

Characterisation of the size and composition of circulating immune complexes in patients with rheumatoid arthritis

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Abstract

The size and composition of circulating immune complexes in the sera of patients with rheumatoid arthritis (RA) were studied in relation to different manifestations of the disease. Circulating immune complexes from the sera of 94 patients (50 with extra-articular disease) and 10 matched controls were fractionated by sucrose density gradient ultracentrifugation. The composition, immunoglobulin and rheumatoid factor (RF) concentrations within each of the fractions were determined by a sensitive enzyme linked immunosorbent assay (ELISA).

Intermediate size (14S-21S) IgG complexes containing RF activity and 22S IgG-IgM RF complexes were found in the sera of 40 patients with RA, while intermediate size complexes of self associated IgG RF and larger size complexes (>22S) of IgG RF and IgM RF were associated with extra-articular features of RA (50% of extra-articular disease). Complexes containing IgA were found in the sera of many patients with RA, and dimeric IgA RF mainly in patients with extra-articular disease.

These results support the view that whereas small size circulating immune complexes are of no primary pathogenic importance in synovitis, large size (>22S) circulating immune complexes may play a role in extra-articular disease in RA. Current understanding of the formation of large complexes provides a biological explanation for their occurrence and effects.

Immune complexes are thought to play a part in the pathogenesis of synovitis and in the adverse immunological events of autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (RA).^{1,2} Immune complexes have been demonstrated in synovial tissue,³ synovial neutrophils,⁴ synovial fluid,^{5,6} and sera^{7,8} from patients with RA, and these findings have stimulated the development of numerous assay procedures for their measurement. In RA the detection and measurement of circulating immune complexes has been seen as a potential laboratory guide to disease activity and as an indicator of impending or deteriorating extra-articular disease,⁹⁻¹¹ though their pathological significance remains poorly understood.¹

In experimental immune complex disease in animals the pathogenic effect of circulating immune complexes seemed to be dependent on their size and composition.¹² In human systemic

lupus erythematosus the presence or absence of different sizes of circulating immune complexes has been found to correlate with different complications of disease, such as renal involvement,¹³ though others disagree on the details of these findings.¹⁴

In RA synovial fluid immune complexes were found to be of both intermediate (6S-19S) and large (22S-30S) size and to consist of IgG bound to IgG rheumatoid factor (RF).^{15,16} In serum, complexes of 22S size have been detected by analytical ultracentrifugation,^{5,7} leading to the suggestion that these might consist of one molecule of IgM RF bound to five molecules of IgG.¹⁶ Pope *et al* demonstrated 7S-19S complexes of non-complement fixing self associated IgG RF in the sera of three patients with RA,¹⁷ and Moller *et al* identified similar intermediate size circulating immune complexes in four of 11 patients.¹⁸

It would be of interest to elucidate further the nature of serum immune complexes in RA and to relate the size of the complexes and their RF content to clinical manifestations of the disease. We used ultracentrifugation of serum followed by IgA, IgG, and IgM class specific enzyme linked immunosorbent assay (ELISA) measurement of immunoglobulins and RF in different fractions to investigate this problem.

Patients and methods

PATIENTS

Of 94 patients with definite or classical RA (American Rheumatism Association criteria¹⁹), 26 were recruited by a single rheumatologist (JRK), together with 10 normal controls matched for age (within 5 years) and sex. Laboratory studies were conducted without knowledge of their clinical status. On the basis of data obtained from this well defined group a further 68 serum samples supplied by different rheumatologists were examined. Results from these two sets of patients were almost identical and have been amalgamated here. All the patients, taken together, included 44 with articular disease (group A) and 50 with extra-articular disease (group EA) (table 1). These included 21 patients with nodules only, nine with vasculitis only, and six with nodules and vasculitis. A further six patients with vasculitis had a range of other complications, including pericarditis (two), episcleritis (one), scleritis (two), and pulmonary fibrosis (one). Four patients had amyloid and a further four had Sjögren's syndrome. Disease activity (none/mild/moderate/severe), radiographic erosions in the hands (present/absent), and treatment were

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Table 1 Patient characteristics

	Group A Articular disease (n=44)	Group EA Extra-articular disease (n=50)
Age (years)*	55 (13)	59 (12)
Sex (M/F)	8/36	24/26
Disease duration (years)*	10 (9)	12 (10)
Age at onset of symptoms (years)*	45 (14)	46 (16)
Disease activity†		
Inactive	6	7
Mild	13	15
Moderate	17	20
Severe	8	8
Erosions (yes/no)‡	32/12	46/4
Positive latex test for rheumatoid factor	37	47
Treatment		
None	7	2
NSAID§ only	14	9
2nd line drugs	18	9
3rd line drugs¶	5	30

*Mean (SD) as reported by managing doctor.

