0031-3998/87/2202-0123$02.00/0
PEDIATRIC RESEARCH

Vol. 22, No. 2, 1987
Printed in U.S.A.

Persistently Circulating C3 Nephritic Factor (C3 NeF)-Stabilized Alternative Pathway C3 Convertase (C3 CoF) in Serum of an 11-Year-Old Girl with Meningococcal Septicemia—Simultaneous Occurrence with Free C3 NeF

V. WAHN, W. MÜLLER, C. RIEGER, AND U. ROTHER

Universitäts-Kinderklinik, Düsseldorf [V.W.]; Kinderklinik der MHH, Hanover [W.M.]; Universitäts-Kinderklinik, Marburg [C.R.]; and Institut für Immunologie und Serologie, Heidelberg [U.R.], FRG

ABSTRACT. Hemolytic complement was found to be absent in the serum of an 11-yr-old girl (R.N.) with meningococcal septicemia. C1, C4, an C2 were slightly decreased, C3 was absent, C5–C9 within the normal range. B levels immunochemically and electrophoretic mobility of B were normal. C3d was >1000% of a pooled EDTA-plasma standard indicating hypercatabolism of C3. On incubation of the patient's serum with normal human serum activation of C3 occurred even in the presence of 0.04 M EDTA. The amount of C3b generated was, however, greater without any chelating agent or in Mg-EGTA. On gel filtration of the serum two protein containing peaks were found to be responsible for activation of C3: the IgG containing peak was able to activate C3 in normal human serum without chelating agents and in Mg-EGTA but not in the presence of EDTA. The IgM-containing peak activated the third component of complement even in the presence of EDTA. The factor responsible for this phenomenon was termed C3 converting factor (C3 CoF). The IgG fraction of the patients serum caused activation of C3 in Mg-EGTA. However, in the presence of EDTA no activation of C3 could be induced even if physiological concentrations of the patients IgG were added to normal human EDTA-plasma. Thus the activity of the patient's IgG did not differ from typical C3 nephritic factor. The decay of C2 in EAC42 intermediates in the presence of the patient's IgG was uninfluenced indicating that it did not carry autoantibody activity against the classical pathway convertase C4b,2a, an activity recently termed NFc. As the ability of the patient's serum to activate C3 in the presence of excess EDTA was still unexplained further analyses were performed. C3 CoF was found to be a β 2-euglobulin and could be separated from IgG by anion exchange chromatography following euglobulin precipitation. C3 conversion with this material was independent from Ca^{2+} and Mg^{2+}. It was partly heat resistant at 56° C and could not be inhibited by soy bean trypsin inhibitor at 5 mg/ml. In contrast, DFP at 5 mM concentration lead to approximately 50% inhibition indicating that C3 CoF might be a serine protease. Treatment of patient's EDTA plasma with anti-C3 or anti-B totally eliminated C3 CoF activity while antialbumin, anti-C2, anti-C4, or nonimmune rabbit-IgG did not. Precipitation with anti-IgG partly eliminated C3 CoF but absorption of serum with an anti-IgG affinity column totally eliminated C3 CoF; these observations suggest that C3 CoF may be a C3 NeF-stabilized alternative pathway C3 convertase. (*Pediatr Res* 22: 123–129, 1987)

Abbreviations

C3 NeF, C3 nephritic factor
C3 CoF, C3 converting factor
NHS, normal human serum
RNS, serum of patient RN
NHPl, normal human EDTA-plasma
IEP, immunoelectrophoresis
MPGN, membranoproliferative glomerulonephritis
C3 AF, C3 activating factor
ESR, erythrocyte sedimentation rate
WBC, white blood cells
AHG, aggregated human γ-globulin
PBS, phosphate-buffered saline
SBTI, soy bean trypsin inhibitor
DFP, diisopropylfluorophosphate
DIC, disseminated intravascular coagulation
PMNL, polymorphonuclear leucocytes
C-EDTA, complement EDTA

Various states of hypercatabolism of the third component of the complement system, C3, have been described (1). Two of them, the deficiencies of factors I and H, were identified as genetic defects (2, 3). H and I function as regulatory proteins of the alternative pathway by degrading C3b to iC3b which in turn can be cleaved to C3c and C3d by a trypsin-like enzyme (4, 5). The absence of H or I leads to the persistence of C3b which combines with factor B and, in the presence of factor D, forms the alternative pathway convertase C3b,Bb (6). Persistence of this enzyme is associated with continuous activation and consumption of C3. Mg^{2+} is essential for its action.

Besides these two genetic deficiences various states of hypercatabolism of C3 have been identified without evidence of familality. The most common cause is activation of complement by circulating immune complexes (CIC, 7) which depends on

Received October 17, 1986; accepted February 19, 1987.
Reprint requests Prof. Dr. V. Wahn, Universitäts-Kinderklinik, Moorenstr. 5, D 4000 Düsseldorf, FRG.

the presence of both Ca^{2+} and Mg^{2+}. C3 Nef has been found in sera of patients with MPGN, partial lipodystrophy, and meningococcal meningitis (8–10). It is an IgG-autoantibody directed against C3b,Bb (11, 12) which stabilizes the enzyme and prevents its intrinsic decay (13). C3 Nef requires Mg^{2+} for its action.

An analogue directed against the classical pathway convertase C4b,2a has been detected in serum of a patient with acute glomerulonephritis (NFc, 1). It is probably identical with the F-42 autoantibody found in some SLE-sera (14, 15). It seems to prevent the intrinsic decay of C4b,2a as well as the extrinsic decay mediated by C4 binding protein (16). In addition, C4b in the stable convertase C4b,2a is protected from the proteolytic action of factor I (16). C4b,2a, thus stabilized, may activate C3 even in EDTA (17). For the formation of C4b,2a, however, Mg^{2+} is essential.

Two more activators of C3 have been reported. Hypercatabolism type II (18) identified in a patient with recurrent infections was due to a 6s heat-labile pseudoglobulin enzyme which also depended on the presence of Mg^{2+}. C3 AF, a γ-migrating non-IgG activator of C3 found in the cryoprecipitate of a patient with MPGN (19), also required Mg^{2+}.

Thus, except for the nephritic factor of the classical pathway NFc which is an IgG, all the known C3 converting activities depend on Mg^{2+}. Herein we describe a factor (C3 CoF) which does not require divalent cations and is composed of C3, B, and IgG.

