

THE OCCURRENCE DURING ACUTE INFECTIONS OF A PROTEIN NOT NORMALLY PRESENT IN THE BLOOD

III. IMMUNOLOGICAL PROPERTIES OF THE C-REACTIVE PROTEIN AND ITS DIFFERENTIATION FROM NORMAL BLOOD PROTEINS

BY COLIN M. MACLEOD, M.D., AND OSWALD T. AVERY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, October 7, 1940)

Previous studies from this laboratory have shown that serum obtained from patients during the acute phase of certain infectious diseases reacts to form a precipitate with the C polysaccharide of Pneumococcus. In the two preceding papers (1, 2) evidence was presented that the C-reactive substance in "acute phase" serum is protein in nature; certain of the chemical properties which distinguish the so called "C-protein" from normal serum proteins were described, and some of the distinctive features of its reactivity with the test carbohydrate were discussed. Because of these differences in chemical properties and in view of the fact that it is not demonstrable in the blood of convalescents and healthy individuals, the question arose whether this particular protein might not also possess immunological properties specifically different from those which characterize the proteins normally found in human serum. With this object in mind the following experiments were carried out.

The present paper presents the results of a study of the antigenicity and immunological specificity of the C-protein. It will be shown that the chemical individuality of this substance is reflected in the serological specificity of the antibodies to which it gives rise in the rabbit, and that by means of antisera it is possible to differentiate the C-protein from the normal protein constituents of human serum.

EXPERIMENTAL

Preparation of Immunizing Antigens.—The C-protein used for the immunization of rabbits was prepared from blood obtained at autopsy from fatal cases of Pneumococcus lobar pneumonia. The reactive protein was precipitated from the serum by the addition of an optimal concentration of the C polysaccharide. It has been shown that in the C-reaction, flocculation of the active protein is conditioned by the calcium ions normally present

in the serum, and that the precipitate thus formed can be repeatedly washed with a dilute solution of calcium chloride without loss of the reactive protein. Thus a method was available for obtaining the C-protein relatively free from the normal serum proteins and in a form especially suitable for testing its antigenicity in animals.

Human serum known to contain the C-precipitable protein was passed through a Berkefeld filter to remove bacteria and cellular debris. To the filtered serum the C polysaccharide was added in final concentration of 1:20,000. The mixture was incubated 2 hours at 37°C. and overnight in the refrigerator. The precipitate which formed was recovered by centrifugation. In order to remove as completely as possible any normal protein which may have become entrained during flocculation, the precipitate was thoroughly washed 3 or 4 times with large portions of $m/20$ calcium chloride in physiological saline. The washed precipitate containing the C-protein was then emulsified in a volume of $m/100$ aqueous solution of calcium chloride equal to $1/10$ the volume of the original serum. This preparation of the protein in particulate form was used as immunizing antigen.

Other lots of reactive protein prepared in this same manner were further purified by re-solution and reprecipitation. The precipitate after repeated washings with dilute calcium was finely emulsified in physiological saline and redissolved by the dropwise addition of the minimal amount of $N/10$ NaOH necessary to effect solution which occurs at approximately pH 8.5, probably due to the splitting off of bound calcium; a small amount of insoluble material was removed by centrifugation. From the clear supernatant, the protein was reprecipitated by adding an equivalent amount of $N/10$ HCl bringing the solution back to neutral reaction at which flocculation again occurs. The reprecipitated protein was recovered by centrifugation and the same procedure repeated a second time. The precipitate was again washed in $m/20$ calcium chloride in physiological salt solution and then resuspended in a finely divided state in $m/100$ calcium chloride in distilled water. All preparations used for immunization were standardized as to protein content on the basis of total nitrogen present. Merthiolate in final concentration of 1:20,000 was added as preservative.

Immunization of Rabbits.—Rabbits were injected intravenously on each of 5 or 6 consecutive days with 1 cc. of a preparation of the antigen containing approximately 1.5 mg. of protein per cc. 7 days after the last injection the animals were bled. Immune sera prepared in this manner are referred to in the following protocols as anti-CP rabbit serum.

