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Measurement of Immune Complexes with the Liquid Phase C1q Binding Assay: Ten Years Experience in a Routine Diagnostic Laboratory

Peter J. Roberts – Thomson, Sim H. Neoh, Alison Kennedy, Malcolm D. Smith, John O'Donnell and John Bradley.

SUMMARY We describe our 10 years experience in assaying over 15,000 clinical specimens for immune complexes (IC) using the C1q binding assay. Normal ranges were initially established using a large panel of blood donor sera and precision of the assay was optimized by inclusion of heat aggregated IgG (HAGG) as standards. Nevertheless some variability was observed due to variation in C1q binding from batch to batch and with aging of this reagent.

In an empirically selected 2 year period involving over 3,000 clinical specimens, 25% had elevated concentrations of IC. Of these the majority were from patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), other connective tissue disorders, infective endocarditis (IE), diffuse interstitial lung disease (DILD) and vasculitis (VASC). In RA, IE and VASC, significant correlations were observed between concentrations of IC and rheumatoid factor (RF) and the addition of a purified monoclonal RF to normal serum caused increased C1q binding. Longitudinal studies in RA and IE demonstrated a striking decline in IC in response to effective treatment.

We conclude that the measurement of IC provides little additional useful diagnostic information in those diseases associated with high levels of RF but appears more useful in disorders such as SLE, IE and DILD in which RF is absent or present in low concentration. Sequential monitoring of IC in RA and IE reflects response to treatment.

The potential pathological consequences of circulating immune complexes has held the fascination of immunologists ever since von Pirquet suggested their occurrence at the beginning of this century.[1] During the mid 1970's, a number of techniques for the detection of IC were described and considerable interest was generated in their possible value in the diagnosis and management of a number of clinical disorders.[2]

About this time (1976) the Diagnostic Clinical Immunology laboratories at Flinders Medical Centre commenced operations and because of the interest in circulating IC we offered two assays to interested physicians to quantitate IC. One assay was locally developed and was based on a nephelometric technique using a purified monoclonal RF.[3] It soon became apparent that it had limited diagnostic value[4] and was phased out. The second assay was the liquid phase C1q binding assay originally described by Nydegger and colleagues[5] and modified by Zubler *et al.*[6] We chose this assay as it was well characterised, relatively simple and cheap and performed well when compared with a number of alternative assays.[7] We now report our experience over the last 10 years in assaying over 15,000 clinical specimens from a large variety of clinical disorders.

MATERIALS AND METHODS

Purification of C1q

C1q was purified from the serum of a single healthy donor on multiple occasions by the salt precipitation method of Volanakis and Stroud.[8] It was stored in small aliquots at -80°C.

Radioiodination of C1q

Radiolabelling of C1q with ^{125}I was performed every 3-4 months by the lactoperoxidase method[9] to give a specific activity of approximately 1 μCi/μg protein. Radiolabelled ^{125}I-C1q was stored at -80°C.

From the Department of Clinical Immunology, Flinders Medical Centre, Adelaide, 5042, South Australia, Australia.

Address for Correspondence: Dr.P.J. Roberts – Thomson, Department of Clinical Immunology, Flinders Medical Centre, Bedford Park, 5042, S.A., Australia.

Preparation of HAGG standards

Commonwealth Serum Laboratories gammaglobulin (20mg/ml) in phosphate buffered saline (PBS) was heated in glass tubes for 30 minutes at 63°C, aliquoted into small volumes and stored at -80°C. When used it was further diluted in PBS.

C1q binding assay

This was performed with minor modifications according to the method of Zubler *et al.*[6] In brief, test sample (50μl) was incubated with 0.2 M EDTA (100μl) in order to break down the C1qrs complex and to prevent incorporation of ^{125}I-C1q into this complex. ^{125}I-C1q and one ml polyethylene glycol (PEG) (final concentration 2.7%) was then added to the EDTA-test sample. After incubation at 4°C for 1 hour, the mixture was centrifuged at $1500 \times g$ at 4°C for 30 minutes, the supernatant discarded and the precipitate counted in a gamma counter and compared with the total counts precipitated by 10% trichloroacetic acid. This % precipitation of radioactivity (% binding) was then referred to a standard curve constructed from the per cent binding of 6 HAGG standards of known concentration. All values determined were the means of duplicates. Results were expressed in terms of μg of HAGG equivalents per 50 μl of test sample which was therefore equivalent to HAGG equivalents $\times$ 20 μg/ml of test sample. However results were generally expressed as units/ml without using the multiplication factor.

