C4 BINDING TO ARTIFICIAL SYSTEMS

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1. Introduction

Activation of complement by the classical pathway contributes to the non-specific defence of vertebrates against infection; it represents the triggering of a cascade system of proteolytic complex enzymes, $C\bar{1}$, the C3 and the C5 convertases. The C3 convertase is a bimolecular complex of proteins C4b and C2a in which C2a contains the catalytic site able to proteolyse C3 into two biologically active peptides C3a and C3b. C4b is the non-catalytic component of the C3 convertase [1,2].

The cleavage of C4 by $C\bar{1}s$ leads to a metastable activated fragment, C4b, by inducing the transient release of a reactive acyl group, from a thioester linkage in the native molecule; liberation of a free sulfhydryl group accompanies the proteolysis of C4 into C4b and C4a [3,4]. Binding of C4b to acceptor surfaces via the activated acyl group is the first step in the formation of C3 convertase. Both membranes [5] and Ig [6,7] can act as acceptor of C4b; the respective participation of each is as yet not well defined. The efficiency of the binding mechanism is low and only a few percent of the C4b molecules have been shown to be hemolytically active [8]. This raises the problem of the reactivity of bound C4b towards C2 and the regulatory proteins C4bp and I.

A sequential molecular analysis of the different steps involved in the formation of the C3 convertase is necessary to understand the whole sequence. We have studied the binding of C4b to two acceptor systems: Sepharose and sheep erythrocyte stroma. The results reported show that the binding of C4b to acceptors is confined only to molecules produced in close proximity to these acceptors. Sepharose, sheep erythrocyte stroma and particulate Ig act as acceptors taking the $EAC\bar{1}$ system as a reference.

Abbreviations: SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; STI, soybean trypsin inhibitor; Ig, immunoglobulins; Ag, antigen; DFP, di-isopropylphosphorofluoridate; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; PBS, 10 mM phosphate buffer (pH 7.2) containing 145 mM NaCl; $DGVB^{2+}$, 5 mM veronal (barbitone), 142 mM NaCl, 2.5% glucose, 0.05%, gelatin, 0.5 mM $MgCl_2$ 0.15 mM $CaCl_2$ (pH 7.5); EA, erythrocytes sensitized with specific antibodies; EAC1 or $EAC\bar{1}$, EA bearing proenzymic or activated C1; ICC1 or $ICC\bar{1}$, immune complexes bearing proenzymic or activated C1

Nomenclature: Components of complement follow that recommended by World Health Organization (1968); a bar indicates the activated state of a component

2. Materials and methods

All chemicals were of analytical grade.

Human citrated plasma was obtained from the Centre de Transfusion Sanguine (Grenoble) and serum prepared as in [9].

Human complement components were purified as in [10] for $C\bar{1}r$–$C\bar{1}s$ complex, $C\bar{1}r$, $C\bar{1}s$ and [11] for C4.

SDS–PAGE of proteins was as in [9] using 5% (w/v) acrylamide gels. ^{125}I-Labelling of C4 and $C\bar{1}s$-cleaved C4 was by the method in [12] or with lactoperoxidase as in [11]. ^{125}I was measured with a MR 480 Kontron counter in the various solutions or in 1 mm gel slices after SDS–PAGE.

Proteins were estimated according to [13] or from their A_{280} for purified proteins using, respectively, $E^{1\%}_{1\,cm}$ = 10, 11.5, 9.5, 10.6 and 14 for C4 [3], $C\bar{1}r$, $C\bar{1}s$, $C\bar{1}r$–$C\bar{1}s$ complex, and IgG [10].

Antibodies to ovalbumin were raised in rabbits

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according to [14]. Total immunoglobulins were purified by Na_2SO_4 precipitation [15]. Specific antibodies were purified by immunoabsorption chromatography on a column of ovalbumin–Sepharose 4B.

EAC$\bar{1}$ were prepared from sheep erythrocytes (Bio-Merieux) sensitized with rabbit hemolysin (Behring) at a final dilution of 1:1000; EA were suspended in human serum containing 1 mM NPGB at 10^9 cells/ml and incubated for 30 min at 0°C to allow fixation of C1; after centrifugation and washing at 4°C with $DGVB^{2+}$ supplemented with 1.5 mM $CaCl_2$, EAC1 were resuspended in this buffer at 10^9 cells/ml and activated 45 min at 37°C to EAC$\bar{1}$.