C Polysaccharide.—The C polysaccharide was prepared from an R strain derived from *Pneumococcus* Type II. The methods of isolation and purification were the same as those previously described (3).

Serological Reactions.—The precipitation and complement-fixation tests were carried out with anti-CP rabbit sera prepared as stated above; the test antigens used in the serological reactions were, (a) whole serum obtained from patients with acute infections and known to contain the protein reactive with the C polysaccharide; (b) control serum from healthy individuals; (c) purified preparations of the C-reactive protein isolated from patients' serum by salting out with sodium sulfate according to the method described in the preceding paper (2).

Immunological Specificity of the C-Reactive Protein.—In view of the chemical differences which distinguish this protein from the normal serum proteins it was of interest to determine whether corresponding differences exist in antigenic function and serological specificity. For this purpose antisera were prepared by injecting rabbits with washed precipitates of the reactive protein removed from acute phase serum by the C polysaccharide.

The method of preparing the immunizing antigen, the technique for the production of rabbit antisera, and the nature of test antigens used in the

TABLE I
*Precipitins in Sera of Rabbits Immunized with C-Reactive Protein**

Anti-CP rabbit serum Lot No.	Dilutions of test antigens					
	Patient's serum; acute stage of lobar pneumonia			Normal human serum		
	1:2	1:5	1:10	1:2	1:5	1:10
1	+++	+++±	+++±	±	+	+
2	+++	+++±	+++±	—	Tr.	Tr.
3	++++	++++	++++	±	+	±
Control: normal rabbit serum†.....	—	—	—	—	—	—

In this and the following tables the degree of precipitation is expressed by the symbols:

++++ = flocculent precipitate, clear supernatant.

+ = slight turbidity.

Tr. = trace.

* Protein precipitated from acute phase serum by C polysaccharide.

† Pooled sera of normal rabbits.

serological reactions have been described in a preceding section of this paper.

In the following experiment various lots of anti-CP rabbit serum were tested for the presence of precipitins for the C-protein by adding a constant amount of immune serum to serial dilutions of patient's serum known to contain the reactive protein as determined by previous test with the C polysaccharide. As controls the same antisera were similarly tested against normal human serum known to be free from the C-reactive protein. The results of the precipitin tests are presented in Table I.

As shown in Table I the precipitins in anti-CP rabbit serum are highly specific for the C-protein and react only weakly or not at all with the proteins normally present in human serum. The lack of cross-reactions is particularly noteworthy in this instance, since the antisera had not been

previously absorbed with normal human serum before being used in the test. The specificity of the antibodies under these conditions is all the more striking in view of the fact that the test antigens contained in common the normal proteins of human serum.

Furthermore, the results of the precipitin tests demonstrate that the reactive protein precipitated by the C polysaccharide is markedly antigenic and in this form is capable of inciting in rabbits the production of antibodies specifically reactive with the homologous protein in the native state in which it exists in the serum of infected patients.

In the following experiment the precipitins in another lot of anti-CP rabbit serum were titrated by the use of acute phase and normal human sera as test antigens. For purposes of titration the immune serum was first absorbed with normal human serum to remove any cross-reacting anti-

TABLE II

Precipitin Reactions of Anti-CP Rabbit Serum with Acute Phase and Normal Human Sera

Serum of rabbits immunized with C-protein*	Test antigens used	Dilutions of test antigens						
		1:2	1:10	1:20	1:50	1:100	1:500	1:1000
Lot 4	Acute phase human serum	++++	++++	+++	++±	+±	±	—
	Normal human serum	Tr.	Tr.	—	—	—	—	—

* Precipitated from patient's serum by C polysaccharide.

bodies which if present would tend to mask the end point. The particular specimen of acute phase serum used as test antigen was obtained from a patient acutely ill with Type III Pneumococcus pneumonia who at the time had high fever, a marked bacteremia, and massive pulmonary involvement.

The precipitating action of the immune serum in the presence of increasing dilutions of the test antigens is shown in Table II.

