An Intact Phosphocholine Binding Site Is Necessary for Transgenic Rabbit C-Reactive Protein to Protect Mice against Challenge with Platelet-Activating Factor[1]

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C-reactive protein (CRP), an acute phase protein in humans and rabbits, is part of the innate immune system. The role of CRP in host defense has been thought to be largely due to its ability to bind phosphocholine, activate complement, and interact with IgGRs (FcγRs). We have shown previously that transgenic rabbit CRP (rbCRP) protects mice from lethal challenges with platelet-activating factor (PAF). To investigate the mechanism of this protection, we created additional lines of transgenic mice that express either wild-type rbCRP, a variant of rbCRP with altered complement activation activity (Y175A), or a variant of rbCRP unable to bind phosphocholine (F66Y/E81K). In the current study, these lines were challenged with a single injection of PAF and their survival monitored. Mice expressing wild-type and Y175A rbCRP were protected against challenge by PAF whereas mice expressing F66Y/E81K rbCRP were not. Treatment with cobra venom factor did not affect survival, confirming the results with the Y175A rbCRP variant and indicating that complement activation was not required to mediate protection. Both wild-type rbCRP and Y175A rbCRP were capable of binding PAF in vitro whereas F66Y/E81K rbCRP was not. Although other interpretations are possible, our results suggest that the protective effect of rbCRP against PAF is due to sequestration of PAF. ***The Journal of Immunology,*** **2005, 175: 1192–1196.**

C-reactive protein (CRP)[3] is an acute phase protein in rabbits and humans whose plasma concentrations can increase up to a 1000-fold, depending on the severity of an inflammatory stimulus. The dramatic changes in circulating levels of CRP along with a plethora of in vitro and in vivo data have led to the hypothesis that CRP plays an important role in host defense and inflammation (1). Many of the effects of CRP are believed to be mediated through three well-described properties: 1) binding to phosphocholine (PCh); 2) the ability to activate the classical complement pathway through direct interaction with C1q; and 3) interaction with IgG receptors (FcγRs).

CRP was discovered due to its ability to bind PCh moieties within the pneumococcal C-polysaccharide of *Streptococcus pneumoniae* (2, 3). Besides being associated with various bacteria, PCh is also found in eukaryotic cell membranes as a constituent of sphingomyelin and phosphatidylcholine. In eukaryotic membranes, the PCh moieties are exposed and available for binding by CRP only in necrotic and apoptotic cells (4–7).

CRP consists of five identical, noncovalently associated ~23-kDa protomers arranged symmetrically around a central pore. Each CRP protomer has a recognition face where PCh can be bound in a Ca^{2+}-dependent manner. Each protomer is oriented in the same direction so that all the PCh binding sites are on the same face of the pentamer. The opposite face of the pentamer, the effector face, contains the binding site for C1q and the site where FcγRs are presumed to bind. The cocrystal structure of CRP and PCh has been solved by x-ray crystallography and indicates that PCh interacts with two residues of CRP, Phe^{66} and Glu^{81} (8). Site-directed mutagenesis and in vitro analysis have confirmed the importance of these residues (9, 10). Similar studies have identified Tyr^{175} as a contact residue necessary for PCh-bound CRP to interact with C1q (11).

In vivo, exogenous and transgenic human and rabbit CRP (rbCRP) have been shown to have protective or anti-inflammatory effects in a variety of models. In mice challenged with a lethal dose of *S. pneumoniae*, CRP has been shown to be protective (12–15). This protection requires an intact complement system (15, 16) but does not require interaction with FcγRs (15). CRP has also been shown to have protective effects in mice challenged with endotoxins (17), an effect requiring FcγRs (18).

CRP also protected mice challenged with platelet-activating factor (PAF) (17). PAF is a PCh-containing lipid mediator of inflammation that is released in response to bacterial LPS (19, 20). PAF is known to induce the aggregation of platelets as well as to activate inflammatory cells, including neutrophils and macrophages (reviewed in Refs. 21–23). It participates in allergic responses, anaphylaxis, and endotoxic shock (reviewed in Refs. 21 and 22). Many of the biological effects of PAF are thought to be mediated by the production of inflammation-associated cytokines, including IL-1β, TNF-α, IL-6, and IL-8 (reviewed in Ref. 24).

