Circulating immune complexes in diabetics with severe microangiopathy: evaluation by two different methods

Domenico Andreani, Umberto Di Mario, Clara Galfo, Luigi Ventriglia and Mario Iavicoli

Department of Endocrinology, University of Rome, Italy

Abstract. An investigation on circulating immune complexes (AgAb) was carried out in 80 diabetics with severe microangiopathy and in 71 diabetics without microvascular lesions.

The duration of the disease, the type of diabetes, the type of treatment and the main localization of microangiopathy (retinopathy and nephropathy) were taken into account. AgAb were detected by two different methods: the solid phase Clq binding test (ClqSP) and the conglutinin binding test (KgBt).

AgAb detected by ClqSP were increased both in prevalence and quantities in diabetics with severe microangiopathy regardless of the duration of the disease and the type of diabetes. Long standing diabetics without microangiopathy had similar prevalence of AgAb as normal controls. The presence of AgAb was not in correlation with the type of treatment and was similar in diabetics with retinopathy and in those with nephropathy. When AgAb were detected by KgBt, they were found with higher prevalence in diabetics than in normal controls but no correlation with microangiopathy was observed. AgAb, detected by KgBt, were higher in long standing type 1 diabetics.

Since the two methods detect different AgAb it is concluded that AgAb present in diabetics seem to be heterogeneous and part of them are related to the presence of microangiopathy.

Much controversy still exists regarding the mechanism and factors involved in the pathogenesis of diabetic microangiopathy. Whilst various processes have been shown to be involved in diabetic microvascular lesions, certain findings appear to be of particular interest: vascular lesions present in immune complex diseases share morphological aspects with those observed in diabetes (Blumenthal et al. 1964; Bloodworth 1968; Andreani et al. 1971), diabetic like vascular lesions can be experimentally induced by immune mechanisms (Mohos et al. 1963; Zampa & Mancini 1965; Andreev et al. 1970), circulating immune complexes have been described in diabetics (Irvine et al. 1977; Kumar & Quismorio 1978; Ludwig et al. 1978; Delespesse et al. 1980) mainly in those with retinopathy (Irvine et al. 1978b; Bodansky et al. 1980).

In this study the presence of circulating immune complexes (AgAb) in serum of diabetics has been investigated in order to establish the correlation between AgAb and severe microangiopathy, either proliferative retinopathy or advanced nephropathy. Attention has been focused especially on patients with long standing diabetes not presenting microangiopathy. The influence of the type of diabetes and of the antidiabetic treatment on AgAb levels was also studied. Since AgAb are heterogeneous and different methods detect different types of complexes, we used two sensitive methods differing in principle to detect them.

Materials and Methods

Selection of patients

One hundred and fifty-one diabetics were selected according to the presence of microangiopathy and the

Address of principal author: Prof. Domenico Andreani, Endocrinologia (II Clinica Medica), Policlinico Umberto I°, 00161 Rome, Italy.
duration of disease among those attending our Diabetic Out-Patient Department.

Since many factors theoretically might influence the levels of AgAb in diabetics, such as the duration of diabetes and the type of treatment (Di Mario et al. 1981), to minimize the interference of other variables, the relation between the presence of microangiopathy and AgAb positivity was studied in diabetics in 2 quite different, well defined and rather uncommon conditions.

At first we studied patients who after many years of diabetes showed no sign of diabetic complications and we compared them with diabetics who at the same distance from diagnosis showed proliferative retinopathy or severe nephropathy. With this purpose 92 diabetics at more than 15 years after diagnosis were studied: (Group I) 31 had no sign whatsoever of diabetic microangiopathy whereas 30 showed proliferative retinopathy without marked nephropathy and 25 had severe nephropathy with various degrees of retinopathy.

Thereafter diabetics with apparently a precocious onset of severe retinopathy were studied and their levels of AgAb compared with those found in diabetics with no sign of diabetic complications at similar distance from diagnosis. Fifty-nine patients were assembled for this investigation: (Group II) 19 diabetics manifested the onset of severe retinopathy within 3 years of diagnosis whereas 40 had no sign of microangiopathy at comparable distance from diagnosis.

