Immunology, 1973, **24**, 259.

The Alternate Pathway of Complement Activation

THE ROLE OF C3 AND ITS INACTIVATOR (KAF)

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(*Received 8th August* 1972; *accepted for publication 14th September* 1972)

Summary. The immunochemical depletion from serum of C3b inactivator (KAF) has been performed using purified $F(ab')_2$ antibody. *In vitro* KAF depletion leads to spontaneous activation of the 'alternate pathway of complement fixation' as evidenced by conversion of glycine-rich β-glycoprotein, depletion of cobra venom factor–C3 proactivator and conversion of C3.

The status of the complement system following *in vitro* KAF depletion accurately mimics that found *in vivo* in the unique KAF-deficient patient.

The activation of the alternative pathway whether by KAF depletion or by conventional alternative pathway activators is prevented by the immunochemical depletion of C3. It therefore appears that the alternative pathway is essentially a C3b-feedback pathway which is normally controlled by the activity of KAF.

INTRODUCTION

C3 fixation is of major biological significance in the complement sequence (Müller-Eberhard, 1968). There are now considered to be two physiological complement pathways for generating C3-splitting enzymes ('C3-convertases'): the classical convertase—$C\overline{42}$ generated by the sequential reaction of complement-fixing antibody C1, C4 and C2; and the so-called alternate pathway convertase (Pillemer *et al.*, 1954; Gewurz, 1972).

The components of the alternate pathway are not yet fully clarified. Its activation results in the splitting of the normal human serum protein, glycine rich β-glycoprotein (GBG) (Boenisch and Alper, 1970; Alper, Boenisch and Watson 1972) or C3 proactivator (Götze and Müller-Eberhard, 1971) to split products with α (glycine rich α-glycoprotein (GAG) and γ glycine rich γ-glycoprotein (GGG) mobilities. Partially purified GGG has been shown to contain C3 convertase activity (Götze and Müller-Eberhard, 1971). Fragmentation of GBG is achieved by GBGase (Alper and Rosen, 1971; Rosen and Alper, 1972) or C3 proactivator convertase (Müller-Eberhard and Götze 1972), this activity requiring the presence of a fragment of C3. An inhibitor of GBGase has also been found in normal serum (Alper and Rosen, 1971; Rosen and Alper, 1972).

A third, presumably non-physiological pathway for generation of a C3 convertase involves a factor from cobra venom (Nelson, 1966) and (a) normal serum factor(s). (Müller-Eberhard, 1967; Müller-Eberhard and Fjellstrom, 1971). This cobra venom factor (CVF) combines with a serum factor in the presence of magnesium to generate a C3 convertase. The serum factor has been identified with GBG (Götze and Müller

Eberhard, 1971) but more recent work suggests that other serum factors may also be required (Alper, Goodkofsky and Lepow, 1972b and c; Hunsicker, Ruddy and Austen, 1972). The term cobra venom factor C3 proactivator (CVF-C3PA) is used in this paper for the serum factor(s) required to generate a C3 convertase with cobra venom factor.

The fixation of C3 is subject to homeostatic control in at least two ways. Firstly both the classical and alternate pathway convertases have short half lives (Müller-Eberhard, 1968; Marcus, Shin and Mayer, 1971; Gewurz, 1972) and second there exists in serum an inactivator of fixed C3.

C3b inactivator (KAF) (Tamura and Nelson, 1967; Lachmann and Müller-Eberhard, 1968; Ruddy and Austen, 1969) inhibits the haemolytic and immune adherence activities of fixed C3b. KAF is not depleted during its presumably proteolytic reaction on C3b and

TABLE 1
ABBREVIATIONS

Abbreviation	Meaning	Other names
KAF	Conglutinogen-activating factor	C3b inactivator
GBG	Glycine rich β glycoprotein	C3 proactivator, factor B*
GAG	Glycine rich α glycoprotein	
GGG	Glycine rich γ glycoprotein	C3 activator
GBGase	Glycine rich β glycoproteinase	C3 proactivator convertase
CVF	Cobra venom factor	
CVF-C3PA	Cobra venom factor C3 proactivator	?C3 proactivator
NHS	Normal human serum	
C2D	Genetically C2 deficient human serum	
CFD	Complement fixation diluent	
R(KAF)	Serum immunochemically depleted of KAF	
R(ovalbumin)	Serum to which ovalbumin is added and then immunochemically depleted	
R(C3)	Serum immunochemically depleted of C3	

* Blum, Pillemer and Lepow (1959).

it results, possibly with an additional DFP-inhibitable protease (Lachmann, Elias and Moffett, 1972) in the cleavage of fixed and fluid phase C3b (Ruddy and Austen 1971; Abramson *et al.*, 1971). Although it has been reported that KAF can be identified with C3 proactivator (Cooper, 1971) it has been shown to be distinct from both glycine rich β-glycoprotein and from cobra venom factor C3 proactivator (Ruddy, Hunsicker and Austen, 1972; Lachmann, Aston and Nicol, 1973).

