Restoration by Purified C3b Inactivator

of Complement-Mediated Function In Vivo in a Patient

with C3b Inactivator Deficiency

JOHN B. ZIEGLER, CHESTER A. ALPER, FRED S. ROSEN,
PETER J. LACHMANN, and LISA SHERINGTON

From the Immunology Division, Department of Medicine, Children's Hospital
Medical Center, the Department of Pediatrics, Harvard Medical School, and
the Center for Blood Research, Boston, Massachusetts 02115; and the
MRC Research Group in Serum Complement, Department of Immunology,,
Royal Postgraduate Medical School, London W12, England

A B S T R A C T In a patient with lifelong increased susceptibility to infection and multiple abnormalities in complement-mediated functions, the infusion of normal plasma had been seen to produce a prolonged partial correction of serum abnormalities. It was subsequently shown that the patient was genetically deficient in the C3b inactivator and that immunochemical depletion of C3b inactivator from normal serum resulted in abnormalities similar to those found in the patient's serum, including alternative pathway C3 activation.

Highly purified C3b inactivator was obtained from the euglobulin fraction of normal human serum, sterilized by filtration, and infused intravenously. Partial or complete correction of almost all the known serum abnormalities was obtained. C3b almost disappeared from the serum within 4–5 h, as did Factor $\bar{D}$ activity. Native C3, C5, and serum hemolytic activity rose to normal or near-normal levels over 4 days and were sustained for another week. Factor B, properdin, opsonic activity, and bactericidal activity reached a level

at least two–five times that found before the infusion within 24 h and fell over the next 5 days.

These observations prove the primary role of C3b inactivator deficiency in the patient's disease and demonstrate clearly the crucial role in vivo of C3b inactivator in modulating alternative pathway activity.

INTRODUCTION

In 1970 a patient was described (1) with a lifelong history of increased susceptibility to bacterial infection. Complement-mediated functions were defective, and serum levels of the third component of complement (C3) were reduced. C3 was present primarily as an inactive conversion product, C3b. The infusion of 500 ml of normal plasma corrected the serum abnormalities, and this effect was demonstrable for 17 days (2), suggesting that the plasma provided a potent and/or long-lived protein in which the patient was deficient. Subsequently, the patient has been shown to be deficient in the C3b inactivator (KAF) (3), and evidence that this deficiency is primary has been provided by a family study which suggested the patient to be homozygous for an autosomal recessive gene for KAF deficiency (4). Table I lists the biochemical abnormalities which have been recognized in this patient. The demon-

TABLE I

Biochemical Abnormalities in a Patient with
Deficiency of the C3b Inactivator

Molecule or activity	Synonyms	Abnormality
C3b inactivator	KAF	Undetectable
Factor $\bar{D}$	GBGase; C3 proactivator convertase; C3PAse	Detectable
Factor B	GBG; C3 proactivator; C3PA	Reduced
CoF-binding protein	Considered by some authors to be Factor B	Undetectable
C3	Factor A	10% or normal
C3b	Factor A	Increased; found on red blood cells
Histamine		Increased in urine

stration that KAF is an inhibitor of the alternative or properdin pathway[1] of C3 activation (4) indicated the mechanism by which a primary KAF deficiency was responsible for the patient's abnormalities. Present knowledge of the mechanisms involved in the alternative pathway of C3 activation (Fig. 1), though incomplete, provide a basis for explaining the role of the KAF in this system and in the pathogenesis of the abnormalities found in the patient.

C3b or a similar fragment of C3 appears to be a necessary component of the enzyme $\bar{D}$, the function of which is to cleave Factor B thereby activating it. The active form of Factor B, $\bar{B}$, is probably the larger cleavage product Bb (GGG), and an additional product, Ba (GAG), has been identified. $\bar{B}$ either directly cleaves C3 or does so via an unidentified intermediate protein (X). Thus, KAF, by cleaving and inactivating C3b or a C3b-like fragment, is clearly a potent inhibitor of the alternative pathway. Whether $\bar{D}$, as well as C3b, is a substrate for KAF remains speculative at this time.