Diabetics presenting slight microangiopathy or patients with chronic disorders with primary or secondary immune aspects were excluded from the investigation.

Personal and clinical data of the patients are shown in Table 1. Part of the patients were type I diabetics and the remainder type II patients. Type II diabetics with an overweight of more than 20% of their ideal body weight were not included in the study in order to minimize the number of variables.

One hundred and eighty-nine blood donors, age range 18–59, were considered as controls for the evaluation of the limit of positivity in AgAb assays.

**Evaluation of microangiopathy**

The presence and degree of retinopathy were assessed in all the diabetics by retinal fluorescein angiography and the results were evaluated by an ophthalmologist. Patients with severe retinopathy, included in the study, showed either proliferative retinopathy or vitreal haemorrhages. Nephropathy was evaluated on the basis of BUN, serum creatinine levels and proteinuria. BUN > 50 mg/100 ml, serum creatinine levels > 2 mg/100 ml, and/or proteinuria > 2 g/l were considered as an index of the kidney involvement.

**Detection of AgAb**

The solid phase Clq binding test (ClqSP) (Hay et al. 1976) and the conglutinin binding test (KgBt) (Casali et al. 1977) were used to detect circulating AgAb.

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### Table 1

<table>
<thead>
<tr>
<th>Group of patients</th>
<th>Type I diabetics</th>
<th>Type II diabetics</th>
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<td>No. of patients</td>
<td>Age mean (years)</td>
<td>No. of patients</td>
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<tr>
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<td>8</td>
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<td>40</td>
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**Presence of microangiopathy**

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<tbody>
<tr>
<td>M</td>
<td>Without microangiopathy</td>
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<tr>
<td>I</td>
<td>40</td>
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<tr>
<td>II</td>
<td>40</td>
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**Insulin treated**

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<th>Age mean (years)</th>
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<tr>
<td>16</td>
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<tr>
<td>23.1 (9.4)**</td>
<td>42.5 (13.2)**</td>
<td>55.4 (10.2)**</td>
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**No. of patients**

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**Sex**

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* Years. ** Standard deviation.
ClqSP is based upon the affinity of the first component of complement (Clq), bound to polystyrene tubes, for the Fc region of complexed immunoglobulins. Clq was isolated as described by Yonemasu & Stroud (1971). The ability of of staphylococcal protein A, labelled with $^{125}$I, to interact with IgG1,2,4 was used to reveal AgAb bound to Clq.

The affinity of conglutinin, fixed to polipropylene tube walls, for the complex bound C3 is the basis of KgBt. Conglutinin a bovine protein, was isolated according to Casali et al. (1977). Again $^{125}$I-labelled staphylococcal protein A was used to reveal tube bound AgAb.

These methods reveal different types of complexes: Clq detects mainly medium size AgAb in antigen excess whereas conglutinin detects large size AgAb near the equivalence point. As already known in most cases the concordance between these two techniques is rather low (Lambert et al. 1978).

As in most studies on circulating AgAb (Hay et al. 1976; Theophilopoulos et al. 1976; Casali et al. 1977; Irvine et al. 1978a,b) quantitation in both methods was obtained by reference of sample duplicates to the uptake of serially diluted heat aggregated immunoglobulins G (AHG). Results were expressed in micrograms of aggregate equivalents per millilitre of undiluted serum (μg AHG Eq/ml).

Since the values of AgAb in the normal population show a positive peaked asymmetric distribution, we have chosen the 90th percentile of the values of the normal population as the limit of positivity (Irvine et al. 1978a; Di Mario et al. 1980). In ClqSP all blood donor samples were assayed in the same experiment. Out of these samples ten sera were chosen to statistically represent the entire blood donor population; their median and inter-quartile range were the same of the total group and one value was above the limit of positivity. Aliquots kept frozen of these ten sera were chosen to statistically represent the entire blood donor population; their median and inter-quartile range were the same of the total group and one value was above the limit of positivity. Aliquots kept frozen of these ten sera were then assayed in the same experiment and only an inter-assay variability of their values, calculated in per cent of the maximum bound, of less than 10% was tolerated.

