

CONTROL OF THE ACUTE PHASE RESPONSE

Demonstration of C-Reactive Protein Synthesis and Secretion by Hepatocytes during Acute Inflammation in the Rabbit*

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C-reactive protein (CRP)¹ is an acute phase reactant (1, 2), normally present in serum in trace amounts, whose concentration rises rapidly and markedly with tissue injury and inflammation (3). Recent studies have shown this protein to be composed of five subunits in cyclic symmetry, and showing structural homology to the P component of amyloid and to C1t, a serum protein which may be involved in regulation of complement C1 function (4, 5). It seems likely that CRP plays a role in modulating inflammatory and immune responses as a result of its ability: (a) to activate the complement system by reacting with a variety of substrates (6, 7), (b) to inhibit platelet aggregation and mediator release (8), and (c) to bind to T lymphocytes and suppress certain lymphocyte functions (9).

It has been shown by studies of minced rabbit hepatic tissue and of perfused isolated rabbit liver that CRP is synthesized by the liver (10-12). The rate of hepatic synthesis of this protein accelerates rapidly after acute inflammatory stimuli (12). The cell of origin of CRP has not been demonstrated; it is uncertain whether this protein is synthesized by hepatocytes, by Kupffer cells, or by one of the other cell types found in liver (13). It is not known whether increased hepatic CRP synthesis after inflammatory stimulus results from increased formation of this protein by a fixed number of cells or from an increase in number of CRP-forming cells as a result of either cell replication or of recruitment of cells to CRP formation.

The current study was undertaken to answer these questions; such information would permit further studies delineating the precise mechanisms by which tissue injury leads to the CRP response specifically, and would cast light on the acute phase response in general. In addition, since colchicine has been shown to inhibit secretion of a variety of plasma proteins by the cells in which they are synthesized (14-17), the effect of administration of this drug upon cellular localization of CRP during acute inflammation was investigated.

Materials and Methods

Preparation of Peroxidase-Labeled Antibody. Goat antiserum to rabbit CRP (18) was absorbed with lyophilized normal rabbit serum to eliminate trace reactivity with normal serum constitu-

* Supported by U. S. Public Health Service grant AI-12886 and by a grant from INSERM (76 508 17). Performed in part during the tenure of a Senior International Fellowship (F06-TW00091) of the Fogarty International Center of the National Institutes of Health by Dr. Kushner.

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¹ Abbreviations used in this paper: CRP, C-reactive protein; GA, Golgi apparatus; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.

ents. Antibodies against CRP were purified from this antiserum employing glutaraldehyde-polymerized, CRP-rich acute phase rabbit serum as immunoadsorbent (19). Purified antibodies thus obtained were concentrated and labeled with horse-radish peroxidase (Boehringer, Mannheim, Grade I, West Germany) (20). Before use, labeled antibodies were diluted 20-fold with a mixture of 0.9% NaCl and normal rabbit serum without detectable CRP; the latter was used to eliminate nonspecific background staining of tissue.

Induction of Inflammation, Collection of Serum and Tissue, Administration of Colchicine. Adult rabbits weighing about 2 kg were injected with 1.5 cubic centimeters of sterile turpentine into each thigh. Blood specimens were obtained from the marginal ear vein before turpentine injection. Groups of three animals were sacrificed at approximately 8, 16, 24, and 38 h after injection. To inhibit release of CRP by cells, one animal in each group received colchicine (Sigma Chemical Co., St. Louis, Mo., lot 53c1.680) in a dose of 30–50 mg/kg intravenously, 2.5–3.5 h before sacrifice. To compare changes in serum CRP levels in animals which did and did not receive colchicine, blood specimens were obtained from all the animals in each group at the approximate time that one of them received colchicine, and at intervals thereafter until sacrifice. Serum was stored at -20°C before study. CRP concentration was determined by a radial immunodiffusion method, sensitive to $2\ \mu\text{g/ml}$ (21). As controls, three rabbits which had not received turpentine were studied; CRP was undetectable in their sera. Colchicine was administered to one of these animals 3 h before sacrifice.

