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Quantitative evaluation of the terminal C5b-9 complement complex by ELISA in human atherosclerotic arteries

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SUMMARY

A quantitative ELISA using monoclonal and polyclonal antibodies against neoantigens of the terminal C5b-9 complement complex was used to evaluate the presence of terminal complexes in 68 human arterial samples with or without atherosclerotic involvement. Plasma levels of SC5b-9 were directly compared with the corresponding levels eluted from the femoral arteries in six patients undergoing surgical procedures. The plasma concentration of SC5b-9 in these donors was in the range of 30–90 arbitrary units (AU)/ml, equivalent to 100–300 ng/ml SC5b-9 or 45–130 AU/100 mg plasma protein. All the arterial samples contained detectable amounts of C5b-9. The aortic normal and fatty streaks intimae presented a minimum mean value of 65 ± 12 AU/100 mg total protein, in the range of normal plasma SC5b-9 levels. The corresponding media contained significantly higher amounts of terminal complexes (115 ± 30 AU/100 mg protein). Markedly increased levels of C5b-9 were eluted from aortic intimal thickenings (350 ± 100 AU/100 mg protein) and the corresponding media (300 ± 53 AU/100 mg protein). Similar concentrations were found in aortic fibrous plaques (340 ± 80 AU/100 mg protein). The observed correlation between C5b-9 levels and atherosclerotic alterations in arterial walls is suggestive of chronic complement activation with involvement of the terminal complement sequence at these sites. These processes may contribute to progression of the arteriosclerotic lesions.

Keywords C5b-9 complement complex atherosclerosis

INTRODUCTION

Activation of the complement sequence to completion results either in the generation of a pore-forming, cytolytic C5b-9 complex on a target membrane, or of a cytolytically inactive, fluid-phase SC5b-9 complex in serum or plasma (Mayer *et al.*, 1981; Bhakdi & Tranum-Jensen, 1983; Müller-Eberhard, 1984).

Both terminal complexes expose neoantigenic determinants on their surfaces, which permit their differentiation from the native C5-C9 proteins (Kolb & Müller-Eberhard, 1975a; Bhakdi *et al.*, 1978; Bhakdi & Muhly, 1983). If activation occurs in the fluid-phase, plasma S-protein binds to the complex and renders it water-soluble (Kolb & Müller-Eberhard, 1975b; Bhakdi & Roth, 1981). The S-protein has recently been identified as a serum-spreading factor or vitronectin, and its binding to C5b-9 may confer novel biological properties on the terminal complex, such as an affinity for connective tissue matrices (Jenne & Stanley, 1985).

Several immunohistological studies have shown the presence of C5b-9 neoantigens in diseased

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or necrotic human tissues including kidney (Biesecker, Katz & Koffler, 1981; Falk *et al.*, 1983; Parra *et al.*, 1984; Rus *et al.*, 1986b; Hinglais *et al.*, 1986), striated muscle (Engel & Biesecker, 1982), skin (Biesecker *et al.*, 1982; Dahl *et al.*, 1984) and heart (Schäfer *et al.*, 1986). Neoantigens of the terminal complex have also consistently been detected in the media of normal arteries (Hinglais *et al.*, 1986; Schäfer *et al.*, 1986), but deposition in arterial walls appears to be markedly accentuated in arteriosclerotic vessels (Vlaicu *et al.*, 1985a; Rus *et al.*, 1986a). To date, no direct immunochemical demonstration and quantification of terminal complement complexes eluted from normal or pathologically altered tissues has yet been reported.

We have been interested in studying the possible involvement of the terminal complement pathway in the pathogenesis of atherosclerosis. These studies were initiated because immunoglobulins and early complement components were previously shown to be present in atherosclerotic arteries. Quantitative estimates indicated these proteins to be preferentially retained in the atherosclerotic intima compared to other serum proteins (Hollander *et al.*, 1979; Vlaicu *et al.*, 1985b).

Subsequently, immunofluorescence and immunoperoxidase staining as well as immunoelectron microscopy utilizing polyclonal antibodies to C5b-9 neoantigens revealed accumulation of terminal complement complexes in atherosclerotic lesions (Vlaicu *et al.*, 1985a; Rus *et al.*, 1986a). The neoantigen deposits were localized on the cell debris, enmeshed within the connective tissue matrix, and appeared related to the degree of arterial fibrosis and cell necrosis (Rus *et al.*, 1986a). In continuation of these studies, we have now performed quantitative measurements of C5b-9 eluted from normal and atherosclerotic arterial tissues. The results confirm and extend the previous data and further support the contention that the terminal complement pathway undergoes activation in atherosclerotic lesions.

