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Different Patterns of C3 and C4 Activation in the Varied Types of Juvenile Arthritis

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ABSTRACT. Quantitative assays for C3 and C4 activation were carried out simultaneously on blood from children with varied types of juvenile arthritis. Factor VIII-related antigen was also measured as an indicator of vascular damage. In active systemic juvenile arthritis, the C4d/C4 ratio was frequently elevated and was usually associated with elevated C3d/C3 ratios and elevated concentrations of factor VIII-related antigen. Children with chronic polyarticular arthritis, no matter which forms of onset they had had, also had increased levels of the C4d/C4 ratio, C3d/C3 ratio, and factor VIII-related antigen, but these were less consistent and were not associated with each other. In contrast, in pauciarticular arthritis there was a uniquely isolated increase in the C3d/C3 ratio. This work implies that there are different mechanisms responsible for complement activation in the different types and at different stages of juvenile arthritis. (*Pediatr Res* 20: 1332–1337, 1986)

Abbreviations

DIC, disseminated intravascular coagulation
F.VIII R:Ag, factor VIII related antigen
JA, juvenile arthritis
LF, latex fixation
SLE, systemic lupus erythematosus
ARA, American Rheumatism Association

The various types of chronic arthritis in childhood are beginning to be distinguished one from another by objective means such as HLA typing. They now can clearly be discerned as different diseases. Until recently, most immunologic studies did not distinguish individual types of disease in childhood, but have clearly separated them from rheumatoid arthritis, for which reason we use the collective term JA. In most current work they are usually separated only by the clinical nature of the onset, as defined by the ARA criteria (1), *i.e.* systemic, polyarticular, or pauciarticular. These clinical descriptions do not necessarily distinguish groups of children with single or consistent laboratory clues as to pathogenesis.

Several laboratories have looked for evidence for roles of circulating antigen-antibody complexes in JA. Individual assays for immune complexes are usually positive in only 50–75% of sera from children with active systemic or rheumatoid factor negative polyarticular disease (2–4). Moreover, different assays do not correlate well with each other in JA (4), so the significance of circulating complexes is not clear. Sequestered complexes, not measurable in sera, might activate complement and release breakdown fragments into the circulation. Evidence of the presence of these would provide better support for an immune complex theory of pathogenesis in JA than we have had to date.

Concentrations of complement components and CH50 activity in serum have been reported to be elevated in active JA (5, 6). However, since increased production might result in raised serum concentrations even when pathologic degrees of activation are occurring, it is necessary to look for activation products per se. In an earlier study we used a qualitative counterimmunoelectrophoretic assay for C3d in plasma (7), and found an incidence of positive results only loosely correlated with a liquid phase Clq binding assay for immune complexes (3), *i.e.* between 50–70% of plasma samples from children with active disease were positive. This raised a further question as to whether the circulating C3d was due to activation of C3 by the classical or the alternative pathways.

The studies reported herein were started to compare simultaneous quantitative assays for the activation products of C3 and C4 with the aim of determining which pathway might be involved. A recent report that subclinical DIC was common in systemic JA (8) raised the possibility that the complement cascade might be activated by the fibrinolytic/clotting systems, and that this might interfere with interpretation of increased concentrations of C4d. For this reason, we subsequently added a simultaneous study for the presence of F.VIII R:Ag, which may be used as a measure of endothelial cell damage in vasculitic diseases.

MATERIALS AND METHODS

Subjects. The children studied consisted of those attending the Rheumatic Disease Clinic at this hospital who fit the ARA criteria for "juvenile rheumatoid arthritis" (1) and who had, or whose parents had, given permission for extra blood to be withdrawn at the time of a clinically needed venipuncture. For this report they were classified both by the current status of their disease and by the status of their disease at onset, using the ARA definitions for "systemic," "polyarticular," and "pauciarticular" (1). Those patients whose disease had progressed from systemic or pauciarticular onset to a polyarticular form at the time of study are so classified (Table 1). Patients with positive LF tests were classified separately, and patients known to have the HLA B-27 antigen were excluded. Control samples were obtained from five males and three females between 8 and 16 yr of age who were either normal or had been admitted to hospital for orthopedic procedures unrelated to inflammatory disease.