In addition to the protective effects of CRP against PAF in vivo, CRP has been described as having an inhibitory effect on PAF in vitro. CRP reduces the amount of platelet aggregation induced by PAF (25–27). CRP was able to inhibit PAF-induced degranulation and superoxide production in neutrophils (28) and inhibit PAF-induced superoxide production and calcium mobilization in guinea pig alveolar macrophages (29).

As previously described (10), we have generated three lines of transgenic mice to better understand the mechanism through which rbCRP protects mice from a lethal challenge with PAF. These mice

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[3] Abbreviations used in this paper: CRP, C-reactive protein; PCh, phosphocholine; PAF, platelet-activating factor; rbCRP, rabbit CRP; PEPCK, phosphoenolpyruvate carboxykinase; CVF, cobra venom factor.

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express either wild-type rbCRP, a variant of rbCRP incapable of binding to PCh (F66Y/E81K), or a variant of rbCRP with altered complement activation activity (Y175A). In contrast to humans, where plasma levels of CRP can reach levels > 200 μg/ml, plasma levels of mouse CRP rarely exceed 2 μg/ml following inflammatory stimuli (30), making the mouse a good model in which to study the effects of transgenic CRP in vivo. In the present study, transgenic mice expressing rbCRP were challenged with PAF. We found that an intact PCh binding site was necessary for rbCRP to protect mice challenged with a lethal dose of PAF. In contrast, complement activation was not required for protection. The simplest explanation for these findings is that rbCRP inhibits the effects of PAF by binding directly to the PCh moiety of PAF, preventing binding of PAF to its receptor.

Materials and Methods

rbCRP expression in transgenic mice

All animals used in this study were maintained according to institutional guidelines. Expression of transgenic rbCRP was controlled by the rat cytosolic phosphoenolpyruvate carboxykinase (PEPCK) promoter as described previously (31). The PEPCK promoter responds to gluconeogenic signals in hepatocytes and is repressed by carbohydrate-rich diets but induced by fat or protein-rich diets. All animals used in these studies were homozygous for the transgene and backcrossed into the C57BL/6 (B6) background six generations. Blood was drawn via a retro-orbital bleed from each mouse once or twice a day for 14 days. Approximately 50 μl of whole blood were drawn each time. Blood drawn on days 0 and 1 were from mice fed standard chow. Starting on day 1 following the blood draw, mice were fed Custom McGrane high-carbohydrate diet for 5 days (32). During this time period, blood was drawn once per day. On day 6, the mice were switched to an isocaloric protein-rich diet (32) and maintained on this diet through the course of the experiment. Blood was drawn once or twice a day up to day 14. Serum from each blood sample was then used to measure rbCRP levels as described below.

rbCRP assays

Serum samples were obtained from mice via retro-orbital bleeding 1 h before challenge with PAF. rbCRP levels in mouse sera were measured as described previously (10). Briefly, PCh-BSA was prepared according to a previously published method (33). Microtiter wells were coated with 100 μl of PCh-BSA at 10 μg/ml in TBS (pH 7.2) and incubated for 1 h at room temperature. Each well was blocked with 1% BSA in TBS (300 μl) for 45 min at room temperature. rbCRP samples and standards were added at appropriate concentrations in a calcium-containing buffer (TBS with 5 mM $CaCl_2$, 0.1% BSA, and 0.01% Igepal) and incubated overnight at 4°C. rbCRP purified from the serum of a rabbit was used to construct a standard curve. A rbCRP-specific goat polyclonal Ab (G2P, a gift of Dr. I. Kushner Case Western Reserve University, Cleveland, OH) was used to detect bound rbCRP (34). Bound G2P was detected with a goat anti-rabbit IgG (H+L) labeled with HRP (Pierce) followed by the use of a peroxidase substrate kit (Bio-Rad), as per the manufacturer's instructions. Color development was measured at 405 nm in a microplate reader (Molecular Devices). rbCRP levels from 100 ng/ml up to 300 μg/ml are detectable by this assay. Radial immunodiffusion assays were used (35) to measure rbCRP levels in serum from transgenic mice expressing F66Y/E81K. rbCRP levels from 2 to 200 μg/ml were detectable using this method. Circulating levels of rbCRP > 100 μg/ml were achieved routinely 24 h after the shift from a carbohydrate-rich to a protein-rich diet.

