Role of the Property of C-Reactive Protein to Activate the Classical Pathway of Complement in Protecting Mice from Pneumococcal Infection

Madathilparambil V. Suresh, Sanjay K. Singh, Donald A. Ferguson, Jr. and Alok Agrawal

*J Immunol* 2006; 176:4369-4374; doi: 10.4049/jimmunol.176.7.4369

http://www.jimmunol.org/content/176/7/4369
Role of the Property of C-Reactive Protein to Activate the Classical Pathway of Complement in Protecting Mice from Pneumococcal Infection

Madathilparambil V. Suresh,* Sanjay K. Singh,* Donald A. Ferguson, Jr., † and Alok Agrawal2*  

C-reactive protein (CRP) is not an acute-phase protein in mice, and therefore, mice are widely used to investigate the functions of human CRP. It has been shown that CRP protects mice from pneumococcal infection, and an active complement system is required for full protection. In this study, we assessed the contribution of CRP’s ability of activating the classical pathway of complement in the protection of mice from lethal infection with virulent Streptococcus pneumoniae type 3. We used two CRP mutants, Y175A and K114A. The Y175A CRP does not bind C1q and does not activate complement in human serum. The K114A CRP binds C1q and activates complement more efficiently than wild-type CRP. Passively administered, both CRP mutants and the wild-type CRP protected mice from infection equally. Infected mice injected with wild-type or mutant CRP had reduced bacteremia, resulting in lower mortality and increased longevity compared with mice that did not receive CRP. Thus, the protection of mice was independent of CRP-mediated activation of the classical pathway of complement. To confirm that human CRP does not differentiate between human and mouse complement, we analyzed the binding of human CRP to mouse C1q. Surprisingly, CRP did not react with mouse C1q, although both mutant and wild-type CRP activated mouse C3, indicating species specificity of CRP-C1q interaction. We conclude that the mouse is an unfit animal for exploring CRP-mediated activation of the classical complement pathway, and that the characteristic of CRP to activate the classical complement pathway has no role in protecting mice from infection. The Journal of Immunology, 2006, 176: 4369–4374.

The primary binding specificity of the acute-phase reactant C-reactive protein (CRP) is toward phosphocholine-containing substances such as C-polysaccharide (PnC) of the cell wall of Streptococcus pneumoniae (1–3). PnC-complexed human CRP interacts with the complement subcomponent C1q, and subsequently activates the classical pathway of complement in human serum (4–8). In mice, in which CRP is not an acute-phase protein (9, 10), passively administered human CRP has been shown to be protective against lethal infection with S. pneumoniae, as determined by increased survival of and decreased bacteremia in the infected mice (11, 12). Mice transgenic for human CRP are also protected from lethal pneumococcal infection (13). A functioning complement system is required for full CRP-mediated protection (14, 15). The mechanism of action of human CRP in protecting mice from infection is not defined. Complement activation by bacteria participates in such protection; however, there is no conclusive evidence showing that the complement activation by CRP complexes is needed.

Using site-directed mutagenesis at the C1q binding site of CRP, we have engineered two mutant forms of CRP, Y175A and K114A (16–19). Complete biochemical characterization of these CRP mutants has been reported previously, and their overall structure was found not to be different from the wild-type (WT) native and WT recombinant CRP (16, 17). Both mutants bind PnC as avidly as the WT CRP. The Y175A CRP does not interact with human C1q, and hence does not activate human complement. The K114A CRP binds to C1q and activates human complement many-fold better than the WT CRP. In this study, we investigated the participation of CRP-mediated activation of the classical pathway of complement in the protection of mice from lethal infection with virulent S. pneumoniae type 3. Our approach was to compare the protective ability of the WT CRP with that of the two mutants of CRP. If the complement-activating property of CRP is involved in the protection of mice from infection, then the Y175A CRP should not be protective and the K114A CRP should be more protective. Our results indicated that the CRP-mediated protection of mice from pneumococcal infection was independent of the CRP’s ability to activate the classical pathway of complement. We also found that human CRP complexed with PnC did not react with mouse C1q, although activated C3, revealing species specificity of recognition between CRP and C1q and of complement activation by CRP.