There exists a single much studied patient (Alper *et al.*, 1970a; Alper *et al.*, 1970b; Abramson *et al.*, 1971; Alper and Rosen, 1971; Alper *et al.*, 1972b) with an apparent total deficiency of KAF. He has chronic depletion/activation of the alternate pathway, i.e. low C3, GBG, CVF-C3PA and an absence of a GBGase inhibitor and he suffers from a marked susceptibility to pyogenic bacterial infections.

The suggestion that, *in vivo*, KAF deficiency results in the loss of homeostatic control for the alternate pathway has prompted the present work. Using an immunochemical method for depleting KAF *in vitro*, a situation closely paralleling the *in vivo* condition of KAF deficiency has been produced. In this paper the consequences of KAF depletion on the complement system are explored. A preliminary account of this work has previously been presented (Lachmann, 1972a).

Where applicable the nomenclature of complement recommended in W.H.O. (1968) is used. Table 1 explains all other abbreviations used in this paper.

MATERIALS AND METHODS

Serum (NHS) was collected from healthy donors by venepuncture and stored in liquid nitrogen until required.

Serum/EDTA 1/20th volume of 0·2 M NaEDTA pH 7·2 was added to serum.

Recalcification 1/20th volume of 2 per cent $CaCl_2$ plus 2 per cent $MgCl_2$ $6H_2O$ was added to serum/EDTA. This was sufficient to restore complement-mediated immune haemolysis.

Serum/ovalbumin 1/10th volume of 100 or 200 μg/ml chicken ovalbumin (Koch Light) was added to serum.

Buffers. Complement-fixation diluent (Mayer, 1961). Phosphate buffered saline.

C2-deficient serum was obtained from the patient described by Sussman, Jones and Lachmann (1972) and stored at −180°.

Pepsin F(ab')$_2$ antibody (Lachmann, 1971). At optimal proportion an immune precipitate of whole monospecific antiserum with antigen was prepared in 0·01 M/EDTA. This precipitate after washing was digested with 2 per cent w/w pepsin at pH 3·1 for 2 hours at 37°. The digest was neutralized and centrifuged, insoluble material being discarded. The purified F(ab')$_2$ antibody was harvested by 20 per cent sodium sulphate precipitation, pepsin and most other products of pepsin degradation being left in the 20 per cent sulphate supernatant. After removal of the sulphate by dialysis against saline, the antibody was quantified with serum by Ouchterlony analysis (Ouchterlony, 1953), or where possible, optimal proportion precipitation. This method has been used to prepare the F(ab')$_2$ antibodies to human KAF, C3, C4, GBG, C6, IgG and ovalbumin used here.

Functionally purified KAF (Lachmann *et al*, 1973) was prepared from the euglobulin fraction of serum via DEAE cellulose and Sephadex G-200 chromatography.

Antrypol (Bayer 205). Treatment of cell-fixed C3 with 1 mg/ml antrypol for 5 minutes prevents the action of KAF on this C3 but the C3 remains fully active haemolytically. (Lachmann, Hobart and Aston, 1972).

Functionally purified C3 (Lachmann *et al.* 1972) was prepared from the euglobulin fraction of serum by DEAE cellulose and hydroxylapatite chromatography.

Anti-human C7 was the DEAE IgG fraction of monospecific rabbit anti-human C7.

Inulin (Sigma Biochem.). A standard suspension of 50 mg/ml in saline was prepared and diluted as required.

Yeast cell walls (Lachmann *et al.*, 1972). A standard suspension in CFD prepared by a modification from that of Hadding, Bitter-Suerman and Melchert (1969) was used.

Aggregated human γ-globulin 10 mg/ml solution of human γ-globulin heated at 63° for 15 minutes was used.

Complement component assays

KAF. Conglutination—the agglutination of fixed human C3 in sheep cells by bovine conglutinin was determined after the action of serial dilutions of KAF sample on the fixed C3. It was also measured antigenically with a monospecific rabbit anti-KAF.

GBG (Boenisch and Alper, 1970). The conversion of GBG to GGG was determined by immunoelectrophoresis in agar gel at pH 8·6 using a monospecific rabbit anti-GBG.

Conversion is scored: ++++ for total conversion to GGG; +++ 50–100 per cent; ++ 50 per cent; + <50 per cent; (+) trace GGG; O no GGG detectable.

C3 conversion was quantified using two-dimensional antigen–antibody crossed electrophoresis (Laurell, 1965) a sheep anti-human C3 antiserum in agarose gel pH 8·6. A voltage of 4 V/cm was applied for 1½ hours for the first dimension and then 4 V/cm in the second for at least 3 hours. The plate was washed, dried, stained with amido black, enlarged and the areas measured by planimetry to give the per cent C3 conversion to electrophoretically faster inactive C3. A contaminating anti-transferrin was used as a marker.

