THE RELATIONSHIP BETWEEN THE ACUTE PHASE RESPONSE AND ANTIBODY PRODUCTION IN THE RABBIT

I. Correlation between the Early Appearance of Cx-Reactive Protein and Subsequent Antibody Production

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It has recently been shown that an acute phase substance analogous to human C-reactive protein is produced by rabbits (1). The rabbit protein appears in the blood in response to stimuli of the same type which causes the appearance of the human protein. In addition, the chemical properties of the two proteins and the procedures employed for their isolation from serum are similar. The close relationship between the rabbit and human proteins is shown by the fact that both react to form precipitates when mixed with solutions of pneumococcal somatic carbohydrate in the presence of calcium ion. The major point of difference is that the rabbit protein does not precipitate with classical C carbohydrate but reacts under similar conditions with a special form of the carbohydrate, designated Cx (1). The human protein reacts equally well with either form of the carbohydrate. Because of this difference in precipitability, the term Cx-reactive protein has been applied to the rabbit protein.

In view of the evidence that rabbit Cx-protein is analogous to human C-protein, the rabbit system was used as an experimental model for investigation of the biological significance of these acute phase substances. In the present study the relationship between the occurrence of Cx-reactive protein during antigen administration to the subsequent appearance of precipitating antibody has been investigated. These experiments stemmed from an observation made during the course of the immunization of rabbits with purified preparations of human C-reactive protein. Upon testing sera obtained from these animals during the course of antigen administration with pneumococcal Cx polysaccharide, it was found that one rabbit which later developed a high titer of antibody gave a strong reaction with the polysaccharide. Rabbits which failed to develop appreciable antibody did not show the presence of Cx-reactive protein during the period of antigen administration. Further observations suggested that a correlation might exist between the early appearance of Cx-reactive protein and the subsequent development of precipitating antibody to human C-reactive protein. A systematic investigation was undertaken to determine whether this correlation was consistent.

The fact that human C-reactive protein was used as an antigen in these studies and that the measurement of rabbit Cx-reactive protein served as an index of the acute phase response leads to some confusion as a result of the cumbersome terminology. The human protein was employed solely for its antigenic properties, and its other biological properties have no relevance to the present experiments. At the time the initial observations were made, this antigen was being employed in attempts to obtain satisfactory antisera for use in a serological test for rheumatic activity (2). The antigen proved to have properties which made it useful in further experiments on the correlation between the acute phase response and antibody production. Thus, only a small proportion of rabbits immunized with human C-protein produce high titered precipitating antisera, and as a result the relationship to the acute phase response could be more sharply defined. Additional experiments were carried out with a second antigen, human gamma globulin, which is also a relatively weak antigen (3).

Materials and Methods

Immunization Procedures.—In all experiments the antigen, either lipid-free C-reactive protein or human gamma globulin, was given in two or three separate courses of three or four successive daily intravenous injections with 4 or 5 days of rest after each course of antigen administration. Sera from all animals on each day of the experiments, up to 27 successive days, were tested for Cx-reactive protein and the sera obtained on the last day of each rest period were tested for antibody. The sera of three of the four groups of animals immunized with human C-reactive protein were tested for Cx-reactive protein by means of the ring test with carbohydrate. The sera of a fourth group of rabbits immunized with C-reactive protein were tested both with Cx carbohydrate and by capillary precipitin tests with a high titered guinea pig antiserum to Cx-reactive protein. The latter reagent is more sensitive than the carbohydrate but was not available at the time the sera of the first three groups of rabbits were tested. All sera of rabbits which received gamma globulin were tested with the guinea pig antiserum to Cx-protein.

Preparation of the C-Reactive Protein.—The C-reactive protein employed as antigen in these experiments was obtained from several thoracentesis fluids which contained the lipid-free protein. Isolation and purification of the protein were carried out by the method described by McCarty (4), except that crystallization was not attempted. The preparation was tested for traces of normal human serum proteins with a rabbit antiserum to normal human serum, and the concentration of protein in the solution was determined by a spectrophotometric method (5). The amounts of antigen given to four groups of animals in each daily injection were respectively 0.165, 0.170, 0.250, and 1.0 mg.

