Kinetics of C5a Release in Cardiac Lymph of Dogs Experiencing Coronary Artery Ischemia–Reperfusion Injury

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Previous studies of myocardial ischemia suggest that complement activation may play a central role in the inflammatory response during reperfusion. Our previous work has demonstrated neutrophil chemotactic activity to be present in reperfusion canine cardiac lymph after myocardial ischemia and infarction. To evaluate the contribution of the complement-dependent anaphylatoxin C5a to this neutrophil chemotactic activity, rabbit antiserum to canine C5a was prepared. At dilutions >1:500 but <1:2,000, the antiserum abolished the ability of zymosan-activated dog serum to induce a ruffled, bipolar morphology in isolated neutrophils used as a biosay of chemotactic stimulation. This antiserum did not affect similar morphological changes in neutrophils exposed to platelet activating factor (10^{-7}–10^{-4} M) or recombinant human interleukin-8 (10^{-9}–10^{-8} M); thus, we deemed it functionally specific for canine C5a. In a pattern similar to what we previously reported, cardiac lymph collected before a 1-hour ligation of the left circumflex coronary artery had little ability to alter the morphology of canine neutrophils (shape change index, 11.3±4.6, mean±SEM; n=7), but by 1 hour of reperfusion, lymph activated neutrophils significantly in five of seven dogs (mean shape change index, 72.6±17.7; p<0.01). At 2 hours of reperfusion, neutrophil activation by lymph occurred in six of seven dogs (mean shape change index, 103.1±22.2). At 3 hours of reperfusion, cardiac lymph of only three of six dogs caused neutrophil activation, and at 4 hours of reperfusion, this activity was evident in lymph from only two of five dogs. In all cases, however, the neutrophil stimulatory activity of cardiac lymph was inhibited 85–90% by addition of anti-C5a (p<0.01). Preimmunization serum had no effect. Thus, these data indicate that C5a is the predominant chemotactic factor in the first 4 hours after reperfusion of ischemic myocardium in the dog.

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KEY WORDS • neutrophils • complement • chemotaxis • myocardial infarction • reperfusion injury

Local chemotactic factor generation within the ischemic and infarcted myocardium is thought to be one mechanism by which the inflammatory response is enhanced during reperfusion.1–3 In a previous study, we demonstrated neutrophil chemotactic activity to be present in reperfusion canine cardiac lymph from the ischemic myocardium after a 1-hour coronary artery occlusion.4 In our previous study, cardiac lymph collected during reperfusion demonstrated the ability to activate the following proinflammatory functions in neutrophils isolated from the blood of healthy donor dogs: 1) morphological changes (shape change) characteristic of neutrophils exposed to chemotactic factor stimulation, 2) orientation of neutrophils in a gradient of cardiac lymph, 3) increased surface expression of CD11b and CD18 adherence glycoproteins, and 4) increased adherence of canine neutrophils to monolayers of canine jugular vein endothelium. Shape change–inducing activity was evaluated at more frequent intervals than other functions and was found to peak at 1–2 hours after initiation of reperfusion and to disappear at 4–6 hours.4 Chemotactic activity was present only in dogs that demonstrated histochemical evidence of myocardial infarction. Dogs that underwent occlusion but because of collateral blood flow had no evidence of infarction also failed to demonstrate chemotactic activity in cardiac lymph. On the basis of Rossen and colleagues'5,6 observation that dogs with myocardial infarction release proteins of mitochondrial origin into cardiac lymph capable of binding C1q, we postulated that the chemotactic activity in cardiac lymph may be a...
result of complement activation and the release of the complement-dependent anaphylatoxin C5a.

Although studies from our laboratories, as well as those of others, lend strong support to the concept that complement activation is responsible for the chemotactic activation of neutrophils during myocardial ischemia, other neutrophil chemotactic factors have been implicated in this setting as well. These include lipid-derived chemotactic factors such as leukotriene B4 or platelet activating factor (PAF), as well as interleukin-8 (IL-8) released from activated endothelium.

