Function of the activated fourth component of complement (C4b) in activation of C2

W. Vogt, B. Hinsch, G. Schmidt and I. von Zabern

Max-Planck-Institute for Experimental Medicine, Dept. of Biochemical Pharmacology, Hermann-Rein-Str. 3, 3400 Göttingen, FRG

Received 20 May 1982

Complement activation *C3 convertase formation* *Cobra venom* *C4* *C2* *C1*

1. INTRODUCTION

In the course of classical pathway activation the fourth component of complement is cleaved by activated $C\bar{1}$ into C4a (8800 M_r) and C4b (197 000 M_r). It is well known that C4b forms a reversible complex with the second component of complement, C2, with the essential participation of Mg^{2+} [1,2]. Furthermore, C4b serves as an acceptor for $C\bar{2}$, activated by cleavage by $C\bar{1}$; the complex formed, C4b2a,b is independent of Mg^{2+} and has proteolytic activity for C3 and C5. The enzymic site resides in the C2a part of the labile complex [2–4], expressed only when C2a is bound, not after its irreversible dissociation or when generated by $C\bar{1}$ in the absence of C4b.

The involvement of C4b in the process of C2 activation remained still unclear. It has been reported that C2 cleavage by soluble, free $C\bar{1}s$ is not affected by the presence or absence of C4b [4,5]. However, intact $C\bar{1}$, composed of the subcomponents $C\bar{1}s$, $C\bar{1}r$, C1q, cleaves C2 better when C4b is present, in the fluid phase as well as when fixed to a surface [5,6,8].

These experiments were designed to further clarify the role of C4b in the enhancement of C2 activation by intact $C\bar{1}$. Here, we show the abrogation of the enhancement by an inhibitor from cobra venom which we found to bind C4b [7] and demonstrate that C2 cleavage by $C\bar{1}$ and its enhancement by C4b are Mg^{2+} independent. Our results indicate that C4b facilitates C2 cleavage by acting on the $C\bar{1}$ complex, an explanation proposed in [5]. Facilitation of C2 cleavage by interaction of C4b with C2 [6] or by action of C4b as product acceptor [8] seem now to be excluded.

Abbreviations: CVF, cobra venom factor; EDTA, ethylenediamine tetraacetic acid

2. MATERIALS AND METHODS

2.1. *Buffers*

VBSGG is 2.5 mM barbital buffer (pH 7.4) with 0.08 M NaCl, 2.5% glucose and 0.1% gelatin. $VBSGG^{2+}$ contains 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, in addition. Ca^{2+}-VBSGG has 0.15 mM $CaCl_2$, no Mg^{2+}. EDTA–VBSGG contains 0.01 M EDTA. VBS lacks glucose and gelatin, and contains 0.15 M NaCl.

2.2. *Cobra inhibitor*

The inhibitor was isolated from 5 g *Naja naja* venom as in [7].

2.3. *Anti-cobra inhibitor*

Antiserum against the inhibitor was raised in rabbits. The antiserum was passed through a column of protein A–Sepharose (Pharmacia), and the bound IgG was eluted with 0.4 M NaCl in 0.1 M glycine buffer (pH 2.8). Finally the IgG fraction was dialyzed against VBS.

2.4. *Complement components*

$C\bar{1}$ used was prepared from human serum as in [9] without adding DFP. The preparation contained 6.5 mg protein/ml estimated by absorption at 280 nm, assuming $A^{1\%}_{270} = 10.0$. C4 was purified from human serum as in [10]. The preparation contained 25 μg/ml

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

(280 nm). C2 was purified from the supernatant of human serum precipitated with 20% (w/v) Na_2SO_4 by chromatography on SP-Sephadex and DEAE-cellulose [11]. $^{oxy}C2$ was obtained by treatment of C2 with iodine [11]. Complement components used for haemolytic assay of C2, were purchased from Cordis Corp. (Miami FL).

2.5. *Immune precipitate (AgAb)*

A solution of human serum albumin (Behringwerke, Marburg) (0.25 ml containing 1.25 mg) was mixed with 4 ml rabbit anti-human serum albumin (Behringwerke) in the presence of 0.01 M EDTA. The mixture which contained antibody in excess, was left at 4°C overnight. Then the precipitate was washed twice with 5 ml EDTA–VBS, twice with 5 ml VBS containing 1 M NaCl, and twice with VBS containing 4 mM NaN_3. The precipitate was resuspended in VBS–azide at 2.1 mg/ml, estimated by the Folin assay, and was kept frozen in small aliquots at – 70°C.

