Clinical and Developmental Immunology

Serum Autoantibodies: From Identification to Clinical Relevance

Guest Editors: Pietro Invernizzi, Xavier Bossuyt, and Dimitrios P. Bogdanos

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Editorial Board

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2013, Article ID 382069, 3 pages
http://dx.doi.org/10.1155/2013/382069

Editorial

Serum Autoantibodies: From Identification to Clinical Relevance

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Received 21 February 2013; Accepted 21 February 2013

This special issue collates 22 papers (15 original and 7 reviews) reporting on various aspects of serum autoantibody research ranging from the identification of novel disease-related autoantigens up to the clinical significance of autoantibodies.

The recent assurance that indirect immunofluorescence (IIF) is the gold standard for anti-nuclear antibody (ANA) testing from the Task Force of American College of Rheumatology (ACR) [1] has led clinical immunologists to revisit the urgent need for automated IIF platforms, which could guarantee the robust and accurate interpretation of ANA patterns [2, 3]. In this special issue, three papers (two original and one review) have assessed the applicability of automated indirect IIF platforms for autoantibody pattern recognition. This topic is emerging as one of the most important advances in recent years that could pave the way for a new era on IIF testing in autoimmune diagnostics.

D. Roggenbuck et al. have provided an up-to-date overview of the data so far provided using the AKLIDES system and critically discuss the *pros* and *cons* of digital automated indirect IIF platforms for autoantibody detection in systemic rheumatological conditions. J. Voigt et al. report original data on the evaluation of ANA on HEp-2 cells using the EUROPattern Suite automated processing and interpretation system. This is the first time that original data on the performance characteristics of this platform have been published in the form of a full-length paper. Concordant results between visual and automated evaluation by the EUROPattern reached 99.4%. This supports the notion that a precise and reproducible differentiation of positive and negative samples tested by HEp-2 cell lines is met by the developed systems. Discrepancies between manual and automated pattern recognition is largely limited to serum samples with mixed ANA patterns, but the developers of those platforms assure the reader that they will soon overcome the current limitations. J. Damoiseaux et al. evaluated the first automated anti-neutrophil cytoplasmic antibodies pattern recognition system developed using the AKLIDES platform. Discrimination of C-ANCA and P-ANCA is satisfactory but the sensitivity on ethanol-fixed neutrophils needs further improvement.

A considerable proportion of apparently healthy individuals have IIF-detected ANA targeting the dense fine speckles 70 (DFS70) antigen. The clinical interpretation of positive anti-DFS70 antibody associated pattern (DFS) tests has emerged as one of the most important problems that routine laboratories are faced with, as it clearly influences the specificity and the positive likelihood of the ANA tests. In their review paper, M. Mahler and M. J. Fritzler discuss this

topic and also describe a novel immunoabsorption method that can block anti-DFS70 antibody reactivity. An original paper by M. Miyara et al. analyzed the clinical value of anti-DFS70 antibodies in patients subjected to routine ANA testing by IIF and concluded that systemic autoimmune rheumatic disorders are less prevalent in patients with the DFS pattern compared to patients with homogenous or other ANA IIF patterns.

R. Yoshimi et al. review the current data surrounding the clinical relevance and the pathogenic significance of anti-Ro/SSA antibodies in systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), and other autoimmune disease. An original paper by A. Wacker-Gußmann et al. provided data suggesting that foetal magnetocardiography can complement foetal echocardiography as a noninvasive approach to detect early electrophysiological signs of atrioventricular delay in foetuses exposed to maternal anti-SSA/Ro and anti-SSB/La antibodies. Anti-C1q antibodies have been detected in women with SLE who experienced failed pregnancy. An interesting study from Greek investigators demonstrated that anti-C1q antibodies cannot differentiate failed from normal pregnancies. The same investigators have found elevated levels of IL-15 compared to those with missed abortions and healthy intrauterine pregnancies, underlying the diagnostic potential of this marker. Another original study has assessed the clinical relevance of circulating glucocorticoid-induced TNFR-related protein ligand (GITRL) levels in patients with SLE. Chinese investigators found that GITRL levels positively correlate with anti-dsDNA titres and these levels were significantly higher in SLE patients with renal involvement and vasculitis compared to patients lacking these clinical manifestations. Another original article from a Chinese group has found that the titres of carbonic anhydrase III and IV autoantibodies are unusually high not only in patients with SLE and rheumatoid arthritis, but also in patients with other diseases including type 1 and type 2 diabetes.

P. C. Teixeira et al. review the literature and discuss the current knowledge on the role of autoantibodies against apolipoprotein A-1 in cardiovascular diseases. T. Shirai et al. provide an overview on the existing knowledge on the biological significance of anti-endothelial cell antibodies for vascular lesions in autoimmune rheumatic diseases. They also discuss in great detail the principles and applications of identifying autoantigens expressed on cell surfaces, known as serological identification system for autoantigens using a retroviral vector and flow cytometry (SARF). A. Shimatsu and N. Hattori review the literature and discuss the diagnostic, clinical, and pathophysiological features of macroprolactinemia caused by high molecular mass complexes of prolactin with immunoglobulin G (IgG) and in particular anti-prolactin antibodies. In their research article, R. Fu et al. and co-investigators used a proteomic approach based on the membrane of bone marrow cells to identify the antigenic targets of autoantibodies detected in a subgroup of patients with immune-mediated pancytopenia.

A significant contribution comes from L. Mihályi et al. Those researchers provide a meticulous overview of their 40-year long clinical experience in the diagnosis and management of patients with autoimmune bullous dermatosis. In their research paper, A. Patsatsi et al. found that titers of anti-BP180 autoantibodies relate with disease activity in Greek patients with bullous pemphigoid, while a clinical study by Dalmády and colleagues provided data suggesting that autoantibodies targeting mutated citrullinated vimentin may assist the diagnosis of psoriatic arthritis. U. Lindberg et al. found that IgA ANCA specific for bactericidal/permeability-increasing protein (BPI-ANCA) identifies cystic fibrosis patients with adverse outcomesand discuss the pathogenic potential of these autoantibodies.

Finally, four original papers report on the relevance of autoantibodies in autoimmune gastrointestinal and liver diseases. A. Antico et al. assessed the predictive value of combined testing of four serological markers in the diagnosis of autoimmune gastritis. These markers include anti-parietal-cell antibodies (PCA), anti-intrinsic factor antibodies (IFA), anti-*Helicobacter pylori* (Hp) antibodies, blood gastrin levels and are diagnostically useful in the classification of gastritis. Their predictive value is comparable to that of histologically assessed gastric biopsies. A British-German collaborative study found that the presence of Crohn's disease-specific pancreatic autoantibodies targeting the zymogen granule GP2 is largely limited to patients with ileal involvement. A. Kempinska-Podhorodecka et al. report on the influence of immunogenetics and their close interaction with humoral markers of liver autoimmunity. These researchers found that polymorphisms of genes involved in TNF-receptor signalling and in particular those of the TNF-receptor-associated factor 1 (*TRAF1*) do not confer susceptibility to primary biliary cirrhosis (PBC). However, the GG homozygotes have significantly higher titres of PBC-specific autoantibodies directed against gp210 autoantibodies compared to AA homozygotes, suggesting that this gene may immunoregulate the persistence of gp210-specific B-lymphocytes. Another intriguing original paper by C. Radzimski et al. reports the development of a recombinant cell-based IIF assay which allows efficient determination of autoimmune hepatitis-specific autoantibodies against soluble liver antigen. These autoantibodies are important for the confirmation of the diagnosis in patients with suspected autoimmune hepatitis and cannot be detected in routine laboratories by IIF.

The broad range of important topics discussed by the authors participating in this issue further underlines the notion shared by the editors of this special issue that serum autoantibodies remain as one of the most valuable tools for the study of autoimmunity [4–6].

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2013, Article ID 474028, 9 pages
http://dx.doi.org/10.1155/2013/474028

Clinical Study

Higher Levels of Autoantibodies Targeting Mutated Citrullinated Vimentin in Patients with Psoriatic Arthritis Than in Patients with Psoriasis Vulgaris

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Received 12 October 2012; Revised 23 December 2012; Accepted 2 January 2013

Academic Editor: Xavier Bossuyt

Antibodies against citrullinated proteins/peptides (ACPAs), and especially antibodies targeting mutated citrullinated vimentin (anti-MCVs), are novel biomarkers of rheumatoid arthritis (RA). Whereas ACPAs are specific and sensitive markers for RA, there have hardly been any reports relating to ACPAs in psoriatic arthritis (PsA) or in psoriasis without joint symptoms (PsO). The aim of the present study was to investigate the prevalence of anti-MCVs in PsA and PsO. Serum anti-MCV titers were measured in 46 PsA and 42 PsO patients and in 40 healthy controls by means of a commercial enzyme-linked immunosorbent assay. The potential correlations of the serum autoantibody levels with several clinical and laboratory parameters were examined. The anti-MCV levels in the PsA patients were significantly higher than those in the PsO group. Among the clinical variables, the presence of tender knee joints and nail psoriasis was significantly associated with anti-MCV positivity in the PsA patients. Higher anti-MCV titers in the PsO patients were associated with a more severe disease course and with the early onset of psoriatic skin symptoms. Our results suggest that anti-MCVs can be used as novel markers in the diagnosis of PsA and in a subset of PsO patients.

1. Introduction

Antibodies targeting mutated citrullinated vimentin (anti-MCVs) belong in the group of anti-citrullinated protein/peptide antibodies (ACPAs). Antibodies against citrullinated cyclic peptides (anti-CCPs) are the most widely used members of the ACPA group [1–3]. The detection of ACPAs is a specific and sensitive marker for the diagnosis of rheumatoid arthritis (RA) [4–9]. The ACPAs are also of prognostic relevance. ACPA positivity is associated with a faster progression and a poorer outcome in RA [10–13]. Anti-MCVs and VCP2 (a peptide corresponding to the modified Epstein-Barr virus encoded protein 2 (EBNA-2)) are highly sensitive members of the ACPA group [14–16]. The anti-MCVs were recently reported to have higher diagnostic sensitivity than anti-CCPs and rheumatoid factor in RA [17–19], though conflicting results were found in another recent study as concerns the superiority of anti-MCVs over anti-CCPs in the diagnosis of RA [16]. Anti-MCVs are detectable in early RA patients, even before the symptoms are manifest, and are therefore presumed to be of prognostic value. Several recent studies have suggested that the production of these autoantibodies is associated with a faster disease progression and may well serve as a useful predictive marker of severe joint damage [20, 21]. Anti-MCVs target citrullinated vimentin. Vimentin, the main cytoskeletal component of

the mesenchymal cells [22, 23], is not coded by DNA and can only be expressed by posttranslational modification, that is, enzymatic citrullination of the amino acid arginine. Vimentin contains 43 arginine residues, and the citrullination is catalyzed by the enzyme peptidylarginine deiminase found in monocytes and macrophages. Tissue inflammation and cell apoptosis lead to changes in the structure of the protein by enzymatic citrullination and activate the immune system by the increased production of autoantibodies [24]. Recent studies suggest that the enzymatic citrullination and the production of ACPAs may also be associated with other inflammative arthritis-associated autoimmune diseases [25–27].

Psoriatic arthritis (PsA) is a seronegative spondyloarthropathy that develops in up to 30 per cent of patients with psoriasis (National Psoriasis Foundation, http://www.psoriasis.org/). PsA occurs more frequently in subject with the HLA-B27 haplotype [28–30]. PsA has several different clinical phenotypes: oligoarticular, polyarticular, symmetrical, and asymmetrical peripheral joint inflammation or axial involvement [31, 32]. Various systems and criteria have been proposed to aid the diagnosis and classification of PsA [29, 33–37]. Although none of them are accepted unequivocally, the classification criteria described by Moll and Wright [37] and more recently the classification criteria for PsA (CASPAR) have been used most frequently [36]. The wide spectrum of disease expression often makes it difficult to distinguish PsA from RA or other spondyloarthropathies. Currently, there is no specific test that could be used reliably for the diagnosis of PsA. Moreover, a biomarker (or biomarkers) that could distinguish between different clinical phenotypes of PsA or between PsA and psoriasis vulgaris (PsO), or that could be used as a predictive marker for future PsA development in PsO patients, is still lacking.

Because of the several clinical similarities between PsA and RA, and in view of the fact that the anti-MCVs are highly sensitive markers in RA, we set out to investigate the prevalence of anti-MCVs in PsA and PsO patients. Possible associations between the anti-MCV titers and the clinical, and laboratory variables of PsA and PsO patients were also studied.

2. Materials and Methods

2.1. Study Population. This cross-sectional clinical investigation was approved by the Regional and Institutional Human Medical Biological Research Ethics Committee of Albert Szent-Györgyi Clinical Center at the University of Szeged. Informed consent was obtained from all participants in the study. Serum samples were collected at the first regular follow-up visits following the commencement of the clinical study, regardless of the patients' clinical status or treatment.

The PsA group comprised 46 patients (24 women and 22 men) who fulfilled the CASPAR classification criteria for PsA and who had been treated in the absence of any information as to their serologic status regarding antibody reactivities against citrullinated proteins. The basic demographic, clinical and laboratory characteristics on the PsA group are to be seen in Tables 1 and 2. The mean (±standard deviation; SD) age of these patients was 54.3 ± 11.9 years (range: 28–77 years). At the time of sampling collection, the mean disease activity score in 28 joints (DAS28) [38, 39] was 4.51 ± 1 (range: 2.08–6.81); the scores of 6 (13%) patients were ≤3.2 (low PsA activity), 27 (59%) had DAS28 scores between 3.3 and 5.1 (moderate PsA activity), and 13 (28%) had DAS28 scores >5.1 (high PsA activity). All patients had been previously or were currently treated with at least one type of disease-modifying antirheumatic drug (DMARD). The group was heterogeneous as regards the arthritis phenotypes. The Moll and Wright criteria [37] were used to classify the PsA patients into subgroups. Twenty-five patients (54%) had asymmetrical oligoarthritis, 20 (43%) had symmetrical polyarthritis, 8 (17%) had axial arthritis, and 2 (4%) had distal arthritis. There was only 1 patient with arthritis mutilans (2%). Fifteen PsA patients (33%) had distal interphalangeal (DIP) joint inflammation. All 46 patients also displayed psoriatic skin lesions.

Table 1: Basic demographic and clinical characteristics of psoriatic arthritis (PsA) and psoriasis (PsO) groups.

Variable	PsA group ($N = 46$)	PsO group ($N = 42$)
Male : female ratio	24 : 22	31 : 11
Age (mean ± SD; years)	54.35 ± 11.87	45.60 ± 15.72
BMI (mean ± SD; kg/m^2)	29.38 ± 6.41	28.86 ± 9.80
Current smokers (%)	20	12
Age at diagnosis of PsO (mean ± SD; years)	38.91 ± 14.47	28.84 ± 15.82
Age at diagnosis of PsA (mean ± SD; years)	45.26 ± 13.80	—
Disease course severity (mild : moderate to severe)•	0 : 46	6 : 36
Psoriasis guttata (%)	4	20
Arthritis mutilans (%)	2	—
Axial arthritis (%)	17	—
Distal arthritis (%)	4	—
Asymmetrical oligoarthritis (%)	54	—
Symmetrical polyarthritis (%)	43	—
Therapy		
Received MTX therapy (%)	85	57
Received systemic steroid treatment (%)	13	2
Received 311 nm NB-UVB therapy (%)	7	38
Received PUVA therapy (%)	2	31
Received biological therapy (%)	13	52

PsO: psoriasis vulgaris, PsA: psoriatic arthritis, BMI: body mass index, MTX: methotrexate, PUVA: psoralen + ultraviolet A, 311 nm NB-UVB: 311-nanometer narrow-band ultraviolet B. Symmetrical arthritis: bilateral arthritis with a frequency of >50%.

•Patients previously or currently treated with DMARDs, systemic therapy or full-body phototherapy were regarded as "moderate-to-severe" patients, whereas the others were considered to exhibit a "mild" disease course.

Table 2: Clinical and laboratory characteristics of patients in the psoriatic arthritis (PsA) and psoriasis vulgaris (PsO) groups.

