High levels of serum complement factors in juvenile rheumatoid arthritis

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Complement activation can cause phenomena *in vitro* related to inflammation (Müller-Eberhard, 1968), and there is evidence of complement activation in human diseases, including rheumatoid arthritis (RA) in adults (Schur and Austen, 1968; Natvig and Winchester, 1969; Ruddy, Gigli, and Austen, 1972d). Patients with RA usually have diminished levels of complement in the synovial fluid (Hedberg, 1963; Pekin and Zvaifler, 1964), and in some cases low serum levels are found (Franco and Schur, 1971). Complement factors have also been demonstrated in immune complexes in synovial tissues from such patients (Rodman, Williams, Bilka, and Müller-Eberhard, 1967; Munthe and Natvig, 1971).

A few studies of complement factors in sera from patients with juvenile rheumatoid arthritis (JRA) have been reported (Schur and Austen, 1971-72). Wedgwood and Janeway (1953) reported raised values of total haemolytic complement activity (CH_{50}) in eleven patients, but only during the initial active phase. Bianco, Panush, Stillman, and Schur (1971) confirmed this, and also reported a significantly lower CH_{50} level in latex-positive than in latex-negative patients, but the mean values of both groups were close to that of normal adults. Further, they found that three seropositive patients had higher levels of C1q and C3, and significantly lower levels of C4 than thirteen seronegative patients. No reference to corresponding levels in healthy individuals was given. Ruddy, Matsuura, Stillman, and Austen (1971) found supranormal CH_{50} values in five of sixteen patients with JRA, no elevation of C1q, moderate elevation of C4 and C2, and markedly supranormal C9 levels, compared with healthy adults. Since these previous studies did not apparently include adequate controls matched for age and sex, we have studied the complement system in patients with JRA and in matched healthy subjects.

The purpose of this presentation is to give the results of quantitation of CH_{50} , C1q, C4, and C3. Quantitation of C3 activator (C3A or β_2 -glycoprotein II) was included, as a lowered concentration might indicate activation of the alternate pathway of com-

plement activation (Ruddy, Gigli, and Austen, 1972a). In addition, immunconglutinin was titrated, which, being an autoantibody mainly to bound C3 (Lachmann, 1967), should be an indicator of C3-binding *in vivo*.

Materials and methods

PATIENTS AND CONTROLS

34 hospitalized patients with JRA and 34 non-hospitalized healthy controls matched for age and sex were investigated. In both groups the mean age was 9.1 yrs (range 2 to 16). The female:male ratio was 1.8:1. Further clinical and laboratory details are given in a previous paper (Høyeraal, 1973).

COLLECTION AND STORAGE OF SERA

Serum was separated within a few hours and stored at -70°C . until analysed for C1q and C3. The other tests were performed on sera that had been thawed and refrozen. Controls showed that the latter procedure did not influence the results significantly, as previously shown by Ruddy and Austen (1970).

CH_{50} , C1q, C4, C3, AND C3A

The total haemolytic complement activity was determined as described by Mayer (1967). One unit was defined as the amount of serum that would lyse 50 per cent. of a standardized suspension of sensitized sheep erythrocytes.

The components C1q, C4, C3, and C3A were quantitated by single radial immunodiffusion technique (Mancini, Carbonara, and Heremans, 1965). A pool of human sera was used as reference. Its concentrations of the first three components were kindly determined by Dr. P. H. Schur of Harvard Medical School.

Rabbit anti-C1q serum (223) was kindly provided by D. Wiger, B.Sc., working at our institute. 0.7 per cent. agarose gel (A37 from l'Industrie Biologique Française S.A., Gennevilliers, France) in 0.05 M barbital buffer (LKB, Sweden), pH 8.6 with 0.01 M EDTA was used. 10 μl . antiserum was mixed with 5.5 ml. gel. Samples of 5 μl . were applied in wells with a diameter of 3 mm. The plates were read after 3 days.

C4 quantitation was performed by use of M-Partigen-C4 (β_{1E} -Globulin immunodiffusion plates (Behringwerke, Marburg, Germany), and the plates were read after 2 days.

For C3 quantitation rabbit-anti β_{1C}/β_{1A} -globulin (Behringwerke, Marburg, Germany) in 1 per cent. agarose gel (A45 from l'Industrie Biologique Française S.A., Gennevilliers, France) with 0.025 M barbital buffer (LKB, Sweden), pH 8.6 containing 0.01 M EDTA was used. 0.15 ml. antiserum was mixed with 4.85 ml. gel. Serum was diluted 1 in 2 in phosphate buffered saline, pH 7.2. The plates were read after 24 hrs.

