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The activation of the alternative pathway C3 convertase by human plasma kallikrein

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Summary. Human plasma kallikrein can replace factor D for the activation of the alternative pathway C3 convertase of human complement. The factor B cleavage patterns by factor D and kallikrein are indistinguishable. The ability of kallikrein to cleave factor B is influenced by the magnesium ion concentration and the C3b concentration. Factor D is about ten-fold more effective on a molar basis, for the alternative pathway C3 convertase activation than is kallikrein. The physiological role of the action of kallikrein on the alternative pathway C3 convertase is discussed.

INTRODUCTION

Component C3 is the central protein of both classical and alternative pathways of human complement (for reviews see: Reid & Porter, 1981; Müller-Eberhard & Schreiber, 1980). In the alternative pathway, component C3 is activated by the complex protease C3 convertase, which has been represented as $\overline{C3bBb}$ (Vogt, Dames, Schmidt & Dieminger, 1977; Müller-Eberhard & Gotze, 1972). The active site of C3 convertase is the Bb subunit, which is an unusual serine protease (Christie, Gagnon & Porter, 1980; Mole & Niemann, 1980).

The formation of the alternative pathway C3 convertase requires the magnesium ion-dependent assembly of C3b and factor B followed by cleavage of the factor B subunit by factor D to yield the active C3 convertase $\overline{C3bBb}$ with the release of the Ba fragment (Vogt *et al.*, 1977).

Factor D, the enzyme responsible for the activation of the alternative pathway C3 convertase, is a 25,000 Dalton single chain serine protease that is present in plasma in an activated form (Brade, Nicholson, Bitter-Suermann & Hadding, 1974a; Lesavre & Müller-Eberhard, 1978; Volanakis, Schrohenloher & Stroud, 1980; Davis, 1980; Johnson, Gagnon & Reid, 1980; Volanakis, Schrohenloher & Stroud, 1977). Although it has been established that trypsin can replace factor D for the alternative pathway C3 convertase activation (Brade *et al.*, 1974a), several other plasma proteases including plasmin and thrombin have only negligible capacity for this (Lesavre & Müller-Eberhard, 1978). The action of kallikrein on the alternative pathway C3 convertase has not been investigated.

Kallikrein is a plasma serine protease of 85,000 to 88,000 Daltons that originates from an inactive precursor, prekallikrein, as a result of limited proteolysis by the activated Hageman factor (factor XIIa) (Mandle & Kaplan, 1977; Bouma, Miles, Beretta & Griffin, 1980; Heimark & Davie, 1979).

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Kallikrein is the enzyme that is responsible for the release of bradykinin, a vasoactive peptide, from high molecular weight kininogen (Han, Kato, Iwanaga & Komiya, 1978). Kallikrein serves to activate the Hageman factor (factor XII) of blood coagulation (Fujikawa, Heimark & Davie, 1980). Kallikrein has also been implicated in fibrinolysis (Kaplan, 1978; Mandle & Kaplan, 1977; Wuepper, 1973).

This report provides evidence that kallikrein can activate the alternative pathway of human complement.

MATERIALS AND METHODS

Materials

The chromogenic substrate S2302: H-D-prolyl-L-phenylalanyl-L-arginine p-nitroanalide dihydrochloride was purchased from Kabi Diagnostica, Stockholm, Sweden. Coomassie brilliant blue R, imidazole, soybean trypsin inhibitor were obtained from Sigma Chemical Corporation, Poole. Sephacryl S300, superfine, and Sepharose CL4B were products of Pharmacia Fine Chemicals, Hounslow. Magnesium chloride, sodium chloride, and sucrose were obtained from Fisons Ltd, Loughborough.

Methods

Human component C3 was purified by the method of Tack & Prahl (1976) as modified by Parkes, DiScipio, Kerr & Prohaska, (1981). Human antithrombin III was purified as described by Thaler & Schmer (1975). Human plasma kallikrein was purified using affinity chromatography on soybean trypsin inhibitor-Sepharose by the procedure of Nagase & Barrett (1981). Factor D was purified by the procedure previously described (DiScipio, 1981).

