# Activated complement C3: A potentially novel predictor of progressive IgA nephropathy

Jörg Zwirner, Michael Burg, Matthias Schulze, Reinhard Brunkhorst, Otto Götze, Karl-Martin Koch, and Jürgen Floege

*Institute of Immunology, University of Göttingen and Division of Nephrology, Medizinische Hochschule, Hannover, Germany*

**Activated complement C3: A potentially novel predictor of progressive IgA nephropathy.** In the search for a serologic marker of disease activity, we measured concentrations of activated C3 (actC3, that is, neoantigens developing after C3 activation on breakdown products), C4-C3 complexes and soluble C5b-9 (sC5b-9) in one or two plasma samples from adult patients with IgA nephropathy (IgAN, $N = 50$) or Henoch-Schönlein purpura (HSP, $N = 4$). As controls, 20 patients with non-immune renal disease, but comparable age, degree of proteinuria, renal dysfunction and prevalence of hypertension were studied. Compared to controls, actC3 levels were elevated in 30% of the patients with IgAN and one of the HSP patients. C4-C3 complexes were elevated in only 8% of the IgAN patients, and sC5b-9 levels were within the control range in all IgAN and HSP patients. In IgAN patients with elevated actC3 levels, proteinuria and hematuria were more pronounced than in those with normal levels. Elevated plasma concentrations of actC3 at the first presentation correlated with subsequent deterioration of renal function both in patients with initially normal and already impaired renal function ($r = -0.56$, $N = 44$, $P = 0.003$). The five IgAN patients with elevated actC3 on both occasions of obtaining plasma showed the most rapid loss of renal function. We conclude that mainly alternative pathway complement activation can be demonstrated in patients with IgAN and HSP. In IgAN patients the presence of complement activation is associated with more severe renal disease. Further studies are warranted to examine the clinical usefulness of actC3 as a predictor of the subsequent course of IgAN.

IgA nephropathy (IgAN), presenting as isolated glomerular disease (primary IgA nephropathy) or as a part of Henoch-Schönlein purpura (HSP), is among the most common types of glomerulonephritis worldwide [1–3]. About 20 to 30% of the patients with IgAN will develop progressive renal failure within 10 to 20 years after the onset of disease [4–7]. Limited data suggest that a similar percentage may also apply to HSP in adults [8, 9]. Epidemiologic studies have identified that some clinical parameters, such as the degree of proteinuria, or the presence of hypertension or impaired renal function, are associated with an increased risk of progressive renal disease, but these parameters are relatively non-specific and not particularly reliable for the individual patient [4–7]. At present, no serologic tests are available that can be employed to assess disease activity in IgAN or to predict renal outcome.

The ability of IgA to cause renal injury via activation of the complement system has long been controversial [10]. Recently, convincing *in vivo* evidence has been presented that, in the rat, dimeric and polymeric IgA can activate complement via the alternative pathway to induce glomerular damage [11, 12]. In patients with IgAN or HSP, complement activation has been shown to occur both at the glomerular as well as systemic levels and has been implicated in the pathogenesis of renal disease [1, 2, 13–15]. In the present study we investigated whether an assessment of systemic complement activation correlates with clinical indices of renal disease activity, and in particular, whether it has a predictive value for the subsequent course of the disease. Such data could not only strengthen the evidence for a pathogenetic role of the complement system in IgAN, but could also lay the basis for future therapeutical trials by tailoring treatment to high risk patients.

In the current study we have employed some novel assays, which allow a sensitive detection of complement activation. The study was specifically designed to preclude any *ex vivo* complement activation after obtaining blood samples and to avoid the assessment of low molecular weight complement components, whose plasma concentration might be influenced by renal dysfunction. Consequently, the following complement components were chosen: (1) activated C3 (actC3) [16], that is, neoantigens which develop on C3 breakdown products following activation of either pathway; (2) C4-C3 complexes [17], a marker of classical pathway complement activation; and (3) soluble C5b-9 (sC5b-9) [18], that is, the late membrane atttack complex which is formed following activation of either pathway.

## Methods

### Patients

Plasma samples of three groups of patients were analyzed: (A) 50 patients with primary IgAN; (B) 4 patients with HSP; and (C) 20 patients with various non-immune renal diseases (Table 1). Plasma samples obtained from 60 healthy persons served as normal controls. In all patients of groups A and B the diagnosis of IgAN had been established by renal biopsy, which exhibited typical findings upon light microscopical, immunohistological and electron microscopical examination (performed by U. Helmchen, University of Hamburg, Germany). HSP was diagnosed in cases with a biopsy proven IgAN and typical systemic symptoms. Patients with liver disease, chronic inflammatory disease of the

**Table 1.** Characteristics of the patients studied

| | Group | | |
| --- | --- | --- | --- |
| | IgAN (N = 50) | HSP (N = 4) | Non-immune renal disease[a] (N = 20) |
| Sex m:f | 39:11 | 2:2 | 14:6 |
| Age *years* | 36 ± 12 (18–69) | 33 ± 13 (18–50) | 43 ± 17 (18–74) |
| Follow-up[b] *months* | 29 ± 22 (4–87) | 33 ± 21 (3–49) | none |
| Percent of patients with hypertension | 82% | 75% | 80% |
| Serum creatinine *μmol/liter*[c] | 199 ± 221 (60-1582) | 133 ± 52 (62-188) | 172 ± 103 (70-450) |
| Proteinuria[c] *g/24 hr* | 3.5 ± 3.5 (0.0–13.2) | 3.4 ± 2.3 (1.5–7.3) | 2.4 ± 3.2 (0.0–10.1) |
| Hematuria[cd] *million erythro./ 24 hr* | 42 ± 41 (0–100) | 57 ± 44 (2–93) | 20 ± 32 (0–100) |

Data are absolute or relative frequencies or means ± SD and (range)
[a] Includes hypertensive renal damage, Alport's syndrome, polycystic kidney disease, thin basement membrane disease and reflux nephropathy
[b] Clinical follow-up after obtaining the first plasma sample (available in 44/50 patients with IgAN and 4/4 patients with HSP)
[c] At the time of obtaining the first plasma sample
[d] Groups significantly different ($P < 0.05$ by Kruskal Wallis testing)

skin, gastrointestinal tract or respiratory tract, malignancies, or other systemic diseases were excluded from the study. No patient showed clinical symptoms of infections at the time of the examination.