CVF-C3PA. The undiluted sample was incubated for 15 minutes at 37° with DEAE-cellulose purified CVF (Ballow and Cochrane, 1969). Five volumes of human plasma in 0·01 M EDTA were added and after a further 2 hours at 37°, C3 conversion was estimated electrophoretically. A control using CFD instead of CVF was included. The per cent conversion due to the addition of CVF was converted using a standard curve to the amount of CVF-C3PA expressed as a percentage of the amount in normal human serum

Whole complement (CH50 units/ml). An adaptation of the Mayer technique (Mayer, 1961) was used.

$C\overline{142}$ CH(142) units/ml (Thompson, 1972). 0·2 ml serial dilutions of serum were incubated with 0·05 ml per cent sensitized sheep cells for T_{max}, 6 minutes. 0·2 ml 1/20 guinea-pig complement in 0·01 M EDTA was added and after 30 minutes at 37° the volume was made to 1 ml and the per cent haemolysis calculated from the optical density at 415 mμ of the cell supernatant.

C3 was measured haemolytically using sheep EAC142 and dilute ammonia-treated guinea-pig complement (Lachmann and Liske, 1966).

Preparation of cells with fixed C3. EAC142 was made with EA and yeast-treated guinea-pig complement. Incubation of washed EAC142 with purified C3 for 15 minutes at 37° results in EAC143.

RESULTS

1. THE EFFECT OF KAF DEPLETION

A. *Immunoabsorption of KAF with $F(ab')_2$ anti-KAF*

Human serum was depleted at optimal proportion with $F(ab')_2$ anti-KAF. This required 4 volumes of serum to 1 volume of the $F(ab')_2$. As a control, chicken ovalbumin added to serum at 20 μg/ml, (a concentration thought to approximate to that of KAF) was depleted with $F(ab')_2$ anti-ovalbumin. After incubation for 30 minutes at 37° the sera were assayed for complement components (Table 2). The depletion of KAF appeared to activate the alternate pathway of complement. Thus GBG (Fig. 1) and CVF-C3PA were totally lost and C3 (Fig. 2) was largely inactivated functionally and electrophoretically. The classical pathway, as mirrored in the C142 titre was only slightly reduced.

The depletion of ovalbumin produced no significant changes apart from a small drop in CVF-C3PA titre indicating that the effects seen on KAF depletion could not be due solely to activation of the alternate pathway by the $F(ab')_2$ antigen reaction.

B. *The effect of the re-addition of KAF to KAF-depleted normal (NHS) and C2-deficient (C2D) serum*

For this experiment the depletion of KAF was performed in EDTA in an attempt to

TABLE 2

IMMUNO-ABSORPTION OF KAF WITH $F(ab')_2$ ANTI-KAF

Component	Activity		
	Serum +20 μg/ml ovalbumin* $F(ab')_2$ anti-KAF R(KAF)	Serum +20 μg/ml ovalbumin $F(ab')_2$ anti-ovalbumin R(Ovalbumin)	Serum +20 μg/ml ovalbumin saline NHS
KAF (conglutination) titre	0(<0·1)	10^3 (100)	10^3 (100)
Ovalbumin (Antigenically)	+	0	+
CH50 units/ml	60(55)	110(100)	110(100)
CH(142) units/ml	242(74)	301(92)	326(100)
C3 titre	400(38)	950(90)	1060(100)
Per cent C3 conversion (Laurell technique)	83	<5	<5
GBG conversion (Antigenically)	++++	0	0
CVF-C3PA Per cent of standard serum	0(<14)	44 (61)	72 (100)

* Per cent in parentheses.

1 Part $F(ab')_2$ antibody or saline was added to 2 parts serum/ovalbumin at time zero. After 30 minutes at 37° complement levels were determined as described in the Material and Methods section.

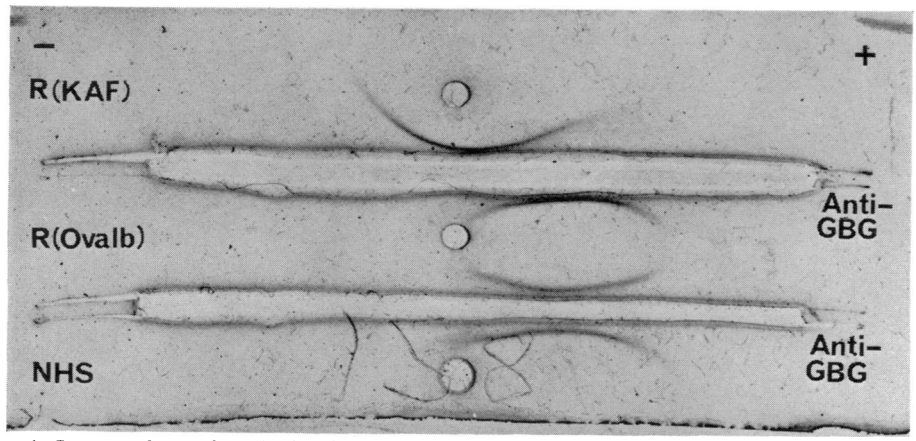

FIG. 1. Immunoelectrophoresis showing complete (++++) conversion of GBG to its breakdown products GGG in R(KAF).

