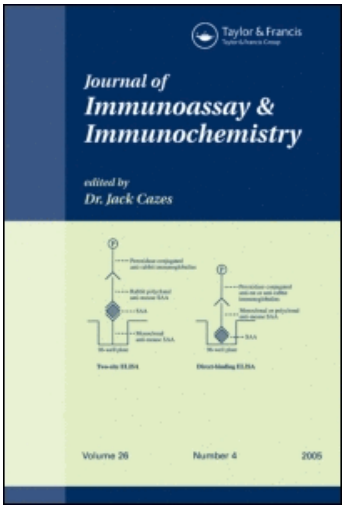

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IMMUNOQUANTITATION OF CYTOCHROME c IN CARDIAC PERFUSATE**

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ABSTRACT

An indirect three step ELISA has been assessed in order to detect the possible release of cytochrome c, a mitochondrial protein, from isolated and perfused guinea-pig heart. The ELISA described in this study is sufficiently sensitive and accurate to measure extracellular cytochrome c.

KEY-WORDS: ELISA, cytochrome,

INTRODUCTION

Cytochrome c is a hemeprotein consisting of a single polypeptide chain (molecular weight 12400) located in the mitochondrial intermembrane aqueous space. Its physiological role consists in shuttling electrons from complex III (coenzyme QH_2-cytochrome c reductase) to complex IV (cytochrome c oxidase) of

the respiratory chain (1). Cytochrome c is a very well known protein and its structure has been determined in a great number of species including man (2,3).

Being the only water-soluble component of the respiratory chain and for this reason and in view of its small molecular mass, it may be lost from the organelle under pathological conditions such as a mitochondrial damage induced by ischemia-reperfusion (4,5). We have set up an immunological method to quantitatively determine cytochrome c in heart effluent.

ABBREVIATIONS: HRP, horseradish peroxidase; PBS, phosphate buffer saline; TPBS, phosphate buffer saline containing 0.2% tween 20; BSA, bovine serum albumine; ELISA, enzyme-linked immunosorbent assay; TBS, tris buffer saline Tris/HCl 0.05 M pH 7.4; TTBS, tris buffer saline containing 0.05% tween 20; O-PD, orthophenylendiammine; LDH, lactate dehydrogenase.

MATERIALS AND METHODS

Materials

Goat antirabbit horseradish peroxidase conjugated-IgG , 4-chloro 1-naphtol and O-PD were obtained from BioRad.

Antibody preparation and characterization

Cytochrome c is a weak immunogen and, in order to obtain specific antisera, the horse heart protein (type VI from Sigma) was polymerized with glutaraldehyde prior to administration to rabbits, following Jemmerson and Margoliash (6). 250 µg of polymeric cytochrome c, emulsified in 0.5 ml of complete Freund's adjuvant (50/50 v/v), were injected subcutaneusly in New Zealand rabbits. The same amount of antigen in incomplete Freund's adjuvant was injected weekly for three times, and, after an interval of two weeks, a final boost was given. After four days

the rabbits were bled from ear artery and antisera titers determined by Ouchterlony double diffusion method. At the end of the second immunization cycle, antisera with the highest titer were collected and stored frozen at -20 °C.

The selectivity of anticytochrome c serum was determined by using a modified immunoblotting method of Towbin et al. (7), which involves the electrophoretic separation of guinea pig heart homogenate proteins and transfer to nitrocellulose paper. Briefly, after blocking non-specific binding sites of the nitrocellulose with 3% gelatin in TBS for 1 h, anticytochrome c serum was added at a dilution of 1/200 and incubated with gentle shaking overnight at room temperature. After extensive washing of the paper with TTBS the goat antirabbit peroxidase conjugated-IgG (diluted 1/1000) was added and incubated for 1 h. The excess of second antibody was removed by TTBS washing and the reaction was developed by adding 4-chloro 1-naphtol and stopped by soaking the paper in water.

Purification of cytochrome c from guinea pig heart.