†Disease activity represents the overall nature of the disease at the time serum samples were taken, assessed on the basis of articular index, duration of morning stiffness, pain, number of joints affected, and the erythrocyte sedimentation rate.

‡Definite erosion established by radiography.

§NSAID=non-steroidal anti-inflammatory drug.

||Penicillamine, gold, or antimalarial drugs.

¶Cytotoxic drugs or steroids, or both.

recorded by the clinician managing the patient. Normal controls are designated group C.

C1q BINDING ASSAY

The C1q binding assay of Zubler *et al.*²⁰ was used, modified according to Bourke *et al.*²¹

C1q SOLID PHASE ELISA

The C1q solid phase assay was that described by Hay *et al.*,²² modified for an ELISA. Bound immune complexes were disclosed with horseradish peroxidase conjugated goat F(ab)₂ anti-human γ , α , or μ heavy chain antibodies (Kallestad Diagnostics, Texas, USA).

The results were expressed in immune complex units according to the following formula as suggested by Wager *et al.*²³:

$$\text{Immune complex units} = (S492 - X492) / SD$$

where S492=absorbance of the test sample at 492 nm; X492=mean absorbance of 10 normal human sera tested on the same plate; SD=standard deviation of the absorbance of the 10 normal sera.

SOLID PHASE ANTI-C3 ASSAY

Solid phase anti-C3 was determined by an ELISA with the solid phase sensitised with goat F(ab)₂ antihuman C3 (Cappel Laboratories, Cochranvilles, PA, USA) at 14 $\mu\text{g/ml}$ and 100 $\mu\text{l/well}$ in phosphate buffered saline. Parallel duplicate wells sensitised with normal goat F(ab)₂ were used as controls. The results were recorded as immune complex units as described for the C1q solid phase assay after subtracting optical density values for binding to normal goat F(ab)₂ from that for binding to anti-C3 sensitised wells.

ELISA FOR THE MEASUREMENT OF RHEUMATOID FACTORS AND IMMUNOGLOBULINS

Rheumatoid factor was measured as described

in detail elsewhere.²⁴ Briefly, ELISA microtitre plates sensitised with rabbit gammaglobulins (at 50 $\mu\text{g/ml}$ and 45 $\mu\text{l/well}$) in phosphate buffered saline were used to measure IgG, IgA, and IgM RFs by reference to pooled RA sera with known concentrations of RF as measured by nephelometry and haemagglutination.

For measurement of immunoglobulins in sera and sucrose gradient fractions a similar approach was used with plates sensitised with F(ab)₂ of antisera to γ , α , and μ heavy chains (Kallestad Diagnostics, Texas, USA).

SUCROSE DENSITY GRADIENT CENTRIFUGATION

Sucrose density gradient centrifugation was carried out as described in detail by McCarthy *et al.*²⁵ Briefly, sucrose gradients (10–30% w/v) were prepared in 16.5 ml polycarbonate tubes with phosphate buffered saline pH 7.4 as the base buffer, and 500 μl samples of serum, which had been diluted 1:2 with the same buffer, were layered on top of the gradient.

Sucrose gradients (10–30% w/v) prepared using 0.105 M glycine-HCl buffer and overlaid with the same volume of serum were centrifuged in parallel to assess the nature of circulating immune complexes in each fraction.

The gradients were centrifuged at 4°C for 19 hours at 23 500 rpm in an MSE 25 ultracentrifuge and fractionated by flotation (60% sucrose) using an ISCO fractionator into 25 fractions of 0.6 ml. Fractions from the acid glycine-HCl gradients were immediately neutralised to pH 7.4 by adding 0.6 ml of 0.2 M Na₂HPO₄ solution. The mean sedimentation coefficient of each fraction was determined as described by McCarthy *et al.*²⁵ Fractions comprising 7S, 8S–13S, 14S–21S, 22S–30S, and >30S were pooled and used in the various assays.