Plasma. Blood was collected in commercially obtained tubes containing EDTA (Becton-Dickinson, Rutherford, NY) sepa-

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Table 1. *Patient characteristics by disease type and course*

	Age range (yr)	Sex distribution	
		M	F
Systemic	2⁴⁄₁₂–17	8	3
Systemic → polyarticular	5²⁄₁₂–16	3	4
Polyarticular, LF (−)	2–15	0	14
Polyarticular, LF (+)	10–18	0	4
Pauciarticular → polyarticular	7⁶⁄₁₂–20	0	7
Pauciarticular	6⁶⁄₁₂–17	3	10

rated by centrifugation at room temperature within 4 h and stored at −70° C until analyzed. In total, 97 samples from 56 children were studied.

Measurement of C3 and C3d. The plasma C3 concentrations were measured by radioimmunodiffusion on commercially obtained agarose plates (Calbiochem-Behring, La Jolla, CA). Our normal samples gave values for mean ± SD of 92 ± 16 mg/dl. The range of 2 SDs around the mean (60–124 mg/dl) compares well with the manufacturer's values (55–120 mg/dl). The C3d concentration was measured by an adaptation of the rocket immunoelectrophoretic methods of Bourke *et al.* (9) and Wahn (personal communication). Intact C3 was precipitated from the plasma by mixing with an equal volume of 24% polyethylene glycol, 6000 mol wt (Sigma Chemical Co., St. Louis, MO) at 4° C. The C3d in 7 μl of the supernatant was determined by one dimensional rocket immunoelectrophoresis on agarose plates containing anti-C3d prepared on 2″ × 3″ glass slides using a mixture of 4.93 ml of 1% agarose (low EEO, Sigma) and 22.5 μl anti-human C3d antibody (lot no. 010D, DAKO, Accurate Chemical, Westbury, NY). The electrophoresis was performed at 50 V for 18 h at 4° C. The plates were washed thoroughly in 0.1 M NaCl two times followed by one wash in distilled water. The dried plates were stained with Coomassie Brilliant Blue R250, and the "rocket" heights measured from well to apex in mm. Known quantities of C3d, previously prepared in this laboratory (7), were used as standards intermittently. The rocket heights of standards and of repeated normal plasmas never varied more than 1 mm on different runs. The C3d/C3 ratio was calculated by dividing the length of the C3d rocket by the square of the diameter of the precipitin ring in the radial immunoassay for C3 of the same sample. This provided the most directly derived ratio, and one with a convenient value for statistical analysis, but not one which reflects a ratio of weights or a true molar ratio. The mean and SD of results from eight normal children were 0.41 ± 0.04, so a value greater than 0.49 was considered abnormal.

Measurement of C4d/C4 ratio. The rocket immunoelectrophoretic method of Milgrom *et al.* (10) was used as described except that the antibody dilution in agarose was 1/15 and the electrophoresis was at 25 V for 18 h at 4° C. In this assay the ratio of C4d to C4 concentrations is measured from photographs of the agarose plates by planimetric analysis of the areas of double "rockets" emanating from a single well, since the antibody in the agarose detects both C4d and C4 in the plasma. Eight normal children's plasmas gave a value for mean and SD of 1.025 ± 0.046, so values greater than 1.12 were considered elevated. In addition, the plasma C4 concentration was measured by radioimmunodiffusion on commercially obtained plates (Calbiochem-Behring). Our values for mean and SD was 32 ± 13 mg/dl, creating a larger range of 2 SDs around the mean (6–58 mg/dl) than indicated as normal by the manufacturer (20–50 mg/dl). It is to be noted that the C4d/C4 ratio obtained in this way does not represent a true weight or molar ratio and is partly dependent on the total C4 present (10), so that it was not possible to calculate absolute C4d concentrations using this ratio.