2.6. *Cellular intermediates*

EAC$\overline{1}$ and EAC$\overline{14}$ cells used for studies on cleavage of C2, were prepared with sheep red cells, rabbit amboceptor, $C1^{gp}$ and $C4^{hu}$ [11]. For the preparation of EAC$\overline{4}$, used in C2 binding experiments, EAC$\overline{14}$ were shaken for 10 min at room temperature with EDTA–VBSGG, centrifuged, washed once with VBSGG, twice with $VBSGG^{2+}$, and resuspended in $VBSGG^{2+}$.

2.7. *Estimation of C2*

Binding and cleavage of C2 was assessed by measuring residual C2 activity in supernatants of incubated samples. C2 was assayed by immune haemolysis using EAC$\overline{14}$, Cordis $C3^{hu}$, $C5^{hu}$ and C6–$C9^{gp}$. Since the cobra inhibitor interfered with the haemolytic C2 assay, all samples which did not contain it during the incubations (controls), received it after the reaction. Then anti-cobra inhibitor-IgG was added to all samples in slight excess.

3. RESULTS

3.1. *Effect of cobra inhibitor on binding of C2 to EAC$\overline{4}$*

EAC$\overline{4}$ (2.5×10^7 cells) were incubated with 0.45 μg C2 in 200 μl total vol. $VBSGG^{2+}$, for 10 min at 20°C. After centrifugation, < 10% of the added C2 was recovered in the supernatant. The cell sediment was then treated with 200 μl EDTA–VBSGG for 10 min, and the new supernatant obtained after centrifugation was supplemented with an equal volume of 0.01 M $CaCl_2$ containing 0.011 M NaOH to neutralize the EDTA without lowering pH. It contained 56% of the originally added C2, in haemolytically active form. The C2 had been bound to EAC$\overline{4}$ and was released by EDTA. When the incubation of C2 with EAC$\overline{4}$ was carried out in the presence of 130 μg/ml cobra inhibitor hardly any C2 was bound; >95% were recovered in the supernatant, and no measurable C2 activity was released from the cells by EDTA–VBSGG (results are means of 2 expt).

3.2. *Effect of cobra inhibitor on cleavage of C2 by EAC$\overline{1}$ and EAC$\overline{14}$*

EAC$\overline{14}$ (2.5×10^7 cells) were incubated with 0.45 μg C2, for 5 min at 30°C, in 100 μl total vol. $VBSGG^{2+}$. Only 6% of the added C2 were recovered in the supernatant, and no activity could be eluted from the cells by EDTA–VBSGG. Thus the loss of C2 activity must have been due to nearly complete cleavage. In the presence of cobra inhibitor (130 μg/ml) the cleavage of C2 by EAC$\overline{14}$ was reduced to only 51%.

EAC$\overline{1}$ cells cleaved C2 much less efficiently, as expected. Incubation of 0.45 μg C2 with 2.5×10^7 EAC$\overline{1}$ in 100 μl (40 min 30°C) led to a loss of 39% C2 activity. Cobra inhibitor (130 μg/ml) had no inhibitory effect; the C2 consumption in its presence was 37%. These results (means of 2 expt) indicate that cobra inhibitor abrogates the enhancing function of C4b on C2 activation by the C$\overline{1}$ complex.

3.3. *Effect of Mg^{2+} on cleavage of C2 by C$\overline{1}$ and C$\overline{14}$, and on generation of C3 convertase, C$\overline{42}$*

To further study the effect of C4b and of Mg^{2+} on the activity of C$\overline{1}$ against C2 we chose preformed immune precipitate (AgAb), as a system which could be used in a reproducible manner, from which any free C$\overline{1}$s could be easily eliminated and which unlike red cells would not release unwanted Mg^{2+} into the medium.

