THE LIVER AS THE SITE OF C-REACTIVE PROTEIN FORMATION*

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C-reactive protein (CRP) is an acute phase serum protein that can be precipitated by the somatic C polysaccharide of pneumococci in the presence of calcium ions (1, 2). Analogous proteins have been found in the monkey and rabbit (3) and were shown to be immunologically related to the human CRP (4). The rabbit protein is called CxRP because it reacts only with the Cx-carbohydrate of pneumococci. The human CRP has been characterized immunoelectrophoretically as a rather long precipitation line in the fast γ-region (5).

The two different methods that have been used in the study of the site of CRP formation have given conflicting results. With the use of immunohistochemical techniques Kushner and Kaplan (6, 7) found localization of CxRP only to sites of inflammation, such as in skeletal (6) and heart (7) muscle fibers undergoing necrotic changes. No evidence of CxRP presence was obtained for any other tissue, including the liver. Such a localization seemed suggestive of local synthesis of CxRP or of its release from tissue constituents during degeneration. However, with the technique of incorporation of C^{14}-amino acids by tissues in vitro, Thorbecke et al. (8) and Asofsky et al. (9) found CRP production only in liver tissue from monkeys stimulated to produce this acute phase protein.

Since the methods of stimulation for CRP production employed in both these studies were different, the present experiments were undertaken to examine further the site of CRP and CxRP formation in vitro using an experimental design similar to that of Kushner and Kaplan (6), and including tissue cultures of liver as well as of degenerating muscle.

In a previous study using the technique of C^{14}-amino acid incorporation in vitro (9, 10), it was found that infection of mice with staphylococci greatly

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stimulated the production of various serum proteins by the liver. A similar effect can be obtained in mice with injection of endotoxin or subjection to blood loss (8). An additional aim of the present study was, therefore, to compare different methods of in vivo stimulation, in rabbits and monkeys, with respect to their effects on CRP and other serum protein production, including a few other acute phase proteins, by the liver.

Materials and Methods

Animals.—Attempts to stimulate CxRP production in male adult New Zealand *rabbits* were made by the following methods: (*a*) Injection into both gastrocnemius muscles of either 0.5 ml (low dose) or 2 ml (high dose) paratyphoid-typhoid vaccine (PT vaccine, Wyeth Laboratory, Marietta, Pa.) combined with some India ink in order to identify the site of injection; (*b*) injection of turpentine (2 ml), or croton oil (0.5 ml) into the gastrocnemius; (*c*) intravenous injection of 5 μg of a purified *Brucella equi* endotoxin; (*d*) removal of 60 ml blood from the ear. The animals were killed by exsanguination at various intervals after these treatments (see Table I). Time of appearance of CxRP in the serum was determined on repeated small bleedings from the ear.

Stimulation of CRP production in female *rhesus monkeys* was attempted by (*a*) intravenous injection of *E. coli* endotoxin (100 to 250 μg, Difco Laboratories, Detroit, Ill., 4 animals); (*b*) intramuscular or subcutaneous injection of 0.5 to 2 ml of an overnight culture of pneumococcus Type II (3 animals); (*c*) removal of 1 lobe of the liver (partial hepatectomy)[1] 5 days prior to sacrifice (1 animal).

Liver cultures from fetal monkeys, late in the gestation period, were kindly donated by Dr. A. M. Silverstein, Wilmer Institute of Ophthalmology, Johns Hopkins School of Medicine, Baltimore, Md.

Fresh sterile *human tissues* were obtained through biopsy or at the time of surgery. Thoracic duct lymph was obtained through the kindness of Dr. A. E. Dumont, Department of Surgery, New York University School of Medicine, New York. The tissues originated from patients with varying illnesses, some infectious, some tumorous in nature. Fetal livers were obtained from fetuses of 20 to 25 wk gestation (as judged by length).

