IMMUNOLOGIC CROSS-REACTIONS AMONG MAMMALIAN ACUTE PHASE PROTEINS*

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Tillet and Francis (1) in 1930 found that sera obtained from patients early in the course of pneumococcal lobar pneumonia contained a substance which precipitated with the somatic C polysaccharide of pneumococci. Abernathy, MacLeod, and Avery (2–4) subsequently found this substance to be a protein, and defined many of its properties. Because it was found in the sera of patients acutely ill with other diseases and since its reaction with C polysaccharide required ionized calcium (2), it seemed unlikely that this "C-reactive protein" was an antibody. Abernathy (5) found that the sera of monkeys infected with pneumococci also contained a material which gave a precipitin reaction with C polysaccharide. Löfström (6, 7) and Anderson and McCarty (8) described the presence in acute phase rabbit sera of an analogous material, "Cx-reactive protein." The work of Anderson and McCarty (8) and Wood *et al.* (9–12) has demonstrated the close physicochemical and biological similarity between human C-reactive protein and rabbit Cx-reactive protein, but the source and function of these acute phase proteins remain obscure.

Both C-reactive and Cx-reactive proteins are apparently antigenically distinct from all normal serum proteins, since antibodies directed against these acute phase proteins do not react with normal sera (4, 8, 9, 13). Some indication of an antigenic similarity between the acute phase proteins of different species had been found by MacLeod and Avery (4), who showed that a rabbit antiserum against human C-reactive protein reacted with acute phase monkey sera. It was the purpose of the present study to further explore the immunologic similarities between the acute phase proteins of the rabbit, monkey, and man.

Materials and Methods

Preparations.—Pneumococcal *Cx polysaccharide* was prepared by the method of Anderson and McCarty (8). Crystalline human *C-reactive protein* (*CRP*) was prepared by the method of Wood, McCarty, and Slater (14) from a concentrate of human pathological transudates

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kindly supplied by Schieffelin & Company, New York. The preparations were recrystallized and one preparation was twice recrystallized. *Crystalline Cx-reactive protein* (CxRP) was prepared by a modification of the method described by Anderson and McCarty (8): 2 mg. of Cx polysaccharide was added to each 100 ml. of acute phase rabbit serum and the mixture was incubated at 37°C. for 2 hours and stored for 48 hours in the cold. The suspension was then centrifuged, the precipitate suspended in 10 to 20 ml. of physiological saline and dissolved by dropwise addition of 1 M sodium citrate. This solution was layered over an equal amount of chloroform and was shaken gently in the cold for 3 hours. The aqueous phase was cleared by centrifugation and dialyzed against physiological saline solution containing 0.001 per cent calcium chloride, until a heavy white precipitate had formed. The precipitate was collected by centrifugation, washed three times with physiological saline containing 0.001 per cent calcium chloride, and then suspended in 15 ml. saline and redissolved by the dropwise addition of 1 M sodium citrate. The solution was dialyzed against saline containing calcium as above, and after a precipitate had formed the washing procedure was repeated twice. The protein-polysaccharide complex was then dissolved in about 2 ml. of saline by the addition of sufficient sodium citrate, and a solution of sodium sulfate saturated at 37°C. was added dropwise until cloudiness first appeared, whereupon it was placed at 37°C. Over a period of 3 to 4 days, the material acquired a silky sheen and upon microscopic examination was found to contain small, needle-shaped crystals resembling those described by Anderson and McCarty (8). After centrifugation, the crystalline material was dissolved in about 2 ml. of water and recrystallized by addition of saturated sodium sulfate to the point of incipient turbidity, followed by incubation at 37°C. The preparation used throughout most of the experiments to be described was similarly recrystallized a second time. The preparations were finally dialyzed against isotonic phosphate buffer pH 7.0, and stored at −20°C. until needed.

