Clin Exp Immunol 1995; 100:514–518

Composition and biological behaviour of immune complexes isolated from synovial fluid of patients with juvenile rheumatoid arthritis (JRA)

J. N. JARVIS, M. M. DIEBOLD*, M. K. CHADWELL*, M. IOBIDZE* & H. T. MOORE*
*Department of Paediatrics, Division of Immunology/Rheumatology, Wayne State University School of Medicine and the Children's Hospital of Michigan, and *Wayne State University School of Medicine, Detroit, MI, USA*

(Accepted for publication 31 January 1995)

SUMMARY

Data published from *in vitro* studies have shown that IgM-rheumatoid factor (RF)-bearing immune complexes possess several biological features that may contribute to their pathogenicity. However, no studies have demonstrated that such complexes exist at sites of inflammation in children with rheumatoid disease. We used two methods of sequential column chromatography to purify immune complexes from synovial fluids of children with JRA. We demonstrate that high molecular weight complexes contain IgM-RF, have not bound C4 *in vivo*, but activate the classical pathway *in vitro*. In contrast, complexes which have bound C3 *in vivo* do not contain IgM-RF and are weak complement activators *in vitro*.

Keywords immune complexes juvenile rheumatoid arthritis complement rheumatoid factor

INTRODUCTION

JRA is a multisystem disease characterized by inflammation of synovial membranes. Three characteristic subtypes (systemic, polyarticular, and pauciarticular) are recognized based on clinical presentation [1].

A well documented feature of each of the JRA subtypes is the presence of circulating immune complexes [2,3]. This is compatible with the finding of elevated levels of complement activation fragments in JRA plasma, particularly in children with active disease [4]. Since active disease in children with JRA (particularly those with polyarticular disease) correlates with the presence of IgM rheumatoid factors (IgM-RF) detected by several different methods [5,6], we have speculated that IgM-RF-bearing immune complexes may be involved in the pathophysiology of chronic synovial inflammation in JRA.

We have isolated high molecular weight immune complexes from plasma of children with polyarticular JRA and have shown that they have interesting characteristics which might involve them in a chronic inflammatory process: these complexes have not fixed complement *in vivo*, but briskly activate the classical complement pathway *in vitro* [7]. These data are consistent with findings using model IgM-RF-bearing immune complexes *in vitro* [8,9], and suggest that such complexes might be inefficiently cleared by complement-mediated mechanisms for immune complex removal [10], while maintaining significant phlogistic properties.

An important consideration in understanding the immune pathogenesis of rheumatoid disease in either adults or children is the fact that there may be important differences in the biological behaviour of immune complexes or rheumatoid factors in joints compared with those in the circulation [11]. These studies were therefore undertaken to examine specifically the composition and biological behaviour of synovial fluid immune complexes isolated from children with JRA.

Correspondence: James N. Jarvis MD, Immunology/Rheumatology, Children's Hospital of Michigan, 3901 Beaubien Blvd., Detroit, MI 48201, USA.

PATIENTS AND METHODS

Patients/blood and synovial fluid specimens

Synovial fluids were obtained from nine children (three males and six females) with active JRA. Pertinent characteristics of these patients are given in Table 1. These included three patients (two females and one male) with polyarticular JRA and six patients (two males and four females) with pauciarticular disease [1]. All three children with polyarticular JRA were negative for IgM-RF by latex agglutination, although one of the three (patient 9) had IgM-RF detected by ELISA [6]. Both boys with pauciarticular disease were HLA-B27-negative. Disease duration ranged from 1 to 14 years. All patients were receiving non-steroidal anti-inflammatory drugs at the time synovial fluid and plasma specimens were obtained. One patient was on oral gold. None of the patients was receiving steroids or methotrexate.