Cytochrome c was purified from 5 g of heart tissue according to the procedure of Brautigan et al. (8). The ratio of absorbance of the dithionite reduced protein at 550 nm and 280 nm was 1.25, indicating a purity higher than 90%. All cytochrome c concentrations were determined spectrophometrically by using an extinction coefficient of 27.6 $mM^{-1}cm^{-1}$ at 550 nm (9) for the reduced protein.

Preparation of tissue homogenate.

Guinea pig hearts (2 g) were minced and homogenized (1 g / 10 ml) in phosphate buffer 0.1M pH 7.4, in a glass-teflon potter and centrifuged at 800xg for 10 min. The pellet, containing

cellular debris was discarded and the supernatant was divided into two aliquots. One was frozen and stored at -70 °C and subsequently used for determination of total proteins (10). The second aliquot was centrifuged at 9000 x g for 20 min. to isolate mitochondria.

ELISA of cytochrome c

Polystyrene microtitration plates (96 wells from Costar) were coated with 100 µl/well of different known amounts of horse heart cytochrome c dissolved in perfusion medium (see below) or 100 µl of the effluent from perfused guinea pig heart. The binding of cytochrome c to polystyrene wells was more efficient in 0.05 M phosphate buffer pH 7.4 in comparison to 0.1 M carbonate buffer (pH 8.6) and 0.1 M acetate buffer (pH 4.5). Overnight incubation at 37 C with the coating antigen ensured maximal binding of the protein. The next day plates were washed twice with 300 µl of PBS and the remaining non-specific adsorption sites were blocked by incubation with 0.3% BSA in PBS for 30 min at room temperature. Then the wells were filled with 100 µl of antiserum diluted 1:750 in PBS and allowed to stand for 1 h at room temperature. At the end of incubation period wells were emptied and the excess unbound antibody was removed by washing with 300 µl/well TPBS three times. Following this, 100 µl of peroxidase-conjugated coupled second goat antirabbit antibody diluted 1000 fold in PBS was added. The immunoreaction was allowed to proceed for 1 h at room temperature. The wells were finally washed five times using TPBS. O-PD and hydrogen peroxide solution was prepared according to Engvall (11); 100 µl of this solution were pipetted into the wells and used as chromogen substrate for the enzymatic reaction. The reaction was stopped by

adding 100 µl of 2 N H_2SO_4. The duration of the incubation with the substrate was kept constant for all wells within a single polystyrene plate, but varied between different plates and never exceeded 5 min. Optical absorbance was read in a Titertek Multiskan Instrument (Flow Laboratories) at 492 nm and absorbance values varied from 0.2 to 1.8. Each determination was always performed in triplicate.

Perfused guinea pig heart.

Male guinea-pig hearts (250-350 g body weight) were used for the experiments. Hearts were excised within 1 min and assembled in a non recirculating Langerdoff perfusing apparatus. The perfusiom medium had the following composition (mM): NaCl 137, KCl 2.7, $MgCl_2$ 0.11, $CaCl_2$ 1.8, $NaHCO_3$ 12, NaH_2PO_4 0.42 and glucose 5.0, equilibrated with 97% O_2 and 3% CO_2 (pO_2= 650 mmHg), the perfusion temperature was 37 °C. Other details are described in Franconi et al. (12). After 45 min of stabilization lactate dehydrogenase activity in the effluent was immediatly determined according to Wroblesky and LaDue (13); for cytochrome c determination, the effluent was stored frozen at -70 °C. Stabilization time was selected on physiological parameters (developed tension, resting tension, and electrocardiogram). The following criteria of exclusion were used: LDH activity over 10 mU/ml, arrhythmic hearts, and hearts which have a heart beat less than 190 and higher than 260.

