

DIFFERENTIAL PRODUCTION OF IgM AND IgA RHEUMATOID FACTORS IN PERIPHERAL BLOOD AND SYNOVIUM OF PATIENTS WITH RHEUMATOID ARTHRITIS

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ABSTRACT

Total IgM, IgA, and IgM-rheumatoid factor (RF) and IgA-RF were quantitated in the serum and synovial fluid of 35 seropositive and 8 seronegative rheumatoid arthritis (RA) patients, using a sensitive enzyme-linked immunosorbent assay (ELISA). Mononuclear cells isolated from peripheral blood and synovial fluid of three seropositive patients were also stimulated *in vitro* with phorbol myristate acetate (PMA) and culture supernatants collected for measurement of total immunoglobulin (Ig) and RF. Our results demonstrated that as a proportion of total Ig, IgM-RF and IgA-RF were significantly higher in the synovial fluid of seropositive patients compared to their serum level. Similar results were observed for *in vitro* stimulated culture supernatants from the same patients. This difference, however, was not evident in the seronegative group of patients.

These results indicate that synovium could be considered as the original site of RF production in seropositive RA patients, and that different repertoires of B-lymphocytes may be involved in RF production in peripheral blood and synovial tissues of affected joints.

Keywords: Rheumatoid factor, Rheumatoid arthritis, Synovial fluid, Enzyme-linked immunosorbent assay (ELISA).

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease of unknown etiology. Production

of RF, an autoantibody of specificity for the F_C portion of human IgG, is a characteristic feature of RA.¹

As an Ig molecule, RF can be produced in different isotypes; IgM, IgA, IgG and IgE-RF have so far been reported.² Rheumatoid factor has been proposed to be implicated in some of the pathologic manifestations observed in RA.^{1,3} Production of RF, however, is not restricted to RA and is detected in a wide spectrum of

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diseases, including viral, parasitic, autoimmune and chronic inflammatory diseases, as well as some lymphoid neoplasia.^{4,6} It is also found in a low titer in a significant proportion of normal individuals, particularly the elderly population.^{5,6} This suggests that RF may contribute to some physiologic functions, such as antigen presentation, elimination of antigen-antibody complexes, regulation of immune responses and enhancement of phagocytosis.^{1,7-9}

There is evidence to suggest that the RF produced in RA is quantitatively and qualitatively different from that produced in other clinical conditions.^{5,6,10} The fact that as opposed to the physiologic RF, a proportion of the RF pool in RA may be produced locally within the affected joints, supports the latter notion.^{1,3} However, local selective production of RF within synovial tissues of RA patients has not been extensively studied and remains controversial.

The present study was designed to address this issue by quantitative analysis of IgM and IgA-RF produced *in vivo* and *in vitro* by peripheral blood and synovial fluid lymphocytes from patients with RA.

MATERIALS AND METHODS

Patients and clinical samples

Thirty-five seropositive (SP) and 8 seronegative (SN) RA patients who attended the Rheumatology Clinic of the University Hospital of Hazrate Rasool in Tehran were studied. Some of the samples were also collected from Imam Reza Hospital of Mashhad. Diagnosis of RA was based on the criteria outlined by the American Rheumatism Association.¹¹ Seropositivity was confirmed by commercial latex agglutination kits (Pajoohan Ltd., Tehran, Iran). The major clinical and physical characteristics of the patients are listed in Table I. The majority of the SP and SN patients were under chemotherapy at the time of sampling (31 and 5 patients, respectively). Both groups of patients received similar drugs, though different combinations of drugs

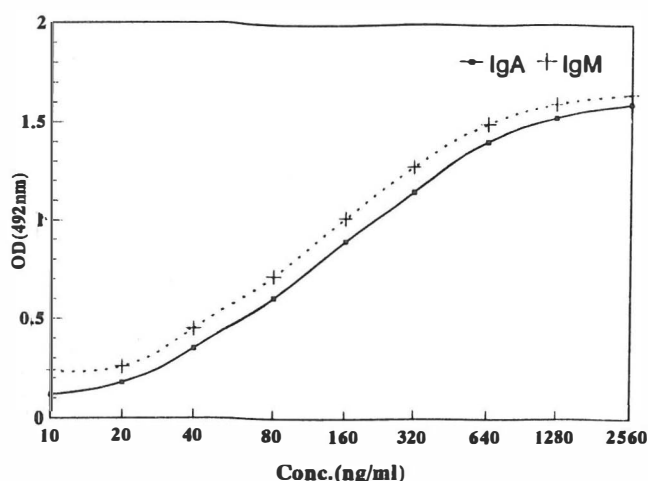


Fig. 1. Standard curves for measurement of total IgM and IgA.

were prescribed to each individual patient. The most commonly used drugs were cyclophosphamide, prednisolone, chloroquine, piroxicam, indomethacin, sulfasalazine, methotrexate, diclofenac and aspirin. Serum and synovial fluid were collected simultaneously from all patients and stored at -20°C until use.

