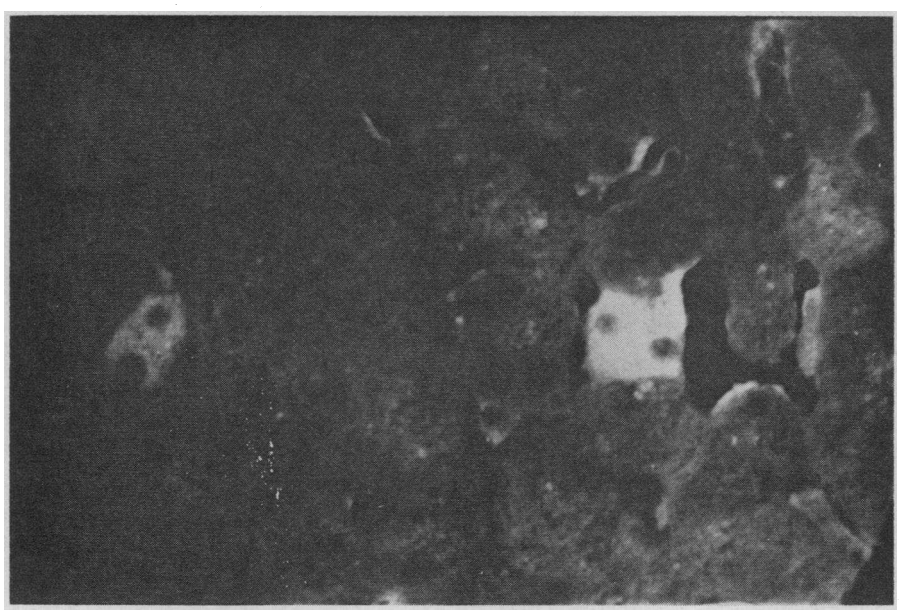

FIG. 3. SPECIFIC FLUORESCENCE IN NORMAL CANINE LIVER (0 HOURS). Cells containing haptoglobin are detected by specific apple green fluorescence. Note the difference in intensity of cytoplasmic fluorescence (between the parenchymal cell pairs) and its finely granular appearance. Several Kupffer cells containing thin rims of specific fluorescent cytoplasm can be seen scattered throughout the section. (× 525.)

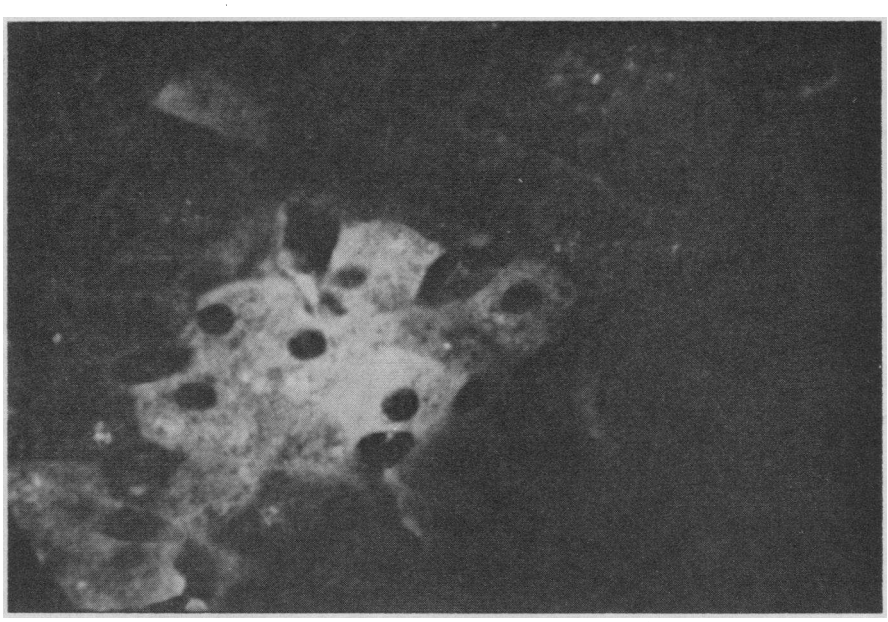

FIG. 4. SPECIFIC FLUORESCENCE IN CANINE LIVER AT THE TIME OF MAXIMAL HAPTOGLOBIN SYNTHESIS AFTER TURPENTINE INJECTION (65 HOURS). Note the prominent cluster of positive-staining parenchymal cells. (× 525.)