STATISTICAL ANALYSIS

One way analysis of variance was used to test for trends in relation to clinical status of the groups. Student's *t* test was used to examine the differences between each pair of groups.

Results

RHEUMATOID FACTOR AND CIRCULATING IMMUNE COMPLEX CONCENTRATIONS

In the 44 patients with articular disease alone raised concentrations of IgG RF, IgA RF, and IgM RF were found in 29 (66%), 27 (61%), and 40 (91%) patients respectively. For the 50 patients with extra-articular disease raised concentrations of RF were noted in 44 (88%), 42 (84%), and 47 (94%) (table 2).

Circulating immune complexes were raised in most patients. Although different assays gave slightly different results, circulating immune complexes were raised more commonly in extra-articular disease (98%, 76%, and 62% by C1q binding assay, C1q solid phase, and anti-C3 assays; see table 2) than in articular disease (59%, 37%, and 20%). This was true for all three isotypes measured.

When serum was fractionated by density gradient centrifugation (table 3, columns 4–8)

Table 2 Rheumatoid factor (RF) and circulating immune complex concentrations and their prevalence in controls (C) and patients with rheumatoid arthritis with articular (A) and extra-articular (EA) disease

	Group C (n=10)		Group A (n=44)		Group EA (n=50)	
	Mean (SD)	% Positive*	Mean (SD)	% Positive*	Mean (SD)	% Positive*
RF†						
IgG	1.2 (0.8)	10	2.8 (2.9)	66	3.5 (4.0)	88
IgA	0.4 (0.7)	0	2.6 (3.0)	61	3.5 (4.0)	84
IgM	2.0 (2.1)	0	4.0 (4.1)	91	5.0 (5.4)	94
Any combination	—	10	—	91	—	96
C1q binding assay‡	4.5 (1.7)	0	21.0 (19)	59	50.0 (28)	98
C1q solid phase§						
IgG	0.04 (0.5)	0	1.4 (2.7)	37	2.3 (3.0)	60
IgA	-1.0 (0.5)	0	0.1 (2.1)	16	2.0 (3.1)	46
IgM	0.8 (1.1)	0	0.6 (2.0)	9	0.9 (1.8)	40
Any combination	—	0	—	37	—	76
Anti-C3 assay§						
IgG	-0.7 (1.5)	0	0.2 (1.6)	16	1.6 (2.4)	46
IgA	-1.7 (0.5)	0	-1.1 (2.1)	9	0.3 (3.0)	18
IgM	0.9 (1.8)	0	0.1 (1.2)	11	0.3 (2.2)	14
Any combination	—	0	—	20	—	62

*Percentage of individual values above the upper limit of normal (defined as mean +2SD for all subjects).

†In log₁₀ ng/ml.‡Percentage of total trichloroacetic acid precipitable ¹²⁵I-C1q.

§In immune complex units (IC).

Table 3 Distribution of immunoglobulin isotypes after sucrose density gradient ultracentrifugation. Values are given as mean (SD)

Isotype	Patient group‡	Whole serum concentration (mg/ml)	Proportion (%) in gradient zones indicated				
			7S	8S-13S	14S-21S	22S-30S	>30S
IgG	C	8.8 (1.8)	76.4 (4.5)	18.1 (0.7)	1.9 (0.7)	1.1 (0.3)	1.3 (1.2)
	A	11.9 (3.9)*	74.6 (6.6)	19.1 (4.9)	3.3 (1.8)*	1.2 (1.0)	1.8 (0.8)
	EA	14.2 (11.3)*	71.4 (6.6)*†	20.7 (4.9)	4.3 (2.5)*†	1.4 (0.9)	1.9 (0.8)
IgA	C	1.8 (0.8)	75.6 (7.1)	18.0 (5.4)	4.1 (3.5)	1.0 (0.2)	1.3 (0.3)
	A	3.2 (1.4)*	70.8 (7.3)	21.0 (4.8)	5.1 (2.7)	1.3 (0.4)*	1.8 (0.6)*
	EA	4.9 (2.7)*†	70.4 (6.6)*	21.1 (4.5)	5.3 (2.4)	1.2 (0.5)*	1.9 (0.5)*
IgM	C	1.8 (1.0)	7.1 (4.0)	20.5 (8.7)	56.8 (8.0)	10.9 (4.6)	4.7 (3.3)
	A	1.6 (1.0)	8.0 (3.9)	20.2 (5.7)	53.4 (6.0)	14.7 (8.2)	3.7 (1.3)
	EA	2.3 (1.9)	9.5 (4.2)	18.6 (5.0)	51.6 (7.3)	16.4 (7.6)*	3.9 (2.7)

*Significantly different from group C (p<0.05).