Histologic, Immunocytochemical, and Ultrastructural Studies. Immediately after sacrifice by air embolus, slices of liver about 2 mm thick were placed in chilled 10% paraformaldehyde (22) in sodium phosphate buffer 0.1 M, pH 7.4, fixed for 8 h at 4°C , and then washed for 48 h at 4°C in several changes of buffer. $8\text{-}\mu\text{m}$ thick specimens were cut with a cryostat and were incubated with the peroxidase-labeled antibodies for 1 h at room temperature. After incubation, sections were washed and peroxidase was demonstrated by the method of Graham and Karnovsky (23). The sections were postfixated in 1.5% osmium tetroxide solution buffered with Veronal buffer, pH 7.2 for 20 min, dehydrated in graded ethanols, placed on a plastic sheet, embedded in Epon and studied under a light microscope. Ultrathin sections were then made at the levels of the cells in which a reaction was noted by light microscopy and were examined without further staining in a Siemens Elmiskop IA electron microscope. To establish specificity, staining was abolished by addition to the peroxidase-labeled antibody of either CRP-rich acute phase rabbit serum or of CRP, purified by the method of Riley and Coleman (24).

Results

Serum CRP Concentrations. CRP could not be detected in serum at the time of turpentine injection in any animal studied. After intramuscular injection of turpentine, serum CRP concentration rose sharply (25) to a plateau at 38 h (Fig. 1); study of specimens obtained between 38 and 72 h in other rabbits not reported here showed no subsequent rise. When colchicine was administered during the period of rapid rise in serum concentration, during the first 16 h after stimulus, the rate of rise was found to slow markedly (Fig. 2). Such an effect could not be studied after 16 h since the rapid rate of rise in serum CRP concentration abated after this initial period in all animals (Fig. 1).

Immunohistologic and Ultrastructural Findings—Colchicine not Administered. On light microscopy the reaction indicating the presence of CRP was expressed as dark brown deposits usually diffusely distributed in the cytoplasm of hepatocytes (Fig. 3 A). CRP was not detected in nuclei of these cells. No staining of Kupffer cells nor of other hepatic cell types was noted. Nonspecific peroxidase staining of erythrocytes was found.

At 8 h after turpentine injection, in animals not receiving colchicine, CRP was detected in the cytoplasm of a few hepatocytes in some periportal areas (Fig. 3 B), with mild to moderate intensity of staining. Most of the tissue was devoid of CRP-containing cells. By 16 h, larger numbers of more intensely stained cells were detected in many periportal areas and elsewhere in the peripheral portion

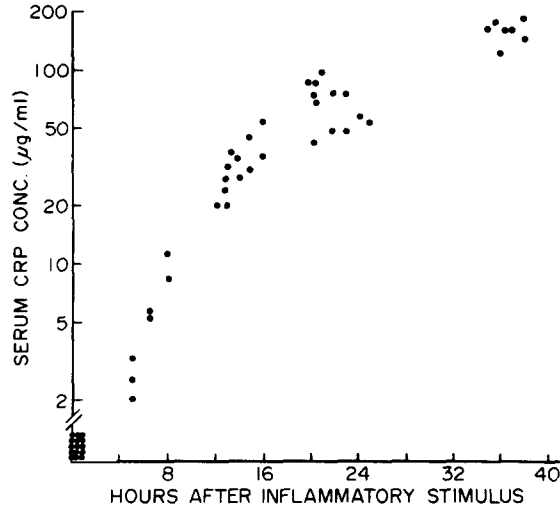


FIG. 1. Composite serum CRP response after intramuscular turpentine injection in 15 rabbits. Turpentine was administered at zero time; CRP was undetectable in sera of all animals before injection. Blood specimens were obtained periodically until time of sacrifice. Serum CRP concentrations found in all samples, except those after colchicine administration, are shown.