RESULTS

Immunoblotting

Immunoblotting experiments were carried out in order to detect any cross reaction of the antiserum with myocardial

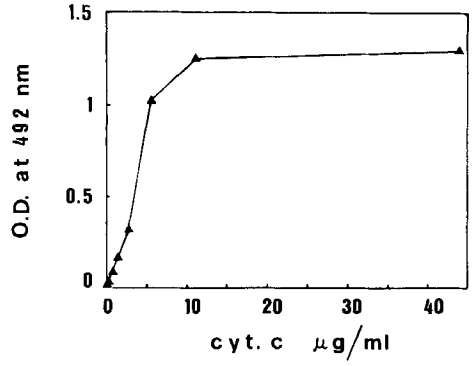

FIGURE 1) **Titration curve of antiserum elicited against cytochrome c.**

Horse cytochrome c at different incubation amounts was added in the wells and antiserum anti cytochrome c (diluted 1/750) and goat anti-rabbit horseradish peroxidase conjugate IgG was used. The linear part of the curve shown in the Figure was calculated by least squares method and regression analysis.

proteins. The dilution of the anticytochrome c serum was at 1/200 (this assay had a detection limit of 100 ng of cytochrome c). No immunoreactive species were detected by Western blot of either cytoplasmatic or mitochondrial protein (data not shown).

ELISA

An indirect three step ELISA (14) was used to determine titers and specifity of antisera and to measure the cytochrome c concentration.

Coating of the wells.

Figure 1 shows the results obtained when anti-cytochrome c serum was added to different amounts of horse heart cytochrome c. A linear relationship ($r>0.98$) between the peroxidase activity and cytochrome c concentrations was obtained in the range 0 to 6 µg /ml. The detection limit (DL) of the method was 0.09 ug/ml;

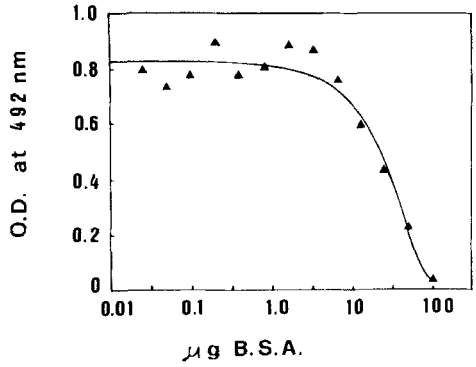

FIGURE 2) **Effect of BSA on cytochrome c adsorption.**

280 ng/well of cytochrome c were adsorpted on polystirene wells in presence of BSA at indicated amounts. Each point was the mean of three independent determinations. In the absence of BSA the reading of 280 ng of cytochrome c at 492 nm was 0.85 O.D..

it was calculated on the basis of the absorbance of the buffer as zero standard plus 2 S.D.) (15). Half maximal binding to the polystyrene was achieved at 3 µg/ml. Saturation was reached above 10 µg/ml. The variability of each point of the standard curve (n=30) in a single assay was between 2.5 and 15 %, higher variability was observed at the lowest cytochrome c concentration.

It is well established that different proteins have specific percentages of adsorption on the polystyrene surface, independent of the absolute amount added (16,17); exogenous addition of aspecific proteins does not interfere with binding of protein within its region of independent binding (16). Therefore we studied interference of BSA on cytochrome c binding to the polystyrene well. A constant amount of horse cytochrome c (280 ng/well) was adsorbed in the presence of increasing amounts of albumin (0 to 1 mg/ml). The results indicate that the recovery

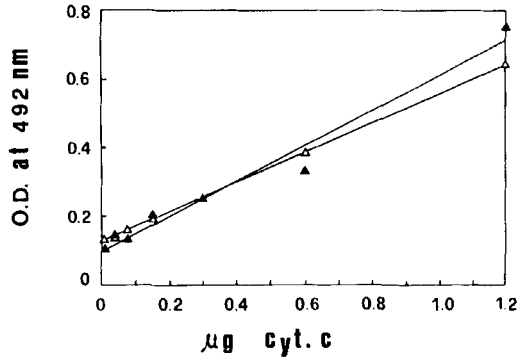

FIGURE 3) **ELISA cross-reactivity beetwen guinea-pig and horse cyt c.**

Antiserum diluted 1/750 was added to 100 µl of guinea-pig (△) and horse cyt c (▲) at the indicated amounts. Each point was the mean of three different determinations.

of cytochrome c was higher than 95% up to 6250 ng of BSA per well. At higher amounts of BSA the recovery of the antigen lineary declines (Fig. 2).