Proteins were coupled to Sepharose 4B as in [16]. After activation of Sepharose 4B with CNBr (1 g CNBr for 5 ml packed Sepharose), proteins were coupled overnight at 4°C in 0.1 M $NaHCO_3$, 0.1 M NaCl. The C$\bar{1}$r–C$\bar{1}$s complex was coupled in 10 mM triethanolamine–HCl, 100 mM NaCl, 5 mM $CaCl_2$ (pH 8.0) and non-covalently bound proteins washed out by 1 M propionic acid containing 1 M NaCl. Sepharose was resuspended in twice its packed volume of buffer for subsequent use.

C$\bar{1}$sDFP was obtained by blocking the active site of C$\bar{1}$s by incubation for 1 h at 37°C adding 5 mM DFP at 0 and 30 min. Calcium-dependent binding of C$\bar{1}$s to Sepharose–C$\bar{1}$s, Sepharose–C$\bar{1}$sDFP and Sepharose–C$\bar{1}$r, in 50 mM Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$ (pH 7.4), was done by incubation for 15 min at 4°C with C$\bar{1}$s in equivalent amount to the Sepharose-bound protein.

To prepare Sepharose–ICC$\bar{1}$, 1 ml packed Sepharose–ovalbumin, corresponding to 4 mg bound ovalbumin, was suspended for 15 min at 4°C in 10 ml 50 mM Tris–HCl, 150 mM NaCl (pH 7.4) containing 15 mg of anti-ovalbumin Ig. The suspension was washed at 4°C by 30 ml 50 mM Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$ (pH 7.4) and 20 ml of the same buffer containing 1 mM NPGB, then incubated for 30 min at 4°C in 20 ml human serum containing 1 mM NPGB. After this incubation the Sepharose was washed at 4°C by 20 ml 50 mM Tris–HCl, 100 mM NaCl, 50 mM $CaCl_2$, 1 mM NPGB (pH 7.4) and 30 ml of the same buffer without NPGB. Sepharose–ICC1 was then resuspended in the last buffer, incubated for 30 min at 37°C, filtered and this step was repeated once leading to the activation of Sepharose–ICC1 to Sepharose–ICC$\bar{1}$. The same protocol was used to prepare Sepharose–Ig anti-ovalbumin C$\bar{1}$ from Sepharose–Ig anti-ovalbumin. The activation steps were in 50 mM Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$ (pH 7.4).

Sheep erythrocyte stroma was obtained by hypotonic lysis of sheep erythrocytes [17]. To prepare stroma–C$\bar{1}$s, a 0.5 mg protein/ml suspension of stroma in PBS was incubated for 17 h at 20°C with 1% (v/v) glutaraldehyde, washed, resuspended at the same dilution in PBS containing C$\bar{1}$s and incubated for 5 h at 20°C. Unreacted glutaraldehyde was saturated by 112 mM lysine.

To measure C4b binding, particles were incubated with ^{125}I-labelled C4 for 30 min at 37°C. Particles were separated by centrifugation and washed repeatedly until the radioactivity of the supernatants reached background level. Proteolysis of C4 was assessed from SDS–PAGE of the first supernatant.

The different systems used are shown in table 1.

3. Results

3.1. *C4b binding to EAC$\bar{1}$*

C4b binds to EAC$\bar{1}$ (fig.1); SDS–PAGE of the supernatants at the end of the incubation showed that all the C4 is proteolysed. In contrast, with EA, only a low level of fixation of C4 and fluid phase C$\bar{1}$s-cleaved C4 was measured. The binding of C4b does not show a linear variation with increasing amounts of EAC$\bar{1}$: this may reflect a disparity between the

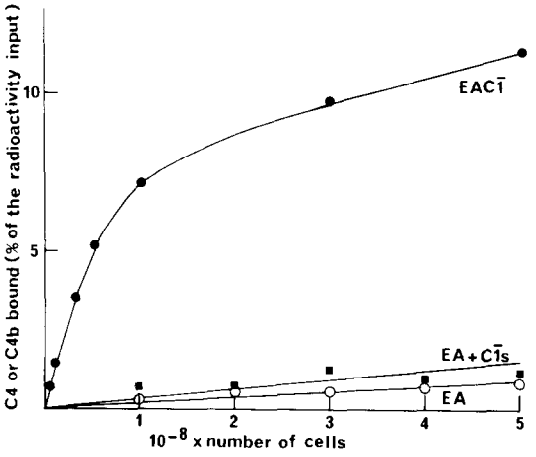

Fig.1. C4b binding to EAC$\bar{1}$: 5 μg ^{125}I-labelled C4 were incubated as in section 2 in $DGVB^{2+}$ supplemented with 1.5 mM $CaCl_2$ with varying concentrations of EAC$\bar{1}$ (•), EA (○) or EA + 2.5 μg C$\bar{1}$s (▪). Washes were with the incubation buffer as in section 2.