Materials and Methods

CRP mutants

The construction, expression, and characterization of the CRP mutants Y175A and K114A have been described earlier (16, 17). For stable expression of CRP mutants, Chinese hamster ovary cells were transfected with the mixture of 10 µg of mutant CRP cDNA-p91023 construct and 2 µg of pSV2neo vector (Invitrogen Life Technologies) using the FuGENE 6 reagent (Roche). The pSV2neo vector carrying neomycin resistance was used as the helper plasmid because the vector p91023 does not harbor a drug selection marker (20). At 96 h posttransfection, stably transfected cells were selected by growth for another 2 wk in the culture medium supplemented with Gentamicin sulfate. Chinese hamster ovary cell lines for both mutants were isolated by a series of subcloning steps.
Purification of CRP

The WT native human CRP from pleural fluid and the mutant CRP from the culture medium were purified in three steps: a Ca^{2+}-dependent affinity chromatography on a phosphocholine-Sepharose column (Pierce), followed by anion-exchange chromatography on a MonoQ column (GE Healthcare) using the Biologic Duo Flow Protein Purification System (Bio-Rad), as described earlier (21). All CRP were judged pure based on the denaturing SDS-PAGE analysis. The native structure of the CRP mutants was confirmed by gel filtration.

Mice

C57BL/6J mice (Jackson ImmunoResearch Laboratories) were brought up and maintained according to protocols approved by the University Committee on Animal Care. Both male and female mice were 8–10 wk old when used in experiments.

Bacteria and infection of mice

All growth medium was purchased from Difco. Virulent S. pneumoniae type 3, strain WU2 (obtained from D. Briles, University of Alabama, Birmingham, AL), was stored as stock cultures at −80°C in Todd-Hewitt broth supplemented with 0.5% yeast extract and containing 10% glycerol. Bacteria were made more virulent by sequential i.v. passages in mice. Virulent bacteria were plated onto tryptic soy agar plates supplemented with 5% sheep blood and incubated for 18 h at 37°C in candle extinction jars. Bacteria for protection experiments were collected from late log-phase broth cultures, washed, and resuspended in normal saline. Concentration of bacteria was estimated from comparison with a McFarland opacity standard (22). Inocula were kept on ice. Mice were injected i.v. with 10^6 CFU bacteria in 100 μl of normal saline, and the injection was started within 5 min of diluting the bacteria. The dose of 10^6 CFU bacteria was chosen because, in preliminary experiments, 10^7 CFU bacteria killed <50% of mice, while 10^8 CFU bacteria killed all mice. The density, purity, and viability of the bacteria were tested by plating on tryptic soy blood agar plates to confirm that all animals got the same CFU of inoculum at the start of the experiment.

Protection experiments and bacteremia

Mice were injected i.v. with 150 μg of WT or mutant CRP in 150 μl of TBS, 30 min before injecting bacteria, as described above. The dose of 150 μg of CRP with 10^8 CFU bacteria was chosen because, under these conditions, the protection of mice with WT CRP was similar to the previously published results (11, 12). Survival of mice was noted twice per day for 10 days. To determine bacteremia (CFU/ml), blood was collected daily for 5 days from the tip of the tail vein of each surviving mouse. Blood was diluted in normal saline, plated on blood agar plates, and incubated for 18 h at 37°C in candle extinction jars before the colonies were counted. The plotting and statistical analyses of the data were done using the GraphPad Prism 4 software.

Binding of CRP to live virulent S. pneumoniae

Virulent bacteria (10^7 CFU) were incubated with increasing amounts of WT and mutant CRP in a final volume of 200 μl of Todd-Hewitt broth. The bacteria, in the presence of CRP, were grown for 4 h at 37°C. Then the bacteria were pelleted, washed with TBS three times, and resuspended in TBS containing 10 mM EDTA to elute the bound CRP. After 10 min, the supernatants were recovered by centrifugation. ELISA (21) was used to determine the amount of CRP in the supernatant.

