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CHARACTERIZATION OF AUTOANTIGENIC SITES ON ISOLATED DOG HEART MITOCHONDRIA

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SUMMARY

1. Anti-heart mitochondria autoantibodies were developed in serum from dogs following experimental myocardial infarction.

2. Heart mitochondria frozen and thawed repeatedly in a sucrose/Tris-chloride buffer retained both their functional integrity as measured by the respiratory control ratio and their ability to serve as an antigen in a complement fixation test. Mitochondria frozen and thawed in a potassium chloride/Tris-chloride buffer lost both their functional integrity and their autoantigenic activity after one freeze-thaw cycle.

3. Extraction of the heart mitochondria with acetone/water mixtures to remove phospholipids from the membrane led to a complete loss of the ability of the mitochondria to react in the complement fixation test but did not affect the ability of the membranes to bind autoantibody in absorption experiments.

4. Treatment of the mitochondrial membranes with increasing concentrations of trypsin caused a loss of up to approximately 50 % of the membrane protein with a gradual decrease in the autoantigenic activity of the membrane without impairment of the ability of the membrane to bind autoantibody.

5. Removal of up to 90 % of the sialic acid of the mitochondrial membrane with neuraminidase resulted in a considerable increase in the complement-fixing autoantigenic activity of the membrane without changing the apparent ability of the membrane to bind autoantibody in absorption experiments.

6. Exposure of mitochondrial membranes to autoantibody and complement caused an inhibition of both an inner mitochondrial membrane enzyme, i.e. cytochrome oxidase (48 %) and an outer mitochondrial membrane enzyme, i.e. NADH-cytochrome *c* reductase (rotenone insensitive) (37 %).

INTRODUCTION

Various studies have been conducted to characterize the temporal development and specificities of autoantibodies produced following acute hepatocellular necrosis [1–5]. Although the primary intracellular autoantigenic loci were reported initially to be in the mitochondrial fraction, subsequent studies in our laboratory indicated that

apparently unique autoantibodies develop to each liver subcellular membrane except the plasma membrane following various forms of liver cell damage [5]. Anti-heart autoantibody development following cardiac cell damage has not been as well delineated in the literature as has the liver system. Autoantibodies directed toward subcellular membranes derived from cardiac tissue develop in high titers in the serum of dogs which have undergone experimental myocardial infarction [6–8]. Various subcellular membranes react with these autoantibodies, the outer mitochondrial membrane being the most reactive on a mg protein basis. Inner and outer mitochondrial membranes possess autoantigens with little or no cross-reaction. The generation of an autoantibody showing specificity for an individual subcellular membrane represents an intriguing possibility for probing the surface structure of the membrane using immunological procedures. Considerable information has been developed concerning the antigenic mosaics on the surface of the erythrocyte [9–11] (i.e. using the blood group antigens) and other types of cellular membranes [12–14] (i.e. using the histocompatibility antigens). The present study represents an attempt to characterize the properties of the autoantigenic sites on cardiac mitochondrial membranes using autoantibodies developed following experimentally induced myocardial infarction in dogs.

MATERIALS AND METHODS

Serum samples containing autoantibody directed toward isolated dog heart mitochondrial membranes were obtained from dogs in which acute myocardial infarction had been induced by the injection of Dowex microspheres directly into the left coronary artery by a procedure described previously [6, 7]. The temporal development of the anti-heart mitochondria autoantibody was assessed by a micro-complement fixation assay using a microtiter apparatus (Cooke Engineering Co., Alexandria, Virginia). The specific details of this procedure also have been described previously [7].

A more accurate spectrophotometric complement fixation assay was used in most of the experiments detailed in this paper [8]. Serum used in this assay was diluted appropriately in barbital-buffered saline, pH 7.3, containing sodium chloride, 0.142 M; sodium barbital, 5.0 mM; magnesium chloride, 0.5 mM; calcium chloride, 0.15 mM and bovine serum albumin 0.2 %.

To 0.1 ml of diluted serum was added 0.1 ml of a minimal hemolytic dose of guinea pig complement, followed by 0.1 ml of the appropriate antigen in the same buffer described above. The mixture was shaken and incubated for 30 min in a 37 °C water bath, after which 0.1 ml of the sensitized sheep cell suspension ($1 \cdot 10^8$ cells/ml) was added. The mixture was incubated for 1 h in a 37 °C water bath with shaking every 15 min. At the end of the incubation period, 0.7 ml of a 1 : 20 dilution of a solution containing sodium chloride, 0.3 M and EDTA, 0.2 M was added to each tube, and the tubes were then centrifuged at $500 \times g$ for 5 min. The supernatants were read at 412 nm and the absorbances were recorded. The percent lysis was determined by comparison of the experimental values with that of a 100 % lysis control (containing 0.1 ml each of barbital-buffered saline, complement, antigen and sheep red blood cells). The 50 % hemolytic end-points were determined by graphing by the von Krogh equation as described by Kabat and Mayer [15].

Mitochondria were prepared from dog cardiac tissue obtained from mongrel

dogs at sacrifice using fractionation and centrifugation procedures described previously [7]. The composition of the solution which was used in each mitochondrial preparation depended upon the individual experiment and is noted in each case. The protein concentrations of the mitochondrial preparations were estimated using the procedure described by Lowry [16].

