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SYNTHESIS AND SECRETION OF SERUM AMYLOID PROTEIN A (SAA) BY HEPATOCYTES IN MICE TREATED WITH CASEIN[1]

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SAA is an acute phase reactant and precursor of the major constituent of secondary amyloid fibrils. Studies were done to demonstrate the *in vivo* synthesis of SAA by hepatocytes. CBA/J mice were injected with 10% casein and serum and hepatic levels measured by radioimmunoassay at 8, 18, 24, and 32 hr. SAA serum levels were elevated by 8 hr (633 ± 49 U/ml, with peak levels at 18 to 24 hr 1933 ± 232 units). Liver tissue levels peaked at 439 ± 37 U/gm at 24 hr and then decreased. In casein-treated animals given colchicine, which inhibits cellular release of protein, there was a blunting of the serum response and a concomitant accumulation of SAA in hepatic tissues (2375 U/gm at 24 hr).

Immunohistochemistry with specific antiserum to protein AA showed localization of SAA in hepatocytes starting in periportal areas at 8 hr, and spreading over the entire lobule by 24 hr, when staining was maximal. Colchicine markedly enhanced staining and correlated with hepatic tissue levels of SAA. No localization in spleen sections was found, and minimal levels of SAA were found in spleen tissue by radioimmunoassay.

These data are consistent with hepatic synthesis of SAA mediated by a humoral factor that is generated as part of an inflammatory response. Relatively low levels of hepatic SAA are present in casein-treated animals, because SAA exits from the cell very rapidly to give high serum levels within 18 to 24 hr. These kinetics are almost identical to those seen with C-reactive protein in the rabbit, where a humoral factor generated by inflammatory response is also postulated.

Systemic secondary amyloidosis is the result of extracellular deposition of a fibrillar proteinaceous material of which the main constituent is protein AA, a single chain polypeptide of 76 amino acids and m.w. 8400 (1–4). This protein is believed to be derived from a serum protein, serum amyloid protein A (SAA),[4] which shows immunologic cross-reactivity with AA (5–7). Although antiserum to AA detects SAA in serum fractions with m.w. of 80,000 to 200,000, all of the antigenic determinants that are common to SAA and AA can be isolated from serum under dissociating conditions as a 12,500 dalton protein (8–10). This small serum protein is homologous at the amino terminus with fibril protein AA. It is assumed, although not proven, that proteolytic cleavage of SAA towards the carboxy terminus leads to the generation of the 8400 dalton AA protein (11, 12).

Studies have been reported from several different laboratories suggesting that SAA is synthesized in a number of tissues. These include fibroblasts (13, 14), lymphoid cells of the spleen (15, 16), and polymorphonuclear leukocytes (17). Analysis of tissue homogenates from mice treated with endotoxin has shown the highest levels in liver, with somewhat lower levels in kidney (18). SAA, although present in all normal sera, behaves as an acute phase reactant with serum levels increasing several hundred fold at times of acute inflammation (18–22). This behavior is not unlike C-reactive protein that is now known to be synthesized in the liver, probably under the direction of a humoral factor produced by the inflammatory process (23). Recent studies with ethionine, a blocker of hepatic protein synthesis, also suggest an hepatic origin for SAA (24). For these reasons, we have taken a new look at the liver as the possible site of synthesis of SAA.

MATERIALS AND METHODS

Animals. Eight- to 10-week-old CBA/J female mice were obtained from The Jackson Laboratory (Bar Harbor, Maine).

Chemicals. Casein was obtained from Worthington Biochemicals and prepared as a 10% solution in sodium bicarbonate (22). Colchicine was obtained in sterile ampules with 1 mg/2 ml (Eli Lilly Company, Indianapolis, Ind.).

Antiserum. Antiserum to murine protein AA was prepared in New Zealand White rabbits as previously described by immunization with tissue AA in complete Freund's adjuvant (25). Antiserum from one rabbit was used for both radioimmunoassay and immunofluorescent studies (22). For immunofluorescent studies, antiserum was absorbed with lyophilized normal liver and spleen powder.