Of the IgAN and HSP patients studied, one to two plasma samples (total of 98 samples) were obtained. The time elapsed between the two plasma samples ranged from 1 to 61 months. A single sample was obtained from all patients with non-immune renal disease. To avoid *ex vivo* complement activation, 10 ml blood was drawn into tubes containing 20 mM EDTA and 100 μM benzamidine (both from Sigma, Deisenhofen, Germany), centrifuged within one hour after obtaining the sample, and the plasma was then immediately aliquoted and stored at −70°C until the assay. Storage durations of the plasma samples obtained the three patient groups were not significantly different (range 12 to 96 months), but were below 12 months in the samples obtained from normal controls.

Data obtained at each patient visit included measurements of serum creatinine, creatinine clearance as well as a quantitative determination of 24-hour urinary protein and erythrocyte excretion using standard methodology. For the purposes of calculations microhematuria of greater than 100 million erythrocytes per day was set as 100. No patient showed macrohematuria at the time of presentation. In 44 (88%) of the IgAN patients clinical follow-up of at least six months including at least five determinations of serum creatinine were available. These data served to calculate the slope of 1/creatinine.

### Antibodies and reagents for ELISAs

The following antibodies and reagents were used in the ELISAs: peroxidase-conjugated monoclonal antibody M4d2 against human complement component C4 that recognizes C4, C4b, and C4d [15]; monoclonal antibody M4c3 against the γ-chain

of C4, C4b and C4c [15]; monoclonal antibody I3/15 against a neoepitope on human C3b, iC3b, C3dg [16]; monoclonal antibody W13/15 against a human C9 neoantigen [18]; peroxidase-conjugated rabbit IgG anti-human C3d (Dakopatts, Hamburg, Germany), biotinylated polyclonal goat antibody against human C6 [19]; peroxidase-coupled streptavidin (Amersham, Braunschweig, Germany).

### ELISA for the measurement of activated C3

This assay was performed as described elsewhere [16]. Microtiter plates (Immunoplate Maxisorp; Nunc, Wiesbaden, Germany) were coated overnight with 100 μl of antibody I3/15 at 5 μg/ml in 0.05 M carbonate buffer pH 10.6. After washing in phosphate buffered saline (PBS, pH 7.2) non-specific binding capacity was blocked with 0.05 M carbonate buffer pH 10.6 containing 1% (wt/vol) gelatin. After washing with PBS/0.05% Tween-20 100 μl samples in duplicate 1:50 dilutions in PBS/0.05% Tween-20/0.02 M EDTA were added for two hours at room temperature. After washing with PBS/Tween-20 100 μl of peroxidase-conjugated rabbit anti-human C3d (Dakopatts) in PBS/Tween-20 were added and incubated for another one hour at room temperature. Finally, streptavidin-horseradish peroxidase-conjugate was added for one hour at room temperature. Determinations of actC3 were performed by colorimetric analysis of the peroxidase-mediated hydrolysis of 2 mM 2,2'-azino-di-(3-ethyl benzthiazolinesulfonate) (ABTS, Boehringer Mannheim, Germany) in substrate buffer (100 mM sodium acetate, 50 mM sodium phosphate, pH 4.2) containing 2.5 mM $H_2O_2$. Absorbance was measured at 410 nm (reference wavelength 490 nm) using a microplate photometer (ThermoMax; Molecular Devices, Menlo Park, CA, USA). ActC3 concentrations in the samples were calculated from calibration curves obtained by plotting the absorbance of purified protein C3b on a double log scale. C3b was generated as described recently [16]. Since purified C3 fragments C3b, iC3b and C3dg have been shown to give identical signals on a weight basis the amount of activated C3 in samples was expressed as μg C3b/iC3b/C3dg per ml.