Measurement of F.VIII R:Ag. F.VIII R:Ag was measured by rocket immunoelectrophoresis using commercially obtained agarose plates containing 0.2% sheep antiserum to human F.VIII R:Ag, barbital buffer pH 8.3–8.7, and a small chamber in which the plates were suspended gel-side down (all from Helena Labs, Beaumont, TX). Electrophoresis was at a constant 16 mA per plate and run at room temperature for 4 h. Initial determinations showed that EDTA plasma produced similar results (94 ± 16%) as the citrated plasma specified by the manufacturer (83 ± 14%). The resulting rockets were measured from well to apex and compared to results obtained from a standard curve generated from a serially diluted sample of a reference pool of normal plasma supplied by Helena Labs. Results were expressed as "percent activity," determined for the normal pool by the manufacturer against a permanent standard. Values for mean and SD from our normal samples, 110 ± 37%, produced a greater range than specified by the manufacturer (50–160%).

Autoantibodies and Clq binding. Serum samples were obtained simultaneously with the plasma samples from 44 patients (nine systemic, eight systemic-onset turned polyarticular, 14 polyarticular, and 13 pauciarticular) and autoantibodies were studied separately at the University of California at Davis as part of a study which has been published separately (11). The autoantibodies measured were antinuclear antibodies on HEp-2 cells, IgM and IgG rheumatoid factor by ELIZA, anti SS-DNA, and anticollagen types I and II. A solid phase Clq binding assay was also performed on the sera.

Statistics. Most of the statistical computations were performed using the "Number Cruncher Statistical System" software program (Hintze JL, Kaysville, UT) and advice from the Department of Biostatistics of Stanford University. Differences of values for means and variances of groups were compared by standard parametric tests including analysis of variance, and then *t* tests, as appropriate. When the variance ratio (F test) between groups was not small enough for these to apply, the Mann-Whitney test was used. In addition, correlation coefficients of the values of the total data in each group and of serially derived data from individual patients were calculated.

RESULTS

Systemic JA. The results of each assay on the first obtained sample from each child with persistent systemic JA are shown in Figure 1, and the means and SDs in Table 2. The most consistent finding was an increased C4d/C4 ratio, occurring in 78% of the samples. High values were frequently found for direct measurement of C3d, 56%; C3d/C3 ratio, 56%; and F.VIII R:Ag, 63%. The means and variances for the values of all these assays differed from normal with $p < 0.05$. C3 and C4 concentrations were not different from normal. Elevations of C3d concentration, the C3d/C3 ratio, and F.VIII R:Ag concentrations were usually associated with elevations in the C4d/C4 ratio, but, the values of the results of these asssays were not statistically correlated. Among all the groups of JA, the highest individual values and the highest mean value for F.VIII R:Ag were in systemic JA patients, and these values statistically correlated to erythrocyte sedimentation rate, $r = 0.8$, $p < 0.01$. No other correlations were noted between patients or on repeated samples from the same patients when disease activity varied. Elevated C4d/C4 values were present in some children with apparently inactive disease or in disease which had lost systemic features and become chronically polyarticular (Fig. 2) but all the other assays were essentially normal in these circumstances.

Pauciarticular JA. In patients with pauciarticular disease, only the C3d/C3 ratio was elevated (Fig. 3). This difference from normal was significant at $p < 0.05$, but was not associated with statistically abnormal values in any of the other assays, including absolute values for C3d or C3. The abnormal ratios were a result of a tendency for C3 values to be below and C3d values to be above the respective means. To be more certain that C4d/C4 ratios were not elevated in persistent pauciarticular JA an additional 16 patients were studied by this assay alone. All were

