In Vivo Studies of Serum C-reactive Protein Turnover in Rabbits

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ABSTRACT We determined the plasma half-life of the acute phase protein C-reactive protein (CRP) both in normal rabbits and in rabbits that had received inflammatory stimuli. Rabbit CRP was purified from acute phase serum by Cx-polysaccharide affinity chromatography, radiolabeled, and rendered pyrogen-free. Six unstimulated rabbits were injected intravenously with ^{125}I-CRP prepared by the lactoperoxidase method and four were injected with CRP labeled by methylation using [^{14}C]formaldehyde. Blood samples were obtained at 0.25 h and at intervals thereafter. Plasma half-life of CRP was calculated from the data generated during the first 12 h, by which time an average of 86% of labeled protein had disappeared from the blood stream. The mean half-life for CRP was 4.45±0.2 h, with no significant difference ($0.40 < P < 0.45$) between ^{125}I- and ^{14}C-labeled CRP. In six animals stimulated with either endotoxin or turpentine 24 h before injection of labeled CRP, a mean half-life of 5.8±0.6 h was found, not significantly different ($0.30 < P < 0.35$) from unstimulated rabbits. We equated fractional catabolic rate to fractional disappearance rate, since the rate constant for passage of CRP from vascular to extravascular compartment can be assumed to be relatively small compared to the observed fractional disappearance rate. Fractional catabolic rate was independent of serum CRP concentration; average fractional catabolic rate in all 16 animals was 14±0.8% h^{-1} of the plasma pool. We were able to estimate rate of CRP synthesis, based on steady-state assumptions of pool sizes in those rabbits whose serum CRP levels did not change substantially during the period of study. Values as low as 6.7 μg/kg per h in the unstimulated animals and as high as 560 μg/kg per h in the stimulated animals were found.

INTRODUCTION

Although C-reactive protein (CRP)[1] has been detected in the plasma of many species (1, 2), it has been shown to behave as a striking acute phase reactant in only man (3), monkey (4), and the rabbit (5). In man and rabbits, CRP concentration rises rapidly after tissue injury or infection (6, 7) and the magnitude of the response is remarkable; concentrations as much as 1,000 or more times normal levels may be attained. The magnitude of the CRP response in both of these species appears to parallel the degree of tissue injury. With recovery from tissue injury or infection, serum CRP concentrations in both species fall rapidly. Human CRP has been found to recognize and bind to a variety of ligands, and by so doing, to initiate multiple effector functions that have been associated with the inflammatory response and with immune-related activities (8).

CRP in both man and rabbit is a pentraxin, a molecule made up of five subunits arranged in cyclic symmetry (9). Substantial amino acid homology (10) and immunological cross-reactivity (11) between rabbit and human CRP have been shown, and in neither species is the protein glycosylated. Molecular weight differs only slightly in these two species (12). There are only minor differences in binding properties between human and rabbit CRP; both bind strongly to phosphocholine (12). In contrast, some of the C-reactive proteins described in other species are glycoproteins, some are hexamers, and most do not demonstrate marked acute phase reactivity (1, 2).

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[1] *Abbreviations used in this paper:* CRP, C-reactive protein; CxPS, Cx-polysaccharide; SAP, serum amyloid P protein.

Because of the similarities in physiologic behavior and biochemical characteristics of CRP in man and rabbit, studies of CRP metabolism in the rabbit have served as models for human CRP metabolism during the acute phase response. Such studies have shown that the liver is the site of CRP synthesis (13), and that the hepatocyte is the cell of origin within that organ (14). After tissue injury the number of hepatocytes that can be shown to be synthesizing CRP has been found to increase with time. Rates of CRP synthesis by isolated perfused livers from stimulated animals have been shown to exceed rates of synthesis by livers obtained from unstimulated animals and to reflect the serum CRP levels attained at the time of liver isolation (15).

