

Macrophage-mediated tumoricidal activity generated by human C-reactive protein (CRP) encapsulated in liposomes is complement-dependent¹

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Human C-reactive protein (CRP) incorporated into multilamellar vesicles (CRP-MLV) has been shown to activate peritoneal exudate cells (PECs) to inhibit tumors. This activity is comparable to that of another macrophage-activating agent, liposomal muramyl-tripeptide (MTP-MLV). The present study shows that the complement system is involved in the CRP-MLV-induced tumoricidal response. Normal mice were depleted of complement and treated with CRP-MLV or MTP-MLV. PECs were later harvested and mixed with T241 tumor cells. After incubation, this mixture was injected into the footpads of normal mice. Sacrificed animals were evaluated for tumor growth and lung metastases. These were significantly inhibited in mice injected with T241 tumor cells mixed with PECs from donors treated with CRP-MLV, but this antitumor activity was lost in mice that received a mixture of tumor cells and PECs from donors depleted of complement before the treatment. Complement depletion, however, had no effect on the antitumor activity of MTP-MLV-induced PECs.

Index terms: C-reactive protein • Complement • Macrophage activation

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C-reactive protein (CRP) is a well-known, acute-phase reactant whose serum levels rise markedly, 100- to 1000-fold, during acute inflammation. Although the existence of this protein molecule has been known for over 50 years, its pathophysiologic role is not yet clear.

CRP activates the classical complement pathway when it is bound to phosphocholine-containing substances,^{1,2} suggesting that one function of CRP may be opsonization. In vitro studies, have shown that CRP or C3b alone could

promote adherence of C-polysaccharide-coated erythrocytes to monocytes and macrophages, but both CRP and C3b were necessary for phagocytosis.^{3,4} CRP and complement have been shown to be effective opsonins in vivo by their ability to enhance blood clearance of intravenously injected *Streptococcus pneumoniae* organisms in mice.^{5,6}

Our studies have focused on CRP's macrophage-activating property. We demonstrated recently that CRP, when administered in liposomes, activates macrophages to generate tumoricidal activity both in vivo^{7,8} and in vitro.⁹ We suspected that CRP's induction of macrophage-mediated tumoricidal activity might require participation of the complement system. In this study, using the Winn neutralization assay, we evaluated the tumoricidal activity of CRP-treated peritoneal exudate cells (PECs) from animals depleted of complement by cobra venom factor (CoVF). The activity of PECs from CRP-MLV-treated donors was also compared with the activity of another known macrophage-activating agent, MTP-MLV. CoVF pretreatment abolished the tumor-inhibitory effect of CRP-activated PECs, suggesting that the complement system is necessary for encapsulated CRP to activate macrophages.

Materials and methods

Animals: Inbred C57BL/6J mice, 10 to 12 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME).

Tumor: Lewis T241 fibrosarcoma was originally induced by dibenzanthracene and has been maintained in our laboratory as previously described.⁷ This tumor was found to be free of infectious murine leukemia virus and 12 other common viruses (Microbiological Associates, Inc., Bethesda, MD).

Preparation of liposomes: Multilamellar vesicles (MLV) were prepared from phosphatidylcholine (PC) and phosphatidylserine (PS) (Avanti Biochemicals, Inc., Birmingham, AL) and CRP, encapsulated as previously described.⁷ We determined the efficiency of protein encapsulation by encapsulating ¹²⁵I-CRP, radiolabeled by Bolton and Hunter's method. The final preparation contained 0.7 μ g CRP per 2.5 μ m phospholipid per 0.1 mL phosphate buffer saline (0.01 M phosphate in 0.15 M NaCl₂, pH 7.4). We prepared liposomes incorporating MTP-PE (CGP 19835; Ciba Geigy, Ltd., Basel, Switzerland) by evapo-

rating 5 μ g MTP-PE per μ mol phospholipid and dispersing the dry film in phosphate buffer saline.⁹

C-reactive protein (CRP): CRP was purified to homogeneity from serous fluids as previously described,^{7,11} and the purity of the final preparation was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The endotoxin level was assayed by the limulus amoebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, MA) and was less than 0.01 ng per μ g CRP.