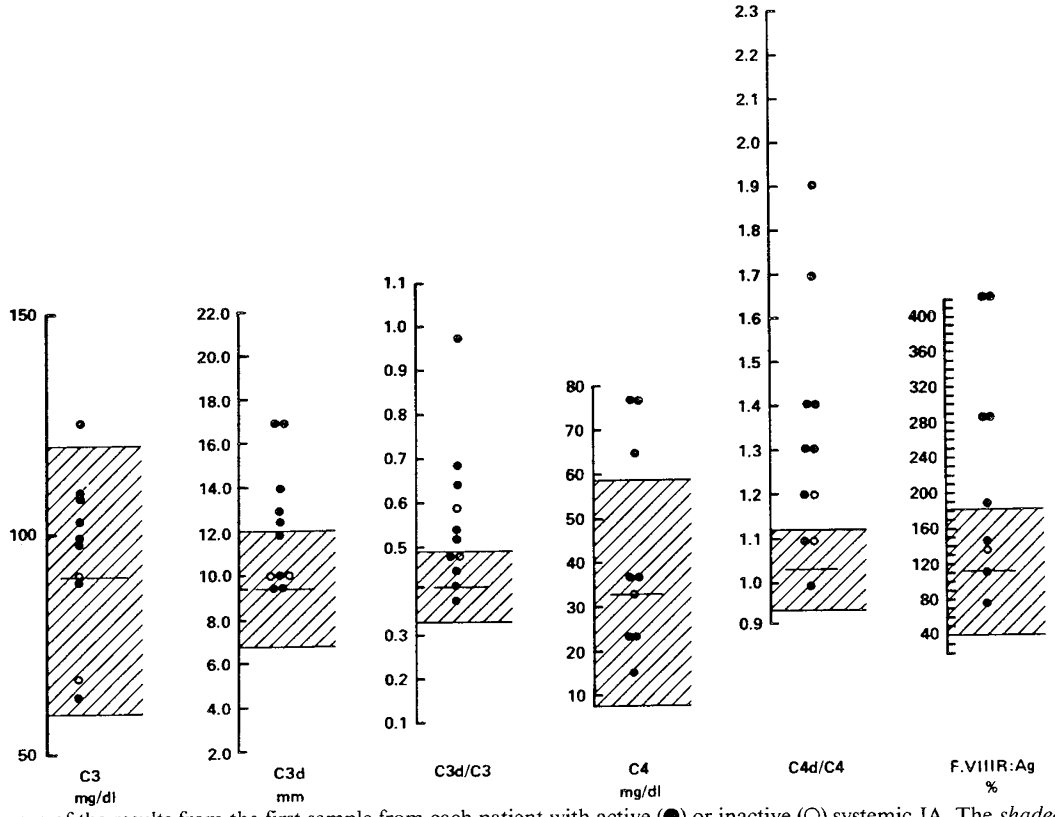

Fig. 1. Scattergram of the results from the first sample from each patient with active (●) or inactive (○) systemic JA. The *shaded area* represents the mean ± 2 SDs of the normal samples. The most frequently abnormally elevated results were of the C4d/C4 ratio. Elevations of C3d, C3d/C3 ratio, or of F.VIII R:Ag were usually associated with elevated C4d/C4 results, but absolute values were not correlated. The values for F.VIII R:Ag were the highest found in any type of JA.

Table 2. *Means and SDS of values for each of the assays in the first sample from each child with active forms of each type of JA**

	C3 (mg/dl)	C3d (mm)	C3d/C3	C4 (mg/dl)	C4d/C4	F.VIII R:Ag (% activity)
Normal	92 ± 16	9.6 ± 1.3	0.41 ± 0.04	32 ± 13	1.03 ± 0.046	110 ± 37
Systemic	91 ± 30	13 ± 2.9†	0.56 ± 0.18†	45 ± 25	1.4 ± 0.28‡	240 ± 130†
Systemic → polyarticular	84 ± 6.9	11 ± 1.1	0.47 ± 0.05	37 ± 4.9	1.4 ± 0.37	180 ± 13
Polyarticular, LF (−)	85 ± 14	12 ± 2.5†	0.60 ± 0.14‡	40 ± 19	1.3 ± 0.27†	160 ± 61
Polyarticular, LF (+)	81 ± 18	13 ± 4.6	0.68 ± 0.35	30 ± 16	1.9 ± 1.0†	140 ± 75
Pauciarticular → polyarticular	92 ± 18	11 ± 3.1	0.53 ± 0.14	35 ± 11	1.2 ± 0.31	130 ± 60
Pauciarticular	87 ± 11	11 ± 1.6	0.56 ± 0.11†	34 ± 14	1.03 ± 0.05	110 ± 40

* The number of samples studied in each group are the same as those in the figures indicating active disease. Statistical significance was calculated by performing analysis of variance of all values followed by either a *t* test if the variance ratio allowed or by the Whitney-Mann test if the variance difference was too large.