CRP metabolism has not been studied directly in vivo. Studies of rate of increase in serum CRP level after acute myocardial infarction in man (6), and following intravenous endotoxin administration in rabbits (7) have shown average serum doubling times of ~8 h in both species. Following attainment of peak CRP levels, concentrations have been noted to fall at variable rates. In some rabbits injected with endotoxin, half-disappearance times as rapid as 4.5 h have been observed (7). Observations such as these have suggested that plasma turnover time of this protein may be relatively rapid compared with most plasma proteins. In addition, serum CRP levels in patients receiving dramatically effective therapeutic agents (such as intravenous corticosteroids in systemic lupus erythematosus and penicillin in gonococcal arthritis) have similarly shown relatively rapid decline (16).

In the present study we used isotopic techniques to determine the serum half-life of CRP in both rabbits that had received inflammatory stimuli and in unstimulated rabbits. Because of the rapid half-lives we observed, it was possible to make use of steady-state assumptions to compute approximate in vivo rates of CRP synthesis in some rabbits, both stimulated and unstimulated, during the period of study.

METHODS

Animals. New Zealand white male rabbits weighing between 2 and 4 kg were used. Those rabbits injected with radioiodinated CRP were given ~4 mg KI and 10 mg NaCl orally for 3 d before starting the experiment. Rabbits in which inflammatory states were induced received either 1 ml turpentine in each thigh or 5 μg bacterial lipopolysaccharide (LPS; *Escherichia coli* serotype no. 055:85:Sigma Chemical Co., St. Louis, MO) intravenously 24 h prior to injection of labeled protein.

Preparation of radiolabeled CRP. Cx-polysaccharide (CxPS) was extracted from Type II pneumococcus (kindly supplied by E. Gotschlich) (5) and coupled to epoxy-activated Sepharose 6B. A CxPS-Sepharose 6B column (0.5 × 4 cm) was washed with 0.15 M Tris-0.05 M $CaCl_2$, pH 7.0. Between 0.5 and 1 ml of acute-phase rabbit serum, containing ~50 μg of CRP, was applied to the column. After unbound protein was washed through the column, CRP was eluted with 0.05 M Na citrate buffer, pH 7.0. Passage over a Sephadex G-200 column of rabbit CRP prepared in this manner showed that >95% of the recovered protein eluted at a volume identical to that at which native rabbit serum CRP is eluted (17). Sodium dodecyl sulfate electrophoresis of the protein on polyacrylamide gels revealed a single band corresponding to the approximate subunit molecular weight of CRP (M_r, 21,500).

Rabbit CRP, prepared as described above, was iodinated using the lactoperoxidase method as performed by Volanakis (18). ^{14}C-labeled CRP was prepared by reductive methylation of lysyl residues (19). Radiolabeled CRP prepared by these two methods was repurified by passage over CxPS-Sepharose 6B, using autoclaved buffer. This procedure also served to remove pyrogen as well as nonprotein bound isotopes. Analytical gel filtration on Sephadex G-200 of aliquots of ^{125}I-CRP prepared in this manner showed that >95% of total radioactivity was in a single peak corresponding to the known molecular weight of rabbit CRP, while the remainder eluted in a volume corresponding to that of the internal volume of the column. In one experiment ^{125}I-CRP was further purified by preparative gel filtration on Sephadex G-200. In this experiment 97% of total protein radioactivity eluted in a volume corresponding to that of native CRP, while the remainder eluted in the void volume. ^{125}I-CRP eluted from the Sephadex G-200 was then again passed over a CxPS column to free it of pyrogenic activity and as a concentration step before injection into a rabbit (rabbit 6).

The average minimum specific activity of ^{125}I-CRP was 0.45 μCi/μg protein. This indicated that an average of 0.02 atoms of radioiodine were attached per molecule of protein. For ^{14}C-CRP, the average specific activity was 0.0023 μCi/μg protein or five substitutions of lysyl residues with ^{14}C per molecule of protein.

In vivo studies. Six unstimulated rabbits received ^{125}I-CRP (2.25 μCi; 5 μg) and four received ^{14}C-CRP (0.069 μCi; 30 μg) intravenously in a volume of 1 ml. Of the six stimulated animals studied, four received turpentine intramuscularly and two received LPS. 24 h later two of the turpentine-injected rabbits were injected intravenously with ^{14}C-CRP while the remaining four rabbits received ^{125}I-CRP in the same doses administered to unstimulated rabbits.