CASE REPORT

The family history of patient R.N. was unremarkable as was her own history until the age of 11 yr. Ten days prior to admission to the hospital she had an episode of otitis media which was treated with amoxycillin for 3 days. One week later she became febrile again and began to vomit. In addition pain and tenderness of the left knee and some finger joints occurred.

On physical examination after admission she had a temperature of 40.4° C and a generalized maculopapulour, partially petechial rash. We noticed marked lymphadenopathy and slight hepatosplenomegaly. Meningeal signs were absent.

Laboratory findings. ESR 42/72, red cell count normal, WBC 26 900/mm^3 with 2 bands, 84 polymorphs, 1 eosinophil, 7 lymphocytes, and 6 monocytes. Platelets 163 000/mm^3. Coagulation tests revealed no evidence of DIC. Rheumatoid factor and antinuclear antibodies were negative, circulating immune complexes (in the first serum) by Clq binding assay were slightly increased. Total hemolytic complement was hardly detectable, C4 immunochemically was normal, C3 undetectable. Blood cultures revealed *Neisseria meningitidis.* CSF: 30 leucocytes/mm^3 (24 PMNL, 6 mononuclear cells), cultures sterile. Evidence of renal disease was found neither at the time of admission nor during the 3 yr observation period following recovery.

Treatment was initiated with ampicillin (300 mg/kg body weight/day) and tobramycin (5 mg/kg body weight/day) and continued with penicillin G after identification of meningococci. Clinical improvement and normal temperature was achieved within 24 h. After 10 days of treatment antibiotic therapy was discontinued.

After complete recovery from her illness (for now approximately 3 yr) CH 50- and C3-levels remained undetectable and therefore further analyses of the complement system were performed (see "Results"). Analyses of the parent's sera revealed normal total hemolytic complement activity and normal levels for C3.

MATERIALS AND METHODS

Sera. Venous blood was obtained and allowed to clot for 30–60 min at room temperature. After centrifugation serum was kept in aliquots at −70° C until use. EDTA-plasma was prepared similarly using EDTA-coated tubes. (NHS and NHP1) was obtained from 30 healthy volunteers from the clinic staff and pooled. These pools served as reference standards for all complement determinations.

Titrations of total hemolytic complement and individual components were performed according to Mayer (20). Cellular intermediates EA, EAC4, EAC1, and EAC1,4 were prepared in our laboratory as described (21). EAC1-7 and functionally purified C components were purchased from Cordis (Miami, FL) or prepared according to Rapp and Borsos (22). Alternative pathway lysis was measured using rabbit erythrocytes in Mg-EGTA buffer according to Platts-Mills and Ishizaka (23).

Immunochemical determinations of C3, C4, and B were performed by single radial immunodiffusion according to Mancini *et al.* (24) using commercially available plates (Behring, Marburg, FRG). Clq, I, H, and P were quantitated by the same technique. Antisera to Clq, I, and H were obtained from Miles (Elkhart, IN), to P from Atlantic Antibodies (Scarborough, ME). C3d was measured in EDTA-plasma by rocketelectrophoresis as recently described (21). Prior to the run into an anti-C3d antibody containing gel EDTA-plasma had been treated with 12% polyethyleneglycol 6000 to precipitate C3 and C3b reactive with the anti-C3d antibody. The antiserum to C3d was obtained from DAKO (Copenhagen, Denmark). C3b was detected by crossed IEP as described by Weeke (25). The antiserum to C3 was purchased from Miles. Cleavage products of factor B were identified by IEP (26) using monospecific antiserum to B from Behring. Normal EDTA-plasma served as a negative control and zymosan-activated serum (10 mg/ml, 30 min at 37° C) as a positive control.

If not specifically mentioned in the text all incubations were performed for 30 at 37° C. For heat-inactivation sera were incubated for 30 min at 56° C. A tandem-crossed IEP was performed to determine the electrophoretic mobility of residual C3-antigen present occasionally in some of the patient's reconvalescent sera. For this purpose two wells were filled on the same gel prior to the run in the first dimension. Well 1 contained RNS, well 2 NHS 1:10 diluted after a 5-min activation with heat-AHG at 2 mg/ml and 37° C. This activated serum served as a source for C3 and C3b. AHG was prepared as described (21).

C3 CoF electrophoretic mobility was determined following electrophoresis in 1% agarose in barbital buffer pH 8.6 shown in scheme 1. Following 2 h electrophoresis at 2 V/cm the gel in the center of the slide (containing separated proteins to 100 μl RNS) was cut into 1-mm slices and each slice added to 0.1 ml of PBS. Fractions were eluted from the agarose by shaking for 1 h at room temperature. C3 CoF assay was performed with all fractions. Next horse antiserum to whole human serum (Behring) was added to the troughs and diffusion was allowed for 24 h in a moist chamber before the slide was washed and the precipitates stained with Coomassie blue. C3 CoF mobility was determined by comparing the maximum of C3 conversion in EDTA with the precipitin arcs of regular IEP.

For determination of C3 CoF molecular weight 1 ml of patient's serum was separated by gel filtration using HPLC equipment (LKB, Bromma, Sweden) and a TSK-G SW 4000 blue column (7.5 × 600 mm, LKB). One-ml fractions were collected, and all protein-containing fractions were assayed for C3-converting activity in Mg-EGTA and EDTA as well as for IgG and IgM as internal marker proteins.

The patient's IgG-fraction and normal IgG were isolated by ammonium sulphate precipitation and DEAE 52 anion exchange chromatography as described by Johnstone and Thorpe (27). Further purification was achieved by gel filtration using a Sephadex G-200 column (2.5 × 30 cm) and by affinity chromatography using a Sepharose CL 4B-protein A column (13 × 55 mm, Pharmacia, Freiburg, FRG) according to Patrick and Virella (28). With this material only one precipitin arc was found on IEP against anti-whole human serum and anti-human IgG (from rabbits, Behring). IgG-levels were determined by radial immunodiffusion using commercial plates (Behring) and adjusted to

approximately 12 mg/ml by ultrafiltration using Amicon UM 30 (Lexington, MA) membranes. These IgG-fractions were used for crossed IEP experiments, the NFc and C3 NeF assay.