C3 deposition assays

The assay was performed, as previously described, with some modifications (17). Microtiter wells were coated with PnC (Stans Serum Institut; 40 μg/ml) in PBS overnight at 4°C. After blocking with PBS + 1% BSA and rinsing with buffer A (PBS + 0.1% BSA + 1 mM CaCl_2), purified WT and mutant CRP diluted in buffer B (buffer A + 0.01% Tween 20) were added to the wells. After 1 h at 37°C, the wells were washed with buffer B and rinsed with buffer C (PBS + 1% BSA + 0.15 mM CaCl_2 + 0.5 mM MgCl_2). Normal human serum (1/40) or normal mouse serum (1/20) diluted in chilled buffer C was added to each well and incubated for 30 min at 37°C. At these dilutions of sera, the standard curves generated for the WT CRP were linear in the range of 0.5–5.0 μg/ml. The sera were preabsorbed with PnC-conjugated agarose for 3 h at 0°C to remove any CRP and anti-PnC Abs, as described previously (17). The wells were then washed with buffer C, and either murine anti-human C3d mAb (Quidel; 1 μg/ml) or goat anti-mouse C3 (Cappel; 4 μg/ml) diluted in buffer C was added to each well. After 1 h at room temperature, the wells were washed and developed with either HRP-conjugated goat anti-mouse IgG (Pierce) or HRP-conjugated bovine anti-goat IgG (Santa Cruz Biotechnology). Color was developed with ABTS reagent and measured at 405 nm in a microtiter plate reader (Molecular Devices).

C1q-binding assays

Two C1q-binding assays were used: one for CRP-PnC complex and another for Ag-Ag complex (CRP-anti-CRP complex). For binding of C1q to CRP-PnC, the assay was performed, as previously described, with modifications (16, 17). Microtiter wells were coated with PnC at 10 μg/ml in TBS overnight at 4°C. Increasing concentrations of purified WT CRP diluted in buffer D (TBS + 0.1% BSA + 5 mM CaCl_2 + 0.02% Tween 20) were added to the wells. After 1 h at 37°C, the wells were washed with buffer D and then once with buffer E (10 mM Tris-HCl (pH 7.2) + 1 μM NaCl + 5 mM CaCl_2 + 0.1% BSA + 0.02% Tween 20). Then either 5.0 μg/ml purified mouse C1q (obtained from U. K. Shire, University of Oxford, Oxford, U.K.) or mouse serum (1/2) diluted in buffer E was added to the wells. After 3 h at room temperature, the wells were washed with buffer E and then with buffer D. Rat anti-mouse C1q mAb (Abcam) was used at 1/100 dilution in buffer D to detect bound C1q. After 1 h at room temperature, wells were developed with HRP-conjugated goat anti-rat IgG (Se-rotec). Color was developed with ABTS reagent and measured at 405 nm in a microtiter plate reader (Molecular Devices).

For binding of C1q to Ag-Ag, microtiter wells were coated with CRP at 10 μg/ml in TBS overnight at 4°C. Increasing concentrations of affinity-purified polyclonal rabbit anti-CRP IgG diluted in buffer D were added to the wells. After 1 h at 37°C, the wells were washed with buffer D and then once with buffer E. Then either 5.0 μg/ml purified mouse C1q or mouse serum (1/2) diluted in buffer E was added to wells, and the assay was completed as above.

Results

Y175A CRP mutant, incapable of activating human complement, protects mice from infection

Fig. 1 shows the combined results of three separate protection experiments, using 12–18 mice in each group, with WT, Y175A mutant, and K114A mutant CRP. The extent of WT CRP-mediated