The metabolic integrity of the various mitochondrial preparations was assessed by determining the respiratory control ratio as defined by Chance and Williams[17] using a Clark-type oxygen electrode. The incubation medium for the mitochondria contained potassium chloride, 0.18 M, potassium phosphate, 2 mM, and EDTA, 0.2 mM, at a pH of 7.2. Each incubation contained 2.5 ml of the incubation medium, approximately 5 mg of mitochondrial protein and pyruvate plus L-malate at a final concentration of 2.5 mM. The consumption of oxygen was initiated by the addition of ADP, 0.25 mM.

In the freeze-thaw experiments dog heart mitochondria were rapidly frozen using an acetone/solid CO_2 bath. The mitochondria were thawed following freezing by immersion in a 37 °C water bath.

Absorption of dog serum containing anti-heart mitochondria autoantibody with dog heart mitochondria was accomplished by resuspending a mitochondrial pellet containing 2 mg mitochondrial protein in 1 ml of dog serum. This suspension was incubated for 1 h at 0 °C and centrifuged for 10 min at 14 000 × *g* to pellet the mitochondria. The supernatant from this centrifugation consisted of dog serum depleted to varying degrees of the anti-heart mitochondria autoantibody.

Dog heart mitochondria were extracted with a 12 % (v/v) water in acetone mixture according to the procedure described by Lester and Fleischer [18]. The acetone/water mixture (6.0 ml) was added dropwise to approx. 10 mg of mitochondrial protein. Following a 5 min incubation at room temperature, the mixture was centrifuged at 14 000 × *g* for 10 min; the mitochondrial pellet was resuspended in an identical volume of the acetone/water mixture and the procedure was repeated. The resulting mitochondrial pellet was resuspended in sucrose, 0.24 M, Tris-chloride, 0.01 M, pH 7.4, and washed three times by centrifugation at 14 000 × *g* for 10 min. Control mitochondria were treated in exactly the same manner except that deionized water was substituted for the acetone/water mixture.

Dog heart mitochondria also were extracted with dilute acid by a procedure described by Lenaz et al. [19]. Dog heart mitochondria were washed twice with nine volumes of deionized water at 4 °C. The washed mitochondria were then extracted with nine volumes of 10 mM HCl for 5 min at 4 °C. Following the extraction, the mitochondria were centrifuged at 39 000 × *g* for 20 min. The resulting pellet was washed twice with sucrose, 0.24 M, Tris-chloride, 0.01 M, pH 7.4, and the resulting pellet was resuspended in the sucrose/Tris-chloride buffer. Control mitochondria were subjected to all procedures except that deionized water was used in place of the HCl.

The procedures for treatment of the dog heart mitochondria with trypsin and neuraminidase are described in Results. The cytochrome oxidase activity of the mitochondria was determined using the procedure of Schnaitman and Greenawalt [20]. NADH-cytochrome *c* reductase (rotenone insensitive) was assayed according to the procedure of Sottocasa et al. [21].

RESULTS

Following the induction of acute myocardial infarction in dogs a 19 S, IgM, complement fixing autoantibody reactive with isolated cardiac subcellular membranes developed in the serum of dogs 6–10 days following infarction [7, 8]. Sized microspheres (297–350 μm in diameter [6]) were injected directly into the coronary circulation of mongrel dogs and the severity of the cardiac damage was monitored by changes in serum creatine phosphokinase levels, by evolutionary electrocardiographic changes and by postmortem examination of the cardiac tissue. Fig. 1 illustrates the temporal development of the complement-fixing, anti-heart mitochondria autoantibody in a dog which is representative of the group of infarcted animals. The induced autoantibody persisted in the serum of dogs for 3–6 weeks following the heart damage after which the levels of autoantibody returned to very low or undetectable levels.

Previous studies [7] demonstrated that the principal autoantigenic locus was the mitochondrial fraction of dog heart, the outer mitochondrial membrane being considerably more reactive with the autoantibody on a mg protein basis. In addition, the inner and outer mitochondrial membranes were shown to have unique autoantigenic specificities in absorption experiments.

During the course of these studies it was observed that dog heart mitochondria which were stored frozen in a solution consisting of KCl, 0.18 M, Tris-chloride, 0.01 M, pH 7.4, were nearly unreactive with the anti-heart autoantibody using the complement fixation assay to test the interaction between the autoantibody and the mitochondrial membrane. However, if the mitochondria were stored in a solution containing sucrose, 0.24 M, Tris-chloride, 0.01 M, pH 7.4, the membranes were always strongly autoantigenic in the test system after freezing. This observation suggested that the mitochondrial membrane autoantigen(s) were sensitive to freezing and thawing in buffers containing potassium ions.

An experiment was performed to attempt to substantiate this observation and the results are shown in Tables I and II. Mitochondria were prepared from dog heart by homogenizing half of the tissue in a sucrose/Tris-chloride buffer and the other half in a KCl/Tris-chloride buffer. Following isolation, the mitochondria prepared in each buffer were divided into two equal portions and resuspended in the

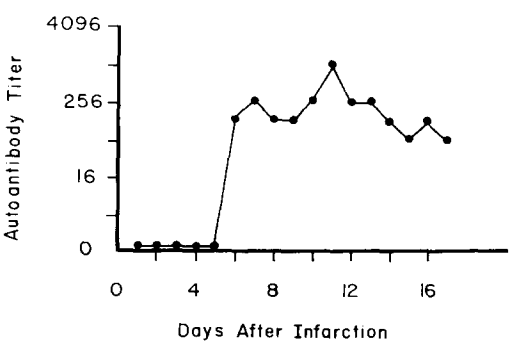

Fig. 1. The temporal development of antiheart mitochondria autoantibody in the serum of a dog following experimental myocardial infarction. Serum samples were obtained and the assay for autoantibody were performed as described in Methods.