Table 1. Patients and JRA subtype

Patient no.	Age	Sex	JRA subtype
1	14	F	Pauciarticular
2	9	F	Pauciarticular
3	9	F	Pauciarticular
4	6	M	Pauciarticular
5	5	M	Pauciarticular
6	3	F	Pauciarticular
7	16	F	Polyarticular
8	4	M	Polyarticular
9	18	F	Polyarticular

Synovial fluid was obtained before intrarticular injection of triamcinolone. Fluid was drawn into sterile, heparinized or calcium EDTA tubes and placed immediately on ice. Cells were separated from the fluid by centrifugation at 4°C and the fluid stored at −70°C until use. Bacterial cultures of all synovial fluids were negative.

Where possible, whole blood, drawn to sterile tubes with calcium EDTA, was obtained concurrently with the synovial fluid samples. Blood was placed immediately on ice and plasma separated by centrifugation at 4°C.

Proteins and antibodies

Goat anti-human IgG, IgM, and IgG, polyclonal rabbit anti-C4, and horseradish peroxidase (HRP)-conjugated rabbit anti-goat and goat anti-rabbit antibodies were purchased from Sigma (St Louis, MO). Human IgG, IgM, and IgA used for RF assays and as positive controls on immunoblots were also obtained from Sigma. Polyclonal rabbit anti-human C3d was purchased from Dako (Carpinteria, CA).

Chromatography media

Sephacryl S-300 was obtained from Pharmacia (Uppsala, Sweden). Staphylococcal protein A immobilized on agarose was obtained from Sigma. Rabbit polyclonal anti-human C3d was leashed to 1,1′-carbonyldiimidazole-activated agarose (Reactigel; Pierce, Rockford, IL) according to the manufacturer's recommendations.

Buffers

Solubilizing buffer for SDS–PAGE consisted of 0·25 M Tris, 10% SDS, 2·0% glycerol, and 5·0% β-mercaptoethanol. Transfer buffer for Western blotting consisted of 20 mM Tris, 150 mM glycine, 0·1% SDS, and 20% methanol pH 8·0.

Purification of high molecular weight complexes from synovial fluids

Synovial fluid was diluted 1 : 10 and placed on a gel filtration column containing Sephacryl S-300 equilibrated with PBS. The first eluted peak was collected and subjected to a second purification step on Protein A agarose equilibrated with PBS. The column was washed extensively and the bound complexes eluted with 0·1 M glycine pH 2·5. Complexes were dialysed against PBS, 0·2% sodium azide, and concentrated by ultrafiltration to 0·5–1·0 ml. Protein concentration of the complexes was determined by the method of Lowry [12].

Affinity chromatography of JRA synovial fluids on anti-C3d-agarose

Synovial fluid was diluted 1 : 10 and subjected to affinity chromatography on Protein A-agarose. The column was washed thoroughly with PBS and the bound protein eluted with 0·1 M glycine pH 2·5. Fractions with eluted protein were identified by optical density at 280 nm. The eluted proteins were dialysed against PBS, then subjected to a second affinity purification step on anti-C3d agarose. Complexes were eluted as above, dialysed against PBS, and concentrated by ultrafiltration to 0·5–1·0 ml.

Alternatively, high molecular weight fractions of synovial fluid were prepared by size-exclusion chromatography on S-300 equilibrated with PBS. The high molecular weight material was then subjected to affinity chromatography on anti-C3d-agarose as above. For convenience, we designate complexes purified by sequential S-300/Protein A chromatography as 'high molecular weight complexes' and complexes purified by sequential Protein A/anti-C3d chromatography as 'C3d complexes'.

SDS–PAGE of purified complexes

SDS–PAGE of proteins eluted from the Protein A-agarose and anti-C3d-agarose columns was performed under reducing conditions in 10% polyacrylamide minigels after the method of Laemmli [13]. Details of this procedure have been published elsewhere [7]. Gels were stained with a commercially available copper staining reagent (BioRad, Hercules, CA) to visualize protein bands.