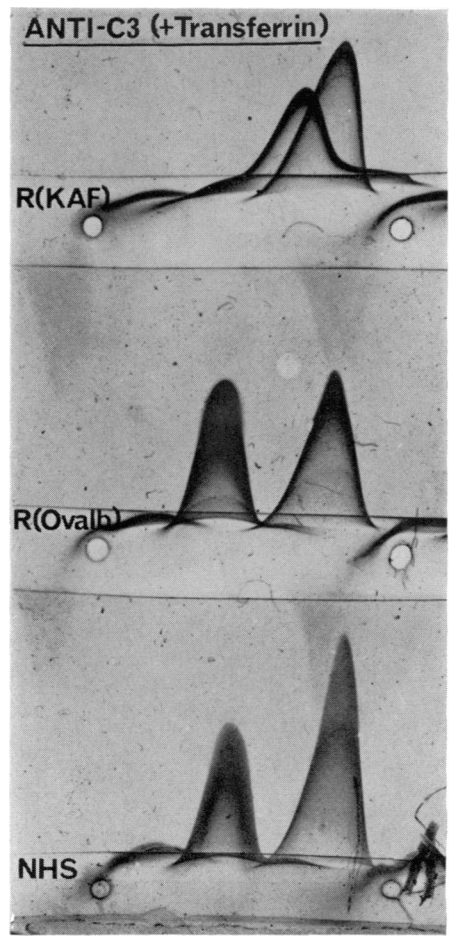

FIG. 2. Two dimensional Laurell electrophoresis for C3. C3 is largely converted to the faster inactive form only in R(KAF). The anode for the first dimension is to the right and to the top for the second dimension.

prevent activation of the alternate pathway during depletion and before the re-addition of the KAF. Serum in 0·01 M EDTA was depleted at optimal proportion with $F(ab')_2$ anti-KAF for 12 hours at 4°. The mixture was centrifuged at 10,000 ***g*** to remove the immune precipitate and calcium and magnesium added to the supernatant in the absence or presence of additional purified KAF. Before recalcifying no GBG conversion was seen in any sample. A control was similarly treated but using saline instead of the $F(ab')_2$ anti-KAF (Table 3).

The results show that some loss of CVF-C3PA and some GBG conversion occurred even in normal human serum treated with EDTA and recalcified. The GBG and C3 conversion was reduced in the controls in C2-deficient serum, suggesting that the classical complement pathway plays some part in this 'spontaneous' activation of the alternate pathway. This topic is discussed later.

Following KAF depletion there was total conversion of GBG and loss of CVF-C3PA in both the normal and C2-deficient sera. In the KAF reconstituted samples, however, there was scarcely more CVF-C3PA and GBG activation than was found in the control sera.

This demonstrates that the activation of the alternate pathway produced by KAF depletion was prevented by the re-addition of purified KAF and did not require the classical pathway. 0·01 M EDTA prevented the activation of the alternate pathway which rules out the possibility that contaminating antibody to GBG or to CVF-C3PA in the anti-KAF might produce the observed effects.

C. *The effect of temperature on the time course of GBG and C3 conversion in R(KAF)*

Immunoabsorption of KAF with $F(ab')_2$ anti-KAF and ovalbumin depletion control as in Experiment 1A was carried out at 4° and 37°. Samples were assayed immediately for GBG and C3 conversion electrophoretically (Fig. 3).

C3 and GBG conversion in KAF depleted serum were temperature dependent and occurred simultaneously. The 50 per cent conversion time for C3 and GBG at 37° was calculated to be 2 minutes. The conversion found in control sera at 37° occurred much more slowly and did not reach 50 per cent conversion within 4 hours.

Similarly, no CVF-C3PA was detectable 15 minutes after the readdition of calcium and magnesium to R(KAF)/EDTA at 37°. At this time the R(KAF) +KAF reconstituted control showed no significant CVF-C3PA depletion.

D. *The demonstration of C3 deposition onto erythrocytes by R(KAF)*

The presence of ABO compatible or autologous normal human red blood cells during KAF depletion resulted in C3 being deposited on the cells where it could be detected by agglutination with a $F(ab')_2$ anti-C3 directed against the A determinant of C3 (West *et al.*, 1966) (Table 4). This is comparable with the situation in the KAF deficient patient. Red blood cells present in control (ovalbumin) depletions could not be agglutinated by anti-C3, and red blood cells added to R(KAF) 20 minutes after the addition of antibody showed only trace agglutination. Incubation of washed C3-coated cells with normal serum or with purified KAF removed the C3 from the cells. The cells remained negative throughout the agglutination with bovine conglutinin.

Agglutination tests using monospecific $F(ab')_2$ antibodies to human γ-globulin, C4, C6, GBG and an IgG anti-C7 were all negative.

