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NEUTROPHIL CHEMOTACTIC ACTIVITY AND C5a FOLLOWING SYSTEMIC ACTIVATION OF COMPLEMENT IN RATS

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Abstract—Using ELISA analysis, rat C5a was stimulated in serum from rats undergoing systemic activation of complement after intravenous infusion of purified cobra venom factor (CVF). Biological (neutrophil chemotactic) activity was also assessed. Serum levels of C5a were directly proportional to the amount of CVF infused. C5a and neutrophil chemotactic activity, peaked by 5 min, then plateaued. In vitro addition of anti-C5a to serum samples of CVF-infused rats totally abolished chemotactic activity, indicating that all biological activity could be ascribed to C5a. Blood neutrophils obtained from CVF-infused animals showed a significant upregulation of CD11b, the increase being reduced (38%) in animals pretreated with anti-C5a. These findings indicate that infusion of CVF into rats produces generation of C5a, all chemotactic activity in serum being related to C5a. The in vivo generation of C5a is, at least inpart, responsible for upregulation of CD11b on blood neutrophils.

INTRODUCTION

Cobra venom factor (CVF) is one of several components found in cobra venom from Naja naja. CVF has properties similar to the activation product of the third component (C3) of human complement, C3b (1–6). CVF binds in the presence of Mg^{++} to factor B, forming a complex (CVF · B). This complex is cleaved into CVF · Bb and Ba by factor D. CVF · Bb acts as a C3 and C5 convertase and is very stable, with a serum half-life of 7 hr at 37°C, as compared to

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C3b · Bb, which dissociates with a half-life of 1.5 min (7). This implies that injection of sufficient doses of CVF into animals leads to extensive complement activation, resulting in complement-depletion (8). In the course of the complement activation cascade, C3 and C5-convertase activity leads to formation of the anaphylatoxins, C3a and C5a (7,9). C5a, a 14 kDa glycoprotein has been known to have potent proinflammatory functions that may be important in leukocyte recruitment (7–11).

Intravascular generation of C5a by infusion of CVF produces neutrophil-dependent lung vascular injury by activating neutrophils, causing accumulation of neutrophils in the lung vasculature and leading to neutropenia, followed by granulocytosis and increasing lung vascular permeability with injury of endothelial cells, intraalveolar hemorrhage, fibrin deposition and interstitial edema (8,12). Recently it has been shown that neutralization of C5a in the CVF model of acute lung injury with anti-C5a IgG could reduce secondary lung injury by 95% and reduce lung MPO-content by 39% in the rat, indicating that C5a plays a key role in neutrophil activation and secondary lung injury (11). Prior to this report, no technology was available for direct measurement of rat C5a after in vitro or in vivo activation of complement in rats. In the present study we measured C5a levels in rat serum as well as serum-chemotactic activity after complement activation by CVF. The data provide quantitative assessments of serum levels of C5a as well as amounts of neutrophil chemotactic activity. They also indicate that CVF-induced upregulation of CD11b on neutrophils is, at least in part, C5a-dependent.

MATERIALS AND METHODS

Reagents. Unless otherwise specified, all reagents were purchased from Sigma Co. St. Louis, Missouri).

Anti-C5a. Rabbits were repeatedly immunized with purified rat C5a to obtain a polyclonal IgG antibody (7). The antibody was shown in recent studies (11) to block neutrophil chemotactic activity in activated rat serum as well as chemotactic activity associated with purified rat C5a but not to block total complement activity (CH50) in whole rate serum (11). The IgG fraction was purified over a 1.5 × 12 cm chromatography column (Bio Rad Laboratories, Hercules, California) made with 2.5 ml of protein G. The column was extensively washed with DPBS pH 7.5, the serum diluted 1:2 with DPBS and applied in 4 ml portions. The column was drained and washed extensively until absorbance readings at 280 nm were below an optical density of 0.02. The IgG fraction was eluted with 0.1 M glycine pH 2.5 into tubes containing 1 M tris pH 8.8. The eluates were dialyzed four times against DPBS (pH 7.5) and finally concentrated using Centricon 100 devices (Amicon Inc., Beverly, Massachusetts) to final concentrations of 5 mg/ml. Reactivity of the purified antibody with purified C5a was shown in Western blot analysis (11).

