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Detection and characterization of immunoconglutinins in patients with systemic lupus erythematosus (SLE): serial analysis in relation to disease course

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SUMMARY

The levels of IgA, IgG and IgM immunoconglutinins (IK) were assessed in sera from 20 patients with SLE which were followed for 8-month periods. At the time of the exacerbation, IgG IKs were significantly increased to 226 ± 90 arbitrary units (mean ± s.e.m.) compared with both the minimum value of 75 ± 28 in the SLE patients and with 31 ± 2 in healthy controls ($P < 0.05$). There was no difference between SLE patients and controls in the levels of IgM and IgA IKs. Most of the SLE patients in this material showed maximal IgG IK levels before exacerbation, but there was no correlation between the clinical disease index and the levels of IgG IK. The specificity of IgG IKs showed a broad diversity for microtitre-fixed C3b, iC3b, C3c and C3dg. The antibodies were of IgG1, IgG3 and in two patients, IgG4 subclass. IgG IKs were correlated to the C3d/C3 ratio which suggested that the IK responses were secondary to C3 activation. In summary, unlike other conditions associated with complement activation where elevated IgM IKs are common, an increase in IgG IK levels was observed. It is possible that this diverging IK response contributes to the pathophysiology of the disease.

Keywords immunoconglutinins IgG IgM complement activation systemic lupus erythematosus

INTRODUCTION

Immunoconglutinins (IK) are autoantibodies against C3 or C4 fragments. Earlier studies have shown that IKs specifically agglutinate particle-bound complement fragments. The agglutination is unaffected by the fluid-phase native protein and its fragments. By using agglutination techniques, IKs have been reported to be mainly of IgM class, but, in addition, both IgG and IgA class have been detected [1]. Elevated levels of IgG IK have predominantly been demonstrated by ELISA [2]. IKs increase in response to acute or chronic inflammations involving complement activation, e.g. infections, and a variety of diseases of autoimmune origin [1,3] such as Crohn's disease, rheumatoid arthritis (RA) [4] and SLE [2].

Analysis of a limited number of sera with high levels of C3-specific IgG IKs from patients with SLE showed that the majority bound to the C3c fragment, some to the C3dg fragment, but none to the C3d fragment [5]. Mapping of the IK epitopes was hampered by the observation that IKs bound poorly to denatured or reduced forms of C3 or C3 fragments.

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The possible functional role of three affinity-purified IgG IKs from the above mentioned SLE patients was also investigated [5]. Two of them, which were specific for C3c, were able to down-regulate the C5-convertase function, to inhibit all three cleavages by factor I in the α'-chain of C3b and to reduce the factor I-mediated release of complement-coated immune complexes from CR1. In contrast, a third IK specific for C3dg only inhibited the third factor I-mediated cleavage. Taken together, these results show that IKs can bind to at least two regions in the C3 molecule as indicated by their binding specificity and their impact on C3 function. The results indicated that IKs might have a potential regulatory role in inflammatory processes.

The purpose of the present study was to further determine the subclasses of C3-specific IgG IKs in SLE patients and to monitor the IK levels in serially followed SLE patients.

PATIENTS AND METHODS

Patients and controls

Twenty patients with SLE taking part in a prospective control programme at the Department of Rheumatology, University Hospital, Lund, Sweden, were studied [6]. The patients were

followed for 8-month periods during which the patients suffered from at least one exacerbation of the disease. During the observation periods the patients were seen five times at regular 2-month intervals for clinical evaluation and blood tests. In patient EB the interval between visit two and three was 6 months. Ten female and ten male healthy blood donors were used as controls.

A validated index (SLEDAI) was used for assessment of global disease activity. The index was slightly modified by exclusion of laboratory items (complement and DNA binding) to allow comparison of clinical disease activity with complement data. The maximal possible score of SLEDAI was therefore reduced from 103 to 101, but in validation studies the scores rarely exceed indices of 25 [7].

Serum and EDTA plasma samples were drawn at each visit and harvested within 2 h of bleeding and stored at −70°C.

Levels of C3, C4, C1q and the C3d fragment in human plasma

Quantitative immunochemical analysis of C3 and C4 was performed by single radial immunodiffusion [8]. C3d was assessed by ELISA according to a modification of the technique used by Bourke *et al.* [9]. C1q was measured by 'rocket immunoelectrophoresis' according to Laurell [10].