Analytical methods. CRP concentrations in sera of normal and stimulated rabbits were determined by single radial immunodiffusion in agar plates (17, 20). The lowest concentration of CRP reproducibly detectable by this method was 2 μg/ml. On certain serum specimens with values less than this, CRP concentrations were determined by radioimmunoassay as described elsewhere.[2]

Where ^{14}C-CRP was used, protein radioactivity in serum was determined according to the trichloracetic acid method of Mans and Novelli (21, 22) in a Packard Tri-Carb 3000 scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). When using radioiodinated CRP, radioactivity in serum samples was determined in a gamma counter. Studies of samples from two rabbits 4 to 8 h after injection of ^{125}I-CRP showed that >94% of serum radioactivity was precipitable in 5% trichloroacetic acid. Gel filtration on Sephadex G-200 of a 4-h serum sample from rabbit 16 and of an 8-h serum sample from rabbit 10 showed that >94% of protein radioactivity eluted in a volume corresponding to the native molecular weight of CRP.

[2] Macintyre, S. S., D. Schultz, and I. Kushner. 1983. *Biochem. J.* (In press).

Protein radioactivity per milliliter serum at each time point was expressed as a percentage of the protein radioactivity found in 1 ml serum at 15 min after injection and was plotted as a function of time on semilogarithmic paper. Linear regression analysis was performed by the method of least squares. The half-life of CRP was determined by extrapolating the straight decay line to zero time, and then reading off the time that corresponded to half the ordinate of the intercept (23).

RESULTS

Studies in unstimulated animals. Fig. 1 demonstrates the composite rate of disappearance of ^{125}I-CRP from serum in six unstimulated rabbits over the period of 12 h after injection. Disappearance of ^{14}C-CRP in four unstimulated rabbits is shown in Fig. 2. The findings in individual unstimulated rabbits are shown in Table I. Correlation coefficients of the curves in individual rabbits ranged between −0.98 and −0.99. No significant differences were observed in disappearance rates of CRP labeled with ^{125}I or ^{14}C.

For the entire group of 10 animals, the average serum half-life was 4.45±0.21 h (mean±SEM) and the average fractional catabolic rate was 15.9±0.8% of the total plasma pool/h. 12 h after injection, an average of 14% of radiolabeled protein present at 15 min was detectable in the serum of these animals; by 24 h, an average of 4% was detectable. Rabbit 6 differed from the other animals in having received ^{125}I-CRP that had been subjected to an additional purification step by passage over Sephadex G-200 before intravenous administration. Half-life of labeled CRP in this rabbit was found to be 3.46 h, a value within the range found in the other unstimulated animals that had received radiolabeled CRP prepared without a gel filtration step.

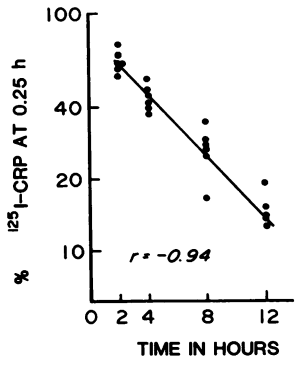

FIGURE 1 Disappearance of radioiodinated CRP from serum in six unstimulated rabbits. Radiolabeled protein was administered intravenously at time zero. Blood samples were obtained from the ear vein at intervals after injection. The percentage of the amount of serum CRP radioactivity present at 15 min was plotted as a function of time on semilogarithmic graph paper and a straight line was fitted by the method of least squares.

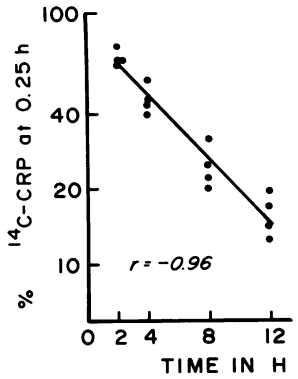

FIGURE 2 Disappearance of ^{14}C-labeled CRP from serum in four unstimulated rabbits. Methods and analysis were as described in Fig. 1.