The selection of patients and the laboratory assays were done by two independent researchers and so the sera were in a random order in the experiments. When a difference of more of 10% in duplicate results was found, the sample was re-tested. The same batch of immunoglobulin G, divided in aliquots and aggregated at 63°C for 20 min, was used throughout the study.

Values greater than 6 μg AHG Eq/ml were considered above the limit of positivity in the ClqSP whereas 4 μg AHG Eq/ml was the limit in the KgBt.

**Statistical evaluation**

The chi square test (X), with Yates’ correction when indicated (Y), Fisher’s exact test (F), Student’s t-test (S) and Cox’s test (C) (Cox 1971) were used in the statistical evaluations of the results as indicated. The quantities of AgAb were expressed as mean ± SEM of values.

When the results in long standing diabetics (Group I) were examined, the prevalence of AgAb, using ClqSP, was similar in patients without microangiopathy (16%) and in normal population (10%), whereas the prevalence of AgAb was found significantly higher in patients with severe microangiopathy (51%) than both in the normal population ($P < 0.001$, Y) and in the patients without complications ($P < 0.005$, Y) (Fig. 1). Also the quantities of AgAb in diabetics with severe microangiopathy were significantly higher ($P < 0.01$, S) when compared to those found in patients without microangiopathy (17.7 ± 3.2 vs 5.7 ± 2.8 μg AHG Eq/ml).

There was a trend for severe microangiopathy to go with medium-high levels of AgAb ($P < 0.02$, C, performed on AgAb values divided as indicated in Fig. 1).

The presence of AgAb studied by ClqSP, was not correlated to the type of diabetes. The AgAb prevalence in type I diabetics with microangiopathy was 52% and in those without microangiopathy 20%. In type II diabetics with microangiopathy...
pathy the prevalence was 50% and in those without microangiopathy 13%.

The prevalences and the quantities of AgAb were similar in patients with proliferative retinopathy alone (50% and 21.3 ± 6.3 µg AHG Eq/ml, respectively) and in patients with nephropathy and various degrees of retinopathy (52% and 15.2 ± 3.2 µg AHG Eq/ml).

When KgbT method was used in patients in Group I, an increased prevalence of AgAb was found both in patients with (39%) and without microangiopathy (36%) in comparison with the normal population \(P < 0.0001\) and \(P < 0.0005\), \(Y\).

Taking into account the type of diabetes, AgAb detected by KgbT were higher in type I diabetics \(P < 0.02\), \(X\). The AgAb prevalence in type I diabetics with microangiopathy was 48% and in those without microangiopathy 47%. The AgAb prevalence detected by KgbT in type II diabetics was respectively 25 and 19%. AgAb presence was the same in diabetics with retinopathy alone (35%) and in those with nephropathy (35%).

When the patients after a short distance from diagnosis (Group II) were considered, using ClqSP method, the prevalence of AgAb appeared to be significantly higher in patients with early retinopathy (63%) than in normal subjects \(P < 0.001\), \(Y\) or in diabetics without microangiopathy (20%) \(P < 0.005\), \(F\) (Fig. 2). Also the quantities of AgAb were different between diabetics with \((15.5 ± 4.2 \mu g \text{AHG Eq/ml})\) and without retinopathy \((3.1 ± 1.1 \mu g \text{AHG Eq/ml})\) \(P < 0.001\), \(S\), and \(P < 0.005\), \(C\) on AgAb values divided as previously described. Taking into account the type of diabetes, the AgAb prevalence using ClqSP was 67% in type I diabetics with microangiopathy and 25% in those without microangiopathy, whereas in type II diabetics with and without microangiopathy was respectively 62 and 15%. There is no difference in AgAb presence using ClqSP between type I and II diabetics.

