

Consumption of Classical Complement Components by Heart Subcellular Membranes In Vitro and in Patients after Acute Myocardial Infarction

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ABSTRACT Experiments were conducted to characterize the antibody-independent activation of complement in human serum by isolated human heart mitochondrial membranes in vitro and to determine whether similar patterns of complement consumption occurred in patients after acute myocardial infarction. Direct evidence for the interaction of C1 and heart mitochondrial membranes was obtained by mitochondria-C1 binding and elution experiments. Exposure of normal human sera to isolated human heart mitochondria at 37°C resulted in the consumption of C1, C4, C2, and C3 without significant consumption of the terminal components of the complement system (C6 through C9). The consumption occurred in the absence of detectable anti-heart mitochondria autoantibody, was demonstrated to be calcium dependent, and was inhibited by either 0.01 M EDTA or ethylene glycol bis(β -aminoethyl ether) N,N,N',N' -tetraacetic acid (EGTA). Although specific absorption of C1q from human sera inhibited the mitochondria-dependent activation of C4, C3 consumption was not affected. These data indicate that the consumption of C4 and C2 likely occurred due to the mitochondrial membrane-mediated activation of C1, but that the consumption of the C3 did not necessarily involve either the classical or alternative complement pathways. After the in vitro characterization of the mitochondria-dependent activation of the complement system, additional studies were performed to determine whether similar consumption occurred in patients after acute myocardial infarction. During a 72-h period after

hospital admission significant decreases in C1, C4, and C3 occurred in six patients after acute myocardial infarction but not in six patients with recent chest pain but no evidence of acute myocardial infarction. These studies suggest that myocardial cell necrosis results in the release of subcellular membrane constituents capable of activating the complement system in the absence of detectable anti-heart autoantibodies; such activation may be responsible in part for the development of acute inflammation and evolution of the infarct size following coronary artery occlusion.

INTRODUCTION

The processes involved in the destruction of myocardial tissue after coronary artery occlusion have been the subject of intensive investigation. Myocardial cell necrosis following coronary artery occlusion is thought to include an initial autolytic and a subsequent heterolytic destruction of myocardial cells in the area of the infarction (1). The autolytic destruction of tissue likely is due to a restricted perfusion of the affected area resulting in a decreased delivery of oxygen and substrates necessary for normal myocardial energy generation. After the initial autolytic tissue damage of heterolytic destruction of myocardial tissue occurs associated with inflammatory cell infiltration into the affected area. During the first 48 h after myocardial infarction the neutrophil is the predominant inflammatory cell within the evolving myocardial lesion and likely is involved in the heterolytic phase of myocardial damage.

There are a variety of factors which could mediate the neutrophilic infiltration following tissue damage,

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one of which is the complement system of serum proteins. Hill and Ward (2) have demonstrated that the neutrophilic infiltration following experimental myocardial infarction in rats was reduced significantly by the prior administration of cobra venom factor, which causes a depletion of the third component (C3) of serum complement. In view of the above, it is crucial to elucidate mechanisms governing the activation of complement after myocardial cell damage.

There are presently three known mechanisms for the activation of the complement system: (a) the classical activation of complement due to the activation of C1 by IgG or IgM antibody; (b) the activation of the alternative pathway by a variety of substances capable of interacting with properdin; and (c) the direct proteolytic activation of C1 and C3 by enzymes such as plasmin or trypsin.

We initiated studies to determine whether anti-heart autoantibodies might mediate the classic activation of complement after acute myocardial infarction. It was shown that IgM, complement-fixing, anti-heart mitochondria autoantibody developed 7-14 days after experimental myocardial infarction in dogs (3, 4); similar temporal production of anti-heart mitochondria autoantibodies was observed in patients after acute myocardial infarction.¹ However, the time-course of autoantibody development precluded its involvement in the initial inflammatory response during the first 2-3 days after myocardial infarction. Subsequently, we demonstrated that all human serum from subjects without prior history of myocardial disease contained a heat-labile, heart-reactive factor which, after the interaction with isolated human heart mitochondrial membranes, led to the activation of serum complement (5). This serum factor was identified as the first component of serum complement which bound directly to the mitochondrial membrane and resulted in an antibody-independent activation of the complement system.

The present studies were designed to characterize further the mechanism(s) for the antibody-independent activation of complement by the heart subcellular membranes and to determine whether a similar intravascular activation of complement occurs in patients after acute myocardial infarction.

METHODS

Preparation of heart mitochondrial membranes. Mitochondrial membranes were prepared from human heart tissue obtained at autopsy within 1 h of the time of death. Portions of the left ventricle were sectioned and washed with a solution containing 0.25 M sucrose and 0.01 M Tris-chloride, pH 7.4, at 0-4°C. This buffer was equilibrated with 100% oxygen before the cardiac tissue was washed. Washed tissue was passed through a meat

grinder (2-mm holes) into approximately 7 vol of the same sucrose-Tris buffer. The resultant mince was disrupted further with a Polytron PT 20 tissue homogenizer (Brinkmann Instruments, Inc., Westbury, N. Y.) at setting 5 for 45 s. The homogenate was centrifuged at 600 g for 10 min. The sediment was discarded and the supernate was recentrifuged at 5,500 g for 15 min. Each mitochondrial pellet was washed twice with the sucrose-Tris buffer (20-25 ml per pellet) and finally resuspended in the same buffer to a final protein concentration of approximately 10 mg/ml. The mitochondrial suspension was frozen rapidly in 0.5-ml aliquots and stored at -70°C. Before freezing, the functional integrity of each mitochondrial preparation was determined by measuring the respiratory control ratio with pyruvate and malate as substrates and ADP as the respiratory stimulant (6). Respiratory control ratios of between 5.0 and 7.5 were normal for these human heart mitochondria preparations.

