Comparison of anti-agalactosyl IgG antibodies, rheumatoid factors, and anti-cyclic citrullinated peptide antibodies in the differential diagnosis of rheumatoid arthritis and its mimics

M.-C. Lu[1], S.-C. Hsieh[2], N.-S. Lai[1], K.-J. Li[3], C.-H. Wu[2], C.-L. Yu[2]

[1]Division of Immunology, Rheumatology and Allergy, Buddhist Dalin Tzu-Chi General Hospital, Chia-Yi; [2]Department of Internal Medicine, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei; [3]Division of Immunology, Rheumatology and Allergy, Department of Medicine, Buddhist Xindian Tzu-Chi General Hospital, Taipei, Taiwan.

Abstract

Objective

Anti-agalactosyl IgG antibodies [anti-Gal(0) IgG] have been regarded as a useful serological marker for rheumatoid arthritis (RA). Our aim was to evaluate the clinical usefulness of anti-Gal(0) IgG in the differential diagnosis of rheumatic disorders that mimic RA compared to rheumatoid factors (RF) and anti-cyclic citrullinated peptide antibodies (anti-CCP).

Methods

Sera were collected from 39 patients with RA, 49 patients with primary Sjögren's syndrome (pSjS), 47 patients with systemic lupus erythematosus (SLE), 65 patients with chronic hepatitis B viral infection (HBV), 68 patients with chronic hepatitis C viral infection (HCV) and 19 normal individuals. RF-IgM was measured by the nephelometeric method, and RF-IgA, anti-Gal(0) IgG and anti-CCP were measured by the respective ELISA assays.

Results

Anti-Gal(0) IgG titers were remarkably elevated in patients with RA (191.0 ± 250.8 AU/ml) compared to pSjS (37.9 ± 42.6 AU/ml), SLE (10.3 ± 13.6 AU/ml), chronic HBV with (36.1 ± 38.4 AU/ml) or without rheumatic symptoms (9.6 ± 19.4 AU/ml), RF(+) chronic HCV without rheumatic symptoms (19.0 ± 14.8 AU/ml), chronic HCV with rheumatic symptoms (15.2 ± 17.4 AU/ml) and healthy individuals (2.6 ± 0.7 AU/ml). The specificity of anti-Gal(0) IgG could be greatly enhanced by elevating the cut-off value from 12 AU/ml to 40 AU/ml (68.6% vs. 85.6%, $p < 0.001$) without significantly compromising its sensitivity (76.9% vs. 61.5%, $p > 0.05$).

Conclusion

The serum titer of anti-Gal(0) IgG is much higher in rheumatoid arthritis than in mimicking diseases. The specificity of anti-Gal(0) IgG is enhanced when the cut-off value is raised. However, anti-CCP remains the most specific biomarker for RA.

Key words

Anti-agalactosyl IgG antibody, anti-cyclic citrullinated peptide antibody, rheumatoid arthritis, primary Sjögren's syndrome, HBV, HCV.

Ming-Chi Lu, MD; Song-Chou Hsieh, MD, PhD; Ning-Sheng Lai, MD, PhD; Ko-Jen Li, MD, MS; Cheng-Han Wu, MD, MS; Chia-Li Yu, MD, PhD.

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Please address correspondence to: Chia-Li Yu, MD, Department of Internal Medicine, National Taiwan University Hospital. No. 7 Chung-Shan South Road, Taipei, Taiwan 100.
E-mail: chialiyu@ ntu.edu.tw

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Introduction

Rheumatoid arthritis (RA) is a common chronic inflammatory arthritis worldwide that can cause serious disability. It is characterized by chronic symmetric polyarticular synovitis that produces rheumatoid factor (RF), one of the important biomarkers for diagnosis, as it is present in the serum of 75-85% of patients. However, many other rheumatic diseases, such as primary Sjögren's syndrome (pSjS), systemic lupus erythematosus (SLE) and mixed connective tissue disease, may also exhibit polyarticular synovitis with or without RF production. Chronic HBV and HCV infection, which can involve rheumatic manifestations and an elevated RF, are endemic in Asia. It is conceivable that RF is primarily associated with RA, but does not exhibit high disease specificity. This autoantibody is also present in the sera of patients with different chronic diseases and even in normal elderly subjects. Although extra-articular manifestations and different serological tests may help us to identify different disorders, a sensitive and specific diagnostic test is needed to differentiate early RA, pSS and arthropathy from chronic HBV and HCV infection. Recently, anti-cyclic citrullinated peptide antibody (anti-CCP) was found to be a unique autoantibody that was extremely specific for the diagnosis and could also predict the prognosis of RA patients (1-5).