† $p < 0.05$ compared to normal.

‡ $p < 0.01$ compared to normal.

normal. Five of seven patients who developed polyarticular disease after a pauciarticular onset had elevated C3d/C3 ratios, but three of these, in addition, had elevated C4d/C4 ratios (Fig. 2). No statistically significant correlations were found between the values of the different assays.

LF (+) arthritis. These four samples included the highest individual values found for C3d concentration, C3d/C3 ratio and C4d/C4 ratio. Only one, from a child with interstitial lung disease, had an elevated value for F.VIII R:Ag (Fig. 4). Although the numbers are too small for statistical study, there appeared to be associations between low concentrations of C4, high C4d/C4 ratios, high concentrations of C3d, and high C3d/C3 ratios.

LF (−) polyarticular JA. All the other children studied had LF (−) polyarticular disease. The pattern of results were similar to those in systemic disease except that the values of F.VIII R:Ag were not as high (Fig. 2). The incidences of elevated values in active disease were C3d concentration, 45%; C3d/C3 ratio, 82%; C4d/C4 ratio, 73%; and F.VIII R:Ag concentration, 45%. However, there was no statistical association or correlation between the values of the different assays, and no consistent patterns were clear. Three patients whose disease had become inactive had normal values for all assays except the C3d/C3 ratio which remained elevated in two. One patient studied four times did not show correlation between changes in her sedimentation rate and any of these assays or between changes in the assays.

C3d/C3 in SLE. Since the assay for C3d/C3 ratio was new, we also studied patients with SLE to validate its usefulness. Ten of 11 samples were abnormal: four from three children with clinically active SLE and low serum C3 concentrations, five samples from three children with clinically active SLE but nor-

