CONCISE REPORT

Deficiency of complement-dependent prevention of immune precipitation in systemic sclerosis

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Background: Previous studies have indicated that complement may be activated or inherently abnormal in systemic sclerosis (SSc), and it has been suggested that immune complex deposition plays a part in the microvascular damage of this disease.

Objective: To study several aspects of the complement system in 24 patients with SSc.

Methods: Complement dependent prevention of immune precipitation (PIP) was measured by a sensitive enzyme immunoassay, levels of C1q, C4, and C3 by rocket immunoelectrophoresis, C4 allotypes by high voltage agarose electrophoresis, and C4A, C4B, and C3d by an enzyme linked immunosorbent assay (ELISA).

Results: PIP was markedly decreased in the patients with SSc ($p < 0.001$). Abnormal complement activation was detected in nine patients as raised levels of the complement split product C3d. However, a relation between low PIP and complement activation was not seen. PIP was significantly lower in patients who carried the C4A*Q0 allotype ($p = 0.03$), and a strong correlation was found between PIP and C4A concentration ($p < 0.00001$). The PIP defect may, at least in some patients, be associated with the initial phase of the disease.

Conclusions: The results show a previously unrecognised functional defect of complement in SSc; the defect correlates with low levels of classical pathway components and, in particular, C4A.

Systemic sclerosis (SSc) is a connective tissue disease (CTD) characterised by fibrosis of skin and internal organs, with skin thickening either restricted to distal extremities and face (limited disease), or affecting also proximal extremities or trunk, or both (diffuse disease).¹ In diffuse SSc, visceral involvement tends to occur earlier and more aggressively. Disease activity tends to be highest initially and is associated with microvascular damage—that is, cold- and stress-induced digital vasospasm followed by vascular occlusion, intimal thickening, and endothelial proliferation associated with perivascular infiltration of mononuclear cells. The advanced stage is characterised by hyperactive fibroblasts which produce proteoglycan and collagen in an uncontrolled manner.

Patients with defective function of classical complement pathway components have an increased incidence of CTD and this is thought to be due to impaired immune complex clearance.² The classical pathway is essential for efficient removal of large immune complexes,³ which otherwise could induce vasculitis after endothelial deposition; C1 and C4 appear to be especially important in this respect.²

A strong association has been found between C4A null alleles (C4A*Q0) and SSc.⁴ C4 exists as two distinct proteins, C4A and C4B. In vitro findings indicate that C4A may be more efficient than C4B in binding to immune complexes and promot-

ing their clearance from the circulation,⁵ and this has been implicated as the cause of the association of C4A*Q0 with autoimmune CTD.²

Microvascular damage is an early and fundamental feature of SSc, but the cause is unknown.¹ Several studies have reported abnormal complement activation and subendothelial deposition of immune complexes in patients with SSc.^{6–9} We have developed a simple, sensitive, and reproducible assay for measuring complement dependent prevention of immune precipitation (PIP).¹⁰ This study aimed at monitoring this and other aspects of complement in 24 patients with SSc who we have followed up over several years.

PATIENTS AND METHODS

Patients

The patients (21 female, three male) were aged 15–85 years, with a disease history of a few months to 20 years; 18 had limited and six had diffuse SSc. All except two had relatively mild disease and only one had renal disease. Patients were evaluated clinically and immunologically when recruited to the study and then followed up for up to nine years. One hundred blood donors served as controls.

The PIP assay

PIP was measured by an enzyme immunoassay.¹⁰ Briefly, alkaline phosphatase (AP, 1/50, 5 μ l) and goat anti-AP (1/5, 5 μ l) were added to serum (40 μ l), and after incubation (37°C, one hour) and centrifugation (5500 $\times$ g, 10 minutes), supernatants were diluted 1:10 in phosphate buffered saline and allowed to react with substrate (*p*-nitrophenylphosphate). Absorbance was converted to arbitrary units (AU) by comparison with a serially diluted reference serum, defined as 100 AU. This assay is sensitive to minor variations within and below the normal range, and assay variability was <10%.

CH₅₀ and complement components

C1q, C4, and C3 were measured by rocket immunoelectrophoresis, and total haemolytic complement (CH₅₀) by standard methods. Complement activation was assessed by monitoring C3d levels by an ELISA, using rabbit anti-C3d for coating and AP-conjugated anti-C3d for catching.¹¹ Absorbance was converted to AU by comparison with zymosan activated reference serum, defined as 100 AU. Specificity for C3d was ensured by precipitating larger C3 fragments from EDTA-treated sera with 22% polyethylene glycol before adding them to the microtitre plate wells.¹¹

Abbreviations: AP, alkaline phosphatase; AU, arbitrary units; C4*Q0, C4 null allele; CTD, connective tissue disease; ELISA, enzyme linked immunosorbent assay; PIP, prevention of immune precipitation; SSc, systemic sclerosis

Figure 1 Complement dependent prevention of immune precipitation in patients with SSc compared with blood donors. The dotted line denotes the 95% lower cut off limit.

C4 allotypes

C4 allotypes were determined by high voltage agarose electrophoresis of carboxypeptidase and neuraminidase treated samples, with subsequent immunofixation and staining.¹² Null alleles were determined by visual scoring of the relative intensities of C4A and C4B bands.

C4A and C4B measurements

Levels of C4A and C4B were measured by an ELISA, using goat anti-C4 for coating and mouse antihuman C4A (RgD1) or C4B

(1228) followed by AP conjugated rabbit antimouse immunoglobulin for catching.¹³ The results were expressed in g/l by comparison with serially diluted reference plasma samples from genotypically homozygous C4A or C4B deficient probands; the concentration of their total C4 had previously been determined twice by rocket immunoelectrophoresis.