Challenge of mice with PAF

PAF (Sigma-Aldrich) diluted in 0.9% sterile saline was injected i.v. into the tail vein of mice at a concentration of 35–60 μg/kg. The appropriate concentration of PAF was determined for each individual experiment using mice expressing high and low levels of wild-type rbCRP. This dose was then applied to animals expressing variant rbCRP. Animals were monitored for 2 h with lethality typically occurring in <1 h. For all experiments, animals expressing low levels of rbCRP were maintained on the carbohydrate-rich diet, and high rbCRP expression was induced by a shift to the protein-rich diet 24–30 h before challenge. Mice treated with cobra venom factor (CVF) (Quidel) were given a single i.p. injection of CVF (30 μg diluted in sterile 0.9% NaCl) 24 h before challenge with PAF. Results were analyzed using the χ^2 test with Yates' correction and considered significant if the p values were <0.05.

Detection of serum C3 by rocket immunoelectrophoresis

Rocket immunoelectrophoresis (36) was used to estimate the amount of C3 in mouse sera. Immunoelectrophoresis was performed on GelBond Film (Cambrex) covered with 9 ml of 1% agarose in Tris-boric acid buffer (pH 8.6) containing 40 μl of rabbit anti-human C3c (DakoCytomation), an Ab known to cross-react with mouse C3c. A field strength of 200 V was applied for 3 h. Precipitin peaks were stained with Coomassie brilliant blue. The amount of C3 depletion was estimated by comparing the height of the "rocket" of a serial dilution of serum from a mouse before injection with CVF with the "rocket" height of serum from the same mouse 24 h after injection of CVF.

Detection of rbCRP binding to PAF

Binding of rbCRP to PAF was demonstrated using a solid phase binding assay. Microtiter wells were coated with PAF at 200 μg/ml in 100% methanol (25 μl/well), dried completely at 37°C, and blocked with 1% BSA in TBS for 45 min at room temperature. Serum from transgenic mice diluted in PAF binding buffer (10 mM TBS, 1% BSA, 5 mM $CaCl_2$, and 1 mM $MgCl_2$ (pH 7.2)) was added to the appropriate well and incubated overnight at room temperature. A rbCRP-specific goat polyclonal Ab (G2P) was used to detect bound rbCRP. Microtiter wells were developed with a HRP-conjugated rabbit anti-goat IgG followed by use of a peroxidase substrate kit (Bio-Rad), as per the manufacturer's instructions. Color development was measured at 405 nm in a microplate reader (Molecular Devices). After each step, the plate was washed four times using the PAF-binding buffer.

Results

Expression of rbCRP is induced by dietary manipulation

As previously described, three lines of transgenic mice expressing either wild-type rbCRP, F66Y/E81K rbCRP, or Y175A rbCRP were generated (10) and backcrossed six generations into the B6 background. Using the rat cytosolic PEPCK promoter involved in gluconeogenesis, hepatic expression of the transgene was induced by dietary manipulation. rbCRP expression was inducible in all three lines of transgenic mice to similar levels. Fig. 1 shows a typical pattern of circulating rbCRP following dietary induction in three mice.

Transgenic mice on normal chow had a constitutive level of rbCRP expression of 10–30 μg/ml. On day 1, the diet was changed to an isocaloric carbohydrate-rich diet that inhibits expression of the transgene from the PEPCK promoter. Forty-eight hours after such a diet change, rbCRP expression levels were typically <10 μg/ml. On day 6, the animals were provided an isocaloric high-protein diet. Transgenic rbCRP expression levels peaked 24–48 h

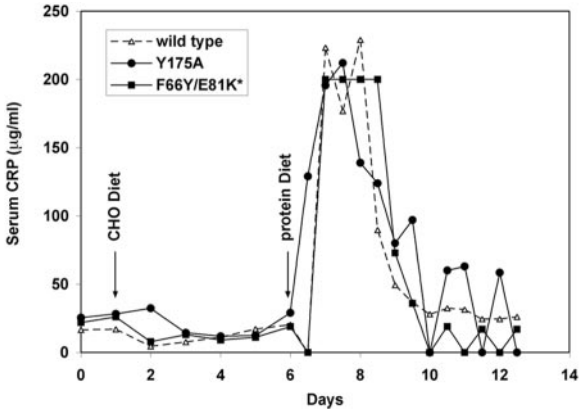

FIGURE 1. Short-term effect of diet on circulating levels of rbCRP in transgenic mice. Mice were provided normal chow up to day 1, a carbohydrate-rich diet (CHO) from days 1 to 6 and a protein-rich diet from days 6 to 14. A representative mouse from each transgenic line is shown. Serum CRP levels were measured as described in *Materials and Methods*. (*, On days 7 and 8, the concentration of F66Y/E81K rbCRP was above the detectable limit of 200 μg/ml using radial immunodiffusion).

