The Role of Late Complement Components and the Alternate Complement Pathway in Experimental Cryptococcosis (37580)

RICHARD D. DIAMOND, JOSEPH E. MAY, MICHAEL KANE, MICHAEL M. FRANK, AND JOHN E. BENNETT

Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Evidence stressing the importance of phagocytic host defense mechanisms in cryptococcosis, particularly early in the course of infection, has been recently reviewed (1). Prior *in vitro* studies have shown that phagocytosis of cryptococci by peripheral blood leukocytes required late complement components and that opsonization proceeded primarily by the recently described alternate complement pathway (2). In the present study, the role of complement in cryptococcosis was further examined in an animal model.

Activation of C3 and the later complement components may occur either by the classical complement pathway through activation of C1, C4, and C2, or by the alternate pathway via activation of properdin and a series of other proteins. To evaluate the relative importance of the classical and alternate complement pathways, cryptococcal infections were produced in guinea pigs with a genetically controlled deficiency in complement component C4 (3). In these animals, the classical complement pathway is nonoperative, so all complement-dependent opsonization must proceed by the alternate pathway. The importance of late complement components (C3-9) was studied using guinea pigs depleted of these components by virtue of treatment with cobra venom factor (CVF).

Materials and Methods. A small capsule cryptococcal isolate previously described (4) (American Type Culture Collection 24067) was grown in Blake bottles on Sabouraud's agar for 5 days, washed 3 times, and suspended in normal saline. C4 deficient "NIH multipurpose" guinea pigs (C4D) and normal animals of the same strain weighing 400–450 g were used. Groups of normal and C4D guinea pigs were depleted of C3-9 with an iv dose of 20 U/100 g animal weight of CVF, as described elsewhere (5). CVF was given either 6 hr (CVF6) or 54 hr (CVF54) prior to challenge with cryptococci. All CVF6 animals surviving at 4 days after infection were retreated with CVF, but no additional CVF was given to CVF54 animals, in order to allow C3-9 levels to recover toward normal shortly after infection with cryptococci. Guinea pigs were challenged with a suspension of 10^9 cryptococci iv. Retroorbital blood samples were obtained without anticoagulant on Day 1 and Day 6 after infection for quantitative blood cultures and C3-9 titers (5). There were seven animals in each group (normal or C4D with and without CVF). On the second day after infection, two animals were sacrificed for quantitative cultures of liver, lung, and brain. Organ samples were weighed, diluted in distilled water, and homogenized with a Ten Broeck tissue grinder. Cryptococci were quantitated by plate counts. Survival of the remaining five animals was quantitated daily. Dead animals were autopsied to verify the presence of cryptococci in smears of brain tissue.

To rule out the possibility of direct toxicity of CVF on phagocytic cells, *in vitro* control studies were done to measure phagocytosis and killing of cryptococci by guinea pig peripheral blood monocytes and neutrophils separated and used as previously described (4). A group of uninfected animals were bled the day after CVF treatment. Separated neutrophils or monocytes from normal and CVF treated guinea pigs were incubated with cryp-

tococci plus either 10% normal or CVF treated serum to provide opsonins.

Results. At the time of infection with cryptococci, all CVF treated animals had no detectable C3-9 in serum. One day after infection, C3-9 titers were still undetectable in all but one of the CVF treated animals (titer in one C4D CVF54 animal was 5.2% of values obtained in the 7 untreated C4D animals). By Day 6 after infection C3-9 titers had increased in surviving CVF54 animals. Titers in normal CVF54 animals were 66.9% of values in corresponding infected normal animals, and titers in C4D CVF54 animals were 65.1% of values in infected C4D animals. In contrast, C3-9 titers on Day 6 remained low in CVF6 animals, which had received a second dose of CVF on Day 3. Normal CVF6 animals had titers of only 5.2% of corresponding normals, and C4D CVF6 animals had 4.6% of values in infected C4D animals.

There was no significant difference in survival after cryptococcal infection comparing normal and C4D guinea pigs ($p < 0.20$, > 0.10 by the Wilcoxon test (6)) (Table I). However, normal or C4D guinea pigs depleted of C3-9 by CVF treatment had equivalent significantly shortened survival after infection with cryptococci ($p < 0.01$ by the Wilcoxon test). Survival was low whether complement levels were kept low by repeated CVF injections during infection (CVF6 group), or if complement levels were allowed to recover after a single CVF injection 54 hr before infection (CVF54 group). It should be noted that there were no deaths in uninfected control normal and C4D animals injected with the same regimen of repeated doses of CVF during the time period of this experiment. The role of late complement components was further explored using quantitative blood and organ cultures (Table I). Normal and C4D animals had significantly lower numbers of fungi in blood 1 day after infection when compared with corresponding CVF6 animals ($p < 0.01$). In the corresponding CVF54 animals, where complement levels may have been recovering, quantitative blood cultures were intermediate or at levels seen with animals which had not received CVF. These differences were accentuated in blood cultures on surviving animals on day 6 after infection. Where complement had been allowed to recover in CVF54 animals, fungi were cleared more effectively from blood than in the CVF6 animals with continuously depressed complement levels. A similar pattern of results was noted in cultures of lung and liver. In contrast, both CVF54 and CVF6 animals had greater numbers of fungi in brain than corresponding normal or C4D animals. CVF54 and CVF6 animals had similar numbers of organisms in brain. The apparent increased clearance of fungi from extraneural sites in CVF54 animals was therefore not accompanied by evidence of decreased fungi in the central nervous system.