Preparation of mononuclear cells and *in vitro* stimulation

Mononuclear cells were isolated from 5-10 mL of heparinized peripheral blood or synovial fluid using Ficoll-Hypaque (Pharmacia, Sweden), as described elsewhere.¹² Briefly, 1/2 diluted peripheral blood or synovial fluid was overlaid on equal volume of Ficoll. After centrifugation the interface layer of mononuclear cells was collected under sterile conditions and washed twice with RPMI-1640 medium (Gibco BRL, Scotland). Following counting and viability assessment, the cells were resuspended in RPMI medium supplemented with 10% heat inactivated fetal calf serum (Gibco BRL, Scotland), 2mM L-glutamine (Sigma, England), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (local pharmacy) and plated at $10^6/\text{mL}$ density in 24-well

Table I. Major clinical and physical characteristics of the RA patients.

| Patients ^a | No. | Sex ^b (M/F) | Age (Yr) | Dis. Dur. ^c (Yr) | Ther. Dur. ^d (Month) |
|-----------------------|-----|---------------------------|---|--------------------------------|------------------------------------|
| SP-RA | 35 | 12/23 | 48.2 \pm 13.4 (23-72) ^e | 5.95 \pm 4.45 (1-17) | 13.8 \pm 11.9 (2-52) |
| SN-RA | 8 | 3/5 | 44.7 \pm 22.6 (20-75) | 8.85 \pm 9.5 (2.5-25) | 60.6 \pm 101 (3-240) |

^aSP-RA: seropositive RA; SN-RA: seronegative RA; ^bF: female; M: male; ^cDis. Dur.: disease duration, mean \pm SD (year); ^dTher. Dur.: duration of therapy (year); ^efigures given in parentheses represent range of age, disease duration or therapy duration.

sterile plates (Nunc, Denmark). Finally, 10 ng/mL of phorbol myristate acetate (PMA) (Sigma, England) was added to each well and the plate incubated in a humidified CO₂ incubator, with 5% CO₂ at 37°C. After 6 days, culture supernatants were harvested and stored at -20°C until use.

Quantitation of total IgM and IgA

Total IgM and IgA were measured in serum, synovial fluid and culture supernatants by a capture ELISA technique, essentially as described previously.¹³ Briefly, 96-well polystyrene ELISA plates (Maxisorp, Nunc, Denmark) were coated with 10 µg/mL of monoclonal antibodies specific for human IgM (AF6) or IgA (2D7), kindly provided by Professor R. Jefferis and Dr. M. Goodall from the Dept. of Immunology, Birmingham University, England (commercially available from Oxoid Ltd., England). Following 90 minutes of incubation at 37°C, the plates were washed three times with PBS buffer containing 0.05% Tween 20 (Sigma, England) and incubated with appropriate dilutions of the samples as above. Finally, appropriate dilutions of F(ab')₂ fragments of HRP-conjugated sheep anti-human IgM or IgA (Dakopatts, Denmark) were added and the plates developed with O-phenylenediamine tetrahydrochloride (OPD) (Sigma, England). Concentrations of IgM or IgA were extrapolated from a standard curve, using optical density (OD) values developed for known inputs of affinity purified IgM paraprotein (Kok) or IgA paraprotein (Sm).¹³

Quantitation of IgM-RF and IgA-RF

The ELISA method employed for measurement of RF has been described elsewhere.¹³ Briefly, ELISA plates were sensitized with 20 µg/mL of DEAE-purified human IgG.⁶ Appropriate dilutions of the samples were added in duplicate and incubated as above. Following incubation of the plates with the isotype specific conjugates (see above), the plates were developed with OPD. The levels of IgM-RF or IgA-RF were