Tissue Cultures and Analysis of Culture Fluids.—The methods for preparing tissue cultures, media, and handling of culture fluids prior to immunoelectrophoresis have been described in detail elsewhere (10–12). 100 to 200 mg of minced tissue was cultured in roller tubes for 24 to 48 hr with 2 ml of a medium containing 1 μc of L-isoleucine-C^{14} and L-lysine-C^{14} each per ml (Institut Pasteur, Paris, France, or Schwarz Bioresearch, Orangeburg, New York) of specific activity 600 to 1000 μc/mg. The modified Eagle's medium contained 0.5% ovalbumin as the only protein. Peripheral leukocytes from rabbits or monkeys were obtained through dextran sedimentation from heparinized blood. Usually, approximately 1 to 3×10^7 leukocytes were cultured in 2 ml of medium. After the culture period, media were dialyzed against 0.015 M phosphate buffer pH 7.2, and concentrated 15- to 20-fold by lyophilization.

These concentrated culture fluids were analyzed by means of autoradiography (10–12) of microimmunoelectrophoretic patterns (13). Appropriate "carrier" sera and antisera were used to develop the lines. For the analysis of rabbit tissue culture fluids the carriers used were normal and acute phase rabbit sera, and the antisera a specific sheep anti-CxRP and sheep anti-whole rabbit serum. The specific antiserum to CxRP was obtained through the kindness of Drs. E. Gotschlich, The Rockefeller University, New York, and H. F. Wood, Department of Pediatrics, Yale University School of Medicine, New Haven, Conn. This antiserum was

[1] We are greatly indebted to Dr. Bruce Hames, Department of Surgery, New York University School of Medicine, New York, for performing the partial hepatectomy for us.

prepared by immunization of sheep with CxRP, isolated by precipitation with Cx-poly saccharide, and complete Freund's adjuvant (14). Appropriate absorption with normal rabbit serum was performed to ensure specificity against CxRP. The sheep anti-whole rabbit serum was prepared for us by Dr. E. Kraft, Otisville Laboratories, New York City Department of Health. The precipitation arcs for albumin, transferrin, IgG, haptoglobin, and β_{1C} were identified by means of their characteristic position and appearance, staining for peroxidase activity (15), binding of $Fe^{59}Cl_3$ (16), and comparison with a specific duck anti-rabbit β_{1C} obtained through the kindness of Dr. H. J. Müller-Eberhard, Department of Experimental Pathology, Scripps Clinic and Basic Research Foundation, La Jolla, Calif. The anti-CxRP showed only one major line upon double diffusion in agar with acute phase rabbit serum, and none with normal rabbit serum. Upon immunoelectrophoresis of acute phase rabbit serum, this line appeared in the γ-region, in a position quite similar to the one described for human CRP (5).

The analysis of human and monkey tissue culture fluids was done with the aid of acute phase human and monkey sera as carrier sera and rabbit antisera to CRP (Schieffelin Co., New York) and to whole human serum, prepared and characterized as described previously (12).

For the immunoelectrophoretic analysis of the concentrated culture fluids, the carrier serum was added once to the antigen well, followed by three additions of the culture fluid. Usually, only one culture fluid was used per slide. After washing and drying of the slides, autoradiographs were made using Kodak Royal Pan film and an exposure time of 2 wk.

Histology.—Representative pieces of cultured tissues were fixed in a 90:5:5 mixture of Zenker's solution, neutral formalin 10%, and trichloroacetic acid 2%. Sections were stained with methyl green-pyronin (17).

RESULTS

Rabbits.—Appearance of CxRP in the serum of the experimental animals resulted from injection of endotoxin, turpentine, croton oil, or PT vaccine, but not from bleeding. The sera of the responding animals showed a line for CxRP upon double diffusion in agar at 24 hr after stimulation. The low dose of PT vaccine induced only a weak response at 24 hr, and further experiments about the temporal appearance of CxRP were, therefore, performed with the higher dose. No significant amount of CxRP could be detected in the sera from animals at 9 to 12 hr, but a prominent line was seen 18, 24, and 48 hr after a high dose of PT vaccine. At 72 hr the amount of CxRP had decreased considerably. A few acute phase sera containing CxRP were also compared in immunoelectrophoresis to a pretreatment serum sample from the same rabbit. A clear cut increase in haptoglobin was evident from the shape and position of the haptoglobin arc.