TABLE I

EFFECT OF SUCCESSIVE FREEZE-THAW CYCLES ON THE RESPIRATORY CONTROL RATIO OF DOG HEART MITOCHONDRIA

Isolation buffer	Resuspension buffer	Number of freeze-thaw cycles				
		Unfrozen control	1	2	3	4
		Respiratory control ratio				
Sucrose/Tris-chloride**	Sucrose/Tris-chloride	4.6*	3.8	3.0	1.9	0
Sucrose/Tris-chloride	KCl/Tris-chloride**	6.0	0	0	0	0
KCl/Tris-chloride	KCl/Tris-chloride	4.2	0	0	0	0
KCl/Tris-chloride	Sucrose/Tris-chloride	5.9	3.7	0	0	0

* Determined with pyruvate + malate as substrates.

** The compositions of the isolation and resuspension buffers were (a) sucrose, 0.24 M, Tris-chloride, 0.01 M pH, 7.4 and (b) KCl, 0.18 M, Tris-chloride, 0.01 M pH, 7.4.

TABLE II

THE EFFECT OF SUCCESSIVE FREEZE-THAW CYCLES ON THE ANTIGENICITY OF ISOLATED DOG HEART MITOCHONDRIA

Isolation buffer	Resuspension buffer	Number of freeze-thaw cycles				
		Unfrozen control	1	2	3	4
		Autoantibody titer				
Sucrose/Tris-chloride**	Sucrose/Tris-chloride	128*	512	64	0	0
Sucrose/Tris-chloride	KCl/Tris-chloride**	64	0	0	0	0
KCl/Tris-chloride	KCl/Tris-chloride	64	0	0	0	0
KCl/Tris-chloride	Sucrose/Tris-chloride	64	64	0	0	0

* Mitochondria were used in the complement fixation test at a concentration of 1 mg/ml mitochondrial protein.

** The compositions of the isolation and resuspension buffers were (a) sucrose, 0.24 M, Tris-chloride 0.01 M pH 7.4 and (b) KCl, 0.18 M, Tris-chloride, 0.01 M, pH 7.4.

isolation buffer or the alternate buffer. The mitochondria were then subjected to rapid freezing in an acetone/solid CO_2 bath and to rapid thawing in a water bath at 37 °C. Following each freeze-thaw cycle, a portion of the mitochondria was removed and the respiratory control ratio was determined using pyruvate plus L-malate as substrates to assess the functional integrity of the mitochondrial preparation. The mitochondria were also tested for their autoantigenic activity in the complement fixation assay, using serum from the dog shown in Fig. 1 which contained a high titer of the anti-heart mitochondria autoantibody. Dog heart mitochondria which were resuspended in either sucrose/Tris-chloride buffer or KCl/Tris-chloride buffer but which were unfrozen maintained reasonably high respiratory control ratios (e.g. 4.2–6.0) and were strongly antigenic when they were used as the test antigen in the complement fixation assay (See Tables I and II). Also, as can be seen in Tables I and II, mitochon-

TABLE III

EFFECT OF SUCCESSIVE FREEZE-THAW CYCLES ON THE ANTIGENICITY OF DOG HEART MITOCHONDRIA RESUSPENDED IN VARIOUS BUFFERS**

Resuspension buffer	Number of freeze-thaw cycles				
	Unfrozen control	1	2	3	4
	Autoantibody titer				
Sucrose/Tris-chloride**	64*	64	64	64	128
KCl/Tris-chloride	64	32	0	0	0
NaCl/Tris-chloride	128	128	128	128	128
Tris-chloride	128	64	64	64	0

* Mitochondria were used in the complement fixation test at a concentration of 1 mg/ml mitochondrial protein.

** The compositions of the various resuspension buffers were (a) sucrose, 0.24 M, Tris-chloride, 0.01 M pH 7.4; (b) KCl, 0.18 M, Tris-chloride, 0.01 M, pH 7.4 and (c) NaCl, 0.18 M, Tris-chloride, 0.01 M, pH 7.4 and Tris-chloride 0.01 M, pH 7.4.

dria which were resuspended and subsequently frozen and thawed in the sucrose/Tris-chloride buffer were somewhat stable to freezing and thawing when assayed for both their respiratory control ratio and for their autoantigenicity. However, mitochondria which were resuspended, frozen and thawed in the KCl/Tris-chloride buffer were found to have lost their functional integrity in addition to losing their autoantigenic activity in the complement fixation test system.