Measurement of anti-heart mitochondria autoantibody. Anti-heart mitochondria autoantibody was assessed by a micro-complement fixation test using a microtiter apparatus (Cooke Engineering Co., Alexandria, Va.). Doubling dilutions of heat-inactivated (56°C for 30 min) human sera were made in 25 μ l of 0.15 M sodium barbital-buffered saline, pH 7.3, containing 0.1% human albumin. Complement fixation was determined by the addition of 25 μ l of a dilution of guinea pig serum containing 1.50 minimal hemolytic doses of complement followed by 25 μ l of isolated human heart mitochondria antigen (1 mg mitochondrial protein/ml). The reaction was allowed to proceed at 37°C for 30 min and then 25 μ l of a 1.5% suspension of sensitized sheep erythrocytes was added and the reaction was allowed to proceed at 37°C for 40 min. The microtiter plates were centrifuged at 1,000 rpm for 5 min and the degree of hemolysis was estimated by visual inspection. The autoantibody titer was defined as the highest dilution of serum at which incomplete hemolysis had occurred. A serum was considered negative for anti-heart mitochondria autoantibody if the titer was less than 1:2. The optimal concentration of the heart mitochondria antigen was determined by checkerboard titrations using a standard serum containing anti-heart mitochondria autoantibody. Appropriate serum and antigen controls were performed simultaneously with the above assays. A positive anti-heart mitochondria autoantibody serum was titrated along with every test in order to ensure uniformity in the sensitivity of the complement fixation test from day to day.

Preparation of complement fixation reagents. Sensitized sheep red blood cells (EA),² Veronal-buffered saline ($\Gamma/2 = 0.15$) containing 0.1% gelatin (GVB), and low ionic strength GVB ($\Gamma/2 = 0.065$) containing 3% glucose (GI-GVB) were prepared according to the methods outlined by Rapp and Borsos (7). Partially purified human C1 was prepared by euglobulin fractionation of fresh human serum as described previously (5). Sera congenitally deficient in the 4th, 6th, or 7th components of complement were ob-

² *Abbreviations used in this paper:* BSA, bovine serum albumin; CH₅₀, total hemolytic complement; CPK, creatinine phosphokinase; EA, sensitized sheep red blood cells; ECG, electrocardiogram; EGTA, ethylene glycol bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid; GI-GVB, low ionic strength GVB ($\Gamma/2 = 0.065$) containing 3% glucose; GOT, glutamic oxaloacetic transaminase; GVB, Veronal-buffered saline ($\Gamma/2 = 0.15$) containing 0.1% gelatin; KCNS, potassium thiocyanate; LDH, lactic dehydrogenase; PBS, phosphate-buffered saline.

¹ Pinckard, R. N., M. S. Olson, and R. A. O'Rourke. Unpublished observations.

tained from C4-deficient guinea pigs, C6-deficient rabbits, and a C7-deficient human, respectively.

Human serum deficient in C3, C4, and C5 but containing stable, oxidized C2 was prepared by treating serum with potassium thiocyanate (KCNS) followed by iodination.³ 1 vol of 2.0 M KCNS was added to 1 vol of fresh human serum and the mixture was allowed to incubate overnight at 0°C. The mixture was dialyzed for 2 h against distilled water and for an additional 2 h each against three changes of phosphate-buffered saline, pH 6.8 (PBS). 9 vol of the KCNS-treated serum were reacted with 1 vol of triatomic iodine (I₃) for 5 min at 0°C. The reaction mixture was dialyzed overnight against PBS and subsequently against GVB. The dialyzed oxy-KCNS-treated serum was aliquoted and frozen in an acetone-dry ice bath and stored at -70°C. The I₃ was prepared by the addition of 0.3173 g of I₂ (Allied Chemical Corp., Morristown, N. J.) to 25.75 g of KI and the resulting solution brought to a final volume of 100 ml with distilled water. Human serum deficient in C3 and C4 was prepared by the treatment of 1 vol of fresh serum with 1 vol of 1.0 M KCNS as described above.

Preparation of cellular intermediates. EAC4 cells were prepared according to the method of Borsos and Rapp (8). EAC1 cells were formed by incubation at 0°C for 15 min of 200-400 effective molecules of human or guinea pig C1 with EA (5×10^8 cells/ml). To this mixture was added 2 vol of the human serum diluted 1:10 with GVB containing 0.01 M EDTA. Alternatively human serum heated to 56°C for 30 min was used in place of the human serum-0.01 M EDTA-GVB mixture. The EAC1,4 cells were washed three times each with 0.01 M EDTA-GVB and with GVB and finally washed two times with G1-GVB.

EAC1,4oxy-2 cells were prepared as described previously.³ EAC1 cells were prepared by the addition of sufficient C1 to 20 ml of 2.5% suspension of EA to yield approximately 400 effective molecules of C1 per erythrocyte. After a 20-min incubation at 37°C, the EAC1 cells were washed 2 times in warm (37°C) GVB and resuspended to the original volume. 10 ml of heat-inactivated (56°C for 30 min) human serum was added and the suspension was incubated 30 min at 37°C. The EAC1,4 cells were washed 3 times and were resuspended to the original volume. 10 ml of oxy-KCNS-treated serum was added to the EAC1,4 cells followed by incubation for 5 min at 37°C. The EAC1,4oxy-2 cells were washed 2 times in G1-GVB and adjusted to a concentration of 1×10^8 cells/ml in G1-GVB.

Complement assays. Total hemolytic complement (CH₅₀), C1, C2, C8, and C9 titrations were performed according to procedures described by Cordis Laboratories (Miami, Fla.) utilizing cellular intermediates and functionally purified human complement components. C4, C6, and C7 titrations were performed by utilizing C4-deficient guinea pig serum, C6-deficient rabbit serum, and C7-deficient human serum, respectively. The C4, C6, and C7 assays were performed by the addition of 0.1 ml of serum dilutions to 0.1 ml G1-GVB followed by the addition of 0.1 ml of optimal dilutions of the appropriate serum deficient in the respective complement component (C4, 1:50; C6, 1:12.5; C7, 1:30). 0.1 ml of sensitized erythrocytes (1×10^8 EA/ml) was added and the suspension was incubated for 120 min at 37°C with intermittent shaking. C3 assays were performed by the addition of 0.1 ml of serum dilutions (prepared in G1-GVB) to 0.1 ml of G1-GVB and 0.1 ml of EAC1,4oxy-2 cells (1.0×10^8

cells/ml). The mixture was incubated at 30°C for 30 min with intermittent shaking. 0.1 ml of a dilution of serum depleted of C3 and C4 but containing excess C5-C9 (references 9, 10; 0.50 M KCNS-treated serum) then was added and the mixture incubated at 37°C for 60 min. At the end of the incubation period for each of the above assays 1 ml of cold saline was added, and after centrifugation the supernates were analyzed spectrophotometrically at 415 nm to quantify the percent hemoglobin released. The 50% hemolytic end-point was determined by the von Krogh equation (7) and the results expressed as the number of functional hemolytic complement component units per milliliter of undiluted serum.

