Complement Breakdown Products in Plasma from Patients with Systemic Lupus Erythematosus and Patients with Membranoproliferative or Other Glomerulonephritis

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ABSTRACT A dynamic estimation of the involvement of the complement system in various diseases was obtained by the direct quantitation of breakdown products of C3 and of properdin factor B. The methods used were based, first, on the separation of native and fragmented molecules according to their molecular size through a precipitation with polyethylene glycol and, secondly, on an immunochemical quantitation, using specific antisera for the major antigens of C3 and factor B. The sensitivity and the specificity of these methods were demonstrated by activation of complement in vitro with generation of C3 and factor B fragments.

A clinical investigation was carried out in 41 patients with systemic lupus erythematosus (SLE), 31 with membranoproliferative glomerulonephritis (MPGN), 26 with other types of glomerulonephritis, and 6 with severe alcoholic cirrhosis of the liver. The following observations were made: (*a*) an elevated plasma level of C3d fragment of C3 was found in 68% of SLE patients, in 87% of MPGN patients, in 62% of patients with other hypocomplementemic nephritis, and in 15% of those with normocomplementemic nephritis, but in only 33% of patients with liver cirrhosis and very low levels of C3; (*b*) a significant difference was observed between the levels of C3 obtained with either anti-"native" C3 or anti-C3c sera for immunochemical quantitation, in patients with SLE or MPGN, indicating the presence of "altered" or fragmented C3 in plasma; (*c*) an elevated plasma level of Ba fragment of properdin factor B was found in 46% of SLE patients, in 67% of MPGN patients, in 50% of patients with other hypocomplementemic nephritis, and in 9% of patients with normocomplementemic nephritis, while the level of properdin factor B was only slightly decreased in these diseases; (*d*) in SLE and MPGN there was an inverse correlation between the levels of C3d and Ba and the level of C3 in plasma. The level of these fragments was directly correlated with the clinical manifestations of SLE; (*e*) some patients with a normal C3 level exhibited an elevated plasma concentration of C3 and factor B fragments, suggesting the coexistence of an increased synthesis with a hypercatabolism of complement components.

Therefore, the quantitation of complement breakdown products by simple immunochemical methods provides additional information concerning the involvement of complement in disease and new features for the evaluation of the intensity of immune reactions during immune complex diseases.

INTRODUCTION

It is now well documented that an alteration in the concentration of complement components occurs in a variety of diseases. Increased levels of complement components and hemolytic activity are frequently found in inflammatory disorders, and striking decreases have been reported in diseases such as systemic lupus erythmatosus (SLE)[1] acute glomerulonephritis, membranoproliferative glomerulonephritis (MPGN), liver disease, and congenital deficiency of complement components (1–3).

Physiological complement activation may proceed through at least two major pathways, both of which lead to the activation of C3 (4). In this respect, C3 is

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[1] *Abbreviations used in this paper:* agg HGG, aggregated human IgG; MPGN, membranoproliferative glomerulonephritis; PEG, polyethylene glycol; SLE, systemic lupus erythematosus.

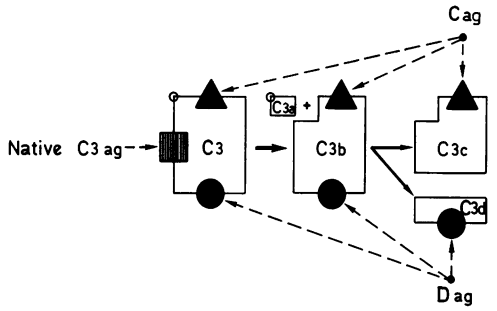

FIGURE 1 Antigenic changes during cleavage of C3 by C3 convertase and cleavage of C3b by C3b inactivator.

a central component of the complement system.[2] Its activation results in the fragmentation of the native molecule of C3 (mol wt 200,000) into C3b (mol wt 190,000) and C3a (mol wt 10,000) (5, 6). C3b is involved in the activation of the late-acting components of the complement system, and the small fragment contributes to anaphylatoxic and chemotactic activity. Further degradation of C3b is generated by C3b inactivator, which cleaves C3b into C3c (mol wt 150,000) and C3d (mol wt 35,000). In the classical complement pathway, activation of C1 by immune complexes leads to the generation of $C\overline{4,2}$ (C3 convertase), which initiates activation of C3 (7). The properdin system is now established as an alternative pathway to C3 activation that can be triggered by nonimmune reactants, such as inulin or bacterial polysaccharides, or by a particular type of immune complex involving IgA antibodies. In presence of C3, activated properdin generates an active enzyme, $\bar{D}$, which acts on properdin factor B. This process leads to a cleavage of C3 and to the activation of the late complement components (C5–C9) (8, 9). Activation of properdin factor B generates two fragments: a large one, Bb (mol wt 75,000) of γ mobility, and a small one, Ba (mol wt 30,000) of α mobility (8, 10). It should be stressed that activation of C3 either through the classical or through the alternative pathway leads to the generation of C3b, which can trigger an amplification system involving components of the alternative pathway: C3b activates factor D and factor B and this leads to further activation of C3 (11).

The involvement of the complement system in human diseases is usually estimated by measuring the plasma level of complement components by hemolytic or immunochemical methods. Unfortunately, a drawback of this approach is the lack of dynamic information provided. Only a static profile of the complement system is obtained and an increased synthetic rate can mask an increased catabolism of complement components.

Turnover studies performed in vivo with radiolabeled complement components allow for a better estimation of involvement of the complement system. Such studies were carried out, especially for C3 in different types of nephritis, with hypocomplementemia; however, controversial results were frequently obtained (12–14). There is some limitation to the use of turnover studies, because degradation products may persist in blood and interfere with evaluation of the catabolism of the native protein (14). Moreover, it is not easy to apply them to the investigation of local activations of the complement system.

An alternative approach to objectivate involvement of the complement system could be provided by methods allowing the detection of breakdown products of complement components. These methods are usually based on the changes of physicochemical properties and of antigenic constitution of complement components occurring during the activation of the complement system. In particular, it is known that the intact C3 molecule bears at least four major antigenic determinants (Fig. 1): one is related to the native configuration of C3 ("native antigen"); A antigen is present on native C3 and on the small a fragment; C antigen is present on native C3, C3b, and C3c fragments; and finally, D antigen is present on native C3, C3b, and C3d fragments. Specific antisera can be raised against each of these antigenic determinants. Similarly, properdin factor B bears two major antigenic determinants (Ba and Bb), which are segregated after cleavage of the native molecule and found either on Ba or Bb fragments. Circulating breakdown products of C3 (15–17) and properdin factor B (18) were demonstrated by immunoelectrophoresis in various types of nephritis and in synovial joint fluids of patients suffering from rheumatoid arthritis. Semiquantitative results were also reported for the presence of C3b and C3c, with the antigen-antibody crossed electrophoresis, in joint fluid of patients with rheumatoid arthritis (19), and in patients suffering from chronic active hepatitis and primary biliary cirrhosis (20).