To determine whether the dog heart mitochondrial autoantigens were stable to freezing and thawing in buffers of different composition, the experiment shown in Table III was performed. Mitochondria were isolated from dog cardiac tissue in sucrose/Tris-chloride buffer and were resuspended prior to freezing in the buffers noted in Table III. The various suspensions of mitochondria were then subjected to freeze-thaw cycles and tested for antigenicity as described above. The results indicated that mitochondria lost their ability to serve as a test antigen in the complement fixation test only when they were frozen and thawed in a buffer containing KCl. Resuspension media containing sucrose/Tris-chloride, NaCl/Tris-chloride or Tris-chloride alone all seemed to preserve significantly the autoantigenic activity of the mitochondrial preparation.

An apparent decrease in the autoantigenic activities of the mitochondrial preparations during freeze-thaw cycles may be due to at least two effects. First, freezing and thawing in potassium chloride solutions may be causing an alteration of ability of the membranes to bind the autoantibody. Alternatively, this treatment may not affect the autoantigenic binding sites but may result in spacial alteration of the membrane leading to a change in the ability of the autoantibody-membrane complex to bind and/or activate complement in the assay system. An experiment to test these two possibilities was performed and the results are shown in Table IV.

Serum containing anti-heart mitochondria autoantibody was absorbed with isolated dog heart mitochondria at a concentration of 2 mg of mitochondrial protein per ml of serum. (See Methods for procedure.) The serum then was tested for autoantibody activity using the complement fixation test with unfrozen mitochondria as

TABLE IV

THE EFFECT OF ABSORPTION OF DOG SERUM WITH FROZEN AND THAWED DOG HEART MITOCHONDRIA ON THE ANTI-HEART MITOCHONDRIA AUTOANTIBODY TITER

Resuspension buffer	Unabsorbed serum	Number of freeze-thaw cycles of absorbing mitochondria				
		Unfrozen	1	2	3	4
		50 % Hemolytic end point of absorbed serum				
Sucrose/Tris-chloride	135*	66**	58	76	78	66
KCl/Tris-chloride	143	72	78	76	80	90

* Unfrozen mitochondria were used as test antigen in the complement fixation test at a concentration of 1 mg/ml mitochondrial protein; the test mitochondria used in the complement fixation test were prepared and resuspended in 0.24 M sucrose, 0.01 M Tris, pH 7.4.

** Serum was absorbed at a mitochondrial protein concentration of 2 mg/ml of serum.

the test antigen. Whereas the unabsorbed serum exhibited a 50 % end point in the hemolytic test system of 135–143, absorption of the serum with heart mitochondria which had been resuspended in either sucrose/Tris-chloride or in KCl/Tris-chloride diminished the autoantibody titer of the serum by approximately 50 %. Sequential freeze-thaw cycles of the mitochondrial suspensions in either of the two resuspending buffers did not change appreciably the ability of the mitochondrial preparation to absorb the autoantibody from the serum. These experiments indicate that freezing and thawing of the mitochondria in KCl-containing buffer resulted in a diminished ability of the membrane-autoantibody complex to activate complement in the test system.

In order to determine whether the autoantigenicity of dog heart mitochondria was stable to thermal treatment, the experiment described in Table V was performed. Mitochondria at identical protein concentrations were incubated for 30 min in water

TABLE V

EFFECT OF THERMAL TREATMENT ON THE AUTOANTIGENIC ACTIVITY OF DOG HEART MITOCHONDRIA

Treatment of mitochondria	Unabsorbed serum	Absorbed serum*
		50 % Hemolytic end points
Unheated control mitochondria	253**	79***
37 °C, 30 min	255	83
56 °C, 30 min	275	85
75 °C, 30 min	Anticomplementary	85
85 °C, 30 min	148	103

* Serum containing dog anti-heart mitochondria autoantibody was absorbed with 2 mg/ml of the appropriate mitochondrial protein.

** The respective mitochondrial suspension was used as antigen in the complement fixation test at a concentration of 1 mg/ml.

*** Mitochondria prepared in 0.24 M sucrose, 0.01 M Tris, pH 7.4 were used as the test antigen in the complement fixation test with the absorbed serum at a protein concentration of 1 mg/ml.

baths at 37, 57, 70 and 85 °C. The heat-treated mitochondria were tested both for their ability to fix complement following the binding of autoantibody, and for their ability to remove autoantibody from serum in absorption experiments.

The results shown in Table V indicate that incubation of mitochondria at 37 and 56 °C for 30 min did not affect significantly the ability of the treated mitochondria to function as antigen in either of the experimental systems used. Mitochondria incubated at 70 °C became anticomplementary when used as antigen in the complement fixation test, but their ability to remove autoantibody from dog serum in absorption experiments was unimpaired. Mitochondria incubated at 85 °C showed a decreased ability both to serve as antigen in the complement fixation test and to remove autoantibody in absorption experiments. These results indicate that the mitochondrial autoantigens which react with the anti-heart autoantibody produced following experimental myocardial infarction are reasonably thermostable to a temperature of 85 °C.

In order to partially characterize the biochemical nature of the autoantigenic property of isolated dog heart mitochondrial membranes various types of extractions and/or treatments were performed. Lester and Fleischer [18] reported that up to 75 % of the phospholipid content of beef heart mitochondria can be removed by extraction with a 12 % (v/v) acetone/water mixture. Dog heart mitochondria were extracted according to this procedure and the extracted mitochondria were tested for antigenicity in the complement fixation assay system and for their ability to absorb anti-heart autoantibody from dog serum in absorption experiments. Dog heart mitochondria also were extracted with dilute acid (HCl) by a procedure described by Lenaz et al. [19]. Lenaz reported that up to 50 % of the protein content of beef heart mitochondria could be extracted by treatment of the mitochondria with 10 mM HCl.