Parekh *et al.* (6) demonstrated that serum from patients with RA contains increased levels of oligosaccharides that lack terminal galactose residues [agalactosyl IgG, Gal(0) IgG]. RF obtained from these patients has been shown to bind better to Gal(0) IgG than galactosyl IgG (7). Anti-Gal(0) IgG therefore could be expected to be relatively specific for RA patients. Similarly to anti-CCP, anti-Gal(0) IgG is generated in the early stages of RA and is correlated with severe joint erosion (8, 9). However, some reports have found that low titers of anti-Gal(0) IgG are present in other diseases, such as SLE and primary Sjögren's syndrome (10-14).

In this study, we sought to determine the cut-off value of anti-Gal(0) IgG for the differential diagnosis of RA from rheumatic diseases that mimic RA and to evaluate the diagnostic usefulness of anti-Gal(0) IgG in comparison with anti-CCP. We compared RA, pSjS, SLE, and chronic HBV and HCV infection with or without rheumatic manifestations and found that low anti-Gal(0) IgG titers were indeed produced by these non-RA patients. Raising the cut-off value of anti-Gal(0) from 12 to 40 AU/ml enhanced the specificity of this autoantibody in the differential diagnosis of RA.

Materials and methods

Patients

Thirty-nine patients fulfilling the 1987 ACR revised criteria for the classification of RA (15), 49 patients fulfilling the American-European Consensus Group criteria for pSjS (16), 47 patients fulfilling the 1982 ACR revised criteria for the classification of SLE (17) and 19 normal individuals were recruited for the present study. In addition, 21 patients with chronic HCV infection and rheumatic manifestations [HCV(+) S(+)], 19 patients with chronic HBV and rheumatic manifestations [HBV(+) S(+)], 47 patients with chronic HCV infection and a positive RF test but no rheumatic manifestations [HCV(+) RF(+) S(-)], and 46 patients with chronic HBV infection without rheumatic manifestation [HBV(+) S(-)] were enrolled (Table I). Chronic HCV infection was defined as positive anti-HCV antibodies in the serum detected by standard laboratory methods and HCV virus was confirmed by RT-PCR. Chronic HBV infection was defined as the presence of HBV surface antigen in the serum detected by standard laboratory tests. The rheumatic manifestations in these chronic hepatitis viral infections are summarized in Table II.

Measurement of anti-Gal(0) IgG antibodies

The serum levels of anti-Gal(0) IgG were determined by a lectin enzyme immunoassay (with some modifications) using human agalactosyl IgG as antigen (Eitest CARF kit, Eisai Co. Ltd, Tokyo, Japan) (12, 18). Briefly, anti-Gal(0) IgG was prepared by the sequential enzym atic digestion of purified human IgG (10 mg/ml) with 1U neuraminidase

Table I. Demographic data on normal subjects, RA patients and patients with conditions that mimic RA.

Diagnosis	Total patients No.	Female patients No. (%)	Age (mean ± SD)
Normal subjects	19	14 (74%)	31 ± 8
Rheumatoid arthritis	39	32 (82%)	50 ± 17
Systemic lupus erythematosus	47	41 (87%)	36 ± 13
Primary Sjögren's syndrome	49	42 (86%)	52 ± 16
HBV (+) symptom (-)	46	13 (28%)	41 ± 12
HBV (+) symptom (+)	19	16 (84%)	46 ± 10
HCV (+) RF (+) symptom (-)	47	27 (57%)	62 ± 13
HCV (+) symptom (+)	21	17 (81%)	59 ± 8

Table II. Rheumatic manifestations in patients with HBV or HCV infection.