### ELISA for the measurement of C4-C3 complexes

This assay was performed as described elsewhere with minor modifications [17]. Microtiter plates (Immunoplate Maxisorp; Nunc) were coated overnight with 50 μl/well of antibody I3/15 against actC3 at 10 μg/ml in PBS. Blocking was performed with PBS/1% bovine serum albumin (BSA) for one hour at room temperature. After washing in PBS, pH 7.2 duplicate samples (50 μl), diluted 1:11 in PBS/0.02 M EDTA/1% BSA, were incubated for two hours at 37°C. After washing in PBS/0.05% Tween-20 peroxidase-coupled antibody M4d2 against C4/C4b/C4d was added and incubation continued for another two hours at 37°C. Finally, wells were washed and 0.3 mM 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) (Fluka, Neu-Ulm, Germany) in substrate buffer (0.1 M citric acid, 0.2 M disodium hydrogen phosphate) containing 2.5 mM $H_2O_2$ was added. The reaction was stopped after two to five minutes by adding an equal volume of 1 M $H_2SO_4$. Absorbance was measured at 450 nm using a microplate reader (ThermoMax; Molecular Devices). A standard curve for the quantitation of C4-C3 complexes was obtained by serially diluting normal human serum activated with 500 μg heat-aggregated human IgG (HAG)/ml serum in PBS/0.02 M
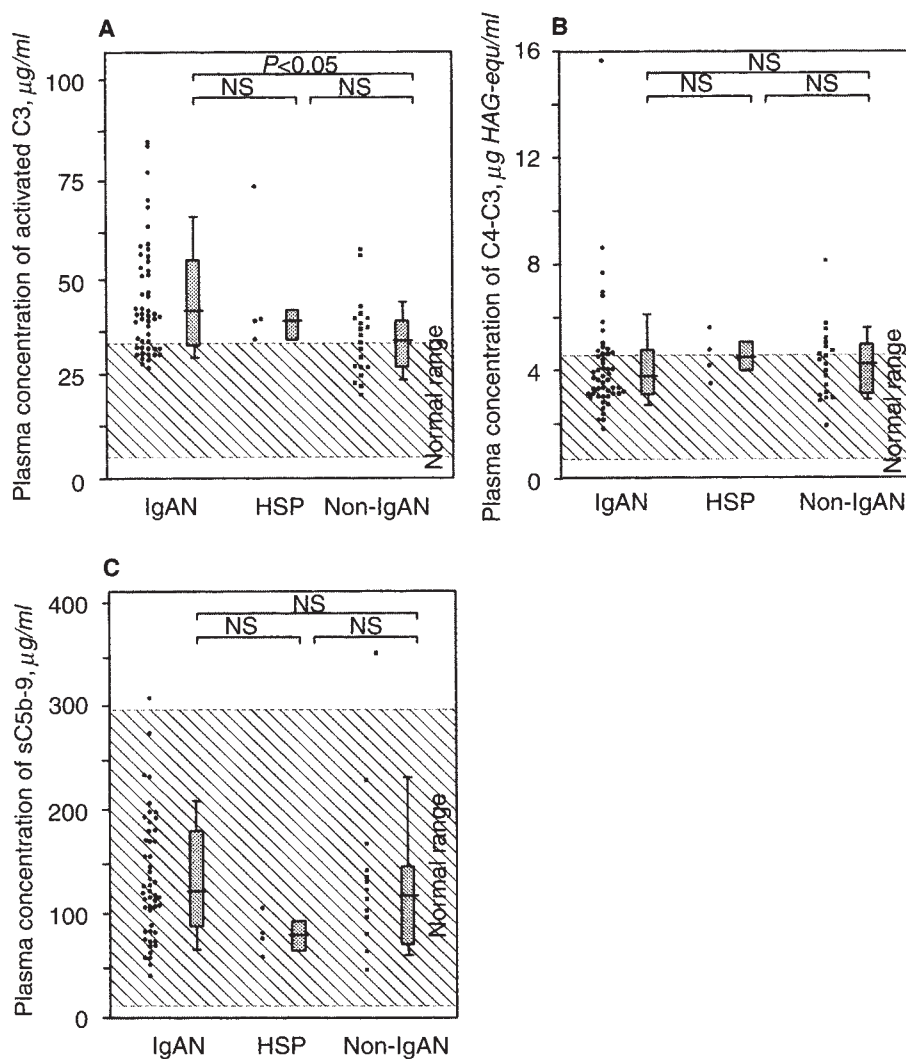
**Fig. 1.** *Plasma levels of (A) actC3, (B) C4-C3 complexes and (C) sC5b-9 in patients with IgA nephropathy, Henoch-Schönlein purpura and non-immune renal disease.* Shown are individual measurements as well as box plots (median, box showing 25th and 75th percentiles, lines indicating 10th and 90th percentiles). Normal ranges (means ± 2 SD) measured in healthy persons are indicated by the hatched areas.

EDTA/1% BSA. The C4-C3 values were expressed as µg HAG-equivalent (Equ)/ml.

### ELISA for the measurement of sC5b-9

The sC5b-9 ELISA was performed essentially as described [18] with minor modifications. Microtiter plates (Immunoplate Maxisorp; Nunc) were coated overnight with 100 µl of antibody W13/15 against a C9 neoantigen at 5 µg/ml in 0.05 M carbonate buffer pH 10.6. Non-specific binding was blocked by incubating the plates with 0.05 M carbonate buffer pH 10.6 containing 1% (wt/vol) gelatin and by incubating plasma samples with heat-aggregated goat IgG (1 mg/1 ml plasma) for 30 minutes. After washing the plates with PBS/0.05% Tween-20 duplicate samples (100 µl of 1:2 dilutions in PBS/0.05% Tween/0.02 M EDTA) were added for two hours at room temperature. After washing with PBS/Tween-20, biotinylated goat anti-human C6 in PBS/Tween-20 (100 µl) was added and incubated for another one hour at room temperature. After a final wash, streptavidin-horseradish peroxidase-conjugate was added and incubated for one hour at room temperature. Finally, TMB was added as has been described for the C4-C3 ELISA. The sC5b-9 assay was calibrated using a

standard of human zymosan-activated serum with known sC5b-9 content (provided by Dr. Thomas Lint, Rush Presbyterian St. Lukes Medical Center, Chicago, IL, USA).

### Statistical analysis

All values are expressed as mean ± SD. Statistical significance (defined as $P < 0.05$) was evaluated using either the Kruskal-Wallis test or Mann-Whitney $U$-test for non-parametric populations. Correlation coefficients were calculated using the Spearman rank test for non-parametric populations. Slopes of 1/creatinine were calculated using linear regression analysis.

### Results

*Presence of alternative or classical pathway complement activation in patients with IgAN and HSP*

As shown in Table 1, the three groups of patients were comparable in their age, frequency of hypertension, degree of renal dysfunction and proteinuria. Kruskal-Wallis testing revealed that of the parameters depicted in Table 1 only the degree of hematuria was significantly different between the three groups.