Variable	PsA group ($N = 46$)	PsO group ($N = 42$)
Anti-MCV positivity (%)	24	8
Level of anti-MCV (mean ± SD; U/mL)	30.32 ± 82.14	8.71 ± 7.41
ANA positivity (%)†	38	Not measured
RF positivity (>9 U/mL; %)†	11	Not measured
Active psoriatic lesions in the skin	100	95
PASI score (mean ± SD)	—	5.84 ± 6.75
Nail psoriasis (%)	28	43
Scalp psoriasis (%)	72	57
Plaques on the face (%)	11	14
Plaques on the upper limbs (%)	61	71
Plaques on the trunk (%)	30	48
Plaques on the perineum (%)	15	7
Plaques on the lower limbs (%)	59	88
Arthritic features		
DAS28 score (mean ± SD)	4.51 ± 1.00	—
DIP involvement (%)	33	—
Erosion (%)	24	—
Tender joint count (mean ± SD)	9.78 ± 5.90	—
Back (%)	48	—
Shoulders (%)	37	—
Elbows (%)	15	—
Wrists (%)	46	—
Hands (%)	67	—
Hips (%)	17	—
Knees (%)	35	—
Feet (%)	61	—
Swollen joint count (mean ± SD)	2.67 ± 3.19	—
Swollen shoulder (%)	0	—
Swollen elbow (%)	2	—
Swollen wrist (%)	9	—
Hand joints (%)	43	—
Swollen knee (%)	11	—
Feet joints (%)	26	—

Anti-MCVs: antibodies against mutated citrullinated vimentin, ANA: antinuclear antibody, RF: rheumatoid factor, DIP: distal interphallangeal, PASI: psoriasis area and severity index, DAS28: disease activity score.

The basic characteristics on the 42 PsO patients are similarly given in Tables 1 and 2. Their mean age was 45.60 ± 15.72 years (range: 18–78 years), and the female : male ratio was 11 : 31 (26% versus 74%). The group consisted of 6 patients with a mild and 36 patients with a moderate-to-severe disease course. The assessment of the severity of the disease course was based on the previous and current antipsoriatic therapy: patients previously or currently treated with systemic (including biological) therapy or full-body phototherapy were considered to have "moderate-to-severe" PsO and the others to have "mild" PsO. At the time of serum sample collection, the mean psoriasis area and severity index (PASI) [40] score was 5.84 ± 6.75 (range: 0.00–34.20), but most patients were on concurrent systemic, biological, or phototherapy. None of the PsO patients had psoriatic joint involvement, as assessed by a trained rheumatologist.

A randomly selected, self-stated healthy group of volunteers ($N = 40$) served as controls (none of them had ever exhibited psoriatic skin or joint symptoms). Their mean age was 45.05 ± 19.56 years (range: 16–82 years) and the female : male ratio was 20 : 20.

2.2. Determination of Anti-MCV IgG by ELISA. Anti-MCV IgG antibodies were analyzed by ELISA (ORG 548 anti-MCV; ORGENTEC Diagnostika GmbH, Mainz, Germany), with recombinant MCV as antigen. The analyses were conducted in accordance with the manufacturer's instructions. As recommended by the manufacturer, patients with anti-MCV titers higher than the 20 U/mL cut-off value were regarded as positive.

2.3. Statistical Analysis. The data on the anti-MCV-positive and negative patient groups were compared by means of the Fisher exact test. Since the data were not normally distributed, the correlations between anti-MCV positivity and the clinical features were determined through the use of nonparametric methods. Nonparametric methods were applied to assess overall group differences via pairwise comparisons (Mann-Whitney U tests) and Spearman's rank correlation coefficient. All statistical analyses were performed with the statistical program SPSS Windows (v15.0). P values <0.05 were considered significant.

3. Results

3.1. Anti-MCV Titers Are Significantly Higher in PsA Than in PsO Patients and Nonpsoriatic Individuals. As anti-MCV positivity is a characteristic hallmark of RA, we first investigated whether anti-MCVs are also associated with a different type of inflammatory joint disease, PsA. Our PsA patients exhibited significantly higher mean serum anti-MCV levels than those of the PsO patients (Figure 1): 30.32 ± 82.14 U/mL and 8.71 ± 7.41 U/mL, respectively. The mean antibody levels of the controls (9.50 ± 4.23 U/mL) and the PsO group did not differ significantly.

With the recommended cut-off value of 20 U/mL, 11 PsA patients (24%) and 3 PsO patients (8%) were found to be positive for anti-MCVs, whereas all of the controls were anti-MCV-negative. The differences between the PsA and PsO groups and between the PsA and control groups were statistically significant ($P = 0.032$ and $P = 0.0009$, resp.) but that between the PsO and control groups was not statistically significant ($P = 0.0848$).

3.2. A Higher Anti-MCV Titer in PsO Patients Is Associated with a More Severe Disease Course. Significantly

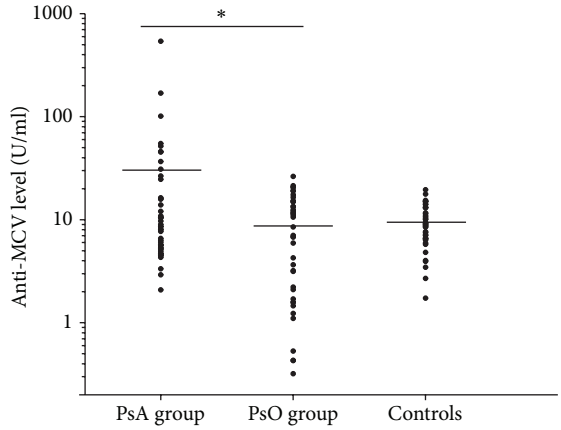

Figure 1: Anti-MCV titers are higher in PsA patients than in patients with psoriasis without arthritis and in healthy volunteers. The plots show the antibody levels of the investigated patients. The horizontal lines indicate the mean levels of anti-MCVs. The mean autoantibody level in the PsA group was 30.32 ± 82.14 U/mL as compared with 8.71 ± 7.41 U/mL in the PsO group and 9.50 ± 4.23 U/mL in the healthy control group. *The difference between the data on the PsA and PsO groups was statistically significant ($P < 0.05$).

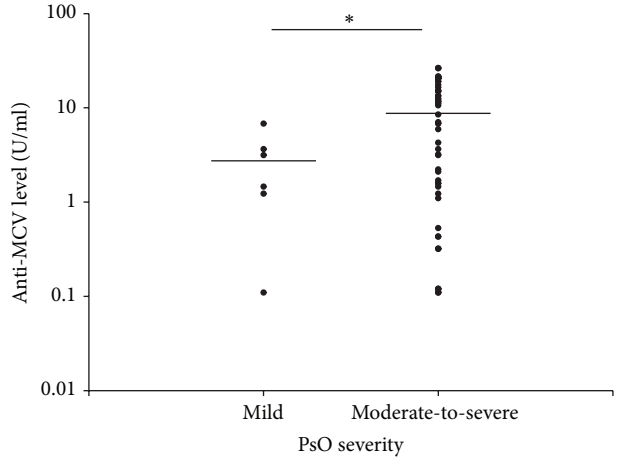

Figure 2: Anti-MCV titers are higher in patients with moderate-to-severe psoriasis than in mild psoriasis. The plots show the anti-MCV levels in moderate-to-severe (previously or currently treated with systemic or phototherapy) and mild (never received systemic or phototherapy) PsO patients. The horizontal lines indicate the mean levels of anti-MCVs. *The difference between the data on mild and the moderate-to-severe psoriasis PsO groups (2.73 ± 2.37 U/mL versus 9.73 ± 7.54 U/mL), respectively, was significant ($P < 0.05$).

higher anti-MCV titers were found in the PsO subgroup with a more severe disease course (9.73 ± 7.54 U/mL versus 2.73 ± 2.37 U/mL; $P = 0.033$) (Figure 2), although in both subgroups the mean titers were below the cut-off level proposed by the manufacturer. Furthermore, the moderate-to-severe PsO patients treated with biological therapy (Figure 3) demonstrated significantly higher anti-MCV levels than those of the moderate-to-severe patients who did not require biological therapy (14.01 ± 6.22 U/mL versus 3.01 ± 3.34 U/mL; $P < 0.01$) (both means were below the proposed cut-off level). On the other hand, the anti-MCV titers did not correlate significantly with the current disease activity (as determined by the PASI and DAS28 scores) at the time of serum sampling (data not shown). It seems, therefore, that the current psoriasis activity is not a critical determinant of the anti-MCV level in PsO. Rather, the PsO patients with the most severe disease course (requiring biological therapy) display the highest anti-MCV levels. A similar analysis was not feasible in the PsA group, which consisted almost entirely of severe cases who were receiving systemic DMARD or biological therapy. Similarly as in the PsO group, the current disease activity, as demonstrated by the DAS28 level, was not associated with higher anti-MCV levels in the PsA group either ($P = 0.843$; data not shown).

3.3. High Anti-MCV Titers in PsO Are Associated with an Early Onset of the Disease. As demonstrated in Figure 4, the anti-MCV levels proved to demonstrate a significant inverse correlation with the age at the onset of PsO ($P = 0.019$; patients who exhibited the early appearance of psoriatic skin symptoms usually presented with higher anti-MCV levels than those of the patients with a late disease onset). In the PsA group, however, no correlation was found between the age at PsA onset and the serum anti-MCV level ($P = 0.096$; data not shown).

3.4. The Presence of Tender Knee Joints and Nail Psoriasis Is Associated with Anti-MCV Positivity in PsA Patients. In order to identify clinical or laboratory features associated with high anti-MCV levels, we examined various parameters (Table 3), PsA patients were subdivided into anti-MCV-positive and negative groups, using the recommended cut-off value of 20 U/mL. A similar experiment, though seemingly reasonable, was not feasible in the PsO group, as the low number of anti-MCV-positives ($N = 3$) did not allow a meaningful statistical analysis in this group.

Only two of these parameters proved to be correlated with high anti-MCV titers. The presence of painful knee joints was significantly more frequent in the anti-MCV-positive patients (63.64% versus 25.71%; $P = 0.032$), and a significantly higher mean anti-MCV titer was detected in the PsA subgroup with painful knees (61.18 ± 133.76 U/mL versus 13.87 ± 20.22 U/mL; $P = 0.013$) (Figure 5). However, there was no correlation, between the presence of painful knees and either the patient's age or the patient's body weight (data not shown).

The second clinical feature that was significantly more frequent in the anti-MCV-positive PsA group was the presence of nail psoriasis (63.64% and 17.14% in the patients with and without psoriatic nail symptoms, resp.; $P = 0.006$). However, when the PsA and PsO patients were subdivided on the basis of the presence of nail symptoms, although a clear tendency was observed toward a higher anti-MCV level in those with nail symptoms, the difference was not statistically significant ($P = 0.305$) (Figure 6).

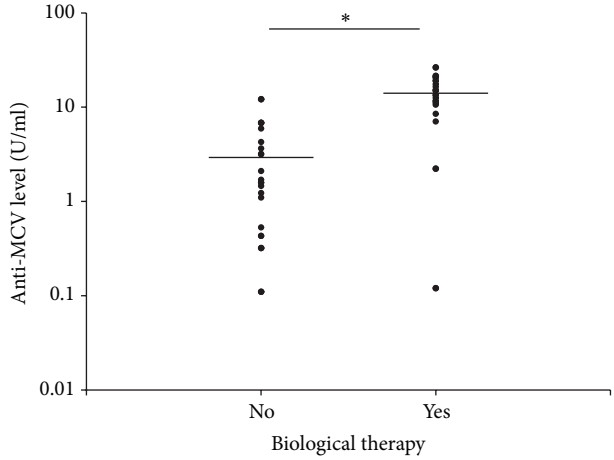

Figure 3: Anti-MCV titers are higher in severe PsO patients treated with biological therapy than in moderate-to-severe psoriasis patients not requiring biological therapy. The plots show the antibody levels of investigated patients. The horizontal lines indicate the mean levels of anti-MCVs. *The PsO patients not requiring biological therapy had significantly lower anti-MCV levels than those treated with biological therapy (3.01 ± 3.34 U/mL versus 14.01 ± 6.22 U/mL; $P < 0.01$).

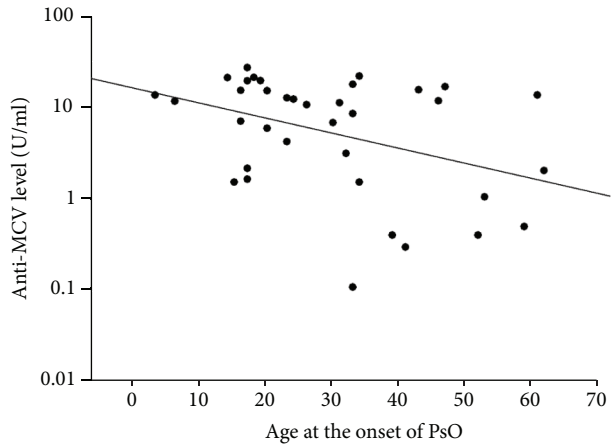

Figure 4: The anti-MCV levels demonstrate a significant inverse correlation with the age at the onset of the disease in the PsO patients. The plots represent the anti-MCV levels of the psoriasis patients and the age at the onset of psoriasis ($P = 0.019$).

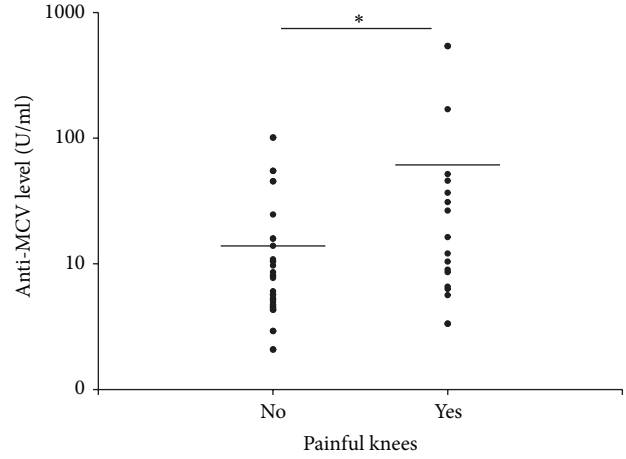

Figure 5: The anti-MCV levels in PsA patients with painful knees are significantly higher than in patients without painful knee. The plots show the antibody in PsA patients without pain of knees (labelled as "No") and with pain of knees (labelled as "Yes"). The horizontal lines indicate the mean levels of anti-MCVs. *The difference between the anti-MCV levels in the two groups (13.87 ± 20.22 U/mL versus 61.18 ± 133.76 U/mL) was significant ($P < 0.05$).

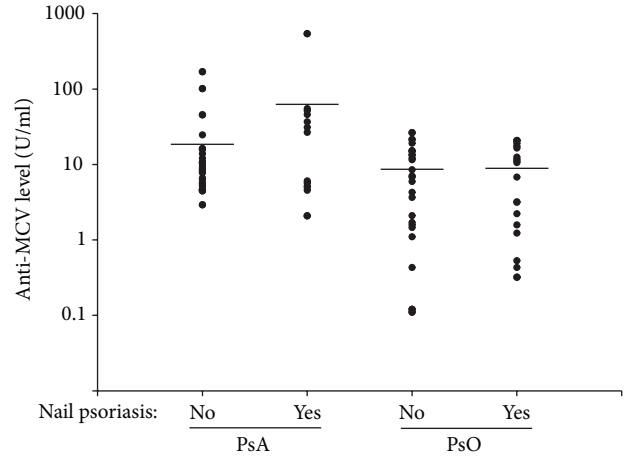

Figure 6: Anti-MCV levels of PsA and PsO patients with and without nail psoriasis. The plots represent the antibody levels of PsA and PsO patients with and without nail psoriasis. The horizontal lines indicate the mean levels of anti-MCVs. The differences between the groups were not significant ($P = 0.305$).

4. Discussion

This study has demonstrated significantly higher anti-MCV titers in PsA patients than in PsO patients or in healthy controls. The mean autoantibody level in the PsA group was 30.3 U/mL, as compared with 8.7 U/mL in the PsO group and 9.5 U/mL in the control group. The serum anti-MCV concentrations, although clearly higher in a subset of PsA patients, were markedly lower than the values of several hundred-to-thousand U/mL reported previously in RA patients [19]. Similar to our observations, modestly elevated anti-MCV titers have been reported in a subpopulation of PsA [18, 41] and in ankylosing spondylitis patients [42]. In our study cohort, 24% (11 out of 46) of the PsA patients were found to be anti-MCV-positive. To the best of our knowledge, anti-MCV levels in PsA have been reported only twice previously, and the results were not in full concordance, the prevalence of anti-MCV positivity in PsA ranging from 3.6% [41] to 15.2% [18]. The cause of the even higher anti-MCV positivity rate in our study population cannot be fully explained. The presence of anti-MCVs was earlier reported to correlate significantly with the disease activity in RA [19, 20]. In our study, the disease activity (as determined by the PASI and the DAS28 scores) was not associated with elevated anti-MCV titers (data not shown), though almost all of our patients

Table 3: Comparisons of clinical findings in anti-MCV-positive and anti-MCV-negative PsA patients.