In the present investigation, a quantitative study of C3 and properdin factor B breakdown products was performed on plasma samples from patients with diseases possibly associated with an involvement of the complement system. This study was carried out with new methods devised for the quantitation of the C3d fragment of C3 and for the Ba fragment of properdin factor B.

METHODS

Patient population. Plasma samples from 41 patients with SLE were collected. 33 SLE patients had a low C3

[2] The provisional nomenclature proposed by the Complement Nomenclature Committee of IUIS for components of the alternative pathway of the complement system has been used in this paper: properdin factors B = C3PA = GBG; Bb fragment = C3A = GGG; Ba fragment = α fragment of C3PA = GAG.

level (−2 SD), as measured by antiserum directed against native C3, and 8 had a normal C3 level. All the patients suffering from SLE with low C3 level had nephritis, typical multisystem inflammatory disease, and positive anti-DNA antibodies, as measured by radioimmunoassay (21). The clinical course and the activity of the disease in the group with normal C3 were less severe. Serial bleedings were performed in six patients.

Plasma samples from 31 children, adolescents, and young adults with MPGN were investigated. Diagnosis was based on histological data on kidney biopsy by light microscopy and immunofluorescence, clinical evolution, and biological results. At the time of our study, six patients had a normal C3 level. None of the patients had anti-DNA antibodies. Plasma samples from 26 patients with other types of glomerulonephritis were collected and studied. The diagnosis was based for most of them on renal biopsies. This group included 18 patients with chronic glomerulonephritis (3 focal, 6 membranous, 4 proliferative, 2 minimal change, and 3 unknown), 2 patients with vasculitis, 2 patients with poststreptococcal glomerulonephritis, 2 patients with anaphylactoid purpura, and one 40-yr-old woman with acute glomerulonephritis that appeared 1 mo after excision of a breast carcinoma. 8 patients had a low level of native C3 and 18 had a normal C3 level. Six patients with alcoholic cirrhosis of the liver, histologically proven, were selected for their low C3 level.

Plasma samples from 25 healthy voluntary blood donors were taken to establish the normal values. All samples were drawn on EDTA (3.5 mg/ml). Centrifugation was carried out at room temperature. The samples were used fresh or after storage at −70°C. For some experiments, suramin (Moranyl; Specia, Paris, France) was added at a final concentration of 1 mg/ml to EDTA plasma samples immediately after separation of plasma.

Complement components. C3 was prepared from the euglobulin fraction of human serum by DEAE cellulose and hydroxypatite chromatography (22). C3c and C3d fragments were obtained after digestion of C3 with Sepharose-bound trypsin (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) (23, 6). After trypsin digestion, the C3 preparation was gel filtrated on Sephadex G200 in 0.3-M NaCl, 0.01-M phosphate buffer, pH 8, in order to separate C3d and C3c fragments. Properdin factor B was isolated by affinity chromatography and DEAE cellulose chromatography (24). Ba fragment of factor B was prepared by digestion of 10 mg of purified factor B on ENZYTE agarose trypsin column (Miles Laboratories, Slough, England) at room temperature. The digest was further separated by gel filtration on Sephadex G200. Fractions of semipurified Bb fragment of factor B were obtained by incubation of 20 cm^3 of normal human serum with 1 cm^3 of inulin 40 mg/ml for 60 min at 37°C. After centrifugation, the supernate was subjected to DEAE cellulose chromatography in 0.2 M phosphate buffer, pH 8. The eluted peak contained IgG and Bb fragment. Further purification of the Bb fragment was done by gel filtration on Sephadex G200. The amount of protein in purified C3, factor B, C3c, C3d, and Ba fractions was determined by the Folin method.

Antisera. All antisera were raised in rabbits. Antisera against native C3 were obtained from two out of four rabbits immunized twice with 0.2 mg of purified C3 in incomplete Freund's adjuvant, with an interval of 3 wk between injections. Samples of these antisera were absorbed with human serum that had been kept for 10 days at 37°C and therefore did not contain any more native C3 antigen. Antiserum against C antigen was obtained after immunization with C3c fragment and did not need any absorption. Antisera against D antigen was obtained by two different methods with similar results. In the first method, 2 mg of native C3 were incubated with normal rabbit serum for 5 days at 37°C to generate C3d fragment, and the mixture was subjected to G200 chromatography. Rabbits were immunized with C3d-containing fractions in complete Freund's adjuvant 2-wk intervals until they developed antibodies. In the second method, rabbits were immunized with C3d fragments obtained by trypsin digestion of native C3 and G200 chromatography. Both antisera were absorbed with a normal human serum that was first incubated for 10 days at 37°C and then heated at 60°C for 60 min. This absorbant reagent still contained C antigen on C3c but no more D antigen, since C3d is heat-labile. All the antisera were checked for specificity in immunoelectrophoretic analysis and by the Ouchterlony test, with fresh, aged, and heated human serum. Each of the samples contained antibodies against one antigenic determinant of C3. They cross-reacted with similar antisera kindly provided by Miss van der Giessen (Central Laboratory of the Amsterdam Blood Center).

Antiserum against factor B was raised by immunization with 0.2 mg of purified factor B in incomplete Freund's adjuvant, three times, at 2-wk intervals. This antiserum reacted with native factor B and with the fragments Bb and Ba. Antibodies specific for Ba antigen were obtained in the following way: pure factor B was left for 1 wk at room temperature and then was used for rabbit immunization in the usual way. Antisera were absorbed with fractions enriched with Bb fragments and were shown to react with native factor B and with Ba fragment but not with Bb fragment. Antisera specific for Bb fragments were obtained by immunization with partially purified Bb fragments, and further absorption with human IgG bound to activated Sepharose was carried out.