Antigens.—Crystalline acute phase proteins were obtained as described above. *Normal human sera* were obtained from healthy volunteers in this laboratory. *Acute phase human sera* were obtained from patients on the medical wards of Bellevue Hospital, New York. *Normal rabbit sera* were obtained from several apparently healthy animals. *Acute phase rabbit sera* were obtained from rabbits 30 hours after intracutaneous infection with Type I pneumococcus (SVI). *Normal monkey serum* was obtained from an apparently healthy *Cercopithecus callitrichus*. *Acute phase monkey serum* was obtained from this animal 24 hours after intracutaneous infection with a culture of Type II pneumococcus.

Antisera.—Rabbit anti-human CRP serum I was kindly supplied by Dr. Harrison F. Wood, and *rabbit anti-human CRP serum II* was prepared in this laboratory by immunizing a rabbit with 1.2 mg. of lyophilized twice recrystallized human C-reactive protein in complete Freund's adjuvants, followed by two intravenous "booster" injections of 0.5 mg. of the same protein. *Sheep anti-human CRP serum* was kindly supplied by Schieffelin & Company. *Sheep anti-rabbit CxRP serum I* was provided by Dr. Harrison F. Wood, and *sheep anti-rabbit CxRP serum II* was obtained from a sheep that had been immunized with 2.5 mg. of twice recrystallized Cx-reactive protein in complete Freund's adjuvants and boosted 3 weeks later with 2 mg. of the same material in oil. *Cat anti-human CRP serum* was obtained from a cat that had been immunized intraperitoneally with 1 mg. of lyophilized human C-reactive protein in complete Freund's adjuvants. The cat received two subcutaneous booster injections of 0.5 mg. of this same material suspended in oil.

Immunological Methods.—The capillary microprecipitation method of Swift, Wilson, and Lancefield (15) was employed, and double diffusion studies in agar were performed according to the method of Ouchterlony (16), employing 1 per cent Difco agar in normal unbuffered saline containing 1:10,000 merthiolate as a preservative. *Passive cutaneous anaphylaxis* was produced according to the technique described by Ovary (17). Albino guinea pigs weighing 250 gm. were injected intradermally with 0.1 ml. of test antiserum. After a period of 3 to 6

hours, injections of 0.5 ml. of 1 per cent Evans blue were given to test for any non-specific increased capillary permeability at the prepared skin sites. Thirty minutes later the animals were injected intravenously with antigen and sacrificed after ½ hour. The skins were removed and any areas of blue discoloration at the injection sites were measured in millimeters.

Sensitization of Guinea Pigs.—Solutions of crystalline acute phase proteins of optical density of 0.100 at 280 mμ were emulsified in a syringe with equal amounts of Freund's adjuvant containing 5.0 mg./ml. of *Mycobacterium butyricum*. Guinea pigs weighing 300 to 400 gm. received injections of 0.2 ml. of emulsion into the hind foot-pads. In other experiments the animals were sensitized with whole serum of which they received 0.2 ml., emulsified with an equal amount of complete adjuvant, in all four foot-pads. Serum was obtained from these animals on the 11th day after the sensitizing injections, and the antibody content of these sera were tested as describe above. Skin tests were performed on the 12th day following

TABLE I

Capillary Precipitin Reactions

Antiserum	CRP	Acute phase human serum	Normal human serum	CxRP	Acute phase rabbit serum	Normal rabbit serum	Acute phase monkey serum	Normal monkey serum	Cx polysaccharide
Rabbit anti-CRP I...	++	++	−	−	−	−	+	−	−
Rabbit anti-CRP II..	++	+++++	−	−	−	−	+	−	−
Sheep anti-CRP.....	++	++	−	+	++	−	++	−	−
Cat anti-CRP.....	+++	+++	−	++	+	−	++	−	−
Sheep anti-CxRP I...	++	+++	−	+++	+++++	−	++	−	−
Sheep anti-CxRP II..	+++	+++++	−	++	+++++	+	++	−	−
Normal sheep serum..	−	−	−	−	−	−	−	−	−
Normal cat serum....	−	−	−	−	−	−	−	−	−

