

Differential Expression of Rheumatoid Factor-Associated Cross-Reactive Idiotypes in Iranian Seropositive and Seronegative Patients with Rheumatoid Arthritis

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ABSTRACT

Introduction: High levels of rheumatoid factors (RF) are detectable in serum of the majority of patients with rheumatoid arthritis (RA), but 5-10% of patients remain seronegative (SN). Despite clinical and genetic similarities between these two subsets of RA, it has been proposed that they may be regarded as distinct clinical entities. **Methods:** In the present study a panel of monoclonal antibodies (mAb) recognizing RF-associated cross-reactive idiotypes (CRI) linked to the V_H1 (G8), V_H4 (LC1), V_K3b (17-109) and a mAb recognizing the V_K3 subgroup (C7) of immunoglobulin variable region (IgV) gene products were used to quantitate the level of expression of these gene products in serum and synovial fluid of 35 seropositive (SP) and 8 SN RA patients by capture ELISA. **Results:** While the concentration and relative proportion of the IgV are recognized by the mAb G8, 17-109 and C7 were significantly higher in serum and synovial fluid of the SP RA, compared to the SN-RA patients (G8, p = 0.009; 17-109, p = 0.0001; C7, p = 0.001). The CRI recognized by the mAb LC1 was highly represented in serum and synovial fluid of the SN-RA patients. There have been no significant differences in the level of expression of these IgV gene products (other than the product recognized by C7 mAb in SP patients) between serum and synovial fluid of either group of patients. **Conclusion:** Our results suggest that the expressed repertoire of Ig V_H and V_K genes in these two subsets of RA is differentially regulated and may be influenced by selective mechanisms leading to positive or negative selection of certain genes. *Iran. Biomed. J. 11 (1): 7-13, 2007*

Keywords: Rheumatoid arthritis (RA), Rheumatoid factor (RF), Cross-reactive idiotypes (CRI), Seronegative (SN), Seropositive (SP)

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease that afflicts primarily, the synovial membrane. It is widely believed that local production of immunoglobulins, including rheumatoid factor (RF), in the synovium participates in the pathogenesis of tissue injury in RA [1, 2]. RF is not specific for RA, but in patients with symmetric polyarthritis, detection of RF has clinical implications. RA associated with production of RF has been known to be a more complicated entity. Moreover, extra-articular RA is seen almost exclusively in RF positive patients [3, 4]. Basically, two types of the RA disease including seropositive

(SP) and seronegative (SN) RA have been distinguished. SN RA is generally less aggressive compared to the SP type, but otherwise exhibits very similar features of joint involvement. Although genetically related, these two types of RA have been reported not to be identical [5, 6].

Immunological aspects of RA have been studied extensively. However, mechanisms underlying production of RF have remained to be clarified. Two alternative mechanisms, antigen selection and polyclonal activation, have been suggested as possible causes of sustained B-cell activation and autoantibody production [7].

Findings of mutated RF and production of autoantibodies to local antigens in the synovium,

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such as collagen type II, imply involvement of antigen-driven immune responses in pathogenesis of the disease [8, 9]. However, a significant proportion of RF found in RA patients does not seem to be antigen-driven. Genetic abnormalities within B-cell repertoire and/or superantigen stimulation have been accounted as alternative pathways responsible for RF production [10, 11]. Distribution of V_H families within the repertoire of B-cell subsets is expected to follow the germline complexity. However, non-random V_H and V_L gene utilization has been reported in certain autoimmune and lymphoproliferative disorders [11-13]. Other studies have suggested that enrichment of a B lymphocyte subpopulation characterized by the ability to produce natural autoantibodies e.g. $CD5^+B$ cells may contribute to the pathogenicity of RA disease [14]. Evidence in favor of either mechanism has been sought by analysis of the genetic basis of autoantibody production in humans, using serological and molecular techniques [15]. Identification of the expressed immunoglobulin variable region (IgV) genes and their products may have the way to elucidate the contributing mechanisms of RF production in SP RA patients [16].