The examination of culture fluids with autoradiography of immunoelectrophoretic patterns showed that only livers from appropriately stimulated rabbits incorporated C^{14}-amino acid into CxRP. The livers from normal rabbits, from rabbits stimulated 3 to 9 hr previously, and from the bled rabbit did not produce detectable labeling of CxRP. However, the liver culture fluids from animals stimulated 16 to 24 hr previously with PT vaccine, croton oil, or turpentine did show CxRP labeling (Table I, Fig. 1).

It can be seen in Table I that the various methods of stimulation also re-

sulted in increased production of other serum proteins by the liver, including albumin, transferrin, haptoglobin, and β_{1C}. It should be noted, however, that the increase in other serum protein production preceded detectability of CxRP labeling by several hours, and that loss of blood stimulated other serum protein production but not CxRP formation.

Degenerating muscle tissue from the site of injection of turpentine or PT vaccine did not incorporate C^{14}-amino acid into CxRP (Fig. 2), regardless of the

TABLE I

In Vitro Formation of CxRP and Other Serum Proteins by Liver Tissue Removed from Rabbits after Different Stimuli

Stimulus	Time after stimulus	No. of animals	Serum protein labeling*				
			Albumin	Hp	β_{1C}	Transferrin	CxRP
	hr						
None		2	+ (1) W+ (1)	W+ (1) − (1)	+ (1) W+ (1)	+ (1) W+ (1)	−
Blood loss	16	1	++	++	++	++	−
Paratyphoid low dose	3–12	3	W+ (2) − (1)	W+ (1) − (2)	+ (2) W+ (1)	W+ (1) − (2)	−
	24	1	+	+	+	+	W+
Paratyphoid high dose	3–9	2	++	++	++	++	−
	18–24	2	++	++	++	++	+
	24	1	+	+	++	W+	W+
Endotoxin	18	1	++	++	++	++	W+
Turpentine or croton oil	16–24	2	+	++ (1) + (1)	++	+	+ (1) W+ (1)

* Labeling of albumin, haptoglobin (Hp), β_{1C}, transferrin, and Cx reactive protein (CxRP) is graded according to the intensity of the autoradiographic image. ++ = very strong; + = definite; W+ = weak or very weak; − = absent labeling. The numbers in parentheses represent the numbers of animals from each group in that grade.

time interval after injection at which the tissue was taken (3, 6, 12, 16, 18, 24, and 48 hr). An occasional muscle culture showed labeling of a protein in the γ-region of the immunoelectrophoresis slide with a double arc appearance, shown by anti-whole rabbit serum but not by anti-CxRP. This protein has not been further identified, and represents neither IgG nor CxRP. Other tissues cultured from animals which showed significant labeling of CxRP by the liver, included spleen and peripheral blood leukocytes, and lung macrophages. None of these cultures showed labeling of CxRP (Fig. 2). Spleen produced labeling of IgM,