In vitro activation of complement. In vitro activation of the complement system was performed by adding either heat-aggregated human immunoglobulins (agg HGG) at various concentrations, or inulin (20 mg/ml) to normal human serum. 1 ml of agg HGG or a ml of inulin (20 mg/ml) was added to 9 ml of normal human serum. The mixture was shaken twice during the incubation (1 h at 37°C) and then centrifuged for 5 min at 2,000 *g*. aggHGG were prepared by incubation of human IgG (Swiss Red Cross, Bern) at 62°C for 20 min.

Quantitation of C3 and properdin factor B. Serum or plasma levels of C3 were measured by radial immunodiffusion with either anti-native C3 or anti-C3c antisera. Properdin factor B was quantitated similarly with either anti-Bb or anti-Ba antisera (18). Standard reference curves were obtained with various concentrations of purified C3 and factor B or serial dilutions of a calibrated plasma pool. The standard error for the quantitation of these components was 2.9% for C3 and 3.4% for factor B.

Hemolytic titrations of C3 were carried out with EAC14 cell intermediates and purified C2, C5, C6, C7, C8, and C9 complement components, obtained from Cordis Laboratories (Miami, Fla.).

Solubility studies of C3, properdin factor B, and their fragments in polyethylene glycol (PEG). The solubility of native C3, factor B, and their fragments in the presence of various concentrations of PEG was studied. PEG, with an average molecular weight of 6,000 (DAB 7—Siegfried, Zofingen, Switzerland) was dissolved at various concentrations between 2 and 40% (wt/vol) in 0.01 M EDTA and 0.1 M borate buffer, pH 8.3. 1 vol of plasma or serum activated by agg HGG or inulin was mixed with 1 vol of

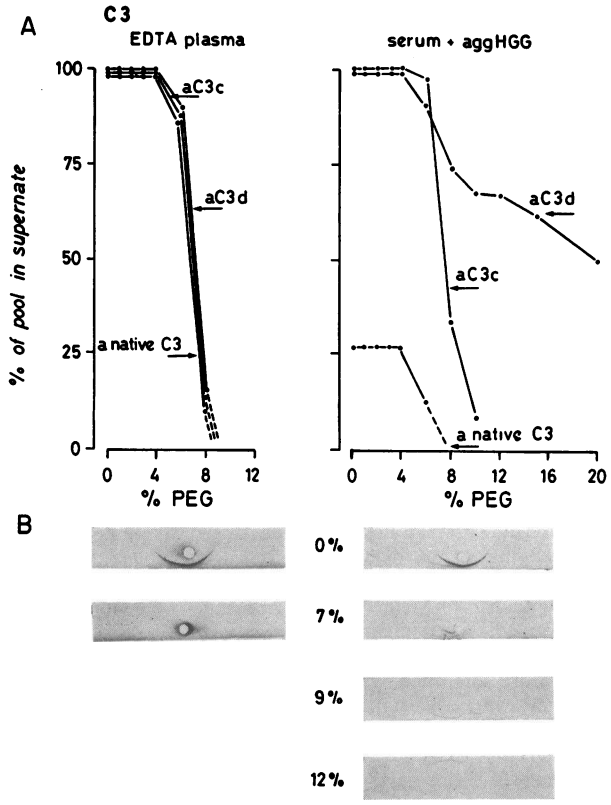

FIGURE 2 (A) Concentration of C3 and its breakdown products measured with antibodies specific for native, C, and D, antigens of C3 in supernates obtained after precipitation (at various concentrations of PEG) of EDTA plasma and serum incubated with agg HGG. (B) Immunoelectrophoretic analysis of these supernates with an antiserum reacting with native, C, and D antigens of C3: only C3d fragment was detected in 12% PEG supernate. Anode is on the left.

PEG at various concentrations. The mixture was left at 4°C for 3 h, then centrifuged at 1,200 *g* for 30 min. The supernates were collected and their antigenic content was analyzed by immunoelectrophoresis and by radial immunodiffusion with 1.5% agar in diethylbarbiturate acetate buffer, ($\mu = 0.1$), pH 8.2, 0.005 M EDTA, and specific antisera against native C3, C3c, C3d, Ba, and Bb.

For C3, similar solubility curves were obtained with all three antisera when EDTA plasma was mixed with various concentrations of PEG (Fig. 2). C3 antigens were no longer detected in the supernates at PEG concentrations higher than 9%. The precipitation pattern observed with serum activated by agg HGG differed considerably from that observed with EDTA plasma. First, a similar solubility curve was obtained with anti-native C3 but the level of this antigen was very low compared to EDTA plasma. Secondly, there was a slight increase in the solubility of C3 antigen when it was measured with anti-C3c antiserum. Thirdly, when C3 or C3 breakdown products bearing the D antigen were studied, the PEG solubility curve obtained suggests the existence of two types of molecule: one with a poor solubility in PEG and another one quite soluble even at high concentrations of PEG.

The nature of the C3 antigens present in the supernate, after precipitation of activated serum at various concentrations of PEG, was further defined by immunoelectrophoretic analysis. It was found that 8% PEG supernate still contained C3c and C3d while 11% PEG supernate contained only C3d.

The same methodology was applied to the study of properdin factor B (Fig. 3). Similar solubility curves were obtained with both anti-Ba and anti-Bb antisera when EDTA plasma was treated with various concentrations of PEG. Factor B antigen was no longer detected in the supernate at PEG concentrations higher than 12% (Fig. 3). When serum previously incubated with inulin was studied, Bb and Ba antigens could be separated in PEG: Bb antigens were largely precipitated at 16% PEG, while Ba antigens were still soluble at higher concentrations. Immunoelectrophoretic analysis confirmed the full precipitation of native properdin factor B at 12% PEG, the increased solubility of Bb fragments, and the persistence of soluble Ba fragments at concentrations of PEG higher than 14%.