Euglobulin was precipitated by dialysis of 5 ml of the patient's serum against 2 mM phosphate buffer pH 7.0, once for 2 h and, after buffer exchange, for 16 h at 4° C. The euglobulin obtained was washed once using the same buffer. Following this precipitation step C3 CoF could not be dissolved at normal ionic strength and normal pH. It was only partly soluble in 0.5 ml of 0.2 M carbonate-bicarbonate buffer pH 9.6. Insoluble material was removed by centrifugation. Dissolved supernatant contained only 20% of the total initial C3 CoF activity. It was next dialyzed against 0.07 M phosphate buffer pH 6.3 for 2 h at 4° C before it was applied to a Ultropac TSK DEAE column (150 × 7.5 mm, LKB) equilibrated with the same buffer. After elution of the IgG-peak (void volume) using this buffer the "non-IgG" fraction was eluted by markedly increasing the ionic strength using 1 M NaCl dissolved in starting buffer. Protein concentrations were recorded by measuring absorbance at 280 nm. The major peaks were pooled, concentrated by ultrafiltration, and assayed for IgG, C3 NeF, and C3 CoF activity by crossed IEP against anti-C3 antiserum after incubation of the fractions with NHS and the required chelating agent. In a representative experiment the IgG fraction after ultrafiltration had a volume of 0.35 ml and a protein content of 0.67 g/dl, the non-IgG fraction a volume of 0.8 ml, and a protein content of 0.11 g/dl. The overall yield for C3 CoF in the latter fraction was approximately 8%, the specific activity was about 8-fold higher than in original patient's serum.

C4b,2a stabilization assay. Basically the method of Halbwachs *et al.* (1) was used. Partially purified components C1 and C2 were prepared as described by Rapp and Borsos (22). C2 was oxidized.

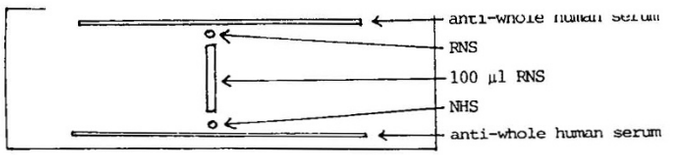
Incubations. In the standard assay for C3 CoF activity 1 volume of the patient's serum was mixed with EDTA at a final concentration of 0.04 M before 1 volume of NHS was added. The mixture was incubated for 30 min at 37° C before it was subjected to crossed IEP against anti-C3 antibody. If fractions eluted from the columns were tested for C3 CoF a 10:1 or 20:1 ratio (volume fraction: volume NHP1) was chosen to compensate dilution effects. EDTA was always added at a final concentration of 0.04 M. If incubations were performed in Mg-EGTA buffer a 0.02 M concentration of EGTA and 0.005 M for Mg^{2+} was used. The temperature dependence of C3 CoF action was determined by performing the standard assay at 0, 10, 20, 30, and 37° C.

To find out if SBTI (Sigma, Munich, FRG) could diminish C3 CoF activity it was added at various concentrations between 0.1 and 5 mg/ml to the standard reaction mixture prior to incubation at 37° C. The same procedure was used for DFP (Sigma).

C3 NeF activity was measured by activation of C3 in NHS by either heat-inactivated patient's serum or purified RN-IgG in Mg-EGTA buffer using crossed IEP.

Next attempts were made to eliminate C3 CoF from RNS by addition of monospecific antisera. The following antibodies were used (if concentration was required it was done by ultrafiltration, dilutions were made in PBS): IgG-fraction of rabbit anti-human IgG (h− and l− chain, Cappell Lab., concentrated 10-fold), IgG-fraction of goat anti-human C3 (Cappell, diluted 1:30), IgG fraction of goat anti-human C4 (Beckmann Instr., Munich, FRG, concentrated 4-fold), IgG fraction of rabbit anti-human factor B (Behring, concentrated 8-fold, sheep anti-human C2 (Seward Lab., London, England). Two volumes of RNS were mixed with 1 volume of 0.2 M EDTA before 2 volumes of antiserum were added.

After 15 min at room temperature and 15 min at 0° C the precipitates were removed by centrifugation. In the control experiments physiological saline, nonimmune rabbit IgG and an IgG fraction of rabbit anti-human albumin (Behring, concentrated 8-fold) was used instead of antiserum. The remaining supernatants (under the conditions chosen free of the precipitated antigen by double diffusion, except for IgG and albumin) were next added to NHPl at a 2:1 (v/v) ratio and the mixture incubated for 30 min at 37° C before crossed IEP against anti-C3 was performed (C3 CoF assay). The areas under C3 and C3b peaks were calculated planimetrically.



Scheme 1. Set up of wells in agarose for determination of C3 CoF electrophoretic mobility.

Affinity chromatography. Because IgG could not be completely eliminated by immune precipitation an affinity column was set up: 120 mg of IgG-fraction of rabbit anti-human IgG (Cappell) were coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) at 7 mg/ml gel according to the instructions of the manufacturer. The resulting column size was 16 × 65 mm. One-half ml of RNS was added and eluted with PBS at 30 ml/h. The material excluded from the column was concentrated by ultrafiltration to 3.29 g/dl and assayed for IgG, C3 NeF, and C3 CoF.

RESULTS

Results of hemolytic titrations. Total hemolytic complement was hardly detectable and alternative pathway lysis undetectable. Components C1, C4, C2, and C5 were decreased and C3 was present in traces immunochemically and functionally. These traces could be identified as native C3 by tandem crossed IEP. C7 was slightly elevated and the other components were within the normal range [normal ranges have been reported (21)]. B, P, I, and H were normal (immunochemically) and C3d-levels extremely elevated (above upper level of our standard curve). The hemolytic activity of C1, C4, and C2 was undetectable if EAC142-intermediates were not washed with $ggvb^{2+}$ before C-EDTA was added as a source of C3-C9.

Figure 1 shows the results of crossed IEP and IEP of C3 and B, respectively, in NHS following incubation with the patient's serum under various conditions. In the presence of Mg-EGTA the electrophoretic mobilities of both C3 and B were shifted to the position of the respective activated component. In the presence of EDTA, however, C3 was activated while B retained its normal electrophoretic mobility. This cleavage of C3 in normal human EDTA-plasma induced by RNS led to the definition of C3 converting factor, C3 CoF (Fig. 2). B cleavage could be induced in RNS if C3 (3 CH 50 U/ml, final mixture) was added prior to incubation instead of NHS as a source of C3. In contrast to cleavage of C3, however, B cleavage depended on the presence of Mg^{2+} (Fig. 3) suggesting activation via the alternative pathway.