Experimental protocol. CBA/J mice were injected subcutaneously with 1 ml of 10% casein. Groups of six mice were sacrificed at 0, 8, 18, 24, and 32 hr. Similar groups of saline-injected mice were used as controls. Three mice from each group received 1 mg of colchicine by i.p. injection 3 hr before

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[4] Abbreviations used in this paper: SAA, serum amyloid protein A; CRP, C-reactive protein.

sacrifice. Mice were killed by cervical dislocation and bled via the jugular vein. Livers and spleens were removed immediately, washed free of blood, and weighed. Portions of liver and spleen were snap-frozen with Dry Ice-acetone for immunohistochemistry. Tissues for SAA determinations were processed without freezing.

Immunohistochemical staining. Sections (8 to 10 μ) of liver and spleen were prepared on a cryostat, placed on gelatinized slides, and fixed briefly with alcohol. The sections were washed in phosphate-buffered saline (PBS) and then incubated with anti-AA antiserum diluted 1:4 for 30 min at room temperature. After the three washes in PBS, rhodamine-labeled goat anti-rabbit IgG (Cappel Laboratory, Cochranville, Pa.) was applied for 30 min at 4°C. The sections were then washed with PBS, mounted in glycerol, and observed in a fluorescence microscope with vertical illuminator (Zeiss Instruments Company, Germany). Peroxidase-labeled goat anti-rabbit IgG was also used as second antibody in a similar fashion. These sections were stained with diaminobenzidine and observed by light microscopy. Some sections were treated with human serum having high titer antinuclear antibody (homogeneous pattern) and then stained with fluorescein-labeled goat anti-human IgG before staining for protein AA.

Radioimmunoassay. Radioimmunoassay was performed as described previously by using rabbit anti-murine AA and ^{125}I-labeled AA (22). Pooled serum from mice treated with casein was used as a standard and SAA levels were reported as microliter units of that standard. Serum from experimental and control mice was obtained at the time of sacrifice. Blood samples were allowed to clot, centrifuged, and stored at −70°C until assayed. Liver and spleen tissues were washed with saline to clear blood from the organs, homogenized with a polytron (Brinkman), and extracted with saline. Aliquots were used for determination of SAA level, which was reported as units SAA/g of wet tissue weight.

RESULTS

Serum and hepatic tissue levels of SAA. Mean serum level of SAA in untreated CBA/J mice was 27 ± 16 U/ml. SAA measurements in homogenized hepatic tissue of untreated mice was 10 ± 4 μ/gm wet weight. After one subcutaneous injection of casein, the serum level rose to a mean of 633 ± 49 U/ml at 8 hr and peaked at 18 hr (1933 ± 232 U/ml) (Fig. 1). By 32 hr serum SAA level had dropped to 1150 ± 102 U/ml. In the same group of mice sacrificed at varying intervals, hepatic tissue levels of SAA rose over the same time course as did the serum levels. At 8 hr liver SAA level was only 76 ± 14 U/gm wet weight, but rose to 390 ± 16 U/gm by 18 hr. The mean peak level of SAA was 439 ± 37 at 24 hr. By 32 hr the hepatic SAA concentration had fallen to 267 ± 40 U/gm of wet weight.

Effect of colchicine on serum and hepatic tissue levels of SAA. In colchicine-treated animals the mean serum SAA level was only 395 ± 16 U/ml 8 hr after receiving casein (Fig. 2). The peak SAA serum level was 750 ± 62 U/ml at 24 hr compared with a peak serum level of 1933 ± 232 U/ml in mice that did not receive colchicine. SAA level fell to 516 ± 58 by 32 hr. At the same time, hepatic tissue levels of SAA rose to 397 ± 12 U/

