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Antibody-independent activation of the complement system by mitochondria is mediated by cardiolipin

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Non-immune activation of the first component of complement (C1) by the heart mitochondrial inner membrane has been investigated. Cardiolipin, the only strong activator of C1 among phospholipids, is present in large amounts in the heart mitochondrial inner membrane. We therefore studied its contribution to C1 activation by mitochondria. The proteins of the mitochondrial inner membrane were found to activate C1 only weakly, in contrast with the phospholipid fraction which induces strong C1 activation. Furthermore, the digestion of mitochondrial inner membranes with proteolytic enzymes did not affect C1 activation. Additional support in favour of cardiolipin being the responsible activator came from competition experiments with mitochondrial creatine kinase (mt-CPK) and adriamycin, known to bind to cardiolipin. Both mt-CPK and adriamycin displaced C1q from the mitochondrial inner membrane. In addition, C1q displaced mt-CPK bound to mitoplasts.

INTRODUCTION

C1, the first component of complement, is a Ca^{2+}-dependent complex of C1q and two proenzymes: C1r and C1s [1]. Upon C1q binding to an activator (antibody–antigen complexes or non-immune activators), C1r is autocatalytically activated [2] and concomitantly cleaves C1s into its active form. The latter cleaves C4 and C2 and thus initiates the classical pathway of complement [3]. $C\bar{1}$-inhibitor, a plasma protein, prevents C1 from spontaneous activation and inhibits C1 activation by several non-immune activators referred to as 'weak' activators [4,5]. Recently, C1r was found not to be required for C1s cleavage in the presence of certain non-immune activators, such as *Escherichia coli* rough strains and cardiolipin vesicles, referred to as 'strong' activators [5,6]. Previous reports have demonstrated that heart subcellular membranes and mitochondrial membranes have the capacity of activating C1 directly in the absence of antibodies [7,8]. In addition, human heart mitochondria were found to be 'weak' activators, i.e. they activated C1 only in absence of $C\bar{1}$-inhibitor, while human heart mitoplasts (mitochondria lacking the outer membrane) are 'strong' activators. The latter induce C1s cleavage within C1 and a mixture of C1q and C1s at a molar ratio of 1:2 ($C1qs_2$) regardless of the presence of $C\bar{1}$-inhibitor [5]. These results suggest that the activating agent of mitochondria is located on the inner membrane. The question as to the identity of the activating agent located on the inner membrane of mitoplasts has not yet been clearly resolved. The present study shows that cardiolipin is the component responsible for non-immune C1 activation by damaged heart mitochondria and mitoplasts.

MATERIALS AND METHODS

Proteins

C1q, C1r, and C1s were purified according to published methods [9,10]. The functional purity of each component was assessed as described previously [6]. Briefly, the presence of C1r in the purified C1s and C1q was ruled out by the absence of C1s cleavage in the presence of EDTA, whereas significant cleavage of C1s was observed in the presence of both C1r and EDTA. Purified C1q and C1s were labelled with $Na^{125}I$ (Amersham International) using Iodogen-coated glass vials (Pierce) [11]. $C\bar{1}$-inhibitor was a gift from M. Schapira, Cantonal Hospital, Geneva. Bovine serum albumin and ovalbumin were purchased from Sigma. Trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and proteinase K (EC 3.4.21.14) were obtained from Boehringer Mannheim. Protein concentrations were determined according to Lowry *et al.* [12] with BSA as a standard.

Lipids

Phospholipids from rat heart mitoplasts were extracted three times with chloroform/methanol (3:1, v/v) and dried in a spherical glass vial. TBS (1 ml/mg of phospholipid) was added and sonicated in a bath sonicator for 5 min. The phospholipids were determined by t.l.c. [13] and calculations based on published values [14]. Adriamycin was purchased from Sigma.