Results of protein determinations indicated that the extraction of dog heart mitochondria with 10 mM HCl resulted in the removal of 32 % of the total mitochondrial protein (data not shown). Control mitochondria were subjected to all procedures except that deionized water was used in place of the HCl or acetone/water for extraction. The control mitochondria showed no appreciable loss of protein. The control mitochondria and the extracted mitochondria were tested for antigenicity in the

TABLE VI

THE EFFECT OF EXTRACTION WITH WATER, HCl, AND ACETONE/WATER ON THE AUTOANTIGENIC ACTIVITY OF DOG HEART MITOCHONDRIA

Extraction procedure	Unabsorbed serum*	Absorbed serum**
	50 % Hemolytic end points	
Untreated control	253	79
Water	205	80
Acetone/water	No activity	95
HCl	Anticomplementary	55

* 1 mg/ml of the appropriate mitochondrial suspension was used as antigen in the complement fixation test.

** Serum containing dog anti-heart mitochondria autoantibody was absorbed with 2 mg/ml of mitochondrial protein, then tested in the complement fixation test with 1 mg/ml of mitochondria which had been prepared and stored in 0.24 M sucrose, 0.01 M Tris, pH 7.4.

complement fixation test and for their ability to remove anti-heart autoantibody from dog serum in absorption experiments.

The results of these various extraction experiments are shown in Table VI and indicate that extraction of dog heart mitochondria with acetone/water resulted in the complete loss of autoantigenic activity when tested in the complement fixation test; however, these acetone/water-extracted mitochondrial membranes were still able to remove anti-heart autoantibody from dog serum when used as the absorbing species in absorption experiments. Treatment of dog heart mitochondria with dilute HCl caused the mitochondria to become anticomplementary when tested in the complement fixation test, but seemed to increase their ability to absorb anti-heart autoantibody when compared with the control mitochondria which were extracted with water.

TABLE VII

THE AUTOANTIGENIC ACTIVITY OF DOG HEART MITOCHONDRIA FOLLOWING TREATMENT WITH TRYPSIN

Trypsin concentration (μg/mg mitochondrial protein)	Mitochondrial protein remaining (mg)	Unabsorbed serum*	Absorbed serum**
		50 % Hemolytic end points	
0	9.1	213	85
1	6.0	165	89
10	5.5	135	84
50	5.5	100	88
100	5.1	Anticomplementary	95

* 1 mg/ml of the trypsin-treated mitochondrial suspension was used as antigen in the complement fixation test.

** Dog serum containing anti-heart autoantibody was absorbed with 2 mg/ml of the trypsin-treated mitochondrial preparation, and the serum was tested for the presence of autoantibody with 1 mg/ml of an untreated mitochondrial suspension prepared and stored in sucrose/Tris-chloride buffer.

TABLE VIII

THE AUTOANTIGENIC ACTIVITY OF DOG HEART MITOCHONDRIA FOLLOWING NEURAMINIDASE TREATMENT

Neuraminidase concentration (μg/mg mitochondrial protein)	% Sialic acid removed	Unabsorbed*	Absorbed**
		50 % Hemolytic end point	
0	12	213	66
1	56	270	70
10	84	400	73
50	88	400	67
100	92	400	65

* 1 mg/ml of the neuraminidase-treated mitochondrial suspension was used as antigen in the complement fixation test.

** Dog serum containing anti-heart autoantibody was absorbed with 2 mg/ml of the neuraminidase-treated mitochondria, then tested in the complement fixation test with 1 mg/ml of untreated mitochondria.

In order to determine the susceptibility of the mitochondrial autoantigens to enzymatic treatment, the experiments described in Tables VII and VIII were performed. Freshly prepared mitochondria were incubated at 37 °C for 30 min in a buffer consisting of KCl, 0.18 M, Tris-chloride, 0.01 M, pH 7.0, which contained 0, 1, 10, 50 and 100 μg trypsin/mg mitochondrial protein. Following the incubation period the mitochondria were centrifuged at 14 000 × *g* for 15 min. The mitochondrial pellets were washed extensively in sucrose, 0.24 M, Tris-chloride buffer, 0.01 M, pH 7.4. The results shown in Table VII show that trypsin treatment resulted in the removal of up to 45 % of the total mitochondrial protein. The effect of the removal of the mitochondrial protein by trypsinization on the antigenicity of the mitochondria is also shown in Table VII. Trypsin digestion of dog heart mitochondria resulted in a decrease in the ability of the mitochondria to function as antigen in the complement fixation test; however, only a slight decrease in the ability of the mitochondria to remove anti-heart autoantibody from dog serum was found in the absorption experiments.

Mitochondria were treated with neuraminidase to determine the effect of the loss of sialic acid residues on the autoantigenicity of the mitochondrial membranes. A modification of the method of Bosmann et al. [22] for the treatment of liver and cerebral cortex mitochondria with neuraminidase was used. Mitochondria were incubated in a buffer which consisted of KCl, 0.1 M, citrate, 0.05 M, pH 5.0, containing neuraminidase at concentrations of 0, 1, 10, 50 and 100 μg/mg mitochondrial protein. The mitochondria were incubated at 37 °C for 30 min followed by centrifugation at 14 000 × *g* for 10 min. The supernatants were removed and the sialic acid content was determined using the method of Warren [23]. The mitochondrial pellets were washed extensively in sucrose, 0.25 M, Tris-chloride, 0.01 M, pH 7.4, and were resuspended in the same buffer.