If ABO compatible, paroxysmal nocturnal haemoglobinuria (PNH) red cells, were

TABLE 3

THE READDITION OF KAF TO KAF DEPLETED NORMAL (NHS) AND C2 DEFICIENT (C2D) SERUM

Component	Activity							
	NHS/EDTA				C2D/EDTA			
	$F(ab')_2$ anti-KAF R(KAF)EDTA		NHS/EDTA		$F(ab')_2$ anti-KAF C2DR(KAF)/EDTA		C2D/EDTA	
	KAF				KAF			
	CaγMg				CaγMg			
	R(KAF)	R(KAF)+KAF	NHS+KAF	NHS	C2DR(KAF)	C2DR(KAF)+KAF	C2D+KAF	C2D
GBG conversion antigenically	++++	+++	++	++	++++	0	0	0
CVF-C3PA (per cent standard serum)	0	76	130	88	0	80	105	80
Per cent C3 Conversion (By Laurell technique)	80	60	35	50	39	11	4	25
KAF titre by conglutination	0	750	3000	750	0	800	1500	1500

One part $(Fab')_2$ anti-KAF or saline was added to 4 parts NHS or 3 parts C2D/EDTA. After immune precipitate removal 1 part purified KAF or saline was added to 2 parts of serum. Calcium and magnesium were added at time zero and the samples were incubated for 30 minutes at 37° before assay of components.

used in this system, 12 per cent lysis of these complement-sensitive cells occurred in the R(KAF). A similar experiment was performed using sheep erythrocytes and depleting of KAF normal human serum previously absorbed with sheep erythrocytes. Three per cent lysis was found. This lysis, although small, also indicated that some activation of the later components of complement resulted from *in vitro KAF* depletion.

E. *The effect of complement inhibitors—EDTA, heat, and ammonia on alternate pathway activation by KAF depletion*

(i) 0·01 M EDTA, as shown in section 1B totally prevented alternate pathway activation indicating a calcium/magnesium requirement. At least three stages are now known to be inhibited by 0·01 M EDTA in the complement sequence. The formation of $C\bar{1}$, the formation of $C\overline{42}$, and the activation of GBG by GBGase.

(ii) Sera heated for 30 minutes at 56° with total destruction of GBG antigenically and of CVF-C3PA functionally shows no C3 conversion following KAF depletion. This treatment is also known to destroy Clr and C2 in the classical pathway and GBGase in the alternate pathway.

(iii) Serum treated with 1/20 volume of 14 per cent ammonia for 45 minutes at 37°, neutralized and dialysed against saline ('C-NH3') showed 40 per cent of its C3 to be converted. The addition of zymosan was unable to increase this C3 conversion indicating total inactivation of the ammonia-sensitive factor(s) in the alternate pathway. The addition of $F(ab')_2$ anti-KAF to C-NH3 was unable to cause CVF-C3PA consumption or GBG breakdown or to increase C3 conversion. An ammonia-sensitive factor, therefore, is necessary for the effects of KAF depletion. The known ammonia-sensitive complement factors are C3, C4 and factor A (Pensky, Wurz, Pillemer and Lepow, 1959).

2. THE IMMUNOCHEMICAL DEPLETION OF C3

The studies above show that KAF depletion causes activation of the alternate pathway at a point prior to heat sensitive, calcium/magnesium requiring, and ammonia-sensitive steps.

KAF could act in at least two ways (Fig. 4). It could be an inhibitor of an early step in the alternate pathway preventing the activation of GBG and CVF-C3PA. 1. Alternatively, we need propose no new substrate for KAF but visualize the lack of inactivation of C3b as the trigger with C3b as an alternate pathway activator. 2. The two possibilities are not mutually exclusive. To test the latter hypothesis, C3 was immunochemically depleted with a $F(ab')_2$ anti-C3 before and after KAF removal.

A. The C3 in serum was depleted with a $F(ab')_2$ anti-human C3 at optimal proportion in EDTA with removal of the immune precipitate (Table 5). A control had saline added instead of $F(ab')_2$ anti-C3. The sera were recalcified and a portion depleted of KAF. The R(C3) when depleted of KAF, R(C3/KAF), failed to show any GBG conversion or CVF-C3PA depletion. Further the R(C3) on its own when recalcified did not show the levels of GBG conversion and CVF-C3PA depletion seen in normal serum treated with EDTA and recalcified.