Of the 10 unstimulated animals studied, 4 showed no CRP response following injection of radiolabeled protein, serum CRP levels remaining <2 μg/ml throughout the period of study. The other six had very mild CRP responses, CRP levels rising by 2–11 μg/ml. It is conceivable that the procedure of intravenous injection itself was sufficiently traumatic in these animals to induce a CRP response (7) or that some pyrogen was inadvertently administered with radiolabeled CRP, despite our precautions.

Studies in stimulated rabbits. A composite graph illustrating disappearance of labeled CRP from serum in six stimulated rabbits is shown in Fig. 3. Table II shows the serum half-life values and fractional catabolic rates of CRP determined in six stimulated animals. No significant difference in half-life or fractional catabolic rate was observed between turpentine and LPS-stimulated animals. These values did not differ significantly from values obtained in normal, unstimulated rabbits ($0.35 > P > 0.30$). For stimulated animals an average of 21 and 9% of radiolabeled protein present at 15 min was detectable in serum at 12 and 24 h after injection.

Over the period between 2 and 12 h after injection of radiolabeled CRP, during which disappearance curves were generated, changes in serum CRP levels ranged between −10 and +25% (Table II).

Relationship of fractional catabolic rate to serum CRP concentration. Fig. 4 shows the relationship between fractional catabolic rates and average serum CRP concentrations during the periods of study. Linear regression analysis of the points yielded a slope (−0.0005) that did not differ significantly from that of a horizontal line ($P = 0.036$) indicating that the fractional catabolic rate of CRP did not vary regardless of serum concentration.

TABLE I

CRP Half-life and Fractional Catabolic Rate in Unstimulated Rabbits

^{125}I-Labeled CRP			^{14}C-Labeled CRP		
Animal	Half-life	FCR*	Animal	Half-life	FCR*
	h	h^{-1}		*h*	h^{-1}
1	4.28	0.162	7	4.12	0.168
2	5.03	0.138	8	5.25	0.132
3	4.51	0.154	9	5.33	0.130
4	4.62	0.150	10	4.58	0.151
5	3.38	0.205			
6	3.46	0.200			
Mean±SEM	4.21±0.025	0.168±0.011		4.82±0.29	0.145±0.009

* FCR, fractional catabolic rate determined according to the equation $K = \frac{0.693}{\text{half-life}}$.

Computation of rate of CRP synthesis. The amount of CRP disappearing from the vascular compartment in a given period of time is equal to fractional catabolic rate × total plasma volume × serum CRP concentration. Precise estimation of fractional catabolic rate and of rate of CRP synthesis would require knowledge of the rate at which intravascular CRP passes into the extravascular space. Such information is not available. However, this rate constant has previously been determined for serum albumin in rabbits and found to be ~4% h^{-1} (24); the corresponding rate constant for CRP would be expected to be even lower, since this molecule is considerably larger than albumin (17). Since rate of passage of CRP from vascular to extravascular space can thus be presumed to be relatively small compared with the fractional disappearance rate observed in these studies, it would have only a small influence on the calculation of fractional catabolic rate. We therefore equated the fractional disappearance rate with the fractional catabolic rate of rabbit CRP and computed synthesis rate based on this value.

In the steady state, the amount of protein disappearing from plasma per unit time is equal to that synthesized and entering the vascular compartment during the same time period. In these studies it was possible to calculate the approximate mass of CRP synthesized in those rabbits in which only modest or no changes in serum CRP concentration were observed over the period during which fractional catabolic rates were determined. For purposes of these calculations, total plasma volume in the rabbit was taken to be 45 ml/kg body wt (25).

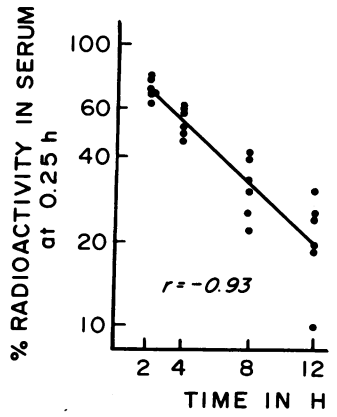

FIGURE 3 Disappearance of radiolabeled CRP in six stimulated rabbits. Rabbits received ^{14}C-CRP or ^{125}I-CRP intravenously 24 h after injection of either turpentine intramuscularly or LPS intravenously.

