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In vivo clearance studies of the terminal fluid-phase complement complex in rabbits

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SUMMARY

The present study was directed at obtaining information on the *in vivo* elimination rate of SC5b-9, the terminal fluid-phase product of complement activation. A sandwich ELISA based on the use of mono- and polyclonal antibodies was constructed that permitted quantitation of rabbit SC5b-9 in plasma. Rabbit serum was activated with inulin *in vitro* to generate SC5b-9, and the activated serum was applied intravenously in normal and C6-deficient rabbits. Elimination of SC5b-9 in normal rabbits was rapid, half-life in the range of 30–50 min. No differences were noted between the clearance of homologous rabbit and heterologous human SC5b-9. SC5b-9 concentrations returned to basal levels 2–3 h after application. Plasma of C6-deficient rabbits contained no SC5b-9 and these animals displayed an even more effective clearance capacity for the complex. Quantitative considerations indicated that basal plasma SC5b-9 levels in healthy animals result from a spontaneous turnover rate of approximately 0·2% of C5-C9 components per h. When multiple doses of SC5b-9 were injected in sequence, the same half-life and total elimination time were found as with single-dose experiments. The results demonstrate the existence of an effective clearance mechanism for SC5b-9, consistent with recent findings that SC5b-9 plasma levels are very low not only in healthy adults, but also in the majority of patients with complement-consuming diseases.

Keyword terminal fluid-phase complement complex in-vivo clearance

INTRODUCTION

SC5b-9 is the terminal, fluid-phase product of complement activation (Kolb & Müller-Eberhard, 1975; Podack & Müller-Eberhard, 1978; Bhakdi & Roth, 1981; Bhakdi & Tranum-Jensen, 1987). It is generated both during fluid-phase activation, e.g., by soluble immune complexes (Bhakdi *et al.*, 1988) and when complement activation occurs on particulate surfaces, e.g., target cells or artificial activator surfaces such as dialysis (Hugo *et al.*, manuscript in preparation) and oxygenator membranes (Fosse, Mollnes & Ingvaldsen, 1987; Salama *et al.*, 1988). Accurate quantitation of plasma SC5b-9 levels has only recently become possible through the advent of sensitive enzyme-linked immunosorbent assay (ELISA) systems constructed with a combination of mono- and polyclonal antibodies (Mollnes *et al.*, 1985b; Hugo, Krämer & Bhakdi, 1987). Recent data indicate that plasma levels of SC5b-9 in healthy humans are very low, generally in the range of 100–300 ng/ml [0·01–0·03 nM (Hugo *et al.*, 1987)]. This is to be compared with normal concentrations of around 1000 nM of native C5-C9 components. Transient increases in SC5b-9 levels to peak values of 1–3 nM have consistently been noted during haemodialysis (Hugo *et al.*, manuscript in preparation) and cardiopulmonary bypass (Salama *et al.*, 1988) due to complement activation on the artificial membrane surfaces. The SC5b-9 levels normalize within 30–60 min after termination of dialysis or bypass, indicating that an effective mechanism exists for elimination of small quantities of SC5b-9 in the human organism.

The present investigation was undertaken in order to obtain more detailed information on the elimination rate of SC5b-9 *in vivo*. These studies are called for because the terminal complement complex is currently being considered as a potentially useful marker for complement-consuming processes e.g., in immune complex disease. According to some reports, SC5b-9 levels are significantly raised in plasma of patients suffering from systemic lupus erythematosus (SLE) and Guillain-Barré syndrome (Falk *et al.*, 1985; Sanders *et al.*, 1986; Koski *et al.*, 1987). Other investigators (Mollnes, Froland & Harboe, 1985a; Horigome *et al.*, 1987) generally fail to confirm the former contention, a result that might seem surprising in view of the fact that overall complement consumption and reduction of C3/C4 levels can be very pronounced in the active phase of SLE (Alper & Rosen, 1967; Sliwinski & Zvaifler, 1972; Ruddy *et al.*, 1975).

In this investigation we determined the elimination rate of SC5b-9 in rabbits, using an ELISA to measure the plasma levels

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of the terminal complex. This system was given preference over the application of radioiodinated SC5b-9 because it circumvents potential artefacts deriving from deiodination processes *in vivo*. We show that a powerful clearance mechanism for SC5b-9 exists and this would explain why significantly elevated levels of the terminal fluid-phase complement complex are seldom encountered in immune complex disease.