Variable	Anti-MCV-positive PsA patients (N = 11)	Anti-MCV-negative PsA patients (N = 35)
Sex ratio (male : female)	3 : 8	21 : 14
Age (mean ± SD; years)	57.91 ± 9.26	53.23 ± 12.48
Current smoker (%)	9	23
Age at diagnosis of PsO (mean ± SD; years)	44.27 ± 13.73	37.23 ± 14.48
Age at diagnosis of PsA (mean ± SD; years)	46.55 ± 15.78	44.86 ± 13.34
PsA severity (mild : moderate to severe)	0 : 11	0 : 35
Psoriasis guttata (%)	9	3
Arthritis mutilans	9	0
Axial type (%)	27	14
Distal type (%)	9	3
Asymmetrical oligoarthritis (%)	64	51
Symmetrical polyarthritis (%)	36	46
Therapy		
Received local steroid treatment (%)	73	83
Received sulfasalazine (%)	27	20
Received systemic steroid treatment (%)	27	9
Received 311 nm NB-UVB therapy (%)	18	3
Received PUVA therapy (%)	0	3
Received MTX therapy (%)	73	89
Received biological therapy (%)	18	11
DIP involvement (%)	18	37
Erosion (%)	18	26
Level of anti-MCV (mean ± SD; U/mL)	**102.41 ± 150.99**	**7.67 ± 3.77***
ANA positivity (%)†	50	33
RF positivity (%)†	0	14
DAS28 score (mean ± SD)	4.49 ± 0.98	4.52 ± 1.02
Psoriatic skin lesions		
Nail psoriasis (%)	**64**	**17***
Scalp psoriasis (%)	64	74
Plaques on the face (%)	9	11
Plaques on the upper limbs (%)	55	63
Plaques on the trunk (%)	36	29
Plaques on the perineum (%)	9	17
Plaques on the lower limbs (%)	55	60
Arthritic features		
Tender joint count (mean ± SD)	7.27 ± 3.58	10.57 ± 6.29
Back (%)	36	51
Shoulders (%)	36	37
Elbows (%)	18	14
Wrists (%)	45	46
Hands (%)	45	74
Hips (%)	0	23
Knees (%)	**64**	**26***
Feet (%)	73	57
Swollen joint count (mean ± SD)	3.45 ± 2.94	2.43 ± 3.27
Swollen shoulder (%)	0	0
Swollen elbow (%)	9	0
Swollen wrist (%)	18	6
Hand joints (%)	45	40
Swollen knee (%)	27	6
Feet joints (%)	18	29

PsO: psoriasis vulgaris, PsA: psoriatic arthritis, anti-MCVs: antibodies against mutated citrullinated vimentin, BMI: body mass index, PASI: psoriasis area and severity index, DAS28: disease activity score, DIP: distal interphalangeal, HLA B27: human leukocyte antigen B27, ANA: anti-nuclear antibodies, RF: rheumatoid factor, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, MTX: methotrexate, PUVA: psoralen + ultraviolet A, nm: nanometer. Symmetrical arthritis: bilateral arthritis with a frequency of >50%. †The current values in these cases related to at least in 60%. For the other values, the data were complete: 100%. *There were significant differences between the anti-MCV-positive and negative groups ($P < 0.05$).

were actively treated with DMARDs or biologicals at the time of sample collection. In the anti-MCV-positive patients separately, the average number of swollen joints was relatively low (3.45 ± 2.94) and the disease activity reflected by the DAS28 score was moderate (4.49 ± 0.98).

Nail psoriasis and tender knee joints were observed significantly more frequently in the anti-MCV-positive PsA patients than in the anti-MCV seronegatives (64% versus 17%). It has recently been recognized that distal DIP joint disease in PsA is associated with diffuse inflammation that envelops the nail root and adjacent bone [43]. Thus, nail matrix inflammation and therefore psoriatic nail changes result from PsA enthesitis, and consequently nail psoriasis reflects DIP joint enthesitis. More recently, it has been demonstrated that nail involvement in psoriasis is directly correlated with systemic enthesitis, as the enthesopathy scores are significantly higher in PsA patients with nail disease than in those without it [44]. Overall, the association of anti-MCV positivity with psoriatic nail symptoms in our study population may indicate that a high anti-MCV level is a marker of systemic enthesitis in PsA. However, we could not confirm this hypothesis through a direct comparison of the enthesopathy scores with the anti-MCV titers, as the presence of subclinical enthesitis was not recorded at the time of sample collection in our study. Whether the increased number of PsA patients with tender knee joints within the group of anti-MCV seropositives is an epiphenomenon, or reflects a clear pathogenetic association, requires further investigations.

Although the mean anti-MCV titer of the PsO patients did not differ from that for the healthy controls, when the PsO patients were divided into moderate-to-severe and mild

groups, higher anti-MCV antibody titers proved to be significantly associated with a more severe disease course. Furthermore, those treated with biological therapy had significantly higher anti-MCV levels than the moderate-to-severe psoriasis patients who did not require biological therapy. Although the mean anti-MCV titers were below the proposed cut-off level, these findings imply that, within the group of PsO patients, higher anti-MCV levels may distinguish those patients with a more severe disease course. Whether these patients have significant subclinical joint involvement potentially detectable with highly sensitive imaging methods is unclear. Furthermore, we cannot exclude the possibility that these patients will eventually develop clinically evident PsA.

As regards the role of (biological) therapy in ACPA levels, some studies have reported significantly decreased serum RF and anti-CCP levels in RA patients in response to 6–12 months of TNF inhibitor therapy [45–47]. Nicaise Roland et al. observed significantly decreased anti-MCV levels after 18–24 months of anti-TNF treatment in RA [48]. Several other studies, however, did not detect marked changes in anti-CCP levels after 22, 30, or 54 weeks of infliximab treatment in RA [49–51]. We are not aware of any literature report of increased anti-MCV (or ACPA) levels in patients treated with biological therapy. Even though a definite conclusion cannot be drawn from these findings, these studies indicate that anti-CCPs are most probably not influenced (or at least are not increased) by anti-TNF-α therapy and are a relatively stable hallmark of RA. In view of the generally low (lower than the manufacturer-recommended cut-off value) anti-MCV titers in our PsO population, it seems unlikely that biological treatment would significantly modify the anti-MCV levels. If therapy had an effect on anti-MCV levels, this would result in decreasing titers in patients on biological therapy. In our opinion, therefore, the higher antibody titers in the group of PsO patients treated with anti-TNF therapy are not directly related to the treatment, but rather to the underlying severe disease course leading to the use of biologicals. Indeed, anti-MCV positivity is associated with a more severe disease course and a poor radiographic prognosis in patients with early RA [19].

The idea that a more severe disease course in PsO is associated with higher anti-MCV level was supported by the finding that the anti-MCV levels displayed a significant inverse correlation with the age at the onset of psoriatic skin symptoms. It is well known that patients with early-onset PsO usually have a more significant genetic background (HLA-cw6) and, among others, develop PsA more frequently [52, 53]. Early-onset PsO is frequently associated with a more severe disease course, and patients with early-onset PsO are therefore potentially more likely to suffer from PsA and more severe skin symptoms.

In conclusion, our study suggests that anti-MCVs, apart from being biomarkers of early RA, can also be used to differentiate a subset of PsO patients. As the differentiation of early and mild forms of PsA can pose a significant challenge in some cases, the detection of anti-MCV positivity can aid the diagnosis of PsA, especially in patients with psoriatic nail symptoms and tender knee joints. Furthermore, high levels of anti-MCVs in PsO patients without clinically manifest arthritis may distinguish patients who are more likely to experience a severe disease course and potentially require biological therapy. However, this study needs to be extended to large groups of both PsA and PsO patients in order to confirm these associations.

Acknowledgments

This work was supported by the following grants: OTKA NK77434, OTKA K73548, TÁMOP-4.3.1-B09/KONV-210-0005, and TÁMOP-4.2.2-B-10/1-2010-0012. The authors thank Éva Dósa-Rácz Viharosné for the statistical analysis.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2013, Article ID 703759, 8 pages
http://dx.doi.org/10.1155/2013/703759

Research Article

Clinical Phenotypes of Patients with Anti-DFS70/LEDGF Antibodies in a Routine ANA Referral Cohort

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Received 31 October 2012; Accepted 19 December 2012

Academic Editor: Xavier Bossuyt

Objective. To analyze the clinical value of anti-DFS70 antibodies in a cohort of patients undergoing routine antinuclear antibodies (ANAs) testing. *Methods*. Sera with a dense fine speckled (DFS) indirect immunofluorescence (IIF) pattern from 100 consecutive patients and 100 patients with other IIF patterns were tested for anti-DFS70 antibodies by a novel chemiluminescence immunoassay (CIA) and for ANA by ANA Screen ELISA (both INOVA). *Results*. Among the 100 patients with a DFS IIF pattern, 91% were anti-DFS70 positive by CIA compared to 3% in the comparator group ($P < 0.0001$). The CIA and IIF titers of anti-DFS antibodies were highly correlated (rho = 0.89). ANA by ELISA was positive in 35% of patients with the DFS IIF pattern as compared to 67% of patients with other patterns ($P < 0.0001$). Only 12.0% of patients with DFS pattern and 13.4% with DFS pattern and anti-DFS70 antibodies detected by CIA had systemic autoimmune rheumatic disease (SARD). Only 5/91 (5.5%) patients with anti-DFS70 antibodies had SARD and their sera were negative on the ANA Screen ELISA. *Conclusion*. Although anti-DFS70 antibodies cannot exclude the presence of SARD, the likelihood is significantly lower than in patients with other IIF patterns and should be included in test algorithms for ANA testing.

1. Introduction

The presence of antinuclear antibodies (ANAs), directed against intracellular antigens, is a hallmark of systemic autoimmune rheumatic diseases (SARDs) [1]. The indirect immunofluorescence (IIF) assay is among the most commonly used routine methods for ANA detection and was recently recommended as the screening test of choice by a study group of the American College of Rheumatology (ACR) [2]. Anti-dense fine speckles 70 (anti-DFS70) antibodies were initially identified as an ANA IIF pattern from a patient with interstitial cystitis [3], but were later associated with various other conditions (reviewed in [4]).

The typical DFS IIF staining pattern is recognized as uniformly distributed fine speckles throughout interphase nuclei and on metaphase chromatin [5]. The antigen was initially termed DFS70 according to the IIF pattern and the apparent molecular weight in immunoblot assays, but the primary target autoantigen was eventually identified as the lens epithelium-derived growth factor (LEDGF) [6] and more recently as the DNA binding transcription coactivator p75 (reviewed in [4]). DFS70/LEDGF is highly expressed in prostate tumour tissues [7] and has a number of physiological functions including serving as a cofactor for human immunodeficiency virus replication through an interaction with viral integrase [8].

Since the first description, anti-DFS70/LEDGF antibodies (hereafter referred to as anti-DFS70) have been reported in patients with a variety of chronic inflammatory conditions (reviewed in [4]), in cancer [7], and even in certain healthy individuals (HI) [9]. Dellavance et al. evaluated over 10,000 ANA positive samples by IIF and immunoblot and reported that anti-DFS70 antibodies were common among ANA-positive individuals with no evidence of SARD and that

among autoimmune patients bearing this autoantibody, over half had evidence of autoimmune thyroiditis [10]. The highest prevalence of anti-DFS70 antibodies has been reported in patients with Vogt-Harada syndrome (66.7%) [11] and atopic dermatitis (AD, 30%) [3, 12] followed by HI (~10%) [4, 9], while its prevalence in SARD is significantly lower (~2-3%) [4]. Considering the prognostic and long term outcome of individuals with anti-DFS70 antibodies, it was recently reported that none of 40 HI with isolated anti-DFS70 reactivity developed a SARD within an average 4-year followup [13]. Therefore, it was suggested that the presence of isolated anti-DFS70 antibodies could be taken as strong evidence against a diagnosis of SARD such as systemic lupus erythematosus (SLE) [9, 13–15].

The low prevalence of anti-DFS70 antibodies in patients with SARD is interesting and represents a potentially important biomarker that can be clinically used to discriminate SARD from ANA-positive HI and/or other inflammatory conditions such as AD. The reasons underlying the observed relative low prevalence in SARD are unclear but may include the impact of therapeutic interventions (i.e., corticosteroids, immune suppression).

Since ANAs and related autoantibodies are generally considered useful biomarkers for SARD and are included in the classification criteria for SLE [16] and systemic sclerosis (SSc) [17], ANA testing on HEp-2 substrates outside a proper clinical framework may yield a sizable portion of ANA-positive individuals without consistent evidence of SARD, purportedly leading to inappropriate referrals to tertiary care specialists, as well as anxiety in patients and physicians alike [13] and, perhaps, inappropriate and potentially toxic therapies [18]. A proper understanding of the clinical relevance of the full spectrum of autoantibodies detected in a diagnostic laboratory becomes even more crucial because of compelling evidence that autoantibodies may precede the clinical onset of SARD for many years [19–21]. Therefore, the concept of utilizing anti-DFS70 antibodies as a diagnostic or prognostic discriminator of ANA positive subjects with and without SARD is appealing. Accordingly, the principal aims of this study were to determine the frequency of anti-DFS70 antibodies in samples showing a DFS staining pattern (against a control group with other patterns) and then to investigate the prevalence SARD in the two groups.

2. Materials and Methods

2.1. Clinically Defined Samples. Sera of 100 consecutive patients that were referred to a single hospital (Pitie-Salpetriere, Paris, France) with a DFS IIF pattern on HEp-2000 cell substrates (ImmunoConcepts) with titers higher or equal to 1:80 were the primary focus of this study. Sera of 100 patients with a positive ANA and other IIF patterns (homogeneous, $n = 20$; speckled, $n = 20$; homogeneous and speckled, $n = 20$; nucleolar, $n = 20$; speckled/nucleolar, $n = 10$; centromere, $n = 10$) were used as the comparator group. Samples were collected during an audit period from 7th of December 2011 to 25th of April 2012 and were from a spectrum of hospital departments. A diagnosis of SARD in patients was retrospectively analyzed by clinical chart review of medical records and was established according to the disease criteria for the respective disease and as described previously [22]. Patient identity was not disclosed and the data were anonymously used in accordance with the latest version of the Helsinki Declaration of human research ethics. Collection of patient samples was carried out according to local ethics committee regulations and ethical approval was obtained from the "CPP - Ile de France- VI" at the Pitié-Salpêtrière Hospital. No consent was needed from any patients involved in this study. It was a retrospective study, without modification in the followup of patients.

2.2. Indirect Immunofluorescence (IIF). IIF was performed using HEp-2000 cells (ImmunoConcepts) using secondary anti-human IgG (H + L) supplied by and according to the manufacturer's instructions. The screening dilution was 1 : 80. Reading and interpretation of the IIF patterns was done by an experienced technologist on a Leica DM LB2, camera DFC 300FX, logiciel IM500, and a 40x objective.

2.3. Chemiluminescent Anti-DFS70 Assay. All samples were tested for the presence of anti-DFS70 antibodies by a novel chemiluminescence immunoassay. The QUANTA Flash DFS70 assay is a novel (CIA) (research use only) that uses recombinant DFS70 (expressed in *E. coli*) coated onto paramagnetic beads and is designed for the BIO-FLASH instrument (Biokit s.a., Barcelona, Spain) [23]. The principles and protocols of the assay system have been previously described [24, 25]. In brief, the relative light units (RLUs) are proportional to the amount of isoluminol conjugate that is bound to the anti-human IgG, which in turn is proportional to the amount of anti-DFS70 antibodies bound to the antigen on the beads. Using a standard curve, all RLU values are converted into calculated units (CU). Samples with antibody titers above the analytical measuring range (AMR, 3.2–450.8 calculated units, CU, cut-off = 20 CUs) were prediluted 1 : 20 and retested to determine the exact anti-DFS70 antibody concentration.

2.4. Detection of Other Autoantibodies and ANA/DFS70 Antibody Score. Antinuclear antibodies were determined in all samples using the QUANTA Lite ANA Screen ELISA (INOVA), which is a semiquantitative ELISA for the detection of ANA. The antigens include chromatin (dsDNA and histones), Sm/RNP, SS-A/Ro60, SS-B/La, Scl-70/topoisomerase I, centromere, PCNA, Jo-1, mitochondria (M-2) and ribosomal-P protein, as well as extracts from HEp-2 nuclei and nucleoli [26, 27]. Samples were tested according to the manufacturer's instructions and were considered positive when values were larger or equal to 20 units. The ANA/DFS70 Score was calculated by dividing the ANA ELISA by the DFS70 CIA results and was expressed in calculated units (CUs).