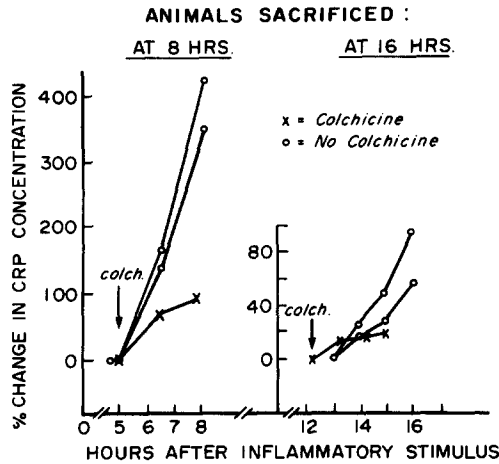


FIG. 2. Effect of colchicine on change in serum CRP concentration. Percentage increase is compared in the one animal receiving colchicine and two animals not receiving colchicine in two groups of animals: those sacrificed at 8 h and those sacrificed between 15 and 16 h after turpentine administration. Time of colchicine administration is indicated by an arrow. Zero on the ordinate indicates serum CRP concentration at the time of colchicine administration and at a comparable time in the control animals. In each instance the noncolchicine treated animals continued to show a marked rise in CRP concentration while colchicine administration was followed by a substantially diminished rate of rise.

of hepatic lobules (Fig. 3 C).

The maximum number of CRP-containing cells was seen at 24 h. Moderate numbers of positive hepatocytes were found in midportions and periphery of hepatic lobules (Fig. 3 D). Positive staining was also found in vessel lumina,