MATERIALS AND METHODS

Proteins

The fluid-phase terminal complement complex SC5b-9 was generated in freshly drawn human and rabbit serum by addition of 2% (w/v) particulate inulin (Serva, Heidelberg, FRG) and incubation for 16 h at 37°C (Bhakdi & Roth, 1981). Rabbit C5b-9(m) membrane complexes were prepared and purified from complement-lysed sheep erythrocytes as described (Bhakdi, Muhly & Roth, 1983).

Animals

Either normal rabbits or C6-deficient rabbits (kindly provided by Drs K. and U. Rother, Heidelberg) were used. They were maintained on a standard pelleted diet and water *ad libitum*.

Experimental procedure

Rabbits were secured in a rabbit restrainer. A bolus of 7·5-ml inulin-activated serum (IAS) containing approximately 2 mg SC5b-9 was injected in a peripheral ear vein. Blood samples (0·5 ml) were drawn in 10 mM EDTA from the middle ear artery and obtained before and at various time intervals after injection of the IAS. Plasma samples were kept on ice and SC5b-9 was measured in duplicate by ELISA on the same day immediately after termination of blood collection. Aliquots were frozen at −20°C for repetition of the assays.

Antibodies

Polyclonal antibodies were raised by immunizing a sheep with rabbit C5b-9(m). The sheep antibodies were affinity purified as described (Bhakdi & Muhly, 1983; Bhakdi *et al.*, 1983). Monoclonal antibody 3B1 directed against a neoantigen of human C5b-9 was isolated from hybridoma culture supernatant fluids. This antibody clone cross-reacts with a C9-neoantigen of the rabbit terminal complement complex (Hugo, Jenne & Bhakdi, 1985).

ELISA for rabbit C5b-9

The ELISA procedure followed a recently published method for quantifying human SC5b-9 and C5b-9(m) (Hugo, Krämer & Bhakdi, 1987). The purified monoclonal antibody (2 μg/ml) was adsorbed onto micro ELISA plates (Nunc, Wiesbaden, FRG) to capture SC5b-9. Bound antigen was detected using the polyclonal, affinity-purified sheep antibodies (diluted 1:1000) followed by biotinylated anti-sheep IgG (Amersham, Braunschweig, FRG; diluted 1:1000). Assays were developed with streptavidin biotinylated horseradish peroxidase complex (Amersham; diluted 1:1000) using *o*-phenylenediamine (Sigma, Munich, FRG) as chromogen. Blocking and washing steps, incubation times and buffers were exactly as described (Hugo, Krämer & Bhakdi, 1987). SC5b-9 concentrations were expressed in arbitrary units (AU/ml) since highly purified rabbit SC5-9 was not available for calibration. A dilution of 1:100 (in dilution buffer)

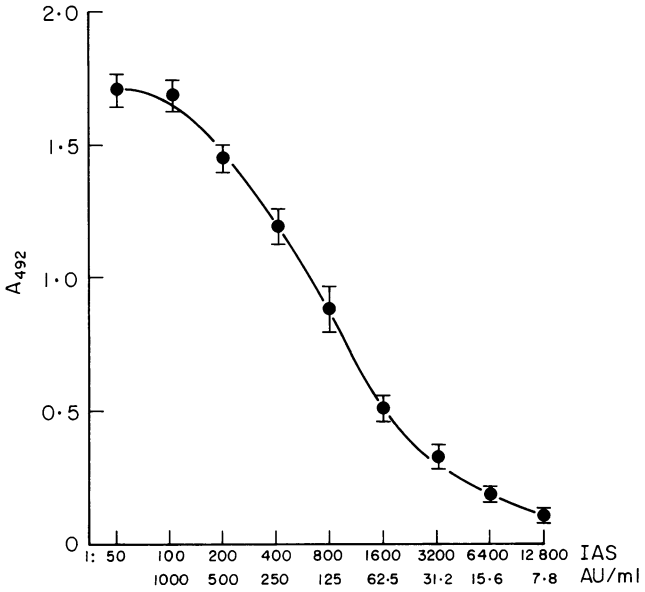

Fig. 1. Sandwich ELISA for rabbit SC5b-9 calibrated with IAS. The abscissa shows the serum dilutions and the corresponding AU/ml. The mean values ± s.e.m. of 10 separate experiments are indicated. The computer generated fit is shown by a smooth curve.