The extinction coefficients that were employed in these studies were as follows: component C3, $A^{1\%}_{280\text{ nm, 1 cm}}$ 9·7 (Tack & Prahl, 1976); human antithrombin III, $A^{1\%}_{280\text{ nm, 1 cm}}$ 5·7 (Kurachi, Schmer, Hermodson, Teller & Davie, 1976); kallikrein, $A^{1\%}_{280\text{ nm, 1 cm}}$ 10·6 (Nagase & Barrett, 1981); factor B, $A^{1\%}_{280\text{ nm, 1 cm}}$ 12·7 (Curman, Sandberg-Tragardh & Peterson, 1977). The extinction coefficient of factor D was assumed to be 10·0.

The haemolytic activity of C3 was assayed as described by Tack & Prahl (1976).

Amidase activity of plasma kallikrein was assayed by incubation of 50 μl of a solution of 1 mM Kabi substrate S2302 with up to 10 μl of test sample. After incubation at 37° for 30 min the sample was diluted to 400 μl, and the absorbance at 405 nm was monitored.

Sepharose-C3b was made by covalently coupling 500 mg of C3b to 100 ml of Sepharose CL4B by the method of Cuatrecasas (1970). Soybean trypsin inhibitor Sepharose was prepared similarly using 200 mg of soybean trypsin inhibitor with 50 ml of Sepharose CL4B.

Human factor B was prepared by a modification of the procedure described by DiScipio (1981). The initial stages of the purification were $BaCl_2$ treatment of plasma, 5%–12% polyethyleneglycol precipitation, DEAE-Sephadex column chromatography and CM-Sephadex column chromatography. After the CM-Sephadex column factor B was directly passed through a soybean trypsin inhibitor-Sepharose column (1·5 cm × 10 cm). The factor B was dialysed against 15 mM-imidazole HCl buffer pH 7·3,50 mM NaCl, 10 mM–$MgCl_2$. The sample was applied to a Sepharose-C3b column (2·6 cm × 20 cm) and the factor B was specifically eluted with 10 mM imidazole-HCl buffer pH 7·3, 50 mM NaCl, 10 mM EDTA. The yield was about 15%.

SDS-polyacrylamide gels were made by the method of Weber & Osborn (1969) as modified by Kisiel *et al.*, (1976). The gels were stained with Coomassie Brilliant Blue R.

Gel filtration of kallikrein and factor D was performed by using a Sephacryl S300 column (2·6 cm × 95 cm) equilibrated with 10 mM imidazole-HCl buffer pH 7·3, 0·15 M NaCl. Fractions were assayed for C3 convertase activating activity and kallikrein specific amidase activity. The diffusion coefficient of kallikrein was estimated by gel filtration (Ackers, 1964) using a calibrated series of standards: IgM, $D_{20,w} = 1{\cdot}7 \times 10^{-7}$ cm^2/sec; apoferritin, $D_{20,w} = 3{\cdot}6 \times 10^{-7}$ cm^2/sec; IgG $D_{20,w} = 4{\cdot}0 \times 10^{-7}$ cm^2/sec; catalase, $D_{20,w} = 4{\cdot}2 \times 10^{-7}$ cm^2/sec; bovine serum albumin $D_{20,w} = 5{\cdot}9 \times 10^{-7}$ cm^2/sec; ovalbumin, $D_{20,w} = 7{\cdot}8 \times 10^{-7}$ cm^2/sec.

Sucrose gradient ultracentrifugation was performed by centrifuging a 200 μl sample of kallikrein over a 10%–40% sucrose gradient in 10 mM imidazole-HCl buffer pH 7·3, 0·15 M NaCl at 35,000 r.p.m. in a Beckman SW 50·1 rotor for 12 hr at 10°. Fractions were assayed for kallikrein-specific amidase activity and C3 convertase activating activity. The sedimentation coefficient standards were apoferritin, 17·6 *s*; catalase, 11·2 *s*; C3, 9·0 *s*; factor H, 5·5 *s*; ovalbumin, 3·0 *s*; soybean trypsin inhibitor, 2·3 *s*.

The cleavage of factor B. Factor B (50 μg) was

incubated in 150 μl of 10 mM imidazole-HCl pH 7·3, 0·15 M NaCl with 2·5 μg of kallikrein or 0·1 μg factor D at various concentrations of $MgCl_2$ for 120 min at 37°. The samples were denatured in SDS and subjected to SDS-polyacrylamide gel electrophoresis. The gels were scanned by employing a Gilford gel scanner. The percentage of factor B converted to Bb and Ba fragments was estimated from results derived from plots of the optical density at 540 nm across the length of the gels. The influence of C3b on the cleavage of factor B was investigated under the same conditions using 8 mM magnesium chloride.

