PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 167, 493–497 (1981)

C-Reactive Protein as a Mediator in the Lysis of Human Erythrocytes Sensitized by Brown Recluse Spider Venom[1] (41203)

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Abstract. Hemolytic reactions, frequently associated with envenomization by the brown recluse spider, *Loxosceles reclusa,* are poorly understood. Using an *in vitro* model some fundamental observations on venom-induced hemolysis have been characterized which indicate that C-reactive protein could be a mediator in the natural toxic process. Washed human type O erythrocytes, when added to small amounts of brown recluse spider venom, show no direct lysis; however, when added to normal fresh, blood group-compatible adult human serum, these venom-sensitized cells are destroyed. The ability to lyse cells is complement dependent, but individual sera show quantitative differences which are not related to the levels of functional complement. Fresh human cord sera contain adequate levels of complement but are unable to lyse venom-sensitized erythrocytes. Experiments were done in which reactive fresh adult human serum was immunoabsorbed with specific rabbit antibodies to human C-reactive protein. This absorbed human serum lost about 65% of the ability to lyse venom-sensitized erythrocytes. These results were highly suggestive that C-reactive protein played a role in this *in vitro* hemolytic reaction. Definitive experiments were done by adding measured amounts of purified human C-reactive protein to nonhemolytic fresh normal cord serum. Amounts as small as 0.192 μg/ml allowed this serum to become hemolytic. C-Reactive protein has recently been shown to activate complement, effect clotting mechanisms and influence T lymphocytes, properties which could be important in the regulation of immunopathological mechanisms. The observation that C-reactive protein plays a necessary role in the *in vitro* complement-dependent lysis of venom-sensitized erythrocytes suggests a possible role for this protein in natural human envenomation by the brown recluse spider.

Human envenomization by the brown recluse spider, *Loxosceles reclusa,* usually results in a characteristic, gangrenous skin lesion at the site of the bite (1). Infrequently, more severe systemic manifestations, apparently associated with the development of intravascular hemolytic reactions and disseminated coagulopathy, can produce complications leading to death (2, 3).

The mechanisms of venom toxicity have not been well characterized although skin lesions similar to those seen in humans have been reproduced in certain species of experimental animals (4, 5). Histologically the lesions in rabbits and guinea pigs injected intradermally with standardized preparations of brown recluse spider venom (BRSV) show features similar to those seen in the Arthus and Shwartzman reactions (6, 7), inflammatory processes known to be dependent on complement activation and polymorphonuclear leukocyte infiltration. Even more similarity is indicated by the studies of Smith and Micks (6), which reported a need for complement and polymorphonuclear leukocytes in the normal development of BRSV-induced skin lesions in rabbits and guinea pigs.

The probable importance of complement in envenomization was first suggested by the *in vitro* studies of Kniker and Morgan (8) and Kniker *et al.* (9). It was shown that small amounts of BRSV (less than 1 μg venom protein/ml), when added to com-

[1] Supported by funds provided by the Graduate School, University of Arkansas for Medical Sciences and the Medical Research Service, Little Rock Veterans Administration Medical Center, Project No. 5833-01.

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0037-9727/81/080493-05$01.00/0

plement in a cell-free, fluid phase system, rapidly led to the inactivation of immune hemolytic complement activity. More recently it has been reported that BRSV interacted with and subsequently inactivated several of the individual complement components including C1, C4, C2, C3, and C5 (10).

Other *in vitro* studies have shown that small amounts of BRSV, when added to washed human erythrocytes, form a venom–erythrocyte complex (11, 12). At concentrations of 2.5 μg venom protein or less, no direct venom-specific hemolysis could be measured; however, venom-sensitized erythrocytes were agglutinated specifically by venom antisera prepared in rabbits (11), or these cells were lysed specifically in the presence of fresh, normal, blood group-compatible adult human sera (12).

The lysis of venom-sensitized erythrocytes (EV complexes) has been shown to be complement-dependent (13). Specific complement inhibitors, when added to fresh normal human adult sera, were effective in removing the ability to lyse EV complexes. These and other data indicate that although complement, including C1, C4, and C2, is necessary for EV lysis, it is not a limiting factor. Marked variations in the ability of undiluted individual sera to lyse EV could not be related to differences in total hemolytic complement levels (12, 13); sera diluted 1:10 were unable to lyse EV although adequate complement levels were demonstrated (12); and most conclusively, it was shown that all fresh, normal, human cord sera tested contained adequate levels of functional hemolytic complement, but were unable to lyse EV complexes (13).