†Significantly different from group A (p<0.05).

‡C=controls, A=patients with articular disease; EA=patients with extra-articular disease.

about 75% of the IgG was found in the 7S fraction, with about 20% in the 8S-13S fraction, and a small proportion in each of the other fractions. This general pattern was the same for all three groups, but there was a significant decrease in the amount of 7S IgG in group EA and a corresponding increase in the 14S-21S fraction. Group A also had an increase in the 14S-21S fraction, but this was not to the same extent. The absolute values of these increases in immunoglobulin concentrations are greater than might be suggested by the proportionate distributions alone, and represent increased

proportions of a larger total immunoglobulin as indicated in the table.

A similar pattern was obtained for IgA (table 3), except that the increase occurred in the heavier 22S-30S fractions, and was similar for both patient groups.

Most of the IgM was detected in the 14S-21S fraction and was distributed similarly for all subjects, though group EA showed a slightly increased proportion in the 22S-30S fraction.

Serum RF concentrations were also higher in patients than in controls (table 4, column 3). This was so to a much more marked degree than

Table 4 Size distribution of rheumatoid factor (RF) isotypes after sucrose density gradient ultracentrifugation. Values are given as mean (SD)

Isotype	Patient group‡	Whole serum concentration (µg/ml)	Total (ng/ml) in gradient zones indicated				
			7S	8S-13S	14S-21S	22S-30S	>30S
IgG RF	C	0.02 (0.01)	6.4 (6.9)	5.2 (6.0)	0.4 (1.3)	0	0
	A	0.63 (0.8)*	20.5 (41.0)*	23.8 (53.4)*	4.2 (10.4)*	0.8 (2.7)*	0.5 (2.5)
	EA	3.2 (10.00)*†	58.7 (75.8)*†	108.4 (234.5)*†	144.4 (146.8)*†	9.6 (35.3)*	2.2 (4.0)*
IgA RF	C	0.003 (0.005)	0	0.01 (0.04)	0	0	0
	A	0.4 (1.0)*	2.4 (5.1)*	24.0 (54.2)	14.0 (40.0)*	2.0 (9.5)	0.1 (0.7)
	EA	3.2 (10.00)*†	29.8 (144.4)*	163.5 (575.0)*†	59.3 (152.6)*†	10.1 (18.6)*	1.3 (4.0)
IgM RF	C	0.1 (0.01)	2.0 (6.3)	4.0 (8.4)	20.0 (23.1)	2.0 (6.3)	0
	A	10.0 (12.6)*	10.5 (18.0)*	157.3 (268.2)*	920.4 (3057.1)*	229.8 (765)*	22.6 (66.4)*
	EA	100.0 (251)*†	64.5 (188.4)*†	1135.6 (3034)*†	4359.4 (12030)*†	1163.5 (3029)*†	80.4 (193.2)*†

*Significantly different from group C (p<0.05).

†Significantly different from group A (p<0.05).

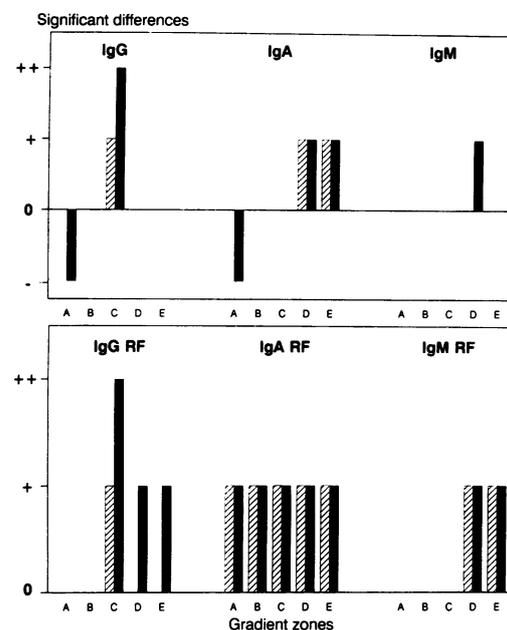
‡C=controls; A=patients with articular disease; EA=patients with extra-articular disease.

with total immunoglobulin. In control subjects the IgG RF was distributed about equally between the 7S and 8S-13S fractions. In group A there was slightly more in the 8S-13S fractions, while in group EA there was a very significant increase in the 8S-13S fraction. In the higher molecular weight fractions there was a slight increase in IgG RF in group A but once again a large increase in group EA.

