# Quantitative measurement of SC5b-9 and C5b-9(m) in infarcted areas of human myocardium

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## SUMMARY

Previous immunohistochemical work has indicated that terminal C5b-9 complement complexes are selectively deposited in infarcted areas of human myocardium. In the present study, we sought to quantify C5b-9 levels in myocardial tissue, and to differentiate between the membrane-bound C5b-9 (m) and the cytolytically inactive SC5b-9 complex. Paired tissue specimens from infarcted and non-infarcted myocardium were obtained from 36 autopsies. The homogenized and washed tissues were extracted with $n$-octyl-$\beta$-D-glucopyranoside (octylglucoside) detergent, and the concentrations of C5b-9 in the extracts were determined by ELISA. Membrane-derived C5b-9 (m) and SC5b-9 were differentiated from each other on the basis of their characteristic sedimentation behaviour in sucrose density gradients. It was found that infarcted myocardial tissue contained on average an approximately three-fold higher concentration of C5b-9, compared with non-infarcted tissue. This increase was due in part to an increase in levels of C5b-9 (m). The results corroborate previous immunohistochemical data and show that complement activation occurs to completion with the generation of potentially cytotoxic C5b-9 complexes in infarcted myocardial tissues.

**Keywords** terminal complement complex myocardial infarction

## INTRODUCTION

Subsequent to primary tissue damage invoked by ischaemia, there is evidence that secondary, heterolytic damage may occasionally ensue during myocardial infarction (Bulkley & Hutchins, 1977). Discussions regarding the cause and nature of such heterolytic damage currently centre around oxygen radical generation (McCord, 1985). Two existing yet widely ignored lines of evidence also suggest that the complement system is involved in the pathogenesis of these processes. Firstly, experimental studies have indicated that infarction areas are smaller in complement-depleted animals compared with normocomplementaemic controls (Maroko *et al.*, 1978). Secondly, complement activation apparently occurs in infarcted myocardium because deposition of complement components is observed selectively at these sites (Pinckard *et al.*, 1980; Schäfer *et al.*, 1986). What initially triggers complement activation is unknown. Possibilities include spontaneous activation due to defects in cell-surface associated complement regulators, and activation by intracellular components such as mitochondria (Pinckard *et al.*, 1973, 1975; Giclas, Pinckard & Olson, 1979; Storrs *et al.*, 1981) when these become exposed to plasma.

