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STUDIES OF ACUTE-PHASE PROTEIN. II. LOCALIZATION OF Cx-REACTIVE PROTEIN IN HEART IN INDUCED MYO- CARDIAL INFARCTION IN RABBITS *

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Cx-reactive protein (CxRP), which appears in the blood of rabbits during inflammation (1), is analogous and immunologically related to C-reactive protein (CRP) of man (2). The site of origin of these acute-phase proteins and the mechanism of their production are problems as yet unresolved.

Previous work from this laboratory has described an immunofluorescent technique for the detection of CxRP in inflamed rabbit tissue (3). After the induction of an inflammatory lesion in the rabbit by the intramuscular injection of typhoid vaccine, CxRP could be found in necrotic myofibers within the inflammatory lesion, and not in any other rabbit organ or tissue studied. At no time was CxRP detected in the cells of the inflammatory exudate. This same localization of CxRP to necrotic myofibers was observed in rabbits made markedly neutropenic by the injection of nitrogen mustard. These observations rendered unlikely the derivation of acute-phase protein from inflammatory cells or from a product of the activity of such cells. Rather, these observations have supported the concept that CxRP has its origin in the tissues undergoing inflammatory or necrotic change.

The present work represents an extension of these findings to inflammatory and necrotic lesions of myocardium. The clinical association of CRP in man with myocardial infarction and rheumatic myocarditis prompted consideration that this acute-phase reactant might be derived from injured myocardial myofibers. Accordingly, the

immunohistochemical localization of CxRP was investigated in rabbits in which infarction of the myocardium was produced by coronary artery occlusion. The results of these studies indicated that in such lesions, CxRP was localized to, and probably had its origin in, cardiac myofibers undergoing necrotic changes.

MATERIALS AND METHODS

Preparation of antiserum. CxRP was prepared as a purified, delipidated, CxRP-Cx carbohydrate precipitate as previously described (3), and was solubilized in 1 per cent NaCl containing 0.0001 M sodium dihydrogen ethylene diamine tetraacetate (EDTA). This solubilized precipitate will henceforth be referred to as CxRP. A goat antiserum to CxRP was prepared by an immunization procedure that included several courses of alum-precipitated CxRP given as previously described (3), followed by a single booster of 90 μ g of protein N administered subcutaneously and intramuscularly in Freund's adjuvant. A highly reactive antiserum was obtained that yielded a minimal reaction with normal rabbit serum in precipitin tests in capillary tubes. After absorption of antiserum with lyophilized normal rabbit serum, the antibody content of the serum (CxRPA) as determined by quantitative precipitation was 910 μ g antibody N per ml. Agar gel diffusion studies employing this CxRPA revealed a single strong band appearing within 1 day and a second very weak band appearing after 8 to 10 days when CxRPA reacted against acute-phase rabbit serum or against CxRP, the same pattern seen with previous antisera prepared without the use of Freund's adjuvant (3).

Immunofluorescent techniques. Preparation of fluorescent conjugates, absorption procedures, fluorescence microscopy, fixation and staining techniques, and criteria for immunohistochemical specificity have all been described previously (3).

For study of the histological appearance of sites revealing immunofluorescent staining, a double-staining technique was used. After sites of staining specific for CxRP were photographed, the coverslips were floated off the slide, the section was stained with hematoxylin and eosin, and the site of CxRP localization was identified.