Estimations of the amount of precipitate in the capillary tubes were made after refrigeration at 4°C. for approximately 48 hours. The height of the column of precipitate at the bottom of each tube was measured in millimeters. +, 1 or 2 mm. precipitate; ++, 3 or 4 mm. precipitate; +++, 5 or 6 mm. precipitate; ++++, 7 or 8 mm. precipitate; +++++, 9 or more mm. precipitate.

the sensitization procedure, by intradermal injection of 0.1 ml. of crystalline C-reactive or Cx-reactive proteins of an optical density of 0.100 at 280mμ. In one experiment the animals were also tested with Cx polysaccharide by the intradermal injection of 0.1 ml. of a 1 mg./ml. solution. Delayed hypersensitivity was manifested by indurated, erythematous skin reactions measuring at least 5 mm. in diameter, appearing in 8 hours and reaching maximal size in 18 to 24 hours.

EXPERIMENTAL

Cross-Reactivity as Demonstrated by Precipitation Reactions

The results of the interactions between antisera against C- or Cx-reactive protein and various antigens are listed in Table I. It can be seen that sheep antisera against either human C-reactive protein or rabbit Cx-reactive protein reacted with both acute phase proteins. The possibility that this cross-reac-

tivity might have been due to denaturation products occurring as a result of the purification procedures was excluded by the observation that the cross-reactions were also observed with native acute phase sera as antigens. These same antisera did not react with Cx polysaccharide, probably present as a minor contaminant of the purified acute phase protein preparations. An antiserum against human C-reactive protein prepared in a cat also cross-reacted with rabbit Cx-reactive protein and with rabbit and monkey acute phase sera.

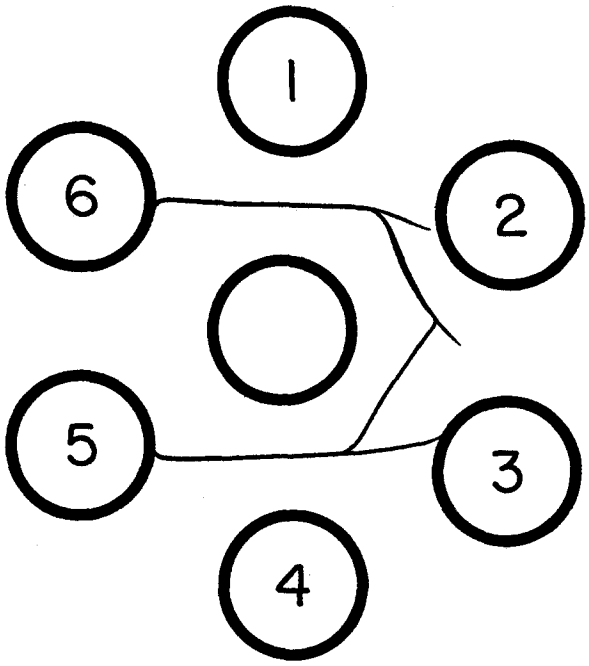

FIG. 1. Tracing prepared from Ochterlony plate, showing lines of precipitation between anti-human CRP serum and acute phase sera from human, monkey, and rabbit. The center well contained sheep anti-human CRP serum. Well 1 contained human acute phase serum; well 2, monkey acute phase serum; well 3, rabbit acute phase serum; well 4, human acute phase serum; well 5, normal rabbit serum; and well 6, normal human serum.

It is to be noted that rabbit anti-human CRP serum did not react with the homologous Cx-reactive protein or with rabbit acute phase serum but, as previously described (4), did react with monkey acute phase serum.

In order to study further the immunological relationships between the acute phase proteins, the technique of double diffusion in agar (16) was employed. While these studies also indicated that the acute phase proteins cross-react, it was clearly demonstrable that the acute phase proteins are not identical, well defined spurs marking the intersections of lines of precipitate. Figs. 1 and 2 illustrate the reactions exhibited by sheep anti-CRP and anti-CxRP sera.