Reagents

The monoclonal antibodies (RgD1 and 1228) were a kind gift from Dr Peter Schneider. AP was from Sigma, anti-AP from Cappel, and all other antibodies from Dako.

RESULTS

PIP

Complement dependent PIP was found to be markedly defective in the patients with SSc ($p < 0.001$), and 12 patients were below the lower normal limit (fig 1). No difference was found between patients with limited and diffuse disease.

Complement components

The functional defect found in the PIP assay did not result from a major complement deficiency detected by CH_{50} , C4, C1q, or C3 measurements (figs 2A–D). Levels of C3d were raised in nine patients (fig 2E) but were not associated with low PIP.

C4 allotypes

C4A or C4B null alleles were found in 13 patients, including one with homozygous C4A*Q0. However, the allelic frequencies were not significantly different from those found in an Icelandic population (Arason GJ, unpublished data). PIP was significantly lower in patients with C4A*Q0 than in the remaining patients ($p = 0.03$), whereas this function was normal in patients carrying C4B*Q0 (Fig 3).

Changes in PIP during patient follow up

Levels of PIP were measured in serum samples collected serially from 15 of the patients. An increase in PIP was seen in 10 patients during the observation time (nine years) and this was associated with diminished disease activity (data not shown).

Figure 2 Total haemolytic complement and levels of C4, C1q, C3, and C3d in patients with SSc. The dotted line denotes the 95% lower cut off limit, except in the last frame (C3d), where it denotes the 95% upper cut off limit. Open circles denote patients with subnormal PIP.

Figure 3 Association of PIP with the presence of C4A*Q0 in patients with SSc. The dashed line denotes the 95% lower cut off limit. Open circles denote patients with C4B*Q0.

One patient had reduced PIP in three samples taken 0.5, 1, and 2 years before she was diagnosed with SSc. Interestingly, her identical twin sister also had subnormal PIP with no other complement abnormalities. One patient relapsed during the study period, and this coincided with a decrease in PIP. Four patients had low levels of PIP in all samples studied, and three of these carried C4A*Q0.

Correlation statistics

PIP was positively correlated with levels of total C4, C1q, and C3 (in that order) and was much more sensitive to variations in these components than to variation in CH₅₀ (table 1). In an attempt to assess further the importance of C4A in PIP, levels of C4A and C4B were measured in all available samples and compared with the other variables measured. PIP was very strongly correlated with levels of C4A ($p < 0.00001$) but not C4B.

DISCUSSION

Microvascular lesions are thought to have a central pathogenic role in SSc.¹⁻⁹ The mechanism of the vascular damage has not been clarified, but deposition of immune complexes and complement activation has been implicated.^{6-9, 14} A defect in the ability to prevent the formation of large immune complexes might account for these findings. Our results indicate that PIP may be severely impaired in SSc, even when no complement abnormalities are found by conventional methods. As the defect was especially prominent in the early, active phase of the disease, this suggests a role in pathogenesis. Our data and recent reports¹⁵ emphasise the advantage of functional rather than quantitative assays in complement research, and PIP appears to be ideally sensitive in this respect compared with the other available assay, CH₅₀.

Our results suggest that low levels of the C4A protein may contribute to the PIP defect, and this is consistent with the suggested functional differences between the two C4 allotypes—namely, C4A binds more readily to immune complexes whereas C4B binds preferentially to hydroxyl groups on cell membranes, including erythrocytes.⁵ This may also form the basis for the observed association of SSc with C4A*Q0 in some populations.⁴ Interestingly, PIP is not decreased in healthy subjects with C4A*Q0 (Arason GJ, unpublished data), suggesting a contribution from an unknown factor besides C4A. Possibly, the combination of relatively low C4A and C1q may explain this difference.

The relation between low C4A, reduced PIP, complement activation, and immune complex formation is not clear. The defect is at least partially inherent (that is, related to the presence of C4A*Q0), and low PIP might contribute to the initiation of SSc in these patients; it should be noted that PIP was reduced in one patient before her disease was diagnosed as well as in her symptom-free identical twin sister. The defect may be secondary in other patients (perhaps related to transient C4A depletion during an infection) as suggested by patient follow up values and a stronger correlation with C4A levels than C4A*Q0. In any case, defective PIP is likely to take part in a vicious circle involving immune complex deposition and C4A depletion, and thus may promote either disease initiation or progression depending on the nature of the defect.

Our study supports the notion that complement plays a part in the pathogenesis of SSc, and that patients with active disease have impaired complement mediated clearance of immune complexes. The possibility that this defect may contribute to the cause of SSc will be considered in future studies.

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Table 1 Correlation matrix*

	PIP	C1q	C4	C3	C4A	C4B
CH ₅₀						
Corr. coeff.	(0.28)	0.39	0.38	(0.32)	(0.27)	0.26
p Value	(NS)	0.02	0.02	(NS)	(NS)	0.04
PIP						
Corr. coeff.	—	0.43	0.44	0.38	0.65	(0.19)
p Value	—	0.005	0.004	0.01	4×10^{-7}	(NS)
C1q						
Corr. coeff.	—	—	0.676	0.539	(0.26)	0.34
p Value	—	—	2×10^{-5}	0.001	(NS)	0.03
C4						
Corr. coeff.	—	—	—	0.46		
p Value	—	—	—	0.003		

*As determined by pairwise comparison of measurements of 48 samples from 24 patients with SSc.

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