Mitochondria, mitoplasts and mt-CPK

Rat heart mitochondria were isolated according to Crompton *et al.* [15], using buffer A. Rat heart mitoplasts were prepared by swelling fresh rat heart mitochondria in 20 mM-sodium phosphate, pH 7.4, for 15 min at 15 °C. The mitoplasts were then centrifuged at 28000 *g* and washed five times with buffer A. mt-CPK was isolated from rat heart mitochondria according to the method of Font *et al.* [16] modified by Müller *et al.* [17]. Briefly, 80 μl of rotenone (0.5 mg/ml), 80 μl of oligomycin (1 mg/ml), and 160 μl of carboxyatractylate (1 mg/ml) were added to 20 mg of fresh mitochondria suspended in 4 ml of 20 mM-sodium phosphate buffer, pH 7.2. After 15 min incubation at 10 °C the suspension was centrifuged for 10 min at 12000 *g*. The supernatant was

Abbreviations used: C1, first component of human complement; $C1qs_2$, a mixture of C1q and C1s at a molar ratio of 1:2; (mt-) CPK, (mitochondrial) creatine kinase; BSA, bovine serum albumin; TBS, 10 mM-Tris/HCl, pH 7.4, containing 150 mM-NaCl; buffer A, 220 mM-mannitol/70 mM-sucrose/10 mM-Tris/HCl, pH 7.4; PAGE, polyacrylamide-gel electrophoresis.

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centrifuged again for 60 min at 150000 *g* in a 50 Ti rotor (Beckman). The resulting supernatant contained 80% pure mt-CPK as determined by SDS/PAGE. This supernatant was then diluted 10-fold with water and used for binding experiments.

C1 activation

C1 or $C1qs_2$ were reconstituted from purified subcomponents in the presence of 1 mM-Ca^{2+}, 0.3% ovalbumin and ^{125}I-C1s in 10 mM-TBS for 10 min at 4 °C. The concentration of C1 subcomponents and $C\bar{1}$-inhibitor (if present) was twice the physiological concentration: C1q, 140 μg/ml; C1r, 68 μg/ml; C1s, 58 μg/ml; ^{125}I-C1s, 4 μg/ml (30 nCi); $C\bar{1}$-inhibitor, 270 μg/ml. The reaction mixture was prepared by mixing 1 vol. of reconstituted C1 or $C1qs_2$ with 1 vol. of activator. Ater 10 min incubation at 37 °C, the samples were analysed by SDS/PAGE in the presence of 0.1 M-dithiothreitol. After migration each lane of the gel was cut in 2 mm slices which were counted for ^{125}I in a Packard LS-8000 gamma-counter. The C1 and $C1qs_2$ activation was quantified by determining the percentage of C1s-bound radioactivity shifted from the 87 kDa proenzyme to the 59 kDa position of the heavy chain of the activated C1s. Controls in which buffer was substituted for activator were included. In each case specific activation (SA) of C1 and $C1qs_2$ was quantified as follows:

$$SA = \frac{\%\,C1s_{H,act.} - \%\,C1s_{H,spont.}}{\%\,C1s_{total}} \times 100$$

where $\%\,C1s_{total}$ represents the percentage of radioactivity initially present in the 87 kDa native C1s and $\%\,C1s_H$ the percentage found in the 58 kDa C1s heavy chain of C1 or $C1qs_2$ incubated with activator (act.) or with buffer (spont.).

C1q binding

C1q, trace-labelled with ^{125}I, was added in the specified amount to the activator in presence of mt-CPK or adriamycin. The reaction mixture was incubated for 30 min at 15 °C, layered over 250 μl of 10 mM-Tris/HCl, pH 7.2, containing 4% sucrose, 75 mM-NaCl and 1.5% ovalbumin in microfuge tubes and centrifuged at 4 °C for 5 min at 15000 *g*. The tubes were then rapidly frozen and the tips of the tubes containing the activator pellet or the control were cut with a razor blade. Both pellet and supernatant were counted for ^{125}I activity and the amount of bound ^{125}I-C1q was expressed as a percentage of the total ^{125}I input. Non-specific binding of C1q was measured in the absence of activator and was of the order of 8–9%. This non-specific binding appears to be due to C1q aggregation at the low ionic strength (0.075) used in the last step of these experiments.