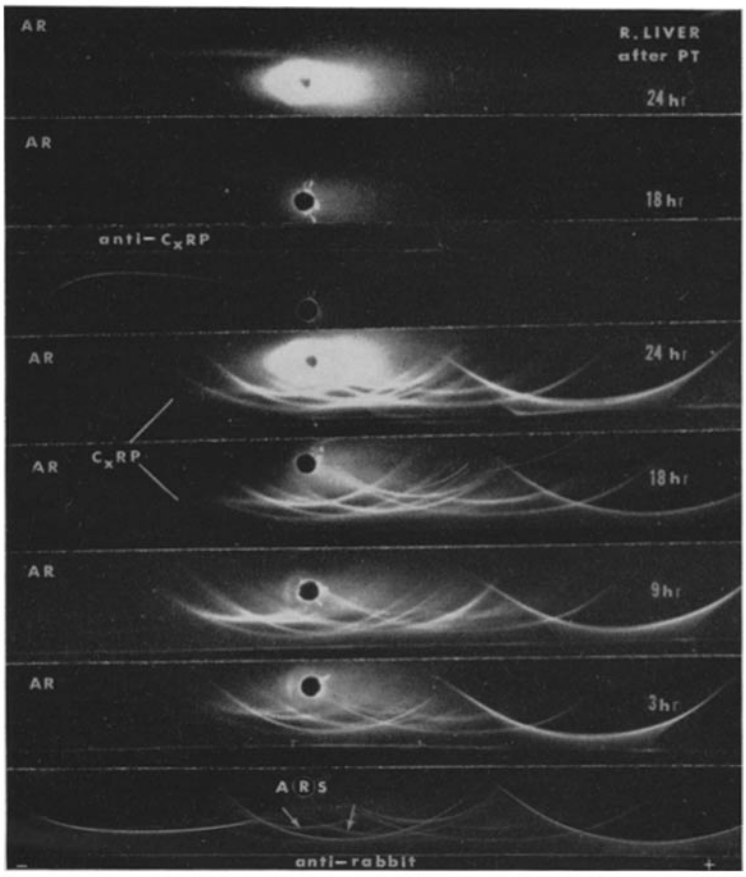

FIG. 1. Autoradiographs of immunoelectrophoretic (IE) patterns made with rabbit liver cultures prepared 3, 9, 18, and 24 hr after a high dose of FT vaccine, and with acute phase rabbit serum as carrier.

Typical carrier IE patterns are included: at the bottom is the IE pattern for acute phase serum developed with sheep anti-acute phase rabbit serum, and the third picture from the top represents the IE pattern of such a serum developed with a specific anti-CxRP. Only the autoradiographs which demonstrated CxRP labeling are shown with this latter carrier pattern. The lines representing β_{1C} and haptoglobin are indicated by arrows in the carrier pattern at the bottom.

IgG, and β_{1C}; peripheral blood leukocytes of IgG only. Labeling of α_{2M} was observed with all cultures, but is considered to be due to binding of other tissue constituents rather than to synthesis of this protein (18).

Attempts were also made to determine whether the presence of degenerating muscle could influence the appearance of labeled CxRP in the liver culture fluid. Since Kushner and Kaplan (6) found localization of CxRP in degenerating

muscle, the possibility existed that muscle would absorb the CxRP formed by the liver. In 2 of the 3 cases in which muscle and liver were cultured in the same roller tube no influence was observed on the degree of labeling of CxRP. In a third case there was a slight reduction.

Monkeys.—Table II shows that among the variety of tissues cultured from monkeys stimulated to produce CRP, liver was the only tissue which showed incorporation of C^{14}-amino acid into this protein. Endotoxin proved somewhat