For C3A quantitation, 0.25 ml. rabbit anti-C3A serum (Behringwerke, Marburg, Germany) was mixed with 4.75 ml. of the same gel and buffer as that used for C3 quantitation. The serum samples were diluted 1 in 4 in phosphate buffered saline, pH 7.2. The plates were read after 24 hrs. Values were expressed as a percentage of those with the reference serum pool.

IMMUNOCONGLUTININ

Titration of immunoconglutinin was performed with sheep erythrocytes sensitized with bovine antibody and horse complement as described (method IIA) by Coombs, Coombs, and Ingram (1961). Before titration with alexinated cells, all sera were tested undiluted with control cells prepared with inactivated horse serum. Negative results with control cells were obtained in all but twelve sera. These became negative following a second absorption with sheep erythrocytes.

STATISTICS

The two-sided Wilcoxon test for pair differences (Diem, 1962) was used for comparing the results in the patient and the control groups. A non-parametric rather than a parametric test was chosen, because some of the factors measured were not normally distributed. When comparing the values in two groups of non-paired individuals, e.g. rheumatoid factor positive and negative patients, a two-sided Wilcoxon test for two samples was used. A few of the complement factors showed a skewed distribution with some difference between arithmetic and geometric means. We therefore decided to use geometric means $\pm$ two standard errors or $\pm$ two standard deviations for all the complement factors. As a geometric scale was used for the titre of immunoconglutinin, in this case the arithmetic means of the $\log_2$ titre were calculated.

Results

The patient group had a higher CH_{50} activity than that of the control group, the geometric means (± 2 S.E.) being 144 units/ml. (135–154) and 105 units/ml. (97–115) respectively (Fig. 1). This difference was statistically highly significant ($P < 0.0001$).

FIG. 1 Serum levels of CH_{50} , C1q, C4, and C3 in 34 patients with JRA and healthy controls. Shaded areas indicate ± 2 S.E. of geometric means, denoted by solid lines. Stippled lines denote ± 2 S.D. of geometric means in control group

Similarly, the patient group had significantly higher serum levels of C4 than the controls, 380 $\mu\text{g./ml.}$ (325–445) and 244 $\mu\text{g./ml.}$ (208–286) respectively ($P < 0.001$).

For C3, the mean levels were 1.78 mg./ml. (1.64–1.94) and 1.29 mg./ml. (1.21–1.37) ($P < 0.0001$).

For C3A, 149 per cent. (136–170) and 90 per cent. (82–97) respectively ($P < 0.0001$) (Fig. 2).

FIG. 2 Serum levels of C3A in 34 patients with JRA and healthy controls.

Shaded areas indicate $\pm 2\text{S.E.}$ of geometric means, denoted by solid lines.

Stippled lines denote $\pm 2\text{S.D.}$ of geometric mean in control group

With C1q there was no difference between the two groups, the values being 145 $\mu\text{g./ml.}$ (124–171) and 142 $\mu\text{g./ml.}$ (126–159) ($P > 0.05$).

The immunoconglutinin activity was very low in both groups, but the patients had a significantly

higher $\log_2$ titre of immunoconglutinin than the controls (Table I).

Among the patients there was no significant difference in the serum levels of CH_{50} , C1q, C4, C3, and C3A, relating to whether the rheumatoid factor test or the indirect immunofluorescence test for antinuclear antibodies were positive or not, or to whether the disease was active or inactive according to the criteria of Ansell and Bywaters (1959). However, the mean immunoconglutinin titre was significantly ($P < 0.05$) higher in the rheumatoid factor positive patients (2.3) than in the rheumatoid factor negative patients (0.8). No significant difference was found between antinuclear factor positive and negative patients or between patients with active and inactive forms of the disease. Nor were there significant differences in the measured factors with regard to the presence or absence of amyloidosis or with regard to treatment or not with prednisone and/or azathioprine, apart from the fact that the ten patients receiving prednisone and/or azathioprine had a significantly ($P < 0.05$) lower C3A level (131 per cent. (128–134)), than those not receiving these drugs (162 per cent. (159–165)). Values for C1q below two standard deviations from the geometric means of the control group were found in only two patients (Fig. 1); one was a known case of hypogammaglobulinaemia, and the other who had normal IgG and IgA, but very high IgM (3.3 mg./ml.), was an acutely febrile case, receiving 35 mg. prednisone daily.

High values were found in four patients for CH_{50} , in none for C1q, in four for C4, in fifteen for C3, in sixteen for C3A, and in seven for immunoconglutinin. No particular clinical or laboratory pattern seemed to be related to high values. The second patient with low C1q, mentioned above, had very high levels of CH_{50} , C4, and C3, but a normal C3A level.