**Table 2.** Spearman rank correlation coefficients between clinical findings and plasma concentrations of actC3, C4-C3 complexes, and sC5b-9 in patients with IgAN ($N = 50$) or non-immune renal disease ($N = 20$)

| Parameter | Patients | ActC3 | | C4-C3 complexes | | sC5b-9 | |
|---|---|---|---|---|---|---|---|
| | | r | P | r | P | r | P |
| Serum creatinine *μmol/liter* | IgAN | 0.17 | NS | 0.19 | NS | 0.18 | NS |
| | Controls | 0.41 | NS | 0.39 | NS | 0.22 | NS |
| Creatinine clearance *ml/min* | IgAN | −0.06 | NS | −0.20 | NS | −0.15 | NS |
| | Controls | −0.39 | NS | −0.22 | NS | −0.29 | NS |
| Proteinuria *g/day* | IgAN | 0.35 | <0.01 | 0.27 | <0.05 | 0.29 | <0.01 |
| | Controls | −0.33 | NS | −0.39 | NS | −0.29 | NS |
| Microhematuria *million erythroc./day* | IgAN | 0.32 | <0.01 | 0.17 | NS | 0.13 | NS |
| | Controls | −0.29 | NS | −0.43 | NS | 0.03 | NS |
| Time since renal biopsy *months* | IgAN | −0.15 | NS | −0.04 | NS | −0.13 | NS |
| | Controls | NA | — | NA | — | NA | — |
| Age *years* | IgAN | 0.15 | NS | 0.24 | NS | 0.14 | NS |
| | Controls | 0.62 | <0.01 | 0.81 | <0.01 | 0.61 | <0.01 |

Abbreviations are: NA, not available, NS, not significant.

Plasma concentrations of actC3 were significantly increased in patients with IgAN and HSP over those with non-immune renal disease (Fig. 1). When the non-immune renal disease group was used to define a control range (mean ± 2 SD), plasma concentrations of actC3 were elevated in one or two samples in 30% of the patients with IgAN and 25% of the patients with HSP. In those IgAN patients where two plasma samples were available, 67% had normal actC3 concentrations on both occasions, 13% elevated concentrations at both occasions and 18% showed elevated concentrations at the first occasion only. Notably, only one IgAN patient (2%) initially presented with a normal actC3 level and had an increased level at the second occasion. In the HSP group both samples with elevated actC3 concentrations were obtained from the same patient within a period of three months.

In contrast to actC3, the plasma concentrations of C4-C3 complexes did not differ significantly between patients with IgAN, HSP and non-immune renal disease (Fig. 1). When the non-immune renal disease group was used again to define a control range, plasma concentrations of C4-C3 complexes were increased in four (8%) IgAN patients and in none of the HSP patients. In three of the four patients, actC3 levels were increased at the same time points as C4-C3 complexes were elevated.

Plasma concentrations of sC5b-9 were not significantly different between patients with IgAN, HSP and non-immune renal disease (Fig. 1), and none of the IgAN samples exceeded the control range as defined by the non-immune renal disease group.

### Correlation of complement activation with clinical status

As we predicted, neither actC3 plasma concentrations nor those of C4-C3 complexes or sC5b-9 showed any correlation with serum creatinines or creatinine clearances in patients with IgAN or non-immune renal disease (Table 2). Similarly, no correlation with a history of macrohematuria (data not shown) or the time elapsed since renal biopsy evolved for any of the three complement components (Table 2). The degree of proteinuria was weakly, yet statistically significantly correlated with levels of actC3, C4-C3 complexes and C5b-9 in patients with IgAN, as were actC3 levels and the degree of microhematuria (Table 2). Patient age correlated significantly with all three complement components in non-immune renal disease patients, but not in the IgAN patients (Table 2).

Compared to IgAN patients with normal actC3 concentrations,

those with elevated levels had a shorter time since renal biopsy (an occasionally used surrogate marker of renal disease activity), exhibited higher proteinuria and more severe microhematuria (Fig. 2). None of these differences reached statistical significance, but all were within the 5 to 6% range of probability (Fig. 2). In the case of C4-C3 complexes, IgAN patients with normal or elevated levels differed significantly only in their extent of proteinuria (Fig. 2).

Six of the 50 patients with IgAN and one of the HSP patients were receiving corticosteroids at one or both occasions of obtaining blood samples, so that 9 of 98 samples were obtained during phases of immunosuppressive therapy. The plasma concentrations of the three complement components were not significantly different between the steroid-treated patients and those without immunosuppressive therapy.

### Predictive value of complement activation for the subsequent course of the renal disease

Figure 3 and Table 3 show that a significant correlation existed between the the level of actC3 at the first presentation and the subsequent slope of 1/creatinine. In subgroup analyses this correlation was found to be mainly due to the patients with normal renal function at presentation ($r = -0.65$; $P = 0.006$) while the correlation in patients with already impaired renal function at presentation failed to reach statistical significance ($r = -0.33$; $P = 0.13$). If the analysis was confined to those patients followed for more than one year ($N = 34$), the correlation between the slope of 1/creatinine and actC3 levels persisted ($r = -0.502$; $P = 0.005$). Table 3 also demonstrates that actC3 levels and proteinuria at the time of the first presentation showed similar correlations with the slope of 1/creatinine, while that of the initial serum creatinine was weaker and hematuria was not significantly correlated with the slope of 1/creatinine.