Protein extraction from rat heart mitoplasts

Rat heart mitoplasts (2 mg) were incubated with 1 ml of 1% Triton X-114 in TBS for 20 min at 4 °C. The tubes were then centrifuged for 5 min at 10000 *g* (4 °C), and the phase separation was induced during 5 min at 37 °C. The aqueous and the detergent phase were separated by centrifugation at 10000 *g* for 5 min. The latter was diluted 10-fold with 50 mM-Na_2CO_3, pH 8.3, and adsorbed on cooled DEAE-Fractogel (Merck) equilibrated in the same buffer. The Triton X-114 was washed out with 2.2 mM-*NN*-dimethyldodecylamine *N*-oxide in 50 mM-Na_2CO_3, pH 8.3. The proteins were eluted in a one-step procedure using a buffer containing 50 mM-Na_2CO_3, pH 8.3, 2.2 mM-*NN*-dimethyldodecylamine *N*-oxide and 1 M-NaCl. The eluted proteins were then dialysed from 48 h against 2.2 mM-*NN*-dimethyldodecylamine *N*-oxide in 50 mM-Na_2CO_3, pH 8.3, and coupled to CNBr-activated Sepharose CL-4B for 16 h at 4 °C. The affinity gel was saturated with 1 M-ethanolamine/HCl, pH 8.3, and the excess detergent removed by extensive washing with TBS. The amount of protein coupled was determined by measuring the difference of protein concentration in the supernatant before and after coupling. The final protein concentration was 3.7 mg/ml in the packed gel.

Table 1. C1 and $C1qs_2$ activation by rat heart mitochondria and rat heart mitoplasts

Rat heart mitochondria and mitoplasts were prepared according to published methods [14,15]. C1 and $C1qs_2$ were reconstituted in the presence of trace-labelled C1s and in the presence or absence of $C\bar{1}$-inhibitor as described in the Materials and methods section, and 5 μl was incubated with 5 μl of rat heart mitochondria or rat heart mitoplasts at a final concentration of 5 mg of protein/ml at 37 °C for 10 min prior to SDS/PAGE analysis. Results are means of three experiments; the S.E.M. was less than 5% of the mean.

Activator	C1 or derivative	Specific activation of C1 or derivative (%)
Mitochondria	C1	52
	C1 with $C\bar{1}$-inhibitor	7.7
Mitoplasts	C1	56.3
	C1 with $C\bar{1}$-inhibitor	54.3
Mitochondria	$C1qs_2$	9.2
	$C1qs_2$ with $C\bar{1}$-inhibitor	4.1
Mitoplasts	$C1qs_2$	54
	$C1qs_2$ with $C\bar{1}$-inhibitor	49.7

Enzymic treatment of rat heart mitoplast surface proteins

Rat heart mitoplasts were reduced in 50 mM-dithiothreitol for 2 h at 37 °C and alkylated for an additional 2 h in 50 mM-iodoacetamide at room temperature. After extensive washing, 10 μl of either trypsin, chymotrypsin or proteinase K (10 mg/ml) were added to 2 mg of rat heart mitoplasts suspended in 2 ml of buffer A containing 1 mM-Ca^{2+} and 1 mM-Mg^{2+}. The mixture was incubated at 37 °C for 12 h and washed five times in TBS. C1 activation was measured as outlined above.