This view depicts the alternative pathway as a potent amplification mechanism, the activity of which is modulated by the action of KAF. C3 activation, whether by classical, alternative, or other pathways, results in the production of C3b, which can initiate $\bar{D}$ activity. Thus, Factor B is cleaved and further C3 cleavage results.

[1] Nomenclature used is that provisionally recommended by the Nomenclature Committee on Complement at a meeting held on July 21, 1974, Brighton, England. Previously employed synonyms are indicated here in parentheses. The sequence of interactions which lead to C3 inactivation without involvement of C1, C4, or C2 is to be known as the alternative or properdin pathway. Its components include Factor B (GBG, C3PA) and Factor D (GBGase, C3PAse). Activated forms are to be indicated by a bar, e.g., $\bar{B}$ ($\overline{GGG}$, C3A). *Other abbreviations used in this paper:* CoF, cobra venom factor; CoFBP, cobra venom factor-binding protein.

Because the role of properdin in the alternative pathway is uncertain, it is not shown. The KAF deficiency, presumed to be the primary defect in the patient, results in persistence of C3b in serum, accumulation of C3b on red blood cells, increased $\bar{D}$ activity, and consumption of Factor B, C3, and perhaps other proteins in the pathway. These changes have, in fact, been induced in normal serum in vitro by depletion of KAF immunochemically (5).

Because other factors are secondarily lacking in the patient's serum, replacement of KAF in vitro does not restore alternate pathway activity. The positive identification of the primary defect in this patient requires the demonstration in vivo that KAF replacement alone is sufficient to correct the abnormalities in alternate pathway activities. In the study described here, a small amount of purified KAF was administered to the patient with a prolonged improvement in complement function and levels of C3 and a transient rise in Factor B.

METHODS

KAF infusion. Highly purified KAF was prepared as previously described (6). The euglobulin fraction of normal human serum was chromatographed successively on DEAE cellulose and Sephadex G-200, concentrated by ultrafiltration, and sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.). The final preparation had a protein concentration of 400 μg/ml, and the KAF activity was 20 times that of normal serum (7). There was a small amount of contaminating IgG. Hepatitis B antigen was not detected. 16 ml of this material with a KAF activity equivalent to 320 ml of normal human serum were infused intravenously
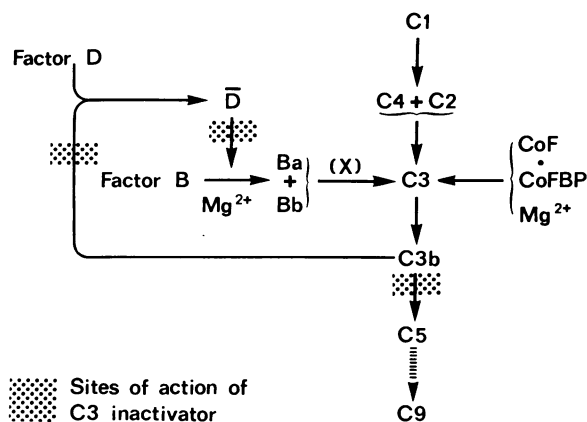

FIGURE 1 Simplified view of alternative or properdin pathway of complement activation. Although the CoFBP is not shown here as part of the alternative pathway, its low levels in the patient can most readily be explained if this protein is part of that system and consumed by the increased activity. Dotted areas represent steps which are probably or definitely inhibited by the C3b inactivator (KAF). Activation of C3 by Factor B may require an unidentified intermediate protein (X).

over a period of 20 min. The patient was well at the time of study and had not had any recent major infective episode. EDTA plasma and serum were collected before and at frequent intervals after the infusion and stored at $-80°C$ until studied.