Clinical manifestation	HCV (n = 21)	HBV (n = 19)
Arthralgia/arthritis, no. (%)	13 (62.0%)	10 (52.6%)
Sicca syndrome, no. (%)	4 (19.0%)	9 (47.4%)
Small vessel vasculitis, no. (%)	3 (14.3%)	4 (21.1%)
Others, no. (%)	2 (9.5%)*	0 (0%)

*One patient with hemolytic anemia and one patient with idiopathic hypereosinophilic syndrome.

(Roche Diagnostics KK, Japan) in 0.1 M acetate buffer for 24 h at 37°C, and then with 0.1U β-galacto-sidase (Seikagaku Corporation, Tokyo, Japan) in 0.1 M citrate-phosphate buffer (pH 7.0) for 48 h at 37°C. Agalactosyl IgG was purified by protein G-conjugated agarose affinity chromatography (ImmunoPure immobilized Protein G, Pierce, Rockford, USA). The elute was dialyzed against phosphate-buffered saline solution (pH 7.0) containing 0.02% sodium azide. Polystyrene microtiter plates (Eisai Co. Ltd, Japan) were then coated with 100 µl of purified agalactosyl IgG (5 µg/ml) at 4°C overnight. After washing with Tris-buffered saline (TBS, 0.01 M, pH7.4), wells were blocked with 150 µl of TBS containing 0.05% bovine serum albumin (BSA, Oriental Yeast OC, Ltd, Japan) at 4°C overnight. One hundred microliters of serum samples diluted 201-fold in the standard dilution solution (0.25% BSA, 50 mM Tris-HCl, 0.15 M NaCl, 0.05% polyoxyethylene-octylphenyl ether, 0.02% p-hydroxylbenzoic acid methyl, 0.5% 2-chloroacetamide, pH7.4) was added. After 60 min of incubation at room temperature (RT), the wells were washed three times and 100 µl of biotinylated *Ricinus communis* agglutinin 120 (RCA120, Seikagaku Corporation) was added. After incubation for one hour at RT and 3 washes, 100 µl of 1:1000X diluted HRP-conjugated streptavidin (Oriental Yeast OC, Ltd, Japan) solution was added. The mixture was incubated for another hour followed by 3 washes. 100 µl of chromogen substrate solution ABTS (2'-azino-3-ethylbenz-thiazoline-6-sulphonic acid, Wako Pure Chemical Industries Ltd., Japan; substrate hydrogen peroxide, Sankyo, Japan) was added. The reaction was halted with 2 mM sodium azide after 30 min of incubation, and the absorbance was read at OD_{405nm} using an ELISA plate reader.

The serum titers of anti-Gal(0) IgG in sera were derived from a standard curve (3.125–50 AU/ml) and expressed in arbitrary units (AU/ml). A normal range was established after measurement of 125 healthy controls, with a cut-off value of 12 AU/ml being the 95th percentile non-parametric setting based on the manufacturer's suggestion.

Measurement of anti-CCP and RF

We used a commercially available second-generation fluoroenzymeimmunoassay test for the anti-CCP determination (Pharmacia Diagnostics AB, Uppsala, Sweden). The assay was carried out according to the manufacturer's instructions. The results of the anti-CCP test were considered positive if the antibody level was greater than 10 IU/ml. RF was measured by laser nephelometry for the IgM isotype (Date Behringer, Marburg, Germany), and a level > 20 IU/ml was considered positive. The RF-IgA titer was measured by a commercially available ELISA kit (INOVA Diagnostics, Inc. San Diego, CA, USA).

Statistical analysis

The study data are presented as means ± SD throughout. The concordance of the results was evaluated by the kappa test and the qualitative variables were compared in the different groups by the non-parametric Wilcoxon rank-sum test. $P < 0.05$ was considered statistically significant.