Heat stability of the suspected activator of C3 was tested and the results are given in Table 1. If NHS is incubated with $ggvb^{2+}$ alone for 30 min at 37° C total hemolytic complement and hemolytic C3 are slightly decreased compared to the nonincubated control (data not shown). Incubation of NHS with heat-inactivated NHS led to similar results. On incubation of NHS with heat-inactivated RNS an almost complete consumption of total hemolytic complement and C3 was observed. If EDTA was added to the latter mixture intermediate values for CH 50 and C3 were obtained. Further experiments with heat-inactivated RNS were carried out by crossed IEP (Fig. 4). We notice that incubation of NHS alone leads to some activation of C3 (*a*). Addition of RNS markedly increases the amount of C3b generated (*b*). Results in Mg-EGTA (*c*) differ only slightly from (*b*). In EDTA, however, most of C3 activation is blocked (*d*) but still demonstrable. The appropriate control for experiment (*d*) is the incubation of NHS in EDTA which does not generate C3 cleav-

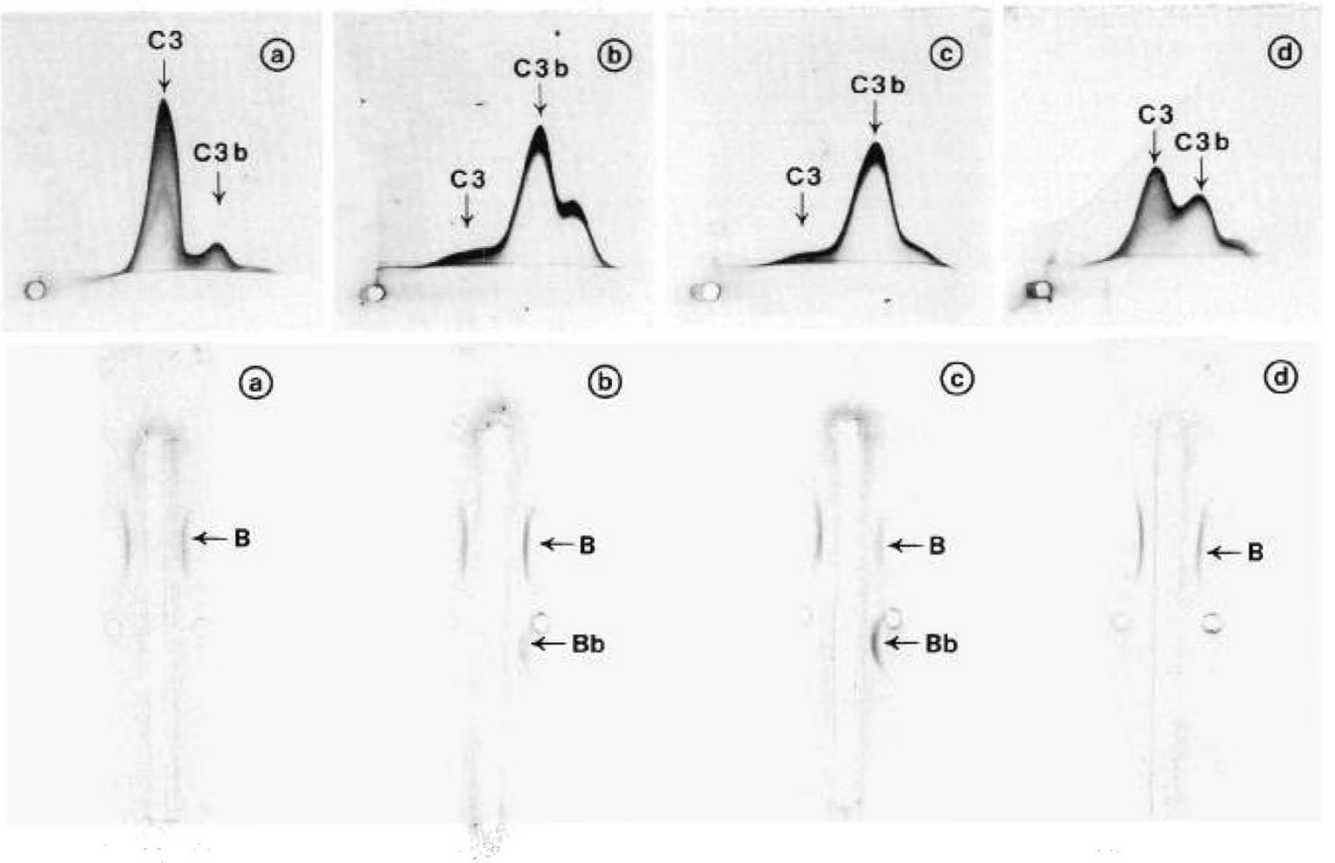


Fig. 1. Activation of C3 (*top*) and B (*bottom*) after incubation (30 min at 37° C) of mixtures of NHS, RNS, and chelating agents. *a* represent NHS + PBS (1:1), *b*, NHS + RNS, *c*, NHS + RNS + Mg-EGTA, and *d*, NHS + RNS + EDTA. On the IEP on the *left* NHP1 is always run as a control for native B.

NHS + 0.04 M EDTA	NHS + R.N. serum + 0.04 M EDTA
Incubation for 30′ at 37°C	

Fig. 2. Definition of C3 CoF. Incubation of a 1:1 mixture for 30 min at 37° C of NHS and RNS in 0.04 M EDTA led to significant generation of C3b not present in either serum alone. To add equal amounts of C3 antigen to both wells in well A PBS was used at the same volume as RNS in well B.