In the present study, expression of cross-reactive idiotype (CRI) associated to certain V_H and V_K gene families was investigated in serum and synovial fluid of Iranian SP and SN RA patients using mAb recognizing the corresponding idiotypes.

MATERIALS AND METHODS

Monoclonal antibodies. The production and characterization of anti-CRI mAb employed in this study has been described in detail elsewhere [1, 17-20]. Anti-CRI mAb are listed in Table 1. Specific mAb for human IgM (AF6) and IgA (2D7) were kindly provided by Prof. R. Jefferis and Dr. M. Godall, Department of Immunology, University of Birmingham, UK. All mAb were of murine origin.

Table 1. Monoclonal anti-CRI antibodies employed in this study.

Antibody	Isotype	Specificity	Reference
G8	IgG1	V_H1 -associated CRI	[17, 19]
C7	IgG1	V_k3 epitop	[18]
17-109	IgG2b	V_H3b -associated CRI	[1]
Lc1	IgG1	V_H4 -associated CRI	[20]

Clinical samples. Sera and synovial fluids were collected from 43 patients (35 SP and 8 SN RA patients, 15 men and 28 women, aged 47 ± 15 years) with classical disease according to the American Rheumatism Association classification. SP of the samples was confirmed using commercial latex agglutination kits (Iran Pajohan, Iran) [21].

Protein purification. Murine mAb were purified from ascitic fluid by ion-exchange chromatography on DEAE cellulose (DE-52; Whatman, Maidstone, UK), or affinity chromatography on staphylococcal protein A-Sepharose 4B (Pharmacia, USA) equilibrated in PBS 0.01 M, pH 7 and 0.1 M Tris/HCl pH 8 at 4°C, respectively. All mAb are of the IgG1 isotype, except 17-109, which is of the IgG2b isotype. $F(ab_2)$ fragments were prepared from the mAb by pepsin digestion and their purity were assessed by SDS-PAGE, as described elsewhere [11]. Polyclonal human IgG was prepared from a normal human Ig preparation as the break through fraction from a DEAE-cellulose column equilibrated and eluted with 0.01M PBS pH 7.

Quantification of total IgM and IgA. IgM and IgA levels in serum and synovial fluid were determined using sandwich ELISA. The procedure has been described elsewhere [22]; in brief polystyrene microtiter ELISA plates (Maxisorp, Nunc, Denmark) were sensitized with 10 μ g/ml of purified monoclonal anti-IgM (AF6) or anti-IgA (2D7) Abs (Oxoid Unipath, Bedford, UK) in PBS, pH 7.4 at 37°C for 2 hours. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS/T) and serum and synovial fluid samples diluted to 1/5000, 1/10,000 and 1/40,000 in PBS/T were added to the wells and further incubated at 37°C for 2 hours. Dilutions of KOK IgMRF and A3 IgA paraproteins were used to construct standard curves for IgM and IgA, respectively. Bound IgM or IgA was revealed using horseradish-peroxidase conjugated sheep anti-human μ and α chains, respectively. The reaction was revealed by ortho phenylene diamine dihydrochloride (OPD) substrate and OD values were measured at 492 nm using a multiscan ELISA reader (Organon Teknika, Boxtel, the Netherlands). The concentration of IgM or IgA in each sample was determined by extrapolation from the standard curves (Fig. 1).

Quantification of IgMRF and IgARF. ELISA plates were sensitized with 100 μ l of DEAE-purified human IgG at a concentration of 20 μ g/ml and samples at dilutions of 1/100, 1/250 and 1/1000 in PBS/T were added in duplicate. Following incubation of the plates with the isotype specific conjugates, the plates were developed with OPD. IgMRF and IgARF concentrations were extrapolated from standard curves constructed from OD values using known input of affinity purified IgMRF paraprotein (Fr) or culture supernatants from IgARF producing hybridoma B27 [23] (Fig. 1).