Consumption of human and mouse complement by CRP-MLV: Liposomes (250 nmol phospholipid) were incubated for 60 min at 37°C with 400 μ L gelatin-veronal buffer with divalent cations (GVB⁺⁺, Cordis Laboratories, Miami, FL, concentration 0.145 M NaCl, 0.00015 M CaCl₂, 0.0005 M MgCl₂, 0.1% gelatin, 0.0031 M barbituric acid, 0.0018 M sodium barbital, pH range 7.3 to 7.6, 0.15 M relative salt concentration) and 100 μ L human complement (Cordis Laboratories, Miami, FL), mouse complement (Cappel Laboratories, Cochranville, PA), or freshly obtained mouse serum from C57BL/6J mice. After incubation, the tubes were centrifuged at 10,000 \times g for 15 min in a refrigerated centrifuge. The supernatant was decanted into tubes held in an ice bath, and the residual complement in the supernatants was measured by the total hemolytic complement assay (CH₅₀) according to Lachman and Hobart's method.¹² In brief, 100 μ L from each supernatant concentration was incubated for 60 min at 37°C with 200 μ L of EA (sheep erythrocytes coated with antibody at a dilution previously determined to be optimal). The degree of hemolysis was determined spectrophotometrically (412 nm), and CH₅₀ units were calculated.¹² The percent consumption was calculated using complement incubated with buffer alone as the control.

Cobra venom factor (CoVF) treatment: CoVF (from *Naja naja kaouthia*) was purchased from Cordis Laboratories (Miami, FL). The mice were given a series of three I.V. injections of 2 units each over a two-day period, according to the method described by Nakayama et al.¹³ The first injection was early A.M., the second was late P.M., and the third injection was early A.M. the following day. Three hours after the third (last) CoVF injection, the mice received CRP-MLV, MTP-MLV, MLV alone, or saline in different groups. Sera were collected from CoVF-treated mice and

were tested in the CH₅₀ assay. Hemolytic activity was markedly diminished in CoVF-treated mice compared with the control mice ($P < 0.001$).

CoVF treatment and MLV treatment: Normal C57BL/6J mice were injected intraperitoneally (I.P.) with 1 mL thioglycolate broth. These mice were then divided into 7 groups. Three groups of mice were depleted of complement by three doses of CoVF I.V. (2 units/mouse) injected over a two-day period as described earlier. Three hours after the last CoVF injection, the three groups received saline alone, CRP-MLV, or MTP-MLV (0.7 µg/2.5 µmol lipid in 0.5 mL PBS), respectively. The four groups of mice that received thioglycolate alone, but no CoVF, were injected with CRP-MLV, MTP-MLV, saline alone, or MLV alone.

Preparation of PECs: Twenty-four hours after injections of liposomal reagents, PECs were harvested from mice in all groups. PECs were collected in phenol red-, calcium-, and magnesium-free Hawk's balanced salt solution. The cells were washed twice, suspended in trypan blue, counted, and used in the Winn neutralization assay to study their tumor inhibitory ability. Differential cell counts were also made on Wright Giemsa-stained cytopsin preparations and nonspecific esterase staining was also done to determine the macrophage concentration. Typically, PECs from donors treated with liposomal reagents consisted of 75% macrophages, 13% granulocytes, and 12% lymphocytes.

Winn neutralization assay: This assay was performed as previously described.¹⁴ Briefly, PECs from C57BL/6J mice that had been injected I.P. with thioglycolate and with MLV reagents 24 hours earlier were mixed with the T241 tumor cells in a ratio of 10:1, centrifuged at 150 × g, and incubated for 30 min at 37° C. The cells (10⁵ tumor cells) were injected in a volume of 0.05 mL per footpad into normal syngeneic mice. Controls received tumor cells alone. When tumors first appeared was noted, and tumor growth was measured at regular intervals. On day 55 the mice were killed, and the feet bearing primary tumors were amputated and weighed. The net tumor weights were calculated by subtracting the weight of feet without tumors amputated at the same location. The lungs were removed, and the metastatic index was calculated as previously described.⁷ All experiments were repeated at least twice or as otherwise indicated.