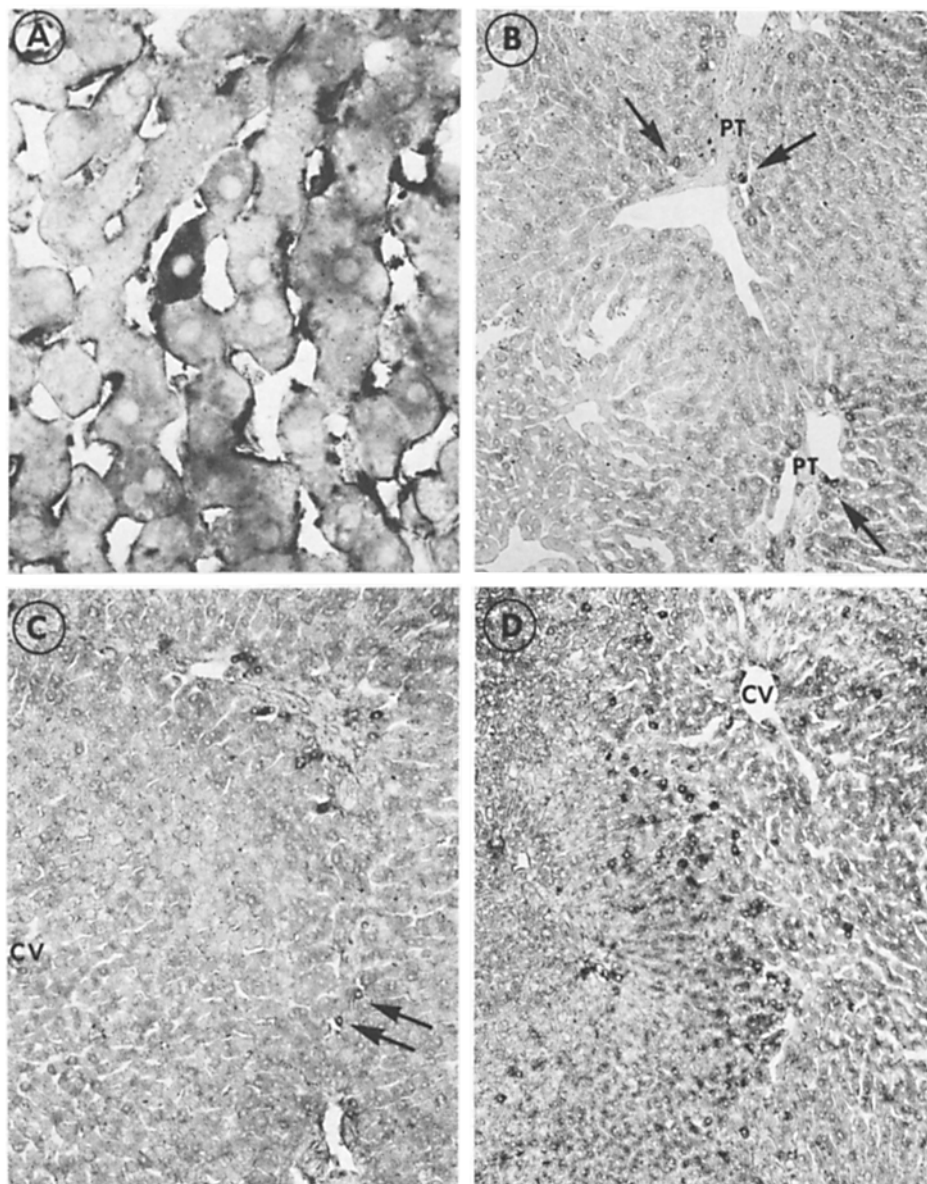


FIG. 3. Hepatic localization of CRP in animals which did not receive colchicine. CV, central vein; PT, portal tract. (A) Diffuse distribution of dark deposits indicating presence of CRP in cytoplasm of hepatocyte 38 h after intramuscular administration of turpentine. CRP is also detected in sinusoidal margins. ($\times 500$) (B) Small number of CRP-containing hepatocytes in periportal sites 8 h after turpentine injection. Arrows indicate several of the more intensely staining cells. Most periportal areas showed no such cells. ($\times 80$) (C) CRP-containing cells are seen 16 h after turpentine injection surrounding a portal tract in upper portion of the figure; two such cells are also seen toward bottom of figure at periphery of lobule (arrows). Larger numbers of more intensely staining cells are found than were seen at 8 h ($\times 95$) (D) CRP-containing cells distributed in midportions and periphery of lobule 24 h after turpentine injection. ($\times 80$).

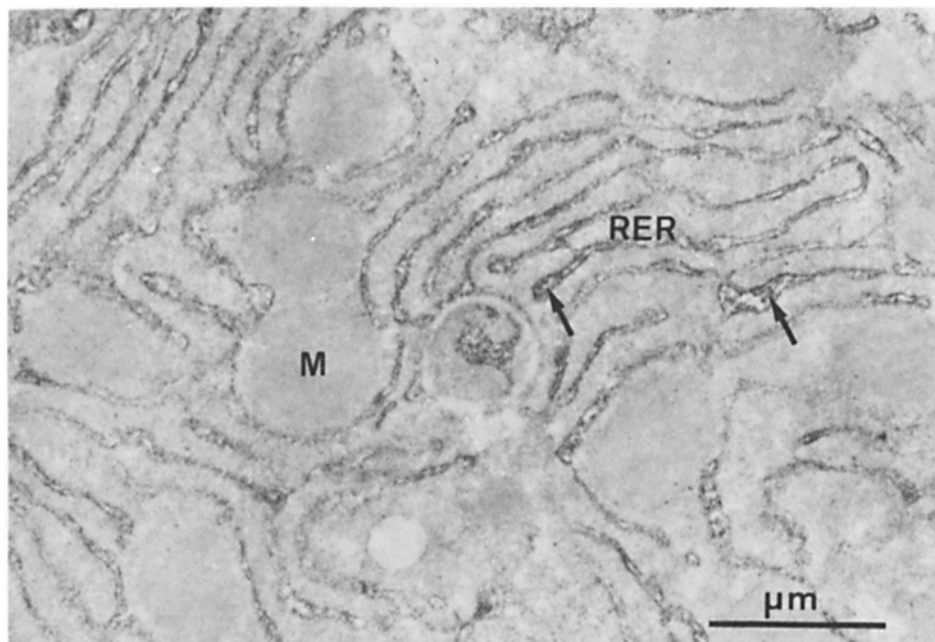


FIG. 4. Electron microscopic localization of CRP in an animal which did not receive colchicine. Part of the cytoplasm of an hepatocyte from a rabbit sacrificed 24 h after turpentine injection showing electron dense deposits reflecting CRP on the membranes of the RER. Dense deposits are also present in the lumen of this organelle (arrow). M, mitochondria. ($\times 29,000$).

sinusoidal margins (Fig. 3 A), and in interfibrillar spaces of portal areas at 24 and 38 h. By 38 h, positive cells were detected in clumps about central veins as well as in midlobular and periportal areas. Somewhat fewer cells appeared to contain CRP than had been noted at 24 h. Since intrahepatic distribution of cells staining for CRP was not random, it was not possible to quantitate the change in number of positive cells with time. After addition of purified rabbit CRP or acute phase rabbit serum to the peroxidase-labeled antibody, all staining, except that of erythrocytes, was abolished.