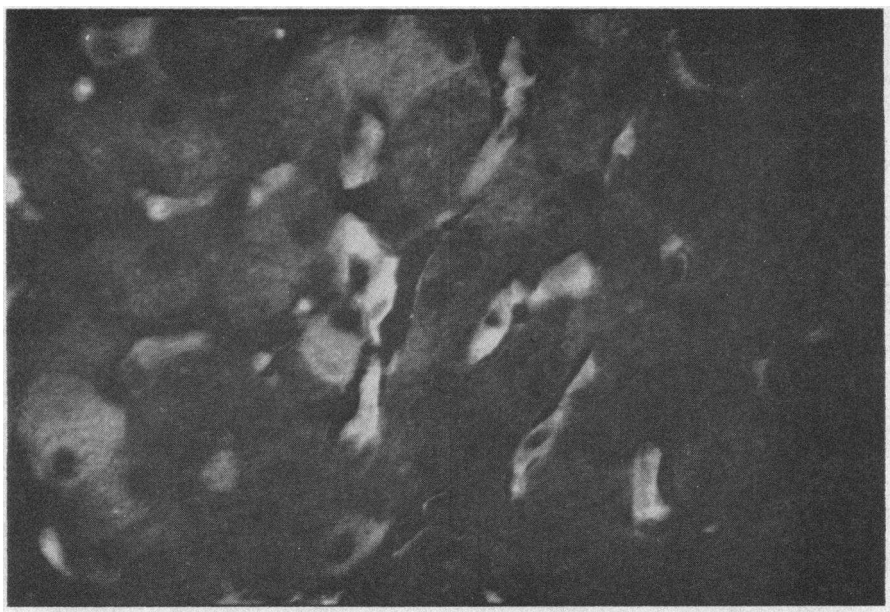

FIG. 5. SPECIFIC FLUORESCENCE IN CANINE LIVER AT THE TIME OF MAXIMAL PLASMA HAPTOGLOBIN CONCENTRATION AFTER TURPENTINE INJECTION (125 HOURS). Plump, brightly staining Kupffer cells are scattered among parenchymal cells showing varying fluorescent intensity. (× 525.)

found the greatest number of haptoglobin-containing hepatic parenchymal cells, about 11.2% of the total. There was a higher percentage of fluorescent binucleate cells (9.1%) at this time, and the fluorescent cells occurred more in clusters (Figure 4). Many more fluorescent Kupffer cells were seen at 65 hours than at 0 hours, and their intensity of fluorescence was estimated at 2 to 3 +. At 125 hours after turpentine injection, when the rate of haptoglobin synthesis had decreased but the plasma concentration was maximal, the number of parenchymal cells staining for haptoglobin had decreased. Only 9.5% of the total parenchymal cells contained haptoglobin, and only 3.6% of these were binucleate. At this time, however, we observed the greatest number of positive-staining Kupffer cells, most of which had a staining intensity of 3 to 4 + (Figure 5). The number of fluorescent macrophage-like cells in the spleen was also greatest at this time although they still occurred singly and sparsely scattered throughout this organ.

In Table III, the sigificance of the differences in specific haptoglobin cellular fluorescence at different time intervals is presented. Between 0 and 65 hours, the increase in number of specific fluorescent cells and binucleate fluorescent cells is highly significant. The decrease in the number of these cells observed at 125 hours compared with 65 hours is also significant. It is evident that at 65 hours there was a significant increase in the ratio

TABLE III

*Significance of differences in specific haptoglobin cellular fluorescence in liver cells at discrete intervals after stimulation with turpentine**

Comparison	$\frac{\text{Fluorescent cells}}{\text{Total cells}} \times 100$	$\frac{\text{Binucleate fluorescent cells}}{\text{Total cells}} \times 100$	$\frac{\text{Binucleate fluorescent cells}}{\text{Total fluorescent cells}} \times 100$
hours			
0 vs. 65	<0.001	<0.001	<0.01
0 vs. 125	<0.001	<0.05	<0.6
65 vs. 125	<0.05	<0.001	<0.001

* Significance of differences expressed as p values.

of the number of specific binucleate fluorescent cells to the total specific fluorescent cells. However, at 125 hours, this ratio had returned to resting levels even though the total number of specific fluorescent cells remained elevated. This indicates that the number of binucleate fluorescent cells returns to resting levels before the number of total fluorescent cells.