Table 1. Consumption of human complement by CRP-MLV

Group	CH ₅₀ units/mL	% Consumption
Human complement* + buffer	22.74†	—
Human complement + MLV	16.79	27.2
Human complement + CRP-MLV	1.97	91.6
Human complement + Zymosan	7.98	65

* Cordis human whole complement.

† Human complement was incubated with buffer, phospholipids, or Zymosan for 60 min at 37° C, centrifuged, and the supernatants were used in CH₅₀ assay.

Statistical analyses: Group results were compared in terms of both mean metastatic index by using the Student's t-test and the median number of metastases per mouse using the Mann Whitney U-test.

Results

Consumption by human and murine complement by CRP-MLV

The ability of human CRP associated with liposomes (MLV) to consume hemolytic human complement was confirmed in this study (Table 1). The capacity of human CRP-MLV to consume mouse complement was also studied (Table 2): CRP-MLV or MLV alone was incubated with mouse complement for different intervals. The supernatants were then tested in the CH₅₀ assay for remaining complement activity using the standard EA used by the clinical laboratory for CH₅₀ assays. CRP-MLV, when incubated with human complement, consumed 91.6% of the available complement (Table 1). In contrast, MLV alone incubated with human complement consumed only 27.2%, and Zymosan, a positive control, consumed 65%. As shown in Table 2, essentially the same pattern of consumption was seen with mouse complement, which was very

Table 2. Consumption of murine complement by CRP-MLV

Group	CH ₅₀ units/mL† Mean ± SEM	% Consumption
Mouse complement* + buffer	16.48 ± 1.19‡	—
Mouse complement + MLV	18.01 ± 4.19	0
Mouse complement + CRP-MLV	1.72 ± 0.28§	90.58 ± 2.63§

* Cappel mouse complement.

† Results represent mean of five different experiments.

‡ Mouse complement was incubated with buffer or phospholipids for 60 min at 37° C, centrifuged, and the supernatants were used in the CH₅₀ assay.

§ $P < 0.001$ compared to group 1 and 2, as determined by t test.

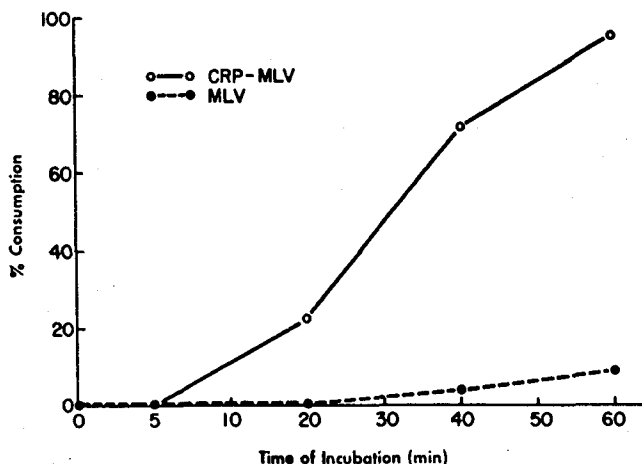


Fig. 1. 250 nmol of phospholipid was incubated with 400 μ L of gelatin veronal buffer with divalent cations (GVB⁺⁺) and 100 μ L of mouse complement for different times. The supernatants were then tested in CH₅₀ assay for remaining complement activity.

efficiently consumed by human CRP-MLV (Fig. 1) in the absence of human C1q, with the optimum incubation time of 60 min. This incubation time was used in all subsequent experiments. These results indicate that murine complement as well as human complement is consumed by human CRP encapsulated in liposomes.

Participation of complement in CRP-MLV-induced tumor inhibition

In an earlier study we showed that CRP-MLV can activate macrophages,⁹ which inhibited tumor growth and pulmonary metastasis of the T241 tumor in the Winn neutralization assay. In this study we have used the Winn assay to determine whether CRP-MLV alone could activate macrophages to be tumoricidal or whether complement was necessary. Mice were first treated I.P. with thioglycolate to induce PEC, then

treated with CoVF to deplete complement, and finally treated with CRP-MLV or MTP-MLV to activate the macrophages. The PECs harvested from these mice were then incubated with tumor cells (T241), and the mixture was transferred to normal syngeneic mice.