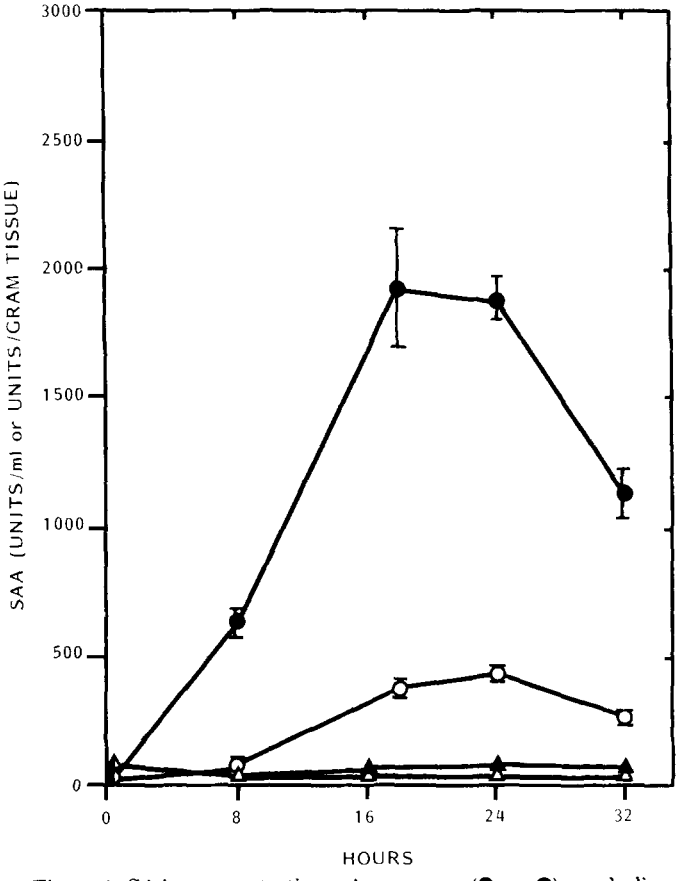

Figure 1. SAA concentrations in serum (●——●) and liver (○——○) of CBA/J mice treated with casein at time zero. Saline-treated control mice gave no rise in SAA levels in either serum (▲——▲) or liver (△——△).

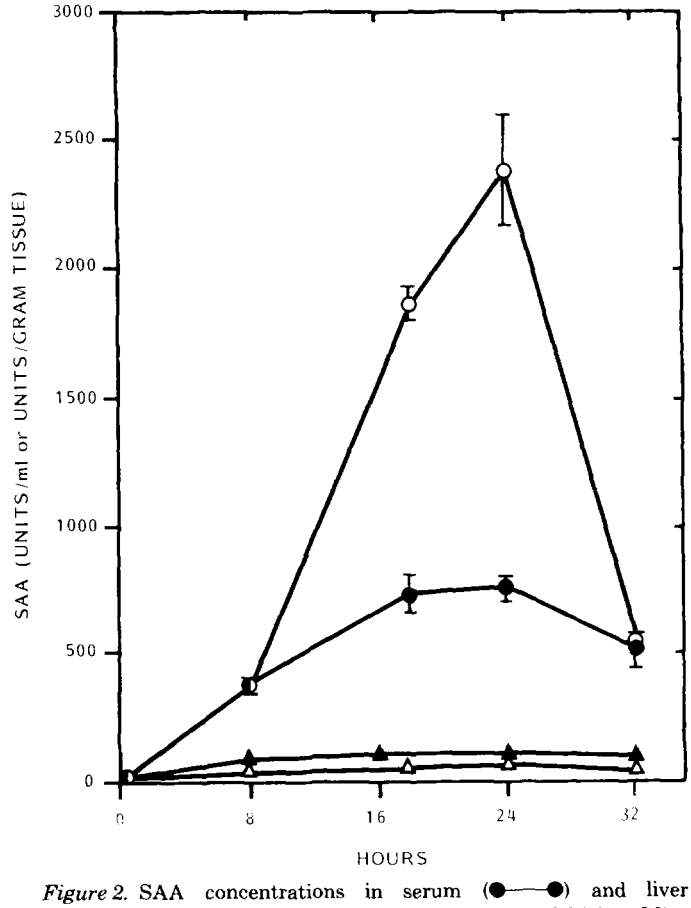

Figure 2. SAA concentrations in serum (●——●) and liver (○——○) of CBA/J mice treated with casein plus colchicine. Mice treated with colchicine alone had no rise in serum (▲——▲) or hepatic (△——△) levels of SAA.