Cross-Reactivity as Demonstrated by Passive Cutaneous Anaphylaxis

Because of the remarkable sensitivity of passive cutaneous anaphylaxis, it seemed desirable to adapt this technique to the study of the immunological similarity between the acute phase proteins. When dilutions of cat anti-human CRP serum were injected intradermally into guinea pigs, a typical passive cutaneous anaphylactic reaction could subsequently be elicited by intravenous

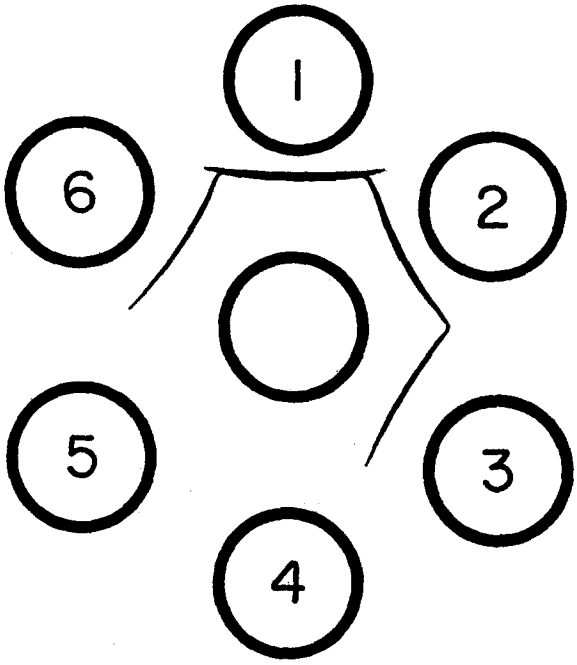

FIG. 2. Tracing prepared from Ouchterlony plate, showing lines of precipitation between anti-rabbit CxRP serum and acute phase serum from human, rabbit, and monkey. The center well contained sheep anti-rabbit CxRP serum I. Well 1 contained rabbit acute phase serum; well 2, monkey acute phase serum; well 3, human acute phase serum; well 4, normal rabbit serum; well 5, normal human serum; and well 6, human acute phase serum. With this antiserum, no spurs were visible at the intersections of the lines of precipitation formed with monkey acute phase serum and human acute phase serum.

administration of human acute phase serum. As shown in Table II, no reactions were obtained when the guinea pigs were challenged with normal human serum, indicating that the antiserum was specific for C-reactive protein. Guinea pigs receiving intradermal injections of this antiserum also showed typical passive cutaneous anaphylactic reactions upon subsequent intravenous administration of rabbit or monkey acute phase sera, while, as shown in Table III, normal sera provoked no reactions. In the cross-reacting systems positive reactions occurred only at antiserum dilutions of 1:200 or less.

TABLE II

Passive Cutaneous Anaphylaxis in Guinea Pigs Injected Intradermally with Cat Anti-Human CRP Serum and Intravenously with Normal or Acute Phase Human Serum

Dilution of cat anti-CRP	Antigen injected intravenously							
	Acute phase human serum (0.25 ml.)				Normal human serum (0.25 ml.)			
	Guinea pig No.				Guinea pig No.			
	1	2	3	4	5	6	7	8
1/100	30*	25	25	40	0	0	0	0
1/500	17	15	14	20	0	0	0	0
1/1000	12	12	11	17	0	0	0	0
1/2000	10	10	5	15	0	0	0	0
1/4000	0	0	0	10	0	0	0	0
1/8000	0	0	0	0	0	0	0	0

* Diameter of reaction in millimeters.

TABLE III

Passive Cutaneous Anaphylaxis in Guinea Pigs Injected Intradermally with Cat Anti-Human CRP Serum and Tested with Rabbit or Monkey Serum Intravenously

Dilution of cat anti-CRP	Antigen injected intravenously												
	Acute phase rabbit serum, 0.5 ml.				Normal rabbit serum, 0.5 ml.			Acute phase monkey serum, 0.5 ml.			Normal monkey serum, 0.5 ml		
	1	2	3	4	5	6	7	8	9	10	11	12	13
1/10								16	15	12	0	0	0
1/20								12	10	8	0	0	0
1/40								8	8	0	0	0	0
1/50	23*	26	20	20	0	0	0						
1/80								11	10	6	0	0	0
1/160								6	7	0	0	0	0
1/200	8	8	10	10	0	0	0						
1/320													
1/400	0	0	0	0	0	0	0						
1/800	0	0	0	0	0	0	0						
1/1600	0	0	0	0	0	0	0						
1/3200	0	0	0	0	0	0	0						

* Diameter of reaction in millimeters.