On electron microscopic study of tissue from animals which had not received colchicine, electron dense deposits indicating the presence of CRP were visible in hepatocytes, and no other cell type, at each time interval studied. In general, deposits were found on membranes of rough endoplasmic reticulum (RER) and, less markedly, in lumina of RER, smooth endoplasmic reticulum (SER), and Golgi apparatus (GA). No CRP was detected on other cytoplasmic organelles. At 8 and 16 h, a small proportion of RER membranes showed the presence of CRP; little or none was found in the lumen of this organelle. At 24 h, CRP was localized to the bulk of RER membranes; the lumen of the RER was also positive (Fig. 4), as was the lumen of the GA. Similar findings, but somewhat less intense and appearing to involve fewer cells, were noted at 38 h. At 16 h and subsequently, CRP was detected in lumina and margins of sinusoids.

Immunohistologic and Ultrastructural Findings—Colchicine-Treated Ani-

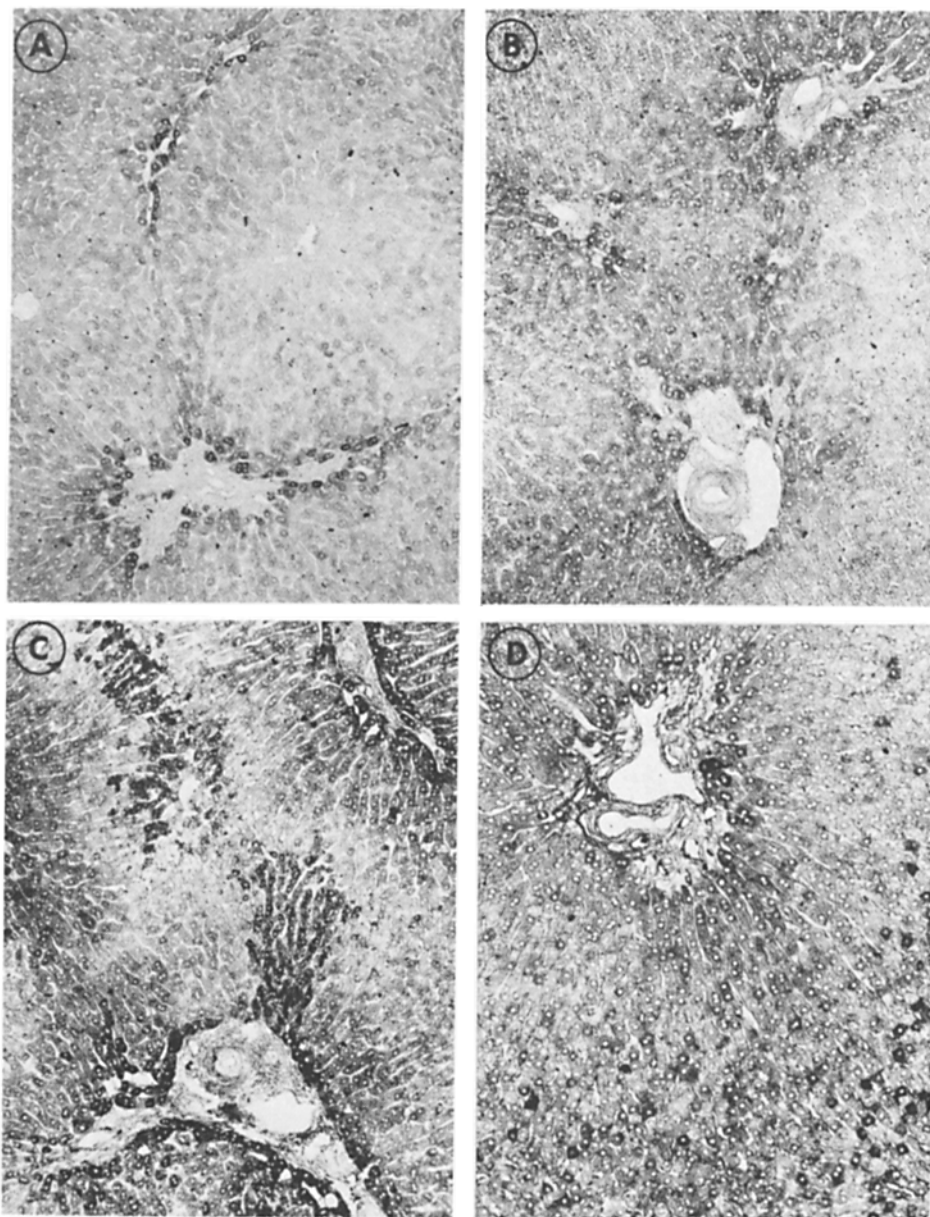


FIG. 5. Hepatic localization of CRP in colchicine-treated animals. (A) CRP detected in hepatocytes at periphery of lobule, 8 h after turpentine injection. ($\times 80$) (B) Larger numbers of hepatocytes containing CRP, surrounding and connecting three portal areas 15 h after inflammatory stimulus. Many cells show heavy perinuclear accentuation. ($\times 85$) (C) CRP-containing hepatocytes are seen in periportal areas and in bands of cells extending into the lobule, 24 h after turpentine injection. Both diffuse cytoplasmic and perinuclear staining are seen. ($\times 80$) (D) 38 h after inflammatory stimulus, CRP is detected in either diffuse cytoplasmic or periportal distribution in virtually all cells of this section. ($\times 110$)