2.5. Statistical Evaluation. Data was statistically evaluated using the Analyse-it software (Version 2.03; Analyse-it Software, Ltd., Leeds, UK). Mann-Whitney U-test and Fisher exact test were carried out to analyze the difference between

groups. Cohen's *kappa* was used to analyze qualitative agreements. The BDT comparator was used to analyze differences between likelihood ratios as previously described [28, 29]. Spearman equation was used to analyze the agreement between the CIA and IIF titers. For all statistical tests P values < 0.05 were considered as significant.

3. Results

3.1. Anti-DFS70 Antibodies and ANA (by ELISA) in Samples with DFS and Other IIF ANA Patterns. Among the 100 patients with DFS IIF pattern, 91% were anti-DFS70 positive by CIA compared to 3% in the comparator group with other IIF ANA patterns ($P < 0.0001$). The positive, negative, total percent agreements, and Cohen's *kappa* were 91.0% (95% Confidence interval; CI 83.6–95.8%), 97.0% (95% CI 91.5–99.4%), 94.0% (95% CI 89.8–96.9%), and 0.88 (95% CI 0.81–0.95), respectively (see Table 1). Receiver operating characteristics (ROC) analysis of anti-DFS70 antibodies demonstrated excellent discrimination between samples with DFS pattern ($n = 100$) and other IIF ANA patterns ($n = 100$) as underlined by an area under the curve value of 0.981 (95% CI 0.960–1.000) (Figure 1). Quantitative comparison of anti-DFS antibody titers by IIF and anti-DFS70 antibodies by CIA showed strong correlation ($P < 0.0001$, rho = 0.89, 95% CI 0.84–0.92).

The ANA Screen ELISA was positive in 67% of patients with other patterns versus 35% in patients with the DFS pattern ($P < 0.0001$). The positive, negative, total percent agreements, and Cohen's *Kappa* (ANA Screen ELISA and other patterns) were 67.0% (95% CI 56.9–76.1%), 65.0% (95% CI 54.8–74.3%), 66.0% (95% CI 59.0–72.5%), and 0.32 (95% CI 0.19–0.45), respectively (see Table 1). ANA titers were significantly higher in samples with other patterns compared to samples with the DFS pattern ($P < 0.0001$, Figure 2(b)).

3.2. Differences in the Referring Physician Pattern of Samples with Dense Fine Speckled Pattern. The samples with the DFS pattern and other IIF ANA patterns were obtained from different referring clinical departments that included internal medicine/rheumatology, neurology, hepatology/gastroenterology, pulmonary diseases, ophthalmology, nephrology, intensive care, haematology, cardiology, infectious diseases, endocrinology, and otolaryngology). In the group with DFS pattern, 58 samples came from internal medicine/rheumatology versus 81 in the group with other patterns ($P = 0.0007$). In contrast, anti-DFS antibodies were more prevalent in samples from neurology (73.1% versus other patterns 34.6%; $P = 0.0193$) and hepatology (72.7% versus 36.4%, P= n.s.).

3.3. Clinical Association of the DFS Pattern and Anti-DFS70 Antibodies. The prevalence of SARD was significantly higher in the group with other ANA IIF patterns compared to the group with the DFS pattern (58% versus 12%, $P < 0.0001$) and to the group with a DFS pattern and confirmed anti-DFS70 antibodies (58% versus 13.4%, $P < 0.0001$). 15/94 (16.0%) patients with anti-DFS70 antibodies had SARD (13 SLE; 1 Sjögren's syndrome; 1 SSc). By comparison, only 5/94 (5.3%) patients with anti-DFS70 antibodies had SARD (4 SLE, 1 SSc) but were negative on the ANA Screen ELISA. Since an intended use of the DFS70 CIA is to confirm anti-DFS70 reactivity in samples showing the DFS pattern, we also calculated the clinical association in this subset of patients. 12/91 (13.4%) patients with DFS pattern and anti-DFS70 antibodies had SARD (10 SLE; 1 Sjögren's syndrome; 1 SSc). Only 5/91 (5.5%) patients with anti-DFS70 antibodies had SARD (4 SLE, 1 SSc) and were negative on the ANA Screen ELISA. The 3 anti-DFS70 positive patients in the control group had high titer (1/640–1/1280) homogeneous ($n = 2$) and homogeneous/speckled ($n = 1$) IIF ANA patterns and all had a diagnosis of SLE.

ROC analyses showed that anti-DFS70 antibodies discriminated between SARD and non-SARD patients (non-SARD patients having higher values, see Figure 3(a)) with an AUC of 0.73 (95% CI 0.66–0.80; $P < 0.0001$). At high titers (199 CU), 25/130 (19.2%) patients without SARD and 2/70 (2.9%) with SARD had anti-DFS70 antibodies. The likelihood ratios (LR+ and LR−) for non-SARD were 6.73 and 0.83, respectively. ANA by ELISA also discriminated between SARD and non-SARD patients (see Figure 3(b)) with an AUC of 0.83 (95% CI 0.77–0.89; $P < 0.0001$). Results are summarized in Table 2. At high titers (131.2 units), 17/70 (24.3%) patients with SARD and 3/130 (2.3%) were ANA ELISA positive. The likelihood ratios (LR+ and LR−) for SARD were 10.5 and 0.76, respectively, and thus more relevant compared to anti-DFS70 antibodies.

3.4. Algorithm of ANA ELISA and DFS70. Next we analyzed if the results derived from ANA Screen ELISA and from DFS70 CIA can be combined in a diagnostic score that improved the differentiation between SARD and non-SARD ANA IIF positive individuals. When using the results of the ANA/DFS70 Score for ROC comparative analysis we found a significantly improved discrimination between SARD patients and non-SARD patients (see Figure 3(c)) with an AUC of 0.84 (95% CI 0.78–0.90; $P < 0.0001$). The sensitivity and specificity at a selected cut-off were 51.4% and 97.7%. The LR+/− ratios for SARD was 22.3 and 0.50. When comparing the ANA/DFS70 Score with ANA ELISA at the same specificity (97.7%), the sensitivity of the ANA/DFS70 Score was significantly higher (51.4% versus 24.3%, $P < 0.0001$).

4. Discussion

Anti-DFS antibodies have been historically associated with interstitial cystitis [5] and atopic dermatitis [30], but they have also been described in various other diseases [4]. Although a distinctive clinical association is unreported, anti-DFS70 antibodies have been proposed as a useful biomarker for the exclusion of SARD [9, 14, 15, 23]. This suggestion has mainly been based on the observation that anti-DFS antibodies are more prevalent in HI than in SARD patients and that anti-DFS positive individuals did not develop SARD after clinical followup of four years [13]. Additional support for the hypothesis came from observations that approximately

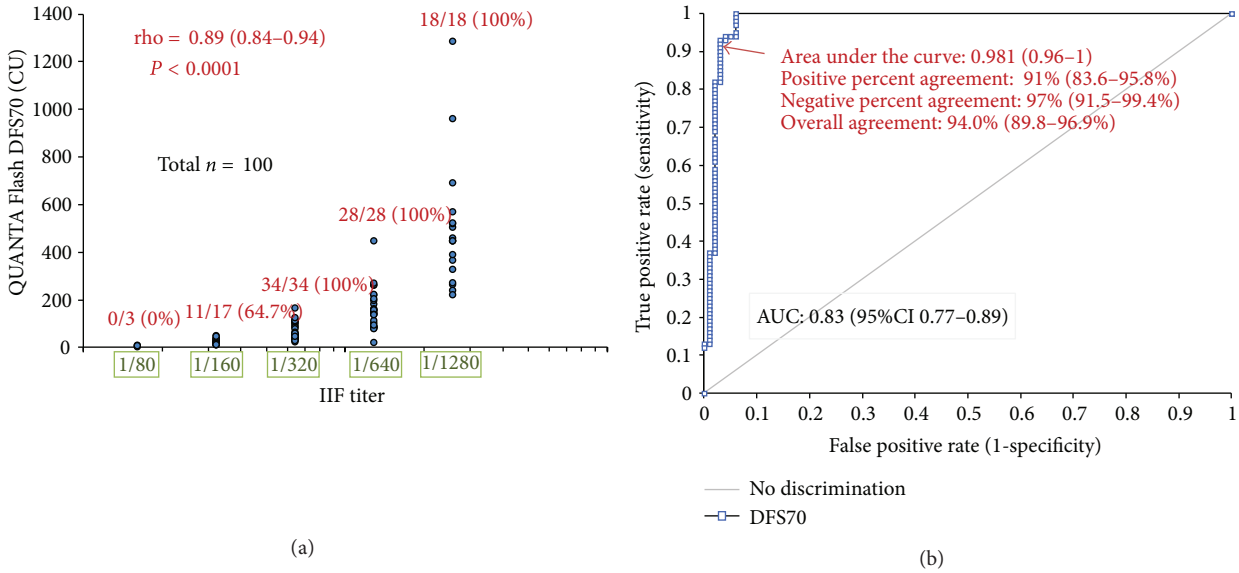

Figure 1: Correlation between dense fine speckled (DFS) pattern by indirect immunofluorescence (IIF) and anti-DFS70 antibodies measured by chemiluminescent immunoassay (CIA). (a) Correlation between the anti-DFS antibody titer by IIF and by QUANTA Flash DFS70. Excellent correlation between the anti-DFS antibody titers by IIF and by QUANTA Flash DFS70 was found using the samples showing the DFS speckled pattern ($n = 100$). Number and percent of the anti-DFS70 antibody positive samples are shown per titer group (cut-off = 20 CU). (b) Receiver operating characteristics (ROC) analysis comparing samples with DFS ($n = 100$) and other IIF ANA patterns ($n = 100$) by means of anti-DFS70 antibodies. Excellent discrimination between samples with DFS pattern and other patterns was observed as underlined by an area under the curve value of 0.981 (95% CI 0.960–1.000).

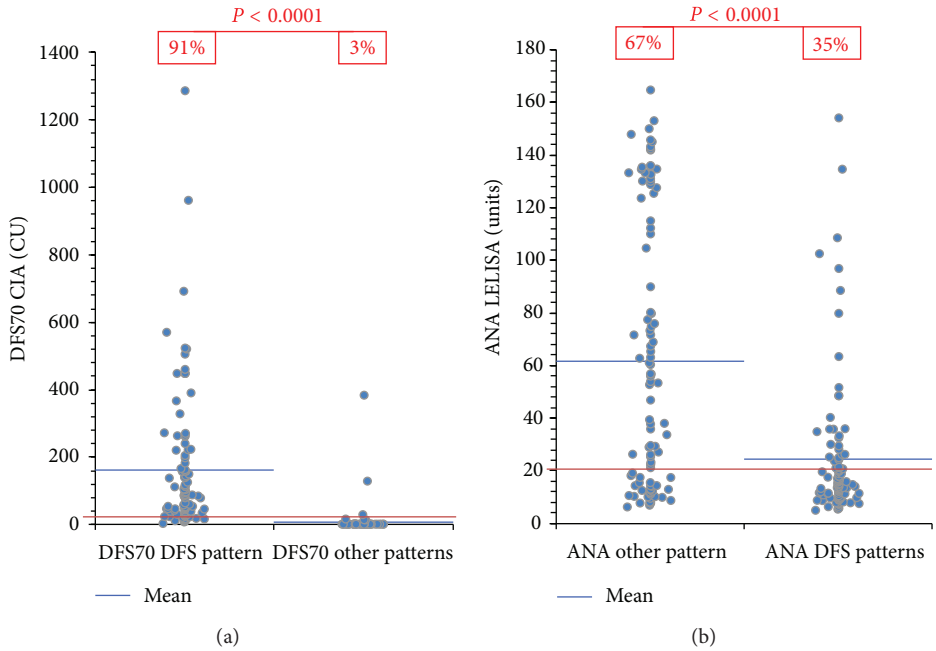

Figure 2: Antinuclear antibodies measured by ELISA and anti-DFS70 antibodies by chemiluminescent immunoassay (CIA) in samples with DFS pattern versus samples with other patterns. (a) Anti-DFS70 antibodies were significantly more prevalent (91.0% versus 3.0%) and their titers higher in samples with DFS pattern compared to samples with other patterns ($P < 0.0001$). (b) In contrast, antinuclear antibodies were significantly less prevalent (35.0% versus 67.0%) and their titers lower in samples with DFS pattern compared to samples with other patterns ($P < 0.0001$). Cut-off values are indicated by the red line.

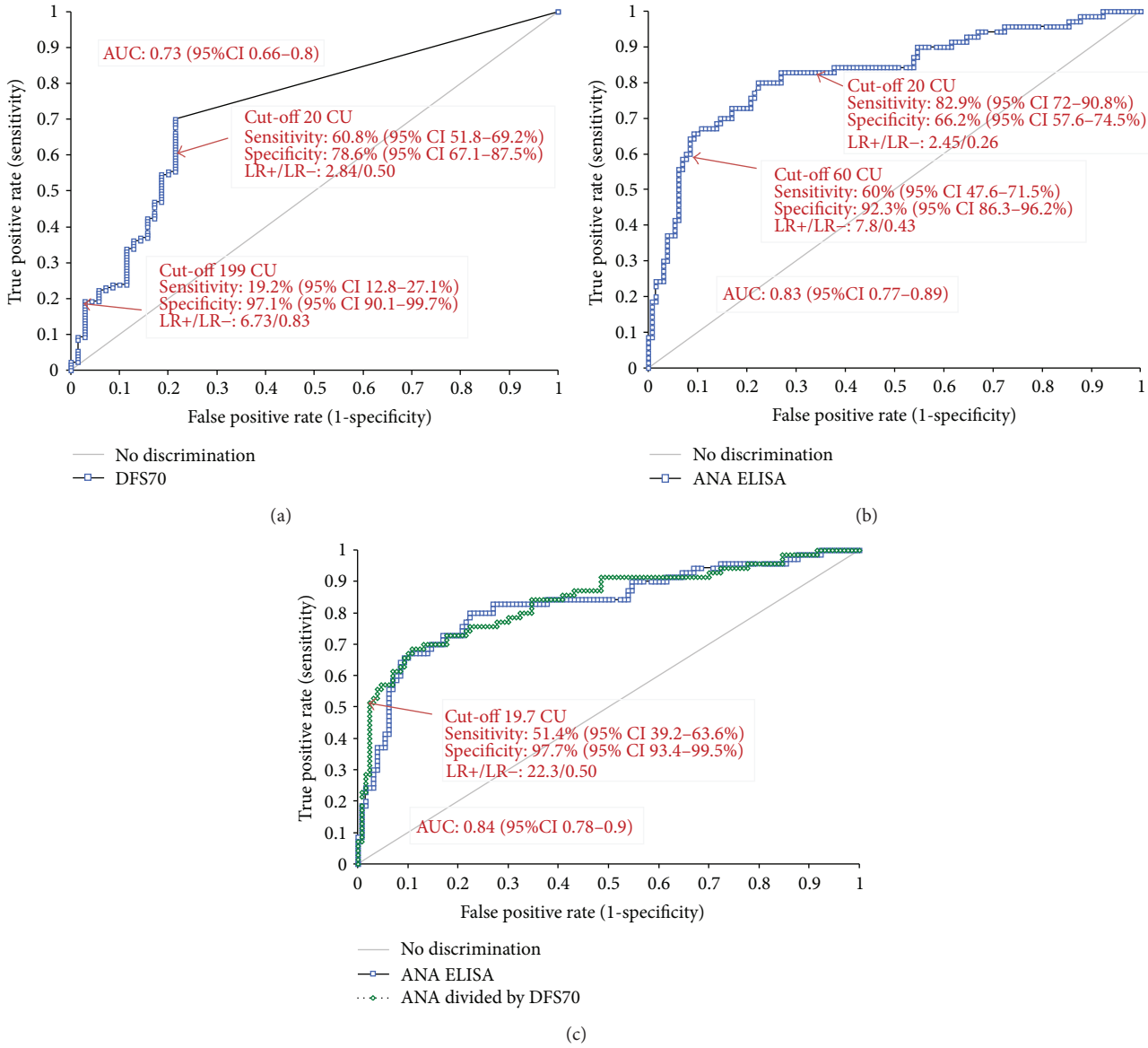

Figure 3: Discrimination between SARD and non-SARD patients using DFS70 and ANA by ELISA. (a) Differentiation of SARD versus non-SARD in all patients ($n = 200$) using DFS70. (b) Differentiation of SARD versus non-SARD in all patients ($n = 200$) using ANA Screen ELISA. (c) Differentiation of SARD versus non-SARD in all patients ($n = 200$) using ANA Screen ELISA and DFS70 Score (ANA ELISA divided by DFS70 CIA).