The data presented in Table II again demonstrate the selective specificity of the precipitins in anti-CP rabbit serum. The results also show that the presence of the reactive protein could still be detected in the patient's serum when the latter was diluted 1 part in 500. In the absence of quantitative data as to the actual amount of reactive protein present in the test serum it is of course obvious that the result does not represent the true titre of precipitins in the immune rabbit serum. The reactions do indicate, however, the relative sensitivity of the precipitin test as a means of demonstrating the reactive protein when present in amounts too small to be detected by the precipitation test with C polysaccharide. In a parallel series of tests the latter reaction was completely negative in all dilutions of the same

serum above 1:20. Because of inherent differences in the nature of the two reactions the use of the polysaccharide test alone may be misleading. In the precipitin reaction the precipitate is composed largely of the antibody globulin from the immune rabbit serum, while in the flocculation test with C polysaccharide, the precipitate consists chiefly of the reactive protein present in the albumin fraction of the patient's serum. Consequently in the latter test a negative result does not necessarily imply the complete absence of the reactive protein since it may be present in amounts too small to yield a visible precipitate with the test carbohydrate. This difference in the sensitivity of the two tests has both theoretical and practical interest; for example, the use of specific antiserum would be the method of choice in any attempt to trace the origin and fate of the reactive protein in the tissues and fluids of the body.

TABLE III

Precipitin Reactions of Anti-CP Rabbit Serum with Purified Preparation of Reactive Protein

Anti-CP rabbit serum Lot No.	Dilutions of test antigen				
	Purified preparation of reactive protein*				
	1:15,000	1:30,000	1:60,000	1:120,000	1:240,000
4	+++	++±	+±	+	Tr.

* Isolated from patient's serum by salting out reactive protein with sodium sulfate, followed by dialysis and reprecipitation (2).

In the following experiment the same lot of anti-CP rabbit serum as that used in the preceding test was titrated against a purified preparation of the reactive protein. In this instance the test antigen was prepared by salting out the reactive protein from acute phase human serum with sodium sulfate followed by dialysis and reprecipitation as described in the preceding paper (2). The dilutions of test antigen are expressed in terms of protein based on the content of total nitrogen in the preparation. The results of the precipitin titration are given in Table III.

As shown in Table III the titre of precipitins in this particular antiserum was 1:240,000 when a highly purified preparation of reactive protein was used. The immune serum was the same lot as that used in the preceding experiment and had been absorbed with normal human serum prior to use in these tests.

The immunizing antigen used in preparing the antiserum consisted of the reactive protein precipitated from patients' serum by C polysaccharide whereas the test antigen was prepared by salting out the serum albumin and subsequently isolating the reactive protein as previously described (2).

The differences in the preparative methods and the fact that the immunizing and test antigens were derived from sera of different patients serve to emphasize the immunological identity of the reactive protein and its differentiation from the proteins of normal human serum. The antigenic quality of the C-precipitable protein is reflected in the high titre of precipitins in the serum of rabbits which had received during a single course of five daily injections a total of only 6.5 mg. of the reactive protein.

As pointed out in the preceding experiment, the greater sensitivity of the precipitin reaction as contrasted with that of the flocculation test with C polysaccharide is again evident in the present instance where the quantitative relationships are more adequately defined. Although not shown in the protocol, it was found in a parallel series that the reaction with the C polysaccharide was completely negative in dilutions of the test antigen above 1:15,000, whereas with immune rabbit serum the presence of the homologous protein could be detected in dilutions as high as 1:240,000.

The amount of reactive protein in the blood during acute bacterial infections is relatively small and varies in individual cases depending to some extent at least upon the nature and severity of the pathological process. As pointed out in earlier studies (4) the concentration of the C-precipitable substance in patients' serum is maximal during the height of the disease and progressively diminishes following the onset of recovery. Abernethy and Francis (5) have shown that in severe cases of pneumonia, the serum may give a positive flocculation reaction when the test carbohydrate is added in dilutions as high as 1:640,000. Reactions of this order compare favorably in sensitivity to precipitin reactions involving the combination of an antigen with its specific antibody. The flocculation of the reactive protein in the presence of the C polysaccharide affords a means of determining roughly the amount of reactive protein present in a given serum; however, for the detection of minute quantities of the protein the immunological reaction with anti-CP rabbit serum is far more sensitive.