after the diet change to levels in the range of 150–225 μg/ml and returned to the baseline levels 2–3 days later. Animals continuously maintained on the carbohydrate-rich diet typically had rbCRP levels < 10 μg/ml. All three lines of transgenic mice showed a similar pattern of induction.

rbCRP expressed in transgenic mice requires an intact PCh binding site to protect against lethal challenge by PAF

Transgenic mice expressing wild-type, F66Y/E81K, or Y175A rbCRP were challenged with a single i.v. injection of PAF. Each line of mice was divided randomly into two groups. One group was maintained on the carbohydrate-rich diet and expressed low levels of rbCRP (<20 μg/ml). The other group was fed the carbohydrate-rich diet for 4–5 days, at which time the diet was changed to the protein-rich diet to induce high levels of rbCRP expression (>100 μg/ml) 24 h before being challenged with PAF.

As shown in Fig. 2, mice expressing high levels of wild-type and Y175A transgenic rbCRP were significantly more likely to survive a lethal challenge with PAF than were littermates expressing low levels of rbCRP ($p = 0.03$ and $p = 0.011$, respectively). In contrast, survival of transgenic mice expressing high levels of F66Y/E81K, the variant incapable of binding PCh, did not differ from that observed in littermates expressing only low levels of F66Y/E81K rbCRP. Survival of the F66Y/E81K mice, regardless of CRP expression levels, was comparable to that observed in mice expressing low levels of wild-type rbCRP. B6 mice ($n = 8$) were also challenged with similar concentrations of PAF; no animals survived.

Protection of rbCRP-expressing mice does not involve complement

In a series of solid phase-binding assays, Y175A rbCRP bound to PCh was incapable of activating complement but could activate complement when bound to polycations or directly adsorbed to a solid phase (10). To more definitively rule out the possibility that complement activation by rbCRP was not playing a role, transgenic mice expressing high levels of wild-type rbCRP were treated with CVF 24 h before challenge with PAF to deplete C3. Rocket immunoelectrophoresis was used to demonstrate the depletion of C3 in these animals (data not shown). Equal amounts of serum from each mouse, obtained before and 24 h after injection of CVF, were subjected to this technique. Twenty-four hours after treatment with CVF, there was a dramatic decrease in rocket size, estimated to be 5% of control C3 levels. Transgenic mice expressing high levels of wild-type rbCRP (>100 μg/ml) and treated with CVF survived challenge with PAF to a similar degree as did those animals expressing high levels of wild-type rbCRP (>100 μg/ml) not treated with CVF (Fig. 3). These findings, along with the observation (Fig. 2) that high levels of Y175A rbCRP protected mice from a lethal challenge with PAF, indicate that complement activation was not required for rbCRP-mediated protection from lethal challenges of PAF.

F66Y/E81K rbCRP is incapable of binding PAF in vitro

PAF contains PCh, and it has been reported previously that CRP is capable of binding PAF (26, 28). We confirmed this binding in a direct manner using a solid phase-binding assay. In this assay, PAF was used to directly coat a solid phase. rbCRP was added to PAF-containing wells and bound rbCRP detected by an Ab specific for rbCRP (G2P). Both wild-type and Y175A rbCRP bound PAF in a dose-dependent manner with similar avidities, but F66Y/E81K demonstrated little binding to PAF (Fig. 4).

Discussion

We have shown previously that transgenic wild-type rbCRP protects mice from lethal challenge by PAF (17). In the present study, we extended these studies to determine whether the PCh binding or the complement-activating properties of CRP contribute to this observed protection. We found that transgenic mice expressing variant rbCRP (F66Y/E81K) incapable of binding PCh are not protected from a lethal challenge of PAF, presumably due to inability to bind the PCh moiety of PAF. Both transgenic mice expressing a variant rbCRP (Y175A) with altered complement activation activity and transgenic mice expressing wild-type rbCRP and treated with CVF were still protected from lethal challenge with PAF, demonstrating that activation of complement is not required for protection from PAF challenge.