Cross-Reactivity as Demonstrated by Delayed Skin Reactions

It was found by Gell and Benacerraf (18) that guinea pigs sensitized either to human or bovine serum albumin in complete Freund's adjuvants subsequently exhibited delayed skin reactivity to both antigens. In the present

study guinea pigs were sensitized with one or the other of the acute phase proteins, and subsequently tested for the development of delayed hypersensitivity to both proteins. Table IV shows the results of sensitization of guinea pigs with

TABLE IV

Reactions of Guinea Pigs Sensitized to Purified Acute Phase Proteins

Animals sensitized to	No. of animal	Delayed reaction to			Antibodies to				
		CRP	CxRP	Cx polysaccharide	CRP	CxRP	Normal human serum	Normal rabbit serum	Cx polysaccharide
CxRP	57-51	−	−	−	−	+	−	+	−
	52	+	+	−	−	+	−	+	−
	53	−	+	−	−	−	−	−	−
	54	+	+	−	−	+	−	+	−
	55	+	+	−	−	+	−	+	−
	56	+	+	−	+	+	−	+	−
	57	+	+	−	−	+	−	+	−
	58	+	+	−	−	+	−	+	−
Adjuvant only	57-59	−	−	−	−	−	−	−	−
	60	−	−	−	−	−	−	−	−
	61	−	−	−	−	−	−	−	−
	62	−	−	−	−	−	−	−	−
	63	−	−	−	−	−	−	−	−
	64	−	−	−	−	−	−	−	−
CRP	57-65	+	+	−	+	−	−	−	−
	66	+	+	−	+	−	−	−	−
	67	+	+	−	+	−	−	−	−
	69	+	+	−	+	−	−	−	−
	70	+	+	−	+	−	−	−	−
	71	−	−	−	+	−	−	−	−
	72	+	+	−	−	−	−	−	−
	73	−	−	−	−	−	−	−	−

Delayed skin reactions were read 24 hours after the skin test injections. Antibodies were determined both by capillary microprecipitation and by passive cutaneous anaphylaxis, and the results of these tests were in complete agreement in the case of each serum. No attempt was made at quantitation of the delayed skin reactions or of the antibody determinations, and the results are expressed above as + (present) or − (absent).

C-reactive protein, Cx-reactive protein, or with adjuvant alone. It is clear that sensitization to either acute phase protein induced delayed sensitivity to both. Table IV also shows that the sera of the sensitized animals contained precipitating antibodies to the acute phase protein used for sensitization. With the doses of antigen used in this sensitization procedure, and the short period of time between sensitization and bleeding, the serological cross-reactivity de-

scribed in the preceding sections was not generally observed, although one of the animals sensitized to Cx-reactive protein did develop antibodies to C-reactive protein.

Since Cx polysaccharide was used in the preparation of both acute phase proteins it seemed possible that the skin reactions observed might have been due to contaminating amounts of this material in each of the acute phase protein preparations, even though delayed hypersensitivity to polysaccharide antigens is unknown. When skin tests were performed with Cx polysaccharide,

TABLE V

Reaction of Guinea Pigs Sensitized to Normal Rabbit Serum and Acute Phase Rabbit Serum

Sensitized to	Animal No.	Delayed reaction to	
		CxRP	CRP
Adjuvant only	1	−	−
	2	−	−
	3	−	−
	4	−	−
	5	−	−
Normal rabbit serum	1	−	−
	2	−	−
	3	−	−
	4	−	−
	5	−	−
Acute phase rabbit serum	1	+	−
	2	+	−
	3	+	+
	4	+	+
	5	+	+

Delayed skin reactions were read at 24 hours, and the results are expressed as + (reaction present) or − (reaction absent).

no delayed reactions were observed. There generally resulted somewhat raised irritative reactions, but these reactions occurred within 2 hours and were less erythematous and less indurated than the delayed skin reactions to the acute phase proteins; furthermore, the animals injected with adjuvant alone showed irritative reactions to Cx polysaccharide to the same degree as did the experimental animals. These considerations appear to exclude Cx polysaccharide as being responsible for the delayed skin reactions to the acute phase protein preparations.