Purified proteins

C3 was purified as described by Hammer *et al.* [11]. Any remaining IgG was removed by affinity chromatography on protein A Sepharose (Pharmacia AB, Sweden). C3b, iC3b, C3c and C3dg were prepared on Thiol-Sepharose (Pharmacia AB, Sweden) as was previously described [12]. Purified IgG1, IgG2, IgG3 and IgG4 were the WHO standard proteins prepared for the WHO immunoglobulin subcommittee (Bern, Switzerland).

ELISAs

Quantification of IKs in human serum. Wells of microtitre plates were coated with 2 pmol of C3 overnight at 4°C. EDTA plasma (100 μl), diluted 1:50 in PBS containing 1% (w/v) bovine serum albumin (BSA) and 10 mM EDTA, was incubated for 60 min at room temperature. The binding of human IgA, IgG and IgM antibodies was detected by anti-human IgA, IgG or IgM, respectively, conjugated with HRP (Dako A/S, Denmark) as described previously [5]. A serial dilution of a serum from a patient with mononucleosis with high levels of both IgG and IgM IKs, was used to construct a standard curve. The levels of IKs are given as arbitrary units, assuming that the standard serum contained 100 000 arbitrary units of IgG and IgM IKs/ml serum.

Binding specificity of IgG IKs to C3 fragments. This ELISA was a modification of the one described above. Wells of microtitre plates were coated with 2 pmol of C3b, iC3b, C3c or C3dg, respectively. The binding was detected with anti-IgG conjugated with HRP.

Subclass specificity of IgG IKs. Wells of microtitre plates were coated with 2 pmol of C3 overnight at 4°C. EDTA plasma (100 μl), diluted 1:50 in PBS containing 1% (w/v) BSA, was incubated for 60 min at room temperature. After washing, the wells were incubated with anti-IgG1 (clone HP6001), anti-IgG2 (clone GOM1), anti-IgG3 (clone ZG4) and anti-IgG4 (clone RJ4) MoAbs (Oxoid/Unipath, UK) for 60 min at room temperature. The binding of MoAbs was detected by anti-mouse immunoglobulins conjugated with HRP (Dako). Standard curves were constructed from serially diluted purified IgG1, IgG2, IgG3, IgG4, respectively, which were allowed to bind to anti-human IgG (3 μg/ml) adsorbed to the microtitre wells.

Table 1. The activity index (SLEDAI) and the levels of IgG immunoconglutinins (IK) and IgM IK of 20 SLE patients at exacerbation

Patient	SLEDAI*	IgG IK†	IgM IK†
AM 59	12	380	43
HG 55	15	170	73
MV 55	25	290	112
TG 51	6	73	26
ACJ 48	7	100	675
IB 28	16	175	15
SA 39	16	63	22
SD 75	12	43	360
KM 11	16	36	115
LD 59	12	28	32
BP 32	10	78	22
CL 73	13	1850	37
UO 44	9	93	400
KI 15	7	175	360
MA 25	7	32	53
SA 48	4	42	68
EB 56	7	55	100
IW 54	2	175	200
MW 53	10	240	32
SK 41	2	152	15

* At exacerbation, defined as maximum activity index (SLEDAI).

† Arbitrary units at maximum SLEDAI.

Statistical analysis

Mean ± s.e.m. are given. Student's *t*-test and the Spearman rank correlation were used.

RESULTS

Longitudinal monitoring of IgA, IgG and IgM IKs in patients with SLE

Twenty patients with SLE were followed during a period of 8 months. During this time, the patients suffered at least one exacerbation of the disease. The maximal SLEDAI and the linked IK values are shown in Table 1. During definite flares the median SLEDAI was 11 (range 2–25).

Levels of IgA, IgG and the IgM IKs with C3 specificity, were aligned according to the major exacerbation. At the time of the exacerbation, IgG IKs were significantly increased to 226 ± 90 arbitary units (mean ± s.e.m.) compared with both the minimum value of 75 ± 28 in the SLE patients and with 31 ± 2 in healthy controls ($P < 0.05$). IgM IK values in the SLE patients (166 ± 56 arbitrary units) did not differ from those of the controls (138 ± 39 arbitrary units). IgA IKs could be detected neither in the controls nor in the SLE patients.