B. Exactly similar results were obtained by making serum to R(KAF) in EDTA and then depleting the C3 before adding calcium and magnesium, R(KAF/C3). When these samples were further incubated after the addition of purified C3 total conversion of

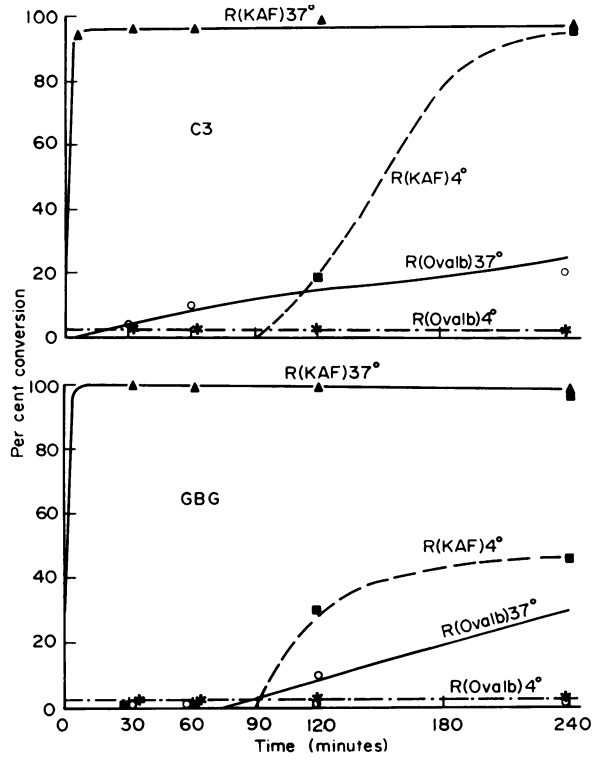

FIG. 3. The effect of temperature on the time course of GBG and C3 conversion in R(KAF). $F(ab')_2$ antibody is added at time zero and C3 and GBG assayed antigenically immediately for conversion at t = 530, 60, 120, 240 minutes.

TABLE 4
C3 DEPOSITION ONTO ERYTHROCYTES BY R(KAF) AND ITS SUBSEQUENT ELUTION BY KAF

Deposition by*	Erythrocyte agglutination titre with	
	Anti-C3	Bovine conglutinin
R(KAF)	8000	0
R(Oval)	0	0
NHS	0	0

ELUTION OF C3 FROM R(KAF) ERYTHROCYTES

Eluting agent†	Erythrocyte agglutination titre with					
	Anti-C3			Conglutinin		
	NHS	KAF	CFD	NHS	KAF	CFD
Time of incubation						
0	8000	8000	8000	0	0	0
3 hours	300	100	8000	0	0	0
7 hours	0	0	8000	0	0	0

* Autologous erythrocytes were added to NHS at a final concentration of 8 per cent after addition of $F(AB')_2$ anti-KAF and incubation for 30 minutes at 37° the cells were washed.

† Incubation of C3 positive cells was carried out at 37° using a final concentration of 5 per cent cells. The medium was enriched with 2·5 per cent glucose.

CVF-C3PA and GBG was found (i.e. in R(KAF/C3)+C3) with total conversion of the added C3. R(KAF)+purified C3 was unable to show any further C3 convertase activity showing that the C3 convertase formed was unstable.

C. The form of C3 necessary to activate the alternate pathway was investigated, by adding to R(C3/KAF) either the cell intermediates EAC43, EAC43 (Antrypol) or EAC43 (KAF); or fluid phase C3 or C3i produced in the supernatant of the reaction of

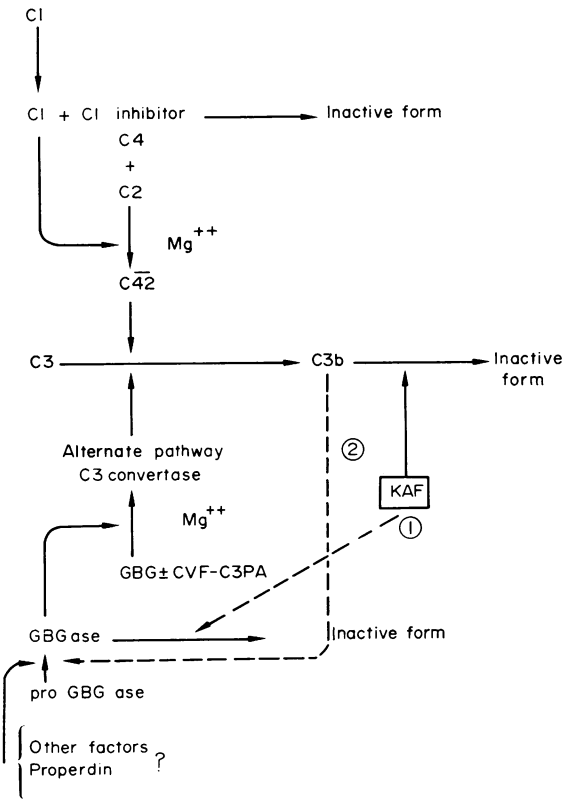

FIG. 4. Possible mechanisms for the inhibition of KAF on alternate pathway activation. 1. KAF as an inhibitor of the alternate pathway. 2. C3b as an activator of the alternate pathway, this activation being inhibited by KAF action on C3b.