When stable or improving renal function was arbitrarily defined as a slope of 1/creatinine of greater than $-5 \times 10^{-5}$ 1/($\mu$mol $*$ month) (Fig. 3), a single elevated actC3 level had a sensitivity of 75% and a specificity of 89% for predicting subsequent progression of the renal disease (Table 4). Specificity and sensitivity of a single actC3 determination exceeded that of various other clinical parameters used to predict progressive renal dysfunction (Table 4).
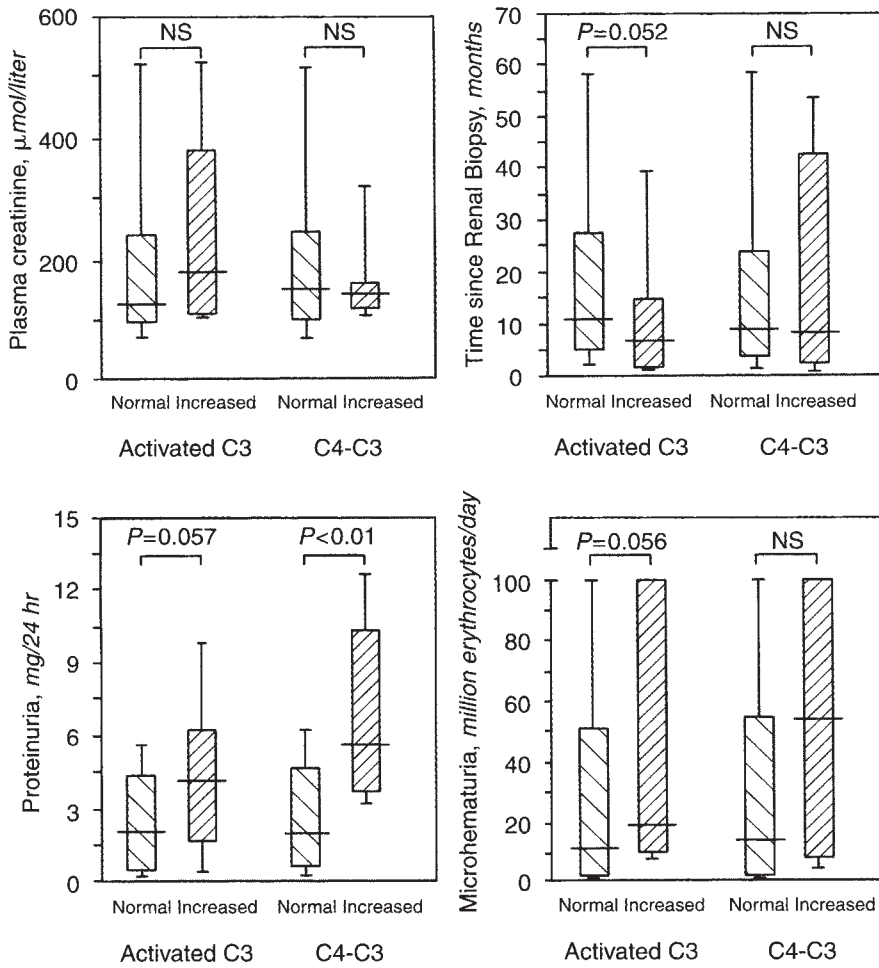
**Fig. 2.** *Comparison of clinical characteristics in IgAN patients at the time of obtaining plasma samples with normal (N = 71) or increased levels of actC3 (N = 19), as well as with normal (N = 84) or increased levels of C4-C3 complexes (N = 6).* Shown are box plots (median, box showing 25th and 75th percentiles, lines indicating 10th and 90th percentiles). In the case of microhematuria, excretion of >100 million erythrocytes per day was set as 100.
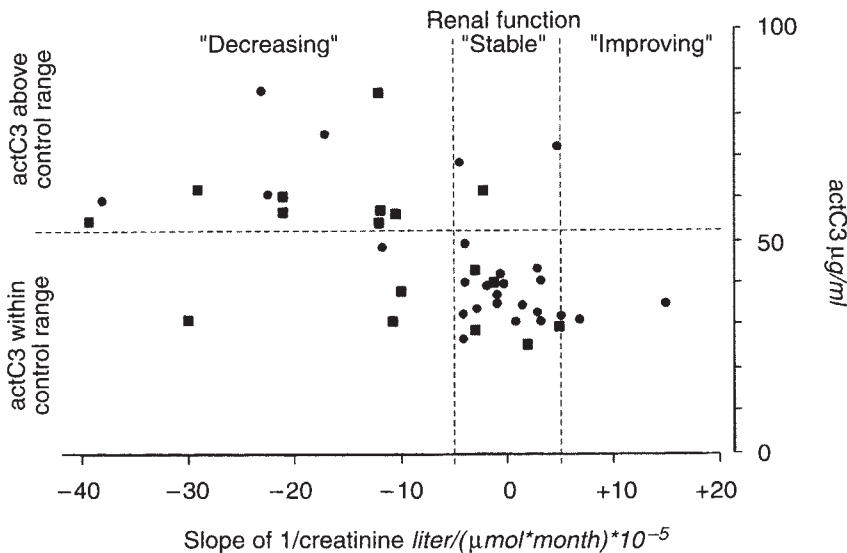


**Fig. 3.** *Correlation between plasma levels of actC3 at the first presentation and the subsequent slope of 1/creatinine in patients with IgAN.* Patients were arbitrarily divided into those with decreasing renal function [slope below $-5 \times 10^{-5}$ l/($\mu$mol * month)], stable renal function [slope between $+5$ and $-5 \times 10^{-5}$ l/($\mu$mol * month)] and improving renal function [slope greater than $+5 \times 10^{-5}$ l/($\mu$mol * month)]. Plasma actC3 levels were separated into those greater than or within the control range, as defined by the mean $\pm$ 2 SD of the non-immune renal disease group. In all the patients represented, the serum creatinine at the time of the first presentation was below a maximum of 407 $\mu$mol/liter. Symbols are (●) Normal renal function at presentation; (■) Decreased renal function at presentation. $r = -0.56$; $P = 0.003$; $N = 44$.

We also assessed whether evidence of complement activation in both blood samples carried a worse prognosis than increased actC3 or C4-C3 complex levels at only one occasion or no increase at all. As shown in Figure 4, IgAN patients with increased actC3 levels on both occasions showed a faster rate of progression than those with an increased level at only the first occasion or no

**Table 3.** Spearman rank correlation coefficients between the slope of 1/creatinine and various parameters in patients with IgAN, in whom more than 6 months of follow-up was available ($N = 44$)

| Slope of 1/creatinine versus | $r =$ | $P =$ |
|---|---|---|
| actC3 levels af first presentation | −0.56 | 0.003 |
| serum creatinine at first presentation | −0.32 | 0.04 |
| proteinuria at first presentation | −0.57 | 0.002 |
| microhematuria at first presentation | −0.17 | NS |

NS is not significant.