kinetics of C4 proteolysis and C4b deposition.

Calculation from the amount of C4b bound by 4×10^8 EAC$\overline{1}$ gives ~4500 molecules C4b bound/erythrocyte.

3.2. *C4b binding to Sepharose–subcomponents of C$\overline{1}$*

Fig.2 shows that Sepharose behaves as an acceptor for C4b; the fixation of C4b increases linearly with increasing concentrations of Sepharose. The link between C4b and Sepharose is resistant to 1% SDS but is sensitive to 50 mM methylamine at pH 10.4 consistent with a nucleophile-sensitive covalent bond. The low binding of C4 to Sepharose–C$\overline{1}$sDFP (table 1, system 2) which does not cleave C4 as compared to Sepharose–C$\overline{1}$s (table 1, system 1) indicates that proteolysis of C4 is a necessary step for its binding.

Assuming that C$\overline{1}$s accounts for half of the total protein bound on Sepharose–(C$\overline{1}$r–C$\overline{1}$s), 0.2 mg C$\overline{1}$s is bound/ml Sepharose–(C$\overline{1}$r–C$\overline{1}$s) and 0.86 mg/ml of Sepharose–C$\overline{1}$s: therefore the results in fig.2 show that Sepharose–(C$\overline{1}$r–C$\overline{1}$s) (table 1, system 3) binds ~7-times more C4b/C$\overline{1}$s than Sepharose–C$\overline{1}$s (table 1, system 1).

As C$\overline{1}$s is able to dimerize in the presence of calcium, Sepharose–dimeric C$\overline{1}$s was prepared either from Sepharose–monomeric C$\overline{1}$s or Sepharose–

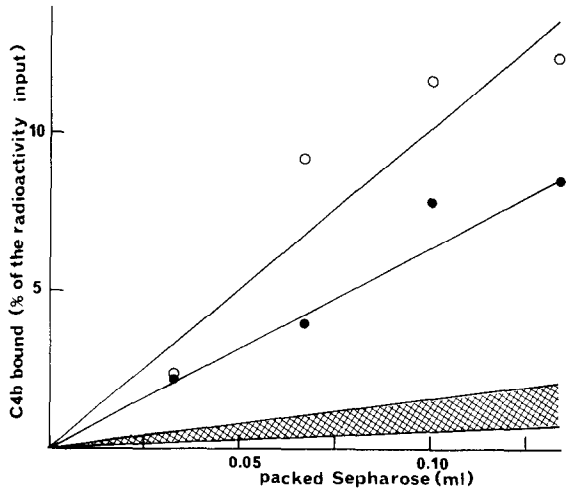

Fig.2. C4b binding to Sepharose–subcomponents of C$\overline{1}$: 5 μg ^{125}I-labelled C4 were incubated as in section 2 in 50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$ (pH 7.4) with varying concentrations of Sepharose–C$\overline{1}$s (•) or Sepharose–($\overline{1}$,$\overline{s}$) (○); the values measured with Sepharose–C$\overline{1}$sDFP, Sepharose–C$\overline{1}$sDFP Ca^{2+}–C$\overline{1}$s, Sepharose–C$\overline{1}$s–Ca^{2+}–C$\overline{1}$s and Sepharose–C$\overline{1}$r–Ca^{2+}–C$\overline{1}$s are comprised in the hatched zone. Washes were with the incubation buffer as in section 2 then with 1% (w/v) aqueous SDS.