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With the use of monoclonal and polyclonal antibodies to C5b-9, we previously demonstrated that terminal complement complexes indeed accumulate selectively in infarcted areas of human myocardium (Schäfer *et al.*, 1986). The potential significance of this finding is two-fold. Firstly, C5b-9 complexes present the most stable antigenic markers for complement activation and their presence unambiguously shows that the complement sequence has been activated to completion. Generation of C5a would attract and activate granulocytes and monocytes and thus contribute to inflammation (Hugli, 1975; Fernandez *et al.*, 1978; Chenoweth & Hugli, 1980). Secondly, the terminal C5b-9 complex itself may be involved in the infliction of tissue damage, the prerequisite being that it be generated in its membrane-bound form C5b-9 (m) rather than as its extracellular, non-functional counterpart SC5b-9 (Bhakdi & Tranum-Jensen, 1987). Differentiation between C5b-9 (m) and SC5b-9 is highly important but cannot be achieved by immunohistochemistry (Bhakdi *et al.*, 1988). In this study, we therefore pursued two objectives. Firstly, we sought to obtain quantitative data on the levels of C5b-9 in myocardial tissues. Secondly, we attempted to determine the nature of C5b-9 complexes deposited in infarcted regions. We present data based on the use of a sensitive and specific ELISA (Hugo, Krämer & Bhakdi, 1987) indicating that C5b-9 is indeed deposited in necrotic areas of human myocardium, partially in the form of potentially cytotoxic, membrane C5b-9 complexes.
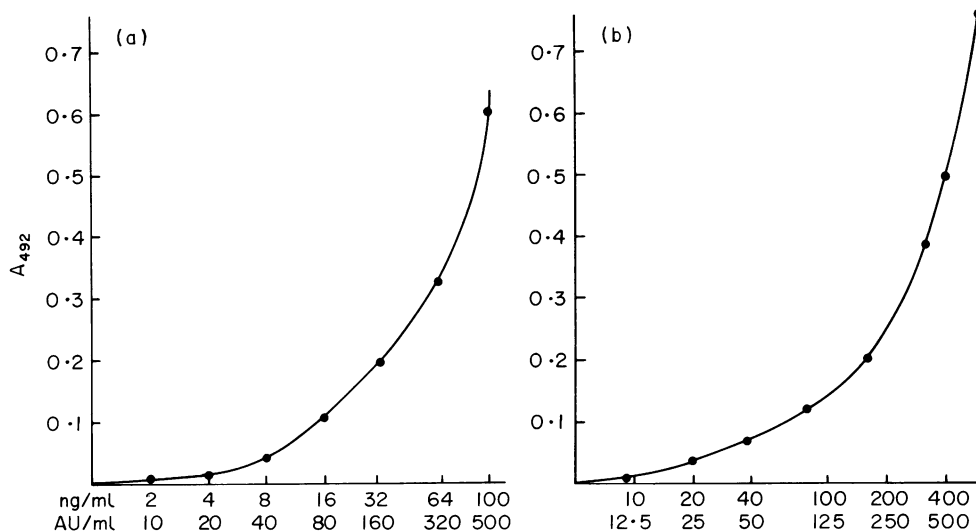
**Fig. 1.** Standard ELISA curves of purified C5b-9 (m) (a) and SC5b-9 (b). The plot shows the mean values of five experiments. An absorbance (A) of 0·5 at 492 nm in the ELISA system was obtained with 100 ng/ml C5b-9 (m) or 400 ng/ml SC5b-9. Samples yielding this absorbance were defined to contain 500 AU/ml C5b-9.

## MATERIALS AND METHODS

### Tissue specimens

We studied 36 autopsies of patients aged 47–97 years (mean 74). The age of the acute infarcts varied between 1 and 8 days (mean 3 days), according to histopathological and clinical findings. Paired tissue specimens were prepared from each autopsy. Tissue blocks were sectioned from the centre of the macroscopically defined infarcted area; control tissue blocks were taken from macroscopically non-infarcted areas; in some cases, tissue blocks were obtained from the periphery of infarcted areas. Histological control examinations were conducted on a section of each specimen to ensure that correct areas had been excised.

Tissue blocks were manually cut into small pieces (approximately 2 mm³) with a scalpel and the wet weight was determined. Afterwards they were suspended in 1 ml saline and briefly sonicated on ice (15 sec, 50 W; Branson Sonifier, Danbury, CT). The homogenates were pelleted by centrifugation (Eppendorf table-top centrifuge) and washed three times with ice-cold saline. The virtually blood-free tissues were then suspended 1 : 1 (w/v) in 130 mM octylglucoside (Sigma, Munich, FRG) and vigorously agitated on an Eppendorf rotation mixer (model 3300) for 20 min at room temperature. Unsolubilized material was sedimented and the detergent extracts were utilized in subsequent analyses.

### Quantification of C5b-9

A sandwich ELISA constructed with the use of a monoclonal antibody to a C5b-9 neoantigen, and affinity-purified polyclonal anti-C5b-9 antibodies were used to quantify C5b-9. The specificity and performance of this assay has been described. The method is sensitive to approximately 20 ng/ml SC5b-9, and 3–5 ng/ml C5b-9 (m) (Hugo *et al.*, 1987). Since the calibration curves obtained with the two complexes differ, it was essential to calibrate the assay with both purified SC5b-9 and C5b-9 (m). These two complexes were prepared and purified as described (Bhakdi & Roth, 1981; Bhakdi & Tranum-Jensen, 1982). As shown in Fig. 1, approximately 100 ng/ml C5b-9(m) and 400 ng/