Standardization

The possible non-specific response of the ELISA assay was evaluated by incubating all the reagents in the absence of the antigen, the primary antiserum or the second antibody. The average value was subtracted from the experimental data.

Experiments to show the degree of cross-reactivity were carried out by using antiserum elicited against horse cytochrome c tested in parallel with horse and guinea-pig cytochrome c. As shown in Figure 3 a substantially identical immunological response was obtained with the two cytochromes; this is consistent with the evolutionary stability of cytochrome c.

The specificity of the assay has been assessed by determining the apparent response with other proteins such as

TABLE 1

Recovery Test

cyt c added (µg/ml)	cyt c measured (µg/ml)	% recovery
0	0.37	-
0.22	0.61	109
0.93	1.30	100
1.34	1.69	98
1.89	2.21	97

Recovery test was performed adding purified cytochrome c to a sample of perfused guinea pig heart. The amounts of cytochrome c amounts were determined before and after the addition, the % of recovery determined.

albumin, gelatin and cytochrome oxidase (another respiratory chain component), hemoglobin and myoglobin (the latter known to be a marker of heart damage). Only myoglobin reacted weakly with the antiserum.

The precision of method was calculated by means of inter-assay and intra-assay coefficient of variation (15). The inter-assay coefficient of variation was calculated using three different samples obtained by three different perfused hearts and the titer of cytochrome c was tested in 7 independent assays. The concentration of cytochrome c ranged from 2.4 to 0.5 µg/ml and the maximal inter-assay coefficient of variation was 24% for the lowest concentration.

The intra-assay coefficient of variation (15) was 12.3 and 8.9% as calculated by performing 10 replicate determinations of the two samples containing different amounts of cytochrome c (0.645 and 1.366 µg/ml respectively).

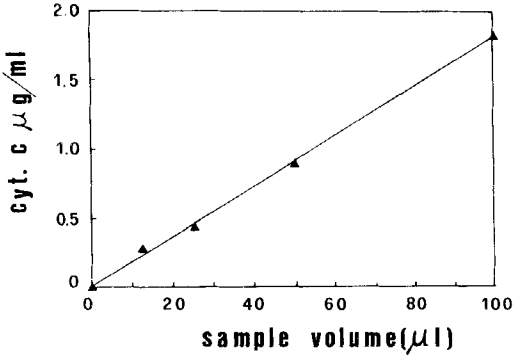

FIGURE 4) **Dilution test.**

A sample obtained from heart effluent was diluted in Tyrode buffer several folds and cytochrome c concentration measured. Cytochrome c concentration has been plotted against volume of sample.

The accuracy of the method was studied a) by recovery test (performed by adding a known amount of exogenous cytochrome c to the sample), b) by dilution tests performed by diluting sample in buffer. These results (shown in Table I and Figure 4) confirm that in our experimental model the assay of cytochrome c is accurate.

DISCUSSION

The assay for cytochrome c described in this paper is rapid and highly specific, requires small amounts of sample (100 µl) and reagent (allowing many tests to be run at the same time), and involves minor sample manipulation (avoiding possible sources of mistakes). Moreover, detection limit of the ELISA described here is sufficient to accurately measure cytochrome c in the heart effluent.

This assay is suitable to probe the status of mitochondria in heart and perhaps, in the future, in the whole animals, avoiding any organelle manipulation. The release of cytochrome c after an insult may be regarded not only as a marker of mitochondrial alteration, but also as a specific marker of respiratory chain efficiency, considering its role in translocating electrons from complex III to complex IV (1). This may be of significance to investigate the mitochondrial performance under pathological condition, and we are presently assessing the cytochrome c relase during hypoxia followed by reoxygenation of perfused guinea pig heart.

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