**Table 4.** Sensitivity and specificity of various parameters to predict the subsequent development of progressive renal insufficiency [defined as a slope of 1/creatinine of less than $-5 \times 10^5$ $1/(\mu\text{mol} \cdot \text{month})$] in patients with IgAN, in whom more than 6 months of follow-up was available ($N = 44$)

| Parameter (at the time of the first presentation) | Specificity % | Sensitivity % |
|---|---|---|
| actC3 level above control range | 89 | 75 |
| Proteinuria >1 g/24 hrs | 44 | 93 |
| Proteinuria >3.5 g/24 hrs | 72 | 69 |
| Elevated serum creatinine | 58 | 69 |
| Significant microhematuria[a] | 16 | 100 |

[a] Defined as >3 million erythrocytes/day

increases at all. In the case of increased C4-C3 complex levels, too few patients showed elevated concentrations to allow a statistical evaluation. However, it is noteworthy that all four patients with elevated C4-C3 complex levels exhibited rapid progression rates (Fig. 4).

## Discussion

Our findings show that systemic evidence of complement activation can be demonstrated in 30% of the patients with IgAN and 25% with HSP. In 6% of the cases with IgAN, complement activation apparently occurred via the classical pathway. However, in the majority of our IgAN and HSP patients we could not detect elevated C4-C3 complex levels, suggesting that in these patients the elevated actC3 levels were the result of alternative pathway activation. In contrast to actC3 and C4-C3 complexes, sC5b-9 plasma concentrations were within the control range in all IgAN and HSP patients. This apparent discrepancy of early yet not late activation of the complement pathway most likely relates to an overall inefficiency of terminal sequence activation and has been observed in other patients with complement-consuming immune complex diseases as well [20].

Four previous studies have also assessed systemic complement activation in patients with IgAN by measuring the complement breakdown products [21–24]. This approach is more sensitive than the measurement of total complement C3 or C4 levels, none of which are decreased in patients with IgAN or HSP [21, 25–27]. Lagrue et al [22] and Sølling [24] detected mild elevations of complement C3d in 50% (5 of 10) and 45% (5 of 11) of their patients, respectively. Wyatt et al [21], using a radioimmunoassay for a neoantigen expressed on fragments of C3, reported increased plasma levels in 73% of 53 adult IgAN patients. These authors also noted elevated C4d/C4 ratios, suggesting classical pathway complement activation, in 28% of their IgAN patients [21]. Finally, Brenchley et al [23], using an ELISA for C3dg, detected increased plasma levels in 45% of 29 IgAN patients. In that study, 17% of the patients also showed increased plasma levels of sC5b-9 [23]. In comparing the results of these studies, it is apparent that all former studies reported a higher prevalence of systemic complement activation than we observed in the current study. Several reasons may underlie this observation. First, instead of using healthy persons as controls, we compared IgAN patients with a non-immune renal disease group, which was comparable in clinical characteristics. This excluded that non-specific effects on the complement system, for example those related to hypertension, proteinuria or age, accounted for our findings. Indeed, actC3 and C4-C3 complex concentrations in several of the non-immune renal disease patients were above the normal range, which is similar to previous findings from our group [28]. On the other hand, the samples from normal persons had not been stored for

similar lengths of time and are therefore not strictly comparable to the patient samples analyzed in the present study. Second, we ascertained that none of the complement components measured were influenced by renal dysfunction. In contrast, Sølling reported that C3d plasma concentrations increased with renal dysfunction, in particular in end-stage renal failure [24]. Similar findings have also been observed for C3a and C4a in patients with IgAN [29]. Consequently, elevated plasma levels of C3d, C3a or C4a in patients with IgAN as observed in these studies could be due to some degree to renal retention. Finally, we ascertained that no complement activation occurred *ex vivo* by adding a relatively high EDTA concentration as well as benzamidine to the blood samples and by rapidly freezing the plasma. These technical issues are of importance, since it is well recognized that any coagulation will activate the complement pathway [19, 30]. Conceivably, coagulation triggered complement activation may be augmented by the presence of immune complexes in the blood. This, for example, could at least partially account for the observation of Kawana and Nishiyama [31], who reported increased sC5b-9 levels in 83% of their serum samples from patients with HSP.

In none of the previous studies described above [21–24] was clinical follow-up available, and the concentrations of the various complement components assessed were therefore only related to the current or past clinical status. Wyatt et al [21] noted no correlation between iC3b-C3d neoantigen concentrations and several clinical parameters, such as proteinuria, presence of chronic renal insufficiency and history of macrohematuria. In contrast, the present study revealed weak, yet statistically significant correlations between actC3 levels, proteinuria and microhematuria. Furthermore, differences of the clinical status of patients with and without elevated actC3 levels were also apparent when those patient subgroups were compared to each other. In a similar approach Brenchley et al [23] described significantly more hematuria in patients with increased C3dg plasma levels than in those with normal levels. These data suggest that elevated levels of actC3 or C3dg identify IgAN patients with high concurrent disease activity. Although we can only describe an association with no proof of causality, the data of the current study support the hypothesis that complement activation, induced by IgA and/or IgG immune complexes is a contributor to the pathogenesis of renal disease in IgAN and, possibly, also in HSP.

The most important finding of the present study was that in patients with IgAN plasma levels of actC3 showed a significant correlation with the subsequent rate at which renal function was lost. This correlation became even stronger when more than one
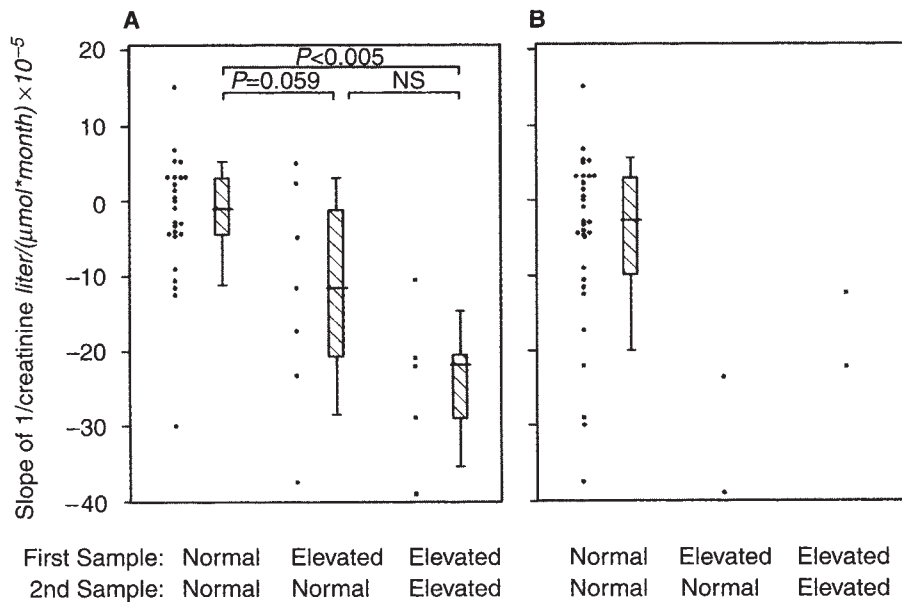
**A**