Quantitation of C3d and Ba fragments. For quantitation of C3d fragments, 0.2 ml of plasma or serum samples were mixed with 0.2 ml of PEG (final concentration, 11%). The mixture was left at 4°C for 3 h and then centrifuged (1,200 *g*, 30 min) to precipitate native C3 and C3b. With a specific antiserum against D antigen, the concentration of C3d was measured in the supernate by radial immunodiffusion. In each plate the standard reference curve was obtained with

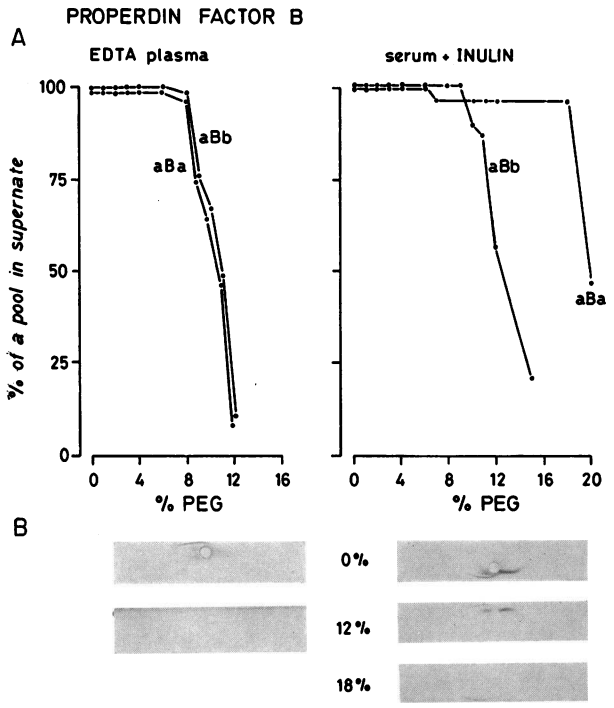

FIGURE 3 (A) Concentration of properdin factor B and its breakdown products measured with anti-Bb and anti-Ba antisera in supernates obtained after precipitation at various concentrations of PEG or EDTA plasma and of serum incubated within inulin. (B) Immunoelectrophoretic analysis of these supernates with an antiserum reacting with both Ba and Bb antigens: only Ba fragment was detected in 18% PEG supernate. Anode is on the left.

various concentrations of purified C3d or of a calibrated serum pool previously activated with agg HGG (5 mg/ml; 1 h at 37°C) and treated similarly with PEG.

For quantitation of Ba fragments of properdin factor B, a similar procedure was used but PEG was added at a final concentration of 18%. The concentration of Ba fragments in the supernate was measured with a specific antiserum against Ba antigen. The standard reference curve was obtained with various concentrations of purified Ba fragments or of a calibrated serum pool previously activated with inulin (2 mg/ml; 1 h at 37°C) and treated with PEG (final concentration 18%). To increase the sensitivity of the radial immunodiffusion method for the detection of Ba fragments, the following procedure was used. Proteins were allowed to migrate for 2 days; then the agar plates were washed with 0.15 M NaCl, 0.01% NaN_3 for 2 days before incubation with sheep anti-rabbit IgG diluted 1/20 in 0.15 M NaCl. The plates were washed again, dried, and stained with amidoschwartz.

Statistical evaluation. Statistical evaluation was carried out according to Student's *t* test and by regression analysis by the method of least squares.

RESULTS

In vitro generation of C3 and factor B breakdown products during complement activation. The significance of the immunochemical quantitation of C3 and properdin factor B fragments in serum was evaluated through a kinetic experiment of complement activation in vitro. 18 ml of normal human serum were incubated with 2 ml of agg HGG (40 mg/ml). At various times, 0.2-ml samples were taken and immediately processed for the quantitation of C3d and Ba fragments. Simultaneously, 0.4 ml samples were collected on 0.05 ml 0.1 M EDTA, pH 8, for immunochemical quantitation of C3 and properdin factor B and for hemolytic titration of C3. The following changes were observed (Fig. 4): there was a decrease in the concentration of C3 measured with anti-native C3 parallel to a decrease of C3 hemolytic activity; there was also a simultaneous appearance of increasing amounts of C3d. The level of C3 as measured with an anti-C3c antiserum decreased by only 15% during the course of this activation. Similarly, there was little modification of the concentration of properdin factor B, as measured with an anti-Bb antiserum, while the amount of Ba fragment increased rapidly during the activation process.

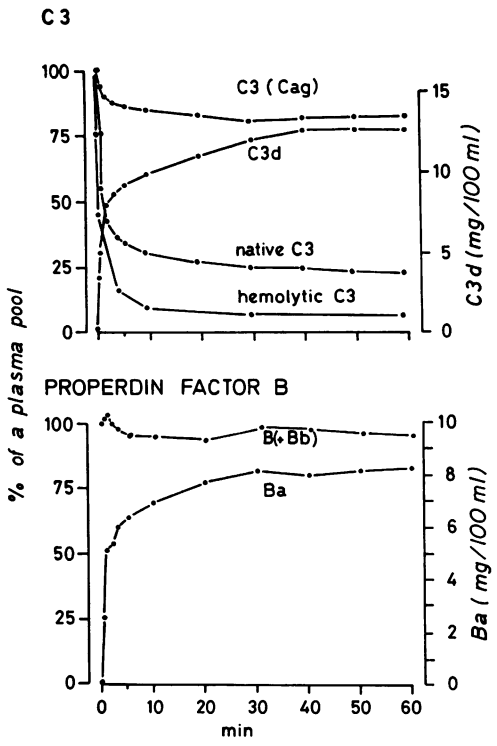

FIGURE 4 Quantitation of hemolytic C3, native C3, C3 (anti-C3c), C3d, properdin factor B (+Bb), and Ba at various times after incubation of normal human serum with agg HGG.

The possible generation of breakdown products during the manipulations preceding the analysis of samples was estimated by comparing various conditions of storage or incubation. C3d and Ba fragments were hardly detectable in EDTA plasma treated with agg HGG (either at 1 or 5 mg/ml) and left for 1 h at 4 or 25°C. The concentration of C3d was still lower than 0.4 mg/100 ml after 24 h but rose to 5.1 mg/100 ml (at 25°C) and to 1.5 mg/100 ml (at 4°C) after 72 h. The concentration of Ba fragments remained lower than 0.7 mg/100 ml after 72 h. There was no significant amount of C3d or Ba generated by repeated freezing and thawing of EDTA plasma.

C3 plasma concentration measured with anti-native C3 and with anti-C3c antisera in patients with SLE and MPGN. The concentration of C3 was measured in plasma samples with antisera directed against either native or C antigens of C3 to evaluate the proportion of circulating C3 that had undergone configuration changes leading to the loss of native antigen. In 25 plasma samples from healthy blood donors, the concentrations of C3 obtained were 135±20 and 137±21 mg/100 ml with anti-native C3 and with anti-C3c antisera, respectively.

In SLE (41 samples) the mean concentrations of C3 were 65±34 and 75±32 mg/100 ml with anti-native C3 and with anti-C3c antisera, respectively. As shown in Fig. 5, for individual samples a higher difference between these two concentrations was observed when the level of C3 was significantly decreased. In some samples, the C3 level was found to be within the normal range with anti-C3c antiserum, while C3 level was clearly reduced when measured with anti-native C3.