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Production of myocardial lesions. Adult, New Zealand, white, male rabbits weighing 3.3 to 4.3 kg were anesthetized with 90 mg of intraperitoneal Nembutal¹ and maintained with intravenous Pentothal² (1 per cent) as needed. After endotracheal intubation via a tracheostomy incision, the animals were maintained on artificial respiration. A thoracotomy was performed through a left lateral chest incision in the fourth or fifth intercostal space. The anterior descending branch of the left coronary artery was identified. Direct epicardial electrocardiographic recordings were obtained with a cotton-tipped, wick electrode of copper wire from the whole anterior and lateral wall of the epicardial surface through the intact pericardium, and were recorded on a 4-channel Cambridge Simpliscribe electrocardiograph. A small portion of the pericardium immediately over the artery was excised and a suture was placed around the artery with an atraumatic needle. The ligature was tightened either directly, completely occluding the artery, or over a probe to allow for quick release if ventricular fibrillation occurred. If the latter did not occur, complete occlusion was effected. Repeat epicardial electrocardiographic leads were then recorded as before the tie. The chest was closed and the animal permitted to come out of anesthesia. After varying intervals, up to 48 hours after ligation, the animals were reanesthetized and maintained on artificial respiration while the thoracotomy incision was reopened and epicardial electrocardiographic recordings were made to ascertain the site of infarction. In some instances, the animals died before a terminal electrocardiogram could be obtained. After sacrifice, tissue was obtained from the heart both distal and proximal to the ligation, from intercostal muscle at the thoracotomy site, and from lung, liver, spleen, kidney, and mesenteric lymph node. The tissue blocks were quick-frozen in a mixture of dry ice and alcohol and stored at -25° C. Tissues were sectioned in the cryostat at a thickness of 4 μ , and the slides were air dried before fixation and staining.

Electrocardiographic interpretation. In the analysis of the electrocardiograms, development of Q waves or enlargement of pre-existing Q waves was considered to be evidence of infarction and necrosis. Elevation of RS-T segments and inversion of T waves were considered evidence of injury and ischemia, respectively.

Determination of serum CxRP concentration. Blood samples were obtained from the marginal ear vein prior to any surgical incision and from the inferior vena cava at the time of sacrifice.

After collection, blood samples were permitted to stand overnight at 4 $^{\circ}$ C, and the serum was separated by centrifugation. Estimation of serum CxRP concentration was performed by the capillary precipitin technique and was graded by the height of precipitin in the column in millimeters (4).

¹ Pentobarbital sodium, Abbott Laboratories, North Chicago, Ill.

² Thiopental sodium, Abbott Laboratories, North Chicago, Ill.

RESULTS

Fourteen rabbits were subjected to coronary artery ligation, and all showed electrocardiographic evidence of myocardial ischemia and injury immediately after ligation (Figure 1). At intervals of 4 to 48 hours after the ligation, prior to sacrifice, terminal electrocardiograms revealed electrical evidence of necrosis in localized portions of myocardium distal to the ligature in all animals surviving this procedure.

Histologic studies of heart tissue taken from the zone of necrosis, demarcated by the electrode recordings, were stained with hematoxylin and eosin. These sections revealed the classical changes associated with myocardial infarction (5-7): loss of cross striations, hyalinization, increased eosinophilic staining of involved myofibers, and loss of nuclei (Figure 2). Homogenization and other evidences of disturbance of fibrillar structure were observed in phosphotungstic acid- and hematoxylin-stained preparations. Vacuolization of myofibers was observed occasionally. The infarcted area generally showed variable infiltration with leukocytes, with interstitial edema.

CxRP was first detected in the serum as early as 4 $\frac{1}{4}$ to 5 hours after arterial ligation (Table I). It was found in the blood in progressively increasing amounts in the remaining interval of the 48-hour observation period after ligation. In immunohistochemical studies, CxRP was first detected in the heart in the zone distal to the ligation at 4 $\frac{1}{4}$ to 5 hours after ligation and was demonstrable only in this zone of infarcted heart tissue for the remainder of the 48 hours of the study. It could not be demonstrated in heart at 2 and 4 hours after ligation. Thus, the localization in tissue and appearance of CxRP in the blood showed a similar time-course.

In the zone of affected myocardium, CxRP was present at the outer edge of myofibers and in fine, round or linear inclusions within the substance of necrotic myofibers (Figures 3-8). Microprecipitate was often present in these sites (Figure 8) as well as occasionally in the interstitium between necrotic myofibers. The presence of CxRP in myofibers was not apparently related to the presence of inflammatory cells. An identical pattern of specific staining was observed in single, scattered myofibers at the periphery of the infarcted

