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Rapid isolation of human complement component C9 to verify the specificity of a haemolytic C9 microassay☆

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ABSTRACT

A sensitive, haemolytic microassay of human complement component C9 was developed. The assay is based on the principle of reactive (C5b6-initiated) haemolysis and uses commercially available C9-depleted serum as reagent for C9. The specificity of the assay was verified by rapid, activity-guided isolation of the haemolytic component from human serum using high-performance liquid chromatography (HPLC) on a system for fast protein liquid chromatography. This isolation yielded a single component with characteristics of C9. The results suggest that rapid, activity-guided isolation as a new application of HPLC can be a useful tool to demonstrate the specificity of a functional assay.

INTRODUCTION

C9 is the last complement (C) component participating in the generation of membrane attack complexes (MAC). It is a single-chain glycoprotein with a relative molecular mass (M_r) of 66 000–71 000 [1,2]. During activation, the component is oligomerized by C5b-8 complexes formed as a consequence of classical and/or alternative C pathway activation. Despite the presumed role of C9 in completing MAC, C9-deficient subjects show substantial classical complement pathway activity in *in vitro* assays using antibody-coated sheep erythrocytes as target cells [3]. This implies that lysis of sheep erythrocytes via the classical C pathway as used by others [1,4] is not the optimum system to determine C9 activity [5]. We therefore searched for other principles that could be applied for the functional assay of C9. C5b6-mediated (reactive) lysis [6] of chicken erythrocytes was tested for suitability. C5b6, prepared by incubating human serum with inulin [7], was combined with low concentrations of commercially available C9-depleted serum as reagent for C9 (R9). The mixture appeared to be a very useful and sensitive tool for determining functional C9 in human serum.

To test the specificity of the method developed, the haemolytic component(s) was (were) isolated from human serum on the basis of activity in the assay. To this end, a rapid separation procedure was developed based on stepwise polyethylene glycol (PEG) precipitation followed by high-performance liquid chromatography (HPLC) on hydroxyapatite (HA), anion-exchange and gel permeation chromatographic columns using an instrument for fast protein liquid chromatography. The identity of the isolated component was confirmed by polyacrylamide gel electrophoresis.

EXPERIMENTAL

Buffers

Veronal-buffered (25 m*M*) saline (750 m*M*) of pH 7.35 ± 0.05 (VSB-5x) [8,9] served as a five-times concentrated stock solution for the preparation of VSB^0, VSB^0-gel (containing 0.1% gelatine and heated at 56°C for 15 min) and EDTA-VB (containing 10 m*M* EDTA).

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Cells

Chicken blood, diluted 50% in Alsever's old solution (114 m*M* citrate–27 m*M* glucose–72 m*M* NaCl, pH 6.1) was obtained from BioTrading (Wilnis, Netherlands) and was used as a source of erythrocytes (ChE). The ChE were stored at 4°C for at least 2 weeks before use. The cells were washed three times with saline (4000 *g*, room temperature, 8 min) before use.

Sera

Human AB serum from a rhesus-positive individual (HS) was obtained from the central blood bank in Utrecht and used as source of either C5 and C6 or C9.

C9-depleted normal HS (R9) was obtained from Cytotech (San Diego, CA, USA) and used as source of C7 and C8.

Functional assay of human C9

Preparation of human C5b6 [7]. Inulin (obtained from BDH, Poole, UK) was washed once with VSB^0 (2500 *g*, room temperature, 10 min). HS (1 ml per 5 mg of inulin) was added to the pellet and the mixture was incubated at 37°C for 30 min. Subsequently, the suspension was chilled on ice, spun (2500 *g*, 4°C, 10 min) and the pellet was washed once with ice-cold VSB^0-gel. After this wash, the pellet was reconstituted in VSB^0 and incubated at 37°C for 30 min. The inulin was removed by centrifugation (4000 *g*, room temperature, 10 min) and the supernatant (containing C5b6) was used directly or after storage in small aliquots at −70°C.

Preparation of ChEC5b6 cells (EC5b6). EC5b6 were prepared by incubating 3×10^8 of ChE per ml of EDTA-VB (4% suspension) with the same volume of C5b6 at 37°C for 45 min. EC5b6 were kept at 4°C until use.