In MPGN, the mean concentrations of C3 were 51±48 and 58±46 mg/100 ml with anti-native and anti-C3c

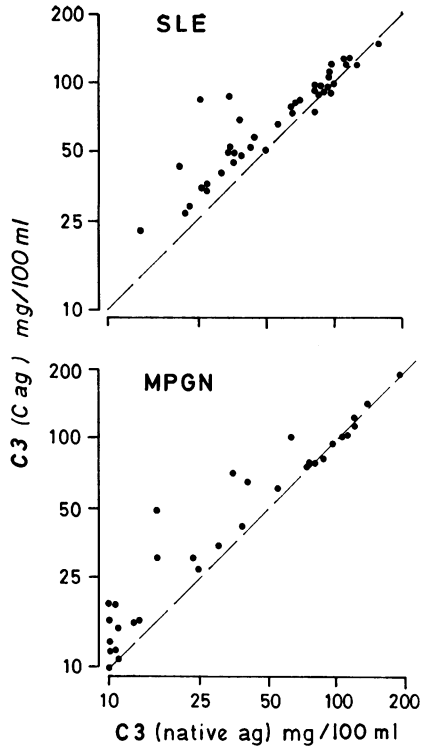

FIGURE 5 Correlation between the levels of C3 measured either with anti-native C3 or with anti-C3c antisera in patients suffering from SLE or MPGN.

antisera, respectively. In patients with C3 within the normal range, no significant difference was observed between the level of C3 measured with both antisera; however, striking differences were observed in samples containing less than 70 mg/100 ml of C3 (Fig. 5). For example, one patient exhibited a C3 concentration

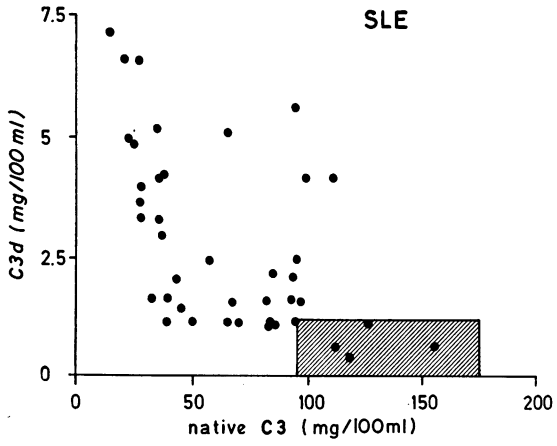

FIGURE 6 Relationship between concentrations of C3d and of native C3 in patients suffering from SLE. Shadowed area indicates the mean ±2 SD range for native C3 and the upper limit for the concentration of C3d measured in plasma from healthy blood donors.

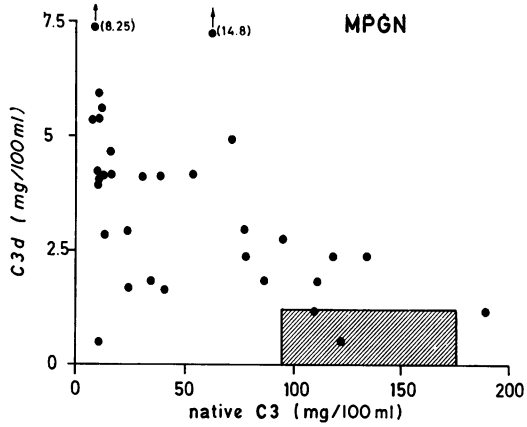

FIGURE 7 Relationship between the concentrations of C3d and of native C3 in patients suffering from MPGN. Shadowed area indicates the mean ±2 SD range for native C3 and the upper limit of C3d concentration measured in healthy blood donors.

of 49 mg/100 ml with anti-C3c antibodies but only 16 mg/100 ml when measured with an anti-native C3 antiserum.

C3d fragments in SLE, MPGN, and other glomerulonephritis. The immunochemical quantitation of C3d was first performed in plasma samples from 25 healthy blood donors. C3d was undetectable in 13 samples, while values lower than 0.7 mg/100 ml were found in 9 samples and lower than 1.2 mg/100 ml in 3 samples.

In SLE patients (41 samples), the mean level of C3d was 2.8±1.9 mg/100 ml. An increased level of C3d (higher than 1.2 mg/100 ml) was observed in 68% of the samples (Fig. 6). There was a significant correlation between the C3d level and the decrease of native C3 concentration ($r = 0.55$, $P < 0.01$). In four patients, relatively high concentration of C3d were found in, plasma, while the level of native C3 was not dramatically decreased. On the contrary, relatively low levels of C3d were observed in five patients in the presence of a native C3 level lower than 20 mg/100 ml. Seven samples with very low values of native C3 had C3d levels higher than 5 mg/100 ml, which represented 8–52% of the concentration of native C3 present in these samples. In these patients, the activity of the disease was particularly marked; all of them presented multi-systemic inflammatory syndrome, lupus nephritis, and high titer of anti-DNA antibodies.

In patients with MPGN (32 samples) the mean level of C3d was 3.7±2.6 mg/100 ml. An increased level of C3d was observed in 87% of the samples (Fig. 7). There was a significant correlation between the C3d level and the decrease of native C3 concentration ($r = 0.35$, $P < 0.05$). In some patients, relatively high concentrations of C3d were found in plasma while the level of native C3 was not dramatically decreased, and in four pa-

TABLE I

Mean Level of C3, Properdin, Factor B, and their Breakdown Products in Patients Suffering from SLE, MPGN, Other Nephritis, Cirrhosis, and in Normal Blood Donors

	No. of samples	C3		C3d	C3d/ native C3	factor B	Ba	Ba/Bb
		a-native C3	a-C3c					
		mg/100 ml	*mg/100 ml*	*mg/100 ml*		*mg/100 ml*	*mg/100 ml*	
SLE	41	65±34	75±32	2.8±1.9	0.074	23.2±6.3	0.55±0.30	0.024
MPGN	32	51±47	58±46	3.7±2.6	0.092	20.2±5.5	0.76±0.40	0.038
Other GN								
N. C3	18	159±30	160±31	<1.0	<0.010	30.9±7.5	<0.30	<0.010
low C3	8	72→16	83±18	2.9±0.9	0.042	22.7±5.1	0.77±0.80	0.028
Cirrhosis	6	50±17	67±22	1.7±0.9	0.026	16.5±5.4	0.35±0.30	0.020
Blood donors	25	135±20	137±21	<1.0	<0.010	30.5±6.0	<0.30	<0.010

tients relatively low levels of C3d were observed in the presence of a native C3 level lower than 40 mg/100 ml. In 13 patients with a concentration of native C3 lower than 20 mg/100 ml, the concentration of C3d represents an average of 40% of the concentration of native C3.