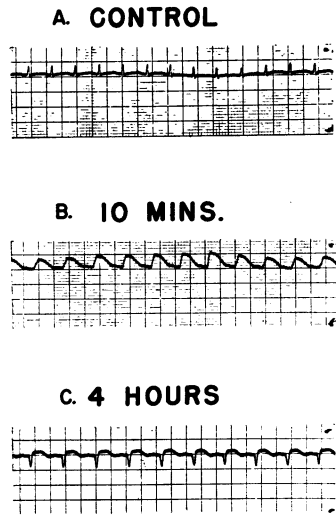


FIG. 1. RABBIT 1065, EPICARDIAL LEAD. A. CONTROL, BEFORE LIGATION. B. 10 MINUTES AFTER CORONARY ARTERY LIGATION. Marked ST segment elevation indicative of injury. C. 4 HOURS AFTER CORONARY ARTERY LIGATION. QS indicative of loss of electrical potentials, considered as evidence of necrosis.

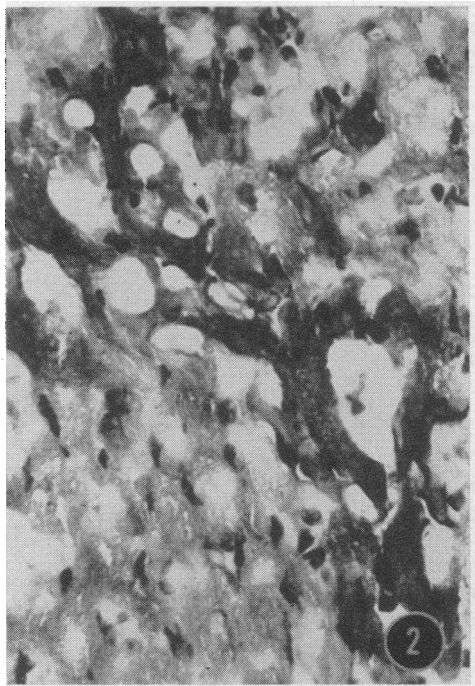


FIG. 2. RABBIT 738, SACRIFICED 48 HOURS AFTER LIGATION OF CORONARY ARTERY. SITE OF INFARCTED MYOCARDIUM. Necrotic myofibers show eosinophilia, hyalinization, and a homogeneous appearance. Frozen section. Hematoxylin and eosin stain. (X 312)

TABLE I
Tissue localization and blood levels of Cx-reactive protein (CxRP), and electrocardiographic findings

Hours after ligation	Rabbit no.	Serum CxRP		Electrocardiogram		Immunofluorescent localization of CxRP								
		Original	Final	Ischemia and injury after ligation	Necrosis in terminal record	Zone of myocardial infarction	Traumatized skeletal muscle	Normal heart	Liver	Spleen	Lymph node	Kidney	Lung	
2	1084	0	0	+	*	0	0	0	0	0	0	0	0	0
4	1065	0	0	+	+	0	0	0	0	0	0	0	0	0
4	1087	0	0	+	*	0	0	0	0	0	0	0	0	0
4½	1092	0	1+	+	*	+	+	+	+	+	+	+	+	+
5	1085	trace	½+	+	*	+	+	+	+	+	+	+	+	+
5½	1090	1+	1+	+	*	+	+	+	+	+	+	+	+	+
8	1098	trace	1+	+	+	+	+	+	+	+	+	+	+	+
8	1082	0	trace	+	+	+	+	+	+	+	+	+	+	+
11	1081	0	trace	+	*	+	+	+	+	+	+	+	+	+
12	1083	heavy trace	3+	+	+	+	+	+	+	+	+	+	+	+
12½	1094	½+	4+	+	+	+	+	+	+	+	+	+	+	+
24	1080	0	3+	+	+	+	+	+	+	+	+	+	+	+
24	1097	heavy trace	2+	+	+	+	+	+	+	+	+	+	+	+
48	738	½+	3+	+	+	+	+	+	+	+	+	+	+	+

* Animal died before terminal record taken.