EAC142 with purified C3. C3 itself, and cell bound C3b were able to activate the alternate pathway in this system (Table 6). Fluid phase C3i also showed alternate pathway activation, but the possibility that this may be due to contaminating unaltered C3 has not been excluded. KAF pretreatment of cell-bound C3b prevented this activation. Rather surprisingly, antrypol treatment of cell-bound C3b, which prevents KAF action on C3b while retaining its haemolytic and immune adherence activities, also inhibited its action in the alternate pathway. This suggested that the site involved in alternate pathway activation was distinct from the haemolytic and immune adherence sites.

3. THE EFFECT OF C3 DEPLETION ON ALTERNATE PATHWAY ACTIVATION BY CONVENTIONAL ALTERNATE PATHWAY ACTIVATORS

The alternate pathway has been shown to be activated by C3b with KAF exerting a strong inhibitory effect on this new biological activity of C3b. The absence of any alternate pathway activation in R(C3) whether produced by KAF depletion or by EDTA and re-addition of calcium and magnesium raises the question of the involvement of C3b in the activation of the alternate pathway by normal alternate pathway activators, e.g. inulin.

TABLE 5

THE IMMUNOCHEMICAL DEPLETION OF C3 PRIOR TO KAF DEPLETION IN NORMAL HUMAN SERUM

	NHS/EDTA $F(ab')_2$ Anti-C3			
	R(C3)/EDTA Ca γ Mg		NHS/EDTA	
	$F(ab')_2$Anti-KAF			
	R(C3)	R(C3 KAF)	R(KAF)	NHS
GBG conversion antigenically	0	0	++++	++
Per cent C3 conversion (Laurell technique)	No C3	No C3	70	30
CVF-C3PA (Per cent standard serum)	95	95	0	70
KAF titre (conglutination)	750	<30	0	750

$F(ab')_2$ anti-C3 was added at optimal proportion (1:1) to NHS/EDTA. After removal of the immune precipitate the sera were recalcified and $F(ab')_2$ anti-KAF or saline added. The samples were assayed after incubation at 37° for 30 minutes.

R(C3) was prepared as above and aliquots were incubated for 30 minutes at 37° with an equal volume of suspensions of inulin (1·7 mg/ml) heat aggregated human γ-globulin (3·3 mg/ml), or CFD. In no case was GBG conversion or CVF-C3PA depletion observed in the absence of C3. The re-addition of C3 to C3 depleted samples resulted in immediate GBG and C3 conversion. By contrast the ability of purified cobra venom factor to react with serum to produce a stable C3 convertase was totally unaffected by C3 depletion.

DISCUSSION

The analysis of a reaction system such as complement can be carried out in various ways. It is possible to build up a system of purified factors and to reconstruct the situation as it occurs in whole serum from them.

We have used the opposite approach by taking whole serum and specifically depleting it of certain individual components using monospecific $F(ab')_2$ antibody and analysing the effect of such depletion on the system.

TABLE 6

THE RE-ADDITION OF FLUID PHASE AND BOUND C3 TO R(KAF, C3)

	R(KAF, C3) +							
	EA	EAC_4	EAC_{43}	EAC_{43} ant	*EAC_{43} KAF,	C3	C3i	CFD
Per cent C3 conversion (Laurell technique)	no C3	no C3	no C3	no C3	no C3	100 per cent	100 per cent	no C3
GBG conversion (antigenically)	0	0	+ +	0	0	+ + + +	+	0
CVF-C3PA (per cent standard serum)	120	104	60	92	100	24	32	78

* Two parts R(KAF), C3) were incubated with 1 part 7·5 per cent cells or 1 part 1·5 mg/ml C3 and C3i for 30 minutes at 37°. The supernatants were assayed for C3, GBG and CVF-C3PA.

A major problem in this approach is the possibility that the complexes involving the $F(ab')_2$ antibodies can themselves activate the complement system. Complement fixation by the $F(ab')_2$ antigen–antibody complexes has been shown to activate the alternate pathway (Reid, 1971). The effect is, however, much reduced if the complexes are not preformed.

Depletion of components such as KAF whose serum concentration is low (probably less than 20 μg/ml) has been shown not to detectably activate complement in this technique. Three types of control have been employed. One involves the depletion of an added independent antigen, e.g. ovalbumin at the same concentration. The second involves depleting in EDTA and removing the precipitate before re-adding calcium and magnesium.

TABLE 7
In vitro AND *in vivo* KAF DEFICIENCY

	KAF-depleted serum *in vitro*	KAF deficiency *in vivo**
KAF	0	0
C3	Rapid conversion	Low level conversion products
C3 on red cells susceptible to KAF	+	+
GBG	Rapid conversion to GGG and GBG	Low or absent
CVF-C3PA	Rapid depletion	Low or not detectable
CH(142)	Small depletion	Normal

* Alper *et al.* (1970a and b). Abramson *et al.* (1971).

This should not allow complement activation by the precipitate. However, the procedure of adding EDTA and recalcifying alone causes some complement activation which must be taken into account. A third control is to re-add the depleted component in purified form and to show that this abolishes the effect of the depletion.