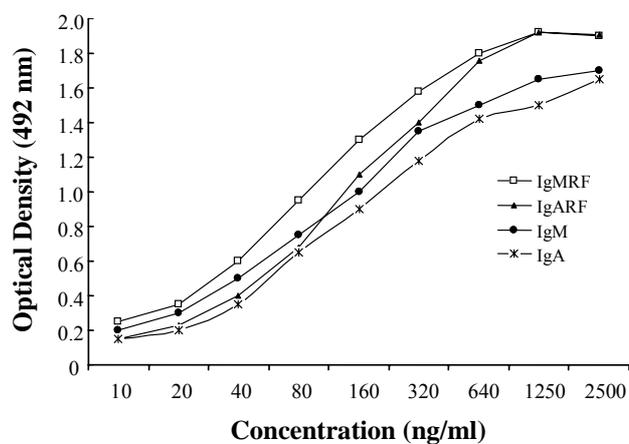


Fig. 1. Standard curves for measurement of total IgM, IgA, IgMRF and IgARF.

Quantification of CRI. A capture ELISA method was employed, as previously described [22]. Briefly, microtiter plates were coated with 5-10 μ g/ml of F(ab)₂ fragment of anti-CRI mAb G8, C7, LC1 and intact mAb 17-109 as first layer. Following 2 hours of incubation at 37°C, plates were washed, as described above. Synovial fluid and serum samples under test were added at dilutions of 1/100, 1/250 and 1/1000 in PBS/T and incubated in duplicate in sensitized plates at 37°C for 2 hours. Bound IgM or IgA was revealed with F(ab)₂ fragments of HRP-sheep anti-human μ and α chains, respectively (Dakopath, Denmark). The concentration of IgM bearing CRI G8, 17-109, C7 and LC1 was extrapolated from standard curves constructed using known inputs of the immunogens Kok and Louckes, respectively (Fig. 2). G8 and C7⁺IgA levels were determined using standard curves constructed from known inputs of paraproteins A3 and A28, respectively (Fig. 2).

Statistical analysis. Comparisons were made using the Mann-Whitney U and Chi-Square (X^2)

tests as appropriate, and differences were considered significant when p values were <0.05 .

RESULTS

Measurement of total IgM, IgA, IgMRF and IgARF. Concentrations of total IgM, IgA, IgMRF and IgARF were measured in serum and synovial fluid of 35 SP and 8 SN RA patients. Serum levels of IgM and IgA in SP patients were significantly higher than that of the synovial fluid ($p<0.0001$ and $p<0.001$, respectively). No significant differences were found between the levels of IgM or IgA in serum and synovial fluid of SN patients. Concentrations of IgMRF and IgARF in serum or synovial fluid were similarly represented in both groups of patients. The levels of both isotypes of RF were several folds higher in serum and synovial fluid of SP patients compared to the SN group (Table 2).

Quantitation of CRI. The levels of IgM-bearing G8, C7, 17-109 and LC1 CRI and IgA-bearing G8 and C7 CRI were determined by capture ELISA and the percentage of total IgM and IgA expressing these CRI were calculated (Table 3). All the CRI⁺IgM (except LC1) were significantly increased in the SP patients compared to SN subjects (G8; $p<0.009$, 17-109; $p<0.0001$, C7; $p<0.001$). (Figs. 3-5, Table 3). No significant differences were found between the levels of LC1⁺IgM, G8⁺IgA and C7⁺IgA in both groups of patients (Figs. 3-5).

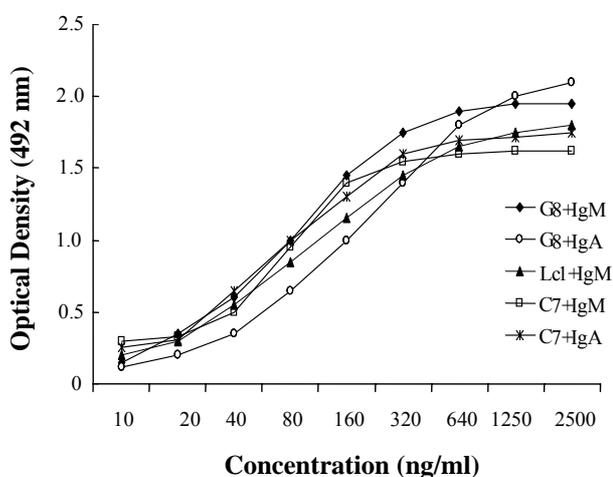


Fig. 2. Standard curves for measurement of CRI⁺IgM and IgA.