Protein quantitations. Concentrations of C3, Factor B, properdin, and C5 were determined by electroimmunoassay using monospecific antisera (8). Crossed immunoelectrophoresis was used to estimate percent C3 conversion: patterns were photographed and enlarged, and the peaks representing native and converted C3 were cut out and weighed as previously described (9). Using total concentrations of immunologically reactive C3 determined by electroimmunoassay, it was possible to estimate native and converted C3 concentrations.

Complement-mediated functions. Opsonic activity was determined by incubating serum with paraffin oil droplets containing oil red O emulsified with bacterial lipopolysaccharide. Opsonization was quantitated by measuring oil red O uptake by neutrophils (10). Hemolytic complement activity was performed by the method of Mayer (11). To assess Factor $\overline{D}$ activity, 7 μl of a preparation of Factor B free of $\overline{D}$ and KAF activity and containing 0.8 mg/ml of Factor B were added to 30 μl of serum and incubated at 37°C for 10 min. Factor B conversion was assessed by immunoelectrophoresis. The cobra venom factor-binding protein (CoFBP) was semiquantitatively assessed as previously described (12). Cobra venom factor (CoF) was isolated from lyophilized cobra venom (*Naja naja*) by Pevikon block electrophoresis and Sephadex G-100 gel filtration (13). [^{125}I]CoF (14) was added to 300 μl of test serum and incubated at 37°C for 30 min. Samples were then run in a 5% polyacrylamide gel at 30 V/cm for 3 h. The presence of CoF-CoFBP complex was assessed by autoradiography. This assay could detect at least 10% of the normal level of CoFBP. The presence of CoF-CoFBP complex was also assessed indirectly by seeking C3 convertase activity: [^{125}I]C3 was added to test serum samples which contained 10 μg/ml CoF and to control samples free of CoF; the samples were incubated at 37°C for 30 min and autoradiographed after electrophoresis in 0.8% agarose gel. Conversion of labeled C3 in samples containing CoF could be directly compared with conversion in samples free of CoF and in appropriate controls. Bactericidal activity of serum (15) was kindly determined by Dr. R. C. Skarnes.

RESULTS

Results of immunochemical determinations of serum proteins are shown in Fig. 2. An initial fall in total C3 concentration during the 4.5 h after the KAF infusion can be seen to be due to a fall in the level of C3b. Subsequently, a rise in concentration of native C3 produced an increase in total C3 which was maintained for 11 days, after which levels fell to those observed before the study. C3b became almost undetectable after the infusion and began to return to prestudy levels on day 9. Native C3, almost undetectable before study, rose rapidly after the infusion and reached a plateau by day 4 which was maintained for an additional 7 days. An eightfold increase in Factor B concentration occurred within 24 h after KAF infusion; but in contrast to the prolonged improvement of C3 concentra-
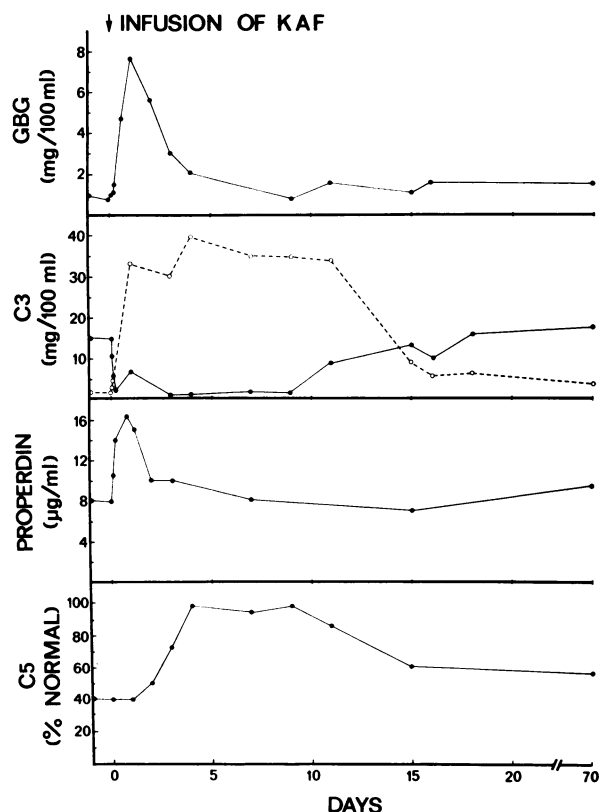