Western blot analysis

High molecular weight immune complexes and complexes eluted from anti-C3d-agarose were subjected to SDS–PAGE and transferred to nitrocellulose. Immunoblotting for IgA, IgM, IgG and C4 was carried out exactly as described previously [7].

Complement activation experiments

Complement activation by synovial fluid immune complexes was assessed by the production of C4d and Bb fragments (measured by ELISA) when 10 μg of complexes were placed in 25% normal human serum (NHS). Details of these assays have been described previously [7]. Results were compared with a positive control consisting of 10 μg bovine serum albumin (BSA)-anti-BSA at 2 × antigen excess. To account for day-to-day variability in the assays, results were expressed as a percentage of C4d produced by BSA-anti-BSA, arbitrarily designated '100%' activation, by the following formula: C4d produced = [C4d] measured − [C4d] in the thermal control.

RF ELISA assays

Immune complex preparations with positive Western blots for IgM and IgA were tested for antiglobulin activity by ELISA as previously described [7].

Statistical analysis

Comparison of C4d activated by high molecular weight or C3d complexes was performed by independent *t*-test using commercially available statistical software (Epistat Σ Services, Richardson, TX).

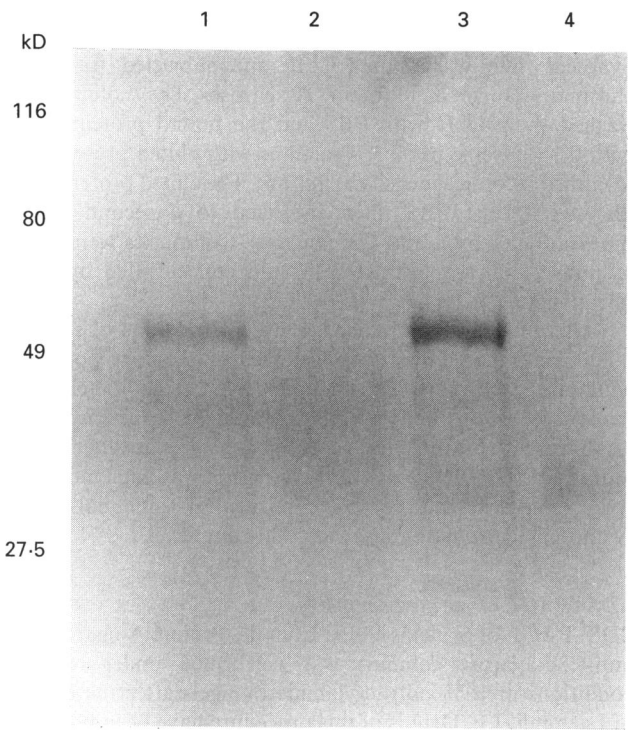

Fig. 1. Copper-stained polyacrylamide gel comparing high molecular weight (lanes 1 and 3) with C3d complexes (lanes 2 and 4) from JRA synovial fluids. A prominent 60-kD band is seen in the high molecular weight complexes only. This band corresponds to IgM on immunoblotting. Smaller 35- and 20-kD bands are also visible in lane 3. Only the 35-kD band is visible in the C3d complexes. Complexes in lanes 1 and 2 came from the same synovial fluid as did those in lanes 3 and 4.

