C-Reactive Protein Protects Mice against Pneumococcal Infection via Both Phosphocholine-Dependent and Phosphocholine-Independent Mechanisms

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The mechanism of action of C-reactive protein (CRP) in protecting mice against lethal *Streptococcus pneumoniae* infection is unknown. The involvement of the phosphocholine (PCh)-binding property of CRP in its antipneumococcal function previously has been explored twice, with conflicting results. In this study, using three different intravenous sepsis mouse models, we investigated the role of the PCh-binding property of CRP by employing a CRP mutant incapable of binding to PCh. The ability of wild-type CRP to protect mice against infection was found to differ in the three models; the protective ability of wild-type CRP decreased when the severity of infection was increased, as determined by measuring mortality and bacteremia. In the first animal model, in which we used 25 μ g of CRP and 10^7 CFU of pneumococci, both wild-type and mutant CRP protected mice against infection, suggesting that the protection was independent of the PCh-binding activity of CRP. In the second model, in which we used 25 μ g of CRP and 5×10^7 CFU of pneumococci, mutant CRP was not protective while wild-type CRP was, suggesting that the protection was dependent on the PCh-binding activity of CRP. In the third model, in which we used 150 μ g of CRP and 10^7 CFU of pneumococci, mutant CRP was as protective as wild-type CRP, again indicating that the protection was independent of the PCh-binding activity of CRP. We conclude that both PCh-dependent and PCh-independent mechanisms are involved in the CRP-mediated decrease in bacteremia and the resulting protection of mice against pneumococcal infection.

Infection with *Streptococcus pneumoniae* is one of the most common causes of community-acquired pneumonia and septicemia worldwide (reviewed in references 1–3). C-reactive protein (CRP) is a plasma protein whose level in the blood is dramatically increased in patients with *S. pneumoniae* infection (reviewed in references 4–8). In experiments using animal models, passively administered human CRP, transgenic human CRP, and murine CRP all have been shown to protect mice against lethal infection with *S. pneumoniae*, as determined by decreased bacteremia and increased survival (9–12). In murine models of infection involving passively administered human CRP, CRP exerted its protective effect only when injected 6 h before to 2 h after the infection but not when mice received CRP 24 h or 36 h postinfection (13, 14). Thus, the CRP-mediated protection of mice requires the presence of CRP in the early stages of infection. However, the mechanism of the protective action of CRP in mice during the early stages of infection is not known.

CRP binds to pneumococci in both serum and Ca^{2+} -containing buffers (14). The binding of CRP to pneumococci is mediated via a Ca^{2+} -dependent interaction between CRP and phosphocholine (PCh) residues present on C-polysaccharide (PnC) of the cell wall of pneumococci (15). Because PnC-complexed CRP activates the complement system in both human and mouse sera (4, 16; reviewed in reference 17), it has been proposed that the mechanism of the antipneumococcal function of CRP involves the ability of CRP to bind to pneumococci through PCh groups present on PnC and the subsequent activation of the complement system by pneumococcus-bound CRP (18). Previously, we have investigated whether the binding of CRP to PCh on pneumococci was required for the protection of mice against pneumococcal infection (14, 19). Employing site-directed mutagenesis, we generated two CRP mutants, F66A/E81A and F66A/T76Y/E81A, both inca-

pable of binding to PCh, and employed them in mouse protection experiments. We hypothesized that if the binding of CRP to PCh were required for the protection of mice against pneumococcal infection, then mutant CRP should not be protective. Two papers were published on these investigations, and the results reported in these papers were contradictory to each other (14, 19). According to one report (14), the CRP-mediated protection was independent of the binding of CRP to PCh. According to another report (19), the CRP-mediated protection was dependent on the binding of CRP to PCh.

In the first report (14), we used 150 μ g of CRP and 10^8 CFU of pneumococci and the F66A/E81A CRP mutant (20). We found that F66A/E81A CRP mutant was as capable as wild-type (WT) CRP in protecting mice against infection. Mutant CRP decreased mortality and prolonged survival of infected mice as well as WT CRP did. The results indicated that the CRP-mediated protection was independent of the binding of CRP to PCh. However, two features of this animal model were not consistent with the other

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previously published models (9, 10); in our model, less than 10^8 CFU of pneumococci did not provide suitable survival and mortality data, and less than 150 μ g CRP was not protective against infection induced by 10^8 CFU of pneumococci.