Undoubtedly PAF-medicated mortality in mice, although rapid, results in the generation of numerous CRP ligands. We cannot rule

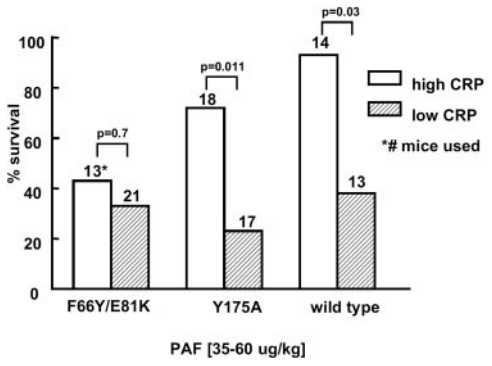

FIGURE 2. Survival following injection of PAF in transgenic mice expressing high levels (>100 μg/ml) or low levels (<20 μg/ml) of rbCRP. Dietary manipulation began 24–30 h before the administration of PAF (35–60 μg/kg). Mice were challenged with an i.v. injection of PAF, and survival was monitored for 24 h with lethality typically occurring within 1 h. The total number of mice used in each group is indicated above the bar. □, Animals maintained on the high-protein diet. ▨, Animals maintained on the carbohydrate-rich diet. Values of p were calculated using the χ^2 test with Yates' correction. Data were considered significant if p values were <0.05.

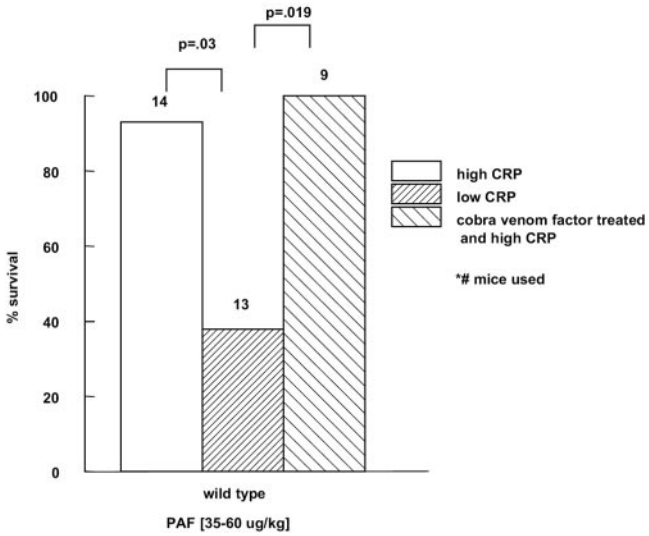

FIGURE 3. Treatment of mice with CVF. Transgenic mice expressing wild-type rbCRP were treated with an i.p. injection of CVF (30 μg) at the same time rbCRP expression was induced with the high-protein diet and were challenged with PAF 24 h later. Survival of mice injected with CVF and challenged with PAF were compared with mice expressing wild-type rbCRP (data taken from Fig. 2).

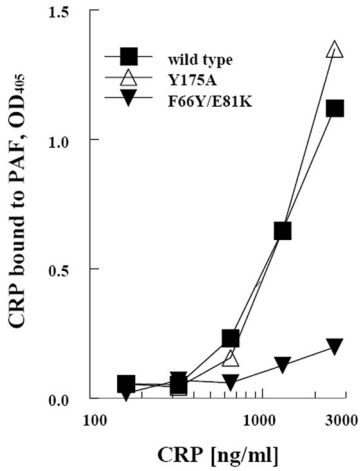

FIGURE 4. Binding of wild type, F66Y/E81K, and Y175A rbCRP to PAF. Microtiter wells were coated with 200 μg/ml PAF. Serum from transgenic mice containing rbCRP at concentrations from 150 up to 2500 ng/ml was added to the appropriate well. Bound rbCRP was detected using the G2P polyclonal Ab followed by detection with a HRP-labeled Ab. The result shown is a representative of two experiments.

out the possibility that our findings are the result of an indirect effect of F66Y/E81K rbCRP interaction with secondary ligands resulting from the challenge. Our previous results have shown the F66Y/E81K variant is still capable of binding many ligands, including polycations and histones, in some cases with an even higher avidity than wild-type rbCRP. It is only defective in binding ligands in a PCh-dependent manner, with PAF as part of this group. The most straightforward interpretation of our results is that wild-type rbCRP and Y175A rbCRP function in this model by sequestering PAF via its PCh moiety, thus preventing it from binding to the receptor for PAF (PAFR). F66Y/E81K rbCRP can no longer bind PAF and therefore does not interfere with the lethal PAF-PAFR interaction. It is conceivable, but less likely, that secondary ligands to which F66Y/E81K binds more avidly than wild type are present and act to abrogate the protective effects normally seen with unbound rbCRP.