Patient selection. The clinical studies were performed on patients admitted to the Coronary Care Unit and Myocardial Infarction Research Unit at the University Hospital, University of California School of Medicine, San Diego. After patient admission, a peripheral venous line (19-gauge angiocatheter) was inserted into a vein in the wrist or arm for subsequent withdrawal of blood samples and for the intravenous injection of medication when needed. The line was kept open with heparin (1,000 U/20 ml flush solution). Blood samples for creatine phosphokinase (CPK), glutamic oxaloacetic transaminase (GOT), lactic dehydrogenase (LDH), and complement determinations were obtained on admission, hourly for the first 7 h, at 2-h intervals for the remainder of the first 24 h, and subsequently at 4-h intervals until 72 h had elapsed. Subsequent blood samples were obtained once daily. All blood samples were allowed to clot at room temperature and the serum aliquots were stored at -70°C for subsequent determination of complement levels and the presence of anti-heart mitochondria autoantibodies. A diagnosis of acute myocardial infarction was based upon the three following criteria: (a) a history of prolonged chest pain typical of acute myocardial infarction; (b) evolutionary electrocardiographic (ECG) changes indicative of acute transmural myocardial infarction; and (c) a rise and subsequent fall in serum levels of CPK, GOT, and LDH in the characteristic pattern of acute myocardial infarction. Six of the patients included in the present studies with evidence of acute myocardial infarction met all three of these criteria. Six other patients were admitted to the Coronary Care Unit for observation because of recent chest pain; none of these patients had evolutionary ECG changes or subsequent elevations in serum enzyme levels. Two of the control patients had angina pectoris without evidence of infarction, two had atypical chest pain with chest wall tenderness, one had a hiatal hernia with reflux esophagitis, and there was no apparent etiology for chest pain in the sixth patient. The four patients without angina subsequently had no ECG changes during progressive exercise treadmill testing and none of the six patients had pleuritis or pericarditis.

RESULTS

C1 binding to heart mitochondria. Direct evidence for the binding of C1 to the mitochondrial membrane was obtained by C1 binding and elution experiments (Table I). Partially purified human C1 was adjusted to an ionic strength of 0.15 followed by incubation at 37°C for 15 min in order to activate C1. Isolated human heart mitochondria (4 mg mitochondrial protein) were resuspended in 0.5 ml of the activated C1 preparation at 0°C. After a 15-min incubation the mitochondria were

³ Boyer, J. T., and P. Wyde. 1975. Hypochlorite-induced alterations of human serum complement. Submitted for publication.

TABLE I
C1 Binding to Heart Mitochondria

	Hemolytic C1
	U/ml
Purified C1	115,000
C1 remaining in the supernate	32,000
C1 eluted from mitochondria	120,000

Isolated human heart mitochondria (4 mg mitochondrial protein) were resuspended in 0.5 ml of a partially purified preparation of activated C1 at 0°C and were incubated for 15 min. The mitochondria were removed by centrifugation and were washed two times with GI-GVB, $\Gamma/2 = 0.065$. After the second wash the mitochondria were resuspended in 0.5 ml of 0.30 M NaCl and were incubated at 0°C for 15 min. The mitochondria were removed by centrifugation and the supernate was diluted with an equal volume of distilled water. Hemolytic C1 assays were performed on the original unabsorbed C1 preparation, the supernate from the mitochondria-absorbed C1 preparation, and the C1 eluted from the mitochondrial membrane.

removed by centrifugation (17,000 *g* for 2 min) and were washed 2 times with GI-GVB, $\Gamma/2 = 0.065$. After the second wash bound C1 was dissociated from the mitochondria by resuspension in 0.5 ml of 0.30 M NaCl and incubation at 0°C for 15 min. The mitochondria were removed by centrifugation and the supernate was diluted with an equal volume of distilled water in order to establish an ionic strength of $\Gamma/2 = 0.15$. Hemolytic C1 assays were performed on the original unabsorbed C1 preparation, the supernate from the mitochondria-absorbed C1 preparation, and the C1 eluted from the mitochondrial membrane. In numerous experiments, one of which is shown in Table I, absorption of C1 preparations with isolated human heart mitochondria always removed greater than 70% of the hemolytic C1 activity; significant C1 activity was recovered from the mitochondria-C1 complex after incubation in 0.30 M NaCl. Similar experiments conducted with fresh human serum also indicated the ability of 4 mg isolated human heart mitochondria to absorb greater than 50% of the C1 from 1 ml undiluted human serum.

Mitochondrial membrane-induced consumption of individual serum components. Studies were conducted to determine which components of complement were consumed after exposure of sera to isolated human heart mitochondria. Isolated human heart mitochondria, 4 mg mitochondrial protein, were resuspended in 0.5 ml of fresh undiluted normal human sera. These sera were documented not to contain detectable complement-fixing, anti-heart mitochondria autoantibody as described in Methods. The mixture was incubated at 37°C for 30 min and the mitochondria were removed by

centrifugation at 17,000 *g* for 2 min. Sera not exposed to isolated human heart mitochondria, but incubated at 37°C for 30 min served as controls. A typical experiment is shown in Table II and demonstrates that significant consumption of C1, C4, C2, and C3 occurred in serum exposed to isolated human heart mitochondria. In contrast, significant consumption of the terminal components of complement (C6 through C9) was never observed. The CH_{50} decreased to a similar degree as the decreases in the hemolytic levels of C2 and C3. When similar experiments were performed in the presence of 0.01 M EDTA, no consumption of C4, C2, or C3 was observed although a significant decrease in C1 was observed.