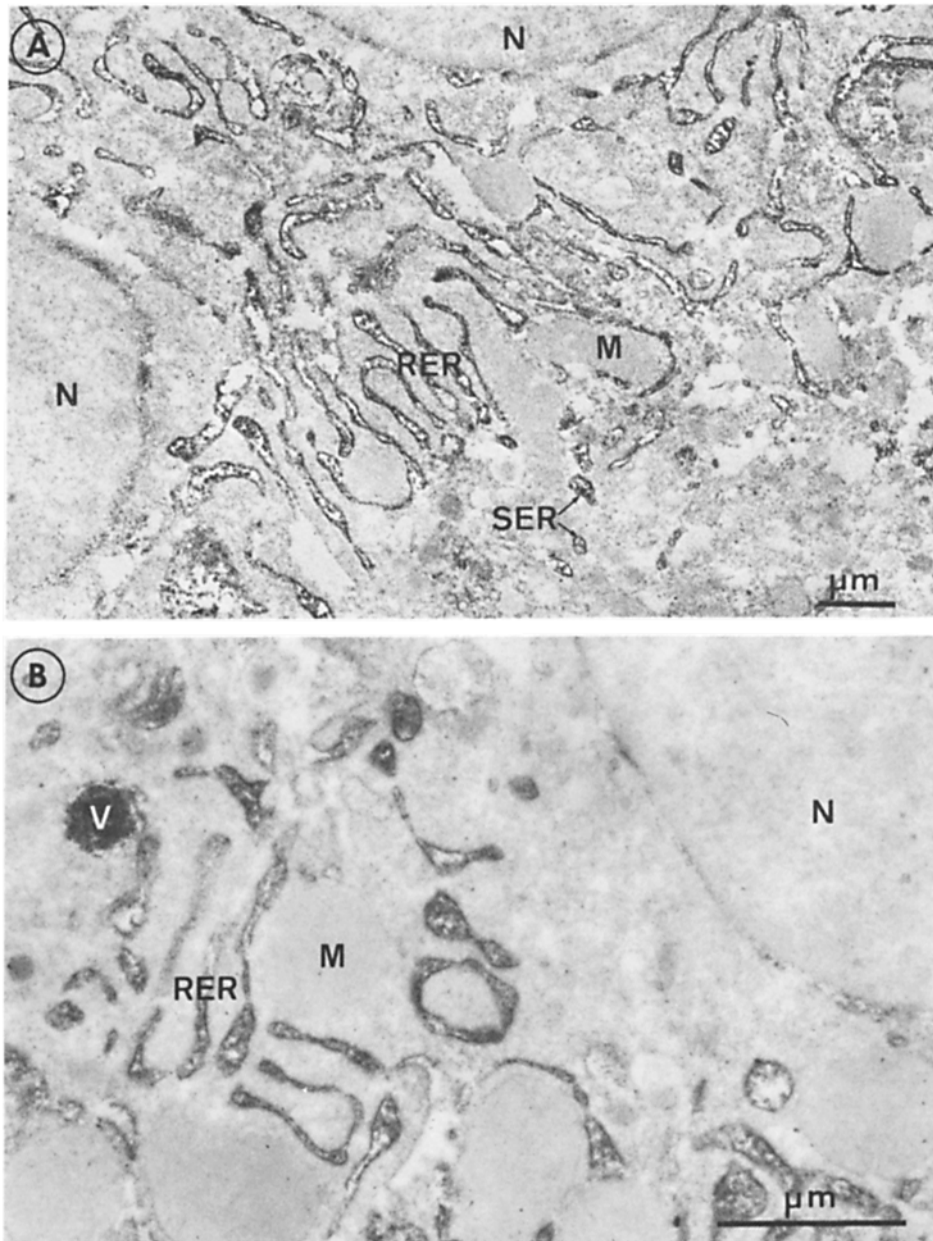


FIG. 6. Electron microscopic localization of CRP in hepatocytes of an animal sacrificed 38 h after turpentine injection. The animal received colchicine 3 h before sacrifice. M, mitochondria; N, nucleus; V, vacuole. (A) Part of a binucleated hepatocyte. Electron dense deposits reflecting CRP are mainly visible on the membranes and in the lumen of the RER located around the nuclei. Some CRP is present in the SER. ($\times 13,000$) (B) A large amount of CRP is visible in the lumen of the RER. CRP is also present in some vacuoles (V). ($\times 31,000$)

mals. In general, light microscopic studies of colchicine-treated rabbits revealed more intense staining reactions and greater numbers of CRP-containing hepatocytes than were seen without colchicine. In addition to diffuse cytoplasmic CRP localization seen in some cells, many other cells showed intense perinuclear staining. Such an intracellular distribution of CRP was only rarely seen in animals not receiving colchicine.

At 8 h after turpentine injection, moderate numbers of CRP-containing cells were found in most periportal areas, often outlining hepatic lobules (Fig. 5 A); intensity of staining was stronger than without colchicine. By 15 h, positive cells, often with perinuclear staining, were found several layers deep, surrounding and connecting portal areas (Fig. 5 B). 24 h after turpentine, bands of positive cells were seen extending from periportal areas into lobules (Fig. 5 C). At 38 h, the peak of the serum response, most but not all hepatic cells were positive for CRP (Fig. 5 D).

Electron microscopic studies of tissue from colchicine-treated animals showed accumulation of CRP on RER, SER, and GA. Intensity of staining was more marked than without colchicine, and was greatest at 24 and 38 h when perinuclear RER staining was most marked (Fig. 6 A, B). In addition, particularly at 24 and 38 h, CRP was visible in cytoplasmic vacuoles of about 0.3-1 μm diameter (Fig. 6 B), at times close to the GA, at other times close to the vascular pole. In some of these vacuoles, usually full of CRP, electron-dense lipid globules were also noted. Sinusoidal lumina did not show staining for CRP in these specimens.