ml SC5b-9 each gave rise to an absorbance of approximately 0·5 (at 492 nm). In the present work, we arbitrarily defined a solution giving rise to this absorbance as containing 500 arbitrary units (AU)/ml or 500 AU/g tissue C5b-9. The total content of C5b-9 in an extract (comprising a mixture of both complexes) was expressed in AU/ml. Following sucrose density gradient centrifugation, the C5b-9 recovered in high molecular weight fractions (25–40 S) were considered to represent C5b-9 (m), and concentrations of this complex were calculated in ng/g tissue using the respective calibration curve. Analogously, C5b-9 that was recovered in 15–23 S fractions was considered to represent SC5b-9 and concentrations were accordingly estimated using the SC5b-9 calibration curve (Bhakdi & Tranum-Jensen, 1981; Hugo *et al.*, 1987).

### Differentiation between C5b-9 (m) and SC5b-9

Tissue extracts (0·5 ml) were applied to linear sucrose density gradients (10–50% w/v, 5 ml total gradient volume) and centrifuged as described previously (Bhakdi & Tranum-Jensen, 1983). Ten equal fractions were collected and examined for C5b-9 by ELISA. In this system, C5b-9 (m) sediments to fractions 1–4 (40–25 S), whereas the SC5b-9 peak is recovered in either fraction 4–5 (23 S) or fraction 7 (15 S) (Hugo *et al.*, 1987). The concentrations of each terminal complex were determined using the respective calibration curves.

## RESULTS

When detergent extracts of human myocardial tissues were analysed for C5b-9, measurable amounts were detected in control specimens, in the range of 160–1245 AU/g tissue (mean 580 AU/ml ± 246 s.d.). Extracts from infarcted areas invariably presented higher concentrations of the terminal complex (Fig. 2), and the mean of 36 determinations was 1539 AU/g tissue (s.d. 1066). Thus, there was an approximately three-fold increase in C5b-9 compared with levels from extracts of non-infarcted areas. In contrast, ELISA analyses of the myocardium of two
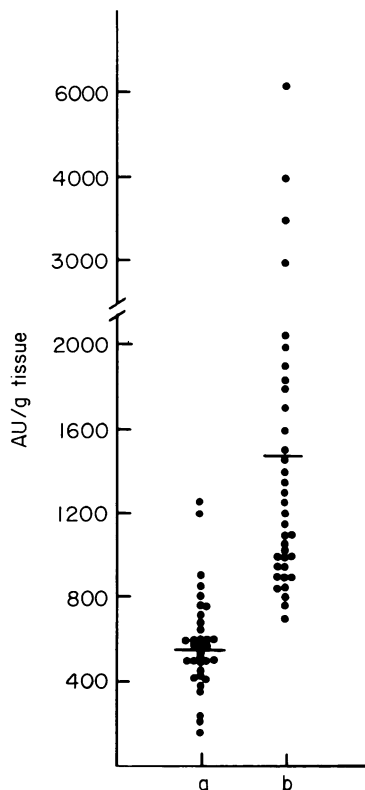
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Fig. 2. Content of C5b-9 in myocardial specimens per g tissue. (a) Control values of tissue extracts from non-infarcted areas; (b) values from tissue sections of histologically confirmed infarcted areas. Each point represents the mean of duplicate measurements; bars indicate mean values (differences are statistically significant, $P < 0.001$, Student's $t$-test).
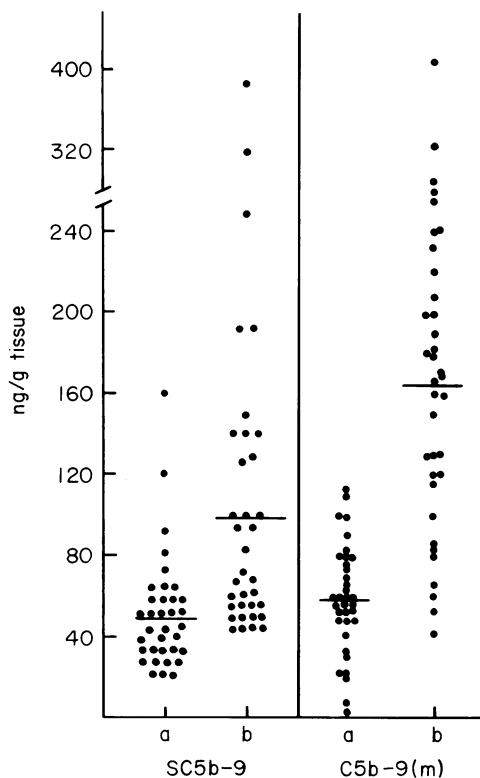