In the second report (19), the animal model was created by using either 25 μ g or 150 μ g of CRP and 5×10^7 CFU of pneumococci; in this study, we used the F66A/T76Y/E81A CRP mutant. We found that, unlike WT CRP, the F66A/T76Y/E81A CRP mutant was not protective at both 25- μ g and 150- μ g doses. Mutant CRP did not decrease mortality and did not prolong the survival of infected mice. The results indicated that the CRP-mediated protection was dependent on the binding of CRP to PCh. However, although not statistically significant, a slight protective effect of mutant CRP was noted at the 150- μ g dose (19). One feature of this animal model also was not consistent with the other published models; in our model, although both doses of WT CRP were protective, the protection was not as much as that seen in other published models (9, 10).

The purpose of the current study was to generate data to explain the findings of the previous two reports (14, 19). In this study, we used different animal models which were consistent with the other previously published models (9, 10) and an F66A/T76Y/E81A CRP mutant that is incapable of binding to PCh. The results indicated that both of our previously published reports were correct and that CRP protects mice during the early stages of infection via both PCh-dependent and PCh-independent mechanisms.

MATERIALS AND METHODS

CRP. The method for constructing the cDNA for CRP mutant F66A/T76Y/E81A has been reported previously (19). Expression of mutant cDNA in CHO cells, isolation of a CHO cell line expressing mutant CRP, and purification of F66A/T76Y/E81A mutant CRP from the cell culture supernatant were performed as described previously (19). Native WT CRP was purified from discarded human pleural fluid as described previously (19). For mouse protection experiments, both purified WT and mutant CRP were treated with the Detoxi-Gel endotoxin-removing gel according to the manufacturer's instructions (Thermo). The concentration of endotoxin in CRP was determined by using the *Limulus* amoebocyte lysate kit (QCL-1000) according to the manufacturer's instructions (Lonza). Binding activity of CRP for PCh was evaluated by using PCh-conjugated bovine serum albumin (PCh-BSA), PnC, and pneumococci as the ligands, as described previously (19), except that CRP was used at concentrations of up to 10 μ g/ml.

Pneumococci. Pneumococci (*S. pneumoniae* type 3, strain WU2) were maintained virulent, stored, and used as described previously (19). The concentration, purity, and viability of pneumococci were confirmed by plating on sheep blood agar.

Mouse protection experiments. Male C57BL/6J mice (Jackson ImmunoResearch Laboratories) were maintained according to protocols approved by the University Committee on Animal Care. Mice were 8 to 10 weeks old when used in experiments. Two separate mouse protection experiments were performed using two batches of purified WT and mutant CRP. Mice first were injected intravenously (i.v.) with either 25 μ g or 150 μ g of WT or mutant CRP in 150 μ l Tris-buffered saline (TBS) containing 2 mM CaCl_2 . The endotoxin content in 25 μ g and 150 μ g WT CRP was 0.77 ± 0.37 endotoxin units (EU) and 4.64 ± 2.19 EU, respectively. The endotoxin content in 25 μ g and 150 μ g mutant CRP was 0.53 ± 0.28 EU and 3.22 ± 1.65 EU, respectively. After 30 min, mice were injected i.v. with either 10^7 CFU or 5×10^7 CFU of pneumococci in 100 μ l of saline. Survival of mice was recorded three times per day for 10 days. Survival curves were generated using GraphPad Prism 4 software. To determine *P* values for the differences in the survival curves among various groups, the

survival curves were compared using the software's log-rank test. To determine bacteremia (CFU/ml) in the surviving mice, blood was collected daily for 5 days from the tip of the tail vein, diluted in normal saline, and plated on sheep blood agar for colony counting. The bacteremia value for dead mice was taken as $>10^8$ CFU/ml, because mice died when the bacteremia exceeded 10^8 CFU/ml. The plotting and statistical analyses of the bacteremia data were performed by using GraphPad Prism 4 software and Mann-Whitney nonparametric two-sample rank test.