Human Gamma Globulin.—Human gamma globulin, fraction II, and fraction II, 1–2 were employed as antigens.[1] The daily doses of antigen in the four experiments were respectively 2.5, 5.0, 10, and 15 mg.

Animals.—Hair brown rabbits weighing between 2500 and 3500 gm. were employed in

[1] The gamma globulin, fraction II, was obtained from the American Red Cross through the kindness of E. R. Squibb & Sons, New Brunswick, New Jersey. The gamma globulin, fraction II, 1–2, was prepared in the laboratory of Dr. E. J. Cohn, Harvard Medical School.

all experiments. A control bleeding was done on each animal to assure the absence of Cx-reactive protein from the serum at the beginning of each experiment.

Pneumococcal Cx Polysaccharide.—This polysaccharide was prepared by rapid lysis and deproteinization of the pneumococcal cells as described by Anderson and McCarty (1). The carbohydrate in concentrations as low as 0.005 mg./cc. gave visible precipitates with acute phase rabbit serum.

Ring Tests with Cx Polysaccharide.—The reactions between serum and Cx polysaccharide were carried out in precipitin tubes by the microprecipitin technique described by Lancefield (6). In each test approximately 0.1 cc. of an 0.1 mg./cc. Cx carbohydrate solution in 0.85 per cent NaCl was carefully layered over 0.1 cc. of the serum being tested. Each determination was controlled with a tube containing the test serum layered with 0.85 per cent NaCl in place of the carbohydrate. After incubation for 1 hour at 37°C., the tubes were inspected for the appearance of a ring of visible precipitate at the interface.

Preparation of Guinea Pig Antiserum to Cx-Reactive Protein.—High titered precipitating antisera to Cx-reactive protein were obtained from 300 to 500 gm. guinea pigs 3 weeks after the subcutaneous injection into two separate abdominal sites of purified Cx-reactive protein incorporated into an adjuvant consisting of an emulsion of two parts of antigen solution and two parts of heavy grade mineral oil to one part of the ointment base aquaphor.[2] 0.5 mg. of dried, heat-killed Jamaica strain tubercle bacilli was incorporated in each 10 cc. of the adjuvant. 1 cc. of the adjuvant was given in each subcutaneous injection.

The test employing guinea pig antiserum for the detection of Cx-reactive protein in serum is considerably more sensitive than the ring test with Cx carbohydrate. A similar difference in sensitivity of the tests has been found with human C-reactive protein. In the latter case, amounts less than 0.005 mg./cc. can be detected with antiserum whereas the C carbohydrate will not give a ring of visible precipitate with sera which contain less than 0.05 mg./cc. of the C-reactive protein (5). Similarly, the Cx carbohydrate will not react to give a ring of precipitate with acute phase rabbit sera which give less than a +++ precipitin reaction with the guinea pig antiserum.

Precipitin Tests with Antisera.—The precipitin tests with antisera to Cx-protein, to C-reactive protein, and to human gamma globulin were carried out in capillary tubes according to the procedure described by Anderson and McCarty (2). This method is based on the capillary precipitin technique for serologic typing of group A streptococci described by Swift, Wilson, and Lancefield (7).

Quantitative Determination of Antibody to Gamma Globulin.—The serum from the final bleeding of each of the 43 animals immunized with gamma globulin in the experiments in which this antigen was employed was set up in capillary precipitin tubes with the following concentrations of the antigen: 10, 5, 2.5, 1.0, 0.1, and 0.01 mg./cc. Equal volumes of antiserum and of antigen were used in each capillary precipitin test. After incubation at 37°C. for 2 hours and refrigeration overnight, the amount of antigen-antibody precipitate in each capillary tube was estimated in terms of a scale from 0 to 8+. The approximate zone of equivalence was obtained in this way for each of the 43 sera.