Fig. 1 demonstrates the rate of consumption of C4 in serum exposed to isolated human heart mitochondria. Fresh human serum was exposed to isolated human heart mitochondria (8 mg mitochondrial protein/ml undiluted serum) at 37°C. At various times samples were removed and centrifuged at 17,000 *g* for 2 min and the C4 titers were determined on the supernates. Sera not exposed to human heart mitochondria but treated in a similar manner served as controls. It can be seen that, immediately after exposure of serum to isolated human heart mitochondria, there was a precipitous decrease in C4 level. The consumption of C4 continued until only 10% of the original C4 level was present 30 min after exposure of the serum to isolated

TABLE II
Consumption of Complement in Human Serum 30 min after the Addition of Isolated Human Heart Mitochondria

Complement component	Serum control	Serum-mitochondria	Percent reduction
	<i>hemolytic U/ml serum</i>		
C1	152,000	60,000	61
C4	1,900,000	185,000	90
C2	7,000	5,600	20
C3	320,000	220,000	31
C6	1,300	1,200	8
C7	60,000	53,000	12
C8	1,480,000	1,530,000	0
C9	440,000	400,000	9
CH_{50}	1,250	910	27

Isolated human heart mitochondria (4 mg mitochondrial protein) were resuspended in 0.5 ml of fresh undiluted normal human serum documented not to contain detectable complement-fixing, anti-heart mitochondria autoantibody. The mixture was incubated at 37°C for 30 min and the mitochondria were removed by centrifugation. Serum not exposed to isolated mitochondria but incubated at 37°C for 30 min served as control. Hemolytic assays were performed on the serum control and serum exposed to mitochondria and the percent reduction of the individual complement components was determined.

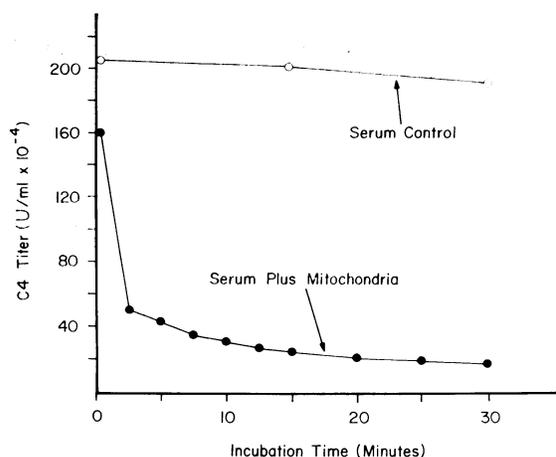


FIGURE 1 Rate of C4 consumption in fresh human serum exposed to isolated human heart mitochondria. Isolated human heart mitochondria (4 mg mitochondrial protein) were resuspended in 1.0 ml of fresh human serum documented not to contain any detectable anti-heart mitochondria autoantibody. The suspension was incubated at 37°C and at various times samples were removed and centrifuged and C4 levels were determined on the supernates. Serum not exposed to human heart mitochondria was treated in a similar manner and served as a control.

human heart mitochondria. As in the previous experiment the presence of 0.01 M EDTA prevented the mitochondria-dependent consumption of C4 in human serum.

Additional experiments were performed to determine whether the mitochondria-dependent consumption of C3

TABLE III
Effect of EGTA upon the Heart Mitochondria-Dependent Consumption of C4 and C3 in Normal Human Sera

Experiment	Complement component	
	C4	C3
	U/ml	
Without EGTA		
Serum control	900,000	200,000
Serum-mitochondria	140,000 (84%)*	125,000 (37%)
Serum-zymosan	650,000 (28%)	55,000 (72%)
With 0.01 M EGTA		
Serum control	710,000	190,000
Serum-mitochondria	720,000 (0%)	175,000 (8%)
Serum-zymosan	800,000 (0%)	92,000 (56%)

Isolated human heart mitochondria (4 mg mitochondrial protein) were resuspended in 0.5 ml fresh human serum in the presence or absence of 0.01 M EGTA at 37°C and were incubated for 30 min. The mitochondria were removed by centrifugation and the hemolytic levels of C4 and C3 were determined.

* Percent reduction when compared to control serum.

occurred as a result of classical activation of complement or was due to activation of the alternative complement pathway. In a representative experiment (Table III) fresh human serum, 0.5 ml, was exposed to 4 mg of human heart mitochondria in the presence or absence of 0.01 M EGTA at 37°C for 30 min. The mitochondria were removed by centrifugation (17,000 g for 2 min) and the hemolytic levels of C4 and C3 were determined. Serum which was not exposed to isolated human heart mitochondria or which was exposed to 4 mg of zymosan served as controls, the latter being performed to demonstrate the activation of the alternative pathway under the experimental conditions. As can be seen in Table III, significant consumption of C4 and C3 occurred in serum not containing EGTA that was reacted with either isolated human heart mitochondria or zymosan. In contrast, no significant consumption of either C4 or C3 occurred in serum containing EGTA that was reacted with mitochondria, but significant consumption of C3 occurred in the serum containing EGTA and zymosan.

Although the above experiments indicated repeatedly that mitochondria-dependent consumption of C3 did not occur through activation of the alternative pathway, they did not establish that the consumption of C4 or C3 by mitochondria occurred through the activation of the classic complement pathway. Therefore, additional experiments were performed to determine whether or not the mitochondria-dependent consumption of C4 and C3 required the activation of human C1 by human heart mitochondria. In a representative experiment (Table IV), fresh human serum was absorbed with rabbit anti-bovine serum albumin (BSA)-BSA immune complexes in slight antibody excess in the presence of 0.01

TABLE IV
Mitochondria-Dependent Consumption of C4 and C3 in Normal and C1q-Absorbed Human Sera

Experiment	Complement component	
	C4	C3
	U/ml	
Normal		
Serum control	970,000	290,000
Serum-mitochondria	130,000 (87%)*	190,000 (34%)
C1q-absorbed		
Serum control	900,000	170,000
Serum-mitochondria	750,000 (17%)	120,000 (30%)

Isolated human heart mitochondria (4 mg mitochondrial protein) were resuspended in 0.5 ml of fresh human serum or human serum depleted of C1q. After incubation at 37°C for 30 min the mitochondria were removed by centrifugation and the C4 and C3 levels were determined.

* Percent reduction when compared to control serum.

M EDTA in order to remove C1q selectively. Three absorptions with BSA-anti-BSA-immune complexes consistently removed greater than 95% of the functional C1 activity. 4 mg isolated human heart mitochondria was resuspended in 0.5 ml of the recalcified C1q-depleted human serum. After incubation at 37°C for 30 min, the mitochondria were removed by centrifugation (17,000 *g* for 2 min) and the hemolytic levels of C4 and C3 were determined. Fresh human sera not absorbed with BSA-anti-BSA immune complexes were utilized in similar experiments and served as controls. As seen in Table IV, removal of C1q significantly decreased the mitochondria-dependent consumption of C4 but did not reduce significantly the consumption of C3.