PCh-binding inhibition assays. Microtiter wells were coated with either 10 μ g/ml of PCh-BSA, 10 μ g/ml of PnC, or 10^7 CFU pneumococci in TBS overnight at 4°C, as described previously (19). The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature. CRP diluted in TBS containing 5 mM CaCl_2 , 0.1% gelatin, and 0.02% Tween 20 (TBS-Ca) was added to the wells. To determine the effects of PCh (Sigma-Aldrich) and dAMP (D6250; Sigma-Aldrich) on the binding of CRP to PCh-BSA, PnC, and pneumococci, CRP was added to the wells in the presence of either 10 mM PCh or 10 mM dAMP. To determine the requirement of Ca^{2+} for the binding of CRP to PCh, CRP was diluted in TBS containing 5 mM EDTA, 0.1% gelatin, and 0.02% Tween 20. After incubating the plates for 2 h at 37°C, unbound CRP was aspirated, followed by washing the wells with TBS-Ca. Rabbit polyclonal anti-CRP antibody (Sigma-Aldrich) and HRP-conjugated donkey anti-rabbit IgG (Thermo) were used to detect bound CRP. Color was developed and the A_{405} was read in a microtiter plate reader, as described previously (19).

RESULTS

At the 25- μ g dose, both WT and mutant CRP protect mice against infection with 10^7 CFU of pneumococci. Figure 1 (left) shows the combined results of two separate protection experiments using 25 μ g of CRP, 10^7 CFU of pneumococci, and six mice in each of the three groups in each experiment. Median survival time (the time taken for the death of 50% of mice) for mice injected with bacteria alone (control group A) was 80 h. The median survival time for mice injected with bacteria and WT CRP (group B) was more than 10 days. The median survival time for mice injected with bacteria and mutant CRP (group C) was 176 h. The *P* values for the difference in the survival curves between groups A and B, A and C, and B and C were 0.001, 0.02 and 0.24, respectively.

Bacteremia values, determined every day for 5 days in each mouse from the protection experiment, are plotted as scatter diagrams (Fig. 1, right). In mice injected with bacteria alone (group A), median bacteremia was approximately 2.5×10^3 CFU/ml of blood 1 day postinfection. In mice injected with bacteria and either WT CRP (group B) or mutant CRP (group C), median bacteremia was $<10^3$ CFU/ml 1 day postinfection in both groups. In group A, bacteremia increased dramatically after day 1, and mice died once bacteremia exceeded 10^8 CFU/ml. In mice administered WT CRP, there was no increase in bacteremia past day 1. In mice administered mutant CRP, there was an increase in bacteremia past day 1, but it reached only approximately 10^7 CFU/ml even after 5 days. On day 1, the *P* values for the difference between groups A and B, A and C, and B and C were 0.03, 0.41, and 0.17, respectively. On day 2, the *P* values for the difference between groups A and B, A and C, and B and C were 0.002, 0.09, and 0.20, respectively. Thus, although the bacteremia values for mutant CRP were not significantly different from that of the control group for both days 1 and 2, the bacteremia values for mutant CRP also were not significantly different from that of WT CRP for both days.

We concluded that both WT and mutant CRP decreased mor-

FIG 1 (Left) Survival of mice infected with 10^7 CFU of *S. pneumoniae* with or without 25 µg of either WT or mutant CRP. CRP was injected first; pneumococci were injected 30 min later. Deaths were recorded three times a day for 10 days. The data are combined from two separate experiments with six mice in each group in both experiments. The *P* values for the difference in the survival curves between groups A and B, A and C, and B and C are 0.001, 0.02, and 0.24, respectively. (Right) Bacteremia in mice infected with 10^7 CFU of *S. pneumoniae* with or without 25 µg of either WT or mutant CRP. Blood was collected from each surviving mouse for the first 5 days after infection. Bacteremia was determined by plating. Each dot represents one mouse. The horizontal line in each group of mice represents the median value of bacteremia in that group. A bacteremia value of $>10^8$ indicates a dead mouse. On day 1, the *P* values for the difference between groups A and B, A and C, and B and C are 0.03, 0.41, and 0.17, respectively. On day 2, the *P* values for the difference between groups A and B, A and C, and B and C are 0.002, 0.09, and 0.20, respectively.

tality and prolonged survival of mice injected with 10^7 CFU of pneumococci, and that the increased resistance to infection was associated with the maintenance of a reduced bacteremia or a slower increase in bacteremia. The results obtained from this animal model (25 µg of CRP and 10^7 CFU of pneumococci) suggest that the CRP-mediated protection of mice from infection is independent of the PCh-binding activity of CRP.