Functional assay of human C9. A variant of the microassay as described by Klerx *et al.* [10] for classical and alternative pathway activation was used. The test was performed in U-welled microtitre plates. Unless stated otherwise, R9 was diluted 1:100 in EDTA-VB before use. A 50-μl volume of R9 was mixed with 50 μl of EC5b6 and a similar volume of C9-containing sample. After incubation at 37°C for 60 min, the remaining cells and ghosts were spun down (2000 *g*, room temperature, 10 min), and 50 μl of the supernates were transferred to flat-bottomed microtitre plates containing 200 μl of water per well. Lysis was read at 405 nm in a Titertek Multiskan PLUS apparatus (Flow Labs., McLean, VA, USA). A mixture of 50 μl of EC5b6 and 100 μl of water served as 100% lysis control.

C9-containing samples were routinely tested in one concentration. For quantification purposes, duplicate dilution series of C9-containing samples were used. C9 activity was expressed in units corresponding to the amount of serum giving rise to 50% haemolysis.

Isolation procedure

The procedure developed to isolate C9 was essentially an HPLC adaptation of existing methods published by Biesecker and Müller-Eberhard [1] and DiScipio and Hugli [2]. The procedure was executed on a system for fast protein liquid chromatography equipped with an LCC-500 controller (Pharmacia, Uppsala, Sweden). To improve the separation characteristics of the procedure, chromatography on hydroxyapatite was performed before anion-exchange chromatography. All purification steps were carried out between 2 and 4°C.

– *Buffers.* All separation buffers contained 1 m*M* phenylmethyl sulphonylfluoride (PMSF) (Sigma, St. Louis, MO, USA) taken from a stock solution of 100 m*M* PMSF in isopropanol. The starting buffer for Mono Q and hydroxyapatite chromatography consisted of 5 m*M* veronal and 100 m*M* NaCl (pH 7.0). Phosphate buffers were prepared with Na_2HPO_4 and KH_2PO_4. Before use, all buffers were filtered through 0.22-μm filters (Millipore, Bedford, MA, USA) and degassed.

– *Polyethylene glycol precipitation.* HS was mixed with a double volume of 12% (w/v) PEG 4000 (BDH) in EDTA-VB and centrifuged at 4000 *g* and 4°C for 1 h. The precipitate was discarded and the PEG concentration in the supernatant was increased to, unless mentioned otherwise, 16% (w/v). The mixture was centrifuged again at 4000 *g* and 4°C for 1 h. The serum PEG precipitate (8–16%) was reconstituted in starting buffer and filtered through an Acrodisc 0.2-μm filter (Gelman, Ann Arbor, MI, USA).

– *Hydroxyapatite chromatography.* Hydroxyapatite (HA) was obtained from Calbiochem (La Jolla, CA, USA). It was poured into a Pharmacia HR 10/10 column and was equilibrated with starting

buffer. After application of 8–16% PEG precipitate of HS, the column was washed with 15 ml of starting buffer. The column was then equilibrated with 15 ml of 80 m*M* phosphate buffer (pH 7.7). C9 was eluted at a flow-rate of 0.5 ml/min (fraction size 2 ml) with 40 ml of 300 m*M* phosphate buffer (pH 7.7), forming a linear phosphate gradient. Fractions containing functional activity were pooled.

– *Dialysis.* The pooled fractions were dialysed against 1 l of starting buffer for 4 h.

– *Mono Q anion-exchange chromatography.* The pooled HA material was loaded on a Mono Q column (Type HR 5/5) (Pharmacia) and eluted with a linear NaCl concentration gradient from 100 to 500 m*M* in 5 m*M* veronal. The flow-rate was 1.0 ml/min and the fraction size 1 ml. Before testing, fractions were diluted 1:3 in EDTA-VB. Fractions containing C9 activity were pooled.

– *Superose 12 gel permeation chromatography.* Further purification was performed on a 24 ml Superose 12 column (Type HR 10/30) (Pharmacia) using EDTA-VB as eluent. The flow-rate was 0.3 ml/min and the fraction size 1 ml.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of fractions was checked by SDS-PAGE (12.5% gel) according to Laemmli [11]. The gels were developed by a silver staining method [12].

Protein determination

Protein concentrations were determined by the Bradford method [13] using BSA (Sigma) as reference.

Statistics

Vertical bars in the figures represent the arithmetic mean ± the standard error of the mean (S.E.M.). Differences from P values of <0.05 were considered to be statistically significant.