Placement of Intra-arterial Catheters and Animal Model of CVF-Induced Lung Injury. Male Long Evans rats (275–300 g, Charles River Laboratories, Portage, Michigan) were anesthetized with ketamine hydrochloride (80 mg/kg, Parke Davis and Co., Morris Plains, New Jersey) and

Xylazine hydrochloride (8 mg/kg, Lloyd Laboratories, Shenandoah, Iowa). Intra-arterial carotid PE-50 catheters (Becton-Dickinson Co., Sparks, Maryland) were placed 24 hr prior to the CVF-experiment by a longitudinal incision over the anterior cervical area, separating the left carotid artery underneath the sternocleidomastoideus muscle from the vagal nerve, followed by temporary ligature of the distal carotid artery and placement and fixation of the catheter in the artery. The catheters were then threaded subcutaneously into the neck, externalized, rolled up and fixed with a tape in order to allow the animals to move freely. The animals were allowed to eat and drink and were used for the CVF experiment on the following day. Cobra venom factor was isolated from lyophilized *Naja naja* venom (ALI corporation, Ann Arbor, Michigan) by ion exchange and gel filtration chromatography (1,8). The animals were anesthetized as described above. Four hundred μl blood were drawn on ice at following time-points: before CVF injection, 1, 2, 5, 10, 15, 20, 25 and 30 min after intravenous CVF injection. After 30 min the animals were sacrificed. Serum was separated and frozen at −80°C.

C5a ELISA. Microtitration plates (Immulon 4, Dynatech Laboratories, Inc., Chantilly, Virginia) were coated with anti-rat C5a at a concentration of 5 μg/ml, incubated overnight and washed three times thereafter. During all subsequent procedural steps, incubation time was 1 hr at 37°C followed by washing of the plate with PBS containing 0.05% Tween-20. Blocking of non-specific binding sites was obtained with 1% milk. Seventy-five μl of the serum samples diluted 1:100 to 1:200 in dilution buffer containing milk as well as a rat C5a standard, with concentrations ranging from 0.049 ng/ml to 100 ng/ml. Ninety μl of biotinylated anti-C5a was used at a concentration of 500 ng/ml and 100 μl of a 1:3000 dilution of streptavidin horseradish peroxidase (Zymed, S. San Francisco, California). Finally, 200 μl peroxidase substrate (o-phenylendiamin hydrochloride) was added. Color development was stopped by adding 3 M H_2SO_4 and the absorbance read at 490 nm using a plate reader.

Neutrophil Chemotaxis Assay. Blood was drawn from normal humans into vials containing citrate [Anticoagulant Citrate Dextrose Solution USP (ACD) Formula A, Baxter Corp., Deerfield, Illinois]. Neutrophils were isolated by Ficoll and Dextran sedimentation (Pharmacia Biotech, ABN, Uppsala, Sweden). Remaining red blood cells were lysed with water. The neutrophils were washed several times with DPBS (with Ca++ and Mg++) containing 0.1% BSA (endotoxin free) and fluorescein-labeled with BCECF-AM (Molecular Probes, Inc., Eugene, Oregon). The cells were washed again and resuspended in Hank's balanced salt solution with 0.1% endotoxin free BSA to a final concentration of 5×10^6 cells/ml. The bottom part of the chemotaxis chamber (Neuro Probe Inc., Cabin John, Maryland) was loaded with 30 μl of the 1:100 serum dilutions in HBSS per well. Formyl-Met-Leu-Phe (FMLP) at concentrations of 10^{-3} to 10^{-9} M was used as a standard chemoattractant. 44 μl of the cell suspension were applied to each well of the top part of the chamber to which a polycarbonate filter with a pore size of 3 μm was attached. The chamber was incubated 30 min at 37°C. Fluorescence was measured in a cytofluorometer (Cytofluor Model 2300, Millipore Corp., Bedford, Massachusetts).

Flow Cytometric Analysis of Neutrophils. Neutrophils (1×10^5), isolated from rat blood, were stained using 5 μg/ml monoclonal antibody to CD11b (1B6c) or an isotope-matched control, (MOPC-21) followed by fluorescence-conjugated goat anti-mouse IgG antibodies (AMAC, Westbrook, Maine) and subsequent fixation in 2% paraformaldehyde solution (13). Neutrophil populations (identified by forward versus right-angle light scatter characteristics) were measured on an FACScan (Becton Dickinson, San Jose, California) in which 10,000 cells per gated determination were counted. Extent of fluorescence intensity (mean channel fluorescence) was analyzed using PC-Lysis software (Becton-Dickinson, San Jose, California).