Table 1: Correlation between DFS and other IIF patterns and anti-DFS70 antibodies by ELISA and CIA.

Assay	DFS pattern	Other patterns	Positive % agreement*	Negative % agreement*	P
DFS70 CIA	91/100 (91.0%)	3/100 (3.0%)	91.0% (83.6–95.8%)	97.0% (91.5–99.4%)	$P < 0.0001$
ANA ELISA	35/100 (35.0%)	67/100 (67.0%)	67.0% (56.9–76.1%)	65.0% (54.8–74.3%)	$P < 0.0001$

*Positive and negative percent agreements were calculated based on the target cohort: For DFS70 CIA the target cohort is the group of samples with DFS pattern and the control cohort is the group of samples with other ANA patterns; for ANA ELISA the target cohort is the group of samples with other ANA patterns and the control cohort is the group of samples with DFS patterns.

30% of ANA positive samples from HI have anti-DFS70 antibodies [13, 31] as determined by IIF compared to 0% in ANA positive individuals with SARD.

Anti-DFS70 antibodies have been reported in approximately 3% of SLE patients [14], but the detection of anti-DFS70 as detected by IIF may be problematic because these sera are usually accompanied by other antibodies such as anti-dsDNA, anti-SS-A/Ro, or anti-Sm, which may mask the DFS IIF staining pattern. In the SLE group reported by Muro et al. [14], 4/7 anti-DFS70 positive SLE were positive for

Table 2: Discrimination of SARD and non-SARD using ANA ELISA, anti-DFS70 CIA, and ANA/DFS70 score (ANA divided by DFS70); different cut-offs.

	SARD	Non-SARD	Sensitivity	Specificity	LR+/LR−
DFS pattern[#]	12/70 (17.1%)	88/130 (67.7%)	67.7% (58.9–75.6%)	82.9% (72.0–90.8%)	2.57/0.25
QUANTA Flash DFS70[#]					
Cut-off 20 CU[&]	15/70 (21.4%)	79/130 (60.8%)	60.8% (51.8–69.2%)	78.6% (67.1–87.5%)	2.84/0.50
Cut-off 199 CU[+]	2/70 (2.9%)	25/130 (19.2%)	19.2% (12.8–27.1%)	97.1% (90.1–99.7%)	6.73/0.83
ANA Screen ELISA[*]					
Cut-off 20 units	58/70 (82.9%)	44/130 (33.8%)	82.9% (72.0–90.8%)	66.2% (57.3–74.2%)	2.45/0.26
Cut-off 60 units[+]	42/70 (60.0%)	10/130 (7.7%)	60.0% (47.6–71.5%)	92.3% (86.3–96.2%)	7.80/0.43
Cut-off 131.2 units[+]	17/70 (24.3%)	3/130 (2.3%)	24.3% (14.8–36.0%)	97.7% (93.4–99.5%)	10.5/0.76
ANA/DFS70 Score[*]					
ANA divided by DFS70 Cut-off 19.7 CU[+]	36/70 (51.4%)	3/130 (2.3%)	51.4% (39.2–63.6%)	97.7% (93.4–99.5%)	22.3/0.50

[*]Positive result (and LR+) considered indicative for SARD; [#]positive result (and LR+) considered indicative for non-SARD; [&] cut off values were previously established; [+]cutoff values were defined based on receiver operating characteristics (ROC) analysis.

anti-SS-A/Ro antibodies, 6/7 were also positive for dsDNA and 2/7 for anti-Sm. In a second study [23], the coexistence of other autoantibodies was similar: 5/7 anti-DFS70 positive SLE patients were positive for anti-dsDNA, and one for anti-Sm antibodies. Only 1/7 SLE patients with anti-DFS70 antibodies had no additional detectable autoantibodies. These data confirm that anti-DFS70 antibodies are rarely observed in SARD and when they are, they are usually accompanied by additional SARD related autoantibodies. In addition, no clinical difference between anti-DFS70 positive and negative SLE patients has been found. These data are consistent with our findings since 13.4% of anti-DFS70 positive but only 5.5% of anti-DFS70 positive/ANA ELISA negative patients had SARD.

The higher prevalence of anti-DFS70 antibodies in HI compared to SARD patients might support the hypothesis that these autoantibodies serve as protective [32] or indifferent or neutral effector [33] autoantibodies. However, further longitudinal studies are required to address this hypothesis. Despite the importance of these previous studies, a significant limitation is that they were based on selected serological cohorts and not on unselected patients for which an ANA test was requested.

The prevalence of the DFS IIF pattern and anti-DFS70 antibodies has been reported to vary significantly [23]. One study reported that 172/21,512 (0.8%) of samples showed the typical DFS pattern by IIF [34] while another investigation showed that anti-DFS antibodies were present in as much as 12.3% of consecutive samples tested for ANA [10]. Although our study does not allow to.

It has been reported that the frequency of anti-DFS70 antibodies in routine laboratories is within the range of other important SARD autoantibodies such as anti-dsDNA antibodies [35, 36]. In addition, it was found that virtually all samples with DFS pattern identified by IIF had anti-DFS70 antibodies by CIA and/or ELISA which is consistent with our findings. In our cohort we found a positive and negative percent agreement of 91.0% and 97.0%, respectively. However, since significant differences have been described between the staining patterns on HEp-2 cells from different manufacturers [35, 37], it remains unclear if the DFS IIF pattern can be recognized with similar accuracy using slides from a variety of manufacturers. Such variations might be attributed to the fixation methods, culture conditions, and/or other processes used for manufacturing the cell substrates [37]. Another variable to consider is the acumen of the laboratory personal in identifying the DFS pattern. Although previous data [23] indicate that the DFS pattern can be identified on slides from a number of ANA kit manufacturers, more samples need to be analyzed to arrive at a conclusion, especially since conflicting results have been published [37].

In our cohort of 200 ANA positive individuals, 130 had no evidence of SARD. Since a positive ANA test result is an important component in the triage and diagnosis of patients with possible SARD, ANA-HEp-2 testing outside a proper clinical framework may yield a sizable portion of ANA-positive apparently HI, causing concern and anxiety in patients and physicians [13], and may lead to prescribing inappropriate and potentially toxic therapeutics [18]. This concern becomes even more important with the recent knowledge that autoantibodies often precede the clinical onset of SARD by many years [19–21]. Hence, samples with DFS staining pattern identified by IIF should be tested for anti-DFS70 antibodies by a specific assay (i.e., ELISA or CIA) and the result should be included in the laboratory report. In addition, it is advisable that clinicians should not overinterpret positive ANA results in patients with anti-DFS70 antibodies alone but should focus on the clinical signs and symptoms complimented by the detection of other disease specific autoantibodies. The observation that ANA by ELISA (QUANTA Lite ANA Screen) is able to differentiate ANA positive patients with SARD from ANA positive non-SARD patients is interesting. It might be assumed that the ANA ELISA does not detect anti-DFS70 antibodies.

The reason that the majority of samples containing anti-DFS70 antibodies are negative on the ANA Screen ELISA requires further investigations. All novel (optimized) cut-off of the DFS70 CIA, the ANA Screen ELISA and the ANA/DFS70 Score were established based on ROC analysis in our cohort of patients and have to be validated in further studies before clinically applied.

Historically, when the ANA HEp-2 test became available in the 1960s, predominantly rheumatologists and clinical immunologists ordered this test. With the eventual recognition that many other diseases with autoimmune features are also associated with ANAs, a broader range of clinical disciplines now order the ANA test. This change in test referral pattern has tremendous consequences for the posttest probability of disease since screening tests with limited specificity (such as IIF ANA) are strongly affected when the pretest probability in a given population decreases. Of note, in our cohort the prevalence of DFS versus other patterns was statistically different in two referral sources. In samples referred from internal medicine/rheumatology, the prevalence of other IIF patterns was higher than of the DFS pattern and in samples referred from neurology the difference was the opposite.

A significant limitation of our study is that most samples came from follow-up visits of the patients. However, since most individuals with ANAs including anti-DFS70 antibodies remain positive for many years [13] it can be speculated that our data is also relevant to the diagnostic setting. Further studies with diagnostic samples are needed to confirm our findings.

Our data confirms previous observations that SARD is less prevalent in patients with a DFS pattern (and anti-DFS70 antibodies) than in patients with other patterns (i.e., homogeneous, speckled, homogeneous and speckled, nucleolar, speckled/nucleolar, centromere). Although the DFS pattern (and anti-DFS70 antibodies) cannot exclude the presence of SARD [38], the likelihood is significantly lower than with other patterns. Therefore, anti-DFS70 antibodies represent an important biomarker that can aid in the interpretation of positive ANA patients and, therefore, should be included in test algorithms for ANA testing. The optimal test algorithm might be laboratory specific being dependent on referral patterns for ANA testing.

Abbreviations

ANA: Antinuclear antibody
DFS: Dense fine speckled
CIA: Chemiluminescent immunoassay
IBD: Inflammatory bowel disease
IIF: Indirect immunofluorescence
LEDGF: Lens derived epithelium growth factor
HI: Healthy individuals
LR: Likelihood ratio
RA: Rheumatoid arthritis
SARD: Systemic autoimmune rheumatic diseases
SSc: Systemic sclerosis
SLE: Systemic lupus erythematosus
SLEDAI: SLE disease activity index
SSc: Systemic sclerosis.

Conflict of Interests

M. Mahler and R. Albesa are employed at INOVA diagnostics selling autoantibody assays. M. J. Fritzler and L. Musset are paid consultants of INOVA. M. J. Fritzler is consultant of ImmunoConcepts and is the Director of Mitogen Advanced Diagnostics Laboratory. M. Miyara receives travel support from INOVA. The other authors have no conflict of interests.

Authors' Contribution

L. Musset and M. Mahler contributed equally to this work.

Acknowledgments

The authors thank Andrea Seaman, Eric Tena (both INOVA Diagnostics), and Laurent Dufat for technical assistance and Silvia Casas for support in the organization of the study.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2013, Article ID 297678, 7 pages
http://dx.doi.org/10.1155/2013/297678

Research Article

Detection and Analysis of Autoantigens Targeted by Autoantibodies in Immunorelated Pancytopenia

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Received 24 October 2012; Revised 19 December 2012; Accepted 23 December 2012

Academic Editor: Dimitrios P. Bogdanos

Previously, we described a group of patients with hemocytopenia who did not conform to diagnostic criteria of known hematological and nonhematological diseases. Most patients responded well to adrenocortical hormone and/or high-dose intravenous immunoglobulin treatment, indicating that cytopenia might be mediated by autoantibodies. Autoantibodies were detected on the membrane of various bone marrow (BM) hemopoietic cells by bone marrow mononuclear-cell-Coombs test or flow cytometric analysis. Thus, the hemocytopenia was termed "Immunorelated Pancytopenia" (IRP) to distinguish it from other pancytopenias. Autoantigens in IRP were investigated by membrane protein extraction from BM hemopoietic cells and BM supernatant from IRP patients. Autoantibody IgG was detected in the BM supernatant of 75% of patients (15/20), which was significantly higher than that in aplastic anemia, myelodysplastic syndrome, or autoimmune hemolytic anemia patients (0%) and normal healthy controls (0%) ($P < 0.01$). Autoantigens had approximate molecular weights of 25, 30, 47.5, 60, 65, 70, and 80 kDa, some of which were further identified by mass fingerprinting. This study identified that a G-protein-coupled receptor 156 variant and chain P, a crystal structure of the cytoplasmic domain of human erythrocyte band-3 protein, were autoantigens in IRP. Further studies are needed to confirm the antigenicity of these autoantigens.

1. Introduction

Over the last decade, we have described a group of patients with hemocytopenia who did not conform to the diagnostic criteria of known hematological and nonhematological diseases, such as aplastic anemia (AA), myelodysplastic syndrome (MDS), paroxysmal nocturnal hemoglobinuria (PNH), megaloblastic anemia (MA), iron deficiency anemia (IDA), anemia of chronic disease (ACD), autoimmune hemolytic anemia (AIHA), or congenital anemia. Anemia, bleeding, and infection are the main manifestations of this hemocytopenia. Most patients had a good response to adrenocortical hormone (ACH) and/or high-dose intravenous immunoglobulin (IVIG) treatment, which indicated that the cytopenia might be mediated by autoantibodies [1–3]. We detected autoantibodies on the membrane of BM hemopoietic cells by bone marrow mononuclear-cell-(BMMNC-) Coombs test [4–6] or flow cytometric analysis [7]. The positive rate was 67% and 86%, respectively [7], indicating that this was an autoimmune disease. We termed this abnormality "Immunorelated Pancytopenia" (IRP). An in-depth study of its pathogenic mechanisms [2, 3] indicated that autoantibodies could inhibit or destroy hemopoietic cells by activating macrophages [8] or complement factors [9] and blocking functional antigens [10]. The production of autoantibodies in this disease may be due to abnormal numbers and altfered functions of B lymphocytes [11], caused by inhibition of regulatory T cells (Treg) [12], T helper (Th) 1, and activated Th2 [13] and Th17 [14] cells. Differentiating IRP from other diseases was beneficial not only for the treatment of these patients but also for treating other bone marrow abnormalities, such as AA, MDS, and AIHA [15, 16].

However, the identity of autoantigens in IRP is not known. The identification of autoantigens in autoimmune diseases, such as systemic lupus erythematosus [17], severe asthma [18], and allergic rhinitis [19], helped develop

targeted therapies. Our study tried to identify IRP-related autoantigens on the membrane of bone marrow (BM) cells by proteomics.

2. Materials and Methods

2.1. Patients. All patients were diagnosed as IRP according to the following features [1]: (1) hemocytopenia or pancytopenia with normal or higher percentages of reticulocyte and/or neutrophils; (2) BM: normal or higher percentage of erythroid cells, erythroblastic islands are easy to see; (3) exclusion of other primary and second hemocytopenia disorders; (4) BMMNC-Coombs test (+) or/and autoantibodies on the membrane of BM hemopoietic cells (+) tested by flow cytometry (FCM). Twenty untreated patients (11 males, nine females) were enrolled in our study with a median age of 29 years (range 14–43 years). All patients were inpatients of Tianjin Medical University General Hospital from February to July 2009. Ten mL samples were taken from their ilia. Thirteen controls (5 AA, 5 MDS, and 3 AIHA) were inpatients of our hospital and were diagnosed according to the international criteria of AA, MDS, and AIHA. Ten normal controls from thoracic surgery were also enrolled in this study. BM samples were taken from their postoperative discarded ribs.

2.2. BMMNC-Coombs Test. BM mononuclear cells rather than peripheral red cells were used to perform the Coombs test [20]. Fresh heparinized BM samples (5 mL) were diluted with phosphate buffered saline (PBS) in a 1 : 1 proportion, layered over the lymphocyte separation medium and centrifuged at a low speed for 20 min. During centrifugation, differential migration resulted in the formation of several cell layers. Because of their density, lymphocytes and other mononuclear cells were found at the plasma-lymphocyte separation medium interface. Cells were recovered by aspirating the layer and washing with PBS three times. Cell suspensions of 4-5 $\times$ 10^6/mL in PBS were prepared for testing. Anti-human serums (IgG, IgA, IgM, and C3) were diluted as a working solution. Working solutions were mixed with cell suspensions in a 1 : 1 proportion, and cultured at 37°C for 30 min. Finally, we observed agglutination by microscopy.

2.3. FCM Analysis. Fresh heparinized BM samples (400 μL) were washed with PBS three times, then separated into four tubes and stained with either mouse IgG1-FITC, mouse IgG1-PE, mouse IgG1-APC as negative control, or stained separately with CD15-FITC, GlyCoA-FITC, and CD34-FITC (BD Pharmingen, San Diego, CA, USA). Anti-human IgG-PE and anti-human IgM-APC (BD PharMingen) were added to each tube. After incubation in the dark for 30 min at 4°C, cells were incubated with 2 mL erythrocyte lytic solution (BD PharMingen) for 10 min at room temperature and washed three times with PBS. Finally, at least 30 000–100 000 cells were acquired and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

2.4. Membrane Protein Extraction. Hemopoietic cells were separated from BM of patients with IRP and healthy volunteers by erythrocyte lytic solution. Membrane proteins were extracted by Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Thermo Scientific Pierce, Rockford, IL, USA). Protein concentration was determined using BCA Protein Assay Kit (Thermo Scientific Pierce). BM supernatant was stored at −80°C until use.