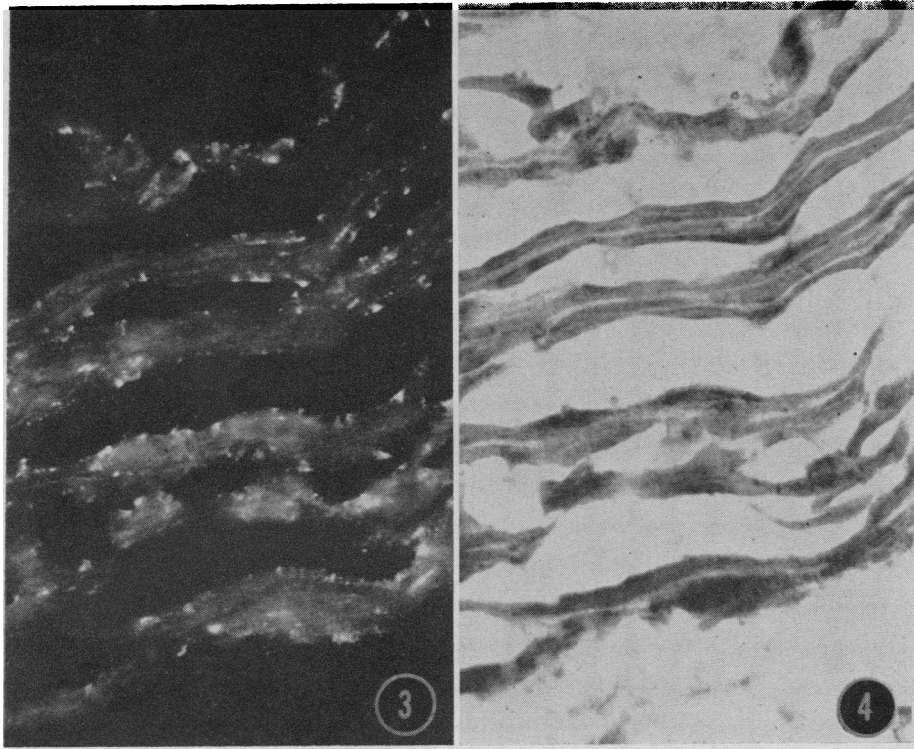


FIG. 3. RABBIT 738, SACRIFICED 48 HOURS AFTER LIGATION. SECTION OF INFARCTED MYOCARDIUM STAINED WITH CxRPA CONJUGATE. CxRP IS LOCALIZED TO EDGES OF MYOFIBERS IN FOCAL DROPLETS AND GRANULES, AND WITHIN SUBSTANCE OF MYOFIBERS. ($\times 312$)

FIG. 4. SAME SECTION AS FIGURE 3. AFTER IMMUNOFLUORESCENT STAINING, COVERSLEIP WAS FLOATED OFF AND TISSUE STAINED WITH HEMATOXYLIN AND EOSIN. NECROTIC MYOFIBERS, EOSINOPHILIC AND HYALINE IN APPEARANCE, WITH LOSS OF NUCLEI. ($\times 312$)

area remote from infiltrating cells (Figure 7). The extent and intensity of the staining reaction for CxRP in the involved tissue increased progressively during the 48 hours of observation.

No CxRP could be demonstrated in normal myocardial tissue proximal to the ligation, nor in the normal-appearing myofibers observed distal to the ligation, even in the presence of infiltration with inflammatory cells. In other organs studied, including liver, kidney, spleen, lung, and mesenteric lymph node, CxRP was observed only in vascular lumens and interstitial spaces. CxRP was also demonstrated in the intercostal skeletal muscle adjacent to the line of thoracotomy incision. The localization of CxRP in this tissue traumatized by surgery was limited to the outer edges of and inclusions within necrotic myofibers. This distribution was similar to that observed previously in inflammatory lesions of muscle induced by injection of typhoid vaccine (3).