It was the purpose of this study, therefore, to develop a monospecific antiserum capable of recognizing and inhibiting the chemotactic activity generated by canine C5a to examine to what extent complement activation and the corresponding release of C5a may be responsible for the chemotactic activity previously demonstrated in postischemic reperfusion cardiac lymph.

**Materials and Methods**

**Purification of C5a**

Canine C5a was isolated by a modification of the methods of Hugli et al. Five liters of fresh frozen dog EDTA plasma was clotted by adding CaCl2 to a final concentration of 10 μM. After clarification at 10,000g for 30 minutes, Plummer’s inhibitor (DL-2-mercaptoethyl-3-guanidinoethyl-thiopropionic acid) was added to a final concentration of 23.7 mg/100 ml serum. After 30 minutes at 37°C, 1 M MgCl2 was slowly added to a final concentration of 3 mM. Cobra venom factor (Naja naja siamensis, Miami Serpentarium) was then added (1 mg/50 ml serum), and the mixture was incubated at 37°C for 2.5 hours. The serum was then cooled to 0°C, and irrelevant proteins were removed by adding 12N HCl to a final concentration of 1N. The precipitated proteins were removed by centrifugation at 10,000g for 30 minutes, and the remaining supernatant was adjusted to pH 5.0 with ammonium hydroxide and dialyzed against 0.1 M ammonium formate, pH 5.0. The supernatant proteins were adsorbed onto Sephadex SP-C25 with a 2.5 x 15-cm column preequilibrated with 0.1 M ammonium formate, pH 5.0. The column was washed with 100 ml of 0.1 M ammonium formate, pH 7.0, and then C5a, C4a, and C3a were desorbed with a gradient consisting of 200 ml 0.1 M ammonium formate and 200 ml of 1 M ammonium formate at a flow rate of 2 ml/min. Fractions containing C5a-like activity were identified by the neutrophil "shape change" bioassay described below. Fractions causing peak shape change activity were isolated between 1.0 and 2.0 M ammonium formate at pH 7.0. Active fractions were pooled, concentrated by lyophilization, and further fractionated by gel permeation chromatography on Sephadex G-75 in 50 mM ammonium formate.

**Preparation of Anti-C5a Antiserum**

Rabbits were immunized by multiple intradermal injections of approximately 100 ng protein homogenized in complete Freund’s adjuvant. Booster injections homogenized in incomplete Freund’s adjuvant were given at 3-week intervals. After the second booster injection, serum was obtained that, when diluted >1:500, could neutralize the ability of zymosan-treated serum to cause bipolar shape change of neutrophils.

Antiserum that, when diluted >1:500 in Dulbecco’s phosphate buffered saline (PBS), inhibited the shape change–inducing activity of zymosan-activated dog serum (ZADS) were pooled and tested for their ability to react with proteins in fresh canine serum and with the putative isolated canine C5a after both had been fractionated under reducing conditions on 15% polyacrylamide gel and blotted to nylon membranes. Lanes A and B are identified as follows: lane A, protein identified by incubation with 1:500 anti-C5a, enzyme conjugated anti-immunoglobulin G, and appropriate substrate; lane B, protein identified by Coomassie blue. In lanes C and D, canine serum was fractionated on polyacrylamide and transferred to nylon as in lanes A and B. Lanes C and D are identified as follows: lane C, transferred proteins identified by Coomassie blue; lane D, proteins identified by incubation with 1:500 rabbit anti-C5a, enzyme conjugated anti-immunoglobulin G, and substrate.

![Figure 1. Immunochemical studies of rabbit antiserum to canine C5a. In lanes A and B, 22 μg isolated C5a containing peak neutrophil shape change–inducing activity was separated under reducing conditions on 15% polyacrylamide and blotted to nylon membranes. Lanes A and B are identified as follows: lane A, protein identified by incubation with 1:500 anti-C5a, enzyme conjugated anti-immunoglobulin G, and appropriate substrate; lane B, protein identified by Coomassie blue. In lanes C and D, canine serum was fractionated on polyacrylamide and transferred to nylon as in lanes A and B. Lanes C and D are identified as follows: lane C, transferred proteins identified by Coomassie blue; lane D, proteins identified by incubation with 1:500 rabbit anti-C5a, enzyme conjugated anti-immunoglobulin G, and substrate.](http://circres.ahajournals.org/doi/fig/10.1161/01.RES.81.5.1519)
ular weight of the 110-kd protein identified in the fractionated canine serum by these rabbit antibodies is consistent with published molecular weights of the C5 α-chain in humans, guinea pigs, and trout.19–21