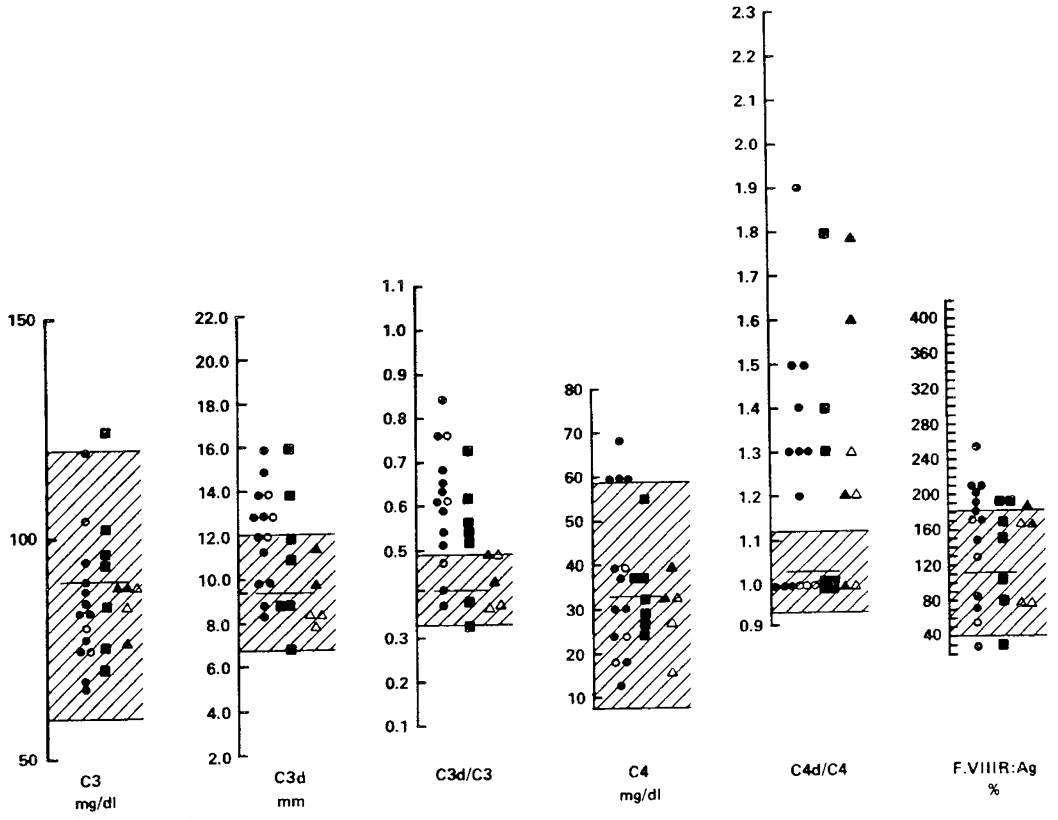

Fig. 2. Scattergram of the results from patients with active (●) or inactive (O) polyarticular rheumatoid factor (−) JA, active pauciarticular-onset turned polyarticular JA (■), and active (▲) or inactive (△) systemic-onset turned polyarticular JA. Elevations of C3d, C3d/C3 ratio, C4d/C4 ratio, or F.VIII R:Ag occurred in a similar pattern despite differences in mode of onset but were neither associated with nor correlated to each other.

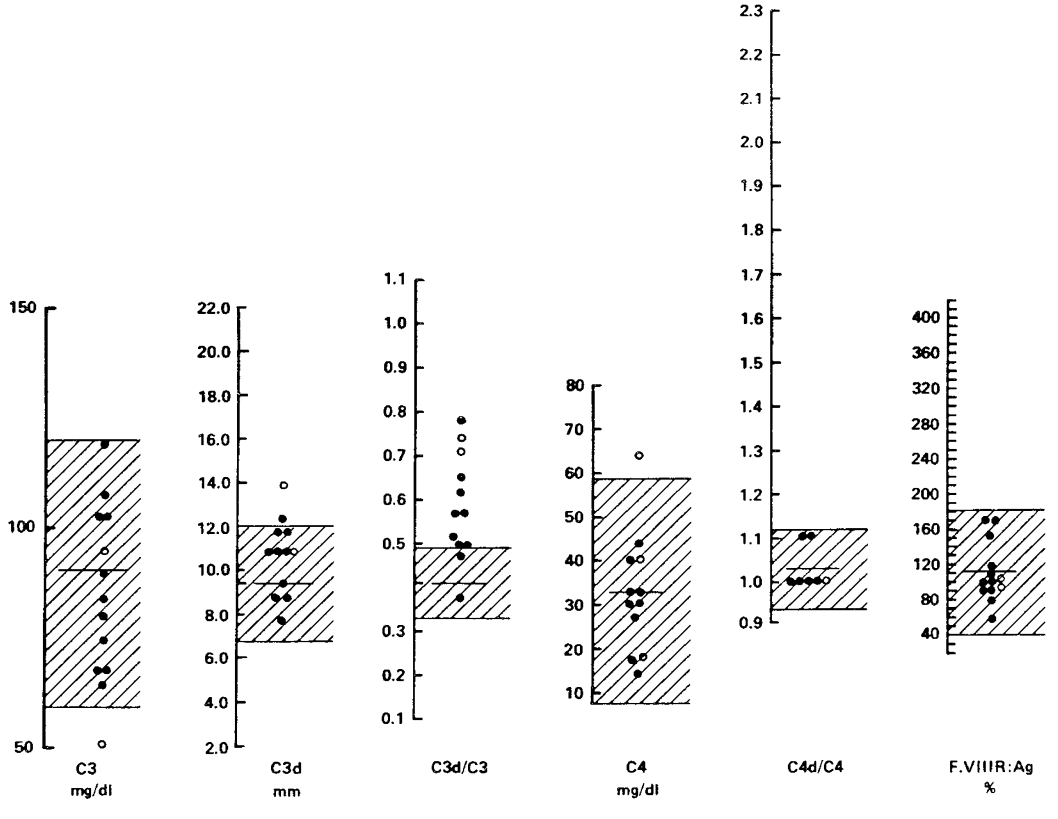

Fig. 3. Scattergram of the results from patients with active (●) and inactive (O) pauciarticular JA. The *shaded area* represents the mean ± 2 SDs of the normal samples. Values for all components and factors were normal in pauciarticular arthritis except for the C3d/C3 ratios which are elevated in a unique pattern.