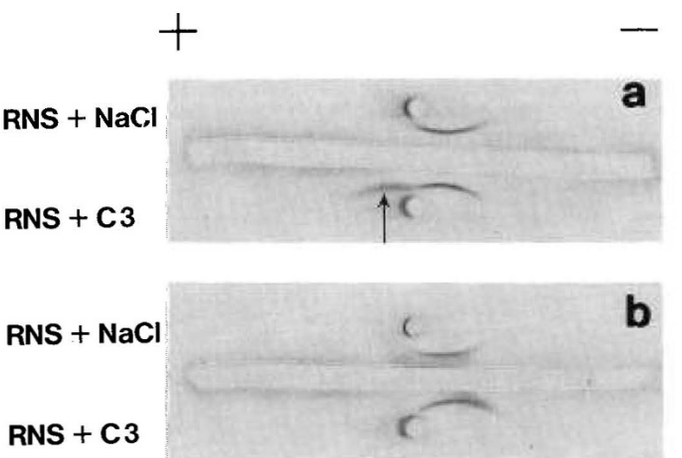


Fig. 3. IEP to detect B activation products following incubation for 30 min at 37° C of RNS with saline or functionally purified C3 (3 CH 50 U/ml) in Mg-EGTA (*a*) or in EDTA (*b*). After addition of C3 B can be activated in Mg-EGTA (*arrow*).

age products (*e*). Comparison of experiment (*d*) with Figure 2 indicates loss of most of C3 CoF activity on treatment at 56° C.

We initially expected only one activator of C3 to be present. Thus, to estimate the molecular weight of this activator RNS was subjected to gel filtration. After separation individual fractions were incubated with NHS and either Mg-EGTA or EDTA for 30 min at 37° C. After crossed IEP against anti-C3 two peaks exhibited activating properties for C3 *in vitro*. In Mg-EGTA C3 was activated in the IgM and IgG region while in EDTA only the IgM-containing fraction was active (Fig. 5).

Further experiments were carried out with patient's IgG and normal IgG isolated under identical conditions. Figure 6 shows that incubation of RN-IgG with NHS induces significant activation of C3. This was observed without chelating agents and in Mg-EGTA. However, if we used EDTA instead no activation of C3 could be achieved. Thus, the activating properties of RN-IgG were not identical with the activity attributed to C3 CoF. RN-IgG markedly activated C3 in Mg-EGTA buffer and was, thus, undistinguishable from C3 NeF. C3 CoF was still unidentified.

Since the patient's serum was able to convert C3 in NHS during incubation in EDTA there was a chance that C3 CoF was identical with an already stabilized classical pathway convertase C4b,2a. Therefore we analyzed whether preformed C4b,2a was stabilized by RN-IgG as reported (1, 16). EAC142 were prepared, washed, and resuspended in EDTA-buffer. One part of resuspended cells was incubated in the presence of RN-IgG, another part with identical concentration of normal IgG isolated in parallel. Samples were taken at intervals, washed, and lysed by the addition of guinea pig serum in 0.01 M EDTA. The decay of C2 in the presence of RN-IgG and N-IgG was identical. NFc activity in patient's IgG was therefore excluded. However, C3 CoF could still be a prestabilized C3 convertase of either pathway. This possibility was next evaluated using monospecific antisera.

If RNS was treated with monospecific antisera prior to C3 CoF assay some antisera markedly reduced it's activity (the amount of C3b generated is expressed as percent C3b area divided by C3 + C3b area). After treatment of RNS with saline 32% C3b were generated, with nonimmune rabbit IgG 35%, and with antialbumin 36%. With anti-C4 it was 34%, with anti-C2 29%, with anti-IgG 13%. After treatment with anti-C3 or anti-B antibody C3 CoF in the supernatant was no longer detectable. These data suggest the presence of C3, B, and IgG antigens in C3 CoF.

As IgG could not completely be precipitated by monospecific antibody it was absorbed out by affinity chromatography. The absorbed material had a protein concentration of 3.29 g/dl. IgG was below the level of detection by radial immunodiffusion (0.8 mg/dl) and C3 NeF and C3 CoF were absent.

On preparative electrophoresis in agarose C3 CoF activity was detected in the β2-region. This also supported the concept that it is no IgG. Of the five temperatures tested most conversion of C3 could be induced at 37° C (Fig. 7) indicating that C3 CoF does not activate C3 in the cold.

We attempted to separate C3 NeF from C3 CoF. We succeeded using euglobulin precipitation and anion exchange chromatography on DEAE 52 using a discontinuous salt gradient to elute fractions. The elution profile is depicted in Figure 8. Peak I contained 630 mg/dl of protein and 463 mg/dl of IgG and

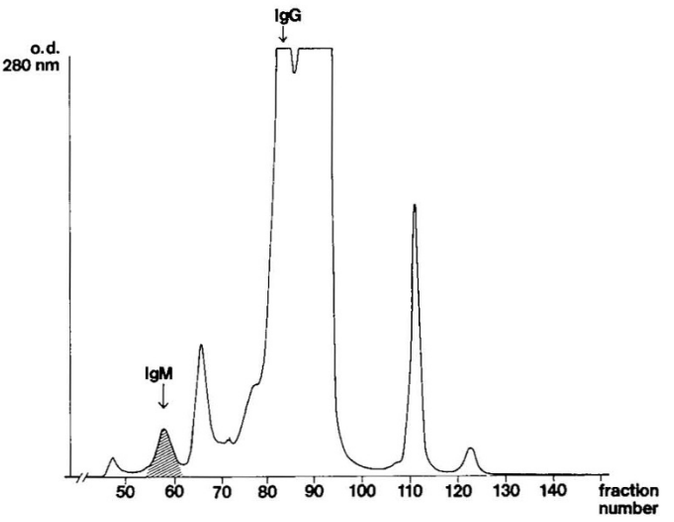


Fig. 5. Elution profile of RNS after separation on a TSK-G SW 4000 blue column (7.5 × 600 mm). Internal markers IgG and IgM are indicated by *arrows*. C3 conversion in Mg-EGTA could be induced by both the IgG and IgM containing peaks and C3 conversion in EDTA by the IgM containing peak only (indicated by the *shaded area*). Thus, it can be expected to have a molecular weight similar to IgM.

Table 1. *Complement consumption induced by RNS following heat-inactivation (30 min at 56° C)**

Hemolytic titer	Mixture, incubated for 30′ at 37°C: NHS + ggvb^{++}	NHS + NHS$_{hia}$†	NHS + RNS$_{hia}$	NHS + RNS$_{hia}$ + EDTA
CH 50 (U/ml)	87	90	15	48
C3 (U/ml)	1400	1900	103	840

* Incubation of NHS with ggvb^{++} or heat-inactivated NHS does not lead to significant loss of hemolytic activity. Incubation with heat-inactivated RNS leads to significant consumption of CH 50 and C3. If EDTA is added at 10 mM prior to addition of heatinactivated RNS intermediate titers for CH 50 and C3 are obtained.