Fig. 4. Differential quantification of SC5b-9 and C5b-9 (m) in myocardial tissue extracts of non-infarcted (a) and infarcted (b) areas. SC5b-9 was determined in fraction 7, C5b-9 (m) in fraction 3 (differences are statistically significant, Student's $t$-test, $P < 0.001$).
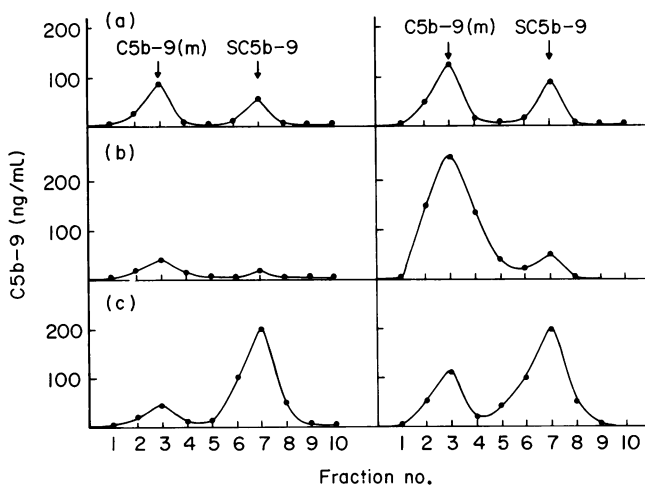


Fig. 3. Centrifugation of myocardial tissue extracts in sucrose density gradients. Three representative examples (a–c). Left panel: data from non-infarcted areas; right panel: corresponding data from infarcted areas.

newborns revealed no measureable amounts of C5b-9. These findings confirm previous immunohistochemical data (Schäfer *et al.*, 1986) and demonstrate that complement activation occurs to completion in infarcted areas of human myocardium. No statistically significant differences in C5b-9 levels were found when data from the centre of the infarcted areas were compared with those from the macroscopically defined periphery of the infarcted areas.

In order to differentiate between C5b-9 (m) and SC5b-9, tissue extracts were centrifuged in linear sucrose density gradients and the sedimentation behaviour of the terminal complexes examined. The method currently represents the only means of differentiating between the two functionally distinct forms of C5b-9 (Bhakdi *et al.*, 1988). Figure 3 depicts typical results obtained, as exemplified by three paired analyses. Normal tissue contained very low amounts of terminal membrane complexes C5b-9 (m), and varying levels of SC5b-9. Infarcted myocardium contained markedly elevated amounts of C5b-9 (m), usually accompanied by raised levels of SC5b-9. For better comparison, the amounts of C56-9 (m) and SC5b-9 were further calculated in fractions 3 and 7 of the sucrose density gradients on the basis of gram of extracted tissue. These fractions were chosen for calculation because they contain the dominant amount either of the membrane complex or the fluid phase complex. Extracts from normal myocardium contained $58 \pm 26$ ng/g C5b-9 (m) and $51 \pm 28$ ng/g SC5b-9. In contrast, the amount of C5b-9 (m) contained in fraction 3 from infarcted

tissue was $167 \pm 78$ ng/g tissue. The levels of SC5b-9 in these extracts (fraction 7) were also raised ($105 \pm 79$ ng/g tissue). It is apparent that the overall increase in tissue-deposited C5b-9 can be attributed to the increased presence of both types of C5b-9, with slight dominance of the membrane complex (Fig. 4).