Dog heart mitochondria were found to contain 2.5 μg sialic acid/mg protein and the effect of neuraminidase treatment of the mitochondria is shown in Table VIII. The control mitochondria, which were treated in the same manner as the experimental mitochondria except they were incubated in buffer which contained no enzyme, lost 15 % of their total sialic acid content while treatment with neuraminidase (100 μg/mg protein) removed up to 92 % of the total sialic acid present on the mitochondrial membranes. There was no loss of mitochondrial protein during the procedure. Treatment with neuraminidase resulted in a significant increase in the ability of the mitochondria to function as antigen in the complement fixation test. However, the same mitochondria did not show a similar increase in the ability to remove anti-heart autoantibody in the absorption experiments.

Finally, an attempt was made to determine whether or not the interaction or binding of anti-heart autoantibody would result in alterations in the activity of various enzymes associated with mitochondrial membranes. Also, the effect of guinea pig complement on the same enzymes was studied. The enzymes tested were cytochrome oxidase, an established inner mitochondrial membrane marker, and NADH-cytochrome *c* reductase (rotenone insensitive), an enzyme found primarily in the outer mitochondrial membrane. Dog heart mitochondria were incubated in the presence of dog anti-heart autoantibody, in the presence of guinea pig complement or in the presence of both complement and autoantibody. The concentration of mitochondria was adjusted to 1 mg/ml and the anti-heart mitochondria autoantibody was present

TABLE IX

THE EFFECT OF ANTI-HEART MITOCHONDRIA AUTOANTIBODY ON THE ACTIVITY OF DOG HEART MITOCHONDRIA CYTOCHROME OXIDASE

Sample	Cytochrome oxidase activity (nmol oxygen/min per mg protein)	% Reduction
Mitochondria + NDS*	440.1†	
Mitochondria + C'** + Ab***	227.2	48.4
Inner membrane + NDS	364.9	
Inner membrane + C'	325.6	10.8
Inner membrane + Ab	321.3	11.9
Inner membrane + C' + Ab	207.7	43.1

* Normal dog serum containing no anti-heart autoantibody at a dilution of 1 : 32.
** Guinea pig complement present at a final dilution of 1 : 32.
*** Dog serum containing anti-heart mitochondria autoantibody present at a final dilution of 1 : 32.
† The cytochrome oxidase was not activated prior to assay.

at a final dilution of 1 : 32 (a concentration which gave a strong reaction in the complement fixation test), and a 1 : 30 dilution of guinea pig complement was used. All dilutions were made in the complement fixation test buffer described in Materials and Methods. Control mitochondria were treated in identical fashion except that the only addition was normal dog serum which did not possess anti-heart autoantibody. No activation of the enzymes was performed prior to assay; all other conditions for assaying the enzymes were conducted as described in Methods.

The results of an experiment in which cytochrome oxidase was assayed in whole mitochondria and in isolated inner mitochondrial membrane are shown in Table IX. In the case of cytochrome oxidase, a reduction in the activity of the enzyme was seen only when complement and autoantibody were present in the incubation medium. Incubation of mitochondria or isolated inner mitochondrial membrane

TABLE X

THE EFFECT OF ANTI-HEART MITOCHONDRIA AUTOANTIBODY ON THE ACTIVITY OF DOG HEART MITOCHONDRIA NADH-CYTOCHROME *c* REDUCTASE (ROTENONE INSENSITIVE)

Sample	NADH-cytochrome *c* reductase activity (nmol/min per mg protein)	% Reduction
Mitochondria + NDS*	0.78†	
Mitochondria + C'** + Ab***	0.64	17.9
Outer membrane + NDS	0.55	
Outer membrane + C'	0.44	20.0
Outer membrane + Ab	0.46	16.4
Outer membrane + C' + Ab	0.35	36.7

* Normal dog serum containing no anti-heart mitochondrial autoantibody at a dilution of 1 : 32.
** Guinea pig complement at a final dilution of 1 : 30.
*** Dog serum containing anti-heart autoantibody at a final dilution of 1 : 32.
† The enzyme activities were obtained without prior activation.

with complement or autoantibody alone did not produce significant inhibition of the enzyme activity.

The results of the incubation of anti-heart autoantibody with dog heart mitochondria or with isolated outer mitochondrial membrane on the activity of NADH-cytochrome *c* reductase (rotenone insensitive) are shown in Table X. Both experiments indicate that the presence of anti-heart autoantibody produced a reduction in the activity of the enzyme when compared to control values. Guinea pig complement also caused a reduction in the enzyme activity observed. The presence of both complement and autoantibody in the incubation medium caused an increase in the inhibition relative to the individual components (36.7 % vs 20 % and 16 % for complement and autoantibody individually, respectively).