TABLE II

Half-Life, Fractional Catabolic Rate, and Changes in Serum CRP Concentrations in Stimulated Animals

Animal	Isotope	Δ[CRP]*	Half-life	FCR‡
			h	h^{-1}
11§	^{125}I	+25	5.57	0.124
12§	^{125}I	+12	6.97	0.099
13§	^{14}C	+14	3.65	0.190
14§	^{14}C	+7.5	5.77	0.120
15‖	^{125}I	−10	5.19	0.133
16‖	^{125}I	+11	7.67	0.090
Mean±SEM			5.80±0.57	0.126±0.01

* Percentage change in serum CRP concentration during the period between 2 and 12 h after injection of radiolabeled CRP.

‡ FCR, fractional catabolic rate.

§ Injected intramuscularly with turpentine (2 ml/animal).

‖ Injected intravenously with lipopolysaccharide (5 μg/animal).

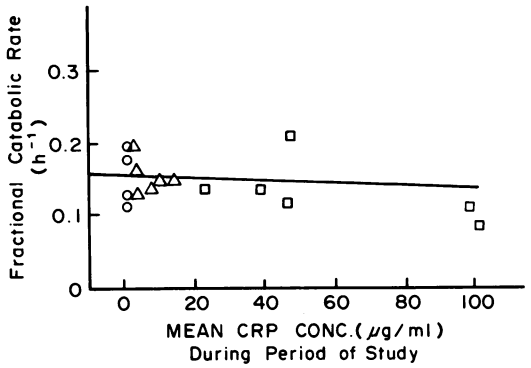

FIGURE 4 Relationship of fractional catabolic rate to serum CRP concentrations. Unstimulated rabbits were classified into two subgroups; nonresponders (○), having undetectable levels of CRP, and weak-responders (△) achieving serum CRP levels up to 11 μg/ml during the period of study. Stimulated animals (□) received either turpentine or LPS as described in the text. Linear regression analysis of these points revealed a slope of −0.0005.

We estimated rates of CRP catabolism and synthesis in the unstimulated animals whose serum CRP concentrations remained <2 μg/ml throughout the period of study. The lowest rate of synthesis, 6.7 μg/kg per h, was found in rabbit 7, which had a fractional catabolic rate of 0.15 h^{-1}, serum CRP level by radioimmunoassay of 0.9 μg/ml, and body wt of 2.23 kg. We performed similar calculations in a rabbit which achieved a serum CRP level of 104 μg/ml 36 h after intramuscular injection of turpentine. This animal's CRP level varied by only 7.5% over the period of study; a rate of synthesis of 560 μg/kg per h was calculated. As indicated above, actual rates of synthesis may be somewhat less than those calculated, because of the influence on computation of fractional catabolic rate of the small amount of passage of radiolabeled CRP from vascular to extravascular space.

DISCUSSION

Our major finding in this study is that the fractional catabolic rate of rabbit CRP is strikingly rapid, both in unstimulated and stimulated animals. The fractional catabolic rate of most plasma proteins is considerably slower (26), although comparably rapid rates have been found, e.g., proconvertin (Factor VII) has been reported to have a half-life of 4–6 h (27). Although it is possible that the rapid rate of disappearance of labeled CRP from plasma that we observed might have resulted from degradation of CRP during radiolabeling or purification, we believe that this is not the case for several reasons. (*a*) The radiolabeled protein was found to have the same molecular size by gel filtration and SDS-polyacrylamide gel electrophoresis as does the unlabeled protein. (*b*) Radiolabeled CRP retained its biologic property of binding to CxPS in the presence of calcium. (*c*) The iodinated CRP was labeled with only a small number of substitutions and the method we used for labeling with ^{14}C is noteworthy for its gentleness (19). Labeling by these two different methods resulted in the same metabolic findings. (*d*) Previous studies of serum CRP changes in rabbits stimulated with LPS suggested that a very rapid fall in serum CRP levels might occur after cessation of stimulation. In one such experiment a serum half-disappearance time as rapid as 4.5 h was noted (7). For all of these reasons our findings must be regarded as valid. This conclusion is further supported by the recent observation that serum amyloid-P component (SAP), a murine acute phase protein in the same superfamily as CRP, similarly shows a relatively rapid half-life of 7.5 to 9.5 h in both stimulated and unstimulated mice (28, 29).