Table 1
Putative representation of the systems used as C4b acceptors

1. Sepharose–C$\overline{1}$s	C$\overline{1}$s covalently bound in the absence of calcium
2. Sepharose–C$\overline{1}$sDFP	C$\overline{1}$sDFP covalently bound as in 1
3. Sepharose < C$\overline{1}$r / Ca^{2+} / C$\overline{1}$s	C$\overline{1}$r–C$\overline{1}$s complex covalently bound in the presence of calcium
4. Sepharose–C$\overline{1}$s–Ca^{2+}–C$\overline{1}$s	C$\overline{1}$s first bound as in 1; second non-covalent binding of C$\overline{1}$s in the presence of calcium
5. Sepharose–C$\overline{1}$sDFP–Ca^{2+}–C$\overline{1}$s	As for 4 but with C$\overline{1}$sDFP in the first binding
6. Sepharose–C$\overline{1}$r–Ca^{2+}–C$\overline{1}$s	C$\overline{1}$r covalently bound first: C$\overline{1}$s bound in the second step, as in 4 and 5
7. Sepharose–Ig–C1q–C$\overline{1}$r–C$\overline{1}$s	Ig covalently bound first: C1 fixed from serum in the second step, then activated to C1
8. Sepharose–Ag–Ig–C1q–C$\overline{1}$r–C$\overline{1}$s	Ag covalently bound first: Ig bound by immune affinity in the second step; then C1 fixed and activated in the third step, as in 7
9. Stroma–C$\overline{1}$s	C$\overline{1}$s covalently bound, in the absence of calcium

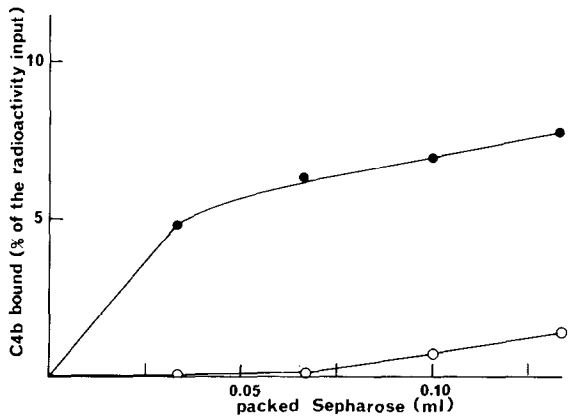

Fig.3. C4b binding to Sepharose–Ig anti-ovalbumin $C\bar{1}$: 5 μg ^{125}I-labelled C4 were incubated as in section 2 in 50 mM Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$ (pH 7.4) containing STI (final conc. 50 μg/ml) with varying concentrations of Sepharose–Ig anti-ovalbumin $C\bar{1}$. Washes were as in section 2 with the incubation buffer without STI then with 1% (w/v) aqueous SDS (•) 2 mg Ig/ml packed Sepharose; (○) 0.2 mg Ig/ml packed Sepharose.

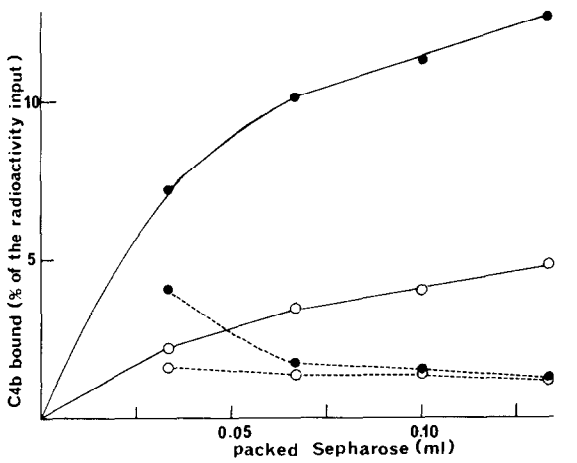

Fig.4. C4b binding to Sepharose–ICC$\bar{1}$: 5 μg (•) or 2.5 μg (○) of ^{125}I-labelled C4 were incubated as in section 2 in 50 mM Tris–HCl, 100 mM NaCl, 5 mM $CaCl_2$ (pH 7.4) containing STI (final conc. 50 μg/ml) with varying concentrations of Sepharose–ICC$\bar{1}$. Sequential washes were as in section 2 with the incubation buffer without STI, 50 mM Tris–HCl, 1 M NaCl (pH 7.4), 1 M propionic acid and 1% (w/v) aqueous SDS. Broken lines indicate the percentage of the input eluted by 1 M propionic acid.

monomeric $C\bar{1}$s blocked by DFP, incubated with soluble $C\bar{1}$s in 5 mM $CaCl_2$. These systems (table 1, systems 4,5) are very inefficient for C4b binding as well as a different Sepharose–$C\bar{1}$r–$C\bar{1}$s system (table 1, system 6) prepared from Sepharose–$C\bar{1}$r incubated with soluble $C\bar{1}$s in 5 mM $CaCl_2$.

