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Role of Complement in C-Reactive-Protein-Mediated Protection of Mice from *Streptococcus pneumoniae*

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Expression of a human C-reactive protein (CRP) transgene has been shown to protect mice from lethal *Streptococcus pneumoniae* infection. In the present study, we used cobra venom factor-induced decomplementation to investigate the role of complement in this CRP-mediated protection. An intact complement system significantly reduced pneumococcal bacteremia at 24 h postinfection and extended median survival time of both CRP-transgenic and nontransgenic mice. However, mortality was significantly lowered only for CRP transgenic mice. The transgene significantly reduced bacteremia for both normocomplementemic and decomplemented mice, but it resulted in a significantly longer median survival time and lower mortality only for normocomplementemic mice. These data suggest that in vivo complement and CRP amplify each other's protective capacity, particularly during the early course of infection.

C-reactive protein (CRP), the prototypic acute-phase protein in humans, binds various pathogens, including bacteria, fungi, and yeasts, and promotes their phagocytosis by human leukocytes (9, 15, 16). In particular, CRP reacts strongly with *Streptococcus pneumoniae*. In contrast to the nearly milligram-per-milliliter levels in plasma attainable in humans during the acute-phase response, mouse CRP is not an acute-phase protein and rarely exceeds 2 μg/ml. This fact has led to extensive studies of the protective effects of administration of human CRP against pneumococcal infection in mice (5, 12, 13, 23). Recently, we extended these observations of CRP-mediated protection by using CRP-transgenic mice as an in vivo model of host resistance (17). We showed that human-CRP-transgenic mice capable of mounting an acute-phase CRP response have reduced bacteremia, increased lifespan, and reduced mortality after infection with *S. pneumoniae* (17). The exact mechanisms involved in CRP-mediated protection in vivo are still unknown, but previous in vitro data strongly suggest that elimination of bacteria is likely mediated through interactions with the complement system and phagocytic cells. For example, it is known that CRP complexed with *S. pneumoniae* cell wall C-polysaccharide is capable of activating the classical pathway of complement (1, 7) and that CRP-coated cells are ingested efficiently by blood-derived macrophages and neutrophils (3, 8, 9).

The relevance of the in vitro complement-activating potential of CRP to its in vivo anti-pneumococcal activity has been indicated by the failure of passively administered CRP to protect *xid* mice depleted of complement (5). This observation supports the contention that the protective effect of CRP against fatal infection with *S. pneumoniae* requires a functioning complement system. Nevertheless, complement-independent pathways for CRP-mediated protection cannot be ruled out, because CRP by itself has opsonic activity (3), and it has been reported to protect decomplemented BALB/c mice from fatal pneumococcal infection (13).

We compared the course of *S. pneumoniae* infection in normocomplementemic CRP-transgenic and nontransgenic mice to that of infection in mice decomplemented with cobra venom factor (CoVF). In this model system, human CRP is produced in vivo by the host in response to infection; therefore, we could more accurately evaluate the contribution of complement in CRP-mediated protection from pathogens. Our results indicate that the presence of the CRP transgene leads to significantly reduced bacteremia in both decomplemented and normocomplementemic mice. However, an intact complement system is clearly essential for full expression of CRP-mediated protection. Thus, CRP and complement appear to act in concert in vivo, one amplifying the protective capacity of the other.

We have described previously (17) the establishment of a breeding colony of human-CRP-transgenic mice by mating transgenic founder males (2) with normal female C57BL/6J mice. Mice were routinely screened for inheritance of the transgene by PCR, performed as described previously (14). This PCR procedure utilizes genomic DNA derived from mouse tails and selectively amplifies a 396-bp product from the coding region of the human CRP gene. Blood expressed because of tail clipping was collected, and 25 μg of purified bacterial lipopolysaccharide (Sigma, St. Louis, Mo.) was immediately injected intraperitoneally to induce an acute-phase response. Fifteen to 20 h later, blood was collected from the tail vein. The concentrations of human CRP and mouse serum-amyloid P component (moSAP) in sera were measured by enzyme-linked immunosorbent assay (ELISA) and by rocket immunoelectrophoresis, respectively, as previously described (10, 17).