With 5×10^7 CFU of pneumococci and CRP at the 25-µg dose, mutant CRP was different from WT CRP in protecting mice against infection. We next changed the animal model in which we used the same dose of CRP as that in the experiment shown in Fig. 1, but we increased the number of pneumococci 5-fold. Figure 2 (left) shows the combined results of two separate protection experiments using 25 µg of CRP, 5×10^7 CFU of pneu-

mococci, and six mice in each group in each experiment. The median survival time for mice injected with bacteria alone (control group A) was 40 h, for mice injected with bacteria and WT CRP (group B) it was 78 h, and for mice injected with bacteria and mutant CRP (group C) it was 46 h. In all three groups, mice began dying at 40 h. In the control group, all mice died by 64 h. In the mutant CRP-treated group, all mice died by 116 h. However, in the WT CRP-treated group, only 75% of mice died in 10 days. The *P* values for the difference in the survival curves between groups A and B, A and C, and B and C are <0.001 , 0.03, and 0.003, respectively. Survival in both WT CRP-treated and mutant CRP-treated groups was significantly different from that for the control group, but the results for the WT CRP-treated group also were significantly different from those for the mutant CRP-treated group.

FIG 2 (Left) Survival of mice infected with 5×10^7 CFU of *S. pneumoniae* with or without 25 µg of either WT or mutant CRP. CRP was injected first; pneumococci were injected 30 min later. Deaths were recorded three times a day for 10 days. The data are combined from two separate experiments with six mice in each group in both experiments. The *P* values for the difference in the survival curves between groups A and B, A and C, and B and C are <0.001 , 0.03, and 0.003, respectively. (Right) Bacteremia in mice infected with 5×10^7 CFU of *S. pneumoniae* with or without 25 µg of either WT or mutant CRP. Blood was collected from each surviving mouse for the first 5 days after infection. Bacteremia was determined by plating. Each dot represents one mouse. The horizontal line in each group of mice represents the median value of bacteremia in that group. A bacteremia value of $>10^8$ indicates a dead mouse. On day 1, the *P* values for the difference between groups A and B, A and C, and B and C are <0.005 , 0.16, and 0.02, respectively. On day 2, the *P* values for the difference between groups A and B, A and C, and B and C are <0.005 , 0.25, and 0.03, respectively.

FIG 3 (Left) Survival of mice infected with 10^7 CFU of *S. pneumoniae* with or without 150 µg of either WT or mutant CRP. CRP was injected first; pneumococci were injected 30 min later. Deaths were recorded three times a day for 10 days. The data are combined from two separate experiments with six mice in each group in both experiments. The *P* values for the difference in the survival curves between groups A and B, A and C, and B and C are <0.005 , <0.005 , and 0.33, respectively. (Right) Bacteremia in mice infected with 10^7 CFU of *S. pneumoniae* with or without 150 µg of either WT or mutant CRP. Blood was collected from each surviving mouse for the first 5 days after infection. Bacteremia was determined by plating. Each dot represents one mouse. The horizontal line in each group of mice represents the median value of bacteremia in that group. A bacteremia value of $>10^8$ indicates a dead mouse. On day 1, the *P* values for the difference between groups A and B, A and C, and B and C are 0.09, 0.005, and 0.31, respectively. On day 2, the *P* values for the difference between groups A and B, A and C, and B and C are 0.02, <0.005 , and 0.85, respectively.