The antitumor effect of CRP-MLV-treated macrophages from mice depleted of complement was compared to that of similarly treated macrophages from mice whose complement system was intact (Table 3). The primary tumor weights and the extent of lung metastases were significantly reduced ($P < 0.01$) in animals treated with tumor cells mixed with PEC from CRP-MLV- or MTP-MLV-treated donors compared with the controls. This tumor inhibitory effect of CRP-MLV-induced macrophages was lost in mice depleted of complement. Complement depletion, however, had no effect on the antitumor activity of MTP-MLV-induced macrophages.

Discussion

Our earlier study showed that murine PECs phagocytize CRP-MLV and subsequently release superoxide anion and demonstrate antitumor activity.⁹ This was in the absence of exogenously added complement, but since most of the complement components can be synthesized by macrophages,¹⁵ the PECs (which were stimulated by thioglycolate) may have locally produced the complement needed to effectively opsonize the CRP-MLV. In a subsequent study, we demonstrated that complement was indeed involved in the CRP-MLV-induced, macrophage-mediated tumoricidal activity.¹⁶

As such, CRP joins a relatively small but diverse group of agents that have been shown to similarly activate macrophages to antitumor activity.¹⁷⁻¹⁹ The present study further differen-

Table 3. Comparison of tumor sizes, tumor weights, and pulmonary metastases in different groups of mice receiving T241 tumor cells mixed with PEC in the Winn assay*

Treatment of PEC donors	Tumor size Mean \pm SEM	Tumor weight Mean \pm SEM	Metastatic index Mean \pm SEM	Median no. Metastases/mouse
No PEC, tumor only	2.3 \pm 1.1	361.0 \pm 224.5	54.0 \pm 20.0	18.5
Buffer:MLV	3.0 \pm 1.5	482.0 \pm 347.0	46.2 \pm 18.6	12.0
Saline:CoVF	2.9 \pm 1.3	381.4 \pm 234.9	44.8 \pm 18.0	15.0
CRP:MLV	0.3 \pm 0.1†	21.0 \pm 18.9†	4.5 \pm 3.5†	0§
MTP:MLV	0.3 \pm 0.2†	27.8 \pm 17.8†	12.2 \pm 6.6‡	0§
CoVF-CRP:MLV	3.0 \pm 1.0	341.0 \pm 152.5	35.3 \pm 14.2	12.5
CoVF-MTP:MLV	0.5 \pm 0.2†	55.5 \pm 42.3‡	9.6 \pm 5.2‡	0§

* Values represent mean data for 10 mice in each group.

† $P < 0.01$ as determined by Student's t-test compared to control group 1.

‡ $P < 0.025$ as determined by Student's t-test compared to control group 1.

§ $P < 0.01$ as determined by Mann-Whitney U-test compared to control group 1.

tiates between their mechanisms of action: CRP-MLV activation is complement-dependent, whereas MTP-MLV is not affected by the depletion of active complement.

Other studies have shown that, although complexed CRP could bind to mononuclear phagocytes,²⁰ both CRP and C3b were required to promote ingestion of the opsonized particles.^{3,4} In vivo studies demonstrate that CRP and complement-coated erythrocytes¹³ and bacteria²¹ are preferentially cleared by macrophages, which subsequently migrate to the liver. Macrophages activated by CRP and complement demonstrate bactericidal activity, providing protection from infection.⁵

The present study confirms Kaplan and Volanakis's findings that, in its complexed form, human CRP activates human complement.¹ They reported that consumption of guinea pig complement by CRP complexes required participation of human C1q, indicating that these complexes do not interact with guinea pig C1q.²² This study shows that human CRP encapsulated in liposomes can activate murine complement in the absence of human C1q. We also present evidence that CRP liposomes in the presence of complement can activate peritoneal-exudate macrophages' tumoricidal activity. Thus, we have added yet another perspective to the continuing investigations into the biologic role of CRP. C-reactive protein may have potential value in cancer therapy as an important biological response modifier.

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