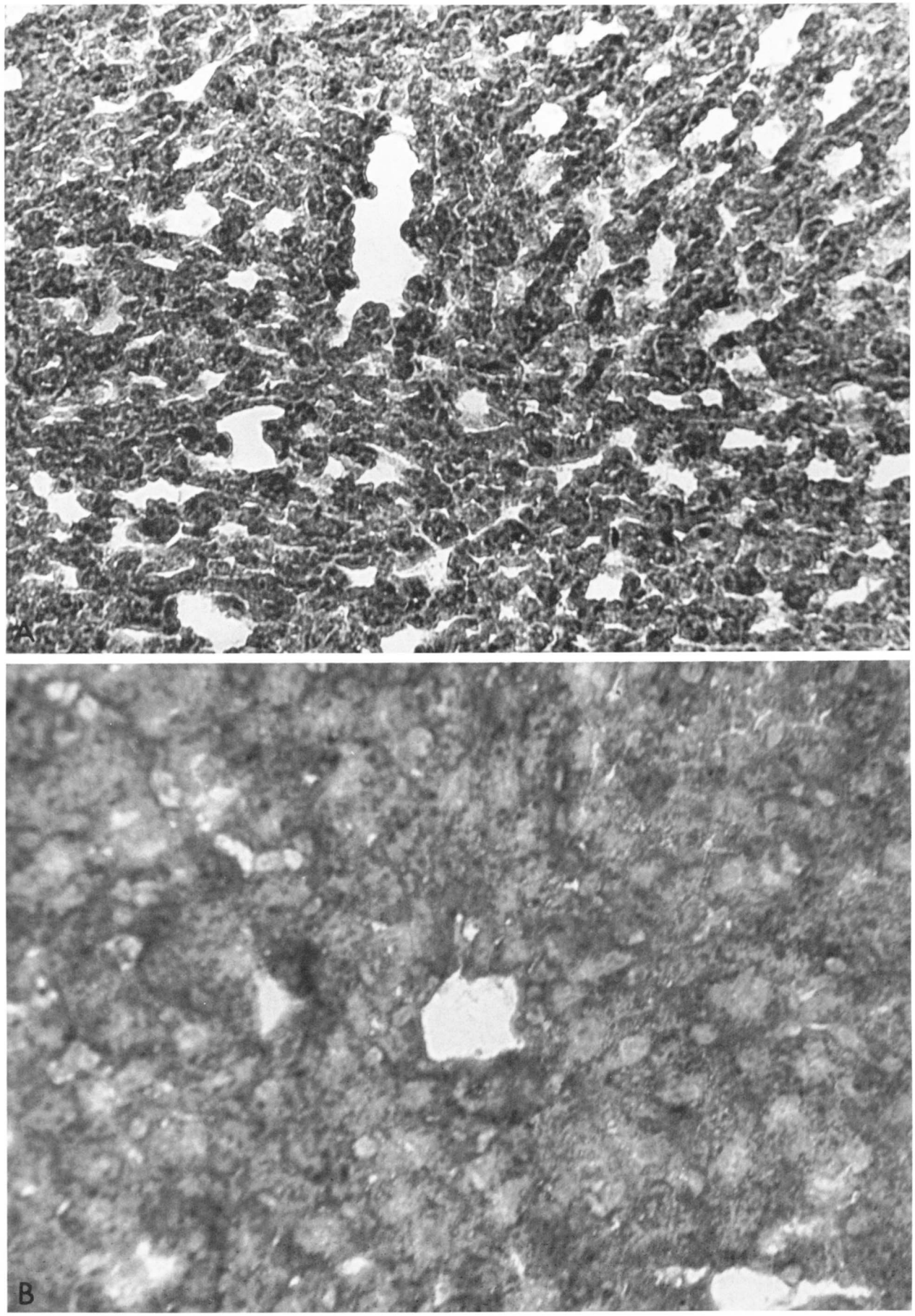

Figure 3. Hepatic localization of SAA in mice treated with casein and colchicine. *A*, section of liver 24 hr after casein injection stained with HRP-labeled antiserum. Dark deposits of reaction product indicate presence of SAA in cytoplasm of most hepatocytes (× 200); *B*, control liver section treated with normal rabbit serum and HRP-labeled anti-rabbit IgG (× 200).

ml wet weight by 8 hr and peaked at 2375 ± 222 U/gm at 24 hr. By 32 hr the mean SAA hepatic tissue level was down to 534 ± 8 U/gm wet weight. Control animals receiving colchicine alone did not have a significant rise in serum or hepatic tissue SAA levels at any point in the course of the experiment.