FIGURE 2 Immunochemically determined serum protein concentrations. In the graph representing C3 concentration, open circles represent native C3 and closed circles represent conversion products (usually C3b). Normal ranges are: Factor B (GBG), 12–56 mg/100 ml; C3, 100–200 mg/100 ml; properdin, 8–30 μg/ml; C5, 41–158% normal.

tion, the Factor B level fell again over the next 3 days to almost preinfusion levels. The properdin level before study was at the lower limit of the normal range at 8 μg/ml. Within 20 h after the KAF infusion, the properdin level had doubled to 16.5 μg/ml. By 2 days, the level had fallen almost to that seen before the infusion. A pyrogenic reaction had occurred during the first 8 h after the infusion; and, since properdin is an acute phase reactant, this observed increase may have been a consequence of the patient's febrile reaction to the injected material. Orosomucoid, a positive acute phase reactant, rose by almost 50% at 3 days, but no other significant changes were found in a screening of six acute phase reactants. Plasma samples obtained after an earlier infusion of whole plasma (2) were, therefore, examined for properdin levels since no pyrogenic response and no change in levels of other acute phase reactants accompanied that study. A similar pattern was seen after the plasma infusion. C5, previously present at 40% of the normal mean level, was restored
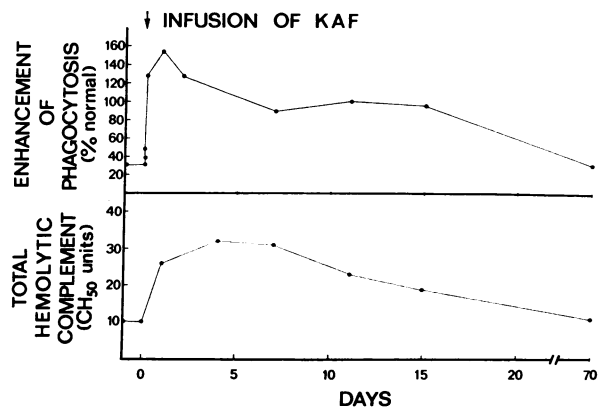
FIGURE 3 Complement-mediated serum functions. Normal range for total hemolytic complement activity is 32–45 CH_{50} units.

to normal concentration after the KAF infusion. The 100% normal level was achieved on day 4 and was maintained until day 9. Samples obtained after the earlier infusion of whole plasma were also examined for C5 levels, and the response obtained was identical in that study.

Hemolytic complement activity was restored to the lower limit of the normal range, the peak of this response occurring at day 4 (Fig. 3). Opsonic activity was restored to greater than normal levels within 4.5 h of the KAF infusion. Normal opsonic activity was observed to have been maintained for at least 15 days (Fig. 3). Bactericidal activity was restored to 50% of normal within 3 h, was 40% of normal at day 3, but by day 7 was undetectable. $\overline{D}$ activity, scored at 4+ before the infusion, was found to have been virtually abolished immediately after the infusion and appeared to return exponentially to preinfusion levels over 3 days. The CoFBP remained undetectable throughout the study, as was the case in the earlier plasma study. CoF-mediated C3 convertase activity was also not detected after the KAF infusion. Serum samples obtained after the infusion of 500 ml of normal plasma did show C3 convertase activity on addition of CoF: the activity was first detectable after 5 h and became maximal at 48 h. When Factor B levels were assessed by antigen-antibody-crossed immunoelectrophoresis in these samples, maximum levels of unconverted Factor B were found at 16 h and were almost undetectable at 25 h. Ba was maximal at 25 h. Electroimmunodiffusion (which measures B + Ba) showed a peak at 24 h, in keeping with the findings after the KAF infusion. Although an infusion of 500 ml of normal plasma had resulted in extensive urticarial eruption, this did not occur after KAF infusion which, unlike the plasma, did not provide C3 substrate for C3a formation.