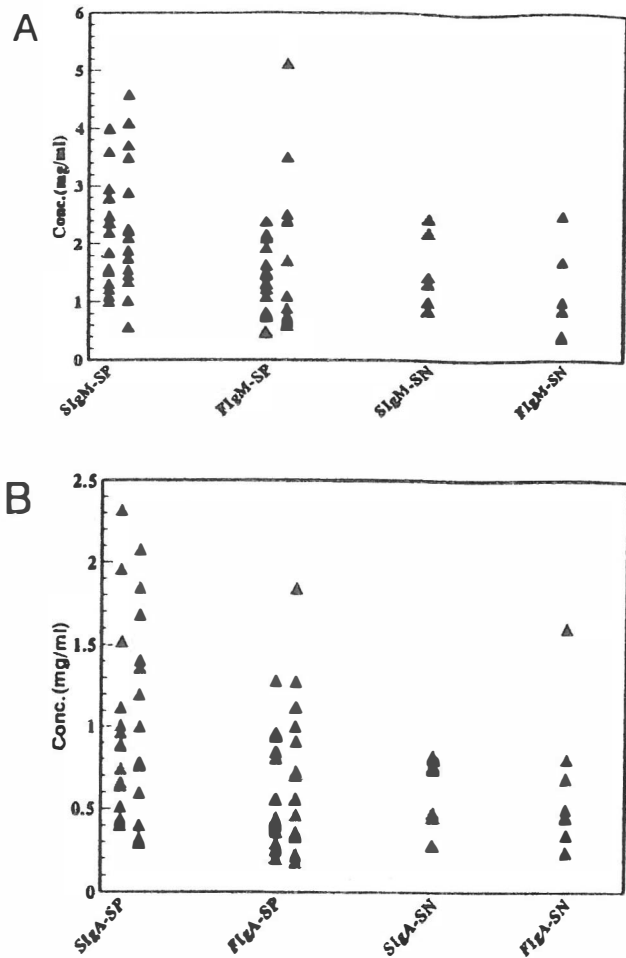


Fig. 2. Concentration of total IgM (A) and IgA (B) in serum and synovial fluid of seropositive and seronegative RA patients. S1gM: serum IgM; F1gM: synovial fluid IgM; SP: seropositive RA; SN: seronegative RA

extrapolated from standard curves, using the OD developed for known inputs of affinity purified IgM-RF paraprotein (Fr) or culture supernatants from IgA-RF producing hybridoma.^{6,14}

Table II. Concentration of total IgM and IgA in serum and synovial fluid of RA patients.

| | SP-RA (n=35) | | P-value | SN-RA (n=8) | | P-value | SP vs. SN (P-value) | |
|-------------|----------------------------|-----------------------|---------|----------------|----------------|-----------------|---------------------|-----------|
| | Srm ^a | Snv. Fld ^b | | Srm | Snv. Fld. | | Srm | Snv. Fld. |
| IgM (mg/mL) | 2.2 ^c (1.02) | 1.45 (0.98) | 0.0001 | 1.44 (0.58) | 1.08 (0.7) | NS ^d | 0.05 | NS |
| IgA (mg/mL) | 0.98 (0.54) | 0.66 (0.4) | 0.001 | 0.64 (0.2) | 0.63 (0.43) | NS | NS | NS |

^aSrm: serum; ^bSnv. Fld.: synovial fluid; ^cfigures given represent mean (SD); ^dNS: not significant.

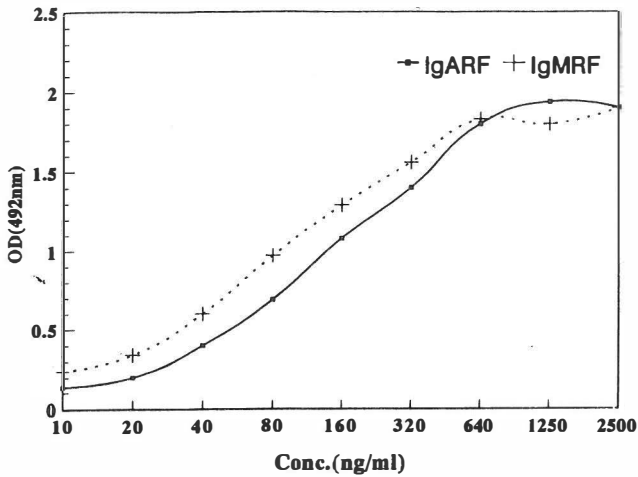


Fig. 3. Standard curves for measurement of IgM-RF and IgA-RF.