DISCUSSION

The localization of CxRP in inflammatory lesions of skeletal muscle produced by an exogenous inflammatory agent, typhoid vaccine, had suggested previously the probable origin of CxRP in this lesion from necrotic muscle fibers. In the present work, coronary artery ligation was selected as a method of producing myocardial injury. In the heart, specific staining of necrotic myocardial cells in the infarcted area was limited to the outer edge, possibly including sarcolemmal or subsarcolemmal segments of the cells, and to inclusions and vacuoles within the sarcoplasm of these cells. This association of CxRP with necrotic but not with normal myofibers is consistent with the hypothesis that CxRP is produced as a result of inflammatory or necrotic changes in cardiac myofibers. The alternative possibility, that the localization described represents secondary deposition of

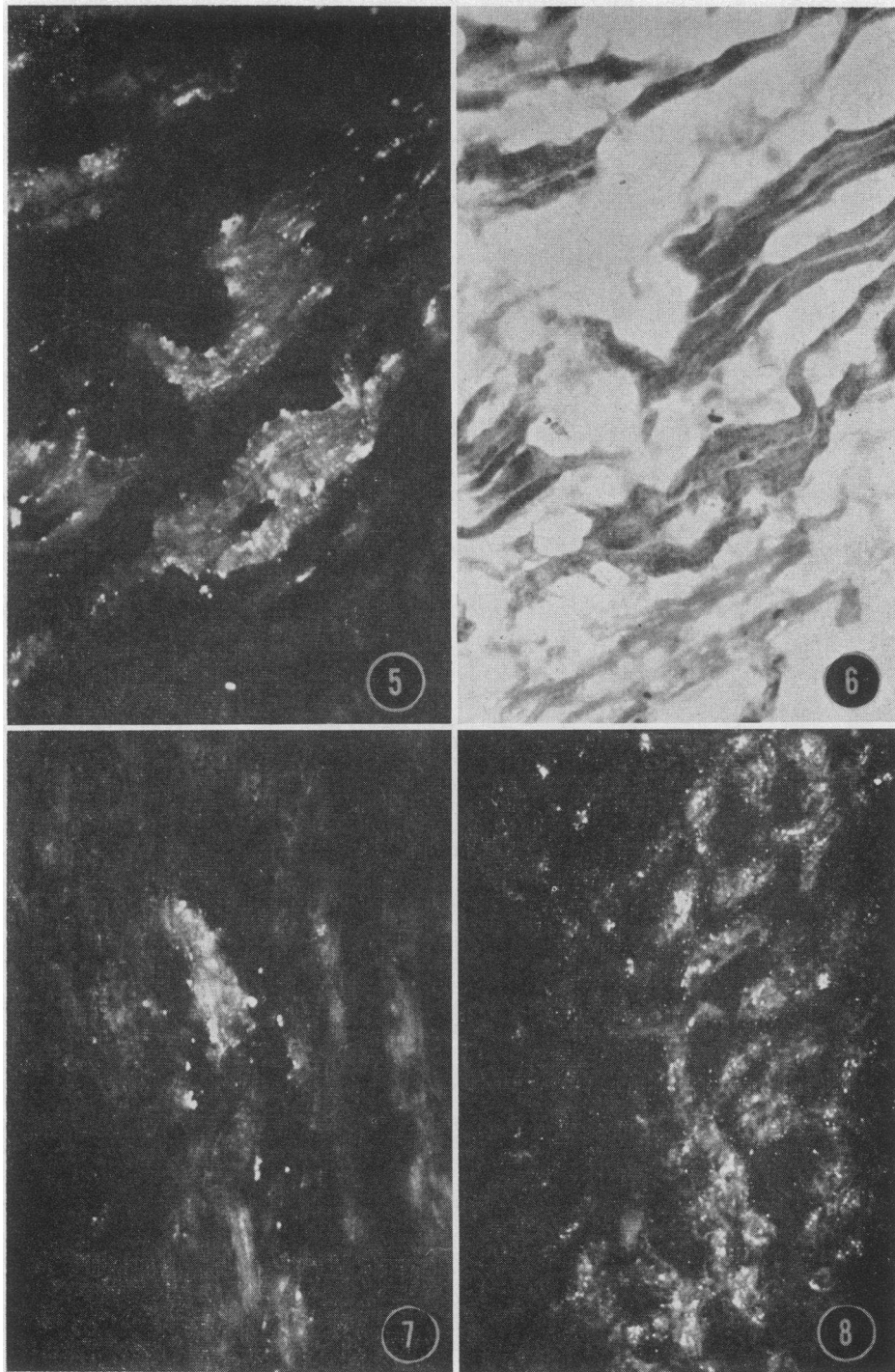


FIG. 5. RABBIT 738, SACRIFICED 48 HOURS AFTER CORONARY ARTERY LIGATION. SECTION OF INFARCTED MYOCARDIUM. CxRP is distributed along the edge and within necrotic fibers. Myofibers with morphologic evidence of necrosis also exhibit a faint diffuse autofluorescence. ($\times 312$)