DISCUSSION

The dramatic changes in C3 and Factor B levels which followed the infusion of less than 10 mg of KAF demonstrate the potent biological activity of this molecule. The partial correction of C3 and Factor B concentrations and restoration of complement-mediated activities indicate conclusively the primary nature of the KAF deficiency of this patient, and the study identifies KAF as the component of normal plasma which was responsible for the correction obtained in an earlier study (2).

The rapid fall in Factor B concentration after day 1, despite maintained C3 concentrations, is more difficult to understand. Similar changes in Factor B levels had been noted to follow the infusion of normal plasma in this patient (2). Two possibilities can be entertained. A fall in Factor B synthetic rate may have occurred in response to KAF infusion and its sequelae. This would result in lower Factor B levels despite lack of Factor B activation. Alternatively, as KAF levels began to fall, a kinetic disequilibrium between different reactions in the alternative pathway may have allowed the rapid return of sufficient $\overline{D}$ activity and C3b to cause rapid inactivation of Factor B and properdin but not enough to make an effective C3 convertase.

The rise in C5 levels which followed both the earlier plasma infusion and the infusion of KAF reported here serves to show that fluid-phase C3 activation is able, albeit inefficiently, to activate C5. This activation was interrupted after the infusion, allowing C5 levels to rise temporarily. Properdin levels are usually at the lower limit of normal in this patient and were observed to double after temporary correction of the metabolic defect. In contrast, proteins known to be consumed in alternative pathway activity are usually almost undetectable (Factor B, CoFBP, native C3). Unlike most of these molecules, properdin underwent only a modest increase in level, a change reminiscent of that seen with C5, a protein which almost certainly has no place in the properdin pathway. A metabolic study using radiolabeled purified properdin [2] showed that the catabolic rate of properdin was normal in this patient, while the properdin synthesis rate was reduced. The observed changes in properdin levels seen in this patient after infusion of whole plasma or KAF may be explained by changes in synthetic rate.

CoFBP was undetectable by a semiquantitative assay which indicates that the level remained below 10% of normal after KAF infusion, and a similar result had followed the plasma infusion. Factor B, by contrast, rose dramatically to approximately 40% of the normal

[2] Ziegler, J. B., F. S. Rosen, C. A. Alper, W. Grupe, and I. H. Lepow. Properdin metabolism in normal subjects and patients. Manuscript submitted for publication.

level. While CoF-mediated C3 convertase activity was maximal at 48 h after the plasma infusion, the peak level of Factor B occurred at 24 h and that of native Factor B was seen even earlier. Vogt, Dieminger, Lynen, and Schmidt (16), using levels of Factor B considerably higher than those occurring physiologically, found that native Factor B forms a loose complex with CoF and that $\bar{D}$ acts on this complex to liberate Ba and form a C3 convertase consisting of CoF and Bb in a firm complex. The demonstration that CoF-mediated C3 convertase can be generated at a time (48 h) when native Factor B is almost absent from serum suggests that CoF can also form a C3 convertase which is independent of Factor B, a conclusion implied by the finding (12) that Factor B and CoFBP protein are distinct molecules. The temporal discrepancy between Factor B and C3 convertase levels is not inconsistent with the findings of Cooper (17) and Hunsicker, Ruddy, and Austen (18), who found that only a small percentage of Factor B molecules are involved in complexes of CoF, indicating heterogeneity of Factor B preparations in their capacity to bind CoF. This can be explained on the basis that the CoF-binding activity is the property of a contaminant of the isolated Factor B. The changes observed in D activity reflect the exponential decay in KAF activity.