Creatine kinase activity measurements

CPK activity was determined by an optical test developed by Roche Diagnostica (Basel, Switzerland). Briefly, 32 μl of the test solution was added to 800 μl of the reagent mixture containing 35 mM-phosphocreatine, 1 mM-ADP, 9 mM-GSH, 0.6 mM-$NADP^+$, 10 mM-AMP, 1200 units each of hexokinase and glucose-6-phosphate dehydrogenase/litre, 100 mM-triethanolamine buffer, pH 7.0, 10 mM-magnesium acetate and 20 mM-D-glucose. The increase in NADPH is directly proportional to the

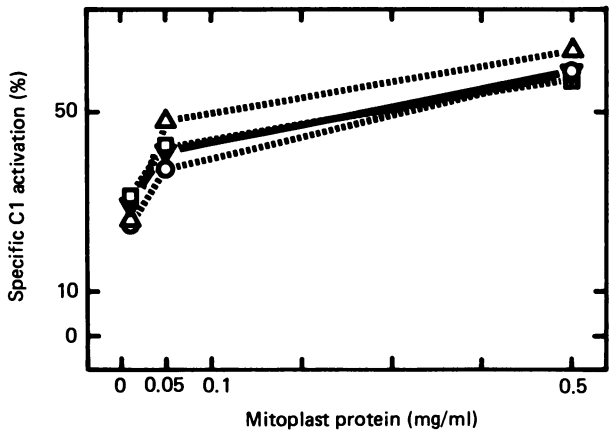

Fig. 1. C1 activation by rat heart mitoplasts treated with different enzymes

Reduced and alkylated rat heart mitoplasts (1 mg of protein/ml) were incubated for 12 h with different proteolytic enzymes at concentrations of 0.05 mg/ml. C1 activation by increasing amounts of the digested rat heart mitoplasts was tested by SDS/PAGE and compared with that of a non-treated control. Points represent the means of three determinations; the S.E.M. was 5% of the mean or less. ▲, Untreated control; △, chymotrypsin; □, trypsin; ○, proteinase K.

CPK activity of the test solution, and can be measured spectrophotometrically at 340 nm. Kinetic measurements were made, and the increase in absorbance was monitored on a chart recorder during 15 min for each test.

RESULTS

Activation of C1 and $C1qs_2$ by rat heart mitochondria and rat heart mitoplasts

We have previously reported that fresh human heart mitochondria belong to the class of weak non-immune activators, in contrast with mitoplasts derived thereof which are strong non-immune activators [5]. In the present study we used rat heart mitochondria and mitoplasts, which can be easily prepared daily from fresh tissue without freezing. In order to assess the C1 activating capacity of rat heart mitochondria and rat heart mitoplasts, we performed both C1 and $C1qs_2$ activation assays in the presence and absence of $C\bar{1}$-inhibitor. The behaviour of rat heart mitochondria and rat heart mitoplasts is identical with that of human heart mitochondria and human heart mitoplasts respectively (Table 1); rat heart mitochondria induce C1s cleavage only in the presence of C1r and in absence of $C\bar{1}$-inhibitor, while rat heart mitoplasts activate C1 and $C1qs_2$, regardless of the presence of $C\bar{1}$-inhibitor.

Activation of C1 by proteinase-treated rat heart mitoplasts

In order to test if mitochondrial proteins are capable of activating C1, rat heart mitoplasts were treated with trypsin, chymotrypsin or proteinase K and their C1 activating capacity was tested in a dose-dependent fashion. Fig. 1 shows that none of the proteolytic enzymes used has an effect on C1 activation by rat heart mitoplasts.

Table 2. C1 activation by rat heart mitoplast phospholipids, proteins, and IgG bound to Sepharose

Rat heart mitoplast phospholipids were extracted with chloroform/methanol (3:1, v/v). Vesicles were prepared by sonication of dried phospholipid-film in presence of TBS (1 mg of phospholipid/ml). Rat heart mitoplast membrane proteins were extracted with Triton X-114 and bound to CNBr-activated Sepharose CL-4B (3.7 mg of protein/ml of packed gel). Excess detergent was removed by extensive washing. Human IgG was bound to CNBr-activated Sepharose CL-45B at 2 mg of protein/ml of packed gel. C1 and $C1qs_2$ were reconstituted as outlined in the Materials and methods section; 50 μl of phospholipid vesicles or 50 μl of packed Sepharose–IgG or Sepharose–mitoplast protein were mixed with 50 μl of reconstituted C1 or $C1qs_2$ and assayed for activation as outlined in the Materials and methods section. Results are means of two determinations; the S.E.M. was 10% of the mean or less.