Complement Fixation by Reactive Protein and Specific Antiserum.— In order to compare further the antigenic specificity of the C-reactive protein with that of normal serum proteins, the corresponding antisera were prepared in rabbits and tested for the capacity to bind complement in the presence of varying dilutions of the C-reactive protein.

The technique used in the complement-fixation test was the same as that described by Pittman and Goodner¹ (6). The anti-CP rabbit serum was prepared as described

¹ The authors take pleasure in thanking Dr. Goodner for his kindness in carrying out these tests.

and was absorbed with normal human serum. The immune sera to the normal proteins in human blood were produced by injecting rabbits intravenously on each of 5 consecutive days with 0.2 cc. of whole serum obtained from a healthy individual. The total amount of normal serum injected was 1.0 cc. per rabbit. 7 days after the last injection the animals were bled and their sera tested for precipitins and complement-fixing antibodies. In precipitin tests the latter antisera reacted with normal human serum diluted as high as 1:80,000.

The C-reactive protein used as test antigen was prepared from patient's serum by salting out with sodium sulfate, followed by dialysis and reprecipitation (2). The amount of protein present was estimated by determination of the total nitrogen. The dilutions of the protein used in tests were made in physiological saline. Results of the complement-fixation tests are shown in Table IV.

TABLE IV
Complement Fixation by Anti-CP Rabbit Serum and Reactive Protein Isolated from Patient's Serum

Sera of rabbits immunized with	Purified preparation of reactive protein*		
	1:5000	1:50,000	1:250,000
C-protein†	+++	++++	+++
Normal human serum	—	—	—

Degree of fixation expressed by the symbols:

++++ = complete fixation of complement.

— = no fixation of complement.

* Isolated from patient's serum by salting out reactive protein with sodium sulfate, followed by dialysis and reprecipitation (2).

† Prepared by precipitating the reactive protein from acute phase serum with C polysaccharide.

As shown in Table IV a purified preparation of the reactive protein in dilutions of 1:5000 to 1:250,000 fixed complement in the presence of an antiserum prepared by immunizing rabbits with the reactive protein in the form precipitated by C. On the other hand mixtures of the reactive protein and an antiserum to normal human serum failed entirely to fix complement. These results confirm those of the precipitin tests and afford further evidence that the C-reactive protein is immunologically distinct from the proteins of normal human serum.

Reactivity of Anti-CP Serum with Acute Phase Monkey Serum.—It has been shown by Abernethy (7) that the serum of monkeys (*Macacus cynomolgus*) acutely ill with experimental Type III Pneumococcus pneumonia precipitates with the C carbohydrate in high dilutions, but that the serum from normal or recovered monkeys fails to react. It was of interest to determine whether the serum of acutely ill monkeys would react specifically with antiserum to the C-reactive protein of acute phase human serum.

Morphinized monkeys (*M. cynomolgus*) were injected intravenously with a virulent strain of Pneumococcus Type I. A severe illness accompanied by bacteremia resulted. The animals were sacrificed on the 3rd day after inoculation. Serum obtained from these animals during the acute phase of the pneumococcal infection reacted strongly in precipitin tests with anti-CP serum, whereas the serum of the same animals before infection gave negligible reactions.

These results show that the antiserum of rabbits immunized with the C-protein of human origin reacts specifically with the similar protein present in the serum of infected monkeys. The difference in the reactions before and after infection leaves no doubt that an immunologically similar protein occurs in the blood of man and monkeys during the course of acute infections—a protein not present in the serum of normal human beings or normal monkeys.

DISCUSSION

The C-reactive protein present in acute phase human serum shows certain differences from the normal serum proteins. As previously described (1, 2) the reactive protein is contained in the albumin fraction of serum, but unlike normal serum albumin it is insoluble in water containing a trace of calcium. Furthermore, it has been shown that the insolubility of the protein under these conditions is due to the presence of lipoid substances associated with the protein, since upon removal of the lipids the protein loses its calcium sensitivity. However, removal of the lipids does not affect the reactivity of the protein with the C polysaccharide.