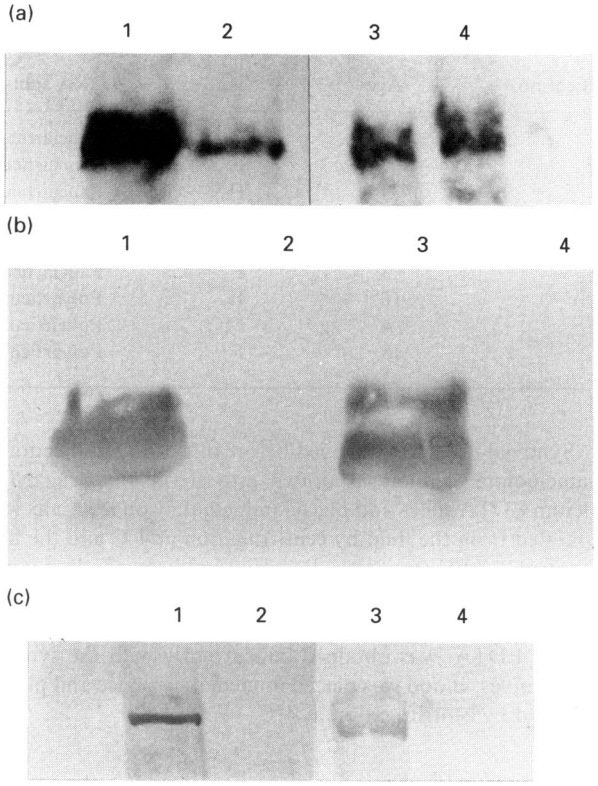

Fig. 2. Immunoblots obtained from the complexes shown in Fig. 1. Lanes 1 and 3 are high molecular weight complexes; lanes 2 and 4 are C3d complexes. (a) Immunoblot for IgG. (b) Immunoblot for IgA. (c) Immunoblot for IgM. Strong blots for IgM are seen in the high molecular weight complexes but not C3d complexes. In addition, strong blots for IgA are seen in the high molecular weight complexes. C3d complexes contain only IgG.

RESULTS

Purification of immune complexes from synovial fluid

High molecular weight IgG-containing complexes were isolated from synovial fluids of all nine patients with JRA using sequential column chromatography on S-300 and Protein A-agarose. Similarly, high molecular weight complexes could be isolated from the plasma of two patients with pauciarticular JRA (patients 1 and 3; see Table 1) and one with polyarticular JRA (patient 7) by the same method (plasma was not available on the other patients). However, when high molecular weight fractions of synovial fluid were prepared on S-300 and subjected to affinity chromatography on anti-C3d-agarose, no bound protein was eluted (data not shown).

Figure 1 shows a representative polyacrylamide gel comparing high molecular weight (lanes 1 and 3) with anti-C3d complexes purified from JRA synovial fluids. High molecular weight complexes have easily visible bands at approximately 60 kD. In addition, smaller 35- and 20-kD bands can be seen in the high molecular weight complexes in lane 3. Only the 35-kD band is visible in lanes containing C3d immune complexes. The approximately 60-kD band corresponds to IgM heavy chain on immunoblotting. The other bands were not specifically identified.

Western blot analysis of immune complexes prepared on Protein-A Sepharose and anti-C3d-agarose

Figure 2a,b,c show representative immunoblots of high molecular weight (lanes 1 and 3) and C3d immune complexes (lanes 2 and 4) taken from synovial fluid of children with JRA. Positive blots for IgG and IgM are seen in high molecular weight complexes. IgA was detected in 5/9 of the high molecular weight complexes. IgA was not detected in complexes from patients 3, 4, 5 and 8. Only IgG was found in C3d immune complexes (lanes 2 and 4).

Immunoblots of serum immune complexes showed identical immunoglobulin content compared with complexes purified from the same patient's plasma. These results were reproduced in the other two patients examined: a girl with polyarticular JRA and a boy with pauciarticular JRA (data not shown).

A faint band for C4 was detected in only 1/9 high molecular weight immune complex preparations (patient 7) and in none of the C3d complexes. This suggests that, if complement was bound to these complexes *in vivo*, it was via the alternative rather than the classical pathway.