In seeking the identity of the limiting factor(s) in fresh serum, a role for C-reactive protein (CRP) was considered. This protein is associated with inflammatory processes and has been reported to interact with complement at the C1 level and subsequently lead to the activation of hemolytic complement activity (14–17). This report presents data which indicate that CRP plays a necessary role in the *in vitro*, complement-mediated lysis of human erythrocytes sensitized with venom from the brown recluse spider.

Materials and Methods. Procedures used to sensitize human type O erythrocytes and to measure venom-specific hemolysis and hemolytic complement levels in compatible sera have been described previously (11–13). The washed erythrocytes in standardized suspensions were sensitized with BRSV (EV). Appropriate controls included similar suspensions of nonsensitized erythrocytes (EC). After washing the cells and restandardizing the suspensions, the cells in 0.2-ml vol were resuspended in 0.2-ml vol of normal, fresh-frozen, blood group-compatible human sera. After 30 min at 37°, specific lysis of EV was determined by measuring the OD at 415 nm of supernatant fluids in a B&L spectrophotometer.

Absorption of human adult serum. Specific rabbit antiserum to human CRP (Calbiochem–Behring Diagnostics) was coupled to CH-Sepharose 4B agarose gel beads using the manufacturer's procedures (Pharmacia). This serum was tested for specificity to human CRP in immunodiffusion studies described below. Normal rabbit serum was coupled to agarose gel beads as appropriate controls. Portions of each preparation were added to separate samples of a fresh adult human serum pool and the mixtures were incubated at 4° for 18 hr. The gel beads were removed by centrifugation and each absorbed serum sample was tested for the ability to lyse EV.

Addition of purified CRP to cord serum. Purified human C-reactive protein was provided by M. B. Pepys. It was purified by affinity chromatography (18) and was characterized as being homogenous by gradient, reduced and unreduced, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; repeated immunization of rabbits and sheep has yielded only monospecific antisera (M. B. Pepys, personal communication). In our laboratory, the CRP was immunodiffused in agarose gels with specific rabbit antiserum to human CRP (Calbiochem–Behring Diagnostic). This was the same antiserum used in immunoabsorption studies of human adult serum. These studies showed a single precipitin band that was the same as the band seen when this antiserum was diffused with the

TABLE I. A POSSIBLE ROLE FOR C-REACTIVE PROTEIN IN THE LYSIS OF HUMAN ERYTHROCYTES SENSITIZED WITH BROWN RECLUSE SPIDER VENOM BY ADULT HUMAN SERUM

	Percentage lysis of EV complexes[a]
Normal serum pool (fresh)	97.4
Normal serum pool (56°C for 30 min)	0
Normal serum pool absorbed with gel beads coated with rabbit antiserum to human CRP	14.0
Normal serum pool absorbed with gel beads coated with normal rabbit serum	81.9

[a] Specific lysis of venom-sensitized erythrocytes as given under Materials and Methods.

pool of fresh adult human sera. A pool of fresh, normal, human cord serum, obtained from the delivery rooms of UAMS University hospital, was also immunodiffused with the rabbit antiserum to human CRP. No precipitin band was evident.

Measured amounts of this purified, human CRP were added to portions of the human cord serum pool. The mixtures were then tested for the ability to lyse EV complexes.

Results. When attempts were made to deplete fresh, reactive, adult, human serum pool of CRP by absorption with specific rabbit antisera to human CRP coupled to agarose gel beads, the ability to lyse EV was greatly reduced. Data presented in Table I show that the overnight incubation at 4° with gel beads coupled with this antiserum reduced the serum's ability to lyse EV by about 65%. Qualitative tests showed that this immunoabsorbed serum contained adequate amounts of residual hemolytic complement activity to lyse effectively antibody-sensitized sheep erythrocytes in a standardized system.

Attempts to remove hemolytic activity from reactive adult human sera by immunoabsorption with antisera to human CRP were highly suggestive that CRP might play a role in this reaction, however, a more definitive approach was undertaken by adding measured amounts of purified CRP to non-reactive fresh cord sera.