2.5. SDS-PAGE and Western Blot. Solubilized proteins were separated using 10% sodium dodecyl sulfate-(SDS-) polyacrylamide gel electrophoresis (PAGE). IgG autoantibodies to BM hemopoietic cells were detected in BM supernatant by western blot. After electrophoresis, the SDS-PAGE gels were stained with Coomassie Brilliant Blue (CBB) or electrotransferred to polyvinylidene difluoride membranes (PVDF) (Bio-Rad Laboratories, Hercules, CA, USA). For PVDF membranes, blocking was performed using 5% nonfat milk at 4°C overnight. Patients' BM supernatant at 1/100 dilution with 5% nonfat milk was applied for 2 h at room temperature. After washing with Tris Buffered Saline, with Tween-20 (TBST), goat anti-human IgG peroxidase conjugate diluted 1/5000 in 5% non-fat milk was applied for 1 h, and electrochemiluminescence (ECL) reagent was used.

2.6. In-Gel Digestion, Mass Determination, and Mass Spectrometry. Protein bands on the gel were stained with CBB, which corresponded to the positive bands on the WB membranes. The recovered gel fragments were digested and analyzed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) at the Academy of Military Medical Sciences. A list of the peptide masses was compiled using the Mascot software program, in which the NCBI protein databases were searched.

2.7. Statistical Analysis. Statistical analysis was performed with SPSS 16.0 statistical software and tested by TANOVA.

3. Results

3.1. Detection of Autoantibodies to BM Hemopoietic Cells. We first tried to understand the overall profiles of autoimmunity in patients with BMMNC-Coombs test positive hemocytopenia. To achieve this, we detected autoantibodies/autoantigens by means of SDS-PAGE and subsequent WB using 43 BM supernatant samples from 20 patients with IRP (Table 1, Figure 1), five patients with AA, five patients with MDS, three patients with AIHA, and 10 normal controls. Proteins extracted from BM hemopoietic cells were separated by SDS-PAGE. Next, the separated proteins were transferred onto membranes and then were reacted with each of the 43 BM supernatant samples by western blot. Autoantibody IgG targeting BM cells were observed in BM supernatant of IRP patients at a positive rate of 75% (15/20), which was significantly higher than for AA, MDS, or AIHA patients (0%) and normal controls (0%) ($P < 0.01$).

Table 1: Profiles of patients with IRP enrolled in this study.

Pt. no.	Age	Sex	BMMNC-Coombs test	FCM analysis
1	42	F	—	CD15+ cells IgG(+)
2	26	M	IgG(+)	CD15+ cells IgG(+), GlyCoA+ cells IgM(+), CD34+ cells IgM(+)
3	34	F	IgM(+)	GlyCoA+ cells IgM(+)
4	40	F	C3(+)	CD34+ cells IgG(+), IgM(+)
5	32	F	IgG(+), C3(+)	CD15+ cells IgG(+), CD34+ cells IgG(+), IgM(+)
6	30	M	—	GlyCoA+ cells IgM(+)
7	23	M	IgM(+)	GlyCoA+ cells IgG(+), IgM(+)
8	27	M	—	CD15+ cells IgM(+)
9	22	M	—	CD34+ cells IgM(+)
10	25	F	—	CD34+ cells IgG(+), IgM(+)
11	14	M	IgM(+)	GlyCoA+ cells IgM(+)
12	14	M	IgM(+)	GlyCoA+ cells IgG(+), IgM(+), CD34+ cells IgM(+)
13	19	M	IgM(+)	CD15+ cells IgM(+)
14	36	F	—	CD34+ cells IgG(+), IgM(+)
15	30	F	IgM(+)	GlyCoA+ cells IgM(+)
16	29	M	IgM(+)	CD15+ cells IgM(+), CD34+ cells IgG(+), IgM(+)
17	30	M	—	CD34+ cells IgM(+)
18	26	M	IgM(+)	CD15+ cells IgM(+), GlyCoA+ cells IgM(+)
19	29	M	—	CD34+ cells IgG(+), IgM(+)
20	43	F	IgG(+)	CD15+ cells IgG(+), IgM(+)

Pt. no.: patient number; Ig: immunoglobulin; FCM: flow cytometry; GlyCoA; glycine coenzyme A; BMMNC: bone marrow mononuclear cell; F: female; M: male.

3.2. Identification of 30 kd BM Hemopoietic Cells Autoantigens. Seven major protein bands reactive to at least one of the samples from the patients with IRP were observed. The approximate molecular weights (MWs) of the seven major protein bands were 25, 30, 47.5, 60, 65, 70, and 80 kDa (Figure 2), and the positive rates were 30% (6/20), 20% (4/20), 15% (3/20), 10% (2/20), 15% (3/20), 10% (2/20), and 5% (1/20), respectively (Table 2). The detection of multiple autoantigens indicated that autoimmunity directed toward various self-proteins may be a common phenomenon in these patients.

We next tried to identify the two major protein bands by LC-MS/MS. Specifically, peptides extracted from the corresponding gel bands after digestion with trypsin were subjected to mass measurement and computer searching. We successfully identified the 30 kDa protein band as two autoantigens, a G-protein-coupled receptor 156 variant, and chain P, crystal structure of the cytoplasmic domain of human erythrocyte band-3 protein (Figure 2).

4. Discussion

IRP is a disease independent from other pancytopenic diseases described in recent years. Infection, anaphylaxis, and pregnancy are suspected risk factors associated with this patient group. A number of patients (72.5%, 145/200) had pancytopenia. The median percentage of reticulocytes was 1.8%. More than half the patients had hyper-BM cellularity with a higher percentage of nucleated erythroid cells in the sternum [21]. Autoantibodies on BM hemopoietic cells were detected by BMMNC-Coombs test and/or flow cytometry. An in-depth study of its pathogenic mechanism showed that IRP is an autoimmune disease where autoantibodies target BM hemopoietic cells. A study in SLE by Fu et al. [22] showed that more than 50% of patients with SLE were observed by BMMNC-Coombs test positive, approximately 60% of which had blood cytopenia. These results indicate that IRP was an autoimmune disease, similar to SLE, but where the target organ was BM.

However, the autoantigens in IRP are unknown. Therefore, we used a proteomic approach, a combination of SDS-PAGE and subsequent mass spectrometry [23]. By screening SDS-PAGE-WB using BM supernatant samples from patients with IRP, AA, MDS, AIHA, and normal controls, we found seven major protein bands in IRP but not in AA, MDS, AIHA, and normal controls. These results also indicated that IRP was different from other known hematological diseases, such as AA, MDS, and AIHA. We often found 3-4 protein bands in individual patients, indicating that autoimmunity is a common phenomenon.

Finally, we identified two autoantigens, G-protein-coupled receptor 156 variant and chain P, crystal structure of the cytoplasmic domain of human erythrocyte band-3 protein by LC-MS/MS in IRP [24]. G-protein-coupled receptor 156 variant belongs to the family of G-protein-coupled receptors (GPCRs) [25–27]. Currently, the structure and function of G-protein-coupled receptor 156 are unknown. However, mutations of GPCRs can cause various kinds of diseases [28]. The activation or suppression of GPCRs are important in nerve conduction and are associated with neurogenic disease, heart disease, metabolic disturbance, and cancer. Furthermore, some types of GPCRs

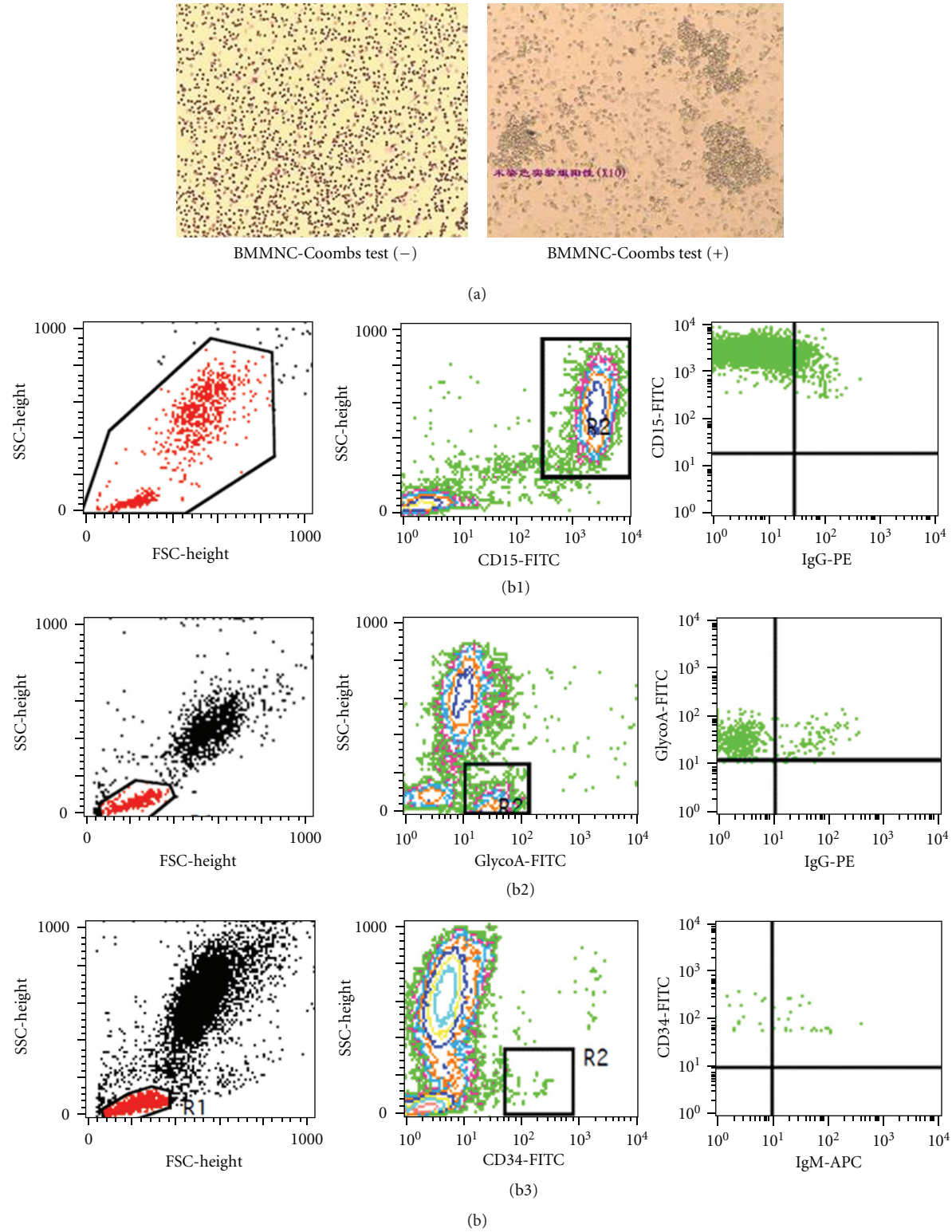

Figure 1: Autoantibodies were detected in IRP patients. (a) BMMNC-Coombs test, (b) flow cytometry analysis. (b1) autoantibodies were detected on granulocytes. (b2) autoantibodies were detected on nucleated erythrocytes. (b3) autoantibodies were detected on stem cells.

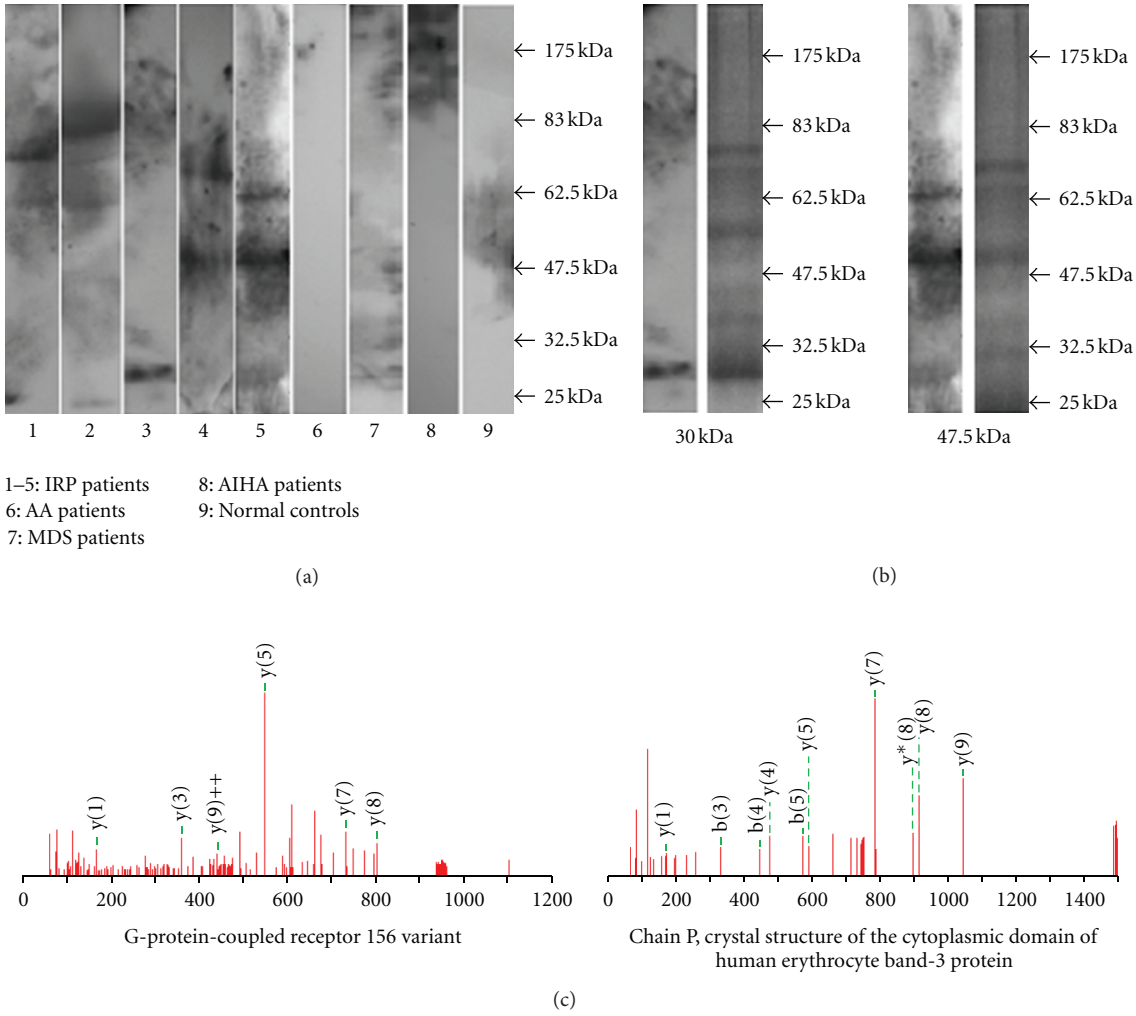

Figure 2: Autoantigens targeted by IgG autoantibody isolated and identified by SDS-PAGE, western blot, and LC-MS/MS. (a) Autoantibody IgG in patients reacted with several autoantigens with approximate MWs of 25, 30, 47.5, 60, 65, 70, and 80 kDa. (b) 30 and 47.5 kDa were identified by LC-MS/MS (L: WB, R: gels stained with CCB). (c) Results of MS.

can inhibit GTPase and, thus, exhibit negative intrinsic activity, and its affinity for receptors is increased following uncoupling from G proteins [29]. Therefore, GPCR 156 variant may play a role in the suppression of proliferation and differentiation of BM hemopoietic cells in BMMNC-Coombs test positive hemocytopenia by preventing binding with ligands due to structural changes, which could inhibit G protein activity. Alternatively, it could induce continuous signal transduction in the absence of ligand binding due to structural changes. The mechanism of GPCR156 variant in this disease requires further study.

Band-3 protein is important for the membrane stability of red blood cells. Abnormalities of band-3 protein are associated with disease, such as hereditary spherocytosis and hereditary erythroblastic multinuclearity with a positive acidified serum test (HAMPAS) [30]. In our study, autoantibodies should not bind to the cytoplasmic domain of normal nucleated erythrocytes, but in some pathological conditions (oxidative damage, acute/chronic virus infection, and increased calcium concentration), the membrane of cells can be damaged, causing deformity. Changes in the membrane of cardiomyocytes in *Helicobacter pylori* (HP) infection have been observed [31]. Guo et al. [32] reported that anti-HP antibodies could have an effect on chain S, the crystal structure of the cytoplasmic domain of human erythrocyte band-3 protein, which causes damage to red blood cells in gastrointestinal ulcers.