DISCUSSION

The development of autoantibodies directed toward various tissue components following cellular necrosis appears to be a normal physiological process [3]. Previous studies in our laboratory demonstrated that hepatocellular necrosis induced in rats by a variety of techniques led to the production of complement-fixing autoantibodies directed toward individual subcellular membranes of the liver [5]. Other studies have demonstrated that the induction of specific cardiac damage in dogs resulted in the production of anti-heart autoantibodies directed toward apparently unique autoantigens located on both the inner and outer mitochondrial membranes of cardiac tissue [8]. Following the initial delineation of the temporal development of the anti-heart autoantibodies, and the subcellular location of the autoantigens, it seemed appropriate to attempt to characterize some of the physical and biochemical properties of the autoantigens associated with the mitochondrial membrane. A detailed characterization of both the anti-heart autoantibodies as well as the mitochondrial autoantigens was considered essential in order to be able to utilize the autoantibody as a possible immunological probe of the surface structure of the mitochondrial membranes from dog cardiac tissue. It was our judgement that the utilization of autoantibodies generated within the same animal or at least the same species may offer a much more specific membrane surface probe than antibodies produced to mitochondrial membrane fragments injected into heterologous species. Also, it was reasoned that the utilization of the autoantibody as a membrane surface probe would offer us unique markers for the mitochondrial membranes of cardiac tissue which would probably not be shared by plasma membranes or serous membranes [5].

Another interesting feature of this system is the fact that our previous studies indicated that at least two populations of autoantibodies were produced following experimental myocardial infarction in the dog. One of these autoantibodies reacted specifically with the outer mitochondrial membrane while the other reacted with the inner mitochondrial membrane, thus allowing a possible distinction to be made between these two distinct portions of the mitochondrion. This apparent difference in specificity of the anti-heart mitochondrial autoantibody population was not exploited in the present study.

Two different immunological properties of the isolated mitochondrial membranes were assessed in the present studies. Firstly, the ability of the isolated mitochondrial membranes to serve as an absorbing antigen in absorption experiments was

tested. Dog serum containing the autoantibody was absorbed with variously treated membrane preparations, merely to assess whether or not the membrane could bind and remove the autoantibody from the serum following high speed centrifugation. Essentially this type of assay would merely determine whether the various membrane treatments used in this study affected the autoantigenic site(s) on the treated membrane preparations.

Secondly, the ability of the membrane to serve as an antigen in a complement fixation test was tested. In this assay not only was it requisite that the autoantibody be able to bind to the membrane but the autoantibody-mitochondrial membrane complex must also allow the fixation of the first component of complement. At least two of the ten antibody-binding sites on the IgM antibody must be bound (combined) to antigen to allow activation of the first component of complement [24]. It was reasoned that if the various physical and enzymatic treatments significantly altered either the integrity or the spacial distribution of the autoantigenic sites on the mitochondrial membranes a significant change may be observed in the ability of the autoantibody-membrane complex to fix complement.

It was observed early in our investigations that the apparent ability of the dog heart mitochondrial membranes to serve as competent antigens in the complement fixation test for the anti-heart autoantibody was very dependent upon the medium in which the membranes were frozen and subsequently stored. Upon further experimental consideration of this observation the data presented in Tables I through IV were obtained. It was found that dog heart mitochondria retained a semblance of their functional integrity as well as their ability to serve as a competent autoantigen in the complement fixation test only when they were frozen and thawed in media which did not contain potassium chloride. If potassium chloride was present during freezing a complete loss of the respiratory function was observed (i.e. ADP did not stimulate the rate of substrate oxidation) and the ability of the mitochondrial sample to serve as an antigen in the complement fixation test was completely lost. Additionally, it was shown (Table IV) that the ability of the frozen and thawed mitochondria to serve as the absorbing antigen in absorption experiments was not appreciably altered by freezing and thawing in either the KCl- or sucrose-containing media.

Although other investigators have studied the effects of freezing and thawing on the ultrastructural and functional integrity of various types of mitochondria [25–29], little or no experimental evidence is presently available as to the effects of various types of suspension media on freeze-thaw induced mitochondrial damage. To our knowledge no information is presently available regarding the effect of freezing and thawing on the immunological properties of any type of mitochondrial membranes.

Our data may be explained by suggesting that freezing and thawing cardiac mitochondria in the presence of K^+ resulted in a redistribution of the autoantigenic determinants on the mitochondrial membrane, resulting in the formation of antigen-antibody complexes incapable of activating complement. Complement activation requires the binding of antibody to two antigenic sites rather closely spaced on the surface of the membrane. Since mitochondria, which were frozen and thawed in the presence of K^+, were able to bind autoantibody, it is possible that the antigenic sites were separated spacially in such a way as to disallow the activation of complement by membrane-bound autoantibody.

The stability of the antigenic sites of dog heart mitochondria to heating was investigated. These data show that heart mitochondria were antigenic in both assays up to 85 °C. Mitochondria which were heated at 85 °C were found to be less able to function as antigen in the complement fixation test as well as in absorption experiments.