AgAb (525 μg) was incubated with 0.3 μg $C\overline{1}^{hu}$ in 730 μl Ca^{2+}–VBSGG, for 15 min in an ice water bath. Then the precipitate was washed twice with Ca^{2+}–VBSGG and resuspended in 250 μl Ca^{2+}–VBSGG. Two 50 μl aliquots of AgAbC$\overline{1}$ intermediate were then treated with 0.3 μg $C4^{hu}$ for 30

Table 1

Cleavage of C2 by AgAbC$\overline{1}$ and AgAbC$\overline{14}$; effect of Mg^{2+}

Intermediate	Mg^{2+} (mM)	% C2 cleavage (mean ± SD)	Difference to (1)
(1) AgAbC$\overline{14}$	4.5	65.3 ± 4.16	
(2) AgAbC$\overline{14}$	0	79.0 ± 4.80	$p < 0.01$
(3) AgAbC$\overline{1}$	4.5	44.3 ± 2.52	$p < 0.001$
(4) AgAbC$\overline{1}$	0	44.0 ± 3.46	
		% ^{oxy}C2 cleavage	
(5) AgAbC$\overline{14}$	4.5	51.5 ± 2.12	$p < 0.02$
(6) AgAbC$\overline{14}$	0	56.0 ± 4.24	

The intermediates (50 μl aliquots) were incubated with $C2^{hu}$ (3 expt) or ^{oxy}C2 (2 expt), in the presence or absence of Mg^{2+}, as indicated. For details see text

Table 2

Generation of C3 cleaving C$\overline{42}$ complex on AgAb; effect of Mg^{2+}

Complex generation	% C3 cleavage (mean ± SD)
AgAbC$\overline{14}$ + ^{oxy}C2	
50 μl + Mg^{2+}	10 ± 0.82
50 μl − Mg^{2+}	0 ± 0
200 μl + Mg^{2+}	33.5 ± 2.5
200 μl − Mg^{2+}	0 ± 0

In 3 expt 50 μl aliquots of AgAbC$\overline{14}$ were incubated with ^{oxy}C2 in presence (4.5 mM) or absence of Mg^{2+}. The complex was then washed and incubated with C3 to assess its convertase activity. In two further experiments 4-times more AgAbC$\overline{14}$ + ^{oxy}C2 was used. For details see text

min at 0°C to obtain AgAbC$\overline{14}$, and 2 others with Ca^{2+}–VBSGG for the same time, these remaining in the state AgAbC$\overline{1}$. Then the aliquots were washed twice with Ca^{2+}, resuspended in 50 μl Ca^{2+}–VBSGG, and 50 μl of a 1:100 dilution of $C2^{hu}$ in Ca^{2+}–VBSGG were added. One AgAb$\overline{14}$ aliquot received 10 μl 50 mM $MgCl_2$ in addition, the other H_2O as control. After 60 min incubation at 30°C the mixtures were centrifuged, and residual C2 was estimated in the supernatants.

Table 1 shows that C4b on the intermediate enhances C2 cleavage by C$\overline{1}$ considerably, and that this effect is quite independent of Mg^{2+}. In its absence the C$\overline{14}$ intermediate cleaves C2 even slightly faster. ^{oxy}C2 is less efficiently cleaved than C2 (confirming [8]), again there is no positive effect of Mg^{2+} on the cleavage.

To test the effect of Mg^{2+} on the formation of a C3 cleaving C$\overline{22}$ complex AgAb was treated as above with C$\overline{1}$, C4 and with ^{oxy}C2 in the absence or presence of Mg^{2+}. The sediment was washed once with EDTA–VBSGG, once with VBSGG, and was then resuspended in 50 μl VBSGG. It was incubated with 50 μl $C3^{hu}$ (Cordis) for 30 min at 30°C and the residual C3 activity in the supernatant was taken as a measure of C3 convertase activity. There was no C3 cleaving activity when Mg^{2+} was missing, i.e., no C$\overline{42}$ complex was formed under these conditions (table 2).

4. DISCUSSION

In [7] cobra inhibitor was shown bind to C4b. We now show that it inhibits binding of C2 to C4b. This is most likely caused by the affinity of cobra inhibitor to C4b, which results in competition with C2 for the binding site, or in steric hindrance of C2 attachment. Since direct cleavage of C2 by EAC$\overline{1}$ is not affected, cobra inhibitor is clearly not an enzyme inhibitor nor does it interact effectively with C2. It does, however, abolish the enhanced cleavage of C2 by the C$\overline{1}$ intermediate. In the presence of cobra inhibitor the cleavage rate of EAC$\overline{14}$ is reduced to that of EAC$\overline{1}$. Similarly, in [8] the increased cleavage rate of C2 by EAC$\overline{14}$ was reduced to the activity of EAC$\overline{1}$ when the C4b on the cells has bound ^{oxy}C2. This indicates that both, ^{oxy}C2 and cobra inhibitor abrogate the facilitating effect of C4b. The decision whether C4b exerts this effect by interaction with enzyme or substrate is possible when the effect of Mg^{2+} is considered. Although it is required for binding of C2 to C4b, it has no facilitating but even some inhibitory effect on C2 cleavage by AgAbC$\overline{14}$, not by AgAbC$\overline{1}$. These results argue against the possibility that C4b facilitates C2 cleavage by substrate modulation; also, against a product acceptor function of C4b [8]; rather, our results point to an effect of C4b on the complex enzyme, C$\overline{1}$.