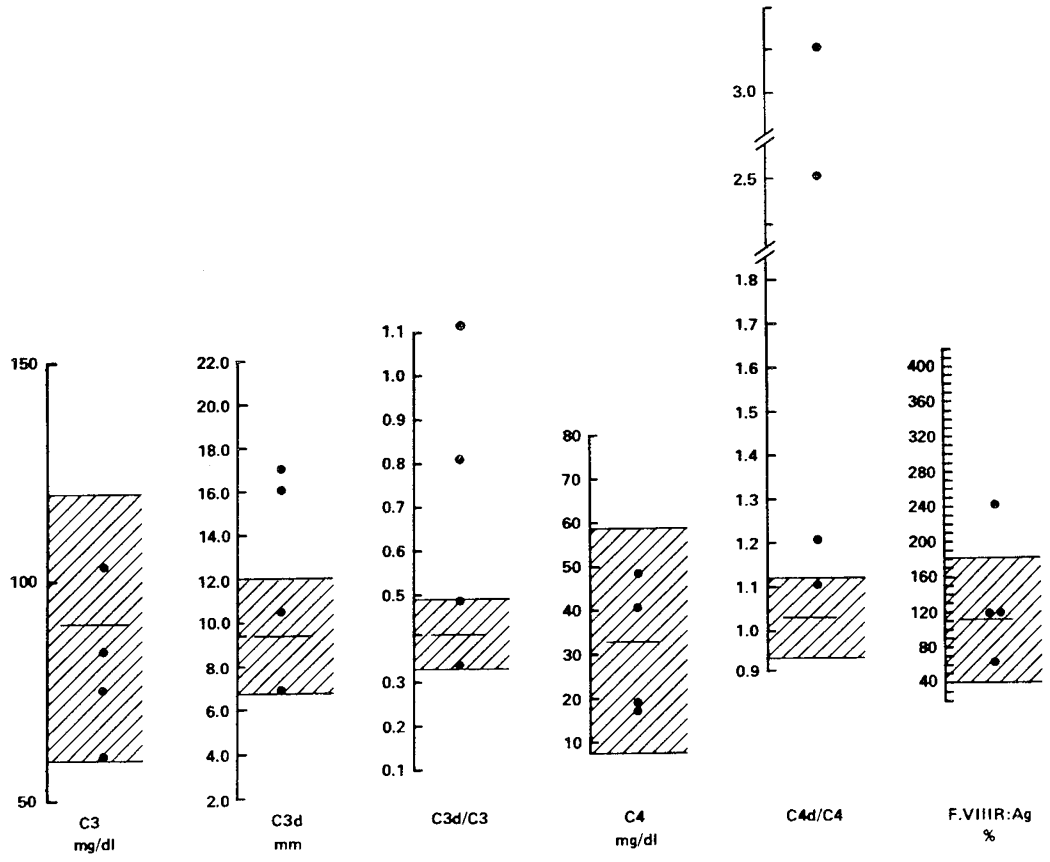

Fig. 4. Scattergram of the results from children with active, LF (+) arthritis. In contrast to systemic disease, C4d/C4 ratios are elevated, but not F.VIII R:Ag concentrations, except in one child with pulmonary vasculitis.

mal C3 concentrations, and one of two samples from two children with clinically inactive SLE. These had the highest range (0.45–1.2), and mean and SD (0.75 ± 0.24) of any group studied.

Correlation with autoantibodies and Clq binding. Antinuclear antibodies were only measured at one dilution so comparison of results with the complement activation assays were done by comparing the means and variances of each assay in samples which were positive *versus* those which were negative. No significant differences were found. Correlation coefficients were calculated for each of the complement activation assays with each of the assays for IgM and IgG rheumatoid factor, antibodies to collagen types I and II, antibodies to SS-DNA, and to the Clq binding assay. Marginally significant negative correlations ($p = 0.05$) were found between C3 and C4 concentrations and concentrations of IgM rheumatoid factor and between C4 and Clq binding in patients with systemic JA. No other significant correlations were found between the antibodies studied or complement activation products, or between Clq binding in serum and complement activation products in plasma.