Activation of C3 convertase as a function of kallikrein or factor D. Component C3 (1 mg/ml) and factor B (180 μg/ml) in 10 mM imidazole HCl buffer pH 7·3, 0·15 M NaCl, 3 mM $MgCl_2$ were mixed with various amounts of factor D or kallikrein for 30 min at 37°. The C3 haemolytic activity was then assayed.

Cleavage of component C3 as a function of factor B concentration was estimated by incubating 70 μg of native C3 with 2·5 μg of kallikrein or 0·1 μg of factor D with various amounts of factor B in 160 μl of 10 mM imidazole-HCl buffer pH 7·3, 0·15 M NaCl, 3 mM $MgCl_2$ for 90 min at 37°. Subsequently C3 haemolytic activity was assayed (Tack & Prahl, 1976).

RESULTS

SDS polyacrylamide gel electrophoresis experiments of factor B and C3 activation

Kallikrein can replace factor D for the alternative pathway C3 convertase activation. Factor B cleavage by factor D or kallikrein and C3 activation by C3 convertases generated from either factor D or kallikrein were studied by SDS polyacrylamide gel electrophoresis (Fig. 1). The results indicated that the splitting of factor B into Bb and Ba fragments by factor D or kallikrein is indistinguishable. Furthermore, there were no apparent differences in the C3b generated by C3 convertases that were activated by either factor D or kallikrein. Under the conditions employed, neither kallikrein nor factor D degrades C3b.

Gel filtration and sucrose gradient ultracentrifugation of kallikrein.

In order to demonstrate that the effect of kallikrein on the alternative pathway C3 convertase was not a result of contamination by factor D or other proteases, the kallikrein was subjected to gel filtration on a Sephacryl S300 column, and the kallikrein was also subjected to sucrose gradient ultracentrifugation. For both procedures fractions were assayed for kallikrein-specific amidase activity and C3 convertase activity. For the gel filtration experiment (Fig. 2), it was observed that kallikrein-specific amidase activity and C3 convertase activity coeluted with a peak at fraction 52 (about 310 ml elution volume). The elution position of factor D on this column was about 350–360 ml. By comparison with a calibrated series of standards, the diffusion coefficient for kallikrein was estimated to be $6{\cdot}0 \times 10^{-7}$ cm^2/sec.

For the sucrose gradient ultracentrifugation experiment (Fig. 3), it was observed that both the kallikrein-specific amidase activity and C3 convertase activating activity cosedimented with an apparent sedimentation coefficient of about 5·5 *s*. Thus if the observed action of kallikrein for C3 convertase activation were due to a contaminant, the contaminant would have a sedimentation coefficient of $s_{20,w} = 5{\cdot}5$ *s* and a diffusion coefficient of $D_{20,w} = 6{\cdot}0 \times 10^{-7}$ cm^2/sec. These parameters differ markedly from the estimated values for factor D, which has $s_{20,w} = 2{\cdot}6$ *s* and $D_{20,w} = 10{\cdot}5 \times 10^{-7}$ cm^2/sec (Volanakis *et al.*, 1977; Brade *et al.*, 1974b; Dieminger, Vogt & Lynen, 1978; Dierich, Hadding, Konig, Limbert, Schorlemmer & Bitter-Suerman, 1974).

The effect of inhibitors on kallikrein

To demonstrate further that kallikrein and not a contaminant was responsible for the observed C3 convertase activating activity, the kallikrein was preincubated for 10 hr with various amounts of soybean trypsin inhibitor or human antithrombin III. Both soybean trypsin inhibitor and antithrombin III inhibited both kallikrein-specific amidase activity and C3 convertase activating activity. Furthermore, one equivalent of antithrombin III could inactivate one equivalent of kallikrein-specific amidase activity or kallikrein C3 convertase activating activity.

