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The Role of C3 as an Opsonin in the Early Stages of Infection[1] (38815)

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(Introduced by M. M. Mayer)

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Activation of the third component of complement (C3) results in its cleavage into two unequal fragments. A number of studies performed *in vitro* have demonstrated that the smaller of the two fragments, C3a, mediates certain aspects of the inflammatory process (1, 2), and that the larger fragment, C3b, acts as an opsonin (3–5). It has been inferred from these *in vitro* observations that C3 plays a significant role *in vivo* in the host's defense against infection. The importance of C3 in the host's defense against infection has received strong support from the identification of patients with C3 hypercatabolism (6, 7) and C3 deficiency (8) who are unduly susceptible to infection.

The present studies were undertaken in order to develop more definitive information on the mechanism by which C3 mediates its protective effect *in vivo*. Since C5 has been shown to play a significant role in the host's defense against infection (5, 9), and since it is activated by enzymes containing C3b as a subunit (10, 11), the possibility must be considered that part of the protective effect of C3 is an indirect one mediated through its role in activating C5. The present experiments were performed in AKR/J mice, completely deficient in C5 (12), in order to separate the direct protective effect of C3 from an indirect effect mediated via its activation of C5. Experiments were also performed to ascertain which of the biological functions of C3a and/or C3b are responsible for the protective effect of C3 *in vivo*. Finally, studies were performed to determine at what stage of infection C3 makes its most significant contribution.

Materials and Methods. Mice. Male AKR/J mice were purchased from the Jackson Laboratory, Bar Harbor, ME, and used when they weighed between 16 and 18 g.

Serum. Normal AKR/J mice were bled from their tails, the serum separated and stored at −70°C.

Bacteria. Type 3 pneumococci, strain IIIS-IR6, of intermediate virulence for the mouse (13, 14), (Pn 3-int), were maintained and grown as previously described (5).

Leukocytes. Swiss Webster mice, purchased from Microbiological Associates, Bethesda, MD, were injected intraperitoneally with 2 ml of a mixture of 2% starch and 4% gluten diluted 1:1 with Trypticase-Soy broth. The exudate leukocytes were harvested 18 hr later as previously described (15).

Serum opsonizing activity. A modification of a previously described phagocytic test was used (15). Briefly, 6.25×10^7 exudate leukocytes and 31.25×10^7 Pn 3-int were added to 0.5 ml of undiluted serum. The mixture was tumbled at 12 rpm at 37°C for 30 min, and serum opsonizing activity was determined by counting the percentage of polymorphonuclear leukocytes that contained pneumococci on a stained smear. The results are expressed as percentage of phagocytosis.

Cobra venom factor. Lyophilized *Naja naja* venom was purchased from Ross Allen Reptile Institute, Silver Spring, FL, and the Cobra Venom Factor (CoVF) purified as previously described (16).

Titration of C3. The C3 titer in serum was measured according to Shin and Mayer (17).

Immunoelectrophoresis. Immunoelectrophoresis was performed in 1% agarose containing 0.01 *M* EDTA on glass slides according to Scheidegger (18). Monospecific rabbit

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anti-guinea pig C3 was kindly supplied by Doctor Anne Nicholson.

Results. Effect of CoVF in vivo on serum C3 titer and serum opsonizing activity. Mice were injected intravenously with 10 μg of CoVF in 0.1 ml of saline initially and again 2 days later. As shown in Fig. 1, within 2 hr after the CoVF was injected the serum C3 titer, as measured in a hemolytic assay, fell to zero, and remained so for 4 days. The same results were obtained when the serum C3 was measured by immunoelectrophoresis using monospecific rabbit anti-guinea pig C3. Concurrently with the drop in C3 titer, the serum opsonizing activity dropped to a level near that of heated serum but began to rise toward normal by the 2nd day.