† Heat inactivated.

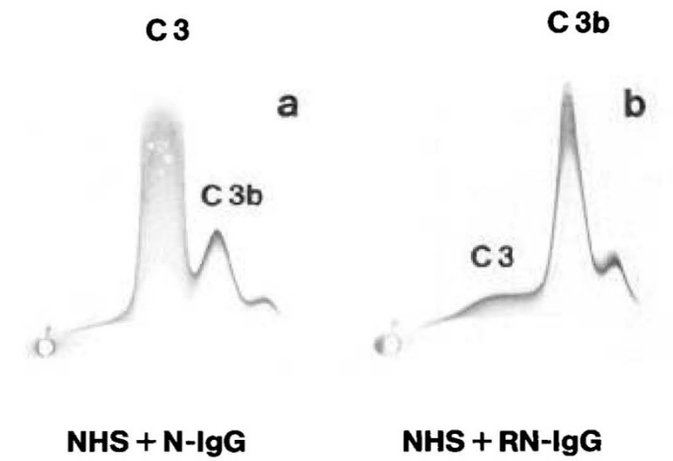


Fig. 6. Incubation of NHS with either normal IgG (N-IgG) or patient's IgG (RN-IgG). While with N-IgG only little activation is achieved (*a*) addition of RN-IgG (*b*) led to almost total consumption of C3.

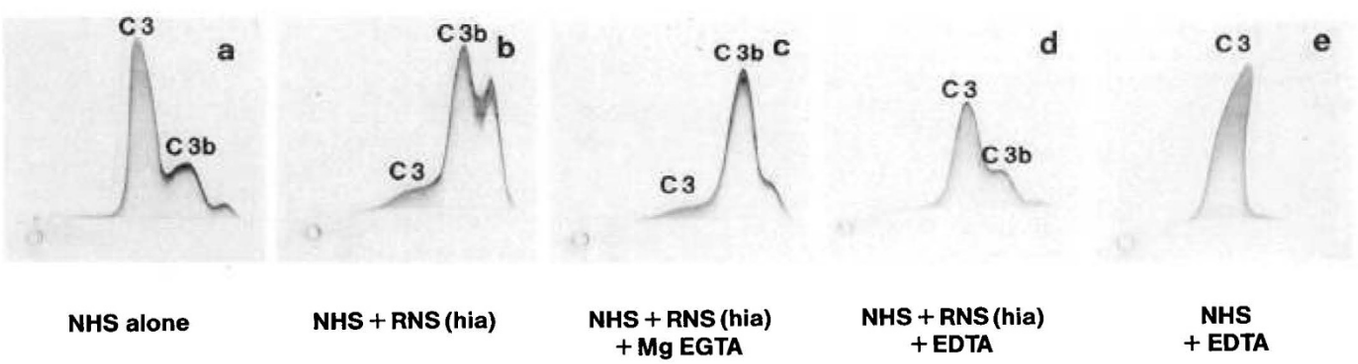


Fig. 4. Crossed IEP against anti-C3 antibody after incubation (30 min, 37° C) of various mixtures: (*a*) contains NHS alone, (*b*) contains a mixture of NHS and heat-inactivated RNS without chelating agents, (*c*) is the experiment (*b*) but with Mg-EGTA added, in (*d*) Mg-EGTA is replaced by EDTA. (*e*) is NHS incubated in EDTA. Incubation of NHS alone in Mg-EGTA was deleted because the result was identical with (*a*).