Statistical Analysis. Data were expressed as mean plus/minus the standard error of the mean (*SEM*). A paired *t*-test was employed to compare the response between two treatments. Two-way analysis of variance in combination with Dunnett's multiple comparisons test was used to determine the significance of differences between controls and multiple experimental groups. Statistical signif-

icance was defined as $p < 0.05$. Specifics regarding details of experiments are contained in the text or in the figure legends.

RESULTS

Relationship Between CVF Amounts and Serum C5a Levels. In rats, receiving intravenous injections of 2, 4, 6 or 8 units of CVF (or saline in the negative group), C5a levels were measured at 5 min. There was a clear dose-response relationship between amounts of CVF infused and C5a generation, with a plateau appearing at 6–8 units CVF (Figure 1). The C5a values for the saline-infused rats and rats infused with 2, 4, 6 or 8 units of CVF were: 4 ± 1, 531 ± 83, 835 ± 45, 1548 ± 236 and 1203 ± 242 ng C5a/, respectively.

In Vivo Suppression of Serum Activities by Anti-C5a. Serum samples were obtained from rats at times 0, 1, 2, 5, 10, 15, 20, 25 and 30 min after intravenous infusion of 4 units CVF. Rats were either pretreated intravenously with 300 μg normal rabbit IgG or anti-rat C5a. C5a content (as determined by ELISA) and neutrophil chemotactic activity were measured. The data are shown in Figure 2. C5a content in serum peaked within the first 2 min, plateauing through the 10 min interval, and then declining (Figure 2A). Chemotactic activity reached a peak within the first 10 min, followed by a slight decline thereafter (Figure 2B). As will be shown in this and in Figure 3, precise profiles of serum con-

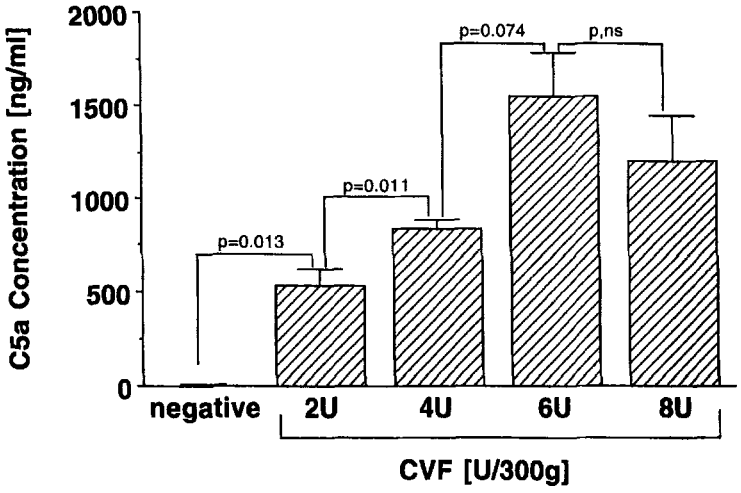

Fig. 1. Serum levels of C5a, as assessed by ELISA, as a function of CVF dose infused into rats. For each data point, $N = 3$.