The determination of the amount of antibody present in selected sera was done by the ninhydrin method described by Kunkel and Ward for estimation of the quantity of antigen-antibody precipitate (8). This procedure, based on the ninhydrin method of Moore and Stein (9), employs small amounts of antiserum and has been shown to be a sensitive and reproducible method.

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[2] Aquaphor is an ointment base manufactured by Duke Laboratories, Inc., Stamford, Connecticut. It contains six parts of eucerite to ninety-four parts of neutral, stable aliphatic hydrocarbons.

RESULTS

Findings with C-reactive Protein as Antigen.—The rabbits which produced Cx-reactive protein in response to injection of human C-reactive protein were the ones which developed high titers of antibody to the human protein (Table I). Furthermore, there was a roughly quantitative relationship between the intensity of the acute phase response and the amount of antibody produced. The time of first appearance of Cx-reactive protein was, in all but one instance, during the first course of antigen injections or during the first rest period.

It can be seen in Table I that one animal in the first group developed a ++ precipitin titer of antibody despite the fact that Cx-protein was not demonstrable during immunization. The failure to detect Cx-protein in this animal may be due to the fact that the tests for Cx-protein were carried out with the ring test using Cx carbohydrate, which is a less sensitive procedure than the capillary precipitin test using guinea pig antiserum.

TABLE I

Correlation between Cx-Protein Response and Development of Antibody to C-Reactive Protein after Three Courses of Antigen Injection

	Group I			Group II				Group III			Group IV		
Amount of antigen in each injection *mg*....	0.165			0.170				0.25			1.0		
Rabbit No.....	1	2	3	4	5	6	7	8	9	10	11	12	13
Cx-reactive protein response during antigen administration (ring test with Cx carbohydrate).....	++++	−	−	++++	−	++	−	++	−	−	++++	++++	−
Capillary precipitin test for antibody to C-reactive protein on last day of experiment.....	++++	++++	−	++	+++++	−	++++	−	++++	±	++	++++	++++	++++	+

In Fig. 1 the results obtained on daily bleedings from each of the 3 rabbits in the third group of animals in Table I are shown in graphic form. In this instance, more detailed data concerning the development of Cx-reactive protein during the course of immunization were obtained by testing for this substance by the precipitin test with guinea pig antiserum. Thus, even the occurrence of trace amounts of the acute phase protein could be detected. As in the other experiments, sera were tested for the presence of antibody on the last day of each rest period. Animal 8 in this group developed a high titer of precipitating antibody to human C-reactive protein and is the only animal of the 3 which developed enough Cx-reactive protein to be detectable with the

less sensitive test using Cx carbohydrate. Animals 9 and 10 did not respond to the antigen throughout the experiment with the production of a sufficient amount of rabbit acute phase protein to be detectable with the carbohydrate, but did produce smaller amounts in response to each course of antigen as indicated by the reactivity with specific guinea pig antiserum.

Findings with Human Gamma Globulin as Antigen.—Four groups of animals, comprising a total of 43 animals, were given human gamma globulin, fraction II, 1–2, or total fraction II on different dosage schedules. Of these

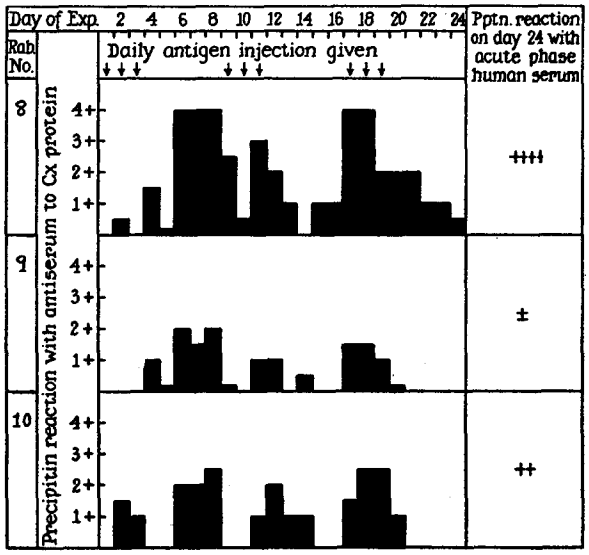

FIG. 1. Cx-reactive protein and antibody responses of 3 rabbits given three courses of 3 successive daily doses of 0.25 mg. C-reactive protein intravenously, each course followed by a 5 day rest period.