However, for the formation of the C3 convertase complex, C4b2a,b, Mg^{2+} is indispensible, in accordance with earlier findings. Not the slightest activity formed in its absence, indicating that activated C2 is unable to bind to C4b under these conditions. Since the convertase complex contains both C2 fragments [3,4], C2a remaining attached to C4b without Mg^{2+}, and since the presence of Mg^{2+} native C2 binds to C4b much more rapidly than cleavage by $C\bar{1}$ proceeds [1], it seems inevitable to conclude that the C4bC2a,b complex forms only when C2 is bound already before cleavage as proposed in [12]. The other, theoretical possibility that C2 is first cleaved, and then both fragments combine with C4b, requiring Mg^{2+} for the attachment but not for the persistence of C2a in the complex, is not equally conceivable; e.g., not well-compatible with the fact that C2a dissociates irreversibly from C4b and, hence, cannot be expected to bind to C4b when preformed. Lastly, both $C\bar{1}$ and C4b are obviously in intimate contact (C4b affects $C\bar{1}$ activity), and C2 when approaching $C\bar{1}$ just cannot escape C4b. The sequence:

$$C2 + C4b \xrightarrow{Mg^{2+}} C4b,C2 \xrightarrow{C\bar{1}} C4b,C2a,b$$

is analogous to the formation of the alternative pathway convertase, C3bBb and CVF,Bb, which also can be generated only when B is bound to C3b or CVF before cleavage by any enzyme [13,14].

In conclusion, our results indicate that C4b enhances C2 cleavage by modulation of the complex enzyme, $C\bar{1}$, as suggested in [5,6]. Since free $C\bar{1}$ esterase, $C\bar{1}s$, cleaves C2 independent of C4b and at about the rate of $C\overline{14}$ [4,5], confirmed by unpublished results) it is likely that in the C1q,r,s complex some subcomponent ($C\bar{1}r$ according to [5]) exerts an inhibitory effect on $C\bar{1}s$ which is eliminated by interaction with C4b. The C3 cleaving complex C4b2a,b probably forms only when C2 is bound before cleavage. Cobra inhibitor inhibits C2 activation by 2 independent consequences of one effect. Its binding to C4b inhibits C4b,C2 formation and abrogates the desinhibition of $C\bar{1}$ by C4b.

REFERENCES

[1] Sitomer, G., Stroud, R.M. and Mayer, M.M. (1966) Immunochemistry 3, 57–69.
[2] Müller-Eberhard, H.J., Polley, M.J. and Calcott, M.A. (1967) J. Exp. Med. 125, 359–380.
[3] Nagasawa, S. and Stroud, R.M. (1977) Proc. Natl. Acad. Sci. USA 74, 2998–3001.
[4] Kerr, M.A. (1980) Biochem. J. 189, 173–181.
[5] Gigli, I. and Austen, K.F. (1969) J. Exp. Med. 130, 833–846.
[6] Gigli, I. and Austen, K.F. (1969) J. Exp. Med. 129, 679–696.
[7] Von Zabern, I., Przyklenk, H., Damerau, B. and Zimmermann, B. (1981) Scand. J. Immunol. 14, 109–120.
[8] Strunk, R. and Colten, H.R. (1974) J. Immunol. 112, 905–910.
[9] Gigli, I., Porter, R.R. and Sim, R.B. (1976) Biochem. J. 157, 541–548.
[10] Gigli, I., Von Zabern, I. and Porter, R.R. (1977) Biochem. J. 165, 439–446.
[11] Vogt, W., Hinsch, B., Schmidt, G. and Von Zabern, I. (1979) Immunology 36, 131–137.
[12] Dodds, A.W. and Porter, R.R. (1979) Mol. Immunol. 16, 1059–1062.
[13] Vogt, W., Dieminger, L., Lynen, R. and Schmidt, G. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 171–183.
[14] Vogt, W., Schmidt, G., Dieminger, L. and Lynen, R. (1975) Z. Immun.-Forsch. 149, 440–455.