## DISCUSSION

In the present investigation we first sought to obtain quantitative data on the amounts of C5b-9 that are present in infarcted *versus* non-infarcted human myocardial tissue. Secondly, attempts were made to differentiate between the membrane form of C5b-9 *versus* its non-cytotoxic, fluid-phase counterpart SC5b-9. These studies were called for because previous immunohistochemical data had indicated that C5b-9 complexes accumulate selectively in infarcted areas (Schäfer *et al.*, 1986). Confirmation of this finding would redirect attention to C5a as an important mediator of inflammatory reactions at these sites (Hugli, 1975; Fernandez *et al.*, 1978). Tentative identification of terminal complexes as C5b-9 (m) would reiterate the possibility that the terminal sequence itself is involved in evoking heterolytic damage to the cells (Schäfer *et al.*, 1986; Bhakdi, 1988).

Analyses using a sensitive ELISA for C5b-9 indicated that normal myocardium of adults contains low levels of C5b-9. Immunohistological data have suggested that terminal complexes accumulate in an age-dependent manner in the connective matrices of arteries (Schäfer *et al.*, 1986), and this would at least partially account for our present findings. In good accord with these data is the fact that C5b-9 complexes could not be detected in myocardium of newborns either by ELISA or by immunochemical staining (Schäfer *et al.*, 1986).

Differentiation between the lytic membrane complex C5b-9 (m) and the inactive fluid-phase complex SC5b-9 is difficult, since the membrane complex harbours small quantities of S-protein and stains positively for this component (Bhakdi *et al.*, 1988). Both complexes express neoantigenic determinants against which monoclonal and polyclonal antibodies can be raised (Kolb & Müller-Eberhard, 1975; Bhakdi *et al.*, 1978; Bhakdi & Muhly, 1983; Mollnes *et al.*, 1985; Hugo, Jenne & Bhakdi, 1985). No neoantigen has thus far been found to be specific for either C5b-9 (m) or SC5b-9. Hence, these complexes can presently only be differentiated on the basis of their different physicochemical properties, e.g. the high sedimentation rate (25–40 S) of C5b-9 (m) as opposed to 16–23 S (fluid-phase SC5b-9 complex). Upon sucrose density gradient centrifugation, small amounts of C5b-9 were recovered in fractions corresponding to the membrane complex, and varying amounts were tentatively identified as SC5b-9 on the basis of the slower sedimentation of the latter. All data available at present indicate that C5b-9 recovered in fractions 5–7 of the gradient represent SC5b-9, and material sedimenting to fractions 1–3 represent C5b-9 (m), the concentration of these two complexes could be estimated with the use of respective calibration curves. We assume that the extraction efficiency is fairly uniform for the different tissue sections and for both complexes. Paired analyses showed that the overall content of C5b-9 was indeed raised in infarcted tissues to approximately three-fold the levels found in non-infarcted areas, and this increase was due in part to an increase in C5b-9 (m).

This study confirms that complement activation occurs to completion selectively in areas of myocardial infarction (Schäfer

*et al.*, 1986). Whether activation takes place on the plasma cell membrane or membranes of intracellular organelles (e.g. mitochondria) is not known. Precise localization and determination of the kinetics of C5b-9 deposition are important, since formation of even a few terminal complexes on the cell surface may suffice to initiate rapid influx of calcium ions (Campbell, Daw & Luzio, 1979). Secondary reactions triggered by calcium influx (Campbell *et al.*, 1981; Seeger *et al.*, 1986), and directly toxic effects of this cation on myofibrils (Ruigrol *et al.*, 1979) may contribute to the pathogenesis of heterolytic tissue damage encountered, for example, in post-reperfusion syndromes (Bulkley & Hutchins, 1977).

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