Bacteremia values, determined every day for 5 days in each mouse from the protection experiment, are plotted as scatter diagrams (Fig. 2, right). In mice injected with bacteria alone (group A), 1 day postinfection, median bacteremia was approximately 1.9×10^5 CFU/ml of blood. In mice injected with bacteria and WT CRP (group B), median bacteremia was 1.5×10^3 CFU/ml 1 day postinfection. In mice injected with bacteria and mutant CRP (group C), 1 day postinfection, median bacteremia was 2.9×10^4 CFU/ml. In group A, bacteremia increased dramatically by day 1, and bacteremia exceeded 10^8 CFU/ml by day 2. In mice administered WT CRP, there was an increase in bacteremia past day 1, but it took 3 days to exceed 10^8 CFU/ml when those mice died. Similar to the control group, in mice administered mutant CRP, bacteremia increased dramatically after day 1, and bacteremia exceeded 10^8 CFU/ml by day 2. On day 1, the *P* values for the difference between groups A and B, A and C, and B and C are <0.005 , 0.16, and 0.02, respectively. On day 2, the *P* values for the difference between groups A and B, A and C, and B and C are <0.005 , 0.25, and 0.03, respectively. Thus, statistically significant differences in bacteremia were observed between the control (group A) and WT CRP-treated groups (group B) for both days but not between the control and the mutant CRP-treated groups (group C) for either day. Also, the bacteremia values for WT CRP were significantly different from that of mutant CRP.

We concluded that, in contrast to WT CRP, mutant CRP did not decrease mortality and did not prolong survival of infected mice due to failure in reducing bacteremia or maintaining reduced bacteremia. The results obtained from this animal model subjected to a markedly greater load of pneumococci indicate that CRP-mediated protection of mice against infection is dependent on the PCh-binding activity of CRP under these circumstances.

Mutant CRP is as capable as WT CRP in protecting mice against infection when large doses of CRP are employed. We changed the animal model again: we used the same dose of pneumococci as that in the experiment shown in Fig. 1, but we increased the dose of CRP by 6-fold. Figure 3 (left) shows the com-

bined results of two separate protection experiments using 150 µg of CRP, 10^7 CFU of pneumococci, and six mice in each group in each experiment. The median survival time for mice injected with bacteria alone (control group A) was 80 h. During this period, survival was 92% in the infected mice treated with either WT or mutant CRP. The median survival time for mice injected with bacteria and either WT or mutant CRP (groups B and C) was more than 10 days in both groups. In the control group, mice began dying at 40 h, and only 9% of mice survived up to 10 days. However, in the WT CRP-treated and mutant CRP-treated groups, no deaths occurred in 64 h, and 75% and 89% of mice survived up to 10 days, respectively. The *P* values for the difference in the survival curves between groups A and B, A and C, and B and C are <0.005 , <0.005 , and 0.33, respectively.

Bacteremia values, determined every day for 5 days in each mouse from the protection experiment, are plotted as scatter diagrams (Fig. 3, right). In mice injected with bacteria alone (group A), 1 day postinfection, median bacteremia was approximately 3.1×10^3 CFU/ml of blood. In mice injected with bacteria and WT CRP (group B), median bacteremia was approximately 200 CFU/ml 1 day postinfection. In mice injected with bacteria and mutant CRP (group C), 1 day postinfection, median bacteremia was 100 CFU/ml. In group A, bacteremia increased dramatically by day 1, and mice died once bacteremia exceeded 10^8 CFU/ml. In mice administered either WT or mutant CRP, there was no significant increase in bacteremia past day 1. On day 1, the *P* values for the difference between groups A and B, A and C, and B and C are 0.09, 0.005, and 0.31, respectively. On day 2, the *P* values for the difference between groups A and B, A and C, and B and C are 0.02, <0.005 , and 0.85, respectively. Thus, the bacteremia values for both WT and mutant CRP were significantly different from that of the control group, and the bacteremia value for mutant CRP was not significantly different from that of WT CRP.