The relative effectiveness of factor D or kallikrein for C3 convertase activation

A comparative study on the effectiveness of factor D

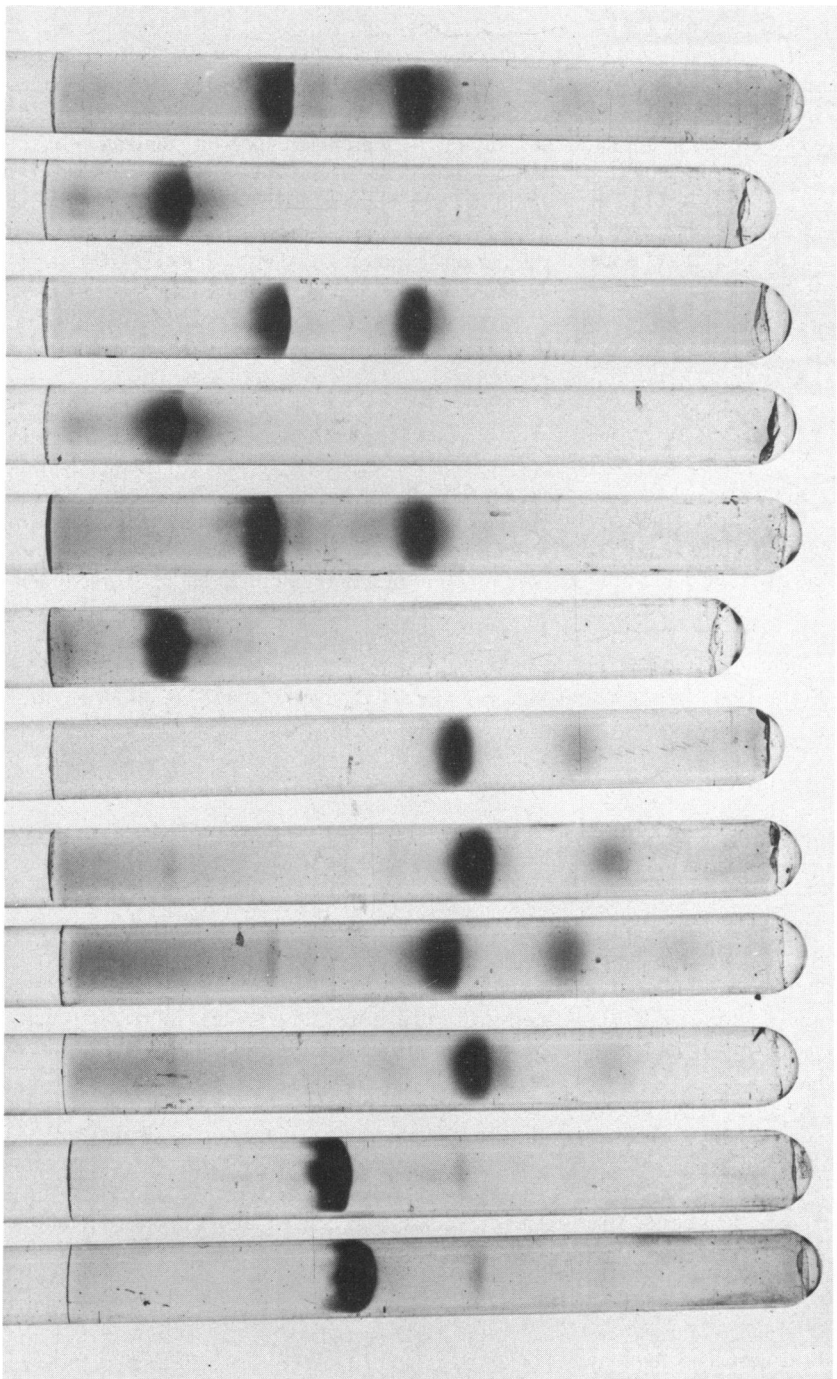

Figure 1. SDS-polyacrylamide gel electrophoresis of factor B cleavage by kallikrein or factor D, and C3 cleavage by C3 convertase formed by action of kallikrein or factor D. The gels consist of 6% polyacrylamide and are loaded with 20–35 μg of protein. Factor B was cleaved to Bb and Ba fragments by incubation of 35 μg of factor B with 10μg of kallikrein or 0·1 μg of factor D with 0·5 μg of C3b in 10 mM imidazole-HCl pH 7·3, 0·15 M NaCl, 3 mM $MgCl_2$. The C3 was cleaved by C3 convertase generated by incubating C3 (30 μg) with 1 μg of factor B and with 0·5 μg of kallikrein or 0·05 μg of factor D in the same buffer. The identity of the gels is as follows: gel 1, factor B (unreduced); gel 2, factor B (reduced); gel 3, Bb/Ba-kallikrein (unreduced); gel 4, Bb/Ba-kallikrein (reduced); gel 5, Bb/Ba-factor D (unreduced); gel 6, Bb/Ba-factor D (reduced); gel 7, C3 (unreduced); gel 8, C3 (reduced); gel 9, C3b-formed by C3 convertase-kallikrein (unreduced); gel 10, C3b formed by C3 convertase-kallikrein (reduced); gel 11, C3b formed by C3 convertase-factor D (unreduced); gel 12, C3b formed by C3 convertase-factor D (reduced).