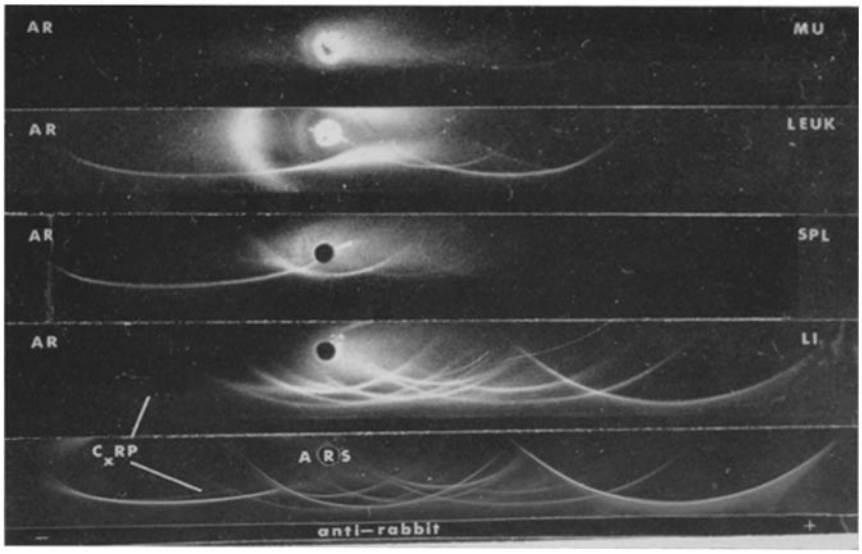

FIG. 2. Autoradiographs of IE patterns made with cultures from liver (LI), spleen (SPL), peripheral leukocytes (LEUK), and injection site muscle (MU), with acute phase rabbit serum as carrier. All these tissues were taken from the same rabbit 18 hrs after injection of PT vaccine. The typical carrier pattern developed with sheep anti-whole rabbit serum is at the bottom. Note that labeling of CxRP occurs only in the liver culture. Labeling of β_{1C} is seen in the spleen and liver cultures as with other species (10–12, 19). The peripheral leukocytes show strong labeling of an α_1-globulin which has not been further identified. IgG labeling is seen in the cultures of spleen and peripheral leukocytes.

more effective than pneumococci in inducing CRP production. Liver from normal adult monkeys never formed CRP, but 1 of the 2 fetal livers studied showed CRP labeling. It was also found that the liver from the normal animal 5 days after partial hepatectomy produced CRP. Fig. 3 shows that CRP production is to some degree correlated with stimulation of other serum proteins by the liver. In the monkey, however, production of serum proteins in the α- and β-region, including β_{1C} (19) was enhanced, but that of albumin and transferrin was not. The livers from the partially hepatectomized monkey and from the fetuses

showed very strong labeling of albumin and transferrin as well as of other serum proteins (Fig. 3, Table II).

There was weak labeling of transferrin in 3 of 7 salivary gland and 1 of 3 mammary gland cultures. The possibility of production of transferrin by ectodermal glands still needs to be studied further, since strong transferrin labeling was also found previously with rabbit and mouse mammary gland cultures (20). In addition to the tissues listed in Table II as cultured from stimulated monkeys and negative for CRP formation, other cultures were also made from ovary,

TABLE II

In Vitro Formation of CRP and Other Serum Proteins by Liver and Other Tissues Removed from Rhesus Monkeys after Different Stimuli

Tissue	No. of animals	Stimulus	Serum protein labeling*											
			Albumin			Transferrin			CRP			IgG		
			+	W+	−	+	W+	−	+	W+	−	+	W+	−
Liver	2	None (fetal)	1	1		1	1		1		1			2
	3	" (adult)		2	1		1	2			3		2	1
	3	Pneumococci		2	1		1	2		2	1		2	1
	4	Endotoxin		3	1		4		2		2		2	2
	1	Hepatectomy	1			1			1				1	
Kidney	7	Various			7			7			7		3	3
Spleen	7	"			7			7			7	7		
Lung	6	"			6			6			6		5	
Lymph node	6	"			6			6			6	6		
Bone marrow	5	"			5			5			5	4		
Intestine	3	"			3			3			3		3	
Salivary gland	7	"			7		3	4			7			7
Mammary gland	3	"			3		1	2			3			3
Peripheral leukocytes	1	"			1			1			1		1	

* The serum protein labeling is graded according to the intensity of the autoradiographic image: + = strong; W+ = weak or very weak; − = absent labeling.

thyroid, brain, and meninges. None of the proteins mentioned in the table was labeled by these organs. Among the tissues that labeled IgG strongly were spleen, lymph node, and bone marrow. Weak labeling of IgG was observed in large parenchymal tissues like liver, kidney, and lung, presumably because of contamination with lymphoid tissue and peripheral blood. Peripheral blood cells in normal monkeys (8) and human beings (21) are known to produce IgG. Cultures from intestinal mucosal lining showed weak labeling of IgG, but much stronger labeling of IgA. This agrees well with results obtained by immunofluorescence (22).