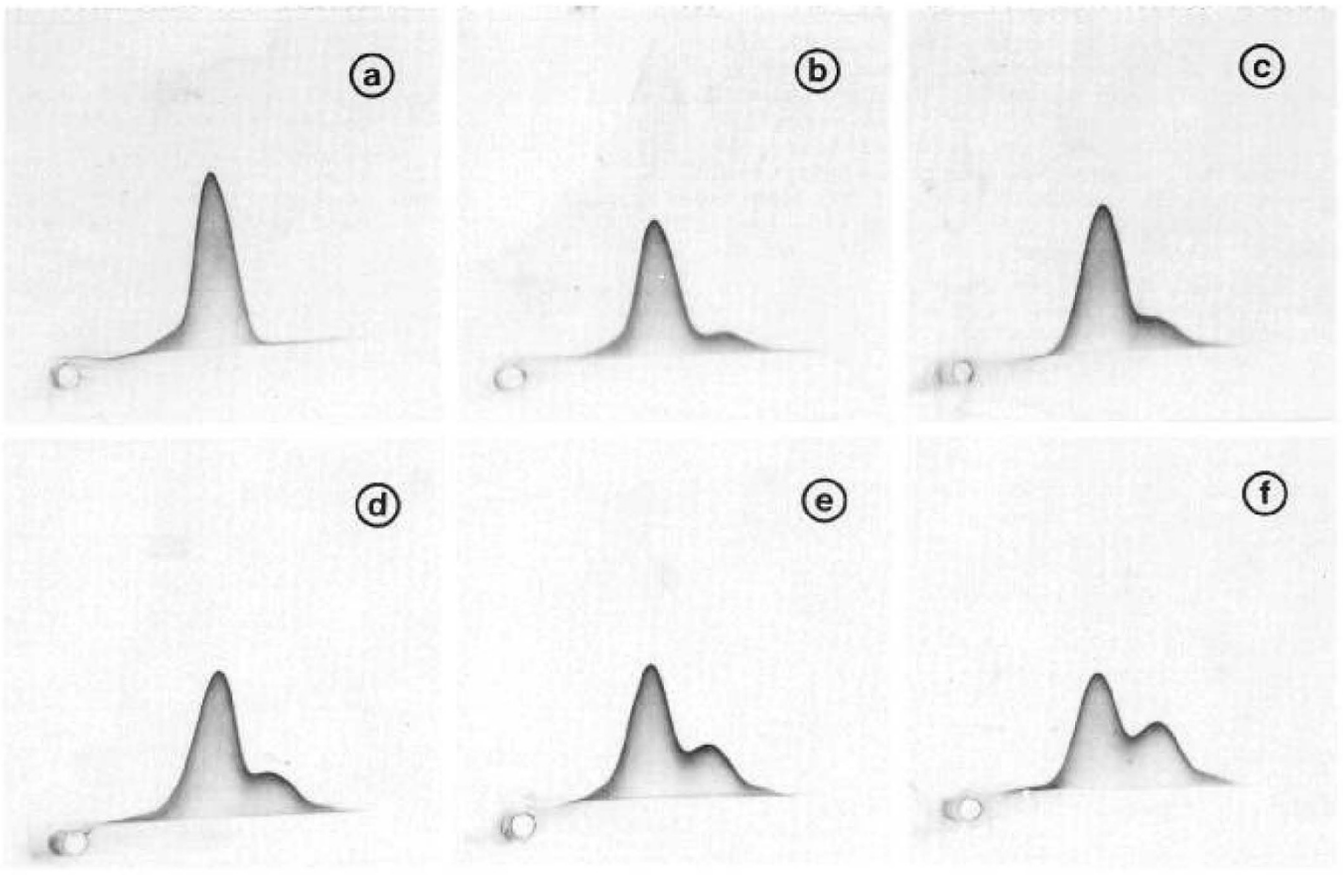


Fig. 7. Influence of temperature on the activity of C3 CoF (standard assay mixture). RNS is one of the recent specimens and not identical with the one in Figure 3. Experiment *a* is the control (NHS + PBS + EDTA), in the others PBS is replaced by RNS before incubation at various temperatures (*b* 0° C, *c* 10° C, *d* 20° C, *e* 30° C, and *f* 37° C).

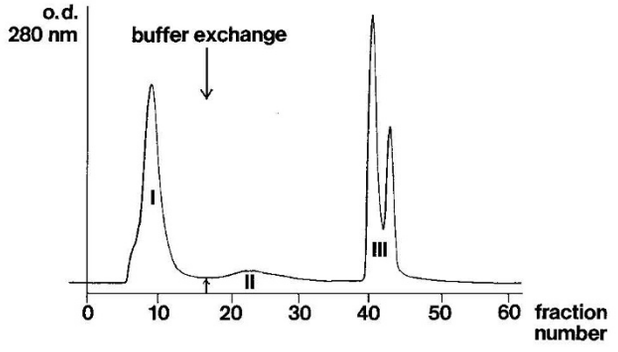


Fig. 8. Separation of C3 NeF and C3 CoF. The patient's euglobulin is dissolved and applied to a DEAE column (as described under "Materials and methods") equilibrated with 0.1 M phosphate buffer pH 7.2. After elution of the first major peak (mainly IgG) the starting buffer is replaced by a 0.1 M phosphate buffer containing 1 M sodium chloride (the buffer exchange is indicated by the *arrow*). All three peaks were pooled, concentrated by ultrafiltration, and assayed for IgG, C3 NeF, and C3 CoF. Peak I contained mainly IgG and C3 NeF activity but was devoid of C3 CoF. Peak II contained traces of IgG as well as C3 NeF but no C3 CoF. Peak III contained only traces of IgG but most of C3 CoF activity. Pool III also activated C3 in Mg-EGTA.

activated C3 in Mg-EGTA but not in EDTA. Peak II contained only little protein and no C3 activator. Peak III contained (after repeated ultrafiltration) 580 mg/dl of protein and only traces of IgG but activated C3 in the presence of 0.04 M EDTA. This strongly suggests that C3 CoF is no IgG.

To exclude that C3 CoF is a trypsin-like enzyme inhibition studies were performed using SBTI. No inhibition was noticed up to a concentration of 5 mg/ml in the final solution. In contrast, DFP produced dose-dependant inhibition of C3 CoF activity up to approximately 50% at 5 mM.

DISCUSSION

In an 11-yr-old girl with meningococcal septicemia hypercatabolism of C3 with undetectable C3 and extremely high levels of C3d persisted as long as 3 yr after complete recovery. This hypercatabolism was due to the presence of two different C3 activators with different biochemical properties.

The first activator was found to be heat stable, Mg^{2+} dependent, and did copurify with IgG. It markedly activated C3 in Mg-EGTA. The decay of the classical pathway convertase C4b,2a, in the presence of RN-IgG, was unaffected. Thus, this first activator did not raise our interest because it seemed to be a well known activator of C3, C3 nephritic factor C3 NeF.

As C3 CoF action is independent from divalent cations it must be different from all Mg^{2+}-dependent activators described. Theoretically, it could be a stabilized C3 convertase of either pathway. If C3 CoF was identical with NFc-stabilized C4b,2a one should expect at least some free circulating NFc in the patients IgG-fraction as described (1); but no such activity was found. In addition, we failed to eliminate C3 CoF by immunoprecipitation with anti-C4 or anti-C2. In contrast, it was completely eliminated by immunoprecipitation with anti-C3 and anti-B and partly eliminated with anti-IgG. Using an anti-IgG affinity column C3 CoF was completely absorbed out together with IgG and C3 NeF. The most plausible explanation for these results is the simultaneous occurrence of free C3 NeF with a C3 NeF-stabilized

AP C3 convertase (C3 CoF). If this is so, it would indicate that if the regular "negative" controls for C3 NeF assays are "slightly positive" C3 CoF may be present. Future studies will have to focus on the mechanism that allows the formation and persistence of C3 CoF.

Sensitivity of C3 CoF to inhibition with DFP further supports the concept that it is a serine protease despite the fact that in serum only 50% inhibition could be achieved while Medicus *et al.* (29) achieved 80% in a serum-free system. In serum DFP might be absorbed to other proteins. An interesting observation was the absence of hemolytic C1, C4, and C2 in RNS when we applied the complement technology generally used. This technology works on the assumption that lysis of EAC142 can be achieved by the addition of C-EDTA as a source of C3-C9. EDTA is said to prevent generation of C3 convertases of either pathway. Serial dilutions of RNS containing C3 CoF obviously activated guinea pig C3 making a C5-C9 reagent of C-EDTA which was unable to lyse EAC142 cells. Washing EAC142 intermediates prior to the addition of C-EDTA eliminated C3 CoF and preserved guinea pig C3 hemolytic activity allowing correct titration of C1, C4, and C2.