In the present paper a further property is described which differentiates the C-reactive protein from normal serum proteins, namely its specific antigenicity. It has been shown that the serum of rabbits immunized with the C-reactive protein reacts specifically not only with purified preparations of protein but also with this protein as it occurs in the serum obtained from man and monkeys during the course of acute bacterial infections. Only negligible reactions occur when normal human serum is mixed with anti-CP serum and this trace of cross-reactivity may be removed by absorption without appreciable loss in the titre of the antibodies to the C-reactive protein.

From these observations it may be concluded that the reactive protein differs in both its chemical and immunological properties from the proteins of normal human and monkey serum.

Observations on the presence of the C-reactive protein in the blood during the acute phase of virus diseases in man are as yet too few to warrant any conclusions as to its occurrence in infections of this nature. Further-

more, there is available at present no evidence of relationship between this protein and the various "soluble antigens" which, separable from the virus, have been found in the tissues and blood of man and animals acutely ill with yellow fever (8), vaccinia (9), psittacosis (10), influenza (11), myxomatosis (12), and lymphocytic choriomeningitis (13). In this connection it should be borne in mind that in acute bacterial diseases of man the occurrence of the C-reactive protein is a general phenomenon and is independent of the specific nature of the etiological incitant. Moreover, in diseases of bacterial origin its demonstration is dependent on certain species relationships, as shown by the fact that a similarly reactive protein has been found only in the blood of immunologically related species such as man and monkeys. An analogous substance may occur in the blood of other species during bacterial infections but its detection would probably require in each instance a selective test reagent.

SUMMARY

The C-reactive protein present in the albumin fraction of the serum of patients during certain acute bacterial infections is highly antigenic upon injection into rabbits. The antiserum thus prepared reacts specifically with this protein and does not react with the proteins of normal human serum. Immunological specificity has been demonstrated by both precipitin and complement-fixation tests.

Antiserum prepared in rabbits to the C-reactive protein from human sources also reacts specifically with the similar protein in the serum of monkeys acutely ill with experimental pneumococcus infection.

By means of immunological reactions it is possible to detect amounts of reactive protein which are too small to yield a visible precipitate in tests with the C polysaccharide.

Certain of the properties are discussed which distinguish the C-reactive protein from the proteins of normal human serum.

BIBLIOGRAPHY

1. Abernethy, T. J., and Avery, O. T., *J. Exp. Med.*, 1941, **73**, 173.
2. MacLeod, C. M., and Avery, O. T., *J. Exp. Med.*, 1941, **73**, 183.
3. Tillett, W. S., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1930, **52**, 895. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1931, **53**, 625.
4. Tillett, W. S., and Francis, T., Jr., *J. Exp. Med.*, 1930, **52**, 561.
5. Abernethy, T. J., and Francis, T., Jr., *J. Exp. Med.*, 1937, **65**, 59.
6. Pittman, M., and Goodner, K., *J. Immunol.*, 1935, **29**, 239.
7. Abernethy, T. J., *J. Exp. Med.*, 1937, **65**, 75.
8. Hughes, T. P., *J. Immunol.*, 1933, **24**, 275.

9. Craigie, J., and Wishart, F. O., *Tr. Roy. Soc. Canada*, Section V, 1935, **29**, 57.
Parker, R. F., and Rivers, T. M., *J. Exp. Med.*, 1936, **63**, 69; 1937, **65**, 243.
Parker, R. F., *J. Exp. Med.*, 1938, **67**, 361.
10. Bedson, S. P., *Brit. J. Exp. Path.*, 1936, **17**, 109.
11. Hoyle, L., and Fairbrother, R. W., *J. Hyg.*, 1937, **37**, 512.
12. Rivers, T. M., and Ward, S. M., *J. Exp. Med.*, 1937, **66**, 1. Rivers, T. M., Ward, S. M., and Smadel, J. E., *J. Exp. Med.*, 1939, **69**, 31; 1940, **72**, 129.
13. Smadel, J. E., Baird, R. D., and Wall, M. J., *J. Exp. Med.*, 1939, **70**, 53; 1940, **71**, 43. Smadel, J. E., and Wall, M. J., *J. Exp. Med.*, 1940, **72**, 389.