Clearly, some questions remain unanswered in this patient. Nevertheless, the crucial place for KAF in the defect in this patient and in controlling the activation of the alternative pathway for C3 activation is beyond dispute.

ACKNOWLEDGMENTS

REFERENCES

1. Alper, C. A., N. Abramson, R. B. Johnston, Jr., J. H. Jandl, and F. S. Rosen. 1970. Increased susceptibility to infection associated with abnormalities of complement-mediated functions and of the third component of complement (C3). N. Engl. J. Med. 282: 349–354.
2. Alper, C. A., N. Abramson, R. B. Johnston, Jr., J. H. Jandl, and F. S. Rosen. 1970. Studies in vivo and in vitro on an abnormality in the metabolism of C3 in a patient with increased susceptibility to infection. J. Clin. Invest. 49: 1975–1985.
3. Abramson, N., C. A. Alper, P. J. Lachmann, F. S. Rosen, and J. H. Jandl. 1971. Deficiency of C3 inactivator in man. J. Immunol. 107: 19–27.
4. Alper, C. A., F. S. Rosen, and P. J. Lachmann. 1972. Inactivator of the third component of complement as an inhibitor in the properdin pathway. Proc. Natl. Acad. Sci. U. S. A. 69: 2910–2913.
5. Nicol, P. A. E., and P. J. Lachmann. 1973. The alternate pathway of complement activation. The role of C3 and its inactivator (KAF). Immunology. 24: 259–275.
6. Lachmann, P. J., M. J. Hobart, and W. P. Aston. 1973. Complement technology. In Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publications Ltd., Oxford, England. 2nd edition. 5.1–5.17.
7. Lachmann, P. J., P. Nicol, and W. P. Aston. 1973. Further studies on the C3b in activator or conglutinogen activating factor (KAF). Immunochemistry. 10: 695–700.
8. Laurell, C.-B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem. 15: 45–52.
9. Alper, C. A., and F. S. Rosen. 1967. Studies of the in vivo behavior of human C'3 in normal subjects and patients. J. Clin. Invest. 46: 2021–2034.
10. Stossel, T. P. 1973. Evaluation of opsonic and leukocyte function with a spectrophotometric test in patients with infection and with phagocytic disorders. Blood J. Hematol. 42: 121–130.
11. Mayer, M. M. 1961. Complement and complement fixation. In Experimental Immunochemistry. E. A. Kabat and M. M. Mayer, editors. Charles C. Thomas, Publisher, Springfield, Ill. 2nd edition. 133–240.
12. Alper, C. A., I. Goodkofsky, and I. H. Lepow. 1973. The relationship of glycine-rich β-glycoprotein to Factor B in the properdin system and to the cobra factor-binding protein of human serum. J. Exp. Med. 137: 424–437.
13. Müller-Eberhard, H. J., U. R. Nilsson, A. P. Dalmasso, M. J. Polley, and M. A. Calcott. 1966. A molecular concept of immune cytolysis. Arch. Pathol. 82: 205–217.
14. McFarlane, A. S. 1958. Efficient trace-labelling of proteins with iodine. Nature (Lond.). 182: 53.
15. Skarnes, R. C. 1967. Leukin, a bactericidal agent from rabbit polymorphonuclear leucocytes. Nature (Lond.). 216: 806–808.
16. Vogt, W., L. Dieminger, R. Lynen, and G. Schmidt. 1974. Formation and composition of the complex with cobra venom factor that cleaves the third component of complement. Hoppe-Seyler's Z. Physiol. Chem. 355: 171: 183.
17. Cooper, N. R. 1973. Formation and function of a complex of the C3 proactivator with a protein from cobra venom. J. Exp. Med. 137: 451–460.
18. Hunsicker, L. G., S. Ruddy, and K. E. Austen. 1973. Alternate complement pathway: factors involved in cobra venom factor (CoVF) activation of the third component of complement (C3). J. Immunol. 110: 128–138.