Complement activation experiments

Figure 3 summarizes the results of complement activation experiments. When high molecular weight complexes prepared on Protein-A Sepharose were incubated with normal human serum, all were more efficient than preformed BSA-anti-BSA at activating the classical complement pathway. The mean percentage of C4 activation in high molecular weight complexes

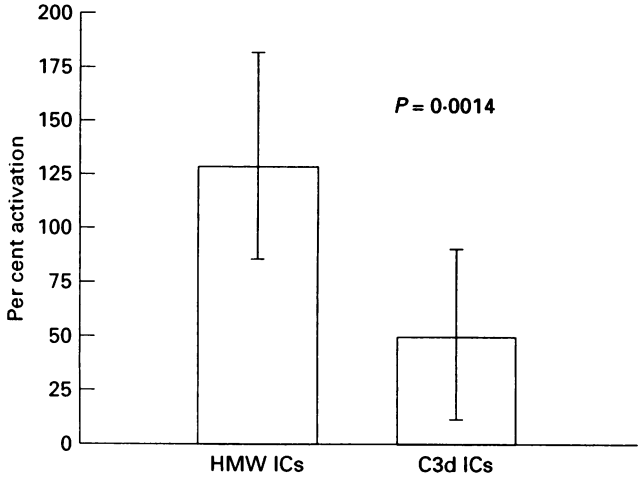

Fig. 3. Results of classical complement activation experiments comparing high molecular weight and C3d immune complexes obtained from JRA synovial fluids. Depicted are results obtained from seven experiments. Results are expressed as percentage of C4d produced compared with a control consisting of bovine serum albumin (BSA)-anti-BSA preformed at ×2 antigen excess (arbitrarily designated '100% activation'). High molecular weight complexes (HMW ICs) produced 133 ± 48% more C4d over the 30-min incubation period than BSA-anti-BSA. In contrast, C4d production was 50 ± 40% of the control in C3d complexes (C3d ICs). These differences were statistically significant ($P = 0{\cdot}0014$).

was 133 ± 48% compared with equal amounts of preformed BSA-anti-BSA complexes. The lowest levels of C4d produced in experiments using high molecular weight complexes were from complexes from a child with polyarticular JRA, whose complexes produced 105% of the C4d produced by equal amounts of BSA-anti-BSA complexes. Of interest is the fact that high molecular weight complexes from this child had a positive immunoblot for C4, suggesting that these complexes had already bound C4 *in vivo*. Consistent with this hypothesis are the findings from complement activation experiments performed with complexes performed by sequential affinity chromatography on Protein A-agarose and anti-C3d Sepharose. C3d complexes were all less efficient than BSA-anti-BSA at activating the classical pathway. The mean percentage of C4 activation by C3d complexes (50 ± 40%) was significantly less than that of the high molecular weight complexes ($P = 0{\cdot}0014$).

Preformed BSA-anti-BSA and C3d complexes were weak activators of the alternative pathway, as measured by Bb production over 30 min. High molecular weight complexes produced 10–15% more Bb than thermal controls (data not shown).

ELISA assays for IgM and IgA-RF

IgM-RF activity was detected in all nine of the high molecular weight immune complex preparations. IgA-RF activity was detected in the five high molecular weight immune complex preparations that were positive for IgA by immunoblotting. There was insufficient material to test these complexes for IgG-RF activity. Because immunoblots from C3d complexes were negative for IgM and IgA, RF ELISA assays were not performed on this material.

DISCUSSION

In vitro investigations have demonstrated that IgM-RF-bearing immune complexes possess several interesting properties: (i) they inhibit the ability of the complement system to regulate immune complex size [14]; (ii) while this may be due to inhibition of the complement activation [15], other studies have shown that complement activation *per se* is unaffected [8]; (iii) these complexes bind poorly to erythrocyte complement receptors [8], due, in all likelihood, to the RF's inhibition of the covalent binding of complement components to the immune complex [9]. Thus, these *in vitro* studies suggest that IgM-RF-bearing immune complexes have considerable pathogenic potential, as they maintain their phlogistic properties while, at the same time, being resistant to complement-mediated mechanisms for immune complex detoxification and removal [10]. Until now, however, there has been no demonstration that such complexes exist at sites of inflammation in rheumatoid disease.