Surgical Instrumentation of the Animal Model

Details of the surgical preparation of this model have been previously described.4,22 Briefly, anesthetized, anesthetized and ventilated animals underwent midline thoracotomy to provide access to the heart and mediastinum. Cardiac lymphatic vessel definition was obtained by injecting Evans blue dye subepicardially into the posterolateral wall of the left ventricle; the largest lymphatic vessel exiting the heart with blue dye was identified and cannulated with a polyethylene catheter (PE-10–90) at a site 2–5 cm from the base of the heart and proximal to the cardiac lymph node(s). Accessory noncardiac lymphatic and tracheobronchial lymphatic connections in this region were subsequently ligated to avoid an admixture of lymph from noncardiac structures. The lymph cannula was tunneled and exteriorized at the base of the neck above the sternum. A hydraulically activated occluding device and Doppler flow probe were then secured around the proximal circumflex coronary artery. Finally, right and left atrial catheters were placed and exteriorized before the chest was closed, and the animal was allowed to recover.

Conscious animals chronically instrumented in this fashion typically have demonstrated “resting” lymph flows from 0.5 to 5 ml/hr. Generally, during complete coronary artery occlusion, lymph flow decreases by 30–50%, and during the hyperemic phase of reperfusion, flow increases from 60% to 100% above baseline. Transit rates for lymph vary depending on lymph flow and the length and diameter of the cannula used, but generally, lymph transit times vary from 2 to 4 minutes.22

Cardiac Lymph Collection

Seventy-two hours after surgical instrumentation, cardiac lymph was collected from awake animals restrained in a nylon mesh sling as previously described.4 Each animal received Talwin (pentazocine lactate) 0.1–0.2 mg/kg i.v. or s.c. for analgesia. Cardiac lymph was collected into polypropylene tubes containing 10 units preservative-free heparin and a suspension of proteinase inhibitors, including 20 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% aprotinin. Collections were made before and during a 1-hour occlusion of the left circumflex coronary artery and at intervals up to 4 hours during reperfusion. Immediately after collection, lymph samples were centrifuged at 6,500g for 1 minute at room temperature, and the supernatant was decanted into a second tube and immediately placed on ice. Fresh lymph samples were evaluated by the shape change bioassay later that same day.

Shape Change Assay

Previous studies have documented that human or canine neutrophils undergo rapid alterations of cell morphology in response to chemotactic stimuli.4,23 Although unactivated neutrophils in suspension have a spherical appearance, after chemotactic stimulation, their shape changes in a way dependent on both the strength and the duration of the stimulus. First, neutrophils remain spherical but become “ruffled,” and later, if the stimulus is sufficient, they assume a polarized configuration characteristic of neutrophils migrating in a chemotactic gradient, with ruffles on one pole and a uropod on the other. This observation was used as a bioassay to assess the shape change–inducing ability of various suspensions of chemotactic factors and cardiac lymph and to assess the inhibitory activity of the putative rabbit anti-canine C5α. With human neutrophils, this bioassay has been shown to be a very sensitive indicator of the presence of human C5a, more sensitive than standard chemotaxis assays and capable of detecting C5a in the 1–10 nM range. In this study, the technique used to isolate canine C5a did not provide a preparation sufficiently clean of other proteins to specifically quantify the sensitivity of canine neutrophils to canine C5a, and canine neutrophils do not respond to human C5a preparations. In parallel assessments of canine and human neutrophils to canine and human preparations of zymosan-activated serum, however, sensitivity appeared to be comparable between the two systems.