![FIGURE 1. Survival curves of mice infected with S. pneumoniae. Four groups of mice, 12–18 mice in each group, were injected with 10^6 CFU bacteria, with or without 150 μg of WT, Y175A, or K114A CRP. CRP was injected first; bacteria were injected 30 min later. Deaths were recorded twice per day for 10 days. The data are combined from three separate experiments. Statistical analyses were performed using one-way ANOVA and Tukey’s multiple comparison test. The p values for the differences among groups A/B, A/C, and A/D are <0.05. The p values for the differences among groups B/C, B/D, and C/D are >0.05. The 95% confidence intervals are: A/B, 0.17–0.94; A/C, 0.17–1.05; A/D, 0.09–0.96; B/C, 0.38–0.49; B/D, 0.46–0.41; and C/D, 0.56–0.39.](http://www.jimmunol.org/Downloadedfrom/347.jpg)
protection of mice from infection was consistent with previous reports (11, 12). The median survival time (the time taken for the death of 50% of mice) for mice injected with bacteria alone (control group A) was 4 days. Less than 28% mortality occurred after 4 days in the infected mice treated with either WT or Y175A CRP. By the end of the sixth day, only 6% of the mice survived in the control group. More than 60% of the mice survived even for 10 days in mice treated with WT or Y175A CRP. Thus, the protective ability of both WT and Y175A mutant CRP was comparable in decreasing mortality and prolonging survival of infected mice. Because the Y175A mutant CRP was incapable of activating human complement, the immediate interpretation of these findings was that the protection of mice conferred by CRP was independent of CRP-mediated activation of the classical pathway of complement.

**K114A CRP mutant, which is more efficient than WT CRP in activating human complement, is not more efficient in protecting mice from infection**

The survival curve for the K114A mutant CRP was similar to that obtained from Y175A CRP (Fig. 1). Within 4 days, survival was >75% in the infected mice treated with K114A CRP. More than 50% of the mice survived even at the end of the 10th day in mice treated with K114A CRP. Thus, the K114A mutant CRP was not better than WT CRP in decreasing mortality and prolonging survival of infected mice. These results again suggested that the CRP-mediated activation of the classical pathway of complement did not contribute to CRP-dependent protection.

**Equal reduction of bacteremia by WT, Y175A, and K114A CRP**

The bacteremia values, determined everyday for 5 days for each surviving mouse from the protection experiment shown in Fig. 1, are plotted as scatter diagram (Fig. 2). In all four groups, 1 day postinfection, bacteremia was <1000 CFU/ml. In the control group A, the bacteremia continued to increase until day 3 and the mice died once bacteremia approached ~10^7 CFU/ml. In the mice administered with either WT or K114A CRP, there was no increase in bacteremia past day 2. In the mice administered with Y175A CRP, there was no increase in bacteremia past day 1. Statistically significant differences in bacteremia were observed between the control and CRP-treated groups by day 2, and the differences persisted until day 4. Based on the median bacteremia values, CRP (WT or mutants)-treated mice had >100-fold fewer bacteria on any day compared with the control group. These results indicate that increased resistance to infection in CRP-treated mice is associated with the persistence of reduced bacteremia regardless of the complement-activating property of CRP.

**Binding of WT and mutant CRP by live and virulent S. pneumoniae**

To verify that the mutations in CRP have not affected the capability of CRP to bind to live S. pneumoniae, we used a CRP-binding assay using live bacteria (Fig. 3). Bacteria were grown in the presence of increasing amount of WT or mutant CRP as shown on the x-axis. Bound CRP is shown on the y-axis. The binding curves were almost overlapping, indicating identical binding of all CRP species to S. pneumoniae.

**Activation of human and mouse complement by human CRP**

Using C3 deposition assays, we next evaluated the ability of human CRP to activate mouse complement. We used WT and mutant CRP and tested activation of complement in human and mouse sera. As shown in Fig. 4A, the WT CRP activated human complement as reflected by CRP dose-dependent deposition of human C3 on CRP-PnC complexes. As reported earlier (16, 17), the Y175A CRP did not activate human complement, while the K114A CRP was more potent than WT CRP in activating human complement. The WT and K114A CRP also activated mouse complement, as indicated by CRP dose-dependent deposition of mouse C3 on CRP-PnC complexes (Fig. 4B). Surprisingly, the Y175A CRP, incapable of activating human complement, activated mouse complement as well as the WT and K114A CRP did. The difference in the activation of complement by human CRP in human and mouse
sera indicated species specificity of CRP. To assess that the PnC by itself did not activate mouse complement, we performed another C3 deposition assay using increasing concentrations of PnC alone (Fig. 4C). As shown, PnC by itself gave high background, which was constant over the entire dose-response range, indicating that PnC by itself did not activate mouse complement. Addition of CRP to PnC enhanced deposition of C3 in a PnC dose-dependent manner. Thus, it was the CRP-PnC complex that was responsible for activating mouse complement.