Pathological samples and control sera

Control sera were obtained from 200 blood donors of known age. Pathological sera were those submitted routinely to the Department for quantitation of IC. The diagnosis was assumed to be that clearly identified on the requesting form. A more detailed study was performed with sera from patients with RA. Patients with inactive disease were defined as having no clinical findings of active synovitis, patients with active disease having active synovitis, patients with rheumatoid vasculitis having evidence of a necrotising vasculitis, mononeuritis multiplex or unexplained skin ulceration, and Felty's Syndrome defined as RA with splenomegaly and neutropaenia. In addition to sera, other pathological fluids including synovial fluid, pleural and pericardial fluid and bronchoalveolar fluid were also studied. All specimens were stored at -80°C prior to assay which was completed within two weeks. RF was measured by rate nephelometry (Beckman's ICS). A monoclonal RF was also studied and was purified from the serum of a patient with a lymphoproliferative disorder as previously described.[3] A monoclonal IgM paraprotein, purified from a patient with macroglobulinaemia, was used as an IgM control.

Statistical analysis

Comparison between variables was performed by linear regression using the method of least squares.

RESULTS

Technical aspects

Batches of C1q initially prepared from different individuals were found to vary in their binding activity to HAGG. Therefore a single donor was selected as the source of C1q as a good binding was consistently achieved. It was also observed that the binding to HAGG would decline over several months despite storage of C1q at -80°C. This together with the natural decay in radioactivity necessitated new batches of C1q being purified and radiolabelled every 3-4 months. These measures reduced considerably the inherent imprecision of the standard curve of C1q binding to HAGG over the period of study.

Determination of upper limits for C1q binding

The mean ± SD C1q binding for 200 individual blood donors was 22.00 ± 3.01%. The mean binding was similar for all age groups studied except for slightly elevated values found for the small number of donors who were older than 60 years. A value of mean + 2SD (= 28% binding) was therefore selected as the upper normal limit. As batches of C1q varied in their binding activity and demonstrated some decline with aging it was necessary to include HAGG standards in each assay. The upper limit of 28% C1q binding was equivalent to a binding of 2 μg of HAGG equivalents and this was therefore taken as the upper normal limit.

To confirm this upper normal limit a further 200 blood donor sera were analysed 5 years later and a very similar upper limit was obtained.

Pathological specimens

Cross sectional studies. Over a ten year period, ICs were measured in over 15,000 samples derived from a large variety of clinical disorders. Approximately 25% of these specimens had elevated concentrations of IC. A 2-year period 1984/85 involving over 3000 specimens was empirically selected for a more detailed study. Approximately 75% of these specimens with elevated concentrations were from patients with RA, 10% with SLE, and the remaining 15% from patients with other disorders. The proportion of patients with elevated concentrations of IC found in various rheumatic, infective and inflammatory disorders are shown in Figures 1 and 2. These specimens made up the majority (98%) with

elevated values seen over this 2-year period. In a separate analysis, patients with RA were subdivided into 4 groups according to their clinical manifestations and the concentrations of IC found are illustrated in Figure 3. High values were found in the majority of patients with active disease, particularly in those with vasculitis. Because of the possibility that RF was contributing to the elevated IC concentrations, we compared the levels of IC found in various rheumatic, inflammatory and infective disorders with the level of RF (Table 1). Significant correlations were found between these variables in RA, IE and VASC, but not in SLE, primary Sjögren's syndrome (SS) and DILD. To verify the involvement of RF in C1q binding, we added a purified monoclonal RF to normal human serum and measured C1q binding. The results (Table 2) indicated that IC involving RF with concentrations found in RA sera will cause the precipitation of radiolabelled C1q in PEG. Immune complexes were also assessed in other biological fluids. The mean ± SD IC level obtained for 36 synovial fluids from patients with RA was 30.3 ± 23.5 units/ml whilst for 16 synovial fluids obtained from other rheumatic disorders was 3.9 ± 3.2 units/ml. Occasional pleural, pericardial and bronchoalveolar fluids gave elevated values of IC in the absence of increased serum levels. The significance of this finding was not determined.