Canine neutrophils were isolated from whole blood of the test animal before occlusion as previously described.4 Suspensions of neutrophils were exposed to various chemotactic factors or suspensions of canine cardiac lymph for 5 minutes at 37°C and then fixed in 1.5% glutaraldehyde. The percentages of cells assuming spherical, ruffled, and bipolar configurations were then determined visually by phase-contrast microscopy. Whereas we previously reported our data only as the percentage of bipolar cells,3 in this study a weighted shape change “index” was determined for each suspension of cells to increase the sensitivity of the assay by including those cells with a ruffled appearance. Thus, spherical cells were scored 0, ruffled cells 1, and bipolar cells 2. By this system, a maximally stimulated suspension of cells with 100% of cells having a bipolar shape would then have a maximal index score of 200.

Chemotactic Factors

ZADS was prepared by incubating zymosan (10 mg/ml) with fresh dog serum for 45 minutes at 37°C, with subsequent heat inactivation of the serum at 56°C for 30 minutes. Zymosan particles were removed by centrifugation, and the remaining serum was diluted to the appropriate concentration with PBS.

Other chemotactic factors used in this study included PAF (1-O-hexadecyl-2-acetoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids, Inc., Alabaster, Ala.) and recombinant human IL-8, (R&D Systems, Minneapolis, Minn.).

Measurement of Infarct Size

Twenty-four hours after ischemia and reperfusion, the dogs were reanesthetized, their original thoracotomy incision was reopened, and the heart stopped by saturated potassium intravenous infusion. After excision of the heart, the left circumflex coronary artery was ligated at the original occlusion site and then cannulated proximally and distally to the site. At a constant perfusion pressure of 100 mm Hg, the distal cannula was perfused with 1.5% triphenyltetrazolium chloride (TTC), and simultaneously, the proximal cannula was perfused with 0.5% Evans blue for 5 minutes. Subse-
quently, the heart was sliced perpendicular to the long axis in 1-cm-thick sections from base to apex. The nonischemic area was identified by Evans blue staining. Within the area at risk, infarcted myocardium was identified by the absence of TTC staining, whereas viable myocardium within the area at risk was identified by a brick red color. Total area at risk, infarcted area, and nonischemic areas were determined in each slice by planimetric analysis as previously described.4,24

Statistics

Pooled data are presented as the mean±SEM except in Figure 3, where the data are presented as mean±SD. In the assay comparing the shape change results of untreated and antiserum-treated ZADS samples (two sample comparisons), statistical significance was determined by use of Student’s paired t test. In the assay using cardiac lymph, the statistical significance of the variation in shape change activity of lymph over time, as well as the significance of the difference between untreated and antiserum-treated lymph, was determined by repeated-measures analysis of variance.

Results

Inhibition of ZADS-Induced Canine Neutrophil Shape Change by Anti-C5a Polyclonal Antiserum

In control experiments, neutrophil suspensions were incubated with ZADS as a source of activated complement. Each aliquot of ZADS was itself preincubated with PBS (negative control), preimmune serum, or postimmune serum for 15 minutes at 37°C (ZADS final concentration, 0.3% or 0.6%; preimmune or postimmune serum final concentration, 1:1,000). The results from these initial studies, depicted in Figure 2, demonstrated that ZADS caused a significant alteration in neutrophil morphology in a dose-dependent fashion consistent with chemotactic activation. This shape change–inducing activity was unaffected by the addition of preimmune serum. With the addition of postimmune serum, however, the shape change response of 0.6% ZADS was significantly attenuated, and the shape change response of 0.3% ZADS was eliminated completely.

Shape Change-Inducing Activity of Interleukin-8 and PAF

As demonstrated in Figure 3, human PAF and recombinant human IL-8 both demonstrated significant shape change–inducing activity when incubated with canine neutrophils. Preincubation of the chemotactic factors with anti-C5a antiserum (1:500) for 15 minutes at 37°C failed to inhibit the activity of either agent, demonstrating functional specificity of the antiserum.

Neutrophil Shape Change–Promoting Activity in Canine Cardiac Lymph

Cardiac lymph was collected from seven animals in this study that demonstrated electrocardiographic evidence of ischemia during coronary artery occlusion as well as subsequent histological evidence of infarction. In the first animal in the series, the presence of myocardial infarction was documented but not quantified. In the six subsequent animals, the area at risk averaged 41±4% of

Figure 2. Graph showing inhibition of canine neutrophil shape change induced by zymosan-activated dog serum (ZADS) by use of rabbit anti-C5a. Aliquots of 0.3% ZADS or 0.6% ZADS were preincubated with either phosphate-buffered saline (PBS), preimmune serum, or postimmune serum (each at 1:1,000 final concentration) for 15 minutes at 37°C. Neutrophils were subsequently added, and the shape change assay was performed as described in the text. Results from six separate experiments are shown. *p<0.001.