RESULTS

A functional assay of human C9 was developed. The assay is based on the lysis of chicken erythrocytes in EDTA by limiting amounts of C9 and excess of both C5b6 and commercially available C9-depleted human serum (R9) as source of C7 and C8. The optimum conditions for the C9 assay were studied by varying the concentrations of both R9 and human serum (HS; source of C9). As shown in Fig. 1, the dose–response curve for R9 showed a biphasic course with an optimum at 0.5 µl; higher concentrations of R9 were inhibitory, probably owing to the presence in serum of a terminal route inhibitor. In further experiments, 0.5 µl of R9 (50 µl, diluted 1:100) per well was used. At this concentration of R9, the optimum amount of HS was determined. Fig. 2A and B show that lysis in the C9 assay again followed a biphasic course, in this instance with an optimum at 160 nl. At low doses (5–160 nl per well) lysis appeared to be linearly dependent on the amount of HS. In some individual sera the optimum was at 50 nl.

To demonstrate the specificity of the assay, C9 was isolated from HS by HPLC on the basis of its haemolytic activity. The following separation techniques were used successively: fractionated PEG precipitation, HA chromatography, Mono Q anion-exchange chromatography and Superose 12 gel permeation chromatography. On stepwise PEG precipitation, no haemolytic activity was recovered in precipitates prepared with percentages of PEG below 8 (not shown), whereas most of the activity was precipitated between 8 and 16% PEG (Fig. 3). Above 16% PEG a slight decrease in activity was observed, probably due to the coprecipitation of a terminal route inhibitor and/or to residual PEG in the precipitate (inhibitory at final test concentra-

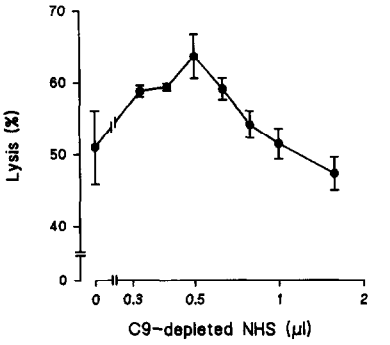

Fig. 1. Dose–effect curve for C9-depleted serum in the presence of a constant amount (50 nl) of human serum in the C9 assay. Circles and vertical bars represent the mean ± S.E.M. ($n=2$–4). The difference between the values obtained with 0 and 0.5 µl of C9-depleted NHS is statistically significant ($p<0.05$). The same holds for the decreased haemolysis above 0.8 µl of C9 reagent ($P<0.025$).

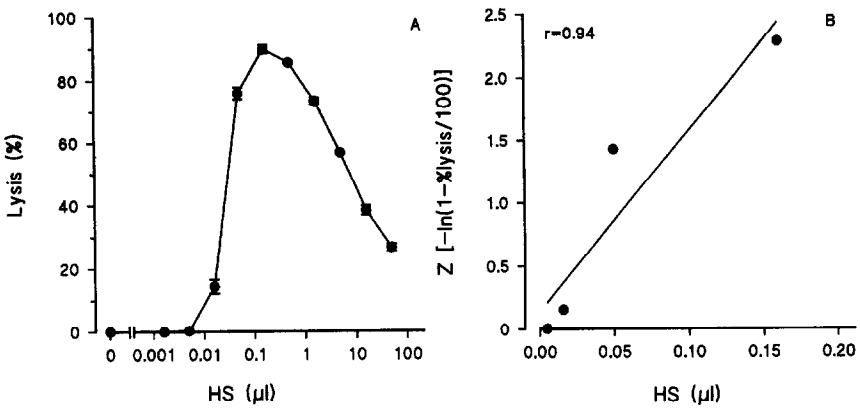

Fig. 2. Dose–effect curve for human serum when tested in the C9 assay in the presence of 0.5 µl of C9-depleted serum (left-hand panel; $n=2$). The right-hand panel shows the kinetics after transformation to "active sites per cell" [$-\ln(1-\%\text{lysis}/100)$] according to Borsos and Rapp [14]. The values for 0.016, 0.05 and 0.16 µl of HS are significantly enhanced in comparison with 0, 0.0016 and 0.005 µl ($P<0.001$). The decrease in activity at serum doses above 0.16 µl is also significant ($P<0.025$).

tions of >1%; not shown). On the HA column, haemolytic activity was eluted at phosphate concentrations between 130 and 300 m*M* (Fig. 4). On Mono Q separation, haemolytic activity was recovered in fractions containing 190–320 m*M* NaCl (Fig. 5). The final chromatographic step on Superose 12 (24-ml column) showed that the haemolytic component was present in the 13th and 14th 1-ml fractions, and in lower concentration in the 15th fraction (Fig. 6). The three active Superose 12 fractions were qualitatively analysed by SDS-PAGE. Fraction 14 showed a single band (Fig. 7), corresponding to an M_r of 74 000. Summarized separation characteristics are given in Table I.