3.3. *C4b binding to Sepharose–Ig anti-ovalbumin $C\bar{1}$*

C4b binds to Sepharose–Ig anti-ovalbumin $C\bar{1}$ (table 1, system 7) (fig.3). Anti-ovalbumin Ig was coupled to Sepharose 4B to generate a system comparable to that described to evaluate C4b binding to Sepharose–ICC$\bar{1}$ (table 1, system 8). From an amount of 0.075 ml packed Sepharose, the amount of bound C4b increases proportionally to the Ig: Sepharose ratio. For lower amounts of Sepharose the hardly-detectable bound C4b, in the case of the lowest Ig concentration, may be attributed to particulate $C\bar{1}$ being limiting.

3.4. *C4b binding to Sepharose–ICC$\bar{1}$*

Analysis of C4b binding to Sepharose–ICC$\bar{1}$ (table 1, system 8) (fig.4) suggests a double localisation: one part of the radioactivity which is eluted by 1 M propionic acid corresponds to C4b binding to the Ig, the other part which is not eluted even by additional washes by 1% aqueous SDS corresponds to covalent binding to Sepharose. Only the binding to Sepharose increases as a function of Sepharose–ICC$\bar{1}$ concentration and appears proportional to the amount of C4 for the two quantities of C4 assayed.

SDS–PAGE of a propionic-acid eluate is shown in fig.5: the distribution of the radioactivity is con-

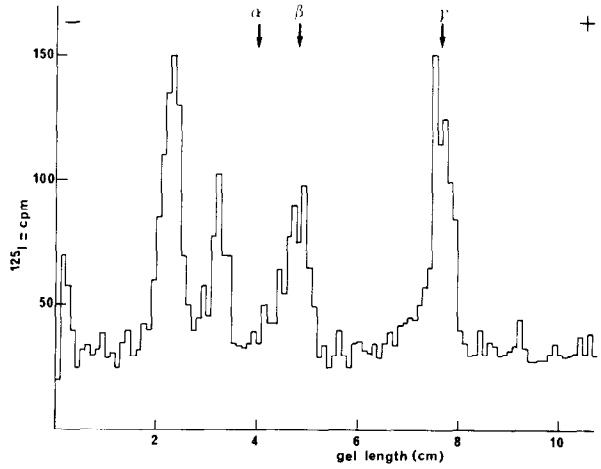

Fig.5. SDS–PAGE radioactivity pattern of a propionic acid eluate from Sepharose–ICC$\bar{1}$ after C4b binding. The propionic acid eluate corresponding to the highest radioactivity eluted in fig.4 was dialyzed against water and lyophilyzed. The lyophilysate was reduced and alkylated. Electrophoresis and counting were as in section 2. The arrows point to the position of the chains of C4.

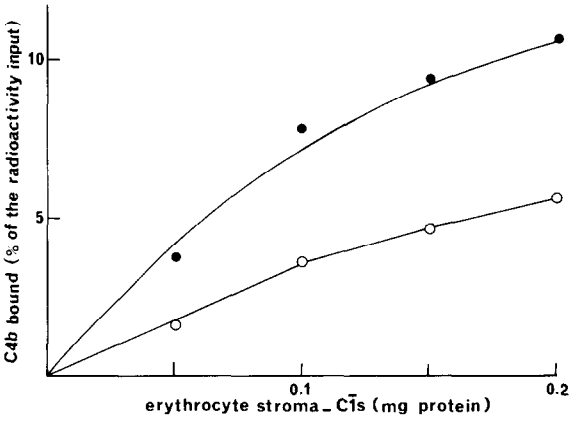

Fig.6. C4b binding to erythrocyte stroma–$C\bar{1}s$: 5 μg ^{125}I-labelled C4 were incubated as in section 2 in 50 mM Tris–HCl, 100 mM NaCl, 5 mM $CaCl_2$ (pH 7.4) containing STI (final conc. 50 μg/ml) with varying concentrations of erythrocyte stroma–$C\bar{1}s$. Washes were as in section 2 with the incubation buffer without STI. For each point the binding of fluid phase $C\bar{1}s$-cleaved C4 was subtracted. The erythrocyte stroma–$C\bar{1}s$ were prepared using 0.16 (○) or 0.32 (●) mg $C\bar{1}s$/mg stroma protein.

sistent with the pattern of radioactivity of ^{125}I-labelled C4b for β and γ chains but shows a very sharp decrease of radioactivity in α′ chain concomitant with the presence of two additional higher-molecular-mass radioactive peaks.