of rabbit IAS yielded an ELISA adsorbance of approximately 1·5 at 492 nm and the concentration of SC5b-9 in this 1% IAS solution was defined as 1000 AU/ml. Undiluted serum or plasma samples gave rise to unspecific absorbance values in the ELISA. This necessitated dilution of the plasma samples. In the given ELISA system, plasma dilution from 1:5 or 1:10 onward could be diluted out in dilution buffer giving reproducible readings. Therefore, the sensitivity of the assay was 5–10 times lower in rabbit plasma samples compared with its performance with purified proteins. Standards were run in duplicate on each micro-ELISA plate. The absorbance was read at 492 nm in a SLT Easy Reader EAR 400 (SLT-Labinstruments, Overath, FRG). Control blanks, whereby the antigen was omitted, were included in every plate. The absorbances from such controls were subtracted from absorbances read for the samples. The concentration of SC5b-9 in the plasma samples was calculated applying the logit-log transformation of the calibration curve (Rodbard & McClean, 1977; Butler, 1988).

RESULTS

The availability of a monoclonal antibody (clone 3B1) that reacted with a neoantigen of rabbit C5b-9 enabled the construction of an ELISA for rabbit C5b-9. The specificity of this assay was guaranteed by the fact that the reactive neoantigen is not exposed on any native plasma protein, and is expressed exclusively on assembled C5b-9 complexes. Fig. 1 depicts a calibration curve obtained with inulin-activated rabbit serum which contained the maximal concentration of generatable SC5b-9. The performance of the ELISA was almost as good as that previously reported for the assay of human C5b-9 (Hugo, Krämer & Bhakdi, 1987). Since highly purified rabbit SC5b-9 was not available for calibration, we expressed all concentrations in AU. The lower detection limit was defined as the lowest SC5b-9 concentration that gave rise to absorbances which were

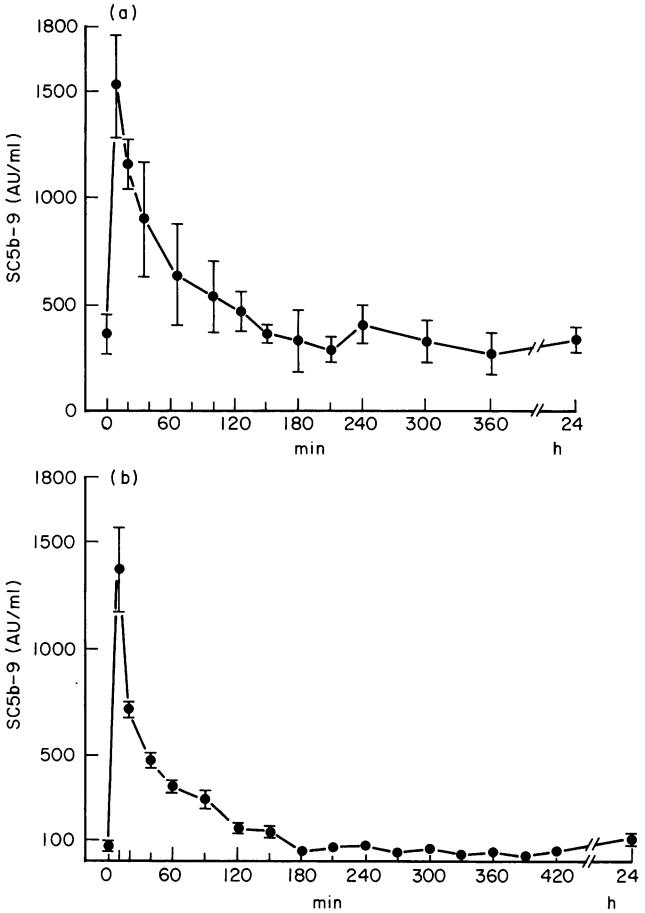

Fig. 2. (a) Elimination of SC5b-9 in normal rabbits. At time point zero, 7·5 ml homologous IAS were applied intravenously. EDTA-plasma samples were collected at the indicated time intervals and the amount of SC5b-9 was determined by ELISA ($n=6$; ±s.e.m.). (b) Clearance of SC5b-9 from the circulation of C6-deficient rabbits. Experimental conditions were the same as for healthy rabbits ($n=6$; ±s.e.m.).