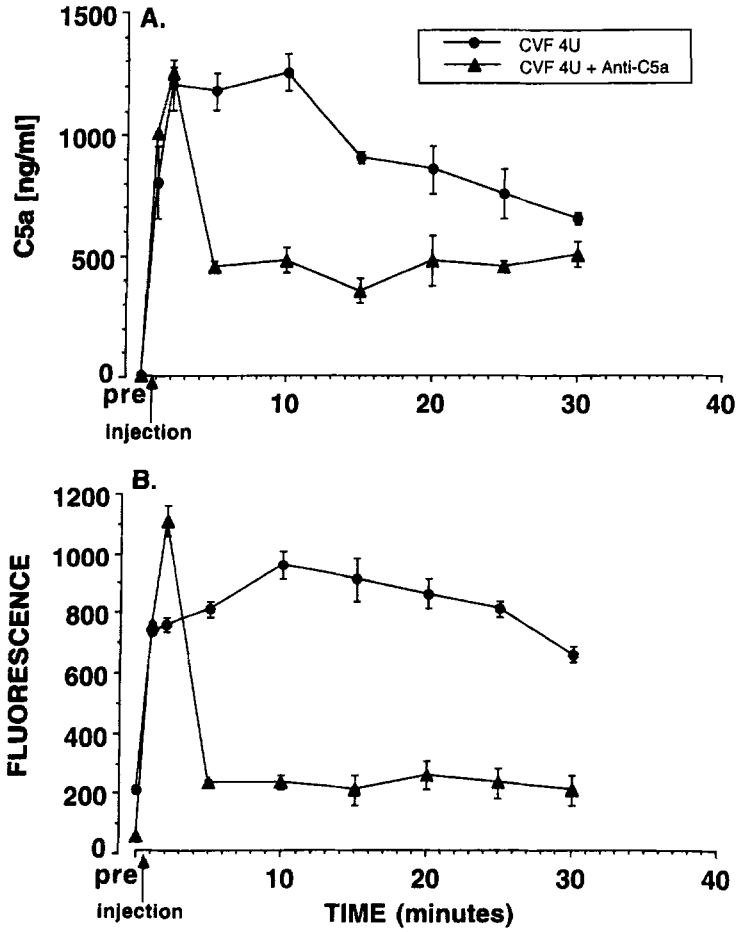

Fig. 2. Time-course for C5a serum levels (frame A) and serum chemotactic activity (frame B) following injection of 4 units of CVF into rats pretreated with 300 μg normal rabbit IgG or 300 μg anti-rat C5a. Serum samples were obtained at 5 min after injection of CVF. For each time point, $N = 3$.

tent of C5a and chemotactic activity vary slightly from experimental group to experimental group, but the overall profiles are quite similar. When animals were pretreated intravenously with 300 μg anti-C5a, the subsequent injection of CVF resulted in no detectable decline in C5a or chemotactic activity in serum during the first 1–2 min, but, thereafter there were significant reductions in both C5a and in chemotactic activity in serum from animals treated with anti-C5a (Figure 2, A, B).

In Vitro Blocking Effects of Anti-C5a on Chemotactic Activity. Five sets of animals ($N = 4$ for each group) were evaluated for effects on C5a and on chemotactic activity following in vitro addition of anti-C5a to serum samples obtained at various time points after infusion in vivo of CVF. The data are shown in Figure 3. The prompt appearance (within 2 min) and plateauing (between 5 and 30 min) of C5a (frame A) and chemotactic activity (frame B) in serum samples was documented. Addition of 50 μg anti-rat C5a rabbit IgG (to 1.0 ml serum) resulted in complete abolition of chemotactic activity (frame B), indicating that all chemotactic activity could be ascribed to C5a.

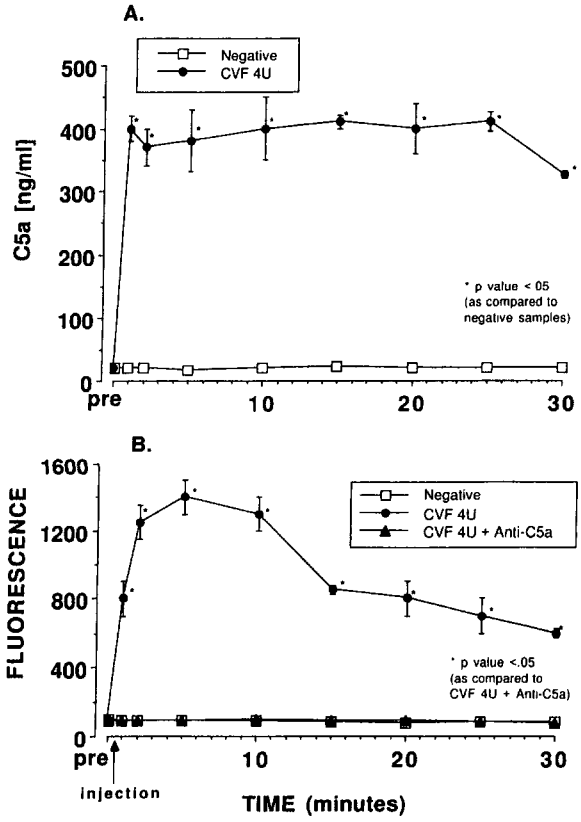

Fig. 3. Time course in appearance of C5a (frame A) and neutrophil chemotactic activity (frame B) in serum of rats injected with 4 units of CVF. Samples were obtained via indwelling arterial catheters. Both antigenic and biological activities in serum could be totally suppressed by in vitro addition to serum of anti-C5a (50 μg/ml). For each measurement, $N = 3$.