Since the level of expression of serum CRP in adult male transgenic mice is higher than that in females (17), we used only male mice 11 to 12 weeks of age weighing 23.5 ± 0.3 g ($\bar{x}$ ± standard error) for the present study. Mice were fed and watered ad libitum and maintained according to protocols established by the Animal Resources Program at this institution. Nontransgenic littermates served as controls. Decomplementation was achieved by injecting intraperitoneally a single dose (30 μg) of purified *Naja naja* CoVF (QUIDEL, San Diego, Calif.) (20, 21). The extent and duration of CoVF-induced decomplementation were monitored by measuring serum C3 levels by ELISA (18). In preliminary experiments, we established that serum C3 levels reproducibly decreased within 4 h after CoVF treatment to less than 3% of initial levels (Fig. 1).

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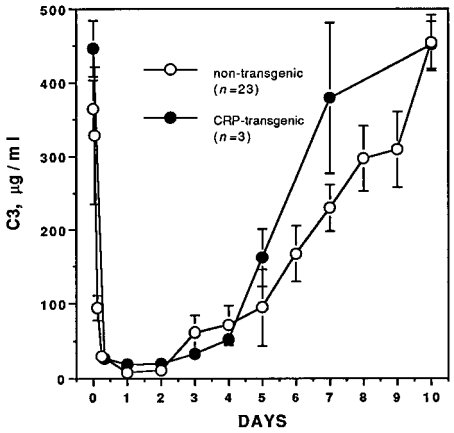

FIG. 1. Concentration of serum C3 ($\bar{x} \pm$ standard error) of mice injected intraperitoneally on day zero with 30 μg of CoVF. The data are pooled from three independent experiments.

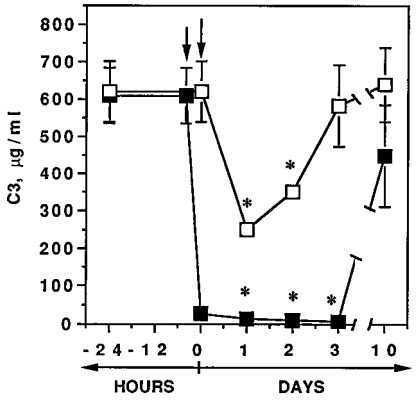

FIG. 2. Concentration of serum C3 ($\bar{x} \pm$ standard error) of mice challenged with *S. pneumoniae*. Mice were injected with saline (□) or CoVF (■) (30 μg intraperitoneally [first arrow]) 4 h prior to challenge with *S. pneumoniae* (60 CFU [second arrow]). The zero hour value plotted for the CoVF group is an estimate based on the known kinetics for C3 depletion by CoVF (Fig. 1). Asterisks indicate a P of <0.05 compared with -24 h values (protected least significant difference test).

By 6 h, many mice had no detectable serum C3, and C3 levels remained below 10% up to day 4. The kinetics, extent, and duration of C3 depletion by CoVF were similar in transgenic and nontransgenic mice.

Four hours after injection of CoVF (or saline as a control), groups of CRP-transgenic and nontransgenic mice were infected. *S. pneumoniae* capsular type 3 strain WU2 cells, maintained virulent by intravenous passage in mice and stored at −70°C (5), were grown and harvested in late log phase. Bacteria were resuspended in Ringer's lactate, and concentrations of bacteria were estimated from A_{420} (an A_{420} of $1 = 2 \times 10^8$ CFU/ml) and confirmed by plating on blood agar. Inocula for infections contained 60 ± 4 CFU of bacteria suspended in 200 μl of lactate solution. Preliminary trials established that this dose of *S. pneumoniae* was approximately 10 times lower than the 50% lethal dose for nontransgenic mice. Daily after infection, blood samples (75 μl) were collected from the retroorbital sinus, serially diluted in Ringer's lactate, and plated on blood agar to determine the number of viable pneumococci. The numbers of pneumococci recovered are presented as means of log-transformed counts ($\log_{10}$ CFU per milliliter of blood). The lower limit of detection of pneumococci was 75 CFU/ml (log = 1.9). The remaining blood was used to determine levels of circulating C3, CRP, and moSAP. Natural deaths of mice were recorded for a 10-day period after inoculation. Survival curves were plotted, and median survival time (MST [i.e., the time to 50% mortality of mice]) was estimated by interpolation.