Results

Anti-Gal(0) IgG titers in different rheumatic diseases

Demographic data for the different disease groups are presented in Table I. The serum levels of anti-Gal(0) IgG antibodies in normal subjects and patients with different diseases are shown in Figure 1. The anti-Gal(0) IgG titer was significantly higher in patients with RA (191.0 ± 250.8 AU/ml) than pSS (37.9 ± 42.6 AU/ml), SLE (10.3 ± 13.6 AU/ml), chronic HBV without rheumatic symptoms (9.6 ± 19.4 AU/ml), chronic HBV with rheumatic symptoms (36.1 ± 38.4 AU/ml), chronic HCV with positive RF but no rheumatic symptoms (19.0 ± 14.8 AU/ml), chronic HCV with rheumatic symptoms (15.2 ± 17.4 AU/ml) and health individuals (2.6 ± 0.7 AU/ml). It is worth noting that patients with primary Sjögren's syndrome had significantly higher anti-Gal(0) IgG levels than normal subjects and non-RA patients ($p < 0.05$). A significant correlation ($p = 0.002$) was found between the titers of anti-Gal(0) IgG and RF in patients with RA ($r = 0.501$) (Fig. 2A). The correlation between anti-Gal(0) IgG and anti-CCP was also significant ($p = 0.045$; $r = 0.389$) (Fig. 2B). Interestingly, 3 RA patients and 4 HBV carriers who were negative for RF exhibited positive anti-Gal(0) IgG. We also checked for the presence of other

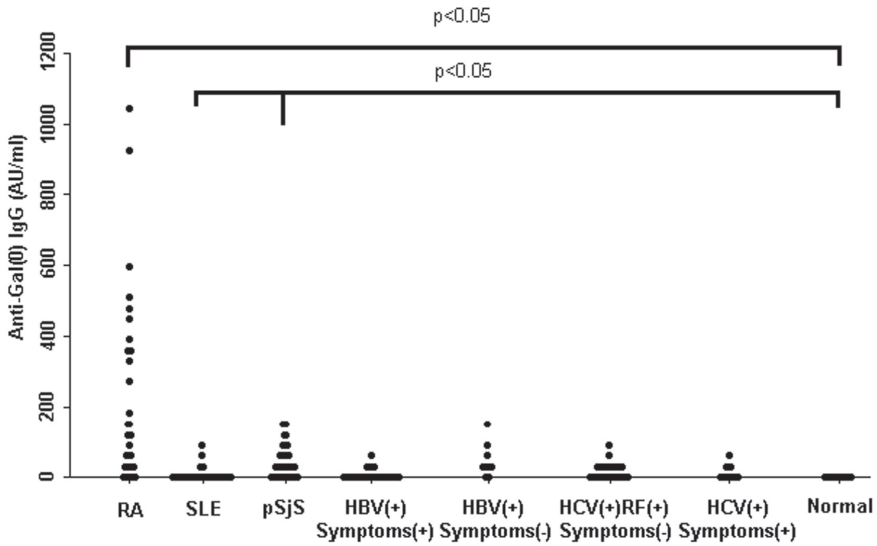


Fig. 1. Comparison of serum anti-agalactosyl IgG antibody [anti-Gal(0) IgG] titers between patients with rheumatoid arthritis (RA) and patients with diseases that may mimic RA. The anti-Gal(0) IgG titer determined by the lectin enzyme immunoassay was significantly higher in patients with RA than in patients with pSjS ($p < 0.01$), SLE ($p < 0.0001$), chronic HBV without rheumatic symptoms ($p < 0.0001$), chronic HBV with rheumatic symptoms ($p < 0.05$), chronic HCV RF(+) without rheumatic symptoms ($p < 0.001$) or chronic HCV with rheumatic symptoms ($p < 0.001$). The anti-Gal(0) IgG titer was significantly higher in patients with pSjS than in those with SLE ($p < 0.01$), chronic HBV without rheumatic symptoms ($p < 0.001$), chronic HCV RF(+) without rheumatic symptoms ($p < 0.05$), or chronic HCV with rheumatic symptoms ($p < 0.01$) (AU denotes arbitrary units).