Activator	C1 or derivative	Specific activation of C1 or derivative (%)
Mitoplast phospholipids	C1	43
	$C1qs_2$	39
Mitoplast proteins	C1	10
	$C1qs_2$	< 2
IgG	C1	65
	$C1qs_2$	< 2

Activation of C1 and $C1qs_2$ by the phospholipids and the proteins from rat heart mitoplasts

As shown by Kovacsovics *et al.* [6,18], at least two phospholipids induce C1 activation: cardiolipin, a strong non-immune activator, and phosphatidylglycerol, which is a weak non-immune activator. Cardiolipin is located essentially in the mitochondrial inner membrane [14]. These considerations prompted us to extract the phospholipid fraction of the mitoplast membrane and to test it for C1 and $C1qs_2$ activation. The extracted phospholipid fraction contained approx. 30% cardiolipin, 35% phosphatidylethanolamine and 35% phosphatidylcholine. As shown in Table 2, both C1 and $C1qs_2$ are specifically activated by the phospholipid fraction of rat heart mitoplasts. In contrast, the protein fraction of rat heart mitoplast membranes bound to Sepharose had little or no effect on C1 and $C1qs_2$ activation. As expected, no binding of C1q was observed to these beads. As a control, IgG was bound to Sepharose C1-4B and induced C1q binding and C1s cleavage only in presence of C1r.

Displacement of mt-CPK bound to rat heart mitoplasts by C1q

mt-CPK is an intermembrane space protein and has been found to bind to cardiolipin on the mitochondrial inner membrane [16]. To test if mt-CPK competes with C1q for cardiolipin in rat heart mitoplasts, the latter were saturated with mt-CPK and then incubated with increasing amounts of C1q; BSA was used as a control. mt-CPK was released into the supernatant solution by C1q in a dose-dependent fashion, while BSA had no effect

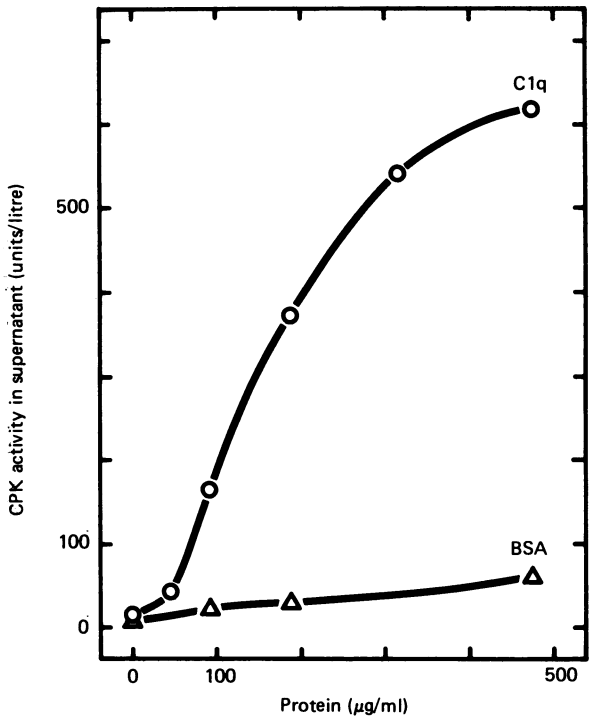

Fig. 2. Competition of mt-CPK and C1q (or BSA) for binding to rat heart mitoplasts

Freshly prepared rat heart mitoplasts (1 mg of protein/ml) were saturated with mt-CPK at an ionic strength of 0.01 and washed prior to the addition of C1q or BSA. Rat heart mitoplasts were centrifuged and the mt-CPK activity in the supernatant was measured as presented in the Materials and methods section. Total CPK activity, extracted at 0.25 ionic strength, corresponded to 800 units/l. Results are the mean of two determinations; the S.E.M. was 5% of the mean or less.