Six experiments involving 60 mice (15 per group) were performed to evaluate the course and outcome of infection with *S. pneumoniae*. As shown in Fig. 2, serum C3 was reduced to 1 to 2% of baseline levels on days 1, 2, and 3 for infected mice pretreated with CoVF. Interestingly, infection of saline-injected mice (controls) resulted in significantly decreased C3 levels. These dropped to 41% of baseline levels on day 1 but returned to within normal range by day 3 (Fig. 2). This transient drop of serum C3 is probably due to complement activation by the bacteria. Serum moSAP responses to *S. pneumoniae* for CoVF- and saline-treated mice were of a similar magnitude and were unaffected by the presence of the transgene (Fig. 3A). Likewise, the CRP response of infected transgenic mice was not influenced by CoVF treatment (Fig. 3B).

Twenty-four hours after infection, bacteremia was significantly lower in both normocomplementemic and decomplemented transgenic mice than in their nontransgenic counterparts ($P < 0.05$; Fisher's protected least significant difference test [Fig. 4]). Similarly, bacteremia of both transgenic and nontransgenic normocomplementemic mice was reduced compared with that of decomplemented mice ($P < 0.005$; protected least significant difference test [Fig. 4]). These differences were still apparent at 48 h postinfection, but the difference between decomplemented transgenic and nontrans-

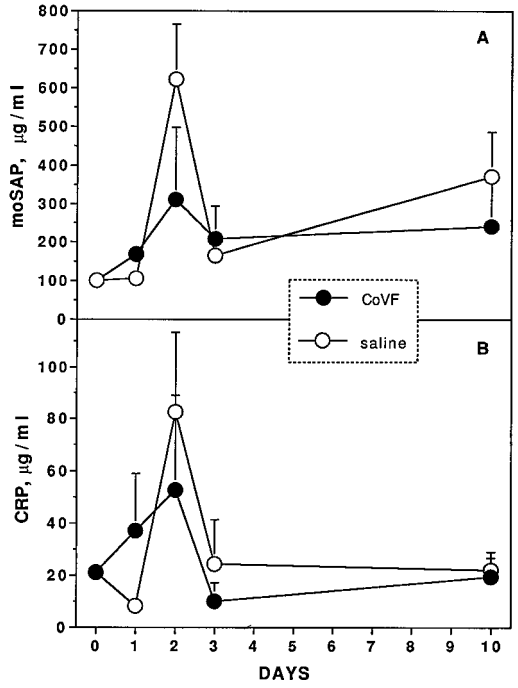

FIG. 3. Concentration of acute-phase serum proteins ($\bar{x} \pm$ standard error) of mice challenged with *S. pneumoniae*. Mice were injected with saline or CoVF (30 μg intraperitoneally) 4 h prior to challenge with 60 CFU of *S. pneumoniae* on day zero.

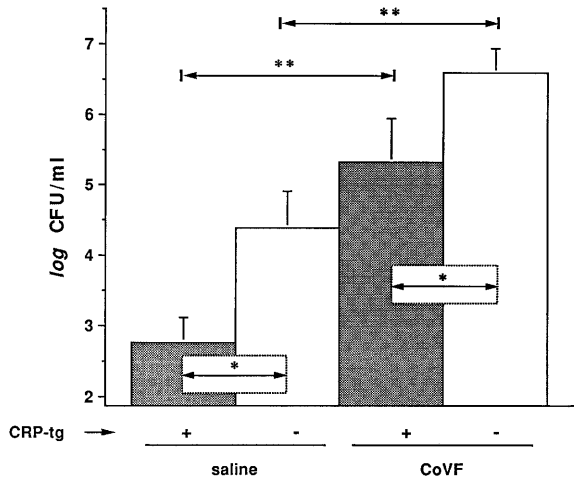

FIG. 4. Bacteremia ($\bar{x}$ ± standard error) of mice challenged with *S. pneumoniae*. Transgenic (CRP-tg$^+$) and nontransgenic (CRP-tg$^-$) mice were injected with saline or 30 μg of CoVF 4 h prior to challenge with 60 CFU of *S. pneumoniae*. Bacteremia was quantified at 24 h postinfection. Asterisks denote significant differences in bacteremia (*, $P < 0.05$; **, $P < 0.005$ [protected least significant difference test]) between the indicated groups ($n = 15$ mice per group).

genic mice was no longer statistically significant (data not shown).