While C3 NeF induced activation of C3 and B without chelating agents and in Mg-EGTA, B remained uncleaved in EDTA. This indicates that C3 NeF requires newly formed C3b,Bb for its action while C3 CoF seems to act independently from either convertase. B in RNS was present in normal concentrations and, despite the presence of C3 NeF, had normal electrophoretic mobility. This can easily be explained as follows. Initiation of the alternative pathway of complement requires native C3 for formation of $C3(H_2O)$. This so-called C3b-like C3 reacts with B in the presence of Mg^{2+} and factor D to $C3(H_2O)$,Bb. B is activated during this step. The patient's serum was devoid of C3 so that initiation of the AP was impossible. However, if additional C3 was provided by either NHS or isolated C3 in the presence of Mg^{2+}, AP activation was facilitated. In EDTA, however, only C3 CoF acted upon C3 to form C3a and C3b while cleavage of B was prevented.

In summary, it seems that under conditions that we are unsure of C3 NeF binds to C3b,Bb to form a chronically circulating AP C3 concertase (C3 CoF). C3 CoF, once formed, acts independently from divalent cations. Its presence should be considered if "negative" controls for C3 NeF turn out to be positive, or if hemolytic titrations of C1, C4, and C2 are close to zero in the presence of normal amounts of antigens.

REFERENCES

1. Halbwachs LM, Leveille M, Lesavre P, Wattel S, Leibovitch J 1980 Nephritic factor of the classical pathway of complement. J Clin Invest 65:1249–1256
2. Thompson RA, Winterborn MH 1981 Hypocomplementemia due to a genetic deficiency of β1H globulin. Clin Exp Immunol 46:110–119
3. Alper CA, Abramson N, Johnston RB, Jandl JH, Rosen FS 1970 Increased susceptibility to infection associated with abnormalities of complement mediated functions of the third component of complement (C3). N Engl J Med 282:349–354
4. Carlo JR, Ruddy S, Studer EJ, Conrad DH 1979 Complement receptor binding to C3b-coated cells treated with C3b inactivator, β1H globulin and trypsin. J Immunol 123:523–528
5. Gaither TA, Hammer CH. Frank MM 1979 Studies of the molecular mechanism of C3b inactivation and a simplified assay of B1H and the C3b inactivator. J Immunol 123:1195–1204
6. Kazatchkine MD, Nydegger UE 1982 The human alternative complement pathway: Biology and immunopathology of activation and regulation. Prog Allergy 30:193–234
7. Theofilopoulos AN, Dixon FJ 1979 The biology and detection of immune complexes. Adv Immunol 28:89–221
8. Spitzer RE, Vallota EH, Forristal J, Sudora E, Stitzel A, Davis NC, West CD 1969 Serum C3 lytic system in patients with glomerulonephritis. Science 164:436–437
9. Sissons JGP, West RJ, Fallows J, Williams DG, Boucher BJ, Amos N, Peters DK 1976 The complement abnormalities of lipodystrophy. N Engl J Med 294:461–465
10. Thompson RA, Yap PL, Brettle RB, Dunmow RE, Chapel H 1983 Meningococcal meningitis associated with persistent hypocomplementemia due to circulating C3 nephritic factor. Clin Exp Immunol 52:153–156
11. Daha MR, van Es LA 1979 Further evidence for the antibody nature of C3 nephritic factor (C3 NeF). J Immunol 123:755–758
12. Scott DM, Amos N, Sissons JGP, Lachmann PJ, Peters DK 1978 The immunoglobulin nature of nephritic factor (NeF). Clin Exp Immunol 32:12–24
13. Daha MR, Fearon DT, Austen KF 1976 C3 nephritis factor (C3 NeF): stabilization of fluid phase and cell-bound alternative pathway convertase. J Immunol 116:1–7
14. Daha MR, Hazevoet HM, van Es LA 1983 Regulation of immune complex-mediated complement activation by autoantibodies (F-42) isolated from sera from patients with systemic lupus erythematosus. Clin Exp Immunol 53:541–546
15. Daha MR, van ES LA 1980 Relative resistance of the F-42-stabilized classical pathway C3 convertase to inactivation to inactivation by C4-binding protein. J Immunol 125:2051–2054
16. Gigli I, Sorvillo J, Halbwachs LM, Leibowitch J 1981 Mechanism of action of the C4 nephritic factor: degradation of the classical pathway C3 convertase. J Exp Med 154:1–12
17. Leibowitch J, Leveille M, Halbwachs L 1979 Description d'une activite enzymatique inhabituelle clivant le 3e composant du complement. C R Acad Sci Paris 288:567–569
18. Alper CA, Block KJ, Rosen FS 1973 Increased susceptibility to infection in a patient with type II essential hypocomplementemia. N Engl J Med 288:601–606
19. Bartlow BG, Roberts JL, Lewis EJ 1979 Nonimmunoglobulin C3 activating factor in membranoproliferative glomerulonephritis. Kidney Int 15:294–302
20. Mayer MM 1961 Complement and complement fixation. In: Kabat F, Mayer MM (eds) Experimental Immunochemistry 2nd ed. Charles C Thomas, Springfield, IL, pp 133–240
21. Wahn V 1982 Intravenöse Gammaglobulintherapie—Messung von zirkulierenden Immunkomplexen und Komplementfaktoren. Thieme, Stuttgart
22. Rapp HJ, Borsos T 1970 Molecular Basis of Complement Action. Appleton Centurty Crofts, New York
23. Platts-Mills TAE, Ishizaka K 1974 Activation of the alternative pathway of human complement by rabbit cells. J Immunol 113:348–358
24. Mancini JA, Carbonara O, Heremans JF 1965 Immunochemical quantitation of antigen by single radial immunodiffusion. Immunochemistry 2:235–242
25. Weeke B 1973 Crossed immunoelectrophoresis. In: Axelsson NH, Kroll J, Weeke B (eds) A Manual of Quantitative Immunoelectrophoresis. Scand J Immunol [Suppl] 1:47–56
26. Day NK, Schreiber RD, Götze O, Müller-Eberhard HJ 1976 Reversible activation of proactivator (factor B) of the alternative pathway without cleavage of the molecule. Scand J Immunol 5:715–720
27. Johnstone A, Thorpe R 1982 Immunochemistry in Practice. Blackwell Scientific Publications, Oxford, pp 44–46
28. Patrick CC. Virella G 1978 Isolation of normal human IgG3. Identical molecular weight for normal and monoclonal gamma-3 chains. Immunochemistry 15:137–139
29. Medicus RG, Götze O, Müller-Eberhard HJ 1976 The serine protease nature of the C3 and C5 convertases of the classical and alternative complement pathways. Scand J Immunol 5:1049