(Fig. 2). Approx. 50% of the mt-CPK activity was recovered in the supernatant solution when 200 μg of C1q were added per mg of rat heart mitoplast protein. The identity of the binding site on the mitochondrial inner membrane for these two components was confirmed, since mt-CPK also released C1q into the supernatant in a dose-dependent manner (Fig. 3). A 50% decrease in C1q binding was measured in presence of 625 unit of mt-CPK/mg of rat heart mitoplast proteins.

Inhibition of C1q binding to rat heart mitoplasts by adriamycin

Adriamycin is an anticancer drug binding specifically to DNA and cardiolipin [19,20]. Müller *et al.* have shown that mt-CPK binding to rat heart mitoplasts can be suppressed by adriamycin [16]. We therefore conducted competition experiments between C1q and adriamycin. Fig. 4 demonstrates the inhibition of C1q binding to rat heart mitoplasts by adriamycin at physiological ionic strength: 50% of the bound C1q was released when 60 μg of adriamycin was added/mg of rat heart mitoplasts.

DISCUSSION

In a previous study we have shown that the antibody-independent C1 activator of human heart mitochondria is

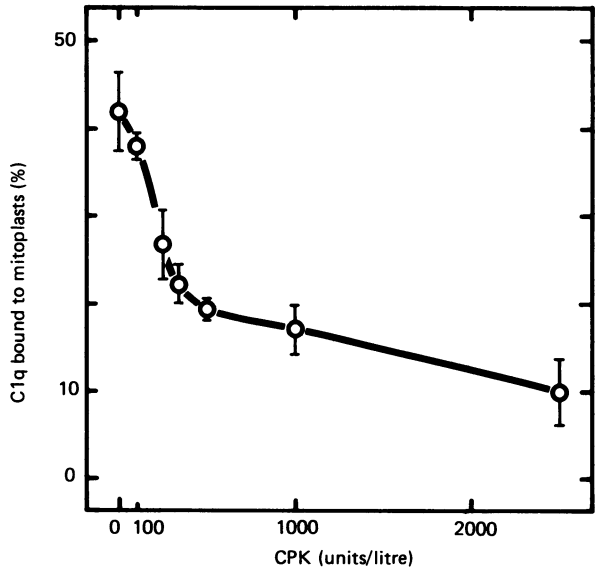

Fig. 3. Displacement of C1q from rat heart mitoplasts by mt-CPK

Freshly prepared rat heart mitoplasts (0.4 mg of protein/ml) were incubated with 23 μg of trace-labelled C1q/ml and increasing concentrations of mt-CPK. C1q binding to rat heart mitoplasts was measured as described in the Materials and methods section. Values are means ± S.E.M.; $n = 3$.

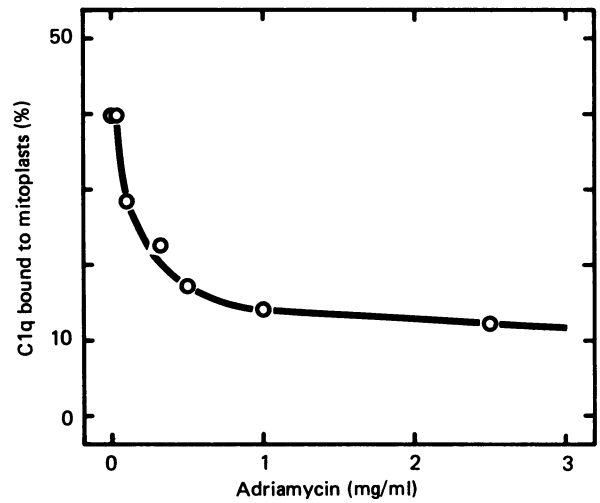

Fig. 4. Competition of C1q and adriamycin for binding on rat heart mitoplasts

C1q (23 μg/ml) and rat heart mitoplasts (3 mg of protein/ml) were incubated with increasing amounts of adriamycin. C1q binding was measured as described in the Materials and methods section. The final ionic strength was 0.15. Results are the mean of three determinations; the S.E.M. was 7% of the mean or less.