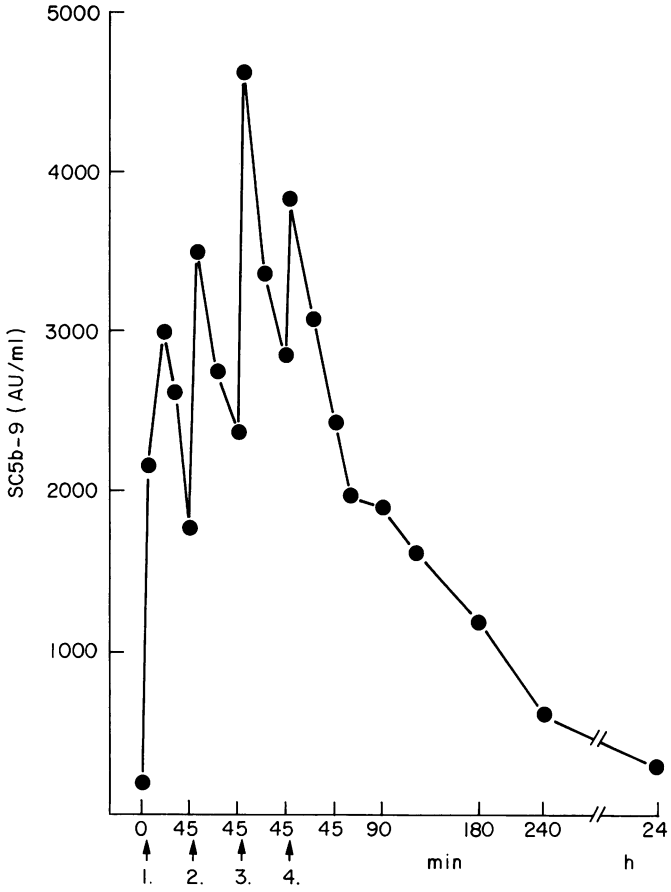

Fig. 3. Elimination of SC5b-9 following repetitive application. Arrows at the abscissa indicate time points of i.v. application of homologous IAS (1–3.: 10 ml IAS, 4.: 5 ml IAS).

statistically different from the background absorbances (control blanks) on a 0·1% level (Student's *t*-test). This value was 10 AU/ml for rabbit IAS. The normal concentration of SC5b-9 in rabbit EDTA-plasma was approximately 300 AU/ml (Fig. 2a; zero time values). This adsorbance background obtained with C6-deficient rabbit plasma corresponding to 50 AU/ml (Fig. 2b; zero time values) was just above the detection threshold, reflecting, essentially, a SC5b-9 level of nil.

Figure 2a depicts the results of elimination experiments obtained in a total of six normal rabbits. During the first 10 min after i.v. application of 7·5 ml homologous IAS, peak SC5b-9 concentrations of approximately 1500 AU/ml were found. Thereafter, a rapid decrease in SC5b-9 levels followed, and basal concentrations of SC5b-9 were always reached 2–3 h post application. The half-life of the administered SC5b-9 amount was in the range of 30–50 min. Administration of the autologous IAS ($n=3$) resulted in the same clearance pattern found for homologous rabbit IAS (results not shown). As a control, non-activated rabbit serum (10 ml of autologous EDTA-plasma) was injected intravenously, and blood samples were collected under the same conditions as for IAS clearance measurements. In these cases, no alterations in basal SC5b-9 levels were observed.

Figure 2b depicts the results of SC5b-9 elimination in C6-deficient rabbits. In this case, two phases in the elimination rate could be distinguished: removal of the complex in the first phase was more rapid, the half-life being approximately 10 min, and the following, second phase exhibited a similar half-life of 30–50 min as was found in normal rabbits. The SC5b-9 complexes were totally eliminated from the circulation in these rabbits within 2–3 hours.