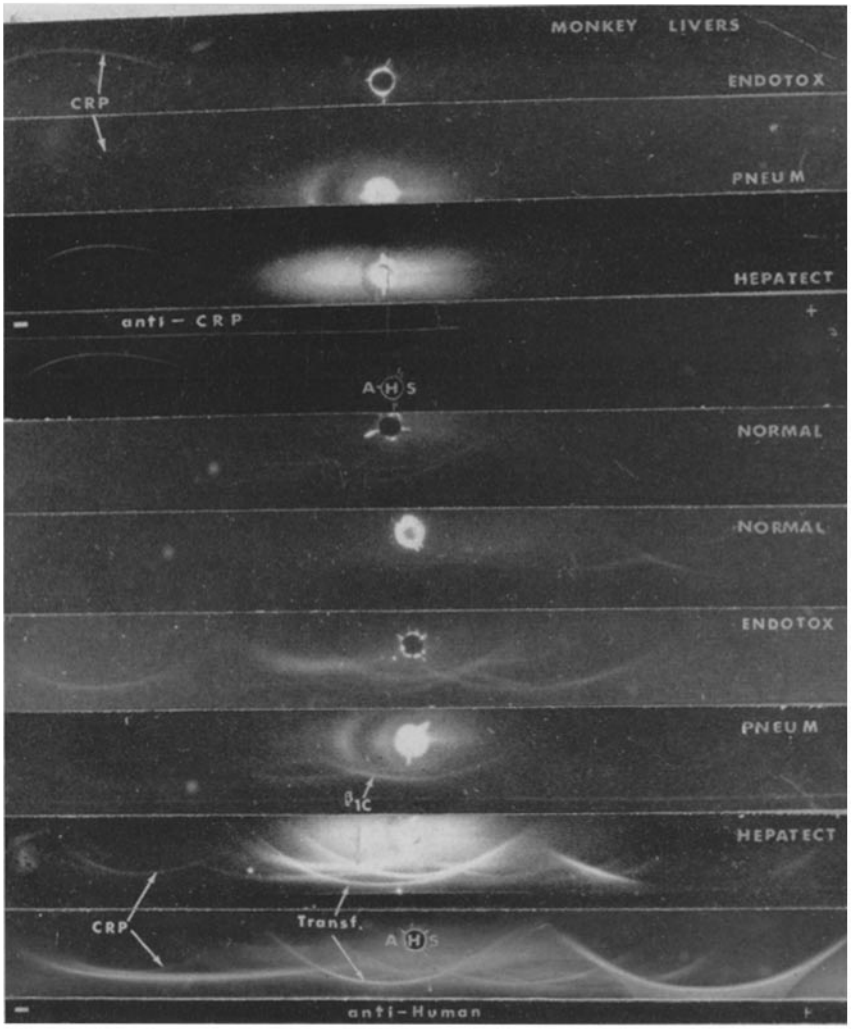

FIG. 3. Autoradiographs of IE patterns made with various monkey liver cultures, and with acute phase human serum as carrier. The monkey livers represented were obtained from 2 normal monkeys, 1 monkey after injection of endotoxin, 1 after injection of pneumococci, and 1 after partial hepatectomy. Typical carrier IE patterns are included: at the bottom is the IE pattern for acute phase human serum developed with a rabbit anti-acute phase human serum, and the fourth picture from the top represents the IE pattern of such a serum developed by a specific anti-CRP serum. Only the autoradiographs that demonstrated CRP labeling are shown with the latter carrier pattern.

Humans Beings.—Human tissues examined for CRP production included bone marrow, spleen, thyroid, mammary gland, thoracic duct lymphocytes, fetal, and adult liver. No labeling of CRP was observed with any of these tissues except for a few livers: 2 of the 6 adult, and 1 of the 4 fetal liver cultures tested