Intravascular consumption of complement components in patients after acute myocardial infarction. Studies were performed to determine whether similar consumption of complement components also might occur in patients after acute myocardial infarction. Serial serum samples were obtained from patients during the 72-h period following hospitalization. 12 patients were included in these studies: 6 patients with unequivocal evidence for acute transmural myocardial infarction; and 6 patients with recent chest pain but who subsequently were shown not to have had an acute myocardial infarction. CH₅₀, C1, C4, C3, and C6 assays were performed on the sera from each patient simultaneously. The maximum percent decreases, during the initial 72-h period following hospital admission, in each respective complement component were determined relative to the levels of the individual complement components determined for the serum sample obtained at admission. The data in Table V show the maximum percent decreases in CH₅₀, C1, C4, C3, and C6 and the times at which these decreases occurred in six control patients

TABLE V
Maximum Percent Decrease in Complement during the 72-h Period Following Hospitalization of Control Patients without Acute Myocardial Infarction

Patient	Complement component				
	CH ₅₀	C1	C4	C3	C6
T. O.	20* (7)	12 (5)	17 (7)	0 (0)	11 (9)
E. E.	17 (4)	11 (4)	14 (36)	15 (8)	10 (36)
K. A.	12 (20)	11 (12)	4 (4)	17 (35)	4 (8)
H. B.	24 (12)	7 (14)	19 (14)	11 (6)	12 (6)
R. A.	0 (0)	25 (4)	ND	14 (10)	5 (2)
R. G.	1 (40)	7 (4)	10 (36)	26 (10)	0 (0)

* Maximum percent decrease observed relative to complement levels of the serum obtained at admission; values shown in parentheses indicate the time (hours) after hospitalization at which the maximum percent decrease in complement occurred. ND, assay not done.

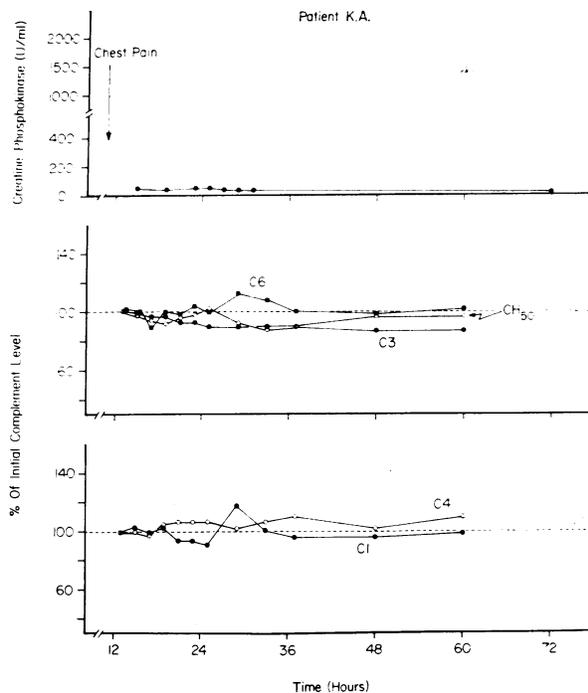


FIGURE 2 Temporal changes in the percent decreases in the levels of C1, C4, C3, C6, and CH₅₀ in relation to the serum CPK in control patient K. A., who did not experience acute myocardial infarction. The time scale in this figure is constructed so that 12 h represents noon on the day of admission and 72 h represents midnight of the 3rd day.

with chest pain but without myocardial infarction. None of the six patients had detectable anti-heart mitochondria antibody in any of the sera studied. These six patients were chosen as controls since the environmental influences and methods for the procurement of blood samples and serum preparation were identical to the six patients with acute myocardial infarction. The data in Table V demonstrate that there were no large decreases in any of the components measured in any of the six patients studied. The mean decreases for the CH₅₀ and C1 were 12% (range 0–24% and 7–25%); the mean decreases of C4 and C3 were 13 and 14%, respectively (range 4–19% and 0–28%); and for C6 it was 7% (range 0–12%). In addition there was no relationship either between patients or within individual patients of the times at which the maximum decreases in individual complement components occurred.

Fig. 2 depicts the fluctuations in the serum levels of CH₅₀, C1, C4, C3, and C6 in a representative control patient. Patient K. A. was admitted 2 h after the onset of atypical chest pain and had no evidence of acute myocardial infarction; i.e. there were neither elevations in the serum levels of CPK, GOT, or LDH, nor evolu-

TABLE VI
Maximum Percent Decrease in Complement during the 72-h
Period Following Hospitalization of Patients
with Acute Myocardial Infarction

Patient	Complement component				
	CH ₅₀	C1	C4	C3	C6
E. H.	ND	65*(41)	38 (41)	33 (24)	0 (0)
G. G.	17 (22)	53 (22)	62 (22)	26 (22)	13 (22)
J. C.	33 (28)	50 (21)	18 (21)	28 (21)	0 (0)
L. C.	13 (18)	27 (18)	32 (18)	29 (22)	15 (4)
A. W.	5 (21)	47 (21)	44 (8)	47 (21)	10 (2)
J. E.	25 (6)	45 (26)	29 (26)	47 (10)	6 (0)

* Maximum percent decrease observed relative to complement levels of the serum obtained at admission; values shown in parenthesis indicate the time (hours) after hospitalization at which the maximum percent decrease in complement occurred. ND, assay not done.

tionary ECG evidence of acute myocardial infarction. No significant fluctuations in the levels of complement components measured were observed over a 50-h period following admission.

In striking contrast to the control patients, significant decreases in the levels of C1, C4, and C3 occurred in every patient with acute myocardial infarction during the 72-h period following hospitalization. These six patients were admitted to the hospital 2-4 h after the onset of chest pain. The initial elevations in serum CPK occurred 3-7 h after admission. None of the six patients with acute myocardial infarction had detectable anti-heart mitochondria antibody at the time of admission nor during the following 72 h of hospitalization; however, four of these six patients developed detectable anti-heart mitochondria antibody during the 2nd wk after hospitalization. The data in Table VI demonstrate that there were significant decreases in complement components C1, C4, and C3. The mean decreases observed were as follows: CH₅₀, 18.5% (range 5-33%); C1, 47.8% (range 27-65%); C4, 37.2% (range 18-62%); C3, 35% (range 26-47%); and C6, 7.3% (range 0-15%). In contrast to the control patients, there appeared to be a relationship between and especially within patients of the times at which the maximum decreases of C1, C4, and C3 occurred. As can be seen in Table VI the maximum decreases of these components occurred approximately 24 h after hospitalization; furthermore, in five of six patients the maximum decreases in C1 and C4 occurred simultaneously and the maximum decrease in C3 generally occurred within hours of the C1 and C4 decreases. Although the maximum decreases in CH₅₀ tended to correlate temporally with the C1, C4, and C3 decreases, no correla-

tion was observed with respect to the relatively small maximum decrease in C6.