Table 2. Concentration of total IgM, IgA, IgMRF and IgARF in serum and synovial fluid of RA patients.

Igs	Groups	SP-RA ¹ (n = 35)		P value	SN-RA ² (n = 8)		P value	SP vs SN (P value)	
		S ³	S.F ⁴		S	S.F		S	S.F
IgM (mg/ml)		2.20 (1.02)	1.45 (0.98)	0.0001	1.44 (0.58)	1.08 (0.70)	N.S	0.0500	N.S
IgA (mg/ml)		0.98 (0.54)	0.66 (0.4)	0.001	0.64 (0.20)	0.63 (0.43)	N.S	N.S	N.S
IgMRF (µg/ml)		121.00 (120.0)	120.00 (145.0)	N.S	4.3 (6.00)	3.06 (4.30)	N.S	0.0001	0.0001
IgARF (µg/ml)		74.00 (89.0)	76.00 (77.7)	N.S	2.75 (5.00)	2.43 (3.50)	N.S	0.0001	0.0001

¹SP-RA; seropositive rheumatoid arthritis, ²SN-RA; seronegative rheumatoid arthritis, ³S; serum, ⁴SF; synovial fluid, N.S; not significant Data given represents mean (SD).

DISCUSSION

SP and SN RA are generally associated with a different clinical outcome, with lack of extra-articular manifestations and less destructive joint involvement in SN RA patients [24]. Production of RF is the distinct feature of SP RA, however the mechanisms underlying production of RF remain largely obscure. Antigen-deiven and/or polyclonal activation of B cells have been suggested as possible causes of RF production [7, 25, 26].

Analysis of the profile of Ig V gene utilization could be an ideal approach to define mechanisms of lymphocyte selection and clonal dominance. The pattern of CRI expression has been demonstrated to reflect restricted Ig V germ line gene utilization with generation of clonally related autoantibodies. CRI

has been used as serological markers for Ig variable regions encoded by germline or minimally mutated germline genes [13, 27]. According to several studies, a correlation has been found between the levels of certain CRI and disease activity in autoimmune disorders. Increased levels of the 9G4 CRI has been demonstrated to be present in a substantial proportion of serum samples from patients with systemic lupus erythematosus to which aspects of disease severity are correlated [28].

In the present study, we investigated the expression of certain RF-associated CRI in the serum and synovial fluid of SP and SN RA patients. The results demonstrated that the concentration of IgM bearing G8 and 17-109 CRI and κ III light chain (recognized by mAb C7) was significantly increased in SP RA patients (Table 3). These results suggest

Table 3. Concentration of CRI⁺IgM and IgA in serum and synovial fluid of seropositive and seronegative RA patients.

Igs	Groups	SP-RA ¹ (n=35)		P value	SN-RA ² (n=8)		P value	SP vs SN (P value)	
		S ³	S.F ⁴		S	S.F		S	S.F
G8 ⁺ IgM (µg/ml)		44.2 (84)	45 (105)	N.S	4.9 (2.2)	3.78 (2.7)	N.S	0.009	0.001
%G8 ⁺ IgM/IgM		2.00	3.1	N.S	0.34	0.36	N.S	0.001	0.0001
G8 ⁺ IgA (µg/ml)		47.3 (72)	32.4 (36)	N.S	47.9 (50.4)	35.16 (23)	N.S	N.S	N.S
%G8 ⁺ IgA/IgA		4.82	4.9	N.S	7.48	4.00	N.S	N.S	N.S
C7 ⁺ IgM(µg/ml)		248.2 (254)	179 (174)	0.002	27.3 (26)	20 (23.5)	N.S	0.001	0.001
%C7 ⁺ IgM/IgM		11.28	12.33	N.S	1.9	1.84	N.S	0.001	0.001
C7 ⁺ IgA(µg/ml)		26.13 (35)	14.2 (22)	0.01	17.06 (17.3)	7.28 (7.5)	N.S	N.S	N.S
%C7 ⁺ IgA/IgA		2.66	2.15	N.S	2.66	1.15	N.S	N.S	N.S
LC1 ⁺ IgM (µg/ml)		497 (424)	258.4 (205)	0.01	545 (178)	343 (342)	N.S	N.S	N.S
%LC1 ⁺ IgM/IgM		22.6	17.8	N.S	37.84	31.75	N.S	0.001	0.03
17-109 ⁺ IgM (OD)		0.64	0.52	N.S	0.08	0.1	N.S	0.0001	0.0001
%17-109 ⁺ IgM/IgM		1.2	1.7	N.S	0.66	0.33	N.S	0.04	0.0001