In patients with other types of glomerulonephritis, two groups were considered. In the group with low C3 level (eight samples), the results obtained were quite similar to those observed in patients with SLE (Table I): five out of eight patients had a C3d concentration higher than 1.2 mg/100 ml. Patients with normal C3 levels (18 samples) exhibited a slight increase of C3d levels with concentrations higher than 2.5 mg/100 ml in three of them. The first patient was a young boy in the remission phase of acute post-streptococcal glomerulonephritis; the second a woman who developed an acute glomerulonephritis after the excision of a breast carcinoma, and the third a patient with chronic glomerulonephritis.

To compare diseases in which a complement activation process may be involved with other diseases in which a low synthesis rate of C3 may be predominant, six cases of severe alcoholic cirrhosis, selected for their low C3 level, were studied. In these patients, the plasma level of C3d was 0.8, 1.2, 1.2, 1.1, 1.7, and 3.3 mg/100 ml. These values represented 2–5% of the level of native C3.

The possibility that C3d detected in pathological samples would be generated in vitro from any C3b fragments during the manipulations was investigated. Suramin was added to eight plasma samples (1 mg/ml) before the processing of the samples, to block the activity of C3b inactivator. No significant difference was found in C3d levels measured in the presence or in the absence of suramin.

Factor B and Ba fragments in SLE, MPGN, and other glomerulonephritis. The immunochemical quantitation of properdin factor B and its Ba fragment was first performed in 25 samples from healthy blood donors. Values of Ba fragments lower than 0.5 mg/100 ml were found in these samples, which represented less than 1.5% of the amount of factor B present in these samples. The mean levels of factor B and Ba fragments in patients suffering from SLE, MPGN, other glomerulonephritis, and cirrhosis are presented in Table I. The mean level of factor B was in the normal range for patients with normocomplementemic glomerulonephritis, but was decreased in all the other groups of patients.

Individual concentrations of factor B and Ba fragments in patients suffering from SLE and MPGN are reported in Fig. 8. Generally the individual values of factor B did not differ strikingly from the normal range, but the concentration of Ba fragments was clearly elevated (more than 0.5 mg/100 ml) in 46% of SLE pa-

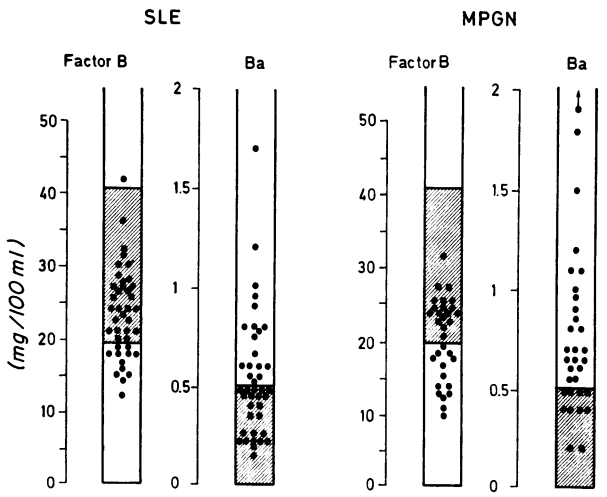

FIGURE 8 Plasma levels of properdin factor B and of Ba fragments in patients suffering from SLE and MPGN. Shadowed areas indicate the mean ±2 SD range for properdin factor B and the upper limit for the concentration of Ba fragments measured in plasma from healthy blood donors.

TABLE II

Correlative Study in Individual Patients Suffering from SLE and MPGN with Either Low or Normal C3

Patients	C3		C3d	C3d/ native C3	Factor B	Ba	Ba/B
	a-native C3	a-C3c					
	mg/100 ml	*mg/100 ml*	*mg/100 ml*		*mg/100 ml*	*mg/100 ml*	
MPGN (low C3)							
E. P.	13.5	16	8.25	0.61	24	0.6	0.03
J. B.	11	11	5.6	0.5	22.5	1.1	0.05
M. J.	22	30	2.9	0.13	12.6	0.7	0.05
B. M.	34	70	1.9	0.04	23.4	0.6	0.03
MPGN (normal C3)							
O. C.	119	122	2.4	0.02	16.5	0.5	0.03
B. D.	122	116	0.5	0.004	24	0.2	0.008
D. M.	110	105	1.2	0.01	24	0.4	0.02
B. M.	111	106	1.8	0.016	27	0.7	0.03
SLE (low C3)							
C. V.	27	35	3.3	0.12	15.6	0.6	0.04
A. M.	37	49	3	0.08	22.2	0.9	0.04
C. V.	65	76	5.1	0.08	31.5	0.7	0.02
D. E.	22	27	5	0.23	18	0.4	0.02
SLE (normal C3)							
L. A.	97	97	1.7	0.02	21	0.3	0.01
P. V.	95	96	1.7	0.02	42	0.3	0.005
B. M.	119	125	0.4	0.003	21	0.3	0.01
B. R.	112	122	0.6	0.005	25	0.6	0.02
Control (mean)	135±20	137±21	<1	<0.01	30.5±6	<0.3	<0.01

tients and 67% of patients with MPGN. An increase of Ba fragments was also found in 4 out of 8 patients with hypocomplementemic glomerulonephritis, in 2 out of 18 patients with normocomplementemic glomerulonephritis, and in 1 patient with liver cirrhosis. The concentration of Ba fragments represented between 2 and 7% of the concentration of factor B in patients with high levels of Ba fragments, and in one particular case of MPGN, a value of 11% was calculated.

In patients with SLE, MPGN, and hypocomplementemic glomerulonephritis, the correlation between factor B and Ba fragment is not significant. However, there was a significant correlation between the level of Ba fragment and the level of native C3 ($r = 0.38$, $P < 0.05$).

Correlative study of C3, C3d, factor B, and Ba fragments during the course of SLE and MPGN. Individual samples were compared for their content of C3d and Ba fragments as well as for C3 measured with anti-native C3 and with anti-C3c antisera. A significant correlation was found between the levels of C3d and Ba ($r = 0.56$, $P < 0.01$). The correlation between these parameters is also illustrated in Table II, in which all data obtained for some individual patients are presented.