Immunofluorescent and immunoperoxidase studies. Indirect immunofluorescent studies in which antiserum specific for protein AA was used showed no localization in either liver or spleen sections of untreated mice. Sections from livers of mice treated with casein showed slight staining of hepatocytes by anti-AA. Spleen sections from casein-treated mice failed to show any localization of tissue antigen with the anti-AA antiserum. The livers from mice receiving casein plus colchicine showed staining with labeled antibody in the hepatic cells by 8 hr. Most intense staining was seen at 18 or 24 hr, and a lesser degree of staining was seen at 32 hr. Controls treated with normal rabbit serum failed to show staining. Staining of rabbit anti-AA-treated sections from animals sacrificed at 8, 18, 24, and 32 hr with peroxidase-labeled goat anti-rabbit IgG showed localization of staining in hepatic cells. Maximal staining was at 18 or 24 hr, and was comparable to the sections stained with fluorescein-labeled antiserum (Fig. 3). In some sections it appeared that periportal and midlobular areas were stained at 8 hr, whereas entire lobules became stained by 18 hr. Control sections of liver from untreated animals and liver sections from mice treated with casein plus colchicine but incubated with normal rabbit serum instead of anti-AA showed no localization of staining.

DISCUSSION

The kinetics of SAA appearance in the serum and liver in mice after an inflammatory stimulus are similar to those seen with C-reactive protein (CRP) in rabbits (18, 22, 23). Both acute phase reactants (SAA and CRP) appear in increased concentrations in liver and blood by 8 hr, peak at 18 to 24 hr, and decreased by 32 hr after a single inflammatory stimulus. The delay in increase of SAA and CRP of 2 to 3 hr and the experiments that have been reported in C3H/HeJ mice suggest that SAA synthesis is controlled by a humoral factor generated by an inflammatory stimulus (26–28). Kushner and Feldmann (23) have used the evidence of CRP appearance in periportal hepatocytes before central lobular hepatocytes as an indication of a circulating factor. CRP kinetics have not been investigated in the mouse system, and therefore only a comparison with the rabbit system can be made at this time.

Studies in other laboratories have suggested synthesis of SAA by embryonic fibroblasts, polymorphonuclear leukocytes, and spleen cells (13–16). In the present study no evidence of SAA synthesis in the spleen, either by measurement with radioimmunoassay or immunofluorescent staining with well-absorbed anti-AA antiserum, was noted. This is in contrast to a report by Watanabe *et al.* (15) showing double immunochemical staining of spleen cells for immunoglobulin and AA.

McAdam and Sipe (18) reported high levels of SAA in liver homogenates after LPS treatment of mice. They failed to find any effect on serum SAA concentration with pretreatment of the mice with colchicine, however. In the present experiments, a blunting of the serum response to casein was noted when animals were given colchicine 3 hr before sacrifice. The amount of colchicine used was very large in comparison with the amount used in experiments for the prevention of amyloid formation during chronic casein treatment (29, 30).

Colchicine has been demonstrated to bind to microtubules of cells and prevent excretion of synthesized proteins (31). In the dosage used, it is likely that this has allowed accumulation of sufficient SAA in hepatocytes to demonstrate the manufacture of SAA by these cells. This is analogous to the situation seen with CRP in rabbits (23). Like CRP, the increase of hepatic tissue levels of SAA corresponds chronologically with the relative decrease in serum level. It appears that synthesized SAA leaves the hepatocyte very quickly and enters the blood. After casein treatment alone, relatively low levels of hepatic SAA are found and only with the use of an agent that blocks excretion, such as colchicine, do high levels of tissue SAA occur. The rapid appearance of high levels in the serum, and a similar rapid disappearance if the inflammatory stimulus is not continued, is consistent with the acute phase nature of this protein.

In the CBA/J mouse it has been demonstrated that levels of SAA that occur after casein treatment are sufficient to suppress antibody synthesis by spleen cells (32, 33). This may be an important factor in the animals' response to antigenic stimulation either from exogenous or endogenous antigens. No effect of SAA on infectious agents or upon the inflammatory reaction has yet been demonstrated. The acute phase nature of SAA and the genetic stability of this protein both suggest that it has an important role in response to antigenic or inflammatory stimuli.

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