Discussion

The immunofluorescent localization of intracellular dog haptoglobin almost exclusively within the liver is consistent with the results of *in vivo* studies in the dog (1, 2) and isolated rat and rabbit liver perfusion experiments (13, 14). Haptoglobin was found in both hepatic parenchymal and Kupffer cells, however, and the problem of distinguishing those cells responsible for synthesis from those containing haptoglobin by virtue of clearance and phagocytosis remains. The finding that the number of fluorescent parenchymal cells was maximal before the plasma haptoglobin concentration reached its peak during the response to inflammation strongly suggests that these cells synthesize haptoglobin. Since the Kupffer cells containing haptoglobin increased in number throughout the 125-hour period of study during which the plasma haptoglobin concentration steadily rose, it seems reasonable to assign a clearance function to these cells. According to Murray, Connell, and Pert, the liver is the principal organ clearing haptoglobin-hemoglobin complexes from the plasma (15). They found essentially no uptake of complexes by the spleen. The technique used in the present investigation does not distinguish haptoglobin from haptoglobin-hemoglobin complexes. Hence, some of the positive fluorescence in the liver and spleen may represent the complex. Although proof is lacking, it seems reasonable to postulate that the clearance of the complex takes place in the reticuloendothelial portion of the liver, i.e., the Kupffer cells. Beyond its complexing with hemoglobin, little is known about the catabolic fate of haptoglobin. Conceivably the Kupffer cells play a role in the destruction of free haptoglobin as well as of that bound to hemoglobin, since the liver is known to catabolize up to 30% of "undenatured" albumin (16) and γ-globulin (17). However, recent evidence (18) indicates that macrophages are capable of synthesizing at least one plasma protein (β_{1C}-globulin). Thus it is conceivable that such cell types might actually synthesize haptoglobin rather than obtain it purely by phagocytosis.

Theoretically, an increase in the concentration of a plasma protein may result from one or more of the following processes: *a*) release from a storage depot, *b*) decreased catabolism, *c*) increased synthesis by each of a fixed number of protein-producing cells, *d*) proliferation of synthesizing cells, and *e*) conversion of nonsynthesizing cells to synthesizing ones. The present studies, like previous ones (2, 19), speak against the presence of a storage depot, at least in the tissues studied, and they provide no direct measure of catabolism. We found an increase in the number of haptoglobin-containing hepatic parenchymal cells during the period of maximal synthesis. This suggests either proliferation of synthesizing cells or the conversion of cells from a nonhaptoglobin-producing stage to an actively synthesizing one. An increased number of binucleate fluorescent cells favors the proliferation hypothesis. The fact that the ratio of binucleate fluorescent cells to total fluorescent cells returns toward resting levels at a rate faster than the total number of fluorescent cells strengthens the argument that proliferation of cells specifically producing haptoglobin has occurred. Increased synthesis by each of a fixed number of synthesizing cells is made less likely by our failure to find an over-all increase in intensity of fluorescence with an increase in haptoglobin synthesis.

The variation in the intensity of fluorescence of the parenchymal cells from one to another within all tissue sections may reflect different phases of haptoglobin synthesis. Possible alternative interpretations, however, include varying rates of maximal synthesis, varying loss of cellular haptoglobin, or antigenic alteration during fixation, among others.

The localization of haptoglobin by specific fluorescence is similar to the observations by other investigators for other specific proteins. Hamashima, Harter, and Coons (20) found albumin in 10 to 15% of human liver cells and fibrinogen in about 1%. Only an occasional cell contained both proteins when combined fluorescein and rhodamine labeling was used. They also found both proteins in Kupffer cells. Barnhart (21) found

bovine prothrombin in unevenly scattered liver parenchymal cells. Further experiments with dogs given vitamin K indicated that 3 hours after receiving the drug, all the parenchymal cells contained prothrombin (22, 23), but only later was a rise in the plasma level observed. These latter findings parallel the observations in the present study of a rise in the number of haptoglobin-containing liver parenchymal cells after stimulation, before the maximal rise in plasma concentration occurred.

Summary

Haptoglobin was localized by specific immunofluorescence in the tissues of dogs during the response to experimental inflammation. Specific fluorescence was found solely in islands of hepatic parenchymal cells and Kupffer cells and in a few macrophage-like cells of the spleen. Kinetic considerations indicate that the parenchymal cells are most likely the sites of synthesis of haptoglobin and that the Kupffer cells and splenic cells are involved in catabolism of haptoglobin-hemoglobin complexes or haptoglobin.

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