located on the mitochondrial inner membrane [5]. We also demonstrated that cardiolipin is the only strong non-immune activator among phospholipids [18] and activates C1s in the absence of C1r [5,6]. Studies on the

detailed mechanism of $C1qs_2$ activation are in progress. Nevertheless, we propose that C1q undergoes a different conformation change upon binding to non-immune activators as compared with immune activators. This may be due to the involvement of different binding domains of C1q to the activators. C1q would then not require C1r for co-operative binding of C1s and thus initiate $C1qs_2$ activation.

Since cardiolipin is present in large amounts in the mitochondrial inner membrane [14], we have proposed that cardiolipin may be involved in activating C1. In the present report we now provide evidence that cardiolipin is responsible for C1 activation on mitoplasts.

In order to localize the activating agent, we fractionated purified mitochondrial inner membranes into a protein and a phospholipid fraction. The protein fraction neither binds nor activates C1. In addition, treatment of rat heart mitoplasts with proteolytic enzymes has no effect on C1 activation, suggesting that the proteins of the mitoplast membrane have no effect on C1 activation. In contrast, the phospholipids extracted from the mitochondrial inner membrane were found to be strong activators of C1. Additional support in favour of cardiolipin being the responsible activator came from competition experiments with mt-CPK and adriamycin, known to bind to cardiolipin [17,20]; C1q and mt-CPK can be displaced by each other from rat heart mitoplasts and adriamycin inhibited the binding of C1q to rat heart mitoplasts.

Cardiolipin represents approx. 20–25% of the phospholipids in rat heart mitoplast membranes. Kovacsovics *et al.* [18] have shown that reverse phase vesicles are C1 activators only when cardiolipin is present at more than 40 mol%. However, cholesterol was shown by the same investigators to reduce the cardiolipin concentration required for C1 activation [18]. Several studies showed that cytochrome *c* oxidase and the ATP/ADP translocator are associated with cardiolipin [21,22]. This, as well as the presence of cholesterol in mitochondria, could account for a lateral phase separation inducing high local cardiolipin concentrations capable of inducing C1 activation.

The binding of mt-CPK as well as C1q to the cardiolipin of the mitochondrial inner membrane may be related to the negatively charged phosphate groups of cardiolipin: mt-CPK is highly cathodic, as compared to cytosolic CPK [23] which we found not to bind to mitochondrial inner membranes (results not shown). The binding of C1q to mitoplasts does not appear to have a physiological function, since mitochondria are intracellular and are normally not exposed to complement. However, when heart myocytes are subject to necrosis, as in ischaemic heart disease, mitochondria have a swollen morphology [19] and their outer membranes are disrupted, exposing the inner membrane to the extracellular fluid. Thus, even though native mitochondria do not activate complement, their preparation [5], especially when frozen and thawed, induces a damage of the outer membrane similar to that observed in mitoplasts.

Several authors have reported that complement may be involved in myocardial infarction; after a primary lesion has been induced by ischaemia complement may be activated by swollen mitochondria in close proximity to bystander cells. Ischaemia is known to cause depletion of ATP, to favour glycolysis and lactic acid production. The resulting low pH may cause reactive lysis of myocytes, further release of mitochondria and thus induce a vicious circle by continuous activation of the complement cascade. Since over 20% of the human myocyte consists of mitochondria, the latter process is probably responsible for the massive decrease of serum complement during myocardial infarction [24]. The finding that necrotic areas of infarcted human myocardium contain large amounts of membrane attack complexes (C5b–9) [25,26] suggests an implication of complement in the pathogenesis of myocardial infarction. The extension of the lytic process to bystander cells and its possible inhibition may become a major factor in controlling this disease.

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