Individual differences in the serial IgG IK levels are shown in Fig. 1. In most patients (17/20) the highest IgG IK concentrations were found before or during flares. In three patients the highest levels of IgG IK were found shortly after the exacerbation. Although there was no significant difference in the mean

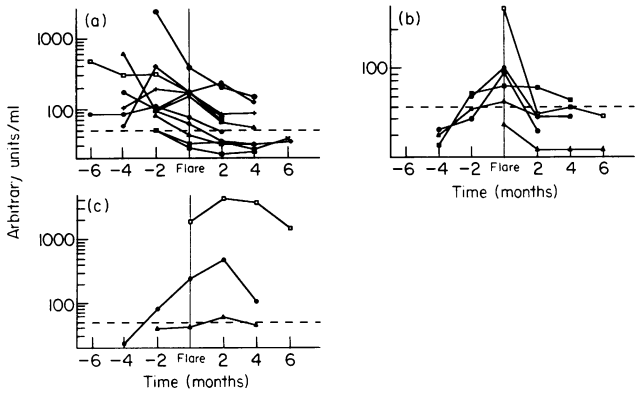

Fig. 1. Changes in IgG immunoconglutinin (IK) levels in 20 SLE patients observed during 8-month periods. Data are aligned after the time of maximum SLEDAI, shown in Table 1, which was defined as the flare. The patients were divided into three categories. In the first (a) which contained 11 patients, the levels of IKs were higher before the exacerbation; in the second group consisting of six patients (b) the levels were at a maximum during the exacerbation and in a third group containing three patients (c), the levels were higher after the exacerbation. The horizontal line in each figure represents the mean + 2 s.d. in 20 healthy individuals.

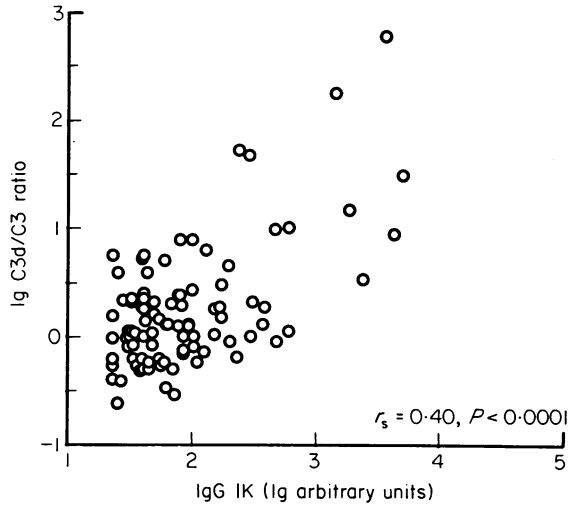

Fig. 2. The correlation between 10_{log} IgG immunoconglutinin (IK) levels and the 10_{log} C3d/C3 ratio.

IgM IK levels before, during or after the exacerbation, the IgM IKs exhibited a different kinetics in that only four patients had increased IgM IK levels before the exacerbation while 11 patients had elevated levels after.

Correlation between the IgG IK levels, activity index and complement activation

No significant correlations were obtained betwen IgG IKs and the concentration of the complement components C1q, C3, C4 or between IgG IKs and the disease activity index SLEDAI. However, IgG IK levels were correlated with the C3d/C3 ratio (r_s = 0·40, P < 0·0001) (Fig. 2).

Specificity of IgG IKs for different C3 fragments

From each patient the serum with the highest IgG IK value was selected for further investigation with regard to binding specificity. The sera were allowed to bind to microtitre plate-fixed C3b, iC3b, C3c and C3dg. In Table 2 it is shown that the 20 different specimens contained IgG IKs with diverse specificities for C3 fragments. The binding was considered positive if the A_{492} was > 0·1. The maximal absorbance observed was 0·8. All IgG IKs recognized C3b, iC3b and/or C3c and six bound to C3dg. The latter detected both C3c and C3dg, indicating recognition of two domains in the molecule. IK in two sera only bound to C3b.

Subclass specificity of IgG IKs

IKs in the 20 sera investigated were allowed to bind to microtitre-fixed C3. Binding was detected with MoAbs to IgG1, IgG2, IgG3 or IgG4. The cut off level was selected at 10 ng/ml. All samples contained IgG1 IKs. In six sera the presence of IgG1 IK was combined with IgG3 IKs and in two with IgG4 IKs, respectively (Table 2). The highest IgG1 IK level was 78 ng/ml. Corresponding values for IgG3 IKs were 17 ng/ml and for IgG4 IKs 13 ng/ml. One sample contained IgG2 IKs in amounts just above the cut off level.

DISCUSSION

Serial analysis of IgG IKs showed that the highest levels of IgG IKs appeared before or during the exacerbation of the disease. This pattern is not uncommon for other autoantibodies, e.g. anti-dsDNA [13]. In the case of IKs, complement activation that is often present early in the SLE exacerbation, is a potential trigger of IK response. A reasonably strong correlation was found between the ratio C3d/C3 and the levels of IgG IKs, suggesting that C3 activation products are the triggering antigens. However, no correlation was found with C1q, C4, and C3. Several reasons might explain why the correlation is not stronger. First, complement activation is present during the whole exacerbation even when IK levels are decreasing, and second, the assessed levels of IKs varied extensively between individuals, which partly can be explained by different IK specificity for different conformational forms of the C3 molecule.