In those few control subjects who had detectable concentrations of IgA RF it was present in fraction 8S-13S. Both patient groups had clearly raised concentrations in all fractions, especially in the 8S-13S and 14S-21S fractions.

IgM RF occurred almost entirely in the 14S-21S fraction in group C but was also present in substantial amounts in the 22S-30S and >30S fractions for both patient groups.

The figure provides a summary of the



Significant differences between patients and controls in concentrations of immunoglobulins and rheumatoid factors of different sizes. Analysis of the results of immunoglobulin and rheumatoid factor measurements (expressed as percentage of total immunoglobulin and rheumatoid factor contents of the gradient fractions) in the gradient zones using one way analysis of variance. The findings are presented as (0) when no differences were noted; (-) for significantly lower concentrations; (+) for significantly higher concentrations ($p < 0.05$), and (++) for very significantly higher values ($p < 0.01$) in the gradient zones for the patient groups compared with controls. The zones are presented as A (7S); B (8S-13S); C (14S-21S); D (22S-30S), and E (>30S). ▨=patients with articular disease; ■=patients with extra-articular disease.

immunoglobulin and RF results, where only the significant differences between the patient and control groups have been plotted. In general, IgG concentrations were increased in the middle range fractions in group A patients and more so in group EA patients. For IgA both patient groups showed increases in the heavier fractions. Concentrations of IgM showed little change from normal.

Rheumatoid factor concentrations, as well as being much greater in the patient groups in absolute values, also showed a difference in distribution according to the density gradient fractions. Whereas both patient groups had increased proportions of IgM RF in the heavy fractions, and both showed increases in IgG RF in the 14S-21S fraction, only group EA patients showed the presence of IgG RF in significant amounts in the heavy fractions (22S-30S and >30S).

The nature of immunoglobulins and RFs in heavy molecular weight fractions was also examined by centrifugation on acid gradient. Serum samples from 16 patients (four from group A and 12 from group EA) were fractionated simultaneously on both phosphate buffered saline (pH 7.4) and glycine-HCl (pH 2.5) gradients. The mean concentrations of IgG (table 5) were significantly increased in the 7S zone and decreased in the 8S-13S and 14S-21S zones for both groups of patients. The concentrations of IgG RF showed a significant increase in the 8S-13S zone only for patients of group A. In marked contrast, in patients from the EA group a significant increase in the concentration of IgG RF was noticed in the 7S zone. This was paralleled by a highly significant reduction in IgG RF in the high molecular weight zones. Similar results were noted for IgA RF and IgM RF (results not presented).

Discussion

Previous studies have shown the importance of size in determining the pathogenicity of circulating immune complexes in human immune complex disease and in animal models.^{12, 13} In this study using ultracentrifugation and class specific ELISA for immunoglobulins and RF we showed an association between large size IgG RF-IgM RF complexes and extra-articular features of RA.

The results show that in general circulating immune complexes in RA may be broadly grouped into small (8S-13S), intermediate

Table 5 Size distribution of IgG and IgG rheumatoid factor (RF) after sucrose density gradient: effect of acid dissociation. Results are given as mean (SD)

Patient group†	pH	Proportion (%) in gradient zones indicated				
		7S	8S-13S	14S-21S	22S-30S	>30S
IgG	A 7.4	67.1 (9.4)	26.1 (7.2)	4.5 (3.5)	1.0 (0.2)	1.3 (0.4)
	EA 2.5	82.2 (6.2)*	13.0 (5.5)*	2.2 (1.2)*	0.7 (0.1)	1.7 (0.2)
	A 7.4	71.8 (7.8)	20.7 (6.2)	4.6 (2.5)	1.2 (0.8)	1.0 (0.3)
	EA 2.5	80.0 (4.6)**	14.5 (4.3)**	2.2 (0.9)**	0.8 (0.2)	1.4 (0.3)
IgG RF	A 7.4	61.3 (11.6)	24.9 (4.3)	2.0 (2.3)	1.1 (1.3)	1.7 (1.9)
	EA 2.5	52.6 (16.9)	43.8 (19.5)**	2.0 (0.7)	0.9 (0.5)	1.2 (1.3)
	A 7.4	50.2 (23.9)	27.5 (11.6)	14.2 (16.2)	7.8 (1.2)	4.9 (5.7)
	EA 2.5	74.9 (12.7)**	16.7 (8.6)**	5.2 (3.8)*	1.2 (1.1)*	1.7 (1.1)*

Significantly different from content of the same zone at pH 7.4: * $p < 0.05$; ** $p < 0.01$. †A=patients with articular disease; EA=patients with extra-articular disease.