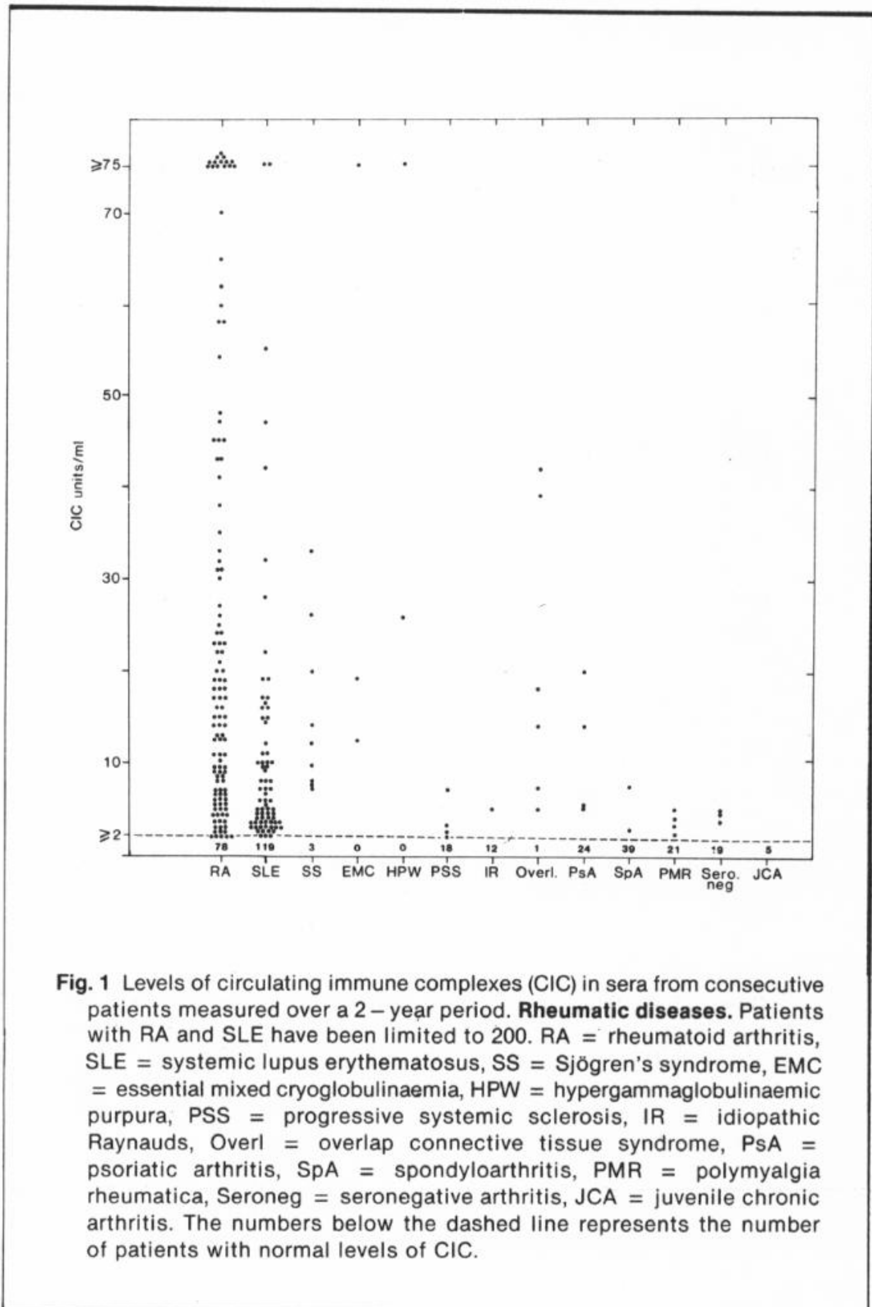

Fig. 1 Levels of circulating immune complexes (CIC) in sera from consecutive patients measured over a 2 – year period. **Rheumatic diseases.** Patients with RA and SLE have been limited to 200. RA = rheumatoid arthritis, SLE = systemic lupus erythematosus, SS = Sjögren's syndrome, EMC = essential mixed cryoglobulinaemia, HPW = hypergammaglobulinaemic purpura, PSS = progressive systemic sclerosis, IR = idiopathic Raynauds, Overl = overlap connective tissue syndrome, PsA = psoriatic arthritis, SpA = spondyloarthritis, PMR = polymyalgia rheumatica, Seroneg = seronegative arthritis, JCA = juvenile chronic arthritis. The numbers below the dashed line represents the number of patients with normal levels of CIC.