We also found that the fractional catabolic rate of CRP is independent of serum concentration and lies roughly between 0.1 and 0.2 h^{-1}. This finding indicates that the same fraction of the intravascular mass of CRP is catabolized per unit time both in unstimulated animals and during the acute phase response. Among human plasma proteins this pattern of catabolism is exemplified by fibrinogen and IgM. Such a phenomenon might be explained by a simple mechanism such as pinocytosis of plasma (26). In contrast, fractional catabolic rates of transferrin and haptoglobin fall as plasma concentration rises, while those of albumin and IgG rise as plasma concentration rises (26).

Approximate rates of CRP synthesis by stimulated animals were calculated from these in vivo studies based on steady-state assumptions. We have previously reported rates of CRP synthesis by isolated perfused rabbit livers (15) and by rabbit primary hepatocyte cultures.[2] By assuming an average of 8.4×10^7 hepatocytes per gram of liver and 40 g of liver/kg body wt (30) it is possible to express rates of CRP synthesis in those studies in terms of micrograms per kilogram body weight per hour. The results obtained in this in vivo study are similar to those thus calculated, when we compare findings in rabbits mounting comparable serum CRP responses: attainment of serum CRP concentrations of 65 to 75 μg/ml, 20–24 h after inflammatory stimuli. Data from all three methods showed rates of CRP synthesis ranging between 410 and 560 μg/kg per h.

We must emphasize that our estimates of synthetic rates do not represent synthetic rates seen in all stimulated and all unstimulated animals. Substantially higher rates of synthesis undoubtedly occur in some

rabbits with more intense stimuli and further evolution of the CRP response. Calculations based on CRP levels in excess of 300 μg/ml, seen in some stimulated rabbits, would yield turnover rates approaching 2 mg/kg per h. For comparison, in normal rabbits albumin synthesis by isolated perfused livers has been found to be ~10.4 mg/kg per h (31). Similarly, while the lower limits of serum CRP levels in normal unstimulated rabbits are not known, if levels <0.1 μg/ml occur in the rabbit as they do in man (32) then rates of synthesis substantially lower than those we calculated in unstimulated rabbits may occur.

SAP was probably a minor contaminant in most of our CRP preparations since it binds to Sepharose in the presence of calcium ion. In the rabbit this protein has been reported to be larger than CRP, with a molecular weight of ~255,000 (33). Chromatography of our radiolabeled CRP on Sephadex G-200 demonstrated <3% of total protein radioactivity eluting in the void volume, indicating that no more than this percentage of radiolabeled protein had a molecular weight >200,000. As much as 5% contamination with other proteins would not significantly alter our physiologic findings, which are based on disappearance of >80% of the label that had been detectable at 15 min after injection. Changes in curves after this much labeled protein had disappeared are largely irrelevant to our major concerns and do not influence our conclusions. In addition, in order to insure that our results were not significantly influenced by the presence of contaminating SAP in our radiolabeled CRP preparations, we added a further purification step of gel filtration on Sephadex G-200 in one experiment in order to remove this protein. Our result in this experiment (rabbit 6) was not significantly different from those obtained in the other nine unstimulated rabbits.

The sites of removal of CRP from the circulation and the mechanisms of its degradation are unknown. That CRP degradation is rapid is indicated by preliminary studies in which as much as 25% of administered protein radioactivity appeared as free radioactivity in the urine by 6 h after injection of radiolabeled CRP. Elucidation of mode and sites of degradation of CRP in stimulated and unstimulated animals may cast further light on its biologic significance.

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