Fig. 3 demonstrates the precipitous decreases in complement components C1, C4, and C3 observed in patient A. W., who had an acute myocardial infarction. The initial decreases in C1, C4, and C3 occurred 4½ h after the onset of chest pain during a period of time when the initial increase in serum CPK levels was occurring. The maximum decreases in C1, C4, and C3 occurred within 24 h and returned to within normal limits during the 3rd day of hospitalization. The CH₅₀ and C6 levels did not fluctuate significantly during the first 48 h after admission, but tended to become elevated on the 3rd day after hospitalization.

Table VII shows a statistical comparison of the maximum percent decreases during the 72-h period following hospitalization in the serum levels of CH₅₀, C1, C4, C3, and C6 between the control and experimental groups. The data, obtained from Tables V and VI, were analyzed by the Student's *t* test for the comparison of means between two groups. These comparisons documented significant decreases in the levels of C1, C4, and C3 ($P < 0.001$, < 0.001 , and < 0.005 , respectively)

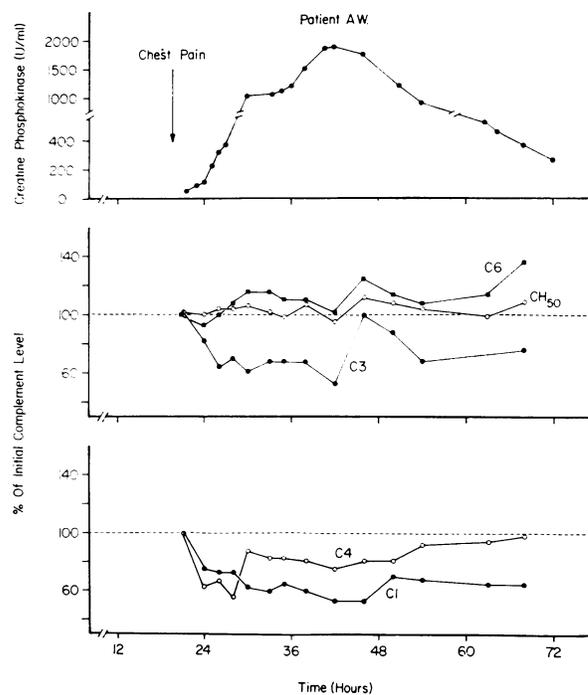


FIGURE 3 Temporal changes in the percent decreases in the levels of C1, C4, C3, C6, and CH₅₀ in relation to the elevation of the serum CPK in patient A. W. This patient was documented to have experienced unequivocal acute transmural myocardial infarction. The time scale in this figure is constructed so that 12 h represents noon on the day of admission and 72 h represents midnight of the 3rd day.

TABLE VII
*Statistical Comparison (Student's *t*) of Maximum Complement Decreases during the 72-h Period Following Hospitalization in Patients with and without Acute Myocardial Infarction*

	Mean \pm SD of percent maximum decrease of complement components				
	CH ₅₀	C1	C4	C3	C6
Patients with acute myocardial infarction	18.6 \pm 10.9 (<i>P</i> > 0.3)*	47.8 \pm 12.4 (<i>P</i> < 0.001)	37.2 \pm 15.0 (<i>P</i> < 0.001)	35.0 \pm 9.6 (<i>P</i> < 0.005)	7.3 \pm 6.4 (<i>P</i> > 0.9)
Control patients without myocardial infarction	12.3 \pm 10.0	12.2 \pm 6.6	12.8 \pm 6.0	13.8 \pm 8.5	7.0 \pm 4.7

* *P* value determined by the Student's *t* test for the comparison of means between two groups; the data utilized in these analyses are given in Tables V and VI.

between the patients with acute myocardial infarction and the control patients without myocardial infarction. No significant differences were noted between the two groups with respect to the maximum percent decreases in CH₅₀ or levels of C6.

DISCUSSION

Recent evidence indicates that myocardial cell necrosis following coronary artery occlusion can be reduced significantly by a variety of therapeutic interventions. Agents which decrease myocardial oxygen demand, increase the supply of oxygen to the myocardium, enhance anaerobic myocardial metabolism, or augment the transport of oxygen and substrates to ischemic cells seems to prevent or to attenuate the autolytic and heterolytic destruction of normal myocardial tissue located in the marginal zone of the infarct (1, 11). Although in-patient deaths from acute myocardial infarction due to primary arrhythmias have been reduced significantly, reduction in the morbidity and mortality due to the destruction of functional myocardial tissue following coronary artery occlusion until recently has not been possible. This latter point is of special importance in view of the correlation between infarct size and prognosis (12). Therefore, it is apparent that the molecular factors governing the evolution of infarct size after coronary artery occlusion warrant further investigation so that appropriate therapy may be developed to limit the degree of myocardial cell necrosis.

The manipulation of factors that affect the development of acute inflammation may prove to be relevant to the above considerations. Interventions have been designed to prevent the autolytic destruction of myocardial cells and the heterolytic destruction of normal myocardial tissue following the infiltration of inflammatory cells. For example, it has been demonstrated that corticosteroid administration can lead to the reduction of infarct size in the experimental animal (13, 14). Presumably the action of corticosteroids is mediated through their ability to stabilize membranes preventing autolysis and by their anti-inflammatory properties

which prevent the heterolytic destruction of normal myocardial tissue caused by infiltrating inflammatory cells, particularly the neutrophilic polymorphonuclear leukocyte. Another promising means of affecting the evolution of infarct size by suitable modification of acute inflammation derives from the ability of cobra venom factor to reduce the amount of myocardial damage after experimental myocardial infarction in dogs (15). Cobra venom factor activates the alternative complement pathway, resulting in significant depletion of C3, which would be expected to prevent a wide variety of inflammatory reactions especially involving the neutrophil. In addition, C3 depletion also would prevent the generation directly or indirectly of a variety of vasoactive substances.