While survival of corresponding groups of normal and C4D animals was similar, there were marked differences in quantitative or-

TABLE I. Guinea Pig Survival and Colony Counts After Infection with Cryptococci.

Group studied	Days survived (range)	Blood cultures (Cryptococci × 10^3/ml)		Organ cultures[a] (Cryptococci × 10^5/g)		
		Day 1[b]	Day 6[c]	Lung	Liver	Brain
Normal	17–26	6.4 ± 1.0	0.7	18.0	1.5	0.3
Normal CVF54	4– 9	10.3 ± 1.1	2.3	9.6	1.5	1.4
Normal CVF6	4– 7	12.1 ± 1.5	33.6	76.4	19.4	1.3
C4D	11–20	4.5 ± 0.5	1.2	1.6	9.4	0.8
C4D CVF54	4– 8	4.1 ± 0.9	4.2	4.0	7.3	6.2
C4D CVF6	3– 7	8.0 ± 1.0	44.6	15.4	36.4	6.3

[a] Values are average from two animals sacrificed on Day 2.

[b] Values are means ± SEM of seven animals.

[c] Values are means of at least three animals, except for normal CVF54 and normal CVF6 groups (only one animal surviving on Day 6).

gan cultures noted between these groups (Table I).

To verify the specificity of action of CVF on serum complement components, *in vitro* control studies were done using guinea pig monocytes and neutrophils. Phagocytosis and killing of cryptococci by cells from CVF treated animals was normal when normal serum was used for opsonization (61.2% killing by monocytes and 53.1% by neutrophils from CVF animals, compared with 54.4% by monocytes and 62.5% by neutrophils from untreated control animals). There was no phagocytosis or killing when normal or CVF cells were used with CVF serum, so it appeared that CVF was effective in depleting opsonins without adversely affecting function of phagocytes.

Discussion. The impaired survival of animals depleted of C3-9 observed in these studies supports the importance of late complement components in host defenses against crytococcosis. A nonspecific action of CVF on host immunity other than serum complement levels was unlikely in these studies. While CVF *in vitro* has been reported to affect cells with C3 receptors (7), CVF *in vivo* did not alter guinea pig peripheral blood leukocyte counts (5), and phagocytic cells in the present study were functionally normal *in vitro*. The rapid death of CVF animals in this study minimizes the importance of the theoretical possibility of an *in vivo* effect of CVF on antibody-producing lymphoid cells with C3 receptors (7). Similarly, while CVF may contain impurities toxic for thymus dependent cells (8), such an effect would not appear important in this study in view of the short survival of CVF treated animals. Therefore, the effect of CVF in these experiments could be best explained by depletion of late serum complement components required for opsonization, since previous studies showed that fresh serum with or without antibody could not kill cryptococci unless phagocytic cells were present (4).

Continuous depletion of C3-9 (CVF6 animals) resulted in high numbers of organisms in blood, lung, liver, and brain. The higher levels of organisms in blood is consistent with the findings of Gilbert *et al.* studying *Escherichia coli* bacteremia in the squirrel monkey (9). In the CVF54 animals, CVF was given only once, 54 hr before infection, to allow C3 levels to recover after challenge with cryptococci. In these animals, there were less fungi in blood, lung, and liver than corresponding CVF6 animals, but the number of organisms in brains of CVF54 and CVF6 animals was almost identical. This suggests that the return of complement components resulted in more efficient killing of fungi from peripheral blood and organs. As noted in earlier studies, complement levels in the central nervous system may be too low for opsonization of cryptococci or generation of chemotactic factors (2). The number of organisms reaching the brain early in the course of infection may then have been a critical factor determining survival, since survival in CVF6 and CVF54 animals was almost identical.

There was no significant difference in survival comparing normal guinea pigs possessing both classical and alternate complement pathways with C4D animals where only the alternate pathway is operative. This also confirms studies which indicated that opsonization of cryptococci proceeded primarily by the alternate complement pathway, though an intact classical pathway was required for optimum phagocytic kinetics (2). Though survival of corresponding groups of normal and C4D animals was similar, there were marked differences in counts of fungi in blood, lung, liver, and brain. The reason for this is unknown, but may reflect differences in local mechanisms of clearance of organisms in normal and C4D animals.

Summary. Duration of survival was comparable in normal and C4D guinea pigs infected iv with cryptococci. Survival was shortened in animals depleted of late complement components by treatment with CVF. Some animals received a single treatment with CVF to allow recovery of complement components after challenge with cryptococci. This resulted in lower counts of fungi in peripheral blood, lung, and liver, but no improvement in levels of fungi in brain, or in survival when compared with animals continuously depleted of C3-9. Late complement components were therefore important in clearance of cryptococci from extraneural sites.

However, once infection was established in brain, complement levels in the central nervous system may have been too low to aid in destruction or organisms.

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