**Human CRP does not interact with mouse C1q**

To determine whether the activation of mouse complement by human CRP was through the classical pathway and whether human CRP differentiated between human and mouse C1q, we investigated binding of WT and mutant CRP to mouse C1q. As shown in Fig. 5A, PnC-complexed human CRP did not bind to purified mouse C1q. However, in the control experiment (Fig. 5B), C1q bound to Ag-Ab complexes in the Ab dose-dependent manner. C1q from the mouse serum was also not recognized by the CRP-PnC complexes (Fig. 5C), although the C1q from the serum bound to Ag-Ab complexes (Fig. 5D). Another anti-C1q Ab, goat anti-human C1q Ab (Calbiochem) that cross-reacts with mouse C1q, gave similar results (data not shown). These data indicated that human CRP lacked the capability to react with mouse C1q and that the activation of mouse complement by human CRP was not through the classical pathway.

**Discussion**

*S. pneumoniae*-activated complement plays the major role in the protection of mice infected with *S. pneumoniae* (23). In this study, we attempted to define the role of CRP-activated complement in the CRP-dependent protection of mice from *S. pneumoniae* infection. We compared the protective ability of three CRP species: WT CRP, Y175A CRP mutant incapable of activating complement in human serum, and K114A CRP mutant capable of activating human complement more efficiently than the WT CRP. Our major findings were: 1) All CRP species, WT, Y175A, and K114A, decreased bacteremia, decreased mortality, and prolonged survival of infected mice, and their protective ability was not significantly different. Also, in vitro, the binding of WT and mutant CRP to S.
pneumoniae was similar. 2) Human CRP activated mouse complement. The Y175A mutant CRP also activated mouse complement in contrast to its inability to activate human complement. 3) The activation of mouse complement by human CRP was not through the classical pathway because the human CRP did not react with mouse C1q.

CRP is an evolutionarily conserved protein and has been found in all animals to date tested for it (24, 25). However, no need was felt to examine species-specific cross-reactivity between CRP and C1q. We found that human CRP did not interact with mouse C1q. Another case of species-specific incompatibility between CRP and C1q was shown 30 years ago when it was observed that human CRP did not interact with guinea pig C1q (26). Indirect evidence indicating that human CRP does not interact with rabbit C1q has also been reported (reviewed in Ref. 27).

It has been shown that human CRP activates complement in human (4, 5), rat (28), and mouse sera (29, and this study), but not in rabbit (27) and guinea pig sera (26). It has also been shown that rabbit CRP does not activate rabbit complement (27). A recent paper showed that neither human CRP nor rabbit CRP activated complement in serum from apolipoprotein E knockout mice, although in this case enzymatically modified low-density lipoprotein was used as the CRP ligand, not the PnC (30). Rat CRP does activate rat complement, but only if it is precipitated with PnC (28, 31). Activation of complement by rat CRP occurs through the classical pathway because it binds to both rat and human C1q (31). Both plaice and lump sucker CRP activate human complement (24), and trout CRP has been shown to activate trout complement (32). Enhanced activation of complement in any given serum by ligand-complexed CRP was considered to be due to the activation of the classical pathway of complement. Our data on human CRP and mouse complement necessitate a change in this view.

The activation of mouse C3 by human CRP without interaction with mouse C1q was unexpected. There are three pathways of complement activation: classical, alternative, and lectin pathway (33). Clearly, the activation of mouse C3 by human CRP was not through the classical pathway; either the alternative pathway or lectin pathway was involved. In case of human complement, it has been shown that CRP inhibits the alternative pathway by enhancing the activity of complement factor H (33–35), and if this inhibitory effect of CRP applies to mouse serum, then the activation of mouse complement by human CRP was not through the alternative pathway either. Thus, our results raise the possibility of direct activation of lectin pathway, at least in mouse serum, by PnC-complexed human CRP. In contrast, because the amplification of lectin pathway requires the alternative pathway, the possible CRP-mediated activation of the lectin pathway may not be amplified due to the inhibitory effect of CRP on the alternative pathway (36, 37).