Data in Table II show that the pool of fresh, normal human cord serum containing functional complement activity was unable to lyse EV. However, when concentrations

TABLE II. EFFECT OF ADDING PURIFIED C-REACTIVE PROTEIN TO NORMAL CORD SERUM POOL IN THE LYSIS OF HUMAN ERYTHROCYTES SENSITIZED WITH BROWN RECLUSE SPIDER VENOM

Concentration of Human CRP Added to Human Cord Serum Pool Containing 234 CH50 Units Complement/ml (μg CRP/ml)	Percentage lysis of EV complexes[a]
0	0
0.02	0
0.192	47.6
0.96	76.7
1.9	90.7
9.5	97.7
48.0	100
96.0	100
Controls	
CRP (96 μg/ml) in serum-free buffer	0
Normal adult human serum pool (fresh)	82.0
Normal adult human serum pool (56°C for 30 min)	0

[a] Specific lysis of venom-sensitized erythrocytes as given in Materials and Methods.

of 0.192 to 96.0 μg CRP/ml were added, cord serum was effectively able to lyse EV.

Discussion. These studies provide evidence that C-reactive protein, a protein long recognized as being associated with inflammatory processes, plays a primary role in the *in vitro*, complement-mediated lysis of human erythrocytes sensitized with brown recluse spider venom. Initial observations showed that the ability of fresh, reactive human adult serum to lyse EV complexes was reduced after immunoabsorption with specific antibodies to CRP. Immunodiffusion studies of the serum containing these antibodies indicated homogeny and the lack of detectable amounts of contaminating serum antibodies. However, other definitive studies were more conclusive. Measured amounts of purified human CRP were added to fresh, nonreactive human cord serum which contained functional complement. Amounts as low as 0.02 μg CRP/ml were unable to lyse EV; however, when 0.192 μg/ml was added, almost half of the sensitized erythrocytes in the test system were lysed. Larger quantities led to complete hemolysis.

Although greatly increased during inflammatory processes, the concentrations of CRP in normal human sera have only been measured recently using radioimmunoassay (19). Although great variation was seen in individual sera, as we had reported in the hemolytic activities of individual human sera, CRP was detected and quantitated in every individual serum tested. The amounts in healthy blood donors varied from 68 to 8200 ng CRP/ml with a median value of 580 ng CRP/ml. The levels in 24 normal cord sera ranged from 10 to 370 ng CRP/ml with a median value of 70 ng CRP/ml.

These values, when correlated with the results we report here, could explain the variation in the ability of individual human sera to lyse venom-sensitized erythrocytes and the reason cord sera were unable to do so. Adult sera have been reported to contain a median value of 580 ng/ml (0.58 μg) CRP/ml; cord sera contain almost 10-fold less (0.07 μg/ml). These values when compared to the data in Table II suggest that the smaller amounts normally in cord sera are inadequate to initiate hemolysis. In contrast the larger, but varying amounts, found in normal adult sera could explain the variations seen in hemolytic ability. These suppositions can be investigated by measuring CRP levels and hemolytic abilities in the same individual sera.

C-Reactive protein has recently been shown to be involved in several physiological functions known to be associated with immunopathological mechanisms. Most notably it has been shown to interact with several substrates, and in a manner similar to the action of antigen–antibody complexes, activate complement at the C1 level (14–17). CRP has also been shown to influence platelet activities, and thus, clotting mechanisms (20). The report (21) that CRP can also bind specifically to T lymphocytes has suggested strongly that this interesting protein could play a fundamental role in the regulation of specific physiological processes during the inflammatory response.

It is exciting to speculate that CRP could play a fundamental role in the toxicity of brown recluse spider venom. The variations in severity of systemic manifestations, i.e., intravascular hemolysis and disseminated coagulopathy, could be associated with increased levels of normal CRP. The subsequent activation of complement, both locally at the site of the bite and systemically through venom absorption, could generate more CRP, additional complement activation and thus even more severe pathological manifestations.

Sincere appreciation to Dr. M. B. Pepys, Royal Postgraduate Medical School, London, is expressed for his providing the purified human C-reactive protein used in these studies.

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Received April 17, 1979. P.S.E.B.M. 1981, Vol. 167.