Sequential studies. C1q binding was quantitated sequentially in many patients particularly those with RA and IE. In RA declining concentrations of IC were observed in response to successful chrysotherapy and a close parallel was observed with RF (Fig. 4). We have described similar changes in IE[10] in response to successful eradication of valvular infection by antibiotics.

Table 1 Correlation between RF and IC in different disease

	RA	SS	SLE	IE	DILD	VASC
n	303	10	26	20	15	14
r	0.66	0.21	−0.1	0.60	0.13	0.77
p	<0.01	NS	NS	<0.01	NS	<0.01

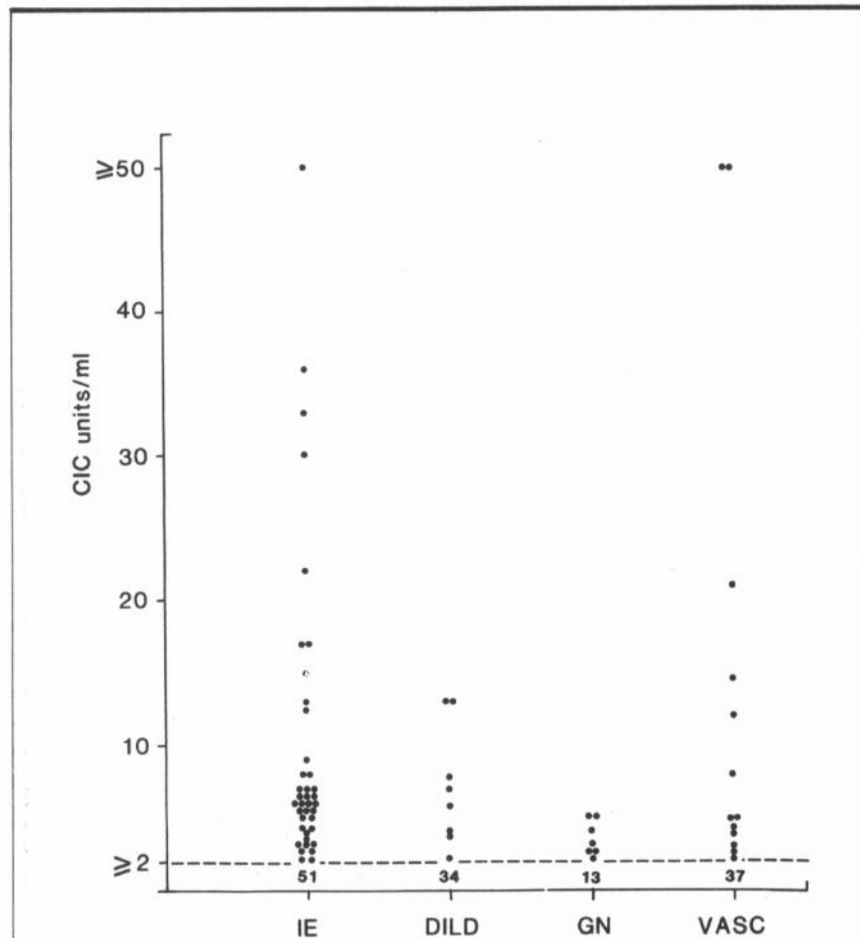

Fig. 2 Other inflammatory disorders. IE = infective endocarditis *(N.B.* in IE, consecutive sera have been charted as many patients had multiple estimations), DILD = diffuse interstitial lung disease, GN = glomerulonephritis, VASC = vasculitis (not associated with a connective tissue disorder). Sera with normal values of CIC are represented by the number below the dashed line.