The importance of complement in the development of acute myocardial inflammation following coronary artery occlusion has been studied in the rat. These studies have demonstrated that the infiltration of neutrophils after experimental myocardial infarction is dependent upon C3 (2). These studies also demonstrated that the C3-dependent neutrophil infiltration likely was initiated by the release of a C3 converting enzyme from damaged myocardial tissue and that such inflammation could be prevented experimentally by the administration of cobra venom factor. The C3-converting enzyme was shown to cleave directly either purified rat or human C3 resulting in the generation of C3 leukotactic factors. C3-converting activity was demonstrated in tissue minces of rat, heart, lung, and spleen (16) and in saline extracts of extensively homogenized rat heart tissue (2).

In view of the possibility that factors released from damaged myocardial tissue may be involved in the activation of the complement system and subsequent development of the acute inflammatory process, our laboratories have been studying a possible immunologic involvement in the pathogenesis of myocardial damage in a variety of cardiac diseases. Our initial interests centered around the temporal development of anti-heart autoantibody after experimentally induced myo-

cardial infarction in dogs (3). However, it was evident that due to the temporal development of the anti-heart mitochondria autoantibody, autoantibody was not involved in the initial inflammatory process occurring within the first 24–72-h period following coronary artery occlusion. Subsequently, we demonstrated a 19S complement-fixing, heat-labile factor present in all normal human serum, which was reactive with human heart mitochondria and sarcoplasmic reticulum. This serum factor reacted with autologous and homologous heart mitochondria but did not react with mitochondria derived from cardiac tissue of other species. Subsequent characterization indicated that this heat-labile factor was the first component of human complement. The C1 reacted with the mitochondrial membrane, was activated, and resulted in an apparent antibody-independent activation of the complement system (5).

The present studies were undertaken to characterize further the mitochondria-dependent activation of complement in human serum and to determine whether similar activation of serum complement also occurred in patients after acute myocardial infarction. Evidence was obtained for the direct binding of partially purified human C1 to the mitochondrial membrane by C1 fixation and elution experiments (Table I). It is likely that the binding of C1 to the mitochondrial membrane occurred through the C1q component since previous studies have demonstrated the ability of isolated mitochondria to selectively remove C1q from human sera in the presence of 0.01 M EDTA (5). These observations, together with the experiments which demonstrated that selective absorption of C1q from human serum prevented the mitochondria-dependent consumption of C4 (Table IV), indicated that direct C1 binding occurred on the mitochondrial membrane with subsequent activation of the C1 molecule.

Incubation of isolated human heart mitochondria with human serum at 37°C resulted in a consumption of C1, C4, C2, and C3 without significant consumption of the terminal components of the complement system, C6 through C9 (Table II). The mitochondria-dependent consumption of C4 was calcium dependent and was inhibited by the presence of 0.01 M EDTA or EGTA, (Table III). These data, together with the significant inhibition of C4 consumption in C1q-depleted serum (Table IV), indicated that the C4 consumption was dependent upon activation of C1 by mitochondria. The inhibition by EGTA of the mitochondria-dependent consumption of C3 indicated that the C3 consumption was not due to activation of the alternative complement pathway by heart mitochondria; under the same experimental conditions significant C3 consumption occurred after exposure of serum to zymosan (Table III). However, no decrease in C3 consumption occurred

in C1q-depleted serum, which suggests that the consumption of C3 was independent of C1 activation (Table IV). These experiments do not completely exclude the possible role of C1 since the C1q-depleted serum contained small amounts of functional C1 and some mitochondria-dependent consumption of C4 was noted. It is unlikely that the decreases in C3 in the serum exposed to mitochondria were due simply to C3 absorption on the mitochondrial membrane for two reasons. First, either EDTA or EGTA inhibited the mitochondria-dependent consumption of C3 in the serum. Second, in contrast to serum not exposed to mitochondria, serum exposed to mitochondria consistently revealed the conversion of $\beta 1c$ to $\beta 1a$. Therefore, it is possible that the mitochondria-dependent consumption of C3 may be analogous to the C3 consumption documented by Hill and Ward (2), who demonstrated a C3-converting enzyme derived from myocardial tissue that enzymatically fragmented human C3. Proteolytic consumption of C3 might explain the lack of C6–C9 consumption as it has been documented that the C3-dependent activation of C5 requires the formation of a $C4\bar{2}3$ complex or $C\bar{3}$ associated with factor \bar{B} of the alternative pathway (17).

After the characterization of the activation of the first four components of human complement by isolated human heart mitochondria, studies were performed to determine whether similar complement decreases occurred in patients after acute myocardial infarction. Sequential serum samples for six patients with unequivocal myocardial infarction were assessed for significant fluctuations in the functional levels of C1, C4, C3, C6, and CH_{50} during the 72-h period following hospitalization. The data demonstrated significant decreases in the functional levels of C1, C4, and C3 but not C6 or CH_{50} in all six patients. The initial precipitous decreases in the functional levels of C1, C4, and C3 occurred simultaneously with the initial elevation in serum CPK (Fig. 3). In general, the maximum decreases in the functional levels of these complement components occurred within 24 h after hospitalization. These maximum decreases in C1 and C4 occurred simultaneously in five of six patients with the maximum decrease in C3 occurring within hours of the maximum decreases in C1 and C4 (Table VI). In contrast, no significant decreases in functional levels of the complement components studied occurred in six control patients with chest pain but without myocardial infarction. The small fluctuations in complement components in the control patients did not change precipitously nor was there any apparent diurnal variation (Table V, Fig. 2). Statistical comparison of the maximum percent decreases in the functional levels of various complement components in the myocardial infarc-

tion and control groups during the first 72 h of hospitalization showed a high degree of significance with respect to C1, C4, and C3 but not with respect to CH₅₀ or C6 (Table VII).

The present studies are the first documentation of significant decreases in the functional levels of individual complement components in patients immediately after acute myocardial infarction. Alterations in total serum complement have been reported previously in patients after myocardial infarction (18). However, in these studies it was reported that patients with myocardial infarction had abnormally high levels of CH₅₀ 2-5 days after myocardial infarction. Recently it has been reported that hypercatabolism of various complement components in a variety of diseases is accompanied by a hypersynthesis of these same complement components (19). In view of the results of the present study it is possible that the elevated CH₅₀ levels previously reported (18) were due to an initial consumption of complement immediately after myocardial infarction with the subsequent compensatory hypersynthesis of these complement components.