CRP could not be detected in liver sections from uninflamed animals who had not received turpentine, whether or not colchicine had been administered.

Discussion

The observation that hepatocytes were the only cells in liver which could be shown by light microscopic studies to contain CRP during the acute phase response strongly suggests that these cells are the site of origin of this protein. This conclusion is confirmed by ultrastructural studies demonstrating localization of this protein to subcellular organelles known to be sites of protein synthesis and secretion: the RER, SER, and GA (26).

The staining of sinusoidal margins seen by light microscopy, 24 and 38 h after stimulus, was found on electron microscopic study to lie in sinusoidal lumina and adjacent margins rather than within cellular processes of Kupffer cells. Failure to find such staining in animals who were not injected with turpentine or who were studied less than 16 h after inflammatory stimulus indicates that this staining represents CRP in the blood of sinusoidal lumina rather than nonspecific binding of antibody to Fc receptors of sinusoidal lining cells.

The current findings of progressive increase in number of CRP-producing hepatocytes with time suggest that the effect of inflammation is to cause hepatocytes which are not producing CRP to begin synthesis and secretion of this protein, presumably as a result of derepression. The alternative explanations: replication of CRP-producing cells or increased CRP synthesis by a fixed number of cells do not seem consistent with our findings. Increased output by a small number of cells is clearly excluded by the demonstration of increasing

numbers of positive cells, with most cells in the liver making CRP at the peak of the response. Replication would have been expected to result in localized nests of CRP-producing cells throughout the liver rather than the diffuse involvement seen. However, it is possible that cell replication may play some role in the CRP response.