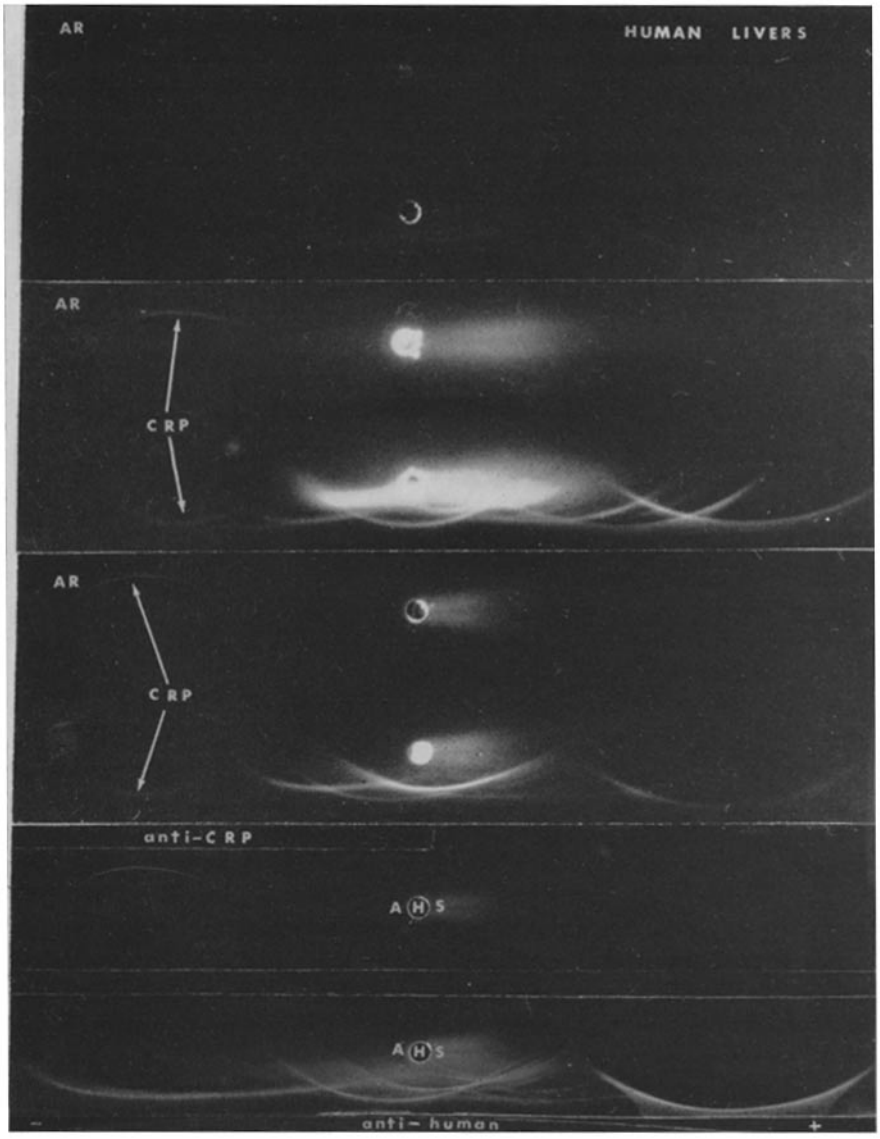

FIG. 4. Autoradiographs of IE patterns made with 3 different human liver cultures and acute phase human serum as carrier. The typical carrier IE pattern at the bottom is developed with anti-whole acute phase human serum and specific anti-CRP. Note the increased labeling of various serum proteins in the 2 livers which show CRP labeling as compared to the one without labeling of CRP.

(Fig. 4.) The diagnosis in one of the adult patients with CRP production was cholecystitis, and in the other enlarged spleen with hypersplenism.

Formation of other serum proteins by the adult liver was much stronger in the 2 liver cultures which also formed CRP than in the other 4. The production of other serum proteins in the 4 fetal liver cultures, however, was very strong in all, and apparently not correlated with formation of CRP.

DISCUSSION

The results presented in this paper strongly suggest that CRP is formed by the liver. Other tissues of the reticuloendothelial system (RES), such as spleen and lymph node, and cell populations such as thoracic duct lymphocytes or peripheral blood leukocytes were all incapable of CRP production. Montella and Wood (23) found that blockade of the RES with thorotrast interferes with the formation of CxRP in rabbits. If cells of the RES are responsible for its formation, the present results indicate that such cells are limited to the liver. Moreover, the high dosages of thorotrast needed to obtain RES blockade might well be toxic also for the liver parenchymal cells. There was no apparent correlation between CRP production and formation of immunoglobulins. This agrees with the observation that CRP formation is normal in agammaglobulinemic patients (24).