The protection of mice by CRP may be through CRP-mediated activation of the lectin pathway of complement. Although the protection of mice from pneumococcal infection mainly involves the alternative pathway, the lectin pathway also plays a role (23, 38). An alternate possibility is that CRP may be acting by preventing the bacteria from escaping the alternative pathway of complement attack. It is known that factor H binds to Hic (factor H-binding inhibitor of complement) present on type 3 pneumococci and leads to reduced activation of the alternative pathway on the pneumococcal surface (39). Because factor H also binds CRP and because both CRP and Hic can bind to factor H simultaneously, it is possible that CRP eliminates the repressive effect of factor H on the activation of the alternative pathway by covering the factor H-Hic complex formed on bacteria. If this hypothesis is true, then the binding of CRP to factor H-coated bacteria will be critical rather than the binding of CRP to bacteria alone. Generation of a CRP mutant that does not activate mouse complement and a mutant that does not bind factor H is in progress to test above possibilities.

There is reason to believe that the CRP-mediated protection of mice from infection does not require CRP-mediated complement activation at all. First, we found that 150 μg of CRP, but not 100 μg of CRP (data not shown), protected mice from infection under our experimental conditions. It is unlikely that the requirement of a relatively higher concentration of CRP is related to the ability of CRP to activate complement. Second, the finding that the decrease in bacteremia during the initial phase of infection involves the alternative pathway (23) suggests that the remaining bacteria acquired resistance to complement attack in general. Third, considering that the t1/2 of injected CRP in mice is only 4 h (40, 41), it is evident that, after a day of infection when the effect of CRP was visible, CRP may not be present in the blood. Fourth, in the CRP-mediated protection of mice challenged with platelet-activating factor, complement was not found to participate (42–44). Taken together, the activation of complement by bacteria, but not the activation of complement by CRP, could be responsible for the protection of mice from infection. We hypothesize that CRP contributes to the protection of mice from infection by acting directly on the effector cells of the immune system. Although the opsonic effects of CRP have been widely described (45), our hypothesis is supported by the finding that the binding ability of human CRP to mouse macrophages through the FcγRs does not contribute to CRP-mediated protection of mice from S. pneumoniae infection (15). Thus, presumably both effector functions of CRP, complement activation and phagocytosis, do not constitute the mechanism of CRP-mediated protection of mice from pneumococcal infection. In contrast, the phagocytosis property of CRP may be essential for CRP-mediated protection of mice from lipopolysaccharide toxicity because it has been shown that FcγRs are required for the CRP-mediated protection of mice from lipopolysaccharide toxicity (46).

Besides the infection model, several strains of mice are also being used to investigate the functions of CRP in atherosclerosis (30, 47, 48). Such studies are performed either by injecting human CRP into mice or by generating transgenic mice for human or rabbit CRP. Our results suggest that a separate study on mouse complement activation by human and rabbit CRP is warranted.

In summary, the property of CRP to activate the classical pathway of complement is irrelevant for the protective function of CRP in mice infected with S. pneumoniae. Our findings indicate that the CRP-dependent antimicrobial effect may also be seen in mice knockout for the classical pathway complement components. In addition, because CRP exhibits species specificity, before using an animal model to explore CRP-mediated activation of the classical pathway of complement, the cross-interaction between CRP and C1q must be evaluated. Certainly, the mouse model is not suitable to explore CRP-mediated activation of the classical pathway of complement.

Acknowledgments

We are grateful to Dr. David E. Briles and Dr. Uday Kishore for the gifts of S. pneumoniae and mouse C1q, respectively. We thank the staff of our Division of Laboratory Animal Resources for help with the mouse experiments.

Disclosures

The authors have no financial conflict of interest.

References