After inflammatory stimulus, the first cells shown to be producing CRP were adjacent to portal areas, with successive appearance, with time, of CRP-forming cells closer to the center of the lobule. Such a sequence could result from a variety of mechanisms. It may be that a circulating mediator, possibly originating at the inflammatory site (2), enters hepatic lobules via portal areas. As has been emphasized by Rappaport et al. (27), hepatocytes at the periphery of the lobule are the first to be exposed to blood-borne constituents and may be the first to respond to such a mediator. Alternatively, portal areas are the site of entry of sympathetic nerve fibers into hepatic parenchyma (28); it may be that neurogenic factors mediate elements of the acute phase response (29). On the other hand, intralobular biochemical (30) or ultrastructural heterogeneity (31) may reflect differences in the capacity of peripheral and centrilobular hepatocytes to respond to the stimuli leading to CRP formation. Ultrastructural differences between these cells have been shown, e.g., human periportal cells contain more RER than do centrilobular cells (31). Such cells may be more readily committed to CRP synthesis.

The ability of colchicine to inhibit secretion of newly synthesized CRP by hepatocytes was demonstrated morphologically. This conclusion is consistent with our findings in serum, in which the rate of rise in CRP concentration was markedly decreased after colchicine administration. Such an effect of colchicine on secretion of other hepatocyte-produced plasma proteins has been shown (14-17). Electron microscopic studies indicated that the perinuclear staining found in large numbers of cells after colchicine administration appeared to represent CRP-filled RER. Such a phenomenon has been noted after colchicine administration when fibrinogen was localized in liver employing peroxidase-labeled antibodies (17). The finding of CRP in cytoplasmic vacuoles, sometimes in association with electron-dense lipid globules, suggests sharing of secretory vacuoles by CRP, very low density lipoproteins, and perhaps other plasma proteins, in a manner consistent with that suggested by Redman et al. (16) and by Palade (26).

The number of cells shown to be synthesizing CRP at the peak of the response is remarkable, particularly since it is known that a number of other acute phase proteins are also being synthesized at an increased rate by the liver at this stage (2). These findings suggest that individual liver cells may synthesize and secrete several proteins at the same time or may alternate between the synthesis of one protein and another over short periods of time.

The demonstration that colchicine-treated animals showed many more and stronger staining positive cells than did untreated animals may partially explain previous difficulties encountered in demonstrating CRP-producing cells in liver employing immunofluorescent methods (18). In those studies, in which colchicine was not administered, the rapid secretion rate in most cells would have greatly diminished the likelihood of detecting positive cells. In addition,

the use of different fixation methods in the current study may have played a role in permitting demonstration of CRP-forming cells.

Summary

To determine the cell of origin of C-reactive protein (CRP) and to cast light on the mechanisms leading to the acute phase response, we used an immunoenzymatic technique to visualize this protein in livers from rabbits at intervals after intramuscular injection of turpentine. CRP was detected only in hepatocytes. 8 h after turpentine injection, CRP was demonstrated in occasional periportal hepatocytes. With time, larger numbers of positive cells were detected successively in perilobular, midlobular, and centrilobular areas. On electron microscopy, CRP was detected in rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), and Golgi apparatus (GA). When colchicine was administered to inhibit cellular secretion of CRP, intensity of reaction and number of CRP-containing hepatocytes were substantially greater than without colchicine, but the sequence of intralobular distribution was similar. At peak serum response 38 h after turpentine injection, CRP could be demonstrated in most hepatocytes. Electron microscopic studies showed accumulation of CRP on membranes and lumina of RER, SER, GA, and in cytoplasmic vacuoles. These findings indicate that CRP is produced by progressively increasing numbers of hepatocytes after inflammatory stimulus and suggest that a mediator, acting initially in portal zones, is responsible for recruitment of cells to CRP production.

We are grateful for the technical assistance of David M. Kushner, Michelle Maurice, and Debra Schultz. The advice and support of Therese Ternynck and Stratis Avrameas were invaluable. Mrs. Helen Morrison's secretarial assistance is acknowledged with gratitude.

Received for publication 3 May 1978.

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