Evidence for CxRP production at the site of inflammation or in degenerating muscle could also not be obtained. Since Kushner and Kaplan could demonstrate CxRP in degenerating muscle fibers only after its appearance in the serum, it may be that this localization should be interpreted as a secondary deposition of CxRP from the blood in cells with enhanced permeability (6). Attempts to show a specific tendency of degenerating muscle to absorb CxRP in the present study were essentially negative. The possibility cannot be excluded that some CxRP becomes demonstrable in degenerating muscle from tissue constituents by breakdown and release rather than by *de novo* synthesis from amino acids. The close correlation between the time of CxRP appearance in the serum and the time after stimulation at which the liver incorporates C^{14}-amino acid into CxRP, suggests that at least part of the serum CxRP is produced by *de novo* synthesis in the liver.

CRP and CxRP are known to be associated with a lipid component (3). No attempts were made in the present studies to extract the lipid from the labeled CRP in culture fluids, primarily because the amount of material was insufficient for such studies. Incorporation of C^{14}-lysine and isoleucine into the complex suggests formation of the protein component, and it seems unlikely that most of the label was incorporated into the lipid fraction.

It has been demonstrated that in cloudy swelling of the liver the increased protein production occurs independently of amino acid pool changes (25). If it is assumed that most of the observations in the present studies are also not sub-

stantially influenced by variations in the amino acid pool of the liver cells, an evaluation of CRP production as correlated to other serum proteins can be made.

Stimulation of CRP production was usually accompanied by enhanced activity of the liver in the labeling of other serum proteins. However, the reverse was not always observed. In the rabbit, stimulation of other serum protein production appeared prior to detectable CxRP production. Asofsky (26) has found that increased activity in serum protein synthesis by the mouse liver occurs as early as 30 min after injection of endotoxin. With liver from fetal monkeys and human beings strong serum protein formation was invariably present, and CRP production only in a few cases. It is not clear from the present study what the nature of the stimulus for CRP production could have been in the fetuses and in the partially hepatectomized monkey. Whole body X-irradiation is also known to induce CxRP formation in rabbits within 2 days (27), and in mice (28) irradiation was found to increase serum protein production by the liver after a similar time interval. The observation that CRP can be produced by fetal liver agrees with the finding of others, that CRP may occur in the serum very early after birth (29).

It is conceivable that the stimuli for production of CRP and other serum proteins are quite different, but that the stimulation of CRP production is usually accompanied by an additional effect on the factors governing the formation of other serum proteins. The usual association between enhancement in the serum of haptoglobin and other acute phase glycoproteins, and occurrence of CRP (30, 31) also indicates that under many conditions their production is governed by similar factors.

SUMMARY

The site of formation of C-reactive protein (CxRP, CRP) has been studied with tissues from rabbits, monkeys, and human beings. Rabbits and monkeys were stimulated to produce the acute phase protein by injection of turpentine, croton oil, endotoxin, paratyphoid-typhoid vaccine, or pneumococci. C^{14}-amino acid incorporation in vitro was demonstrated by means of autoradiography of immunoelectrophoretic patterns made with culture fluids.

It was found that among many different tissues tested liver was the only tissue which incorporated C^{14}-lysine and isoleucine into CxRP or CRP. Only livers taken 16 to 24 hr after various stimuli were active; livers from normal animals or from animals killed 3 to 9 hr after stimulation did not produce detectable amounts of CxRP. Inflamed muscle from the injection site did not show C^{14}-amino acid incorporation into CxRP. Several human tissues were also cultured, and a few liver cultures found to contain labeled CRP.

The formation of CxRP or CRP by the liver was always accompanied by enhanced C^{14}-amino acid incorporation into other serum proteins, but the reverse was not always found.

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