We concluded that mutant CRP was as effective as WT CRP in decreasing mortality and prolonging the survival of mice, and that the increased resistance to infection was associated with the maintenance of a reduced bacteremia. The results obtained from this

- Group A: Bacteria alone (10^7 CFU)
 ■ Group B: Bacteria (10^7 CFU) + 25 μ g WT CRP
 ○ Group C: Bacteria alone (5×10^7 CFU)
 ● Group D: Bacteria (5×10^7 CFU) + 25 μ g WT CRP

FIG 4 Survival of mice infected with 10^7 CFU and 5×10^7 CFU of *S. pneumoniae* with or without 25 μ g of WT CRP. CRP was injected first; pneumococci were injected 30 min later. Deaths were recorded three times a day for 10 days. The data are combined from two separate experiments with six mice in each group in both experiments. The *P* values for the difference in the survival curves between groups A and B, C and D, A and C, and B and D are 0.001, <0.005, <0.005, and 0.03, respectively.

animal model (150 μ g of CRP and 10^7 CFU of pneumococci) suggest that the CRP-mediated protection of mice against infection is independent of the PCh-binding activity of CRP when very large doses of CRP are administered.

The ability of CRP to protect mice against infection depends on the animal model. The combined results of the protection experiments using WT CRP (25 μ g in Fig. 1 and 150 μ g in Fig. 3) are shown in Fig. 4. The median survival time for mice injected with 10^7 CFU of pneumococci (group A) was 80 h. The median survival time for mice injected with 10^7 CFU of pneumococci and 25 μ g of WT CRP (group B) was more than 10 days. The median survival time for mice injected with 5×10^7 CFU of pneumococci (group C) was only 40 h. The median survival time for mice injected with 5×10^7 CFU of pneumococci and 25 μ g of WT CRP (group D) was 78 h. The *P* values for the difference in the survival curves between groups A and B, C and D, A and C, and B and D are 0.001, <0.005, <0.005, and 0.03, respectively. Thus, the protective ability of CRP decreased when the severity of infection was increased, suggesting that the protective ability of even WT CRP decreases with increasing doses of pneumococci.

Comparison of binding of WT and mutant CRP to PCh-containing ligands. We have reported previously that the F66A/T76Y/E81A CRP mutant did not bind to PCh-containing ligands (19). If mutant CRP does not bind to PCh, and if the PCh-binding property of CRP is required for CRP to protect mice against pneumococcal infection, then mutant CRP should not have protected mice against infection regardless of the doses of CRP and pneumococci to set up the animal model. The finding that mutant CRP did protect mice against infection in one defined animal model (150 μ g mutant CRP and 10^7 CFU pneumococci) warranted the reassessment of the PCh-binding activity of mutant CRP. Previously, our PCh-binding assays utilized CRP in the 5-ng/ml to 50-ng/ml range, because this range provided the linear part of the binding curve. We, however, used 10 μ g/ml of mutant CRP to reassess the PCh-binding ability of mutant CRP, using two differ-

FIG 5 Binding of CRP to PCh-containing ligands. Microtiter wells were coated with PCh-BSA (A), PnC (B), and pneumococci (C). The unreacted sites in the wells were blocked with gelatin. CRP diluted in TBS-Ca then was added to the wells. Bound CRP was detected by using rabbit polyclonal anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. Color was developed, and the absorbance was read at 405 nm. A representative of three experiments is shown.

ent PCh-containing ligands, PCh-BSA (Fig. 5A) and PnC (Fig. 5B), and whole live pneumococci (Fig. 5C). As shown, for equivalent binding (A_{405}) of CRP to any of the three ligands, approximately 100 times more mutant CRP was required than WT CRP, indicating that the PCh-binding ability of mutant CRP was approximately 99% less than that of WT CRP.

Because the dose of mutant CRP we used in the mouse protection experiments was only 6 times higher than that of WT CRP and not 100 times higher, it is not possible that the PCh-independent protection was due to the residual PCh-binding activity of mutant CRP in cases where a 6-times-higher dose of mutant CRP was used. Although we do not know if there is an indirect, PCh-independent interaction between mutant CRP and pneumococci *in vivo*, the data obtained from these assays suggest that the protection of mice seen with mutant CRP did not involve PCh-mediated interaction between CRP and pneumococci.