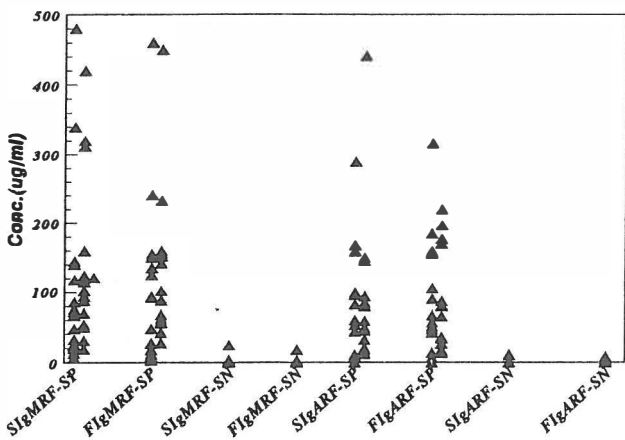


Fig. 4. Concentration of IgM-RF and IgA-RF in serum and synovial fluid of seropositive and seronegative RA patients.

Statistical analysis

Differences between IgM-RF and IgA-RF in serum and synovial fluid of seropositive and seronegative patients were analysed by Students' t-test or Mann-Whitney test, as appropriate. P-values less than 0.05 were considered significant.

RESULTS

Quantitation of total IgM and IgA

Concentrations of total IgM and IgA were measured in serum and synovial fluid of 35 seropositive and 8 seronegative RA patients. The standard curves employed for extrapolation of IgM and IgA titers are illustrated in Fig. 1. Serum levels of IgM and IgA in seropositive patients were significantly higher than that of the synovial fluid ($p < 0.0001$ and $p < 0.001$, respectively, Figs. 2a and 2b, Table II). No significant differences were found between the levels of IgM or

IgA in serum and synovial fluid of seronegative patients. The levels of IgM or IgA in serum or synovial fluid were similarly represented in both groups of patients (Table II).

Quantitation of IgM-RF and IgA-RF

IgM-RF and IgA-RF were measured in serum and synovial fluid of both groups of patients by extrapolation from the corresponding standard curves (Fig. 3). As little as 10 ng/mL of RF was detectable by this ELISA technique. The levels of both isotypes of RF were several-fold higher in serum and synovial fluid of seropositive patients compared to the seronegative group (Fig. 4).

Within each group however, IgM-RF and IgA-RF were similarly represented in serum and synovial fluid (Table III). When taken as a proportion of total Ig, IgM-RF and IgA-RF were found to be significantly higher only in synovial fluid of seropositive patients, compared to their serum. Such differences were not observed among seronegative patients (Table III).

Quantitation of *in vitro* induced IgM-RF and IgA-RF

Lymphocytes isolated from peripheral blood and synovial fluid of three seropositive patients were stimulated *in vitro* with PMA and culture supernatants collected for measurement of total IgM and IgA, as well as IgM-RF and IgA-RF. The *in vitro* induced RF was compared with the *in vivo* results obtained for the same patients. As shown in Table IV, there is a close correlation between the *in vitro* and *in vivo* results, indicating enrichment of both isotypes of RF in the synovial fluid compartment, compared to peripheral blood. However, due to the small size of samples tested, statistical analysis can not be applied.

DISCUSSION

This study was conducted to evaluate the precursor frequency of RF-producing B-lymphocytes in peripheral blood and synovial fluid of patients with seropositive RA, by quantitative analysis of the RF produced in each compartment.

Our results demonstrated that IgM-RF constitutes 5.5% and 8.3% of the total IgM in serum and synovial fluid, respectively, whereas IgA-RF comprises 8% and 12% of the total IgA in the same compartments. These results clearly indicate that RF is actively produced in the local joints. This could either be the result of overstimulation of the specific B-lymphocytes infiltrating the synovium, or selective infiltration of RF-producing lymphocytes within the synovial microenvironment, resulting in a higher proportion of RF production in

Table III. Concentration of IgM-RF and IgA-RF in serum and synovial fluid of RA patients.

| | SP-RA (n=35) | | P-value | SN-RA (n=8) | | P-value | SP vs. SN (P-value) | |
|-----------------------------|--------------|--------------|---------|--------------|---------------|---------|---------------------|-----------|
| | Serum | Snv. Fld. | | Serum | Snv. Fld. | | Serum | Snv. Fld. |
| IgM RF ($\mu\text{g/mL}$) | 121 (120) | 120 (145) | NS | 4.3 (8.2) | 3.06 (6.0) | NS | 0.0001 | 0.0001 |
| % IgM-RF/IgM ^a | 5.5 | 8.3 | 0.02 | 0.3 | 0.28 | NS | 0.0001 | 0.0001 |
| IgA-RF ($\mu\text{g/mL}$) | 74 (89) | 76 (78) | NS | 2.8 (5) | 2.4 (3.6) | NS | 0.0001 | 0.0001 |
| % IgA-RF/IgA | 7.7 | 11.3 | 0.004 | 0.4 | 0.4 | NS | 0.0001 | 0.0001 |