Figure 3. Graphs showing shape change–inducing activity of interleukin-8 (IL-8) (panel A) and platelet activating factor (PAF) (panel B). Preincubation of each chemotactic factor with anti-C5a antiserum (1:500) for 15 minutes at 37°C failed to inhibit the activity of either agent, demonstrating the functional specificity of the antiserum for C5a.
Clq demonstrated Ward8 ity

Subsequently, Clq had been recorded in heart reperfusion, only in cardiac samples of the left circumflex coronary artery. Anti-C5a change-inducing shape change was observed in lymph from all animals tested at each time point. Fractions in parentheses indicate the number of animals demonstrating a positive shape change response over the number of animals tested at that time point. Closed circles indicate the shape change–promoting activity of the same lymph samples preincubated with anti-C5a antiserum (1:500 final concentration). At no time point during the course of reperfusion was there shape change–inducing activity that was not inhibitable by the anti-C5a. PRE, preocclusion; 1 HR, 2 HR, 3 HR, 4 HR, 1, 2, 3, and 4 hours of reperfusion, respectively. *p<0.01 compared with PRE; *p<0.01 compared with untreated (open circles).

**Figure 4.** Graph showing anti-C5a suppression of neutrophil shape change–promoting activity in canine cardiac lymph collected before and up to 4 hours after ischemia and reperfusion from seven animals fulfilling electrocardiographic evidence of ischemia and histological evidence of infarction after a 1-hour occlusion of the left circumflex coronary artery. Open circles show the mean±SEM of the shape change indexes for lymph from all animals tested at each time point.

The left ventricle. Infarct sizes were 16%, 35%, 30%, 3%, 6%, and 9% of the area at risk, respectively. They averaged 16.5±5.4% of the area at risk, or 7.4±2.7% of the total area of the left ventricle.

Shape change assessments were made in cardiac lymph samples collected before occlusion and at 1-hour intervals during reperfusion up to 4 hours. As demonstrated in Figure 4, a significant shape change–inducing response was present in cardiac lymph at 1 hour of reperfusion in five of seven animals. This shape change–inducing response was detected in six of seven dogs and peaked after 2 hours of reperfusion; by 3 hours of reperfusion, shape change–inducing activity was detectable in lymph from three of six animals and by 4 hours in only two of five animals. The three animals with the smallest infarcts did not have shape change–inducing activity detectable beyond 2 hours of reperfusion, whereas the three animals with the larger infarcts had shape change–inducing activity present at 3 hours of reperfusion in one and 4 hours of reperfusion in the other two. The results shown are similar to those previously published from this model. A novel observation recorded here, however, is that the addition of anti-C5a inhibited the shape change–inducing response of cardiac lymph at each of the time points by ≥85%. At no time point was shape change–inducing activity present that was not inhibitable by the anti-C5a.

**Discussion**

Previous studies have provided indirect evidence that complement activation can occur as a result of ischemic injury to the myocardium. Pinckard and associates demonstrated in a series of in vitro experiments that heart subcellular membranes rich in mitochondria bound C1q and were capable of complement activation. Subsequently, Rossen et al demonstrated in coronary artery occlusion experiments in dogs that during reperfusion C1q colocalized with neutrophils in areas that had been made ischemic. Other studies by Hill and Ward demonstrated that extractable chemotactic activity was present in infarcted rat myocardium after coronary artery occlusion and was inhibited by the addition of anti-C3 antibody. Still other animal studies demonstrated that complement depletion with cobra venom factor could reduce myocardial necrosis after coronary artery occlusion. Further this study adds to the above findings by directly implicating C5a as the principal neutrophil chemotactic factor present in the extracellular fluid or microenvironment of the canine myocardium during the first 4 hours of reperfusion after ischemic injury.