Women, both patients and controls, had lower mean levels of all the quantitated factors than men, apart from C3A in the controls (Table II, overleaf). In some the differences were significant. The female : male ratio was about 0.8:1.

Discussion

A group of patients with JRA was found to have significantly higher serum levels of CH_{50} , C4, C3,

Table I Serum immunoconglutinin in 34 patients and controls

Group	No. with $\log_2$ titre							$\log_2$ titre	
	<0	0	1	2	3	4	5	Mean $\pm$ 2S.E.	P*
JRA	6	7	9	5	5	1	1	1.1 $\pm$ 0.5	<0.05
Controls	10	12	2	9	1	0	0	0.4 $\pm$ 0.4	

* = Wilcoxon test for pair differences, two-sided.

Table II Sex ratio of complement factors

Group	Female: male ratio					
	CH ₅₀	C1q	C4	C3	C3A	IK
JRA	0.85**	0.92*	0.75*	0.79**	0.90	0.80
Controls	0.93	0.74**	0.79*	0.86*	1.06	0.10

* = $P < 0.05$, ** = $P < 0.01$ (Wilcoxon test, two-sided).

IK = Immunoconglutinin.

C3A, and immunoconglutinin than a group of controls. No such difference was found for C1q. The high CH₅₀ values conform with reports by Wedgwood and Janeway (1953), Bianco and others (1971), and Ruddy and others (1971). However, our findings do not confirm the difference between active and inactive forms of the disease observed by Bianco and others (1971). Nor were our findings consistent with the differences observed between seropositive and seronegative patients with JRA (Bianco and others, 1971) and with RA (Mongan, Cass, Jacox, and Vaughan, 1969).

The high serum levels of several complement factors found in patients with JRA differ somewhat from those reported in adult patients. Patients with RA usually have normal or only slightly raised levels of CH₅₀. Franco and Schur (1971) reported low values in four per cent., who tended to have a severe form of the disease, including marked functional impairment, seropositivity, and subcutaneous nodules. Although 79 per cent. of the patients with JRA included in this study had an active form of the disease, none had a CH₅₀ value below the mean of the control group.

Interpretations of serum complement levels in pathogenetic states must be made with caution (Cooper and Fogel, 1967; Ruddy, Gigli, and Austen, 1972d). Activation of the complement system is usually associated with lowering of concentration. There are several possible explanations for the raised serum levels found in this study. Perhaps the most likely one is that increased consumption has resulted in increased synthesis with overcompensation. A less likely possibility is hypocatabolism, since hypercatabolism of C3 has been demonstrated in two patients with rheumatoid vasculitis having normal serum C3 levels (Weinstein, Peters, Brown, and Bluestone, 1971), and of C1q in patients with low serum levels of C1q and IgG deficiency (Kohler and Müller-Eberhard, 1970). A low serum level of C1q has been reported in hypogammaglobulinaemia (Kohler and Müller-Eberhard, 1969; Ruddy, Gigli, and Austen, 1972c). A third possibility is a generally increased synthesis of proteins or of acute phase proteins. This is unlikely, as the levels of total proteins,

albumin, and α_1 -antitrypsin were normal in the patient group (Höyeraal, unpublished results).

Since immunoglobulin levels are increased in JRA (Houba and Bardfeld, 1969; Höyeraal and Mellbye, 1974), it is possible that the high levels of complement factors may have an immunological basis.

The values obtained for C3A do not indicate activation of the alternate pathway directly.

The low titres of immunoconglutinin throughout make evaluation difficult, but the significantly higher titres in patients with JRA suggest binding and activation of complement *in vivo*. Tedesco, Corrocher, and Brown (1972) found evidence that immunoconglutinin normally has an amplifying effect on complement fixation *in vivo*, and so potentiates the ability of complement to bring about red cell destruction in rabbits. In this way, immunoconglutinin might be a pathogenetic factor in rheumatoid inflammation (Mellbye and Munthe, 1971).

At least for CH₅₀ and C3 there seems to be little difference in the serum levels of patients over 2 years of age (Geisert, Sacrez, Malgras, Hauptmann, Peter, and Duvivier, 1971; Masi and Vivarelli, 1971). The effects of age and sex variation have been excluded in the present study by the use of matched pairs.

Summary

A group of patients with JRA had significantly higher serum levels of CH₅₀, C4, C3, and C3A than a matched control group, whereas no such difference was found for factor C1q. One explanation for this is increased consumption resulting in increased synthesis with overcompensation. The previously reported higher CH₅₀ levels in active than in inactive disease, and the lower levels in seropositive than in seronegative patients, were not confirmed in this study. The immunoconglutinin titres were low, but the higher level found in the patient group than in the control group may reflect complement-binding mechanisms in this disease.

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