Serial bleeding was performed in five patients; it was found that the level of C3d and Ba fragments increases while the level of native C3 and factor B decreases during acute attacks of SLE, and that clinical improvement was characterized by a normalization of native C3 and factor B and a fall in C3d and Ba fragment levels. The results obtained in two patients are reported in Figs. 9 and 10, The first patient was a woman of 42 yr with a first manifestation of SLE characterized by a butterfly facial rash. On the first admission 2 mo later, she had fever and complained of arthritis. She had positive LE cell preparation, high titer of anti-DNA antibodies, low native C3, slightly decreased properdin factor B, and high levels of C3d and Ba fragments. Urine analysis showed an increased albuminuria and microscopic hematuria and casts. With rest and therapy, which consisted of corticosteroids and azathioprine, biological results improved. This was followed, 1 mo later, by clinical improvement. The titer of anti-DNA antibodies decreased progressively. The level of native C3 and properdin factor B went back to the normal range and C3d and Ba fragment levels decreased rapidly.

The second patient was a young girl of 22 yr, suffering from SLE since 1964, with recurrent attacks of

nephritis. During the time of observation, the patient presented two episodes of acute nephritis in October 1973 and March 1974. On both occasions, the native C3 level fell and C3d was high. Variations of factor B level was less impressive, but Ba fragment level was particularly high on the first attack and was increased during the whole period of observation.

DISCUSSION

It is generally accepted that the complement system is involved in the pathogenesis of SLE and of various forms of glomerulonephritis (1, 2). Besides static measurement of complement components by hemolytic or immunochemical methods, the involvement of the complement system was demonstrated in turnover studies, with radiolabeled complement components, or was evidenced by the detection of breakdown products of complement components in serum. In this respect, C3 was often selected as an indicator of the activation of complement, since C3 is activated by the classical as well as the alternate pathway. Turnover studies carried out with radiolabeled C3 in hypocomplementemic nephritis led to some discrepancies in the interpretation of the results. In some studies, hypercatabolism of C3 was found (13, 25, 26), while in others a decreased synthesis appeared to be predominant (12). Recently, Charlesworth, Gwyn Williams, Sherington, Lackmann, and Peters (14) emphasized the interference of persisting C3d in the interpretation of the curve of disappearance of radiolabeled C3.

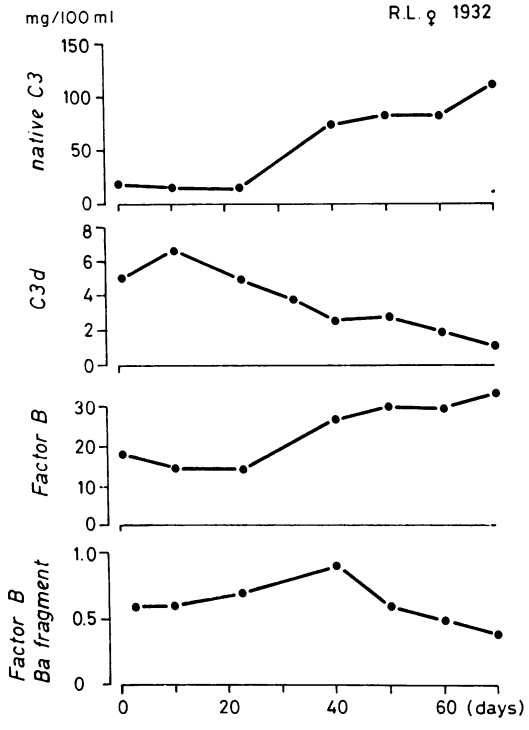

FIGURE 9 Follow-up study of native C3, C3d, properdin factor B, and Ba in a patient with SLE, before and during treatment.

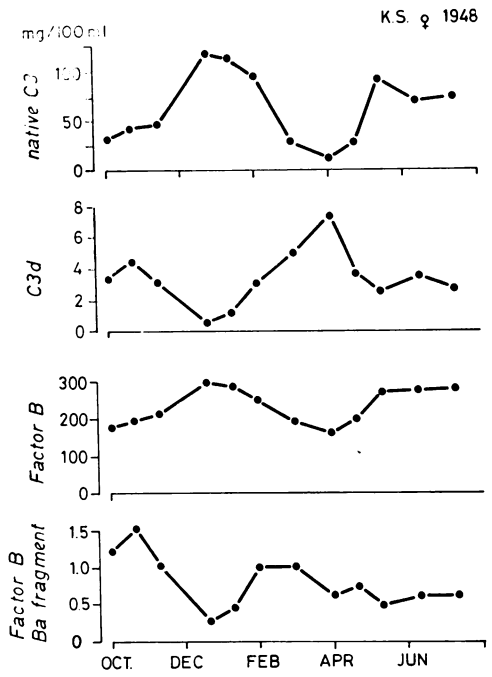

FIGURE 10 Follow-up study of native C3, C3d, properdin factor B, and Ba in a 26-yr-old patient suffering from recurrent attacks of SLE with nephritis.

The detection of breakdown products of C3 provides an alternative possibility for the evaluation of the involvement of the complement system. Most of the techniques previously used take advantage first of the change in mobility of breakdown products of C3 and, secondly, of the change in the antigens present on native C3 and on its breakdown products. Using immunoelectrophoresis, Morse, Muller-Eberhard, and Kunkel (27) have found fragments bearing the C antigen (β1A) in one out of nine patients with SLE. Alper and Rosen (25) did not observe fragments bearing the C antigen in patients with SLE or in progressive glomerulonephritis, but found them in a few patients with acute glomerulonephritis within the first 48 h after the onset of symptoms. Soothill found an altered form of C3 in the fresh plasma of 15 out of 16 patients with acute glomerulonephritis and in some patients with nephrotic syndromes (15). Breakdown products of C3 were also detected by West, Winter, Forristal, McConville, and Davis (16), using an immunoelectrophoretic precipitin method and specific antisera against native C3, C, and D antigens. The presence of C3 breakdown products was reported in the synovial fluid from patients with rheumatoid arthritis and in plasma from patients with

chronic active hepatitis or primary biliary cirrhosis (20). Similarly, breakdown products of properdin factor B have been detected by immunoelectrophoretic analysis in the plasma of some patients suffering from SLE and MPGN (18, 28) and in the synovial fluid of patients with rheumatoid arthritis (29, 30).