The transgene resulted in a significantly increased MST for normocomplementemic mice (Mann-Whitney U test; $P < 0.05$) but not for decomplemented mice (Fig. 5). On the other hand, the MSTs of both transgenic and control normocomplementemic mice were significantly extended compared with those of their decomplemented counterparts (Mann-Whitney U tests; $P < 0.001$ [Fig. 5]). Total mortality on day 10 postinfection was significantly reduced for normocomplementemic compared with decomplemented mice, but only in the presence of the transgene (Fisher's exact test; $P = 0.025$ [Fig. 5]).

The importance of complement in host resistance to *S. pneumoniae* has clearly been established (6, 22). In agreement with these previous data, we observed that the presence of an intact complement system significantly reduced bacteremia and increased the MST regardless of CRP-transgenic status. Our data also show that an intact complement system is necessary for CRP-mediated protection from lethality after infection by *S. pneumoniae*. Correspondingly, significantly lowered mortality for normocomplementemic compared with decomplemented mice was observed only in the CRP-transgenic group. These observations are consistent with the known ability of CRP to initiate activation of the classical pathway of complement leading to assembly of an effective C3-convertase (reviewed in reference 1). Presumably, this results in subsequent generation of host defense-related complement fragments, including the C3a anaphylotoxin and the C3b and iC3b opsonins, which contribute to the elimination of bacteria.

A significant observation is that the presence of the CRP transgene increased early resistance of decomplemented mice to *S. pneumoniae*, as indicated by reduced bacteremia at 24 h postinfection. We cannot completely rule out a role of complement in the protection we observed for CRP-transgenic mice treated with CoVF. However, on the basis of extensive data from the literature indicating the absence of a functional complement system in similarly treated mice, such a role seems highly unlikely. This early protective effect of the transgene can probably be attributed to the direct opsonic properties of CRP. Several investigators have already demonstrated that CRP can mediate phagocytosis by neutrophils and blood-derived macrophages. CRP binds to monocytes through a distinct CRP receptor (19) and to both neutrophils and monocytes through the high-affinity Fc receptor FcγRI (11). The exact role of these receptor molecules in phagocytosis remains unknown. It is of further interest that expression of FcγRI by monocytes and neutrophils is increased during streptococcal infection (4).

In conclusion, in our transgenic mice, the protective effects of CRP against *S. pneumoniae* infection are mediated mainly via activation of complement. However, even in the absence of a functioning complement system, CRP affords some protection. We speculate that the observed complement-independent effects attributable to CRP might be mediated by phagocytosis and intracellular killing of CRP-coated bacteria.

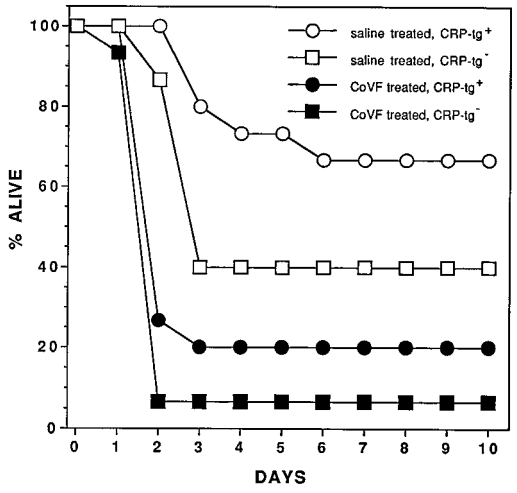

FIG. 5. Survival curves of mice challenged with *S. pneumoniae* ($n = 15$ per group). Transgenic (CRP-tg$^+$) and nontransgenic (CRP-tg$^-$) mice were injected with saline or 30 μg of CoVF 4 h prior to infection with 60 CFU of *S. pneumoniae* on day zero.

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