Table 2 Addition of monoclonal IgM rheumatoid factor to normal serum

Dilution of		Dilution of	% binding	IC (units/ml)
MRF⁺	:	NHS⁰		
1	:	1	76	69
1/5	:	1	70	50
1/25	:	1	42	13
1/125	:	1	20	3
IgM control*	:	NHS		
1	:	1	9	<2
1/5	:	1	9	<2
1/25	:	1	8	<2
1/125	:	1	9	<2

+ MRF = monoclonal IgM rheumatoid factor (4 mg/ml)
0 NHS = normal human serum
* IgM control = purified monoclonal IgM (60 mg/ml)

DISCUSSION

Of the many assays described for measuring IC, the C1q binding assay has several attractions. It is well characterised,[6] is sensitive and has performed well in comparative studies with other assays.[7] Furthermore, despite the fact it does not bind non-complement-fixing IC, it appears to detect IC in most diseases in which an IC pathogenesis has been implicated.[7] It does, however, have certain technical limitations; it requires the regular purification of C1q from a previously identified 'quality' donor, it utilizes radioactivity and hence suffers from the potential dangers inherent in this form of assay and requires the use of an expensive gamma counter. In addition, its precision over prolonged periods of time is difficult to monitor in view of the problem of maintaining stable controls. Nevertheless, we selected this assay for routine IC quantitation and have now assayed over 15,000 clinical specimens.

Over 25% of these specimens had elevated concentrations of IC, particularly those from patients with RA. A more detailed examination of this group confirmed our previous findings[11] that elevated values were particularly prominent in those patients with active disease and a significant correlation was found between IC and RF. Furthermore, the addition of purified RF to normal serum resulted in enhanced C1q binding, implicating RF/IgG complexes as one form of IC to cause increased C1q binding. This association has also been described elsewhere.[12] However, the association of RF with IC was not invariable; in some diseases, *e.g.*, SLE, DILD, elevated C1q binding was seen in the absence of RF while some patients with low level of RF had high levels of IC (*e.g.*, IE and SS). These observations suggest that non-RF containing IC or other substances can also bind C1q and in IE sequential