The present study confirms some of these data but with the addition of a quantitative analysis of the breakdown products of C3 and of properdin factor B in various clinical conditions. Indeed, the new methodology used allowed for a direct quantitation of C3d and of Ba fragments. In the first step, the selective precipitation of native C3, factor B, and of their large fragments by PEG is probably due to a molecular solvent effect (31). The use of specific antisera for D or Ba antigens in the second step of the methods limits considerably the risk of interference by large fragments of C3c or Bb in the immunochemical quantitation of C3d or Ba fragments. Moreover, the quantitation of C3 protein in plasma with both anti-native C3 and anti-C3c antisera provides an indirect estimation of the amount of molecules that carry the C antigen but that are no longer in the form of native C3. It therefore corresponds to C3b and C3c and possibly to some slightly altered C3. One should also note that Bb fragments of factor B that may occur in plasma would interfere with the immunochemical quantitation of properdin factor B, with anti-Bb antisera, since no particular antigenic structure specific for native factor B has been discovered as yet. It is known that some cleavage of C3 or properdin factor B may occur in vitro during the collection and storage of the samples. However, it was found that plasma samples collected on EDTA did not contain significant amounts of C3d or Ba fragments, even after 24 h at 25°C or after addition of aggHGG, or after repeated freezing and thawing. It is also unlikely that a significant part of C3d detected in plasma samples would be generated in vitro after cleavage of some C3b by C3b inactivator. Indeed, the addition, directly after the collection of samples, of suramin, which inhibits the activity of C3b inhibitor, to patient plasma (with a high content of C3d) did not influence the results obtained.

The specificity and sensitivity of the new methods used in this study were demonstrated in experiments based on the in vitro generation of complement breakdown products during the activation of normal serum by aggHGG. The increase in C3d and Ba concentrations was parallel to the decrease of native C3 and of hemolytically active C3. After 1 h, there was a decrease in the concentration of native C3 that corresponded to a change in configuration, or to a cleavage, of 76% of C3 molecules ("altered" C3). During this period, the amount of C3d generated represented 77% of the theoretical amount that would result from a complete cleavage of the altered C3 in its fragments, assuming that 0.175 mg of C3d (mol wt 35,000) would be generated from 1 mg of C3 (mol wt 200,000). The difference between the theoretical and the observed values of C3d is probably due to the persistence of uncleaved C3b and of C3 that has lost its native configuration. The amount of Ba fragment of properdin factor B generated simultaneously corresponded to 90% of the values calculated for a complete cleavage of factor B in this serum. The apparent absence of changes in the level of factor B during such experiments is due to the interference of the Bb fragments with the measurement technique with anti-Bb sera.

The main interest of the clinical investigation of complement breakdown products is to provide an estimate of the catabolic rate of complement components. This is of particular importance when the concentration of some components does not differ significantly from the normal range. The apparent advantages of the quantitative approach used in this study, as compared to the previous qualitative analysis, is the increased sensitivity and the precise measurement, which can be submitted to statistical evaluation. With regard to C3, the quantitation of C3d is likely to reflect a catabolic rate of C3 in patients with SLE or MPGN. Indeed, the highest levels of C3d were observed in patients with the lowest concentrations of C3. These findings also correlate with the results obtained by quantitation of C3 using both anti-native C3 and anti-C3c sera, which provides an estimation of the amount of C3 or fragments carrying the C antigen but no longer carrying the antigen characteristic of the native configuration of C3. However, one should note that in SLE and MPGN this estimate generally represented less than 25% of the concentration of native C3, while the concentration of C3d in these plasma samples often exceeded the concentration that could theoretically be expected after a complete cleavage of the native C3 present in the samples. This result strongly suggests that the C3d fragment detected in this clinical study is actually generated in vivo and is not the result of an activation of C3 during the in vitro manipulations. The relative differences between the two parameters obtained may indicate a higher rate of elimination for C3c than for C3d fragments. It should be pointed out that if these results indicate a hypercatabolism of C3, they are not inconsistent with the possible occurrence of a decreased synthesis of C3. The level of C3 fragments in patients with C3 levels close to or within the normal range was usually low in patients with MPGN, but abnormally elevated in some patients with SLE. The latter are characterized by a higher degree of inflammation, usually associated with an increased synthesis of C3. In such conditions, the increased catabolism of C3 would be partially masked.

The combination of C3 hypercatabolism and normal or elevated C3 levels have also been noted in several patients with SLE studied with radiolabeled C3 (12, 14). The ratio of C3d to native C3 in plasma probably reflects the "catabolic index" of C3. In healthy blood donors, this ratio was lower than 0.01, while values higher than 0.30 were observed in several patients with SLE or MPGN.

The results obtained in patients with other types of glomerulonephritis are of some interest, since elevated levels of C3d were found not only in patients with hypocomplementemic nephritis but also in three patients with normocomplementemic nephritis, suggesting an increased consumption of complement components due to the circulation of immune complexes. Therefore, this parameter may provide additional information concerning the involvement of immune reactions in the development of glomerulonephritis.

The levels of C3d observed in patients with severe alcoholic cirrhosis and with very low levels of C3 indicate the existence of some hypercatabolism of C3. However, these C3d levels represent only an average of 2.6% of the concentration of C3, as opposed to the high values observed in cases of SLE or MPGN with similar levels of C3. It is likely that such patients at late stages of cirrhosis have an impaired synthesis of C3, which plays an important role in the decrease of C3 levels (32, 33).

The clinical investigation of the level of properdin factor B does not frequently reveal a marked decrease of this component. There is a significant decrease in the level of factor B in patients with SLE or with MPGN, but the individual values obtained do not differ very much from the normal range (13, 18, 28). Therefore, it is difficult to assume the participation of factor B either in the alternate pathway or in the C3b amplification system. One drawback of the immunochemical quantitation of properdin factor B is the interference of eventual Bb fragments in clinical samples. It appeared that the direct determination of the concentration in the Ba fragment leads to a better estimation of the involvement of factor B in various diseases, since more than 40% of samples from patients with SLE exhibited a high level of Ba fragment.

Serial and correlative studies carried out in patients with SLE or MPGN demonstrated first an inverse relationship between the concentration of C3 and the native concentrations of C3d or of Ba fragments. Secondly, the positive correlation between the concentration of Ba and that of C3d fragments supports the idea of a general activation of the complement system in these diseases. The results obtained during the follow-up studies emphasize the possible application of the quantitation of complement breakdown products in the clinical investigation of SLE patients. Indeed, there was a direct correlation between the activity of the disease and the levels of C3d and Ba fragments. Such a determination would be of particular interest in cases of SLE with infectious complications.

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