^a% IgM-RF-IgM: Percent of concentration of IgM-RF to total IgM.

synovial fluid compared to peripheral blood. Germinal center-like aggregates of lymphocytes, mostly of the B-lineage, have recently been described in the synovial membranes of RA patients.^{15,16}

Otten et al¹⁷ have recently studied the precursor frequency of RF-producing B-lymphocytes in bone marrow, peripheral blood and synovium of RA patients. Their results displayed no significant difference between the levels of RF produced *in vitro* by mononuclear cells isolated from these lymphoid compartments. They however did not take into account the proportion of RF to total Ig produced by the mononuclear cells. This is a crucial point, without which a final conclusion would not be possible. Our results with regard to the absolute concentration of RF in synovial fluid and peripheral blood is in agreement with those of Otten and colleagues.¹⁷ However, if taken as a proportion of total IgM and IgA, we observe a highly significant increase in synovial fluid RF, compared to peripheral blood (Table III). This was more evident in supernatants of *in vitro* cultured lymphocytes (Table IV). Thus the proportion of RF in culture supernatants of synovial fluid lymphocytes was about 2.5-5 fold higher than that of peripheral blood, approaching 30% of the total Ig. This could be due to dysregulation of T-suppressor lymphocytes, reported in RA patients, particularly in the affected joints.^{18,19} Over-stimulation of T-helper lymphocytes and antigen-presenting cells, characterized by production and release of stimulatory mediators and cytokines in the synovial microenvironment, has also been proposed to be implicated.^{20,21} Internal defects within RF-producing lymphocytes, similar to those reported in (NZBxW) F1 mice, may also contribute to over-production of RF in the synovium of RA patients.²² Very recent observations have revealed the presence of Ig-like molecules within the synovial fluid of seropositive, but

Table IV. Concentration of total IgM, IgA, IgM-RF and IgA-RF in culture supernatants from mitogen-stimulated lymphocytes of three seropositive RA patients

| | <i>In vitro</i> PBL | (ng/mL) ^a Snv. Fld. | <i>In vivo</i> Serum | ($\mu\text{g/mL}$) ^b Snv. Fld. |
|--------------|-----------------------------|-----------------------------------|-------------------------|--|
| IgM | 3810 ^c (1300) | 1910 (1500) | 2400 (350) | 1500 (990) |
| IgM-RF | 226 (165) | 566 (383) | 125 (109) | 100 (35) |
| % IgM-RF/IgM | 6 | 30 | 5.2 | 7 |
| IgA | 5100 (2300) | 2840 (1300) | 1000 (260) | 700 (400) |
| IgA-RF | 103 (113) | 134 (131) | 86 (58) | 120 (54) |
| % IgA-RF/IgA | 2 | 5 | 7 | 17 |

^a*In vitro*: the results represent concentration of total Ig and RF secreted by *in vitro* stimulated lymphocytes isolated from peripheral blood (PBL) or synovial fluid (Snv. Fld.) of three SP-RA patients; ^b*In vivo*: the results represent concentration of total Ig and RF in serum and synovial fluid (Snv. Fld.) collected from the same patients; ^cthe figures given represent mean (SD).

not seronegative, RA patients which can stimulate synovial T-lymphocytes and induce RF production.^{23,24}

Our results of differential production of RF in synovium and peripheral blood have also been reported by others.²⁵⁻²⁷ To our knowledge, however, our results are unique in that previous reports were confined to measurement of a single isotype of RF *in vitro* following stimulation of the lymphocytes with a polyclonal activator. We measured both IgM and IgA-RF *in vitro* and *in vivo*, allowing closer inspection of the synovial tissue as the original site of RF production in RA patients.

The data presented may suggest that the repertoire

of RF-producing B-lymphocytes in the synovium differ from that of peripheral blood. As opposed to synovial fluid, the RF detected in peripheral blood is largely the product of plasma cells resident in the bone marrow. Our recent results with regard to specificity and idiotypy of the synovial and peripheral blood RF are in agreement with the latter proposal.^{28,29}

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