Fig. 2A

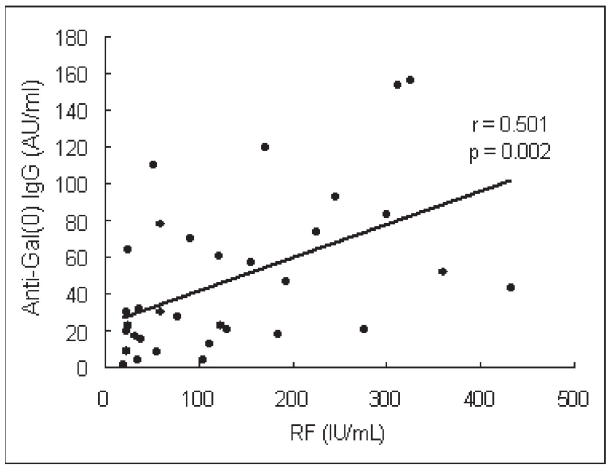


Fig. 2B

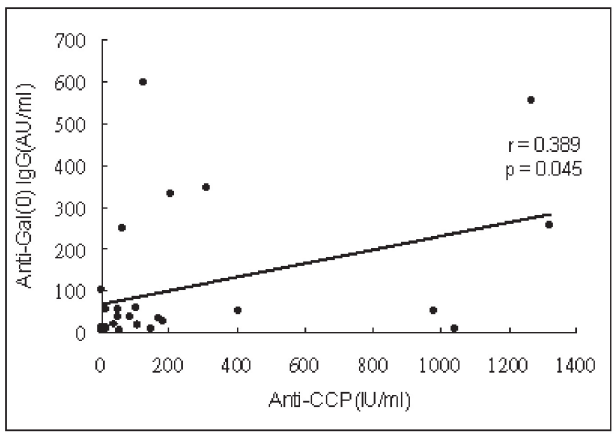


Fig. 2. Correlation among anti-agalactosyl IgG [anti-Gal(0) IgG], RF and anti-CCP antibodies in the sera of patients with rheumatoid arthritis. (**a**) Correlation between anti-Gal(0) IgG antibodies and RF; (**b**) correlation between anti-Gal(0) IgG and anti-CCP.

RF isotypes in these 7 sera, and found only negligible levels of RF-IgA by the ELISA test (data not shown). These results suggest that anti-Gal(0) IgG may appear in seronegative RA patients.

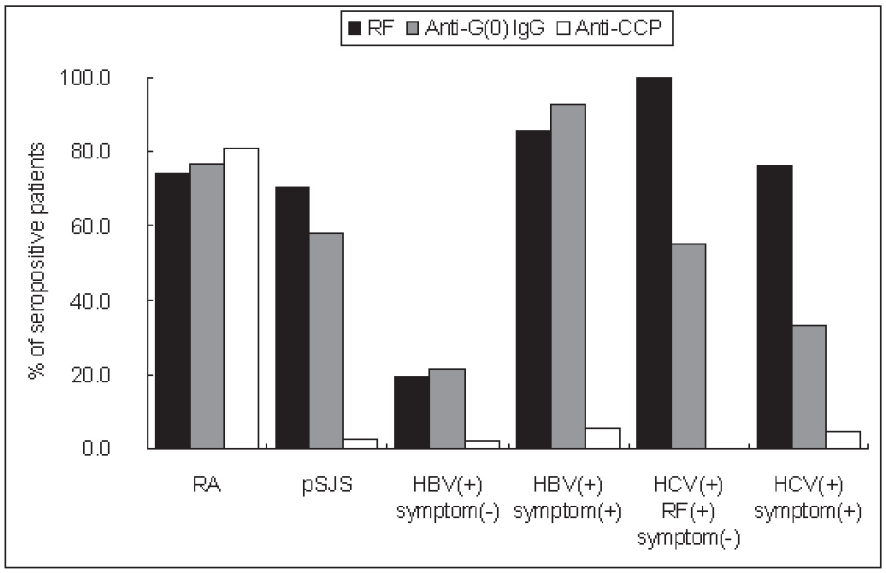
Comparison of anti-Gal(0) IgG, anti-CCP and RF in different disease groups