In our initial report of rbCRP providing protection against challenge by PAF, we postulated that this protection was not due to rbCRP binding to the PCh moiety of PAF, based on indirect data (17). In those studies, we found that simultaneous injection of PAF and lyso-PAF in mice expressing high levels of rbCRP did not alter rbCRP-mediated survival. Lyso-PAF, a biologically inactive PCh containing metabolite of PAF, was used in an amount calculated to be sufficient to bind the PCh binding sites on rbCRP. Our current approach with transgenic mice expressing a variant of rbCRP incapable of binding PCh leads to a contrary conclusion. It is more direct and, combined with the in vitro data (Fig. 4), argues that it is likely that rbCRP mediates protection by binding and sequestering PAF.

This is the first direct demonstration of the importance of the PCh binding site in an in vivo, biologically significant setting. Our data support the hypothesis that CRP affords protection against PAF by direct interaction of CRP with its PCh moiety. There have been other reports that support this hypothesis in which CRP preincubated with PAF abrogated the effects of PAF (25, 26).

Although not universally accepted (37), we (17) and others (18, 38) have demonstrated that transgenic or exogenous CRP provides partial protection from LPS-induced mortality. Because one mediator of LPS toxicity is PAF (19, 20), it is tempting to speculate that a CRP-PAF interaction may contribute to the ability of CRP to provide protection from LPS. We would predict that mice expressing F66Y/E81K rbCRP would be sensitive to an LPS challenge. Experiments are planned to test this hypothesis.

We are not the first to suggest that CRP functions by sequestration through PCh binding. Gould and Weiser (39) published evidence suggesting that CRP expressed in the respiratory tract binds PCh on the surface of *S. pneumoniae* and *Haemophilus influenza*, preventing their attachment to host cells (40). The PCh expressed by these species mediates binding to PAFR, a mechanism by which the bacteria increase their adherence to and invasion of host epithelial cells (41, 42). CRP inhibited adherence of bacteria expressing PCh and their subsequent invasion but had no effect on bacteria without PCh (40).

CRP binding to PCh of PAF may not be the only means through which activation of the PAFR is blunted. The ability of CRP to bind PCh on the surface of necrotic and apoptotic cells may provide a general mechanism to limit the inflammatory response generated by signaling through the PAFR. The PAFR shows a strong preference for the sn-1 ether bond, the sn-2 acetyl residue, and the choline head group of PAF (43). There are a number of PAF-like lipids that are also capable of interacting with the PAFR, including oxidized phosphatidylcholine (reviewed in Ref. 43). In fact, it has been reported that CRP is capable of binding the PCh moiety in oxidized phosphatidylcholine but is unable to bind nonoxidized phosphatidylcholine (7). CRP binding to PCh of oxidized phosphatidylcholine may help limit the damage caused by these oxidatively modified phospholipids.

Although our results suggest that the PCh binding site is important in this model, other aspects of CRP biology may also be involved. It has been shown that CRP prevented binding of PAF to platelets, leading to the speculation that CRP may bind directly to PAFR (27). An alternate explanation may be CRP binding to FcγRs. This seems more likely because transgenic animals expressing F66Y/E81K still showed a significant but minimal amount of protection when compared with B6 mice, which carried no transgene. All of our transgenic mice express at least low levels of rbCRP ($<$10 μg/ml). This low level expression of rbCRP may be sufficient to induce an effect through interactions between rbCRP (variant or wild type) and FcγRs, resulting in survival differences between all three transgenic lines and B6. It should be noted that it is formally possible that the small fraction of the 129 strain genome remaining after six generations of backcrossing is responsible for the differences between the transgenic lines and B6. However, on a random basis, it is unlikely that a common 129 sequence remains in all of the transgenic lines after the breeding which involved multiple breeding pairs.

The use of these transgenic animals expressing variants of rbCRP will allow us to further examine the importance of these two fundamental properties of CRP, PCh binding and complement activation, in a variety of inflammatory models, including protection from bacterial species, both those expressing PCh and those which do not.

Disclosures

The authors have no financial conflict of interest.

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