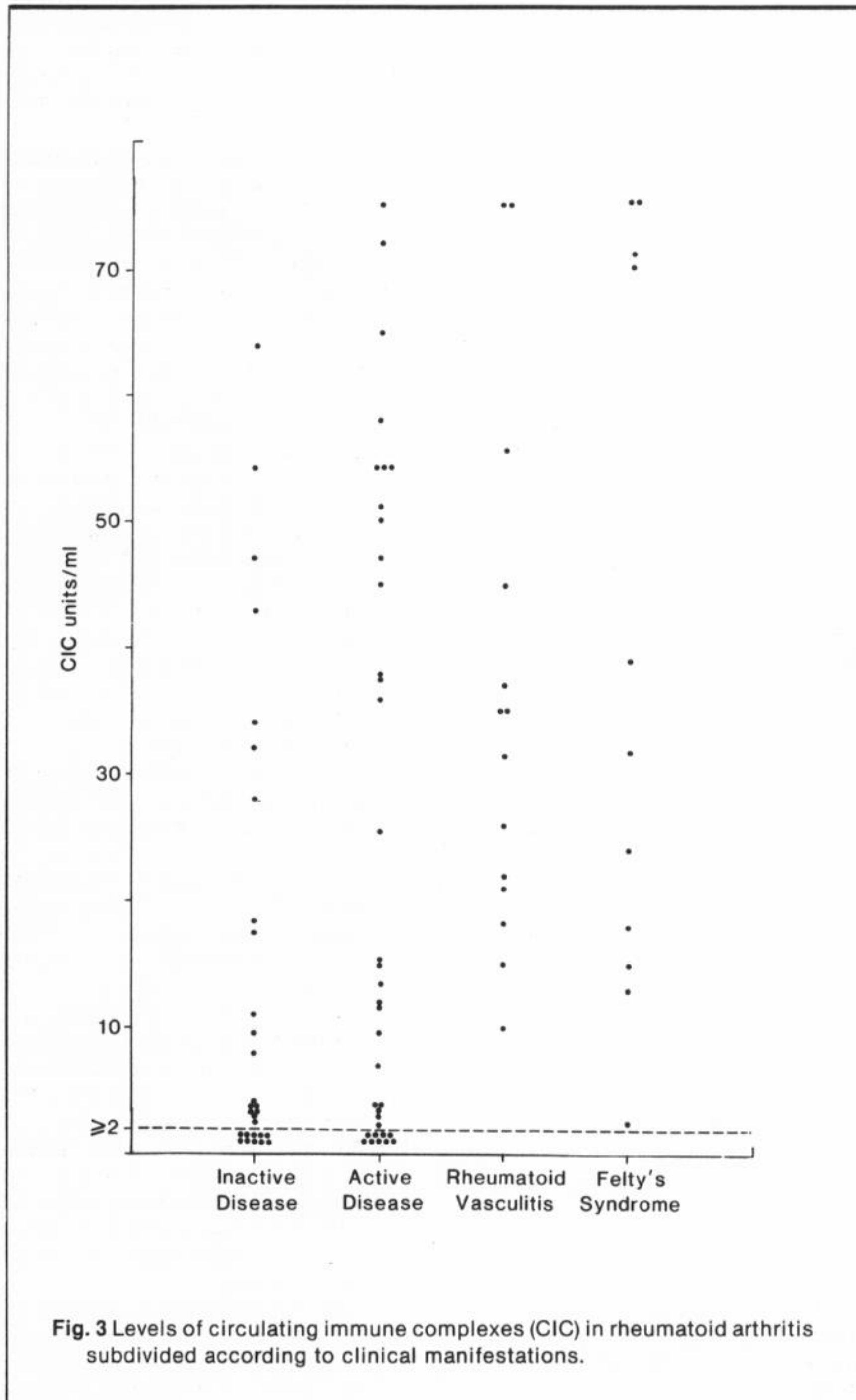

Fig. 3 Levels of circulating immune complexes (CIC) in rheumatoid arthritis subdivided according to clinical manifestations.

studies revealed that a successful response to antimicrobial treatment was reflected by a striking decline in IC concentrations.[10]

In the current study, because of its retrospective nature, we have not specifically addressed the question of sensitivity and specificity of IC quantitation in various clinical disorders. It appeared, however, that the measurement of IC gave little additional useful clinical information in RA above that obtainable by measuring the level of RF. Furthermore, sequential studies in RA revealed a striking parallel between RF and IC as illustrated by the decline in both with chrysotherapy. This once again suggests little additional benefit in assessing IC sequentially in this disorder as compared with RF. However, in other disorders, *e.g.*, SLE, DILD, VASC and IE, when elevated levels were found it was our impression (although not formally addressed in this study) that this finding frequently alerted the attending physician to a possible IC disorder (particularly with IE). Sequential monitoring in these disorders were of value[10] although a number of SLE patients were observed who had persistently elevated concentrations of IC in the absence of overt disease activity. Immune complexes were also detected in other biological fluids apart from serum. Elevated levels were particularly common in rheumatoid synovial fluid, however, in most instances serum levels were also elevated but to a lesser extent. Once again the finding of elevated levels in this circumstance had limited diagnostic value. One group of clinical disorders of which we had minimal experience was cancer. Elevated C1q binding has been reported in a number of different forms of solid and haematological cancer and some investigators have suggested that elevated concentrations of IC has prognostic significance.[13,12] The usefulness of the quantitation of IC in cancer in a routine diagnostic laboratory has yet to be reported.

In conclusion, we feel that the quantitation of IC by the C1q binding assay has diagnostic and prognostic value in a limited number of clinical disorders. In RA, little useful additional information is gained above that achieved by measuring RF. However, in selected disorders, *e.g.*, IE, DILD, VASC, SLE, elevated concentrations may be found which will alert the clinician to a possible IC pathogenesis and determining sequential concentrations may be useful in monitoring response to treatment.