Finally, the distribution of C9 levels in a normal population was studied. To this end, serum samples from 38 healthy students were tested in triplicate. The titres showed a slightly unequal distribution pattern with some deviants at higher levels. Minimum, median, mean and maximum values were 3248, 11 446, 13 741 and 35 313 units/ml, respectively (Fig. 8). The detection limit was 20 units/ml.

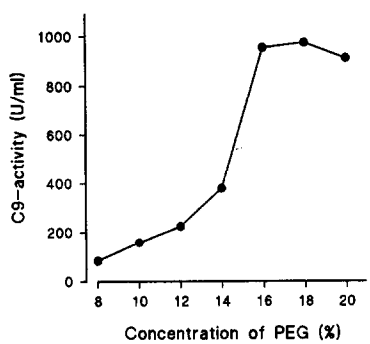

Fig. 3. PEG-precipitation characteristics of functional C9 in human serum. An 8% PEG supernatant of HS was incubated and spun (4000 *g*, 4°C, 1 h) with the indicated percentage of PEG. The precipitates were reconstituted in EDTA-containing buffer and tested for functional activity in the C9 assay.

DISCUSSION

A novel, haemolytic microassay for determining C9 activity in human serum and serum fractions was developed. The assay is based on the lysis of chicken erythrocytes by C9 and an excess of C5b6 and C9-depleted serum (R9) in EDTA. The C9-assay is specific, as it follows first-order kinetics (Fig. 2B) [14], and purification of the functional molecule from human serum yields a single protein with an apparent M_r of 74 000. This is in the range of known M_rs of human C9 [1,2]. The method is also sensitive, as test samples can be applied in high dilution. As the mean C9 level in the population is about 60 µg/ml [15] and the mean level in a random sample of the population was determined to be 13 741 units/ml, the detection limit of the assay (20 units/ml; 0.1%) is about 90 ng/ml. This limit is of

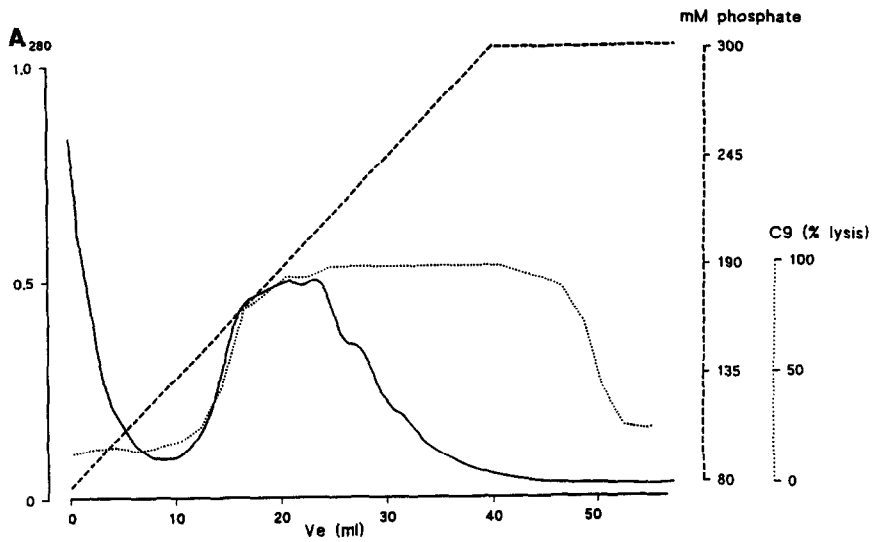

Fig. 4. Elution profile of the 8–16% PEG precipitate on hydroxyapatite chromatography. Fractions were tested for haemolytic activity in the C9 assay.