43 animals, 16 produced precipitating antibody in titers ranging between 0.15 and 0.64 mg. antibody N/cc. of serum, and 27 produced antibody in titer less than 0.15 mg. antibody N/cc. of serum. All but 1 of the 16 animals which produced the larger amount of antibody had shown sufficient Cx-reactive protein in the serum to be detectable with the Cx carbohydrate. Except in the instance of this 1 rabbit, all the 16 animals which produced the higher titer of precipitating antibody had more Cx-reactive protein in their sera over a longer period of time than did the other 27 animals, most of which produced relatively small amounts of Cx-reactive protein in response to the antigen. Table II summarizes the findings obtained with these 43 rabbits.

A more detailed picture of the sequence of events seen in 4 rabbits from one experiment is shown in Fig. 2, in which the results obtained on daily bleedings

TABLE II

Production of Cx-Protein and Precipitating Antibody by Rabbits Given Gamma Globulin as an Antigen

	Group I		Group II		Group III		Group IV	
Amount of antigen in each injection, *mg*.....	2.5		5		10		15	
Total No. of animals.....	9		16		8		10	
Test for Cx-protein	+	−	+	−	+	−	+	−
No. of animals showing indicated Cx-protein response.....	2	7	5	17	2	6	6	4
No. of animals producing high titer precipitating antibody.....	2	0	5	0	2	0	7	0

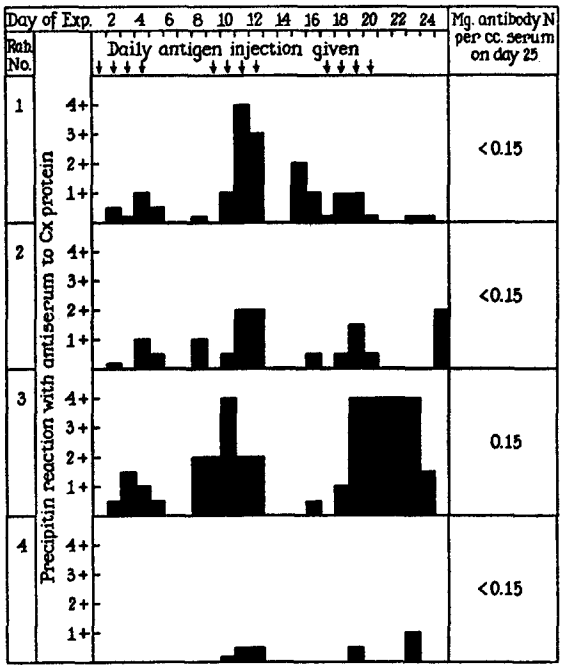

FIG. 2. Cx-reactive protein and antibody responses of 4 rabbits given three courses of 4 successive daily intravenous injections of 10 mg. human gamma globulin, fraction II, 1-2 each course followed by a 4 day rest period.

are shown in graphic form. These animals were given three series of four injections of 10 mg. gamma globulin, Fraction II, followed by three 4 day rest periods.

It is evident from inspection of Fig. 2 that rabbit 3 which had the highest titer of antibody at the end of the experiment produced the largest amounts of Cx-reactive protein in response to the antigenic stimulus. Rabbit 4 which produced almost no antibody responded to the antigen with the production of very little Cx-protein. It should be noted that Cx-reactive protein would have been detectable with the Cx carbohydrate in the sera of only 2 rabbits, Nos. 1 and 3. It would have been detectable on the 11th day only in the sera of rabbit 1, but would have been detectable on the 10th, 19th, 20th, 21st, and 22nd days in the sera of rabbit 3.