(14S–21S), and large (>22S) sizes. About half of all the patients with RA studied had small size complexes. Such complexes are likely to be composed of dimers of self associated IgG RF or one IgG RF complexed to a normal IgG molecule.

Intermediate size IgG complexes (14S–21S) are composed of three to five molecules of IgG and are likely to be arranged in the form of a tetramer of two molecules of self associated IgG RF and two normal IgG molecules according to the theoretical model of Nardella *et al.*²⁶ IgG RF tends to self associate preferentially as thermodynamics favour the formation of two antigen-antibody bonds rather than one. Further polymerisation of self associated IgG RF will proceed with a comparable free energy to that of normal IgG binding. Thus normal IgG will bind to a dimer of self associated IgG RF in preference to other IgG RFs owing to its relatively high concentration in serum and hence the effect of mass action, so effectively terminating further polymerisation and resulting in tetrameric complexes. The increase in RF complexes reported here may result from the higher concentration of IgG RF found in the serum of patients with extra-articular features compared with articular RA. More IgG RF will therefore be available to bind to the self associated IgG RF dimers. Large size complexes (22S–30S and >30S) were characteristically found in the serum of patients with extra-articular features of RA. These complexes are apparently composed of IgG RF dimers polymerised further with other IgG RF molecules. In contrast with intermediate size IgG tetramers such polymers have exposed antigenic sites and thus bind to IgM RF.²⁶

Analysis of the serum samples under dissociating conditions using glycine-HCl gradients showed that the high molecular weight IgG RF was in the form of immune complexes rather than aggregates as aggregated IgG cannot be dissociated under the same conditions.²⁷ Furthermore, the pattern of change of the concentration of IgG and IgG RF supported our hypothesis about the nature of complexes in patients with extra-articular disease. Whereas there is a substantial rise in the concentration of dimeric (8S–13S) IgG RF in patients with articular disease (group A) after acid dissociation, the rise in concentration of IgG RF in patients with extra-articular disease (group EA) was mainly noted in the 7S zone under the same conditions.

Complexes of IgM RF and polymers of IgG RF will form in preference to complexes between IgM RF and normal IgG owing to the low affinity of IgM RF for monomeric IgG and its high avidity for aggregated IgG.²⁸

Previous studies have suggested the association of high concentrations of IgG RF with extra-articular features of RA.²⁹ The precise mechanism by which IgG RF may contribute to the pathogenesis of extra-articular features, and vasculitis in particular, in patients with RA was not clear. Whereas small size circulating immune complexes are apparently of no pathogenic significance in synovitis, the formation of large complexes of IgM RF and polymers of IgG

RF reported here can initiate pathogenic mechanisms which contribute to vasculitis.¹ Large size IgG RF complexes enhance platelet aggregation,³⁰ leading to the release of vasoactive amines and histamine, which in turn increases vascular permeability resulting in the trapping of complexes along the basement membrane.¹² Furthermore, although IgM RF containing complexes inhibit platelet aggregation,³⁰ IgM RF may nevertheless enhance complement activation by IgG RF complexes and reduce solubilisation of such complexes.³¹

The significance of circulating immune complexes containing IgA in the sera of many patients with RA, and of dimeric IgA RF detected in the sera of patients with extra-articular features, remains to be elucidated. Recent studies link increased concentrations of IgA RF with fluctuation of disease activity, poor prognosis, and the development of erosions.^{32–34} Our results also show that IgA RF in RA occurs mainly in the form of polymers. Similar findings were reported for IgA RF in patients with sicca syndrome.³⁵ Longitudinal studies on patients measuring the size of their circulating immune complexes before and after the development of extra-articular features may provide additional evidence as to the pathogenic role of IgA RF complexes.

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