**B**



First Sample: Normal Elevated Elevated Normal Elevated Elevated
2nd Sample: Normal Normal Elevated Normal Normal Elevated

**Fig. 4.** *Relationship between the slope of 1/creatinine and the frequency of detecting elevated levels of* (**A**) *actC3 and* (**B**) *C4-C3 complexes in those patients with IgA nephropathy from whom two plasma samples were available.* Shown are individual measurements as well as box plots (median, box showing 25th and 75th percentiles, lines indicating 10th and 90th percentiles).

plasma sample exhibited an elevated actC3 level. These findings especially applied to patients with normal renal function at the first presentation, suggesting that complement activation in IgAN patients does not occur secondary to established renal impairment. All of the aforementioned observations lend further support to the hypothesis that actC3 plasma levels reflect renal disease activity and that complement activation contributes to the pathogenesis of IgAN. More importantly, our data suggest that assessment of actC3 could represent the first serologic test to predict outcome in patients with IgAN. Compared to proteinuria, which has frequently been used as a risk marker of progressive IgAN [4, 6, 32, 33], the determination of actC3 had a somewhat lower sensitivity but markedly improved specificity for predicting subsequent deterioration of renal function. Conceivably, sensitivity and specificity of actC3 determinations might be enhanced further if more than one plasma sample is analyzed.

In contrast to actC3, other serological tests, such as the demonstration of circulating immune complexes, do not distinguish between patients with deteriorating and stable renal function [24]. The same has been reported for IgA fibronectin complexes [34, 35]. Finally, urinary cytokine excretion, in particular interleukin-6, has been assessed in IgAN patients [36–38]. However, it is not clear whether the increased urinary interleukin-6 excretion in some patients with IgAN reflects increased proteinuria *per se* and whether it is indeed associated with a progressive course of the disease.

We conclude that systemic complement activation, mainly via the alternative pathway, can be detected in about one third of patients with IgAN and in some patients with HSP. Several findings support the notion that complement activation, in particular the detection of increased actC3 plasma levels, reflects high degrees of renal disease activity. Given the association between high actC3 levels and subsequent deterioration of renal function, this complement component could potentially represent a novel marker to non-invasively identify those patients who are most likely to benefit from therapeutic intervention. At present, no established therapy is available for patients with progressive types

of IgAN or HSP [1, 9]. However, if our observation is independently confirmed in other populations of patients, elevated plasma actC3 may become a useful entry criterion in future trials aimed at the evaluation of novel therapeutic approaches to IgAN.

### Acknowledgments

### References

1. GALLA JH: IgA nephropathy. *Kidney Int* 47:377–387, 1995
2. VAN ES LA: Pathogenesis of IgA nephropathy. *Kidney Int* 41:1720–1729, 1992
3. D'AMICO G: The commonest glomerulonephritis in the world: IgA nephropathy. *Quart J Med* 64:709–727, 1987
4. BAILEY RR, LYNN KL, ROBSON RA, SMITH AH, WELLS JE: Long term follow up of patients with IgA nephropathy. *NZ Med J* 107:142–144, 1994
5. IBELS LS, GYORY AZ: IgA nephropathy: Analysis of the natural history, important factors in the progression of renal disease, and a review of the literature. *Medicine* 73:79–102, 1994
6. JOHNSTON PA, BROWN JS, BRAUMHOLTZ DA, DAVISON AM: Clinico-pathological correlations and long-term follow-up of 253 United Kingdom patients with IgA nephropathy. A report from the MRC Glomerulonephritis Registry. *Quart J Med* 84:619–627, 1992
7. D'AMICO G: Influence of clinical and histological features on actuarial renal survival in adult patients with idiopathic IgA nephropathy, membranous nephropathy, and membranoproliferative glomerulonephritis: Survey of the recent literature. *Am J Kidney Dis* 20:315–323, 1992
8. MIURA M, NOMOTO Y, SAKAI H, YAMAMOTO O: An aged patient with Henoch-Schönlein nephritis: A case report and review of the literature. *Intern Med* 31:232–238, 1992
9. ROBSON WLM, LEUNG AKC: Henoch-Schönlein purpura. *Adv Pediatr* 41:163–194, 1994
10. WALDO FB: Role of IgA in IgA nephropathy. *J Pediatr* 116:S78–S85, 1990
11. STAD RK, BRUIJN JA, VAN GIJLSWIJK JANSSEN DJ, VAN ES LA, DAHA MR: An acute model for IgA-mediated glomerular inflammation in

rats induced by monoclonal polymeric rat IgA antibodies. *Clin Exp Immunol* 92:514–521, 1993

12. STAD RK, BOGERS WMJM, THOOMES VAN DER SLUYS ME, VAN ES LA, DAHA MR: In vivo activation of complement by IgA in a rat model. *Clin Exp Immunol* 87:138–143, 1992

13. WYATT RJ: The complement system in IgA nephropathy and Henoch-Schönlein purpura: Functional and genetic aspects. *Contrib Nephrol* 104:82–91, 1993

14. JENNETTE JC: The immunohistology of IgA nephropathy. *Am J Kidney Dis* 5:348–352, 1988

15. ZWIRNER J, FELBER E, HERZOG V, RIETHMÜLLER G, FEUCHT HE: Classical pathway of complement activation in normal and diseased human glomeruli. *Kidney Int* 36:1069–1077, 1989