MATERIALS AND METHODS

Arterial and plasma samples. Twenty-one human thoracic aortae were obtained at autopsy within 6–12 h after death from15 males and six females aged between 22 and 71 years (mean age 60·3). The cause of death was myocardial infarction, stroke or trauma, and no immune-mediated diseases were present in their past history. The samples were rinsed with cold (4°C) isotonic phosphate-buffered saline, pH 7·4, containing 10 mM EDTA (PBS-EDTA), and areas with different degrees of atherosclerotic involvement were carefully dissected. These included: four normal and fatty streak intimae and the four corresponding, inner-thirds of the media; 14 intimal thickenings and the corresponding inner-thirds of the media; 13 fibrous plaques with the corresponding inner-thirds of the media. In six cases of arteriosclerosis obliterans, femoral fibrous plaques were obtained at surgery. These arterial samples were extensively washed with cold PBS-EDTA in the operating room and then immediately processed for elution.

EDTA-plasma samples were obtained from these six patients 1 day before surgery.

The 68 tissue samples were cut into small pieces of about 2 × 2 mm, washed in cold PBS-EDTA buffer containing 100 U/ml of Trasylol (Bayer, FRG) and 0·04 M ε-amino-caproic acid (UMB, Bucharest, Rumania), and then homogenized in a Waring blender at high speed for 30 s at 4°C. The homogenate was then incubated in PBS-EDTA + 3% (v/v) Triton X-100 containing the protease inhibitors for 1 h at 4°C (10 ml extraction buffer/g wet tissue). Supernatants obtained after centrifuging at 6000 *g* for 30 min (4°C) were collected and the extraction procedure was repeated once. The supernatants were pooled, concentrated by ultrafiltration, dialysed against detergent-free buffer at 4°C for 3–5 days, and stored frozen at −70°C. Protein concentrations in plasma and arterial tissue eluates were determined according to Lowry *et al.* (1951).

Antibodies. A monoclonal antibody against a neoantigen of the C5b-9 complex was produced and isolated as described previously (Hugo, Jenne & Bhakdi, 1985). This antibody reacted with both membrane-derived C5b-9 and fluid-phase SC5b-9, but not with any native complement or other serum protein. Affinity-purified rabbit IgG antibodies to C5b-9 were obtained by a membrane absorption-desorption procedure (Bhakdi & Muhly, 1983; Bhakdi, Muhly & Roth, 1983). Donkey

anti-rabbit biotinylated IgG and streptavidin-biotinylated peroxidase complex were obtained from Amersham (Braunschweig, FRG).

ELISA for C5b-9. This assay has been described in detail in a separate communication (Hugo, Krämer and Bhakdi, 1987). In brief, Nunc-Immuno plates (type I, 96 wells, flat bottom, high binding capacity, from Nunc, Wiesbaden, FRG) were coated with the monoclonal anti-C5b-9 neoantigen antibody (2μg/ml final IgG concentration). Samples were applied to the wells, and bound C5b-9 was subsequently detected by using the polyclonal rabbit antibodies as second antibodies, followed by incubation with biotinylated anti-rabbit IgG and streptavidin-biotinylated peroxidase complex. The ELISA was developed with *o*-phenylenediamine and absorbance was read at 492 nm in a Spekol 10 photometer (Carl Zeiss, Jena, GDR). Control blanks were included in every plate, whereby either one of the antibodies or the antigen was omitted. The net absorbance of each sample was obtained by subtracting the average of duplicate control wells from the average of duplicate sample wells. As shown previously, background controls were acceptably low ($A_{492nm} = 0{\cdot}045 \pm 0{\cdot}02$). A standard curve was derived by plotting the net absorbance versus concentration of purified SC5b-9 (Hugo *et al.*, 1987). Since the ELISA-performance differs with membrane C5b-9, and because we have not been able to differentiate between membrane and fluid-phase C5b-9 in the eluted samples in the present study, levels of terminal complexes were expressed in arbitrary units. We defined 1000 arbitrary units (AU) to be that concentration of SC5b-9 that yielded a net absorbance of approximately 1·2 in the ELISA (see Results and Fig. 1).

Further controls for specificity of the assay were performed by pre-incubating the arterial eluates with anti-C5b-9 rabbit IgG, or by replacing the human samples with rabbit arterial eluates.

RESULTS

The present ELISA is sensitive to approximately 20 ng/ml SC5b-9, as determined from a calibration curve by the use of purified SC5b-9 (Fig. 1). An absorbance of 1·2 is elicited by approximately 3·4 μg/ml SC5b-9; membrane-derived C5b-9 elicits the same absorbance at considerably lower concentrations (Hugo *et al.*, 1987). We have presented evidence that neoantigens present in EDTA-plasma derive from the presence of genuine SC5b-9, and that SC5-9 levels in plasma from healthy donors are generally in the range of 250 ng/ml or lower (Hugo *et al.*, 1987). The six plasma samples from patients obtained before surgery in this study were also found to have SC5b-9 levels of 150 ± 10 ng/ml (corresponding to 44 ± 3 AU/ml).