DISCUSSION

The most important findings of this work are the clear differences in pattern of complement activation between pauciarticular JA and systemic and polyarticular JA. The results are consistent with, but do not prove, the possibility that the classical pathway is activated in systemic and polyarticular JA and, in contrast, that the alternative pathway is activated in pauciarticular arthritis. This suggests the need to look for entirely different causes, and possibly treatment, for these different kinds of arthritis. A less distinct difference was the higher values for F.VIII R:Ag in systemic JA than in other forms, probably indicating more severe vascular disease.

However, we do not know the relative sensitivities of these assays in respect to underlying pathologic events. For this reason we do not know whether the primary event in systemic JA is activation of the classical pathway by antigen-antibody complexes which also cause vascular damage, or whether some other cause of vascular damage precipitates the clotting and fibinolytic systems and then the complement system. Nevertheless, the mechanisms involved in C3 activation must be different in systemic JA than in pauciarticular JA.

These data confirm those of Scott *et al.* (8) regarding the incidence of elevated values of F.VIII R:Ag in systemic JA. This appears to be due to endothelial cell damage and presumed DIC. This work extends information regarding this assay to polyarticular and pauciarticular JA. The degree of this abnormality is lower in polyarticular arthritis than in systemic disease, and the incidence is negligible in pauciarticular arthritis. Since endothelial cell damage is an integral part of inflammation, it is possible that the F.VIII R:Ag level is nonspecific. However, F.VIII R:Ag levels were only elevated in one child with LF (+) arthritis, a girl with known pulmonry fibrosis, even though all these children had florid joint inflammation. F.VIII R:Ag concentrations have been found elevated in some other vasculitic diseases, although not universally (12).

In earlier work using a qualitative test for C3d we found elevated results more frequently in systemic JA than in pauciarticular JA (7). In this study we found only slightly elevated C3d concentrations but consistently elevated C3d/C3 ratios in pauciarticular JA. This new and unexpected result reflects the increased sensitivity of using this ratio instead of just a qualitative assay or a simple measurement of concentration. Although not found during the course of this study, high concentrations of C3 have been described in systemic JA (5, 6). If this had been true in our earlier group of patients, and since C3 is breaking down to some degree at all times, the qualitative assay may only have been reflecting the normal catabolism of higher concentrations

of C3. The use of a ratio of C3d/C3, a more accurate measure of increased pathologic activation, is more likely to reflect a true abnormality in pauciarticular JA.

Although five patients were studied on more than four occasions, none changed from active to completely inactive. Within the changes that did take place, we did not find statistical relationships with either sedimentation rates or hemoglobin to any of the assays, except between F.VIII R:Ag and sedimentation rates in systemic JA. The assay for C4d/C4 has shown a good correlation with disease activity in SLE and LF (+) rheumatoid arthritis in adults (10), but our data did not confirm this for JA. Serial studies of patients at the very onset of the various forms of JA would be valuable to clarify this problem. In later stages of the disease, when chronic polyarticular arthritis becomes predominant, abnormalities of immunologic and inflammatory mechanisms appear to become quite nonspecific. The variability of the results of these assays in chronic LF (−) polyarthritis is in contrast to the consistent pattern seen in LF (+) rheumatoid arthritis in adults (10), and adds to the reasons for considering these childhood arthritides to be distinct from true rheumatoid arthritis.

If complement activation had been found to be correlated with a specific autoantibody, we would have had a valuable clue to the pathogenesis of these diseases. The absence of such correlations probably means that these particular antibodies are not primarily involved. The lack of direct correlation between circulating complexes and complement activation products points out the difficulties in using changes in peripheral blood to understand pathologic events in solid tissues. We have studied complement activation products in synovial fluids, and have found similar relationship in pauciarticular fluids between C3a and C4a as reported here for C3d and C4d in blood, but also, no statistical relationship between the presence of complexes and complement activation (15). Even synovial fluid may not accurately reflect what is happening in synovial tissue per se.

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