The positive rate (%) of anti-Gal(0) IgG, anti-CCP and RF in different diseases using 12 AU/ml as the cut-off point is shown in Figure 3a. In patients with RA, when the cut-off value was raised to 40 AU/ml, the positive rate of anti-Gal(0) IgG in RA patients fell from 76.9% to 61.5%, although the difference was still not significant (Fig. 3b). The specificity increased from 68.6% to 85.6%. The difference in the positive rate between RF (74.4%), anti-Gal(0) IgG (76.9%) and anti-CCP (81.0%) was not statistically significant. There was also no significant difference in the positive rates of RF and anti-Gal(0) IgG in patients with primary Sjögren's syndrome (70.3% *vs*. 58.1%), chronic HBV without rheumatic symptoms (19.6% *vs*. 21.7%) or chronic HBV with rheumatic symptoms (89.5% *vs*. 93.3%). RF positivity was higher than anti-Gal(0) IgG positivity in patients with chronic HCV and positive RF but no rheumatic symptoms (100% *vs*. 55.3%) and in patients with chronic HCV with rheumatic symptoms (76.2% *vs*. 33.3%). Anti-CCP was rarely detected in the patients with diseases mimicking RA.

Discussion

This is the first study to demonstrate that serum anti-Gal(0) IgG is markedly elevated in RA compared to other diseases mimicking RA including primary Sjögren's syndrome, SLE, chronic HBV without rheumatic symptoms, chronic HBV with rheumatic symptoms, chronic HCV with positive RF but no rheumatic symptoms, and chronic HCV with rheumatic symptoms. Our results suggest that the sensitivity of anti-Gal(0) IgG is equal to that of RF (76.9% *vs*. 74.4%), whereas the specificity of anti-Gal(0) IgG is significantly higher (68.6% *vs*. 51.%, $p < 0.01$) except in chronic HCV RF(+)S(-) patients

(**a**) Anti-G(0) IgG cut-off value: 12 AU/ml



(**b**) Anti-G(0) IgG cut-off value: 40 AU/ml

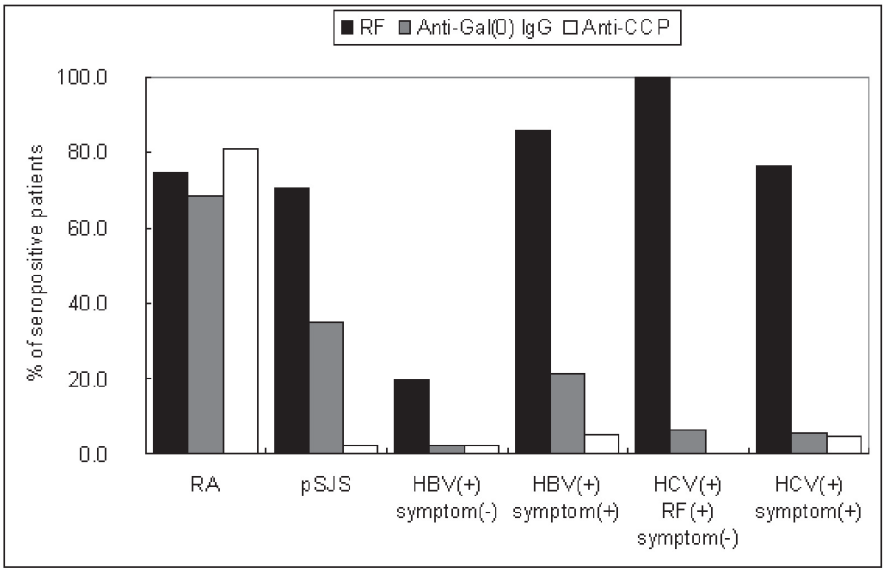


Fig. 3. Changes in the positive rate (%) of anti-agalactosyl IgG [anti-Gal(0) IgG] antibodies using different cut-off values compared to RF and anti-CCP in RA, primary Sjögren's syndrome, chronic HBV and HCV infection. (**a**) The cut-off value of anti-G(0) IgG was set at 12 AU/ml; (**b**) the cut-off value of anti-G(0) IgG was elevated to 40 AU/ml.