Since the sera of guinea pigs immunized with rabbit Cx-reactive protein contained antibodies reacting with normal rabbit serum, the possibility was considered that the results shown in Table IV might have been due to normal serum

protein contaminants in the preparations of Cx-reactive protein. In order to exclude this possibility the following experiment was performed. Fifteen guinea pigs were sensitized: five with normal rabbit serum, five with acute phase rabbit serum, and five with Freund's adjuvant alone. Twelve days later these animals were skin-tested with C-reactive and Cx-reactive proteins in the usual manner, and the results are summarized in Table V. No animal immunized with normal rabbit serum or adjuvant alone had a positive reaction to either acute phase protein. Of the animals immunized with acute phase serum all possessed delayed skin reactivity to Cx-reactive protein and 3 of these animals also showed delayed reactions to C-reactive protein. The small amount of native Cx-reactive protein in the acute phase serum was evidently capable of inducing delayed hypersensitivity.

DISCUSSION

The results of this study show that the acute phase proteins of man, monkey, and rabbit are serologically related, all reacting with antisera made against either the human or rabbit protein. The studies with cat anti-human CRP serum have demonstrated that cat antibody, like that of guinea pig, mouse, rat, rabbit, and man, is able to fix to the skin of guinea pigs and participate in passive cutaneous anaphylactic reactions. Using this antiserum, it was possible to obtain passive cutaneous anaphylaxis with acute phase rabbit or monkey sera. The fact that such cross-reactivity could be demonstrated by passive cutaneous anaphylaxis, despite the sharp specificity of this technique, indicates that these acute phase proteins are probably quite closely related antigenically, and this interpretation is borne out by the double-diffusion precipitin studies. Quantitative immunochemical studies would be helpful in further investigating this point, but the small amounts of purified proteins available have thus far precluded such analysis.

Cross reactivity was also evident in the delayed skin reactions of guinea pigs sensitized to acute phase proteins. However, the cutaneous reactions of delayed hypersensitivity are apparently not mediated by conventional antibodies, and therefore the cross-reactivity observed in these latter experiments may or may not have been an expression of the same antigenic similarity as that revealed by the serologic reactions. These acute phase proteins share the property of reacting with Cx polysaccharide, and it is possible that the Cx-reacting portion of the acute phase protein molecules may be closely similar from species to species. Whether this portion of the molecule is also responsible for the immunologic cross-reactions observed *in vitro* and *in vivo* remains to be determined. Some evidence has been obtained which indicates that Cx polysaccharide is capable of interfering with the precipitation of the acute phase proteins by their antisera, but further work is needed to establish the significance of this finding.

Since the three known acute phase proteins thus appear to be antigenically

similar, analogous acute phase substances in other species may be demonstrable by one or another of the immunological methods described above. Hitherto, attempts at detection of these acute phase proteins in other species have depended either on the demonstration of a calcium-dependent precipitation reaction with pneumococcal Cx polysaccharide or the production of capsular swelling of a specific type of pneumococcus. The ease and sensitivity of immunologic methods should make them useful in such comparative studies.

SUMMARY

Crystalline rabbit Cx-reactive protein has been compared immunologically with the analogous crystalline C-reactive protein of man.

Immunologic cross-reactivity has been demonstrated between the acute phase proteins of man, rabbit, and monkey.

Double-diffusion reactions in agar and passive cutaneous anaphylaxis reactions *in vivo* both indicate that these acute phase proteins are antigenically closely similar but not identical.

Guinea pigs with delayed hypersensitivity to C-reactive protein exhibit delayed skin reactions when tested with Cx-reactive protein and *vice versa*.

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