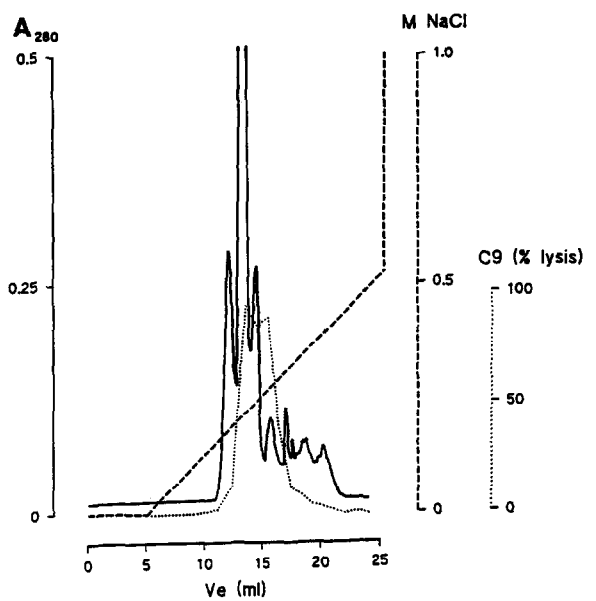

Fig. 5. Elution profile of pooled hydroxyapatite fractions on Mono Q. Fractions were diluted 1:3 in EDTA-VB and tested for haemolytic activity in the C9 assay.

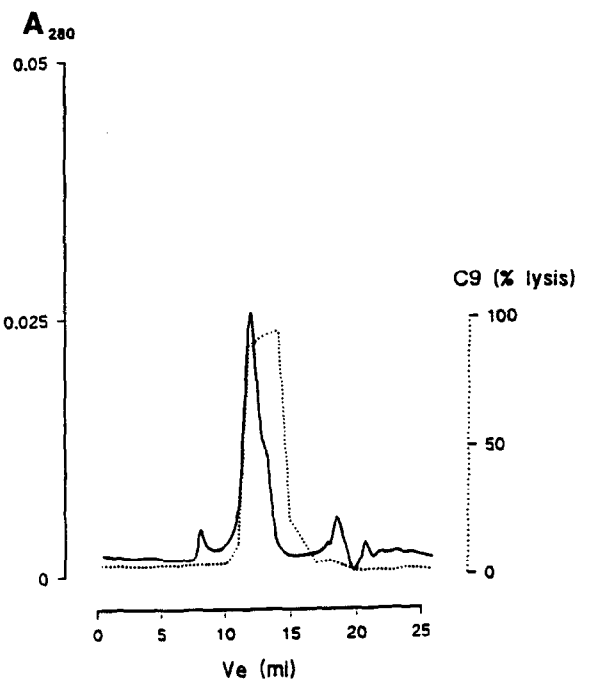

Fig. 6. Separation characteristics of pooled Mono Q fractions on Superose 12. Fractions were tested for haemolytic activity in the C9 assay.

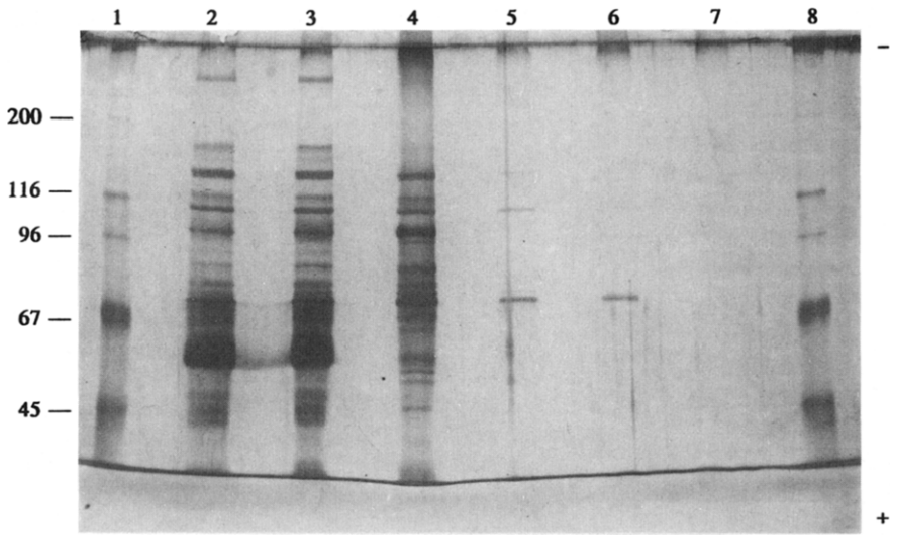

Fig. 7. SDS-PAGE under reducing conditions of C9-containing fractions at different steps of the isolation procedure. Lanes: 1 and 8 = M_r markers (M_r indicated × 10^{-3}); 2 and 3 = pooled hydroxyapatite fractions before and after dialysis; 4 = pooled Mono Q fraction; 5–7 = Superose 12 fraction 13–15.

the same order of magnitude as that of other haemolytic C9 assays used to determine C9 deficiency in patients with meningococcal meningitis [16]. The detection limit of immunochemical assays varies between 100-times higher [16] and 180-times lower [17] than that of the present assay. Finally, the proposed test is easily accessible and inexpensive, because isolated C components are not required, all reagents are commercially available and the most expensive reagent, C9-depleted serum, is needed in only a 0.5-μl amount per well.