Using these different controls we have shown that the consequences of immunochemical depletion of C3b inactivator or KAF are to produce spontaneously a complete activation of the alternate pathway as shown by GBG conversion and CVF-C3PA depletion.

The effects mimic accurately the situation seen in the KAF-deficient patient described by Abramson *et al.* (1971) (Table 7). This patient's serum is entirely analagous to the *in vitro* KAF depleted serum allowing for the rapid clearance of complement breakdown products from the circulation *in vivo*. Both the *in vivo* and the *in vitro* situation clearly indicates the importance of KAF in the homeostatic control of C3 fixation by the alternate pathway fixation.

The mechanism by which KAF removal results in alternate pathway activation has been shown to involve C3b and is not seen in C3 depleted serum. It can be restored to an R(KAF/C3) by fixed C3b. This feedback of C3b into the alternate pathway is a new activity for this component. The site involved is inhibited by KAF and is also blocked by antrypol. It is therefore distinct from the haemolytic and immune adherence sites.

The position of feedback into the alternate pathway is prior to GBG and CVF-C3PA activation as they are both totally inactivated. The second hypothesis in Fig. 4, therefore,

seems to be correct. That KAF may, in addition, exert an inhibitory effect directly on some part of the alternate pathway is not excluded.

The activation of the alternate pathway feedback cycle in the absence of KAF generate nascent C3b which attaches to unsensitized autologous red blood cells *in vitro* and *in vivo* (Abramson *et al.*, 1971), via its short lived hydrophobic-binding site, with further activation of the alternate pathway cycle near the cell membrane surface. The loss of agglutination by anti-C3c following prolonged KAF action on cell bound C3 has been reported (Ruddy and Austen, 1971) and is found here. The lack of conglutination of these C3 positive cells even following KAF treatment may be a question of sensitivity, agglutination by specific antiserum being a more sensitive test for C3 than conglutination.

Significantly, C3 depleted serum will also not support alternate pathway activation by conventional alternate pathway activators, e.g. yeast cell wall, or inulin, i.e. the feedback cycle seems to be an essential part of the alternate pathway.

Using a built up system of purified components, Müller-Eberhard and Götze (1972) have reported that C3 is involved in the activation of the alternate pathway—a finding wholly consistent with our own work. They have claimed that C3 is identical with the ammonia sensitive factor A of the properdin system (Pensky, *et al.*, 1959).

Paradoxically, the alternate pathway for C3 fixation seems to possess an absolute requirement for fixed C3, the product of its reaction. To explain this paradox it may be necessary to postulate that *in vivo* there is a minute degree of spontaneous C3 activation normally efficiently damped by inhibitors. This would be compatible with the high *in vivo* turnover rate of C3 seen normally (Alper and Rosen, 1967). A similar effect must also be present *in vitro*. Sufficient C3b may be generated by thrombin, plasmin or other active serum or tissue proteases (Bokisch, Müller-Eberhard and Cochrane, 1969; Hill and Ward 1969; Ward, 1967) for recycling in the absence of KAF. The effect seen on recalcification of normal or C2 deficient serum may be by way of a metal ion and/or dilution effect on $C\overline{1}$, $C\overline{42}$ or GBGase all of which require metal ions as co-factors. This is an already well-known effect of $C\overline{1}$. The mechanism by which inulin or yeast cell walls set off the C3b feedback cycle is still uncertain. It appears to involve factors other than GBG and CVF-C3PA. Properdin (Blum and Lee, 1972) is one likely candidate. It could be that alternate pathway activators act to stabilize the pathway normally spontaneously turning over. In our hands, predominantly classical complement activators, e.g. aggregated human γ-globulin or rabbit IgM also activate the alternate pathway as indicated by GBG conversion. This GBG conversion is, however, not seen after C3 depletion although there is still substantial $C\overline{142}$ inactivation. This ability of classical pathway activators to activate alternate pathway components by way of the C3b feedback is important in trying to define material as 'classical' or 'alternate' activators. The use of C2-deficient human serum (Nicol and Lachmann, 1973) as a negative control for classical pathway activation is currently being used to circumvent this problem.

The *in vitro* depletion of KAF has thus unmasked an autocatalytic feedback mechanism for C3 fixation involving alternate pathway components and has shown the importance of C3b inactivator in the homeostatic control of C3 fixation.

ACKNOWLEDGMENTS

The authors would like to thank Dr M. J. Hobart and Miss A. Lippok for supplying the purified complement components and Mr R. G. Oldroyd for technical assistance. They

would also like to thank Dr Max Sussman for generously making available C2-deficient serum and Dr M. J. Hobart and Dr D. L. Brown for helpful discussion.

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NOTE ADDED IN PROOF

It has now been demonstrated that KAF can indeed act as GBGase inhibitor (Alper, Rosen and Lachmann, 1972). However, this can also be ascribed to KAF action on C3B whose presence we have found to be essential for GBGase *activity* as well as for its *activation*.

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