¹SP-RA; seropositive rheumatoid arthritis, ²SN-RA; seronegative rheumatoid arthritis, ³S; serum, ⁴SF; synovial fluid, N.S; not significant. OD; optical density; Data given represents mean (SD).

that expression of these CRI is closely related to IgMRF specificity. MAb G8 recognizes a conformational idiotope expressed on V_H1 Ig proteins [11, 29]. Our previous study showed that the concentration of G8-CRI in RA patients was significantly higher than that of normal individuals [7]. The G8 and G6 idiotopes (V_H1-associated CRI) constitute a substantial proportion of the polyclonal IgMRF produced in early synovitis patients [15].

The mAb C7 recognizes a specific epitope on the light chain from the V_K3 subgroup. The V_K3 gene family constitutes 22-30% of the human V_K genes [30]. IgMRF paraproteins have been shown to express the V_K3 light chain more frequently than IgM paraproteins without RF activity (75% vs. 25%) [7, 15].

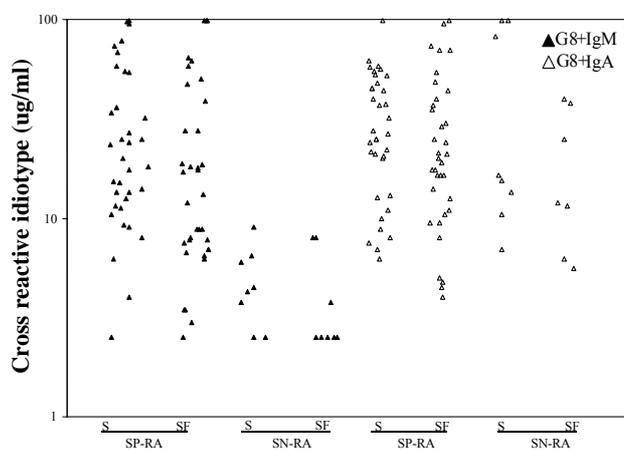


Fig. 3. Distribution of levels of G8⁺IgM and IgA in serum and synovial fluid of seropositive and seronegative RA patients. S, serum; SF, synovial fluid; SP, seropositive; SN, seronegative.

In the present study, we showed that the level of C7⁺IgM was significantly higher in SP RA patients than that of seronegative RA patients (12% vs. 2%). Based on protein and DNA sequencing and serological studies, the VK3 gene products have been classified into V_K3a and V_K3b sub-subgroup. V_K3b sub-subgroup constitutes only a proportion of the V_K3 subgroup of light chain which is recognized by mAb 17-109 [15, 31, 32]. Expression of 17-109CRI in our patients revealed a significant difference between the SP and SN RA patients. This CRI was similarly represented in serum (1.2%) and synovial fluid (1.7%) of SP RA patients (Table 3).