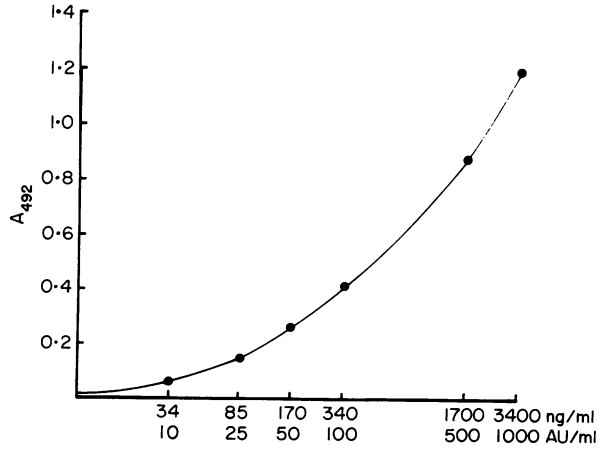

Fig. 1. ELISA-calibration with purified SC5b-9. The concentration of SC5b-9 is given in ng/ml; 1000 arbitrary units (AU) were defined as the concentration that yielded an absorbance of 1·2 in the assay.

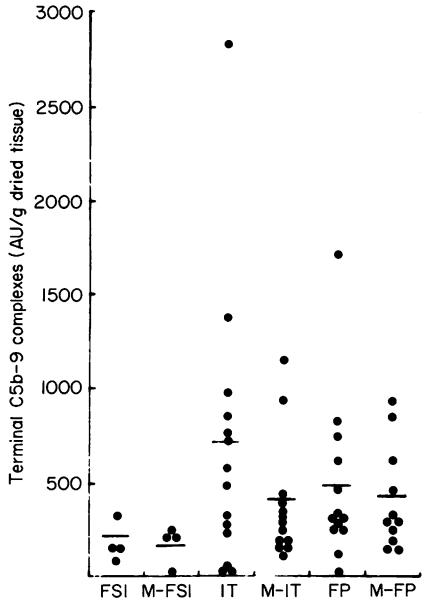

Fig. 2. Concentrations of terminal C5b-9 complexes eluted from arterial samples expressed in arbitrary units/ g dried tissue. FSI, aortic normal and fatty streaks intima; M-FSI, media corresponding to the fatty streaks intima; IT, aortic intimal thickening; M-IT, media corresponding to the intimal thickening; FP, aortic fibrous plaques; M-FP, media corresponding to the aortic fibrous plaques. Bars represent mean values.

Table 1. Quantitative determination of terminal complement complexes eluted from vessel tissues

	Protein concentration (total extracted protein mg/g dried tissue)	ELISA values (AU/100 mg total protein)
FSI	320 ± 19	65 ± 12
M-FSI	136 ± 9	115 ± 29
IT	246 ± 27	347 ± 106
M-IT	136 ± 11	297 ± 53
FP	181 ± 16	338 ± 82
M-FP	167 ± 5	271 ± 88

Abbreviations as in text and Fig. 2.

Since the ELISA-performance differs for SC5b-9 and membrane C5b-9 and because the neoantigen-positive material eluted from arterial samples could not yet be identified or differentiated, ELISA results for arterial eluates were expressed in arbitrary units. By definition, a sample containing 1000 AU/ml of terminal complexes elicited an absorbance of 1·2 in the ELISA. We found that all arterial eluates contained detectable amounts of C5b-9, and the levels per g dried tissue are shown in Fig. 2. Normal and fatty streak intimae (FSI) presented a mean value of 210 ± 55 AU/g, with values ranging between 80 and 360 AU/g. The corresponding inner-third of the media (M-FSI) presented a mean value of 165 ± 47 AU/g, with values ranging between 23–200 AU/g. The aortic intimal thickenings (IT) presented the highest amounts of C5b-9, with a mean value of 700 ± 200 AU/g and values ranging between 80 and 2850 AU/g. The corresponding media (M-IT)

presented values of 410 ± 90 AU/g, with values ranging from 90 to 1150 AU/g. The aortic fibrous plaques (FP) presented values of 485 ± 120 AU/g; similar levels were found in the corresponding media (M-FP) (425 ± 85 AU/g). The femoral fibrous plaques (FFP) obtained during surgery presented C5b-9 levels of 400 ± 175 AU/g. The inner-third media of the femoral arterial tissues, which was not contaminated with blood from the adventitia, was included as a fibrous block in the area eluted as femoral fibrous plaque.