These studies have shown that high molecular weight immune complexes can be isolated from synovial fluids of children with chronic inflammatory arthritis. These complexes are composed of IgM, IgA, and IgG. Some of the IgM and IgA within the complexes possess antiglobulin (rheumatoid factor) activity. Furthermore, even though eight of the nine high molecular weight immune complex preparations had not bound C4b *in vivo*, all efficiently activated the classical complement pathway *in vitro*. All of these findings are compatible with and predictable from *in vitro* work done in our laboratory as well as those of other investigators.

Previous studies from our laboratory have demonstrated a correlation between plasma complement activation and active disease in both pauciarticular and polyarticular JRA [4,16]. However, the correlation between complement activation fragments and circulating immune complexes (measured by ELISA with monoclonal anti-C3d or -C1q antibodies with HRP-conjugated anti-human IgG as the detection antibody [4] or by ELISA using purified C1q as the capture protein [16]) was weak. A stronger correlation is seen between the presence of IgM-RF (detected by ELISA and now known to be common in all subtypes of JRA [17]) and disease activity [6]. Similarly, we have demonstrated the presence of IgM-RF within high molecular weight complexes in JRA plasma [7]. Together, these findings explain the lack of correlation between immune complex levels and JRA disease activity when assays which rely on *in vitro* complement fixation by the immune complexes are used. We postulate that the complexes of interest (i.e. those most likely to be involved in the pathophysiology of the disease) are the high molecular weight, IgM- (and possibly IgA-) bearing complexes which have not bound complement *in vivo*.

One caveat in interpreting our data involves patient selection. The clinical indications for joint aspiration and injection on our service are variable, but include the judgement that functioning can be substantially improved by rapidly decreasing inflammation in a single joint. This rarely occurs in polyarticular JRA, as, when joint inflammation occurs, multiple joints are usually involved. Therefore, these findings may not be applicable to all children, particularly those with polyarticular JRA.

While the studies reported here do not definitively prove that IgM-RF are critically involved in disease pathogenesis in

JRA, they raise some interesting questions. We are intrigued, for example, that other investigators have reported a strong link between the presence of specific HLA class II haplotypes and *in vitro* synthesis of IgM-RF, even in normal individuals [18]. This suggests a link between our findings and our evolving knowledge of the immunogenetics of rheumatoid disease in children [19]. Even if there is no single 'trigger' or initating event in the JRA or common to the different JRA subtypes, we hypothesize that children with JRA may be immunogenetically disposed to synthesize high titres of high-affinity IgM-RF in response to specific stimuli. If this genetic predisposition is also linked to genetically determined inefficiencies in immune complex removal [20,21], net accumulation and/or tissue deposition of immune complexes could occur. The data presented here and by other investigators suggest that, once the deposition of high molecular weight, IgM-RF-bearing immune complexes occurs, their removal may be difficult while their phlogistic potential persists. Each phase of this hypothesis is testable.

We conclude that high molecular weight, IgM-RF-bearing immune complexes can be found in joints of children with active synovitis in both polyarticular and pauciarticular JRA. The composition and biological behaviour of these complexes is consistent with findings that have been described for IgM-RF-bearing complexes formed *in vitro*, and suggest a pathophysiologic role for these complexes *in vivo*.

ACKNOWLEDGMENTS

This work was supported by the Children's Hospital of Michigan Research Endowment Fund, Parents Promoting Arthritis Research for Children (PPARC), and the Wayne State University Medical Alumni Association, which provided a summer fellowship for one of the authors (M.M.D.).

REFERENCES

1 Cassidy JT, Levinson JE, Bass JC. A study of classification criteria for a diagnosis of juvenile rheumatoid arthritis. Arthritis Rheum 1986; **29**:274–81.

2 Moore TL, Sheridan PW, Traycoff RB, Zuckner J, Dorner RW. Immune complexes in juvenile rheumatoid arthritis: a comparison of four methods. J Rheumatol 1982; **9**:395–401.