FIG. 6. SAME SECTION AS FIGURE 5, STAINED WITH HEMATOXYLIN AND EOSIN AFTER COVERSIP FLOATED OFF. Myofibers containing CxRP show necrotic changes. ($\times 312$)

CxRP from the blood into myofibers with altered permeability, cannot be excluded, although it is considered less likely in view of the highly characteristic cytologic distribution of CxRP in segments of myofibers at the periphery of the cell and in inclusions and vacuoles. Were this localization due to penetration of necrotic myofibers by CxRP from the blood or tissue fluids, a diffuse or random distribution in the myofiber might have been expected rather than this delicate, consistent pattern. Further, possibility of the origin of CxRP from other tissues was opposed by the failure of CxRP to be detected in noninfarcted portions of myocardium, or in cells of other organs, including liver, kidney, spleen, lung, and lymph nodes. The observation of CxRP in necrotic skeletal muscle at the site of thoracotomy was consistent with origin of CxRP from this site of tissue injury as well.

The inflammatory stimulus in the present work was endogenous in that it consisted of the tissue response to a local ischemic state. The association of CxRP with necrotic cardiac myofibers in this lesion paralleled observations of CxRP in skeletal muscle necrosis induced with an exogenous agent, typhoid vaccine. Thus, it does not seem necessary to ascribe a special function to exogenous agents in eliciting CxRP, except that necrosis be induced. Other structures of heart and skeletal muscle, including connective tissue and blood vessel walls, did not show localization of CxRP. It would be of interest to determine the distribution of CxRP in other types of inflammation, such as that associated with the hypersensitivity state, in which blood vessels and interstitial tissue are perhaps more directly affected.

Approximately 5 hours elapsed between the inflammatory stimulus and the appearance of CxRP in the blood in some of the rabbits studied. This is more rapid than has been described previously (8), and is three hours earlier than was seen in previous studies after the intramuscular injection of typhoid vaccine. The report by Kroop and

Shachman (9) that the strong stimulus of major surgery evokes a more rapid and more marked CRP response than that seen after minor surgery, might suggest that both the speed and magnitude of the acute-phase protein response are proportional to the severity of the inflammatory stimulus. Other variables to be considered, however, are the kind of inflammatory stimulus and the tissue affected. In the present work, the combined inflammatory stimulation induced by surgical thoracotomy and myocardial infarction may possibly account for the particularly rapid appearance of CxRP in the rabbits studied.

SUMMARY

The histologic localization of Cx-reactive protein (CxRP) was studied in inflammatory lesions of heart tissue induced by infarction secondary to coronary artery ligation. CxRP was found within and around necrotic myofibers, beginning approximately 5 hours after ligation, and could not be found in normal myocardial sites or in other organs studied, except for the intercostal skeletal muscle site traumatized during thoracotomy. These observations are consistent with the hypothesis that acute-phase protein is produced at the inflammatory site as a result of inflammatory or necrotic tissue change. These experimental results direct attention to the possible origin of C-reactive protein (CRP) from injured cardiac myofibers in infarctive and rheumatic inflammation of heart tissue in man.

ACKNOWLEDGMENT

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FIG. 7. RABBIT 738, 48 HOURS AFTER LIGATION. CxRP is detected in an isolated myofiber distant from the mass of necrotic myofibers elsewhere in the section. CxRP is distributed in focal sites at edge and in inclusions within myofiber. ($\times 250$)

FIG. 8. RABBIT 1082, SACRIFICED 8 HOURS AFTER LIGATION. Section of infarcted myocardium. CxRP is demonstrated as a fine microprecipitate on the edges of myofibers showing early necrosis. ($\times 250$)

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