Table 1. Surface Expression of Neutrophil CD11b After Injection of CVF[a]

	Time of Analysis (min)				
Treatment of Animals	0	15		30	
Saline	283 ± 11.5	218 ± 23.8		201 ± 29.0	
		0.02[b]		0.004	
CVF (4 units)	160 ± 14.6	486 ± 128	*N.S.*	691 ± 134	<0.05
		N.S.		0.03	
CVF + anti-C5a (300 μg)	255 ± 27.1	331 ± 56.0		421 ± 85.9	

[a]Data reported as mean channel fluorescence
[b]*P* values, comparing groups as indicated in table

Flow Cytometric Analysis of CD11b in Blood Neutrophils. Blood samples were obtained from rats receiving 4 units CVF intravenously. The neutrophil content of CD11b was evaluated on the basis of flow cytometric analysis at various time points after infusion of CVF. The results are shown in Table 1. Early time points (e.g. 1–10 min) were not analyzed because of the profound neutropenia occurring in animals infused with CVF, making retrieval of adequate numbers of blood neutrophils for reliable analysis difficult. At 15 min, neutrophil content of CD11b was approximately doubled ($P = 0.02$) in CVF-infused rats; anti-C5a treatment of rats caused a modest reduction (approximately 31%) in CD11b content, but this did not reach statistical significance. At 30 min, CD11b content on blood neutrophils was increased by 3.4 fold and, in the in vivo presence of anti-C5a, the level of CD11b fell by approximately 38% ($p = 0.03$) (Table 1). Accordingly, upregulation of CD11b on blood neutrophils after intravenous infusion of CVF can, at least in part, be attributed to C5a. To what extent other factors, such as C5b-9 may be involved, remains to be determined. It should also be noted that probably an even more pronounced upregulation of CD11b might occur on neutrophils that have lodged in the lung microvasculature.

DISCUSSION

Activation of the alternative pathway of complement activation is considered to be an effective defense mechanism against invading microorganisms (6). However, excessive activation may lead to harmful amounts of two potent acti-

vation products, C5a and the membrane attack complex, C5b-9. CVF has long been known to be a potent complement activator that leads to extensive in vivo activation of complement. To our knowledge, no data on levels of C5a generated in vivo are available. CVF is a 140 kDa protein consisting of 3 subunits A, B and C, with molecular weights of approximately 72, 54 and 27–35 kDa, respectively (2). In earlier studies, C5a levels could only be estimated indirectly by measuring neutrophil chemotactic activity in serum from CVF-treated animals (8). The precise contribution to chemotactic activity by C5a was undetermined. By ELISA technique, we have been able to measure C5a presence directly in serum. In the current experiments, the appearance of serum chemotactic activity was parallel to the C5a concentration in serum, leading to the conclusion that, at least within the first thirty minutes after complement activation, C5a is probably the only relevant neutrophil chemoattractant generated. It seems likely that, in CVF-induced lung injury, which is neutrophil-dependent (8), C5a is a key mediator that initiates the chain of events leading to lung vascular injury. One possible mechanism leading to injury might be through the interaction of C5a with lung vascular endothelial cells, causing P-selectin (but not ICAM-1) upregulation (14). In this model of injury, endothelial P-selectin is required for adhesion of neutrophils and subsequent damage to the vascular endothelium produced by oxidants generated from activated neutrophils (8,12,15). In vitro, it has been shown that C5a can induce human umbilical vein endothelial cells to express P-selectin (16). It seems likely that neutrophils form intravascular aggregates, which are induced by C5a. Such events would also cause increased expression of Mac-1 (CD11b/CD18) on the surface of neutrophils as well as oxidant production. The ability of anti-C5a to attenuate, at least in part, these reactions seems likely to be linked to these activation steps. Why only partial reduction by anti-C5a in upregulation of CD11b was found, remains to be explained. The lack of complete suppression might be related to massive levels of C5a generated by infusion of CVF. It is also possible that additional products of the complement system, such as C5b-9, may contribute to upregulation of neutrophil CD11b. This possibility remains to be determined.

The inability of anti-C5a to abolish the rapid increase in C5a within the first two min after CVF injection (Figure 3) is likely due to the overwhelming production of C5a immediately after infusion of CVF. The ability of anti-C5a to suppress completely the presence of chemotactic activity in serum from CVF-infused rats indicates that this activity can be completely attributed to the presence of C5a.

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