(Fig. 3a). In addition, anti-Gal(0) IgG is more specific than RF in the differential diagnosis of RA *vs*. other RF(+) non-RA rheumatic disorders, since the rise in anti-Gal(0) IgG titers in most of the latter groups was in the borderline zone. We totally agree with Dr. Das *et al*. (10) that the cut-off value for anti-Gal(0) IgG needs to be raised for the differential diagnosis of RA and other conditions mimicking RA. Raising the cut-off value to 40 AU/ml had little effect on the sensitivity (74.4% *vs*. 61.5%, $p > 0.05$), but markedly increased the specificity (68.6% *vs*. 85.7%, $p < 0.001$) for the differential diagnosis of polyarthritis.

This study confirmed anti-CCP to be the most specific serum biomarker for RA, as it was rarely detected in the other, RA-mimicking diseases. As shown in Figure 2b, the correlation between anti-Gal(0) IgG and anti-CCP was not sufficiently high, indicating that the two antoantibodies may represent two different biomarker systems. The use in combination of anti-CCP and anti-Gal(0) IgG detection may further increase the sensitivity from 81.0% to 87.2% (not statistically significant) and the specificity from 98.4% to 99.2% in the differential diagnosis of RA and RA-mimicking disorders compared with anti-CCP alone. Therefore, we would suggest that the combined measurements could increase the accuracy of the diagnosis of RA in the clinical setting.

Some of the patients with primary Sjögren's syndrome had elevated anti-Gal(0) IgG, but their titers never exceeded 200 AU/ml. None of these patients fulfilled the criteria for RA. Nonetheless, the possibility that pSjS patients with high anti-Gal(0) IgG could eventually develop RA cannot be absolutely ruled out.

The correlation between anti-Gal(0) IgG and RF-IgM was significant, as shown in Figure 2a, but the correlation coefficient ($r = 0.501$) in our study was lower than that reported by Das *et al*. ($r = 0.918$) (10). This low correlation could be due to racial differences between Taiwanese and Japanese subjects. Interestingly, we noted that among the 287 sera tested, those from 3 RA patients and 4 chronic HBV infected patients were negative for RF but exhibited positive anti-Gal(0) IgG. To rule out the presence of other RF immunoglobulin isotypes, we checked the RF-IgA titer by ELISA in these 7 sera and found it to be negligible (data not shown). However, the eventual presence RF-IgG cannot be excluded completely, since anti-Gal(0) IgG was detected in some of the seronegative RA and chronic HBV infection patients. It remains feasible that anti-Gal(0) IgG is not completely compatible with RF. If so, anti-Gal(0) IgG may indeed represent a novel serum biomarker for RA, to be placed next to anti-CCP.

The region of Asia including Taiwan is endemic for chronic HBV and HCV infection, conditions that may elicit positive RF in the serum and symmetric polyarticular synovitis mimicking RA. In clinical practice it is sometimes difficult to differentiate between early RA, primary Sjögren's syndrome, and arthropathy due to chronic HBV and

chronic HCV infections. We are the first group to study anti-Gal(0) IgG in chronic viral hepatitis sera. RF is no longer considered to be a specific marker for RA, because this autoantibody is frequently induced in other diseases (19, 20). Given the erosive nature of RA, however, early diagnosis and early aggressive treatment are absolutely necessary. Our results show that anti-CCP is the most specific test, followed by anti-Gal(0) IgG, while RF remains the least specific test for differentiating RA from other conditions mimicking RA. Although many authors have shown that anti-CCP is a reliable serological biomarker to distinguish the rheumatologic manifestations associated with Sjögren's syndrome or HCV infection from rheumatoid arthritis (21-24), the present study is the first to demonstrate the positive rates of anti-CCP in HBV-associated rheumatic manifestations.

In conclusion, anti-Gal(0) IgG constitutes a useful serum marker for the differential diagnosis of RA if the cut-off value is raised from 12 to 40AU/ml. However anti-CCP remain the most reliable biomarker for differentiating RA from other diseases such as primary Sjögren's syndrome, or chronic HBV or HCV infection with rheumatic manifestation ± RF. The concomitant measurement of anti-CCP and anti-Gal(0) IgG could further enhance the diagnostic accuracy of these tests for RA.

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