Inhibition of binding of WT and mutant CRP to PCh-containing ligands. We next determined the molecular basis of the residual binding of mutant CRP to PCh-containing ligands. We used inhibition assays in which we used 10 mM PCh and 10 mM dAMP as fluid-phase inhibitors of binding of CRP to immobilized PCh-containing ligands. We also performed assays in EDTA-containing buffers to confirm that the binding of any CRP to PCh-containing ligands was Ca^{2+} dependent, because the binding of CRP to PCh requires Ca^{2+} . As shown in Fig. 6A, the binding of WT CRP to PCh-BSA was completely inhibited by PCh at all concentrations of CRP. The binding also was inhibited by dAMP, although dAMP was a less effective inhibitor than PCh, as reported previously (15). In contrast to WT CRP, the residual binding of mutant CRP to PCh-BSA was completely inhibited by both PCh and dAMP (Fig. 6B), indicating that the binding of mutant CRP to PCh was mediated through the phosphate group of PCh. The weak binding and strong inhibitory power of dAMP suggested that mutant CRP retained the ability to bind to phosphate. Similar results were obtained with PnC and pneumococci for both WT and mutant CRP (Fig. 6C to F). In all assays (Fig. 6A to F), no binding was observed in EDTA, indicating that the binding of mutant CRP was Ca^{2+} dependent and was through the phosphate groups on the ligands.

DISCUSSION

We tested the hypothesis that the mechanism of CRP-mediated protection of mice against *S. pneumoniae* infection during the early stages of infection involves binding of CRP to PCh groups present on pneumococci. We compared the protective ability of WT CRP that binds to PCh and pneumococci with that of a CRP mutant that does not bind to either PCh or pneumococci in mice infected with lethal doses of pneumococci. We used three different animal model setups. Our major findings were the following: (i) in the animal model using 25 μg of CRP and 10^7 CFU of pneumococci, both WT and mutant CRP protected mice against infection; (ii) in the model using 25 μg of CRP and 5×10^7 CFU of pneumococci, WT CRP protected but mutant CRP did not protect mice against infection; and (iii) in the animal model using 150 μg of CRP and 10^7 CFU of pneumococci, mutant CRP was as capable as WT CRP in protecting mice against infection. Thus, by using different animal model setups, it was revealed that CRP works through both PCh-dependent and PCh-independent mechanisms in protecting mice against early-stage pneumococcal infection.

Previously, we initially reported that CRP-mediated protection of mice was not dependent on the PCh-binding property of CRP (14) but subsequently reported that the CRP-mediated protection of mice was dependent on the PCh-binding property of CRP (19). We provided an explanation for these contradictory reports by indicating that the CRP mutants used in the two studies were different (19), namely, F66A/E81A in the first study and F66A/T76Y/E81A in the second study. We also postulated that the contradictory findings were due to differences in the purity of the two CRP preparations employed, frozen and thawed mutant CRP in the first study and freshly purified mutant CRP in the second study (19). Our current findings, however, indicate that the contradictory findings were due to the differences in the setup of the animal model; in terms of median survival time, WT CRP was much less protective in the second study than in the first study. It was important that an animal model be used in which WT CRP

FIG 6 Inhibition of binding of WT (left) and mutant (right) CRP to PCh-containing ligands. (A and B) Inhibition of binding of CRP to PCh-BSA by PCh and dAMP. Microtiter wells were coated with PCh-BSA. CRP at increasing concentrations in TBS-Ca, with and without 10 mM PCh or 10 mM dAMP, was added to the wells. (C and D) Inhibition of binding of CRP to PnC by PCh and dAMP. Microtiter wells were coated with PnC. CRP at increasing concentrations in TBS-Ca, with and without 10 mM PCh or 10 mM dAMP, was added to the wells. (E and F) Inhibition of binding of CRP to pneumococci by PCh and dAMP. Microtiter wells were coated with pneumococci. CRP at increasing concentrations in TBS-Ca, with and without 10 mM PCh or 10 mM dAMP added to the wells. In all experiments, the requirement of Ca^{2+} for the binding of CRP to PCh-containing ligands also was evaluated by using CRP at increasing concentrations in TBS containing 5 mM EDTA. In all experiments, bound CRP was detected by using rabbit polyclonal anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. Color was developed, and the absorbance was read at 405 nm. A representative of three experiments is shown.

showed prolonged protection in terms of median survival time, which was needed to evaluate the protective ability of mutant CRP.