16. OPPERMANN M, BAUMGARTEN H, BRANDT E, GOTTSLEBEN W, KURTS C, GÖTZE O: Quantification of components of the alternative pathway of complement (APC) by enzyme-linked immunosorbent assays. *J Immunol Meth* 133:181–190, 1990

17. ZWIRNER J, DOBOS G, GÖTZE O: A novel ELISA for the assessment of classical pathway of complement activation in vivo by measuring C4–C3 complexes. *J Immunol Meth* 186:55–63, 1995

18. SCHULZE M, DONADIO JV, PRUCHNO CJ, BAKER PJ, JOHNSON RJ, STAHL RAK, WATKINS S, MARTIN DC, WÜRZNER R, GÖTZE O, COUSER WG: Elevated urinary excretion of the C5b-9 complex in membranous nephropathy. *Kidney Int* 40:533–538, 1991

19. WÜRZNER R, ORREN A, POTTER P, MORGAN BP, PONARD D, SPÄTH P, BRAI M, SCHULZE M, HAPPE L, GÖTZE O: Functionally active complement proteins C6 and C7 detected in C6- and C7-deficient individuals. *Clin Exp Immunol* 83:430–437, 1991

20. BHAKDI S, FASSBENDER W, HUGO F, CARRENO MP, BERSTECHER C, MALASIT P, KAZATCHKINE MD: Relative inefficiency of terminal complement activation. *J Immunol* 141:3117–3122, 1988

21. WYATT RJ, KANAYAMA Y, JULIAN BA, NEGORO N, SUGIMOTO S, HUDSON EC, CURD JG: Complement activation in IgA nephropathy. *Kidney Int* 31:1019–1023, 1987

22. LAGRUE G, BRANELLEC A, INTRATOR L, MOISY M, SOBEL A: Evaluation du C3 days dans les nephropathies glomerulaires chroniques primitives. *Nouv Presse Med* 8:1153–1156, 1979

23. BRENCHLEY PE, COUPES B, SHORT CD, O'DONOGHUE DJ, BALLARDIE FW, MALLICK NP: Urinary C3dg and C5b-9 indicate active immune disease in human membranous nephropathy. *Kidney Int* 41:933–937, 1992

24. SØLLING J: Circulating immune complexes and complement breakdown product C3 days in glomerulonephritis and kidney transplantation. *Acta Pathol Microbiol Immunol Scand* 92:213–220, 1984

25. SHULMAN ST, BARRATT TM, SOOTHILL JF: Immunoconglutinin and complement studies in congenital nephrotic syndrome and nephritis of Henoch-Schönlein purpura in children. *Arch Dis Child* 46:838–841, 1971

26. JULIAN BA, WYATT RJ, MCMORROW RG, GALLA JH: Serum complement proteins in IgA nephropathy. *Clin Nephrol* 20:251–258, 1983

27. GARCIA-FUENTES M, MARTIN A, CHANTLER C, WILLIAMS DG: Serum complement components in Henoch-Schönlein purpura. *Arch Dis Child* 53:417–419, 1978

28. OPPERMANN M, KURTS C, ZIERZ R, QUENTIN E, WEBER MH, GÖTZE O: Elevated plasma levels of the immunosuppressive complement fragment Ba in renal failure. *Kidney Int* 40:939–947, 1991

29. ABOU-RAGHEB HHA, WILLIAMS AJ, BROWN CB, MILFORD-WARD A: Plasma levels of the anaphylotoxins C3a and C4a in patients with IgA nephropathy/Henoch-Schönlein purpura. *Nephron* 62:22–26, 1992

30. MOLLNES TE, GARRED P, BERGSETH G: Effect of time, temperature and anticoagulants on in vitro complement activation. Consequences for collection and preservation of samples to be examined for complement activation. *Clin Exp Immunol* 73:484–488, 1988

31. KAWANA S, NISHIYAMA S: Serum cC5b-9 (terminal complement complex) level, a sensitive indicator of disease activity in patients with Henoch-Schönlein purpura. *Dermatology* 184:171–176, 1992

32. NEELAKANTAPPA K, GALLO GR, BALDWIN DS: Proteinuria in IgA nephropathy. *Kidney Int* 33:716–721, 1988

33. DONADIO JV, BERGSTRALH EJ, OFFORD KP, SPENCER DC, HOLLEY KE: A controlled trial of fish oil in IgA nephropathy. *N Engl J Med* 331:1194–1199, 1994

34. JENNETTE JC, WIESLANDER J, TUTTLE R, FALK RJ: Serum IgA-fibronectin aggregates in patients with IgA nephropathy and Henoch-Schönlein purpura: Diagnostic value and pathogenic implications. *Am J Kidney Dis* 18:466–471, 1991

35. BALDREE LA, WYATT RJ, JULIAN BA, FALK RJ, JENNETTE JC: Immunoglobulin A-fibronectin aggregate levels in children and adults with immunoglobulin A nephropathy. *Am J Kidney Dis* 22:1–4, 1993

36. TOMINO Y, FUNABIKI K, OHMURO H, SHIMIZU M, YOKOYAMA K, SHIRATO I, SHIRAI T, TAKAHASHI M, KOIDE H: Urinary levels of interleukin-6 and disease activity in patients with IgA nephropathy. *Am J Nephrol* 11:459–464, 1991

37. GORDON C, RICHARDS N, HOWIE AJ, RICHARDSON K, MICHAEL J, ADU D, EMERY P: Urinary IL-6: A marker for mesangial proliferative glomerulonephritis? *Clin Exp Immunol* 86:145–149, 1991

38. DOHI K, IWANO M, MURAGUCHI A, HORII Y, HIRAYAMA T, OGAWA S, SHIIKI H, HIRANO T, KISHIMOTO T, ISHIKAWA H: The prognostic significance of urinary interleukin 6 in IgA nephropathy. *Clin Nephrol* 35:1–5, 1991