3.5. *C4b binding to erythrocyte stroma–$C\bar{1}s$*

C4b binds to stroma–$C\bar{1}s$ (table 1, system 9) as a function of the amount of stroma (fig.6). The $C\bar{1}s$ to stroma ratio appears to influence C4b binding; doubling $C\bar{1}s$ for the same amount of stroma induces a doubling of bound C4b.

Assuming that the yield of stroma from the starting erythrocytes is 100%, one can calculate that 450 molecules of C4b are bound per erythrocyte, based on the highest value of C4b binding.

4. Discussion

As binding of a metastable activated form of C4 represents a critical step in the formation of the C3 convertase, the binding of this component was examined using Sepharose and erythrocyte stroma as acceptors, in reference to the $EAC\bar{1}$ system.

C4 binds to Sepharose after cleavage by $C\bar{1}s$ bound to the same matrix. When C4 is proteolysed by soluble $C\bar{1}s$ no significant binding occurs, which is also observed with EA as acceptor. Several models in which $C\bar{1}s$ is located at different distances from the matrix (table 1) clearly show that the distance between the protease active site and the acceptor is the limiting factor in C4b binding, as shown for C3b binding to Sepharose–trypsin [18]. $C\bar{1}s$ directly bound to Sepharose (table 1, system 1) leads to a net binding of C4b whereas when it is at some distance no binding occurs. With a spacer as small as $C\bar{1}s$ itself or $C\bar{1}s$DFP (table 1, systems 4,5) only negligible amounts of C4b bind in spite of a total cleavage of C4; in this case the amount of C4b bound is of the same order as when C4 is incubated with Sepharose–$C\bar{1}s$DFP (table 1, system 2) which does not cleave C4. In the case of systems 7 and 8 (table 1) where, respectively, Ig–C1q–$C\bar{1}r$ and Ag–Ig–C1q–$C\bar{1}r$ are between $C\bar{1}s$ and the acceptor Sepharose, the binding of C4 on the matrix can be explained by a suitable orientation of $C\bar{1}s$.

The immediate proteic environment of $C\bar{1}s$ seems to influence the efficiency of C4b binding: when the $C\bar{1}r$–$C\bar{1}s$ complex is covalently linked to Sepharose (table 1, system 3), the system appears more efficient than when $C\bar{1}s$ is bound alone (table 1, system 1). In the same light the efficient binding of C4b reported above for systems 7 and 8 (table 1) can also be attributed to the integration of $C\bar{1}s$ in $C\bar{1}$, in close proximity to $C\bar{1}r$. The role of $C\bar{1}r$ could be either to modulate the active site of neighbouring $C\bar{1}s$ or to orient C4. The low binding of C4b observed with system 6 (table 1) in which $C\bar{1}r$ and $C\bar{1}s$ are sequentially bound can be explained by the only very partial reconstitution of the $C\bar{1}r$–$C\bar{1}s$ complex.

Both Sepharose and Ig participate as acceptor of C4b on Sepharose–$ICC\bar{1}$ (table 1, system 8). However more C4b binds to Sepharose than to Ig; the decrease of Ig-bound C4b with increasing concentrations of Sepharose–$ICC\bar{1}$ suggests that C4b has a higher affinity for Sepharose than for Ig. The electrophoretic pattern of C4b eluted from Ig by propionic acid indicates two high-molecular mass peaks wich could be accounted for by α′–α′ and α′–heavy Ig chain associations as observed with C4b bound to immune complex aggregates [7].

Fixation of C4b on stroma–$C\bar{1}s$ (table 1, system 9) is of the same order of magnitude as on $EAC\bar{1}$. $EAC\bar{1}$ and to a less degree stroma–$C\bar{1}s$ show pseudo-saturation curves for the fixation of C4b with increasing concentrations of acceptor, suggesting a difference between the kinetics of proteolysis and binding of C4b.

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