Lester and Fleischer [18] and Fleischer et al. [30] reported that the extraction of beef heart mitochondria with 12 % acetone/water mixtures resulted in the removal of all of the neutral lipid and 80 % of the phospholipid present in the mitochondrial membranes. The removal of the lipid resulted in the loss of enzymatic activity of several lipid-requiring mitochondrial enzymes. It was shown in these studies that the lipid depletion resulted in the complete disappearance of the outer mitochondrial membrane while the inner membrane was present and retained the unit membrane appearance in electron micrographs. When dog heart mitochondria were extracted with 12 % acetone/water mixtures, a complete loss of activity was observed when the mitochondria were used as antigen in the complement fixation test. However, the autoantibody binding capacity of these mitochondria was reduced only 18 % when compared to control mitochondria. These results suggest that the antigenic sites of the treated mitochondria were protein in nature and were not sensitive to conformational changes which may have taken place upon the extraction of mitochondrial lipid.

Dog heart mitochondria were extracted using the acid extraction procedure described by Lenaz et al. [19] in order to remove protein from the mitochondrial membranes. Extraction of the mitochondria with dilute HCl resulted in the loss of 32 % of the total mitochondrial protein. When the extracted mitochondria were tested in the complement fixation test, they were found to be extremely anticomplementary; however, the extracted mitochondria were found to be more efficient than control mitochondria when tested for their ability to bind autoantibody in absorption experiments. The removal of mitochondrial protein which was soluble in dilute HCl apparently exposed more autoantigenic sites on the mitochondrial surface as evidenced by the increased ability of the mitochondria to remove autoantibody from dog serum in the absorption experiments. The reason for the mitochondria becoming anti-complementary following the dilute acid extraction is not clear. It is possible that the removal of the acid-soluble protein resulted in the non-specific binding of guinea pig complement to the membrane or in the exposure of complement reactive sites on the membranes. Either of these events would result in an antibody-independent utilization of complement and could account for the observed results.

Experiments were performed to investigate the effect of treatment of the mitochondria with neuraminidase and trypsin. The data in Table VIII indicate that neuraminidase treatment caused an increase in complement fixation titers when the mitochondria were used as antigen. A similar increase in the ability of the neuraminidase treated mitochondria to absorb anti-heart autoantibody was not observed. The removal of mitochondrial sialic acid residues resulted in an increased efficiency of the membrane antigen-antibody complexes for activating guinea pig complement. An explanation for this observation may be that neuraminidase treatment of the mitochondria exposed autoantigenic sites normally inaccessible to the autoantibody. Alternatively, neuraminidase may have altered the spacial distribution of autoantigenic sites such that an increased fixation of complement resulted. The presence of a large number of these sites could have resulted in an increased efficiency of com-

plement fixation, but not necessarily in an increase in the ability of the mitochondria to bind autoantibody in absorption experiments.

The treatment of mitochondria with trypsin resulted in the removal of up to 42 % of the total mitochondrial protein. The result of this loss of protein was a significant reduction in the ability of the treated mitochondria to serve as antigen in the complement fixation test without significant effect on the ability of the membrane to bind autoantibody. From this observation, it could be proposed that the autoantigenic sites present on the dog heart mitochondrial membranes are either protein in nature or require the presence of a trypsin-sensitive protein(s) for their integrity. It is also possible that the removal of the trypsin-sensitive protein results in a conformational alteration of the mitochondrial membrane which alters the antigenicity of the sites.

The effect of dog anti-heart mitochondria autoantibody, guinea pig complement, and the combination of autoantibody and complement on the activity of dog heart mitochondrial NADH-cytochrome *c* reductase (rotenone insensitive) and cytochrome oxidase were investigated. The results shown in Tables IX and X indicate that the activities of these enzymes were reduced in both whole mitochondria and in the individual purified membranes in the presence of complement and autoantibody. The possibility that this inhibition was due to a nonspecific reaction with some component of normal dog serum was ruled out since all control incubations were performed in the presence of dog serum which contained no detectable anti-heart autoantibody. While the exact reason for the observed inhibition is not known, several possible explanations may be considered. It is possible that the enzymes themselves are antigenic and that the binding of autoantibody caused the enzyme to become inactivated. Another explanation may be that the binding of the autoantibody to the membrane caused alterations in the configuration of the membrane rendering the enzymes less active. Alternatively, binding such macromolecules as the autoantibody and complement to the membrane may have prevented the access of exogenous substrate to the enzyme. While these explanations are valid for the case of antibody or antibody and complement, they do not necessarily hold for the case of guinea pig complement alone. The reason for the inhibition of the enzymes in the presence of guinea pig complement alone is not as easily explained. Again, it is possible to speculate that the enzymatic inhibition was caused by the binding of guinea pig complement to the mitochondrial membrane, thus hindering the access of exogenous substrate to the enzyme. Since guinea pig complement is not activated by dog mitochondria (dog heart mitochondria in the absence of antibody do not prevent the lysis of sensitized sheep red blood cells by guinea pig complement) the inhibition is probably not due to any direct action by the complement.

Davies and Bollet [31] and Davies et al. [32] reported that antisera produced in rabbits against rat liver mitochondria caused an inhibition of various mitochondrial enzymes. When rat liver mitochondria were tested for NADH-cytochrome *c* reductase activity in the presence of rabbit antisera, the enzyme was found to be inhibited. Their results differ from ours in that the addition of complement to their system either resulted in no change in the observed inhibition or in some instances resulted in a reversal of the enzymatic inhibition.

Studies are currently in progress to further define the biochemical nature of the anti-heart mitochondria autoantigens. Our intent is to isolate, to purify and to perform structural analyses on the autoantigenic species on the mitochondrial membrane.

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