In order to determine whether the effective elimination of SC5b-9 is species-specific, removal of heterologous human SC5b-9 was also followed in rabbits. It was found that the removal rate of human SC5b-9 was virtually the same as for rabbit SC5b-9 (results not shown).

To test the capacity of the elimination system, inulin-activated serum was applied in four sequential doses over a period of 45 min. As shown in Fig. 3, the initial rapid phase of elimination followed after each provocation, and there were no signs of exhaustion or overload of the removal mechanism. Despite this massive application, SC5b-9 was almost normal 3 h after application of the last dose.

DISCUSSION

It is becoming evident that the terminal complement sequence

may contribute to the pathogenesis of various immune and non-immune disease (Biesecker 1983; Groggel, Adler & Rennke, 1983; Morgan, Campbell & Compston, 1984; Schäfer *et al.*, 1986; Niculescu *et al.*, 1987) and SC5b-9 is currently being considered as a potentially useful marker for such processes (Mollnes *et al.*, 1985a, b; Hugo, Krämer & Bhakdi, 1987). Although elevated SC5b-9 levels in patients with SLE or Guillain-Barré syndrome have been reported by some investigators (Falk *et al.*, 1985; Koski *et al.*, 1987), these findings have not been generally confirmed (Mollnes *et al.*, 1985a; Horigome *et al.*, 1987). At this stage there is obviously a pressing need to obtain information on the metabolic behaviour a SC5b-9 in-vivo. With the availability of an ELISA for rabbit SC5b-9, clearance studies of the fluid-phase terminal complement complex in rabbits became feasible.

Low, but readily measurable basal concentrations of SC5b-9 were always found in normal rabbits. The low basal SC5b-9 concentrations in normal rabbit plasma were reminiscent of the low levels found in humans (Hugo *et al.*, 1987) and always well below 0·5% of the maximally generatable SC5b-9 levels contained in inulin-activated serum. Following intravenous application of SC5b-9, it was found that the complex was rapidly removed from the circulation and returned to basal levels in normal rabbits within 3 hours. Basal SC5b-9 levels in normal animals are likely to reflect a dynamic equilibrium between generated and eliminated complexes. The reduction in SC5b-9 levels 60 min post application is approximately 300 AU/ml per hour. We infer from the experiments in C6-deficient animals that this elimination rate would lead to complete clearance of SC5b-9 from the circulation within a few hours. The fact that plasma contains measurable SC5b-9 levels indicates that the terminal complex is continuously formed *in vivo* at a rate corresponding to 0·2–0·3% of maximally generatable SC5b-9.

The fate of SC5b-9 has not been followed. However, since the complex is very large (M_r approximately 1 million), it is most likely to be trapped in the liver and spleen. A preliminary report indicates this to be the case in the rat (Dalmasso & Falk, 1987). Effective removal of the terminal activation product of complement can be compared with that previously demonstrated for C5a or $C5a_{des\ arg}$ or both (Weisdorf *et al.*, 1981; Webster, Larsen & Henson, 1982). Over 50% of these radiolabelled fragments were removed within 2 min after injection into the rabbits, and less than 20% of the label was found in the circulation 30 min after application (Webster, Larsen & Henson, 1982).

Rapid clearance of SC5b-9 is consistent with the observations that levels of SC5b-9 are low not only in healthy individuals (Hugo, Krämer & Bhakdi, 1987), but also in patients with complement-consuming diseases (Mollnes *et al.*, 1985a; Horigome *et al.*, 1987). It is notable that the efficiency of SC5b-9 generation by soluble immune complexes is very low when compared with C3-cleavage (Bhakdi *et al.*, 1988). Raised SC5b-9 levels probably reflect exceptionally massive complement consumption with effective generation of SC5b-9, e.g., during activation on particulate activators such as oxygenator (Fosse, Mollnes & Ingvaldsen, 1987; Salama *et al.*, 1988) and dialysis membrane surfaces (Hugo *et al.*, manuscript in preparation), or in immune complex disease with tissue-deposited antigens (Mollnes & Paus, 1986; Mollnes *et al.*, 1986; Mollnes *et al.*, 1987; Sanders *et al.*, 1987). It is also conceivable that SC5b-9 elevations may ensue as a consequence of massive damage to the eliminating organ(s). If so, a search among patients with severe impairment of hepatosplenic function may be worth undertaking.

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