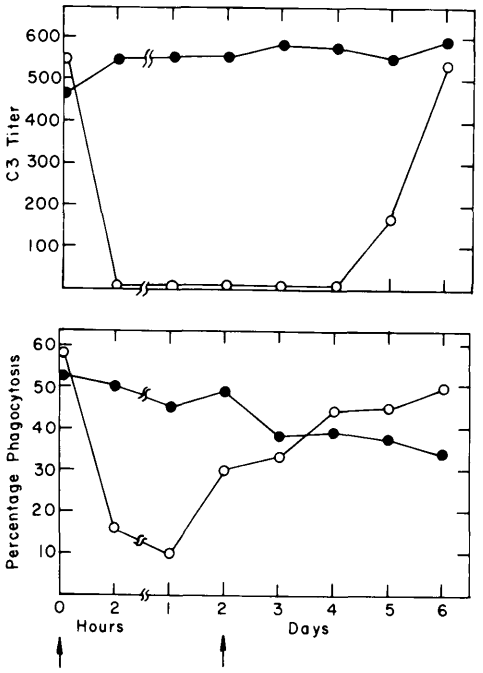

FIG. 1. The effect of CoVF treatment of AKR/J mice on their serum C3 titer and their serum pneumococcal opsonizing activity. The arrows signify the time of an intravenous injection of either 0.1 ml of saline (●——●) or 10 μg of CoVF in 0.1 ml of saline (○——○). The animals in each group were bled from their tails and the pooled serum tested for C3 titer (upper graph) and pneumococcal opsonizing activity (lower graph). Normal serum, heated at 56°C for 30 min, gave 9% phagocytosis.

TABLE I. *In Vivo* PHAGOCYTOSIS OF PN 3-INT IN PREFORMED PERITONEAL EXUDATES OF MICE TREATED WITH SALINE OR CoVF.

Treatment of mice	
Saline	CoVF
15[a]	12
26	9
12	6
23	13
19	10
Mean 19[b]	10[b]

[a] Percentage phagocytosis. Each value represents a single mouse.

[b] $P = < 0.01$ by Student's t test.

In vivo phagocytosis in CoVF-treated mice. Since CoVF treatment decreased the serum C3 titer and serum opsonizing activity, the following experiment was performed to determine if *in vivo* phagocytosis was also affected.

Mice were injected with either CoVF or saline as described above and 2 hr later an acute peritonitis was induced with the starch-gluten suspension. Twenty-two hours later, exudates from either the CoVF- or saline-treated animals were found to contain 1×10^7 leukocytes. Duplicate animals were then injected intraperitoneally with 5×10^7 log phase Pn 3-int in 0.5 ml of broth. Thirty minutes later each animal was sacrificed, the exudate recovered and stained, and the percentage of phagocytosis determined.

As can be seen in Table I, the percentage of phagocytosis was significantly decreased in the exudates from the CoVF-treated animals as compared to that in the exudates recovered from the control animals ($P < 0.01$ by Student's t test). Thus, CoVF treatment decreased *in vivo* phagocytosis.

Effect of CoVF on resistance to pneumococcal infection. Since CoVF treatment depleted mice of serum C3, reduced the serum pneumococcal opsonizing activity, and impaired *in vivo* phagocytosis, the following experiments were performed in order to determine whether these alterations resulted in a decrease in their resistance to pneumococcal infection.

Groups of 8 AKR/J mice were treated

with CoVF or saline as above, and 2 hr later they were challenged intraperitoneally with 10-fold dilutions of log phase Pn 3-int. The mice were observed for 7 days and the LD_{50} was calculated according to the Reed–Muench method (19). It should be noted that the C3 titer did not change in mice challenged with an LD_{50} of pneumococci. As shown in Table II, the LD_{50} was significantly lower in the CoVF-treated mice than in the control animals. However, when the CoVF was injected 6 hr after, rather than 2 hr before the pneumococcal challenge, the LD_{50} was not significantly different from that for the control animals. Thus, CoVF lowered the resistance of the mice to pneumococcal challenge; however, it was effective only during the early stages of the infection.

Since treatment of the mice with CoVF could have affected their host defense in a manner other than through its effect on serum C3 and opsonizing activity, the following experiment was performed.

Pn 3-int were preincubated in either normal or heated (56°C for 30 min) AKR/J serum at a concentration of 2×10^9/ml for 30 min at 37°C. Previous studies have shown that when pneumococci are incubated in normal serum under these conditions, they fix C3 to their surfaces and become opsonized, whereas when they are incubated in heated serum they do not fix C3 and are not opsonized (20). The Pn 3-int were then sedimented by centrifugation and resuspended in broth for use in LD_{50} studies.

As can be seen in Table III, when CoVF-treated mice were challenged with Pn 3-int preopsonized in normal AKR/J serum the LD_{50} was not significantly different from the LD_{50} in control mice. However, when

TABLE II. LD_{50} OF PN 3-INT FOR MICE TREATED WITH SALINE OR COVF.