The subclass determination of the IgG IKs showed restriction to IgG1 and IgG3, and for two patients, to IgG4. Restriction of the autoantibody response to a limited number of subclasses has been reported in other autoimmune diseases and the presented subclass constellation is common [14–18]. IgG1 and IgG3, but not IgG4, activate complement, which suggests that there is a difference in functional properties of these antibody categories. While IKs of the IgG4 subclass will have functional properties restricted to its antigen and Fc receptor binding sites, IgG1 and IgG3 will also be able to amplify complement activation [19].

The impression in our previous study that the individual IK response was at least oligoclonal was supported not only by the above mentioned subclass diversity but also by the observation that IKs in some specimens bound concomitantly to C3c and C3dg. Furthermore, the difference in binding of individual IKs to C3b, iC3b, C3c and C3dg suggests that the epitopes of the individual IKs differ extensively. Different specificity for C3b, iC3b and C3c, which all contain the C3c region, also shows that

Table 2. Binding to microtitre-fixed C3 fragments and subclass composition of individual IgG immunoconglutinins (IKs) measured by ELISA

	C3 fragments				IgG subclasses			
Patients	C3b*	iC3b*	C3c*	C3dg*	IgG1†	IgG2†	IgG3†	IgG4†
AM 59	+	+	+	+	+		+	
HG 55	+	+	+	+	+			
MV 55	+	+	+	+	+		+	
TG 51	+	+	+	+	+			
ACJ 48	+	+	+		+		+	
IB 28	+	+	+		+			
SA 39	+	+	+		+			+
SD 75	+	+	+		+			+
KM 11	+				+			
LD 59	+				+			
BP 32		+	+	+	+			
CL 73		+	+	+	+		+	
UO 44		+	+	+	+			
KI 15		+	+		+			
MA 25		+	+		+			
SA 48		+	+		+			
EB 56		+	+		+	+		
IW 54			+		+		+	
MW 53			+		+			
SK 41			+		+		+	

* A_{492} values > 0·1 are indicated with +.
† Values > 10 ng/ml are indicated with +.

the epitopes are highly sensitive to conformational changes in the molecule.

By using agglutination techniques which preferentially detect IgM antibodies, previous investigators have reported that a substantial number of normal blood donors have detectable levels of IKs [1]. This is a finding that is confirmed in this study where IgM IKs, unlike IgA IKs, are detected in normal individuals by ELISA.

Several studies have also shown that the levels of IgM IKs are elevated but quickly returned to normal without evidence of a secondary antibody response, during infections and exacerbations of autoimmune diseases in humans or after repeated i.v. injections of various bacteria in animals [1,20]. In contrast to other autoimmune diseases, SLE patients have previously been shown to exhibit a decrease in the agglutination titre of IKs during an acute exacerbation, although the titre remains higher than in normal individuals [20]. By using an ELISA technique, we find no increase in the levels of IgM IKs in the SLE patients, but supporting the previous finding, the IgM IK levels tended to be higher after the exacerbation.

A possible explanation for the unexpected decrease in the agglutination titre and the low IgM IK levels during exacerbation of SLE is the significant increase in the levels of IgG IKs. The IgM IK agglutination and the binding of IgM IKs in the IgM IK ELISA might be inhibited by the presence of the high affinity IgG IK antibodies. This mechanism suggests that high IgG IK levels before the exacerbation might conceal a possible increase in the IgM IK titre and that the detected increase in IgM IKs after the exacerbation is a result of a decreasing IgG IK level.

Thus, unlike other conditions which involve repeated complement activation, SLE presents an IK profile consistent with a secondary antibody response with high levels of IgG and possibly IgM IKs, against bound C3 fragments. Since C3 activation fragments have been shown to be important in the generation of a normal antibody response [21], this might be the result of the repeated and very pronounced complement activation typical of the disease.

As previously reported, IgM IKs are present in normal healthy individuals and the levels increase in response to inflammation involving complement activation. This might represent a normal physiological process which regulates complement functions. In SLE patients, however, it seems to be a combined IgM and IgG response, thereby modifying the functional properties of the generated IKs. It is conceivable that this diverging response is involved in the pathophysiology of the disease.

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