We have demonstrated that isolated human heart mitochondria can activate the first four components of human complement in human serum in vitro and that similar decreases in the functional levels of these serum complement components occur in patients after acute myocardial infarction. Although these data indicate the intravascular activation of the first four components of complement after acute myocardial infarction, unequivocal documentation for the intravascular consumption of plasma complement can only be obtained by measuring the fractional catabolic rates of these components (19) and/or documenting the development of circulating complement conversion products. The decreases of individual complement components both in vitro and in vivo in the present studies could not be explained on the basis of the presence of anti-heart mitochondria autoantibody. Whether the release of subcellular organelles from damaged myocardial tissue is responsible for the antibody-independent decrease in the functional levels of individual complement components in patients after myocardial infarction is not known at present. Certainly a wide variety of substances have been reported to activate C1 or C3 in the absence of detectable antibody and include single- and double-stranded DNA, various polynucleotides, proteolytic enzymes such as trypsin and plasmin, endotoxin, certain viruses, polyacations, and C-reactive protein (17). Regarding C-reactive protein, it is noteworthy that the initial and maximum decreases in C1, C4, and C3 reported in the present studies occurred before the reported increase in C-reactive protein and other acute phase reactants in myocardial infarction patients (20).

In view of the recent evidence indicating the role of complement in the development of acute myocardial inflammation and evolution of infarction size (1, 2, 15), the molecular mechanisms governing the activation of complement by heart subcellular organelles warrants further investigation. Such studies might provide new information regarding potential therapeutic interventions which could selectively inhibit the activation of complement after heart damage, thereby decreasing the amount of myocardial cell necrosis following coronary artery occlusion.

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REFERENCES

1. Braunwald, E., and P. R. Maroko. 1974. The reduction of infarct size—an idea whose time (for testing) has come. *Circulation*. **50**: 206-209.
2. Hill, J. H., and P. A. Ward. 1971. The phlogistic role of C3 leukotactic fragments in myocardial infarcts of rats. *J. Exp. Med.* **133**: 885-900.
3. Pinckard, R. N., M. S. Olson, R. A. O'Rourke, J. D. Palmer, R. E. Kelley, and S. Goldfein. 1971. Development of complement-fixing 19S, anti-heart mitochondria autoantibody following myocardial infarction in dogs. *Circ. Res.* **29**: 276-285.
4. Kelley, R. E., M. S. Olson, and R. N. Pinckard. 1974. Characterization of an anti-heart mitochondria autoantibodies produced in dogs following myocardial infarction. *Circ. Res.* **35**: 862-870.
5. Pinckard, R. N., M. S. Olson, and R. E. Kelley, D. H. DeHeer, J. D. Palmer, R. A. O'Rourke, and S. Goldfein. 1973. Antibody-independent activation of human C1 after interaction with heart subcellular membranes. *J. Immunol.* **110**: 1376-1382.
6. Chance, B., and G. R. Williams. 1956. The respiratory chain and oxidative phosphorylation. *Adv. Enzymol.* **17**: 65-134.
7. Rapp, H. J., and J. Borsos. 1970. Molecular Basis of Complement Action. Appleton-Century-Crofts, New York. 164 pp.
8. Borsos, T., and H. J. Rapp. 1967. Immune hemolysis: a simplified method for the preparation of EAC'4 with guinea pig or with human complement. *J. Immunol.* **99**: 263-268.
9. Dalmasso, A. P., and H. J. Müller-Eberhard. 1966. Hemolytic activity of lipoprotein-depleted serum and the effect of certain anions on complement. *J. Immunol.* **97**: 680-685.
10. Cooper, N. R., M. J. Polley, and H. J. Müller-Eberhard. 1971. Biology of complement. In *Immunological Disease*. M. Samter, editor. Little, Brown and Company, Boston. 2nd edition. 289-331.
11. Maroko, P. R., and E. Braunwald. 1973. Modification of myocardial infarction size after coronary occlusion. *Ann. Intern. Med.* **79**: 720-733.
12. Sobel, B. E., G. F. Bresnahan, W. E. Shell, and R. D. Yoder. 1972. Estimation of infarct size in man and its relation to prognosis. *Circulation*. **46**: 640-648.

13. Spath, J. A., Jr., D. L. Lane, and A. M. Lefer. 1974. Preservation of cardiac cellular integrity by methylprednisolone in acute myocardial ischemia. *Am. J. Cardiol.* **33**: 171. (Abstr.)
14. Libby, P., P. R. Maroko, C. M. Bloor, B. E. Sobel, and E. Braunwald. 1973. Reduction of experimental myocardial infarct size by corticosteroid administration. *J. Clin. Invest.* **52**: 599-607.
15. Maroko, P. R., and C. B. Carpenter. 1974. Reduction in infarct size following acute coronary occlusion by the administration of cobra venom factor. *Clin. Res.* **22**: 289. (Abstr.)
16. Hill, J. H., and P. A. Ward. 1969. C3 leukotactic factors produced by a tissue protease. *J. Exp. Med.* **130**: 505-518.
17. Cooper, N. R. 1973. Activation of the complement system. *In Contemporary Topics in Molecular Immunology*. R. A. Reisfeld and W. J. Mandy, editors. Plenum Publishing Corporation, New York. **2**: 155-183.
18. Boltax, A. J., and E. E. Fischel. 1956. Serologic tests for inflammation. Serum complement, C-reactive protein and erythrocyte sedimentation rate in myocardial infarction. *Am. J. Med.* **20**: 418-427.
19. Ruddy, S., C. B. Carpenter, K. W. Chin, J. N. Knostman, N. A. Soter, O. Götze, H. J. Müller-Eberhard, and K. F. Austen. 1975. Human complement metabolism: an analysis of 144 studies. *Medicine (Baltimore)*. **54**: 165-178.
20. Johansson, B. G., C. O. Kindmark, E. Y. Trelle, and F. A. Wollheim. 1972. Sequential changes of plasma proteins after myocardial infarction. *Scand. J. Clin. Lab. Invest. Suppl. 124*. **29**: 117-126.