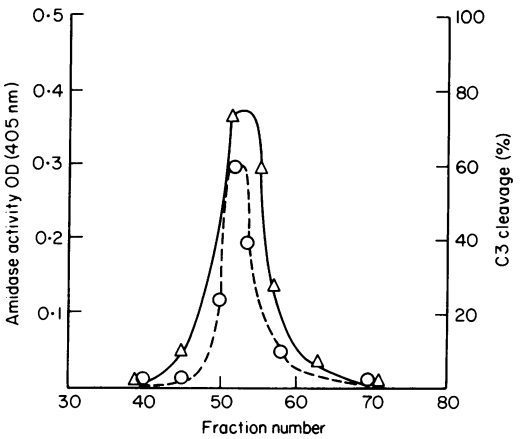

Figure 2. Gel filtration of kallikrein on Sephacryl S300. The kallikrein (150 μg) was subjected to molecular sieving in 10 mM-imidazole-HCl pH 7·3, 0·15 M NaCl on a Sephacryl S300 (2·6 cm × 95 cm), and 5·1 ml fractions were collected. Aliquots (5 μl) were assayed for amidase activity (O—O) using Kabi substrate S2302, and aliquots (30 μl) were also assayed for C3 convertase activating activity (Δ—Δ) by incubating an aliquot from the column with C3 (70 μg), factor B (10 μg) in 100 μl of the same buffer plus 3 mM $MgCl_2$, for 2 hr, and then C3 haemolytic activity was assayed. The elution peak for the kallikrein is about 314 ml. Factor D elutes at between 350—360 ml on this column.

or kallikrein to activate the alternative pathway C3 convertase was performed by incubating C3 (1 mg/ml) with factor B (180 μg/ml) in the presence of 3 mM $MgCl_2$ for 30 min with various amounts of factor D or kallikrein. Under the conditions used, it is observed in Fig. 4 that 50% of the C3 is cleaved by C3 convertase initiated by 0·5 ng of factor D (Fig. 4, panel a) or 17 ng of kallikrein (Fig 4, panel b).

The cleavage of factor B by kallikrein or factor D

The cleavage of factor B by factor D or kallikrein was investigated in the absence of C3b as a function of magnesium chloride concentration. It is observed (Fig. 5) that factor D does not cleave factor B in the absence of C3b, but kallikrein does cleave factor B in the absence of C3b as a function of magnesium ion concentration. No splitting of factor B by kallikrein was observed under these conditions in the presence of 5 mM EDTA. Factor B cleavage by both factor D and kallikrein in 8 mM $MgCl_2$ is a function of C3b concentration (Fig. 6). Thus the kallikrein cleavage of factor B is a function of magnesium ion concentration and C3b. Factor D cleaves factor B only in the presence of magnesium ions and C3b.