TABLE I

YIELDS OF FUNCTIONAL C9 AT THE DIFFERENT STAGES OF THE ISOLATION PROCEDURE

Fraction	Specific activity (U/mg)[a]	Yield (%)	Purification factor
Human serum	866	100	1
8–16% PEG	1479	33	1.7
Hydroxyapatite	41 275	15	48
Mono Q	125 632	10	145
Superose 12	333 492	5	385

[a] Functional C9 was measured by the lysis of chicken erythrocytes (7.5 × 10^6 per well) in the presence of an excess of inulin-generated human C5b6 and 1:300 diluted C9-depleted human serum.

Other functional C9 assays are based on classical C pathway activation and use either isolated C components [1], C8-depleted serum and isolated C8 [5] or 5 μl of C9-depleted human serum per test-tube [4]. Classical pathway activation, however, has the disadvantage that sera from C9-deficient subjects show considerable C8-dependent background activity (3,5], which does not occur in our assay. Other workers [17] use immunochemical C9 assays that detect antigenic, but not necessarily functional, C9.

The reason for the very low background values in our assay may be the use of chicken erythrocytes as target cells. Chicken, unlike sheep, erythrocytes belong to the very few target cells that are sensitive to

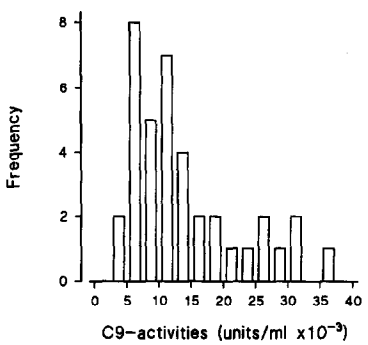

Fig. 8. Distribution pattern of C9 activities as determined in a group of 38 healthy students (20–27 years of age).

human C5b6-initiated or "reactive" lysis [7,18]. The resistance of sheep erythrocytes to reactive lysis by human complement therefore points to a functional cross-reactivity of human and sheep erythrocytes at the level of one of the late complement inhibitors (LCI; C8-binding protein and/or CD59 [19,20]). Recently, we succeeded in showing immunogenic cross-reactivity between human and sheep erythrocytes at that level [21]. This means that sheep erythrocytes could boost an LCI-neutralizing antibody response in mice induced by immunization with human erythrocytes and *vice versa*. In those experiments the anti-LCI antibodies were measured by a functional assay based on the lysis of human erythrocytes by cobra venom factor-activated, homologous complement. Because of the cross-reactivity between human and sheep LCI, the contribution of C9 to lysis of sheep erythrocytes may be relatively low, resulting in a co-determination of C8 in functional C9 assays using these erythrocytes as target cells [1,4,5]. The absence of cross-reactive LCI on chicken erythrocytes may therefore be the explanation for the high sensitivity of these cells to haemolysis by human C9 and their relative resistance to human C8.

The main purpose of isolating C9 from human serum was to show the specificity of the C9 assay. Therefore, a rapid HPLC adaptation of existing methods [1,2] was used. Using this isolation procedure, one fraction was obtained with functional activity and containing a single protein as judged by SDS-PAGE. In this sense, the fast protein isolation procedure for human C9 developed was very successful. For preparative purposes, however, the procedure might not be entirely optimal and could need some adaptation.

The M_r of the functionally active C9 as described in this paper (74 000) is slightly higher than those found by other workers (71 000) [1,22] or 68 000 [2,23]. By gel permeation chromatographic analysis of human C9, however, a higher M_r (80 000) [23], 79 000 [24] or 78 000 [25] is found.

The unequal distribution pattern of C9 levels in the population as observed with our haemolytic assay and especially the higher deviants may be attributed to the ability of C9 to behave as an acute phase reactant [26–28].

In conclusion, our results suggest that rapid, activity-guided isolation of a component by, *e.g.*, HPLC may in general be a fruitful approach to the determination of the specificity of a functional assay for the component involved.

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