The times of appearance and disappearance of Cx-protein showed no uniform pattern in the 43 animals immunized with gamma globulin. Some animals, most of the 16 which attained high antibody titers, produced large amounts of the acute phase protein in response to each course of antigen. Other animals produced the Cx-protein in response to the second and third courses of antigen, and some to the final course alone. The animals which produced little or no antibody showed only trace amounts of Cx-protein in response to any of the antigen courses. In the case of the animals which produced large amounts of the acute phase protein, Cx-protein usually appeared in the serum during the injection period, persisted for several days, and then disappeared from the blood or became greatly diminished in quantity by the 3rd day of the succeeding rest period.

As in the four experiments in which C-reactive protein was employed as antigen, the results of the experiments employing gamma globulin indicate that a correlation exists between the early appearance of large amounts of Cx-reactive protein in response to the antigenic stimulus and the subsequent production of significant amounts of precipitating antibody.

DISCUSSION

The results of the experiments described in this study indicate that some rabbits respond to the intravenous injection of weak antigens of non-bacterial origin with the production of acute phase Cx-reactive protein. In the four groups of animals immunized with human C-reactive protein a consistent positive correlation was found to exist between the early production of the Cx-reactive protein and the subsequent production of high titers of precipitating antibody. Only those animals which showed an early response to the administration of the antigen by the production of their homologous acute phase protein later developed significant levels of precipitating antibody to the antigen.

In the experiments in which 43 rabbits were immunized with human gamma globulin, 16 developed titers of precipitating antibody greater than 0.15 mg. antibody N/cc. of serum. 15 of these 16 rabbits produced large amounts of

Cx-reactive protein. The remaining rabbit, like the 27 which at the end of the experiments had little or no precipitating antibody to gamma globulin, produced very little Cx-reactive protein in response to the injection of antigen. Thus, though the correlation between the early production of Cx-reactive protein by rabbits immunized with gamma globulin and the subsequent production of precipitating antibody to gamma globulin was very good, it was not without exception as was the case in the experiments in which C-reactive protein was used as antigen.

In the experiments using C-reactive protein as antigen, 1 mg. or less was given in each injection. Nine of the animals given gamma globulin as antigen received 2.5 mg. in each injection. In the instances of the administration of these very small quantities of antigen, the appearance of the Cx-reactive protein in the serum was a constant and extremely sensitive early indicator of subsequent antibody response of the treated animals.

It can not be determined from the present studies whether the Cx-protein response plays an essential role in antibody production. It has been suggested that the degree of inflammatory reaction influences the quantity of antibody produced (10–12); if so, the appearance of Cx-reactive protein may be correlated with the amount of antibody merely because it is related to the amount of inflammation produced by the injection of antigen. In this case, the occurrence of Cx-protein would be of no immediate significance to the immune response except in so far as it provided a tool for the prediction of the antibody response. On the other hand, the possibility exists that this particular acute phase protein is directly concerned in the chain of events that lead to antibody formation. Further experiments will be required to elucidate this point. In the following paper it will be shown that substances which induce a Cx-reactive protein response in rabbits will enhance the production of antibody to antigens introduced at a separate site; it is clear, therefore, that inflammation at the site of antigen injection alone is not required.

These results may have practical usefulness in the preparation of antisera to poor antigens which are difficult to obtain in large quantities. In this laboratory it has been possible to conserve the limited supply of human C-protein by giving repeated courses only to those rabbits which showed an early production of large amounts of Cx-reactive protein.

SUMMARY

It has been found that some rabbits respond to the administration of either human C-reactive protein or human gamma globulin with the production of an acute phase substance, Cx-reactive protein. A correlation was found to exist between the amount of Cx-protein produced and the subsequent production of significant titers of precipitating antibody to the two antigens employed.

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