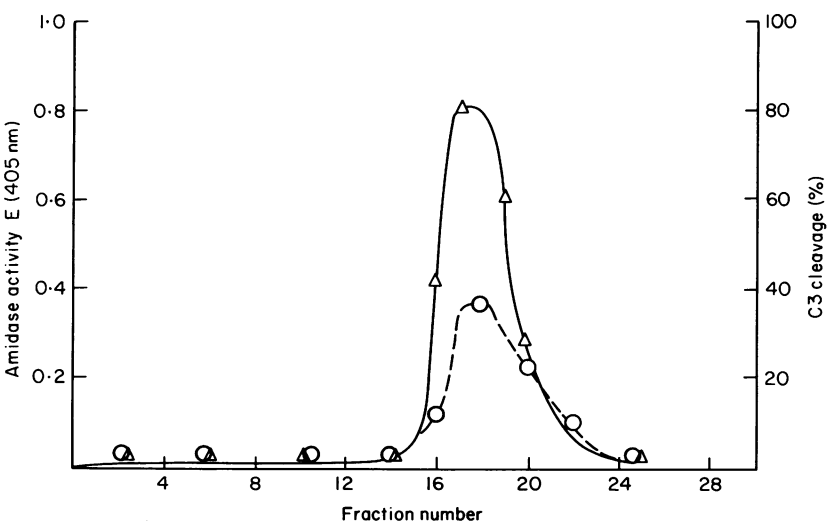

Figure 3. Sucrose density gradient ultracentrifugation of kallikrein. Kallikrein (60 μg) was subjected to sucrose density gradient ultracentrifugation. The gradients consisted of 10%–40% sucrose in 10 mM imidazole-HCl pH 7·3, 0·15 M NaCl, and the samples were centrifuged at 35,000 r.p.m. for 15 hr at 10°. Aliquots were assayed for amidase activity (O—O) and C3 convertase activating activity (Δ—Δ) as previously described. Factor D would peak at about tube 24 in this experiment.

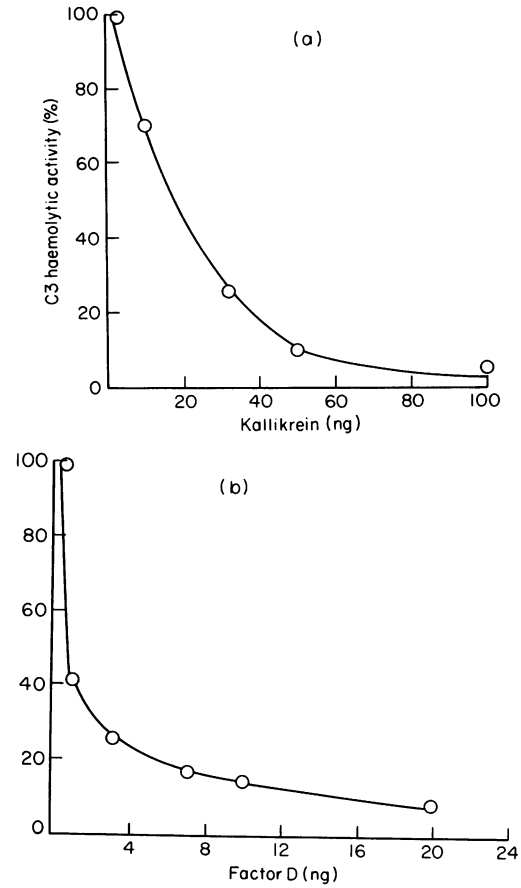

Figure 4. The cleavage of C3 as a function of kallikrein or factor D. Component C3 (100 μg) was incubated with 18 μg of factor B with various amounts of kallikrein (a) or factor D (b) at 37° in 100 μl of 10 mM imidazole-HCl pH 7·3, 0·15 M NaCl, 3 mM $MgCl_2$ for 30 min. Then the haemolytic activity of C3 was measured.

C3 activation by C3 convertase generated by factor D or kallikrein

The C3 cleavage by C3 convertase ($\overline{C3bBb}$) as a function of the amount of factor B added to the reaction is shown in Fig. 7. For both factor D and kallikrein, C3 conversion is a function of factor B concentration, indication that C3 cleavage is a result C3 convertase and not the direct action of the serine proteases factor D or kallikrein. In fact, kallikrein is observed to convert directly a small amount of C3 to C3b (about 5% under the conditions employed) in the absence of factor B, but the extent of C3 cleavage is markedly augmented by factor B.

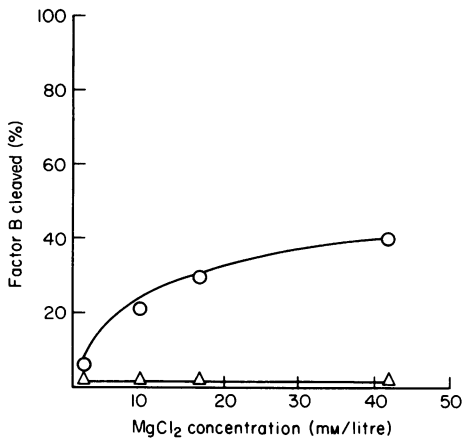

Figure 5. Factor B cleavage by kallikrein or factor D as a function of magnesium chloride concentration. Factor B (50 μg) was incubated with 2·5 μg of kallikrein (O—O) or 0·1 μg of factor D (Δ—Δ) for 120 min in the presence of various concentrations of $MgCl_2$. Then the samples were prepared for SDS-polyacrylamide gel electrophoresis, and after electrophoresis and staining, the factor B cleavage was estimated by gel scanning.