Experiment	Treatment of mice		
	Saline 2 hr before Pn challenge	CoVF 2 hr before Pn challenge	CoVF 6 hr after Pn challenge
1	$10^{7.8}$[a]	$10^{6.3}$	—
2	$10^{7.8}$	$10^{5.9}$	$10^{7.6}$
3	$10^{7.6}$	$10^{6.0}$	$10^{7.5}$

[a] LD_{50} of intraperitoneal challenge.

TABLE III. EFFECT OF PREOPSONIZATION OF PN 3-INT ON THE LD_{50} IN MICE TREATED WITH SALINE OR COVF.

Pretreatment of Pn 3-int	Treatment of mice	
	Saline	CoVF
Heated serum[a]	$10^{7.5}$[b]	$10^{6.3}$
Normal serum	$10^{7.2}$	$10^{7.0}$

[a] 56°C for 30 min.
[b] LD_{50} of intraperitoneal challenge.

CoVF-treated mice were challenged with Pn 3-int preincubated in heated AKR/J serum the LD_{50} was significantly lower than that in control animals. Thus, the effect of the CoVF treatment on decreasing resistance of the mice to pneumococcal infection could be overcome by preopsonizing the pneumococci *in vitro*.

Discussion. The role of C3 in the host's defense against infection has been extentively studied *in vitro*; however, information as to its role in host defense *in vivo* has been incomplete. The present experiments were designed to investigate the mechanism by which C3 mediates its protective effect *in vivo*.

Treatment of AKR/J mice with CoVF led to a rapid depletion of their serum C3, a marked reduction in their pneumococcal serum opsonizing activity and a significant decrease in their *in vivo* phagocytosis. It was not surprising, therefore, that the CoVF-treated mice were from 30 to 80 times more susceptible to pneumococcal challenge than control mice. Since the present experiments were performed with AKR/J mice, genetically deficient in C5, the possibility of an indirect contribution of C3 to host defense, via activation of C5–C9, is not possible. Thus, it is reasonable to conclude that C3 itself makes a significant direct contribution to the host's defense against infection.

It is unclear why the serum opsonizing activity returned to normal before the serum C3 titer did so. It is possible that as yet unidentified opsonically active serum factors, other than C3, were induced by the CoVF treatment. Alternatively, it is possible that the lack of correlation may be due to the

fact that the test for serum opsonizing activity was more sensitive than those for serum C3 and consequently, the return of the C3 was signaled first in the serum opsonizing test. In any case, in the period of time after infection that the CoVF was found to affect the resistance of the mice to pneumococcal infection, both the serum C3 and serum opsonizing activity were markedly reduced.

When CoVF-treated AKR/J mice were challenged with pneumococci that had been first preopsonized *in vitro* they were no more susceptible to infection than control mice. Presumably, the C3b deposited on the pneumococci during preopsonization *in vitro* effectively substituted for that which would have been deposited on them *in vivo* if the native C3 of the mice had not been depleted by the CoVF treatment. This result suggests that the decreased resistance of the CoVF-treated mice was in large part a consequence of their decrease in C3-dependent opsonization. Of course, the biological functions of C3a may also play a significant role in host defense but experiments of a different design might be required to demonstrate its effect.

The CoVF was effective in decreasing the resistance of the mice to pneumococcal infection only within the first 6 hr after bacterial challenge. Thus, the role of C3 in the nonimmune host's defense would appear to be the most significant in the early stages of bacterial invasion. This finding is consistent with previous studies demonstrating that the eventual outcome of acute bacterial infections is determined very early after bacterial challenge (21), and suggests that at least part of that outcome is predicated upon effective C3-dependent opsonization.

Summary. In order to investigate the role of C3 in host defense *in vivo*, normal AKR/J mice, genetically deficient in C5, were depleted of serum C3 by the injection of purified cobra venom factor (CoVF). Concurrent with their C3 depletion, their serum opsonizing activity decreased to a level less than 20% of normal. When these mice were challenged with an intraperitoneal injection of pneumococci 2 hr after the CoVF treatment, the LD_{50} was from 30 to 80 times lower than the LD_{50} in saline-treated control animals. When the CoVF was given only 6 hr after the pneumococcal challenge, the LD_{50} was the same as in the control mice. If the pneumococci were first preopsonized *in vitro* and then injected into CoVF-treated animals, the LD_{50} was the same as that in control animals.

These experiments demonstrate that C3 plays a significant role *in vivo* in the host's defense against infection and that a major part of that role is through its action as an opsonin. Furthermore, these experiments demonstrate that the role of C3 is most significant during the early stages of bacterial invasion.

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