3 Latzka U, Menzel EJ, Granninger W, Steffen C. Immune complexes in juvenile chronic arthritis. Rheumatol Int 1984; **4**:45–48.

4 Jarvis JN, Pousak T, Krenz M, Iobidze M, Taylor H. Complement activation and immune complexes in juvenile rheumatoid arthritis. J Rheumatol 1993; **20**:114–7.

5 Moore TL, Dorner RW, Sheridan PW, Weiss TD, Baldassare AR, Osborn TG, Zuckner J. Longitudinal study of the presence of hidden 19S rheumatoid factor in juvenile rheumatoid arthritis. J Rheumatol 1982; **9**:599–602.

6 Jarvis JN, Pousak T, Krenz M. Detection of IgM rheumatoid factors by enzyme-linked immunoabsorbant assay in children with juvenile rheumatoid arthritis: correlation with articular disease and laboratory abnormalities. Pediatr 1992; **90**:945–9.

7 Jarvis JN, Iobidze M, Taylor H, DeJonge J, Chang S. A comparison of IgG-containing high molecular weight complexes isolated from children with juvenile rheumatoid arthritis and congenital HIV infection. Pediatr Res 1993; **34**:781–4.

8 Ng Y, Peters K, Walport M. Monoclonal rheumatoid factor-IgG immune complexes: poor fixation of C3 and C4 despite efficient complement activation. Arthritis Rheum 1988; **31**:99–107.

9 Jarvis JN, Lockman JC, Levine RP. IgM rheumatoid factor and the inhibition of covalent binding of C4b to IgG in immune complexes. Clin Exp Rheumatol 1993; **11**:135–41.

10 Schiffereli J, Ng Y, Peters K. The role of complement and its receptors in the elimination of immune complexes. N Eng J Med 1986; **315**:488–95.

11 Vaughn JH. Pathogenic concepts and origins of rheumatoid factor in rheumatoid arthritis. Arthritis Rheum 1993; **36**:1–6.

12 Lowry O, Rosenbrough N, Farr A, Randall R. Protein measurement with Folin reagent. J Biol Chem 1951; **193**:265–72.

13 Laemmli VK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 1970; **22**:680–5.

14 Mitchell WS, Naama JJK, Whaley K. IgM-RF prevents complement-mediated inhibition of immune precipitation. Immunology 1984; **27**:1130–6.

15 O'Sullivan M, Amos N, Williams B. Complement-mediated inhibition of immune precipitation in rheumatoid arthritis: studies on the interaction of heat-aggregated IgG and IgM rheumatoid factor. Ann Rheum Dis 1988; **47**:675–80.

16 Jarvis JN, Taylor H, Iobidze M, Krenz M. Complement activation and immune complexes in children with polyarticular juvenile rheumatoid arthritis. J Rheumatol 1994; **21**:1124–7.

17 Moore TL, Dorner RW, Osborn TG, Zuckner J. Hidden 19S rheumatoid factors. Semin Arthritis Rheum 1988; **18**:72–75.

18 Olsen NJ, Stasny P, Jasin HE. High levels of *in vitro* IgM–rheumatoid factor synthesis correlate with HLA-DR4 in normal individuals. Arthritis Rheum 1987; **30**:841–8.

19 Ploski R, Vinje O, Rønningen KS, Spurkland A, Sorkaar D, Vartdal F, Førre O. HLA class II alleles and heterogeneity of juvenile rheumatoid arthritis. Arthritis Rheum 1993; **36**:465–72.

20 Walport MJ, Lachman PJ. Erythrocyte complement receptor type 1, immune complexes, and the rheumatic diseases. Arthritis Rheum 1988; **31**:153–8.

21 Thomsen BS, Heilman C, Jacobsen SEH *et al.* Complement C3b receptors on erythrocytes in patients with juvenile rheumatoid arthritis. Arthritis Rheum 1987; **30**:967–71.