The conclusions in this study are derived from the assumption that the antiserum developed recognizes C5a. The term “C5a” in this study has been used to represent the chemotactically active component of the anaphylatoxin C5. Thus, it should be acknowledged that the polyclonal antiserum, as developed, probably recognizes C5 as well as C5a des-Arg, a less potent but nevertheless chemotactically active metabolite of C5a. The designation of the antiserum as “anti-C5a” is based principally on the antiserum’s functional characterization. The observations that ZADS is a rich source of C5a and that the shape change assay is a valid assessment of chemotactic activity have both been previously documented. In this study, ZADS-induced shape change was clearly inhibited by the antiserum, whereas preimmune serum had no effect. The specificity of the antiserum for C5a was demonstrated by its inability to block the shape change–inducing activity of PAF and IL-8, chemotactic factors that have been implicated as agents possibly involved in attracting neutrophils to the ischemic myocardium.

Further evidence for the specificity of the antiserum is provided by immunochemical analyses that indicated that the immunoglobulin G antibodies in the anti-C5a reacted with a protein in fresh canine serum that has a molecular weight consistent with that of the α-chain of C5, as well as one other, unidentified component. Moreover, in the purified serum fractions that contained the functional activities of C5a, these immunoglobulin G antibodies identified a polypeptide that has the same relative molecular mass (8.5 kd) as porcine


CSa\textsuperscript{29} and nonglycosylated recombinant human CSa\textsuperscript{30} as well as a smaller degradation product. The susceptibility of even recombinant CSa to protease degradation has been previously demonstrated.\textsuperscript{31}

The awake, chronically cannulated canine cardiac lymph model was established as a means to examine changes in the interstitial fluid or extracellular milieu of the myocardium independent of surgical trauma.\textsuperscript{4,22} Previous studies have demonstrated that increases in creatine kinase and phosphorylase after coronary artery occlusion and reperfusion can be demonstrated earlier and with greater sensitivity in the lymph than in peripheral blood.\textsuperscript{32} Additional studies have indicated that ischemia causes platelet activation and thromboxane release into the lymph during reperfusion.\textsuperscript{33} Moreover, the data from this study supplement previous observations with this model that have documented the release of mitochondrial proteins into the lymph that bind Clq and activate complement.\textsuperscript{3,4}

The fact that chemotactic activity in cardiac lymph during early reperfusion appears to be attributable primarily to CSa, however, does not exclude the possibility that other chemotactic factors may be initiated by myocardial ischemia and reperfusion. These other agents may remain localized within the ischemic myocardium or may be released into lymph at concentrations not detectable by the present assay system or at times not tested in this study. Nevertheless, the rise and fall of CSa-mediated chemotactic activity in cardiac lymph closely approximate the time course of canine neutrophil localization previously reported by our group during myocardial ischemia and reperfusion.\textsuperscript{34}

Thus, the data from this study strongly support the concept that complement activation and the release of CSa are key components of the inflammatory response early in the course of reperfusion after myocardial ischemia and infarction. As mentioned above, some of the earliest attempts at suppressing the inflammatory response to ischemic injury have used cobra venom factor, an agent that effectively abrogates circulating complement and is effective in reducing the postischemic inflammatory response but, because it inactivates complement by activating the alternative pathway, also elicits all the manifestations of massive intravascular anaphylatoxin release.\textsuperscript{7} Recently, Weisman et al\textsuperscript{35} have developed a more benign method for suppressing complement activation after ischemic injury by infusing soluble, recombinant type I complement receptors intravascularly at or about the time of coronary occlusion. Our data suggest that anti-CSa antibody reagents may also provide a measure of control of the complement-mediated inflammatory responses induced by ischemia. Suppression of CS activation or neutralization of CSa would be expected to attenuate neutrophil activation. In addition, such reagents would be expected to suppress the release of mast cell histamine, production of thromboxane A\textsubscript{2}, and the generation of leukotrienes LTC\textsubscript{4} and LTD\textsubscript{4} that can also occur as a result of complement activation.\textsuperscript{36-39} Further investigation is warranted to address these interesting possibilities.

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References

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