5. Conclusions

In conclusion, IRP is a bone marrow abnormality different from other known hemopoietic diseases. Here, we applied a proteomic approach to survey autoantigens and identified two autoantigens. Further studies to confirm the antigenicity of these autoantigens, including the use of a recombinant protein and measuring the prevalence of autoantibodies in IRP, are required.

Table 2: Protein bands reactive with BM supernatant samples from IRP patients.

Pt. no.	Protein bands						
	25 kDa	30 kDa	47.5 kDa	60 kDa	62.5 kDa	70 kDa	80 kDa
1						+	
2				+		+	+
3				+			
4		+					
5			+		+		
6	+						
7			+		+		
8	+	+					
9	+						
10	+						
11							
12		+					
13	+						
14							
15							
16			+		+		
17	+						
18		+					
19							
20							

Pt. no.: patient number.

Authors' Contribution

H. Liu and R. Fu have contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grants nos. 30971285, 30971286, 30670886, and 30470749), Research Fund for the Doctoral Program of Higher Education of China (200800620004), Tianjin Municipal Natural Science Foundation (08JCYBJC07800, 09JCYBJC11200), Key Projects in the National Science & Technology Pillar Program during the Eleventh Five-Year Plan Period (2008BAI61B02), Tianjin Science and Technology support Key Project Plan (07ZCGYSF00600), and the Tianjin High School Science and Technology Foundation (200317).

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2013, Article ID 637513, 6 pages
http://dx.doi.org/10.1155/2013/637513

Research Article

Interleukin-15 (IL-15) and Anti-C1q Antibodies as Serum Biomarkers for Ectopic Pregnancy and Missed Abortion

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Received 3 November 2012; Accepted 25 November 2012

Academic Editor: Dimitrios P. Bogdanos

Given the present lack of clinically useful tests for the accurate diagnosis of ectopic pregnancy (EP), there is a need to select out those immunological factors measured in the maternal serum, as potential biomarkers. Our assumption was that C1q/anti-C1q antibody complexes and serum levels of interleukin-15 (IL-15) may play a role in differentiating abortions (MAs) and EPs and normal pregnancies. We assessed whether their measurement could set the diagnosis in a case control study at 6–8 weeks consisting of 60 women with failed early pregnancy (30 EPs, 30 MAs) and 33 women with intrauterine pregnancies. Normal pregnancies contain anti-C1q antibodies more frequently compared to women with failed pregnancies, the lowest levels being found in EPs, but this lacked statistical significance and anti-C1q could not serve as a marker. However EP pregnancies had elevated IL-15 levels that could statistically significantly differentiate them from MAs and IUPs. Furthermore, when assessing IL-15 for the clinically important differentiation between IUP and EP, we found at a cut-off of 16 pg/mL a negative predictive value of 99 with a sensitivity for diagnosing an EP of 92%. According to these results, serum IL-15 is a promising marker differentiating an MA from an EP.

1. Introduction

Unless a normal early intrauterine pregnancy (IUP) is visible by ultrasound, diagnosis can be a challenge [1–3]. When these patients present with pain and/or vaginal bleeding, the differential diagnosis between IUP and missed abortion (MA) or ectopic pregnancy (EP) is very difficult [4, 5]. The fear of intervening in the case of a desired pregnancy without certainty of diagnosis must be carefully weighed against the risk of misdiagnosing a missed abortion (MA) instead of an EP, due to the inherent danger to the mothers suffering an EP of tubal rupture and intraperitoneal haemorrhage.

Pregnancy is a natural example of an immune reaction occurring for a determined time period in the organism which opposes the rules of graft rejection [6]. The semi- or allogeneic fetal components in a normal pregnancy growing in the privileged site of uterus, not only escape maternal immune attack but also are supported by the maternal immune system [6].

Given the present lack of a clinically useful test for the accurate diagnosis of EP, there is a need to select out those immunological factors measured in the maternal serum, that are involved in the potentially disturbed maternal immune system's answer to the semiallogeneic conceptus in failed

pregnancy cases and display the most promise to differentiate abortion (MA) and EP as potential biomarkers [3].

During implantation and early pregnancy, the immunological processes that take place within the uterus are to a great extent modulated by pro- and anti-inflammatory cytokines and their altered expression in the maternal serum may play a role in early pregnancy failure [7].

Successful pregnancy is considered a T helper 1 (Th1)-Th2 cooperation phenomenon, with a predominantly Th2-type lymphocyte response and specific cytokine production [8]. Th2 responses favour a cytokine milieu that promotes the induction of autoantibodies and several studies have attempted to link pregnancy failures and/or neonatal diseases with the presence of specific autoantibodies [9]. There has been an interest in the role played by anti-C1q antibodies, as these autoantibodies have been studied as prognosticators of disease flares and pregnancy outcomes in immune-mediated diseases such as systemic lupus erythematosus (SLE) [10] but not in women with missed abortions or ectopic pregnancies.

Our assumption is that the formation of C1q/anti-C1q antibody complexes may also play a role in pregnancy failures such as MAs and EPs. We based our hypothesis on published reports (reviewed by Girardi et al. [9]) underlying the pivotal role played by C1q in promoting trophoblast invasion of deciduas, a crucial step in normal placental development. Thus, work on experimental models and C1q deficient mice has elegantly shown lack of C1q is characterized by poor trophoblast invasion and pregnancy failure [11].

As anti-C1q antibodies have not been tested as autoantibody markers in MA and EP, we assessed their presence and attempted to relate their appearance with the serum levels of interleukin-15 (IL-15). We have focused on the study of IL-15 as this cytokine is expressed by human placental tissue culture, its serum levels correlate with the duration of the pregnancy and it is maximally expressed during the implantation period in the deciduas [8, 12]. Notably, recurrent abortion cases are characterized by an upregulation of IL-15 expression in trophoblasts [13], suggesting that IL-15 can be a marker for pregnancy failure.

At 6–8 weeks gestational age the clinical differential diagnosis of a failed pregnancy is difficult due to uncertain dates of the last menstrual period or irregular cycles. We therefore set out to assess whether IL-15 serum measurement at 6–8 weeks could contribute to the differential diagnosis between failed pregnancies, whether EP or missed abortions (MA), and healthy intrauterine pregnancies (IUP). We also assessed the simultaneous presence of anti-C1q antibodies, as this could be a potential marker of the underlying immunopathological processes.

2. Materials and Methods

2.1. Subjects. We performed a case control study consisting of 60 patients with failed early pregnancy presenting with mild abdominal pain or vaginal bleeding between 6–8 weeks of gestation, who were admitted to our tertiary centre between January 2009 and October 2010. Among the 60 cases included, 30 women had a ruptured EP, while 30 had MA. Serum samples were collected at the initial visit before treatment. If the clinician was unable to make a diagnosis on this first visit, the patient was admitted and followed up until a diagnosis of a viable intrauterine pregnancy or MA or EP was confirmed. All diagnoses were histologically confirmed. Serum beta HCG and IL-15 were measured in all 60 patients and in a group of 33 women with IUP between 6 and 8 weeks of gestation that served as a control group. EP, MA, and IUP women did not differ in terms of ethnicity (all Caucasian), maternal age (IUP: median 27 years (range 18–39); MA: median 35 years (range 21–45); EP median 32 years (range 26–44)), BMI (IUP: median 24 (range 19.9–31.2); MA: median 25.6 (range 20.7–35); EP: median 26.4 (range 21–34.5)), and smoking history.

The experimental testing complied with the principles laid down in the Declaration of Helsinki. All participating individuals gave informed consent to the work. The project was approved by the Larissa University Hospital Research Ethics Committee.

2.2. Beta HCG Measurement. Serum concentrations of beta human chorionic gonadotropin (HCG-β) were measured by an electrochemiluminescence immunoassay (ECLIA) intended for use on the automated analyzer Modular Analytics E170 (Roche Diagnostics GmbH, Mannheim, Germany). The results were expressed as mIU/mL and the lower limit of detection was <0.1 mIU/mL.

2.3. IL-15 ELISA Measurements. Serum samples were collected at the initial visit before treatment. All samples were processed by centrifuge (1,000 g for 15 minutes), and the supernatants were stored at −80°C until assayed. Serum concentrations of IL-15 were determined by quantitative sandwich ELISA (Quantikine human IL-15, R&D Systems, Minneapolis, MN, USA) according to the instructions of the manufacturer.: Quantikine human IL-15 is a quantitative sandwich enzyme immunoassay technique based on a monoclonal antibody specific for IL-15 which has been precoated onto a microplate. Testing steps have been followed in according to the protocols provided by the manufacturer. A standard curve based on the readings of 7 standards corresponding to 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL, 7.8 pg/mL, and 3.9 pg/mL is used to calculate the test serum concentrations of IL-15. The 250 pg/mL standard serves as the high standard and the calibrator diluent serves as the zero standard (0 pg/mL).

2.4. Anti-C1q Antibody Testing. IgG class anti-C1q antibodies have been tested by ELISA purchased from INOVA Diagnostics (San Diego, CA, USA). Testing has been carried out according to the manufacturer's instructions (http://www.inovadx.com/). Based on the provided leaflet, samples can be classified as negative (<20 AU/mL), weak positive (20–39 AU/mL, moderate positive (40–80 AU/mL), or strong positive (>80 AU/mL).

2.5. Statistical Analysis. Skewed distributed variables are presented as median and interquartile range (IQR) (Table 1).

Table 1: Anti-C1q antibody concentrations in 60 women with failed pregnancies, including 30 women with ectopic pregnancy (EP) and missed abortion (MA) and 33 women with intrauterine pregnancy (IUP). The nonparametric Kruskal-Wallis test was used to identify differences among EP, MA, and viable IUP. To perform pairwise comparisons between groups Mann-Whitney test was conducted determining as critical value for significance $P = 0.0167$ after using Bonferroni correction which determined as critical value of significance for each (Mann-Whitney) test the value 0.0167. Therefore anti-C1q antibody levels are not helpful in differentiating between the three groups since as shown in Table 1 there is no P value <0.167.

		Failed pregnancies (MA and EP)			Normal	P value
Anti-C1q	Median	30.92			38.95	**0.018**[*]
	IQR	17.89–40.79			28.95–59.01	
		Pregnancy outcome				
		IUP	MA	EP		
Anti-C1q	Median	38.95	31.97	18.82		**0.045**[**]
	IQR	28.95–59.01	23.95–39.47	16.32–40.79		

[*]Mann-Whitney test.
[**]Kruskal-Wallis test.

Analysis of variance was conducted in order to perform orthogonal contrasts (Helmert contrasts) comparing IUP versus MA and EP, as well as MA versus EP regarding IL-15. The optimal cut-off points for sensitivity and specificity were calculated by Receiver Operating Characteristics (ROC) curve analyses as the value with the maximum sensitivity plus specificity. According to the design of this study, the area under the curve (AUC) depicts the probability that the single value of IL-15 of a randomly selected patient with a normal pregnancy illustrated in Figure 1 (see below) will exceed that of a single value of a randomly selected patient with an abnormal pregnancy (EP or MA). Median values and interquartile range were calculated and sensitivity, specificity, positive predictive values (PPVs), and negative predictive values (NPVs) for EP were estimated (Table 2).

The nonparametric Kruskal-Wallis test was used to identify differences on bhcg among EP, MA, and viable IUP. To perform pairwise comparisons between groups Mann-Whitney test was conducted determining as critical value for significance $P = 0.0167$ after using Bonferroni correction.

Basic demographic characteristics such as age, BMI were compared using Kruskal-Wallis.

Spearman's rank correlation coefficient (ρ) was used to explore the relationship between beta HCG with the other measures. All statistical analyses were performed in SPSS 15 statistical software (Chicago, IL, USA). A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Diagnostic Accuracy of Anti-C1q Antibodies. Strong (>80 AU/mL) anti-C1q antibody reactivity was found in 4/30, 4/30, and 6/33 women with EP, MA, and IUP, respectively (not significant). Moderate or strong anti-C1q antibody reactivity (>40 AU/mL) was present in 10/30 women with EP, 8/30 women with MA, and 14/33 women with IUP (not significant).

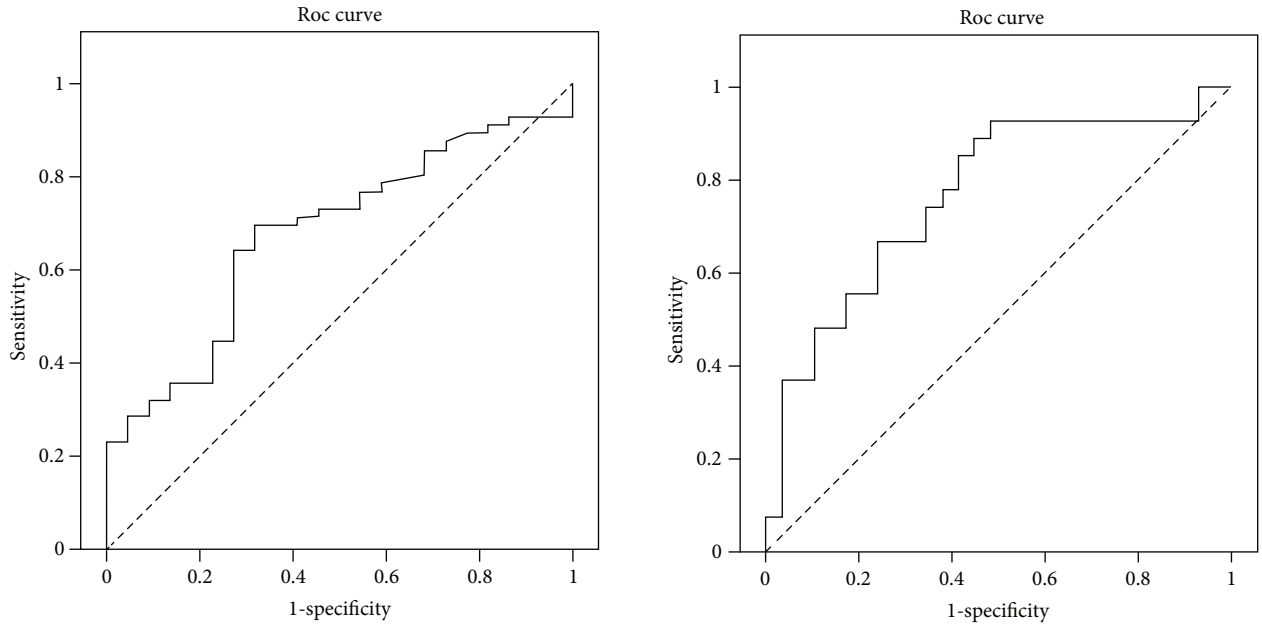
As our data are not normally distributed, the nonparametric Kruskal-Wallis test was used to identify any difference on anti-C1q among groups of pregnancy outcome. However, in order to find out which groups differ, a Mann-Whitney test was performed conducting three pairwise comparisons. Additionally, using Bonferroni correction, as critical value of significance for each (Mann-Whitney) test was determined the value 0.0167. Therefore anti-C1q antibody levels are not helpful in differentiating between the three groups since as shown in Table 1 there is no P value <0.167.

3.2. Diagnostic Accuracy of IL-15. IL-15 concentrations were significantly higher in 60 pregnancy failures (median 19.86, IQ range 16.04–26.97 pg/mL) compared to women with a viable IUP (median 15.06, IQ range 12.74–21.41 pg/mL) and this was highly significant ($P < 0.001$, Table 2).

This is mainly the result of the fact that IL-15 concentrations were significantly higher in women with EP ($n = 30$, median 24.9 pg/mL) compared to patients with IUP ($n = 33$, 15.06 pg/mL), $P < 0.001$ (Table 2). Additionally, IL-15 had the ability to discriminate an EP from MA ($P = 0.015$) (Table 2). The corresponding ROC analyses were calculated and plotted for the diagnostic accuracy of serum IL-15 concentration to discriminate between the groups (AUCs in Table 2) (Figure 1).

IL-15 values were plotted in ROC curves in order to further evaluate their diagnostic accuracy for the diagnosis of healthy IUP and for accurate discrimination of an EP from an MA. All AUCs are shown in Table 2. IL-15 showed high diagnostic accuracy for the discrimination of a viable IUP from an EP with an area under the curve (AUC) 0.818, (Table 2, Figure 1) and at the threshold of 16.1 pg/mL, had a sensitivity of 92% and a specificity of 68%, and a clinically important negative predictive value (NPV) of 0.999 for diagnosing a normal from an EP. According to this, if the IL-15 concentration is less than 16 pg/mL it is highly unlikely that the pregnant woman suffers from an EP.