The concentration of C5b-9 was also related to the absolute protein concentrations in the arterial eluates (Table 1). For comparison, the SC5b-9 plasma levels measured in the six patients before surgery were approximately 210 ng/100 mg total plasma protein or 61 AU/100 mg protein. It is apparent that the levels measured in fatty streaks and normal intimae as well as in the corresponding media were in the range of the plasma concentration. However, C5b-9 levels in all the atherosclerotically altered samples ranged far above the normal concentration. Levels in the femoral fibrous plaques were 182 ± 15 AU/100 mg total protein, corresponding to an approximately 3-fold higher value than found in the plasma of the patients.

DISCUSSION

Previous immunohistochemical studies indicated the presence of C5b-9 in atherosclerotic lesions in human arteries (Vlaicu *et al.*, 1985; Rus *et al.*, 1986a). These findings have now been corroborated and extended by the direct determination of C5b-9 levels in arterial eluates. The sandwich ELISA used for this purpose is highly specific and utilizes a monoclonal anti-neoantigen antibody combined with affinity-purified polyclonal antibodies. Hence, there can be no doubt that we are dealing with genuine SC5b-9 or membrane C5b-9 complexes. At present, however, we have not been able to differentiate between these two essentially different forms of the terminal complex. Because the ELISA-performance is different for SC5b-9 as opposed to membrane-derived C5b-9, it was deemed most suitable to express levels of the terminal complex(es) in arbitrary units and relate these to grams of dried tissue, or to the total amount of protein in an eluate. The latter expression is probably most reliable, especially in view of the fact that the absolute efficiency of protein extraction by the present detergent-elution procedure could not be accurately determined in these small tissue samples.

The terminal complement complex was detected in all tissue eluates, but in significantly varying concentrations. Lowest levels corresponding approximately to plasma SC5b-9 concentrations were found in the normal or fatty streaks intimae; the corresponding media presented somewhat higher values. However, 3- to 5-fold higher levels were found in atherosclerotically altered areas, whereby values in the intimal thickenings and fibrous plaques appeared slightly higher than in the corresponding media. Overall these observations are in good accord with previous findings on earlier-reacting complement components (Hollander *et al.*, 1979; Vlaicu *et al.*, 1985). The origin of the tissue-extracted C5b-9 complexes is presently unknown but three basic possibilities may be discussed. First, they may represent SC5b-9 that is generated in plasma and then deposited in the arterial wall. This possibility must be considered especially because S-protein *alias* serum-spreading factor (vitronectin) may mediate the binding of SC5b-9 to connective matrices via specific binding sites (Jenne & Stanley, 1985). On the other hand, it would then be a somewhat surprising coincidence that earlier complement components are also deposited at the same sites. A second possibility is, therefore, that the C5b-9 complexes are generated as primary products of complement activation *in situ* and represent membrane-bound C5b-9 complexes. Finally, there is a third possibility that at least one population of the complexes represents SC5b-9 that is generated *in situ* as a by-product of complement activation. The concept of genuine generation *in situ* of either membrane or fluid-phase terminal complexes appears attractive because it would be in line with several previous observations. These include (a) the detection of early- and late-acting complement components in atherosclerotic lesions; (b) the observation in the rabbit model that C3-deposition occurs in correlation with the development of cholesterol-induced atherosclerosis (Pang, Katz & Minta, 1979) and (c) the incidence of atherosclerosis is decreased in C6-deficient rabbits (Geertinger & Sørensen, 1975); (d) the presence of extracellular liposomal structures in subendothelial spaces in

the arterial intima of hyperlipoproteinemic rabbits (Simionescu *et al.*, 1986) as well as of cell debris present in atherosclerotic lesions (e.g. Geer & Webster, 1974; Stary, 1977; Joris *et al.*, 1983; Ross, 1986). There is evidence that complement activation may spontaneously occur on necrotic cells (Engel & Biesecker, 1982; Schäfer *et al.*, 1986) and on liposomes (e.g. Alving, Richards & Guingius, 1977). Such activation processes would not only generate terminal C5b-9 complexes, but also result in the local release of complement anaphylatoxins, of which C5a may assume an important role as a chemoattractant and activator of leukocytes (Hugli & Müller-Eberhard, 1978). Dying cells attacked by the cytolytic C5b-9 complex may additionally liberate lipid mediators derived from arachidonate metabolism (Imigawa *et al.*, 1983; Hänsch *et al.*, 1984; Seeger *et al.*, 1986). Taken together, these diverse processes arising through local complement activation could well contribute to the progression of the atherosclerotic lesion.

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