Using KgbT method in patients in Group II, differences in the prevalence of AgAb in patients with early retinopathy (52%) and in those without microvascular involvement (30%) were not statistically significant, though the prevalence of AgAb in all diabetics (37%) was higher than in the control population \(P < 0.005\), \(Y\). No difference in AgAb presence was found between type I and II diabetics.

As already shown (Lambert et al. 1978) the techniques ClqSP and KgbT detect different types of AgAb and also in this work there was not a significant concordance between AgAb values obtained by these two methods: AgAb values were concordantly positive in 15% of the cases and concordantly negative in 41%. 24% of sera showed an AgAb presence only by ClqSP and 20% only by KgbT.

Discussion

In agreement with recent reports (Kumar & Quisnorio 1978; Kumar et al. 1979; Ludwig et al. 1979) circulating AgAb have been found increased in some diabetics. AgAb were referred to be increased in certain conditions, namely type I diabetics at diagnosis (Irvine et al. 1977, 1978a) and long standing insulin treated diabetics (Irvine et al. 1977, 1978b; Charlesworth et al. 1979; Delespesse et al. 1980). In the present study an increase in circulating AgAb has been observed in diabetics with severe microangiopathy.

AgAb detected by ClqSP are increased both in diabetics with proliferative retinopathy and in those with severe nephropathy, irrespective therefore of the organ more overtly damaged.

AgAb (ClqSP) are also increased in patients with early retinopathy, including patients with the so-called malignant microangiopathy (Andreani
and in long standing diabetics with severe microangiopathy, but not in diabetics without microangiopathy comparable for duration of disease.

The entity of microangiopathic changes, therefore, seems related to AgAb levels and not to the duration of the illness.

In patients with severe complications AgAb levels were increased disregarding the type of anti-diabetic treatment; therefore in the presence of complications the type of treatment seems to be of no relevance. Similar conclusion is drawn when the presence of AgAb is correlated with the levels of insulin antibodies in patients with severe microangiopathy, as presented elsewhere by our group (Iavicoli et al. 1980). However, since in randomly selected diabetics AgAb levels were found to be higher in insulin treated diabetics and in negative correlation with insulin antibody titres (Iavicoli et al. 1980), it may be speculated that once complications are established some factors modify the relationship between AgAb levels and type of antidiabetic treatment.

All these findings suggest that the increased prevalence of AgAb, as a consequence of a modification of the dynamics of formation, clearance and tissue deposition of complexes, is related to the microvascular damage that proliferative retinopathy and diabetic nephropathy have in common and probably to the course of the complications, being more marked in patients with rapid development of the pathological changes.

Our selection of diabetics with extreme and unusual conditions, i.e. severe retinopathy within few years of disease and absence of microangiopathy even after many years of disease highlights the correlation between AgAb and microangiopathy (Irvine et al. 1978b). This correlation is likely hidden by the presence of the slight/medium microangiopathy, and may explain the apparent discrepancy with other recent reports (Ludwig et al. 1979; Balestrieri et al. 1979).

When KgBT method was used an elevated presence of AgAb in diabetics was demonstrated but partially discordant results were found. Conglutinin and Clq bind different types of AgAb through unrelated mechanisms. It is likely that the AgAb present in diabetes are heterogeneous and from the characteristics of the two methods used it may be inferred that complexes associated with microangiopathy are formed in antigen excess (Casali et al. 1977), have a low degree of lattice formation (Di Mario et al. 1980) and are potentially harmful (WHO 1977), for instance after a passive trapping or binding into blood vessel walls.

Since only some diabetics with severe microangiopathy have an increase of circulating immune complexes, the true importance of their presence remains to be established.

Nevertheless, this correlation between the presence of circulating AgAb and the occurrence of severe microangiopathy should be kept in mind when attempting to establish the pathogenesis of diabetic microangiopathy.

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References


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