There are two different CRP mutants available, F66A/E81A and F66A/T76Y/E81A, which do not bind to either PCh or pneumococci. However, the F66A/T76Y/E81A CRP mutant binds to phosphoethanolamine much more avidly than the F66A/E81A CRP mutant (19). To purify F66A/E81A CRP, immunoaffinity

chromatography is required, which results in poor recovery of pentameric CRP due to the use of an acidic pH buffer (14). We chose the F66A/T76Y/E81A CRP mutant for this study, because it was easier to purify this mutant by affinity chromatography using a phosphoethanolamine-agarose column with higher recovery of the purified protein. It has been shown previously that the clearance rate of F66A/T76Y/E81A CRP mutant from mouse circulation is similar to that of WT CRP (19). Complete biochemical characteristics of the F66A/T76Y/E81A CRP mutant have been described previously (19).

We found that the binding of mutant CRP to PCh ligands, including pneumococci, was approximately only 1% of that of WT CRP and that the residual binding was mediated only through the phosphate group of PCh. The PCh-binding site of CRP consists of a hydrophobic pocket formed mainly by Phe⁶⁶, Thr⁷⁶, and Glu⁸¹ and two Ca²⁺ ions which are bound to CRP by interactions with seven amino acids from other parts of the protein. The phosphate group of PCh directly coordinates with the two Ca²⁺ ions. The choline group of PCh lies within the hydrophobic pocket. Phe⁶⁶ provides hydrophobic interactions with the three methyl groups of choline. Thr⁷⁶ is critical for creating the appropriately sized pocket to accommodate PCh. Glu⁸¹ interacts with the positively charged nitrogen atom of choline (21–23). Mutant CRP retained its Ca²⁺-dependent binding to phosphate because the mutations in the F66A/T76Y/E81A CRP mutant were not in the Ca²⁺-binding site. Our conclusion that CRP used a PCh-binding-independent mechanism to protect mice against infection also was based on the finding that the amount of mutant CRP required to protect mice against infection was the same as that of WT CRP, despite the drastic difference in the avidity of binding of WT and mutant CRP to pneumococci.

The significance of the PCh-dependent mechanism is the initial and immediate clearance of pneumococci, as has been shown recently with endogenous mouse CRP (12). The definition of the PCh-independent mechanism is not known. We hypothesize that the PCh-independent mechanism involves first a structural change in CRP, which then is followed by the interaction between structurally altered CRP and factor H-bound pneumococci, as we have discussed previously (4, 14, 19). This hypothesis is based on three findings. First, pneumococci are able to recruit factor H, a regulator of complement activation, on their surface, and factor H-coated pneumococci are resistant to attack by the complement system (4, 24). Second, it has been shown that the pentameric structure of CRP is altered in a variety of conditions (4, 25–31), and that structurally altered CRP gains the ability to bind to immobilized factor H (4, 25, 26, 31–35). Third, based on the bacteremia data, 150 µg of mutant CRP was more protective than 25 µg, unlike WT CRP, for which there was no difference between the use of 25 and 150 µg CRP. The half-life ($t_{1/2}$) of CRP in mice is only approximately 4 h (36). Administering a higher dose of CRP allows CRP to stay longer in the host. We hypothesize that the CRP mutant, when given at the beginning of the experiment, undergoes structural changes during its stay in the host to express the factor H-binding property, regardless of the mutations at the PCh-binding site. Once factor H on pneumococci is bound to structurally altered CRP, such pneumococci may not be resistant to complement attack any longer.

In order to protect mice from infection, CRP must bind to pneumococci and that mutant CRP must be doing so *in vivo*, although not through the PCh groups present on the pneumococ-

cal surface. Besides the structurally altered CRP-factor H hypothesis described in the previous paragraph, several other possible mechanisms, including one based on the interaction between CRP and ficolins, have been reported (4, 16, 37–39). As proposed previously (19), the development of infection models involving passively administered human WT and mutant CRP in CRP knock-out (KO) mice and SAP KO mice (12, 40–45), the development of mice transgenic for the F66A/T76Y/E81A CRP mutant, and also the use of a strain of *S. pneumoniae* incapable of recruiting factor H in mouse protection experiments may provide more information on the PCh-independent mechanism of the antipneumococcal function of CRP.

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