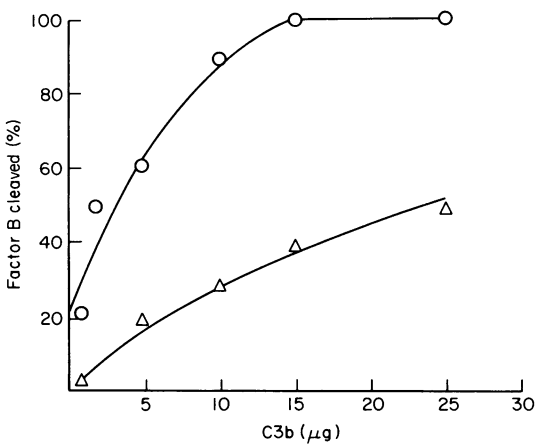

Figure 6. Factor B cleavage as a function of C3b. Factor B (50 μg) was incubated with 2·5 μg of kallikrein (O—O) or 0·1 μg of factor D (Δ—Δ) with various amounts of C3b in 10 mM imidazole HCl pH 7·3, 0·15 M NaCl, 8 mM $MgCl_2$. Cleavage of factor B was determined by scanning of SDS-polyacrylamide gels as previously described.

DISCUSSION

It has been demonstrated in this report that kallikrein can replace factor D for the activation of the alterna-

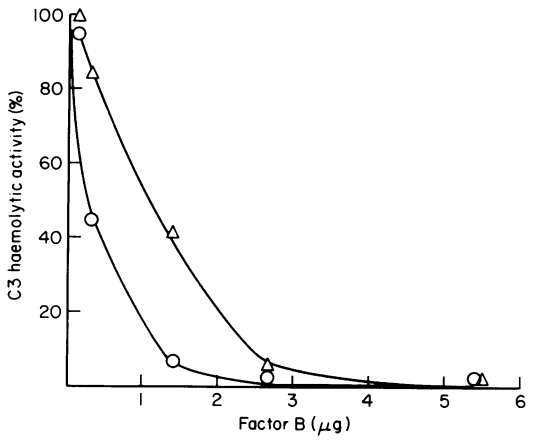

Figure 7. The cleavage of native C3 by C3 convertase generated by kallikrein or factor D as a function of factor B concentration. Native C3 (7 μg) was incubated with 2·5 μg of kallikrein (○—○) or 0·1 μg of factor D (△—△) with various amounts of factor B in a final volume of 160 μl in 10 mM imidazole-HCl buffer pH 7·3, 0·15 M NaCl. The samples were incubated for 90 min at 37°, and then C3 haemolytic activity was assayed.

tive pathway C3 convertase. The kallikrein used in these studies was unlikely to be contaminated by factor D or other proteases as shown by the following facts: (i) the kallikrein was affinity purified by the method of Nagase & Barrett (1981); (ii) the C3 convertase activating potential copurified with the kallikrein specific amidase activity on gel filtration on Sephacryl S300 (Fig. 4), and on sucrose density gradient ultracentrifugation (Fig. 5); (iii) soybean trypsin inhibitor and human antithrombin III inhibited the kallikrein-specific amidase and C3 convertase activating activity. It has been reported that factor D is not inhibited by soybean trypsin inhibitor or plasma protease inhibitors (Lesavre & Müller-Eberhard, 1978), but soybean trypsin inhibitor and antithrombin III have been reported to inactivate plasma kallikrein (Nagase & Barrett, 1981; Vennerod, Laake, Solberg & Stromland, 1976).