The levels of IgM in serum or synovial fluid were similarly represented in both groups of patients (Table 2). Thus, the increased level of IgM bearing G8, 17-109 CRI and C7 epitope in SP RA patients was probably a reflection of the total level of IgMRF

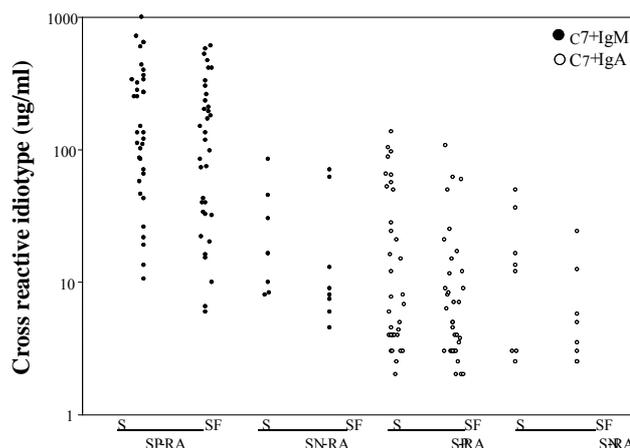


Fig. 4. Distribution of levels of C7⁺IgM and IgA in serum and synovial fluid of seropositive and seronegative RA patients. S, serum; SF, synovial fluid; SP, seropositive; SN, seronegative.

and indicated the association between these idiotypes with RF specificity. The concentration of IgA bearing G8-CRI and V_K3 light chain in serum and synovial fluid was similarly represented in SP and SN RA patients. (Figs. 3 and 4 and Table 3).

These results may suggest lack of association between these CRI and IgA-RF specificity. These CRI were found to be highly associated with IgMRF activity implies that production of IgARF and IgMRF is regulated independently by distinct B cell clones. Reduced expression of the associated-CRI on IgARF identified by the mAb G8 could also be attributed to somatic mutations in the variable regions of the Ig induced by continuous antigenic stimulation.

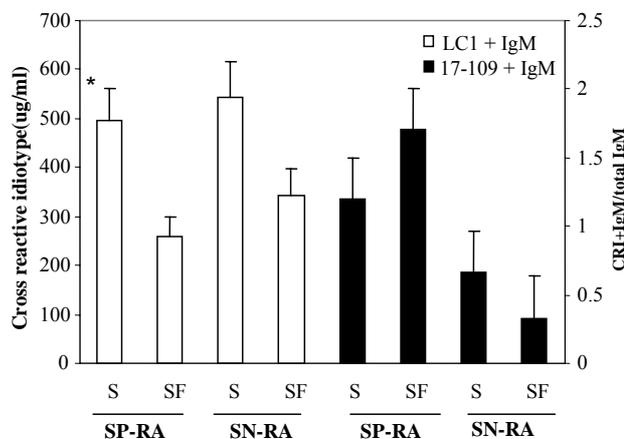


Fig. 5. Concentration of LC1 + IgM and 17-109 + IgM in serum and synovial fluid of seropositive and seronegative RA patients. S, serum; SF, synovial fluid; SP, seropositive; SN, seronegative; *Vertical bars represent standard error of mean.

We also showed that the expression of LC1⁺IgM, revealed a totally different pattern. The concentration of LC1⁺IgM in SN RA patients was higher than that of the SP RA patients, suggesting lack of association with RF activity, or alternatively negative selection for LC1 coding genes in RF production. Our results may appear to be in contrast with the study of Ono *et al.* [20] who reported significant elevations of the LC1-CRI in polyclonal RF from the sera of RA patients. It has been suggested that heavy chains from a small subset of the V_H4 gene family are used by human RF that coexpresses V_K3 light chains concurrently with the 6B6.6-CRI. Most (8 of 9) V_K3-6B6.6 V_H4 RF were identified by mAb LC1, a V_H4-restricted anti-CRI that inhibits RF activity [31]. There was not a significant difference in the relative proportion of the CRI in serum and synovial fluid of SP RA patients. This may imply that RF produced in serum and synovium is structurally related and may be the product of common B cell clones.

Finally, low proportion of RF expressed the probed CRI in SP RA (2-12%), a finding already reported in patients with classical RA [7, 15]. This may imply involvement of polyclonal activation, resulting in maturation of the RF response with recruitment of multiple V genes. Alternatively, specific antigenic stimulation followed by extensive mutation of IgV germline genes may account for low expression of the CRI. Our results suggest that the expressed repertoire of Ig V_H and V_K genes in these two subsets of RA is differentially regulated and may be influenced by selective mechanisms leading to positive or negative selection of certain genes.

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