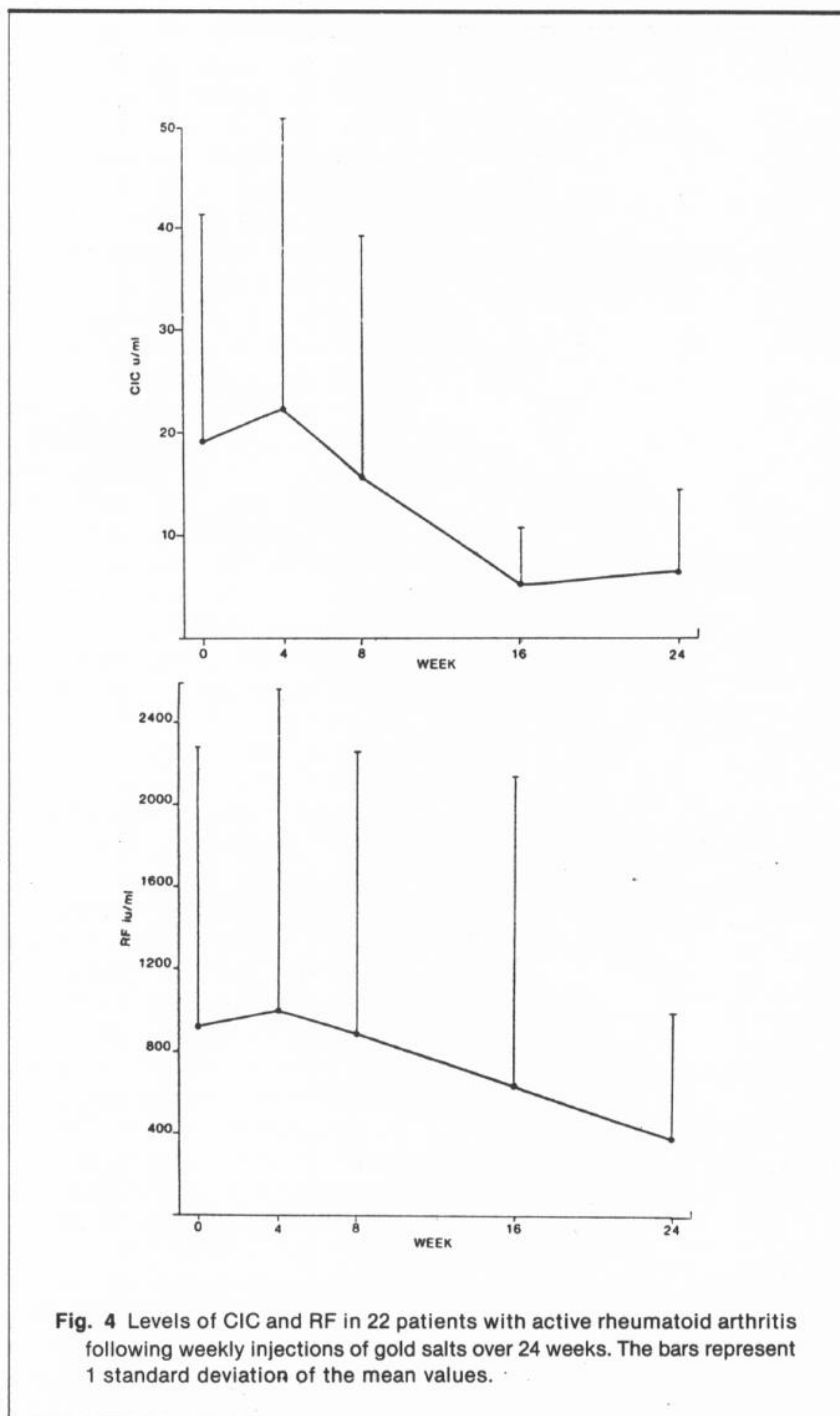

Fig. 4 Levels of CIC and RF in 22 patients with active rheumatoid arthritis following weekly injections of gold salts over 24 weeks. The bars represent 1 standard deviation of the mean values.

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