This report has not established a physiological role for the activation of C3 convertase by human plasma kallikrein. Factor D is about ten-fold more effective, on a molar basis, than kallikrein for the C3 convertase

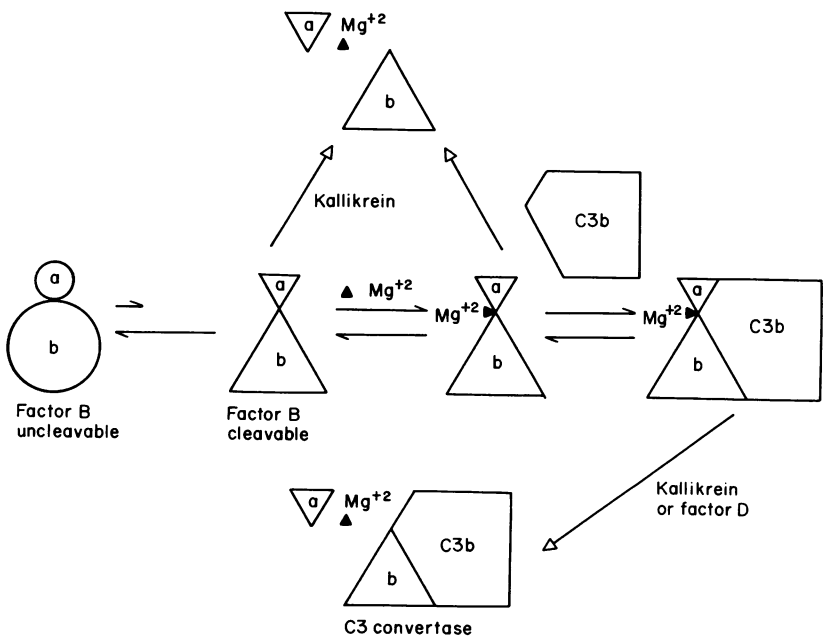

Figure 8. A scheme for the cleavage of factor B by kallikrein or factor D. The factor B is envisioned to exist in equilibrium in two conformational states referred to as uncleavable and cleavable. The binding of Mg^{+2} and C3b shifts the equilibrium, by mass action effects, to the cleavable form.

activation capacity (Fig. 4). The plasma concentration of human factor D has been estimated to be 1–2 μg/ml (Lesavre & Müller-Eberhard, 1978; Lesavre, Hugli, Esser & Müller-Eberhard, 1979), and the plasma concentration of human prekallikrein has been estimated to be 55 μg/ml (Bouma *et al.*, 1980). Thus in plasma there is about as much C3 convertase activating potential associated with prekallikrein as with factor D. Although plasma protease inhibitors can inactivate kallikrein but not factor D (Lesavre & Müller-Eberhard 1978), there may exist circumstances in plasma where sufficiently high amounts of prekallikrein can be activated that the kallikrein which is formed can influence the activation of the alternative pathway C3 convertase before the kallikrein is inactivated by the plasma protease inhibitors.

In plasma factor D is required for the alternative pathway, but at physiological concentration factor D is limiting in the activation of C3 convertase (Lesavre & Müller-Eberhard, 1978). There may exist physiological situations where kallikrein could serve to supplement the free factor D concentration in plasma. This would result in an increase in the rate of activation of the alternative pathway C3 convertase, which could override the controlling influences of complement factors H and I.

Factor D was observed to cleave factor B only in the presence of C3b and magnesium ions. Kallikrein can cleave factor B, in the absence of C3b, as a function of magnesium ion concentration. Furthermore, in the presence of magnesium ions, kallikrein was observed to cleave factor B as a function of C3b concentration (Figs 5 and 6). These data suggest a scheme for the activation of factor B shown in Fig. 8. Under this scheme, factor B exists in an equilibrium between two states, an uncleavable state and a cleavable state. Only the cleavable form binds magnesium ions, and only factor B with bound magnesium ions will bind C3b. Thus the presence of magnesium ions and C3b shifts the equilibrium to the cleavable form of factor B. The scheme shown in Fig. 8 is consistent with the observation that the affinity constant of factor B for C3b is magnesium ion concentration dependent (Kazatchkine, Fearon & Austen, 1979). This scheme of factor B activation predicts that factor B binds magnesium ions with a higher affinity in the presence of C3b.

This report also describes an affinity purification method for human factor B that is devoid of plasma kallikrein. Factor B and kallikrein are of similar size and have similar ion exchange properties. The likelihood of kallikrein contamination of factor B is especially high if serum, instead of plasma, is used as starting material and if contact with glass is not avoided during the course of the preparation.

In conclusion, kallikrein can replace factor D for the activation of the human alternative pathway C3 convertase. Further work will be required to demonstrate if this observation has a physiological significance.

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