3.3. IL-15 Maternal Serum Concentration Relationship to Beta HCG. Since IL-15 was showing to be a promising biomarker we further analysed its serum concentration relationship with bHCG. IUPs had a median bHCG concentration of 59,668 mIU/mL (40,156–87,906 mIU/mL), while MAs had a median of 3000 mIU/mL (1447–5500 mIU/mL) and EPs a



Area	Standard error	Significance	95% Cl lower	95% Cl upper
0.677	0.064	0.015	0.552	0.803

(a) Normal versus failed pregnancy

Area	Standard error	Significance	95% Cl lower	95% Cl upper
0.765	0.065	0.001	0.638	0.892

(b) Missed abortion versus ectopic pregnancy

Figure 1: IL-15 ROC curves of normal versus failed pregnancy (a) and missed abortions versus ectopic pregnancies (b).

Table 2: IL-15 concentrations in 60 women with failed pregnancies, including 30 women with ectopic pregnancy (EP) and missed abortion (MA) and 33 women with intrauterine pregnancy (IUP). Median, interquartile range (IR) in pg/mL, and the Mann-Whitney P value are presented. Additionally sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPP) were calculated and IL-15 values were plotted in ROC curves in order to further evaluate their diagnostic accuracy for the diagnosis of healthy IUP and for accurate discrimination of an EP from a MA. All AUCs are shown.

IL-15 Serum levels	Median (interquartile range) Mann Whitney P	Sensitivity	Specificity	Cut-off point	Direction	AUC	P value (ROC curve)	PPV	NPV
Failed versus normal	19.86 (16.04–26.97) 15.06 (12.74–21.41) $P < 0.001$	0.750	0.682	16.1	Upper	0.725	0.002	0.310	0.935
Abortion versus normal	16.63 (14.56–21.37) 15.06 (12.74–21.41) $P < 0.001$	0.857	0.364	14.0	Upper	0.605	0.207	0.192	0.935
Ectopic versus normal	24.9 (18.59–29.81) 15.06 (12.74–21.41) $P < 0.001$	0.917	0.682	16.1	Upper	0.818	<0.001	0.028	0.999
Ectopic versus abortion	24.9 (18.59–29.81) 16.63 (14.56–21.37) $P = 0.015$	0.861	0.571	16.9	Upper	0.753	0.001	0.118	0.984

median of 1828 mIU/mL with an IQR 1147–2790 mIU/mL (Kruskal-Wallis test, $P < 0.001$).

In order to further explore pairwise comparisons between groups, we conducted Mann-Whitney test using the Bonferroni correction. It was identified that IUPs have significantly higher values of beta HCG compared to MAs and then to EPs, ($P < 0.001$). Between MAs and EPs there was no statistically significant difference ($P = 0.115$).

Spearman's rank correlation coefficient (ρ) between beta HCG and IL-15 in IUPs was 0.033 ($P = 0.884$), in MAs −0.141 ($P = 0.521$) and in EP 0.115 ($P = 0.585$) demonstrating that there is no correlation between IL-15 and bHCG values in all three study groups.

4. Discussion

Currently, there is no stand-alone diagnostic biomarker for tubal ectopic pregnancy that has been adequately tested and yields satisfactory results. The clinical end-point of this study was the potential identification of EP cases by a single serum

measurement of IL-15. In a similar context, we tested for anti-C1q antibodies, as a potential marker of pregnancy failure.

Much to our surprise, we have found that serum samples from women with normal pregnancies contain IgG anti-C1q antibodies more frequently compared to those with failed pregnancies, the lowest level of detectable antibodies being found in women with EP. In our study, 18% (6/33) of women with IUP have shown strong IgG anti-C1q antibody reactivity. This percentage is comparable to that of 20% recently reported in a study testing 30 women with normal pregnancies [14]. Antibodies to C1q are found in SLE (including pregnant women) and a variety of other autoimmune and nonautoimmune conditions, including healthy individuals, and their role in the induction of pathological processes remains elusive [10, 15–20]. Stoyanova et al. [14] have suggested that the presence of anti-C1q antibodies in women with normal pregnancies could be associated with their physiological and pregnancy state or their current hormonal status. This hypothesis needs to be explored further, as our data argue in support of the notion that failed pregnancies and in particular those with EPs are immunologically privileged in respect to the loss of immunological tolerance to C1q.

Nevertheless, the great majority of women, irrespectively of whether they have failed or normal pregnancies, lack autoantibodies against C1q and this needs to be taken into account when exploring the immunobiology of pregnancy in relation to humoral immunity specific for C1q.

Biomarkers which reflect the viability of a pregnancy may be higher in normal IUPs but may not differentiate an abnormal pregnancy in the uterus (miscarriage) versus an abnormal pregnancy in the fallopian tube (EP). Markers reflecting the location of the pregnancy, rather than viability, may therefore be able to differentiate between the two types of nonviable conceptuses: a miscarriage and an EP.

Our analysis has shown that EP pregnancies had increased IL-15 levels that could statistically significantly differentiate them from MAs and IUPs. Furthermore, when assessing IL-15 for the clinically important differentiation between IUP and EP, we found at a cut-off of 16 pg/mL, a negative predictive value of 99 (AUC of 0.818) (Table 2) with a sensitivity for diagnosing an EP of 92%. According to these results, serum IL-15 is a marker that can differentiate an MA from an EP (AUC of 0.753). This comes as no surprise as Toth et al. [13] reported that IL-15 expression is upregulated in placental tissue of disturbed human first trimester pregnancy and trophoblast cells were detected as a main source for IL-15 in women with recurrent miscarriages. The trophoblast invasion in ectopic pregnancy is different from normal pregnancies [21, 22] and this may explain differences related to IL-15 tissue expression and circulating levels of this cytokine [13]. Trophoblast infiltrating the tube or the peripheral NK cells can be the source of the increased levels of IL-15 in EPs compared to IUPs, but this needs to be explored further. This is in accordance to our finding of increased IL- 15 levels in EP which due to its protective effect might explain the surviving of trophoblasts while penetrating the tubal wall. However it must be emphasized that the aim of the present study was not to explore whether increase in IL-15 directly or indirectly relates to the development of EP and MA. Thus, it is uncertain whether the high levels of IL-15 in pregnancy failure are the consequence of the pathological processes that take place or whether their increase participates in the induction of EP, with increased serum levels simply reflecting the involvement of IL-15 in the pathogenesis of the disease. The pathophysiological significance of our observations can only be studied through serial measurements and further tissue expression assessment of these markers in a larger prospective study.

Our group performed a number of studies reporting that maternal serum angiogenic factors vascular endothelial growth factor receptor [VEGF-R1 (flt-1)], VEGF, angiopoetin (ANG-)1 and ANG-2 are potential markers of failed pregnancies—MA and EP, as their levels are significantly decreased in early pregnancy failure (MA or EP) at 6–8 weeks of gestation compared to those found in healthy intrauterine pregnancies [3, 23, 24].

Therefore IL-15 could be an ideal additional biomarker to the already reported angiogenic factors in a multimarker diagnostic kit possibly reflecting different pathogenic mechanisms of pregnancy failure. In our series of failed pregnancies the IL-15 values did not correlate with beta HCG values raising the expectation that these parameters could be HCG-independent biomarkers, which could supplement transvaginal ultrasound and other biomarkers in a future multimarker algorithm.

Of relevance, the median beta HCG value in our EPs was at 1828 mIU/mL, which is a level with a known difficulty in identifying a gestational sac by ultrasound that would exclude an EP.

The possible clinical implications of these findings are that women with threatened abortions and no visible gestational sac could be scheduled for regular follow-up antenatal visits and repeat ultrasounds if the IL-15 level is lower than 16 pg/mL as it has an excellent NPV for EP. Furthermore it could be argued that these women may not need to be admitted as possible pregnancy failures with obvious cost benefits.

Our results on IL-15 must be tested in a longitudinal or retrospective cohort study and validated in a larger prospective study to determine its potential clinical value, ideally in a prospective multicenter study. We hope our findings may contribute to the research that is underway to both identify novel biomarkers and combine new and existing markers into a multiple marker test with the goal of accurately identifying ectopic pregnancies.

Abbreviations

AUC: Area under the curve
EP: Ectopic pregnancy
IL-15: Interleukin 15
IUP: Intrauterine pregnancy
MA: Missed abortion.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2013, Article ID 453058, 10 pages
http://dx.doi.org/10.1155/2013/453058

Review Article

An Innovative Method to Identify Autoantigens Expressed on the Endothelial Cell Surface: Serological Identification System for Autoantigens Using a Retroviral Vector and Flow Cytometry (SARF)

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Received 21 September 2012; Revised 20 November 2012; Accepted 21 November 2012

Academic Editor: Xavier Bossuyt

Autoantibodies against integral membrane proteins are usually pathogenic. Although anti-endothelial cell antibodies (AECAs) are considered to be critical, especially for vascular lesions in collagen diseases, most molecules identified as autoantigens for AECAs are localized within the cell and not expressed on the cell surface. For identification of autoantigens, proteomics and expression library analyses have been performed for many years with some success. To specifically target cell-surface molecules in identification of autoantigens, we constructed a serological identification system for autoantigens using a retroviral vector and flow cytometry (SARF). Here, we present an overview of recent research in AECAs and their target molecules and discuss the principle and the application of SARF. Using SARF, we successfully identified three different membrane proteins: fibronectin leucine-rich transmembrane protein 2 (FLRT2) from patients with systemic lupus erythematosus (SLE), intercellular adhesion molecule 1 (ICAM-1) from a patient with rheumatoid arthritis, and Pk (Gb3/CD77) from an SLE patient with hemolytic anemia, as targets for AECAs. SARF is useful for specific identification of autoantigens expressed on the cell surface, and identification of such interactions of the cell-surface autoantigens and pathogenic autoantibodies may enable the development of more specific intervention strategies in autoimmune diseases.

1. Introduction

Inappropriate humoral and cellular immune responses mediate the tissue damage in autoimmune diseases, and the outcome of an autoimmune disease is influenced mainly by the tissue distribution of target self antigens [1]. The pathogenesis of most autoimmune diseases is highly complex and involves multiple cellular and humoral pathways. One part of the humoral arm of the immune assault is caused by autoantibodies, and the mechanisms of autoimmune damage mediated by many autoantibodies have been studied [2]. Clinically, specific autoantibodies are critical for the diagnosis, classification, and monitoring of autoimmune diseases [2].

Autoantibodies cause damage through a number of mechanisms, including the formation of immune complexes, cytolysis or phagocytosis of target cells, and interference with cellular physiology [3]. The cellular localization of the target antigen is believed to play a critical role in the pathogenetic potential of autoantibodies [4]. Intracellular proteins are preferential targets of autoantibodies in autoimmune diseases, but many questions remain unanswered regarding how autoantibodies against intracellular proteins play pathogenic roles. In contrast, it is generally accepted that autoantibodies against integral membrane proteins are usually pathogenic [1]. Some autoantibodies have been clearly confirmed to be pathogenic in several autoimmune diseases, and a model

Table 1: Prevalence of anti-endothelial cell antibodies.

Disease	% of positive sera
Systemic lupus erythematosus	15–85
Rheumatoid arthritis	0–87
Mixed connective tissue disease	33–45
Systemic sclerosis	15–84
Polymyositis/dermatomyositis	44–64
Antiphospholipid syndrome	0–64
Sjögren's syndrome	24-25
Polyarteritis nodosa	50–56
Microscopic polyangiitis	2–60
Granulomatosis with polyangiitis	19–80
Eosinophilic granulomatosis with polyangiitis	50–69
Takayasu arteritis	54–95
Giant-cell arteritis	33–50
Behçet's disease	14–80
Kawasaki disease	65

for customized and specific therapeutic approaches against a highly pathogenic subset of autoantibodies using small molecules have been reported [5].

In 1971, Lindqvist and Osterland first described autoantibodies to vascular endothelium based on indirect immunofluorescence (IIF) experiments [6]. These autoantibodies were called anti-endothelial cell antibodies (AECAs) and were defined as autoantibodies targeting antigens present on the endothelial cell (EC) membrane [7]. As target antigens of AECAs are present on the ECs, which are always in contact with these circulating antibodies, AECAs have the potential to induce vascular lesions directly. Here, we present a review of AECAs and a novel method for identification of cell-surface autoantigens.

2. AECAs

2.1. AECAs and Disease. The presence of AECAs has been reported in patients with a wide variety of diseases, including collagen diseases (Table 1), inflammatory bowel disease, diabetes, thyroid diseases, thrombotic thrombocytopenic purpura, primary sclerosing cholangitis, interstitial lung disease, chronic obstructive lung disease, uveoretinitis, renal transplantation, Susac syndrome, masked hypertension, and atherosclerosis [8–23]. AECAs are correlated to disease activity in some collagen diseases, and are thought to be critical especially for vascular lesions in collagen diseases [23]. In addition, AECAs have been shown to be clinical signs of vasculitis in patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [24]. AECAs were also reported to play critical roles in several pathophysiological conditions, including pulmonary hypertension, digital ulcers, and gangrene [21, 22].

AECAs are detected even in healthy subjects [25, 26]. These natural autoantibodies interact with living ECs with lower affinity as compared to pathologic AECAs, and their antigens are highly conserved protein families. They contribute to modulate endothelial function with protective anti-inflammatory and anti-thrombotic functions [26].

2.2. Detection and Identification of AECAs. Methods for detection of AECAs have not been standardized, and a number of methods have been reported, including IIF, cell-based-enzyme linked immunosorbent assay (ELISA), flow cytometry, radioimmunoassay, western blotting (WB), and immunoprecipitation [22, 23]. As these each of methods have advantages and disadvantages, use of different technical approaches to obtain more robust data is recommended [7].

Human umbilical vein endothelial cells (HUVECs) are commonly used as a substrate, but antigen patterns of ECs differ among other ECs, passage numbers, and culture conditions [27]. It is also important whether ECs are fixed or not because fixation induces permeabilization of the EC membrane, and intracellular antigens become accessible to antibodies [22]. The results of AECA positivity were therefore not considered in the same light, and the prevalence of AECAs differed among studies (Table 1). Miura et al. recently reported a novel solubilized cell-surface protein capture ELISA for detection of AECAs [28], and further evaluation and standardization are needed.

2.3. Pathogenicity of AECAs. An experimental animal model for pathogenicity of AECAs was reported by Damianovich et al. [29]. In their experiment, BALB/c mice were actively immunized with the purified AECAs from a patient with granulomatosis with polyangiitis. Three months after a booster injection with human AECAs, mice developed endogenous AECAs, and histological examination of lungs and kidneys revealed both lymphoid cell infiltration surrounding arterioles and venules.

AECAs have been shown to be correlated with disease activities, and have the potential to induce vascular lesions because their targets are expressed on ECs that are readily accessible to these circulating antibodies. AECAs are also considered to play roles in the development of pathological lesions by a number of methods as described below [22, 23, 30–32].

The first is the cytotoxicity of ECs through complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). CDC activity of AECAs was reported in patients with SLE, Takayasu arteritis, hemolytic-uremic syndrome, and Kawasaki disease [7, 24, 33–35]. Recently, we confirmed that fibronectin leucine-rich transmembrane protein 2 (FLRT2) is a novel target antigen of AECAs in SLE, which exerts direct cytotoxic effects through CDC [9].

The second is the induction of coagulation. AECAs may exhibit procoagulant effects by the production of tissue factor in SLE and the release of heparin sulfate in systemic sclerosis (SSc) [36, 37].

The third is the induction of apoptosis. AECAs may induce EC apoptosis through CD95 or cross-reaction with anti-phospholipid antibodies [38–40]. Dieudé et al. reported that heat-shock protein (Hsp60) bound to ECs and induced phosphatidylserine exposure and then apoptosis [41].

Margutti et al. identified antibodies to the C-terminus of Ral-binding protein 1 (RLIP76), and these autoantibodies induced oxidative stress-mediated EC apoptosis [42].

The fourth is the activation of ECs. AECAs were reported to induce the secretion of interleukin (IL)-1β, IL-6, IL-8, and monocyte chemotactic protein-1, (MCP-1), and the expression of adhesion molecules such as E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) [8, 24, 31], which cause leukocyte recruitment and adhesion.

Alard et al. reported that recognition of cell-surface adenosine triphosphate (ATP) synthase in the low pH microenvironment contributes to intracellular acidification of ECs, which may induce cell death and trigger inflammation [43].

As described above, there is a great deal of evidence that AECAs play pathogenic roles in collagen diseases. Identification of targets of AECAs is required because (a) antigen-specific detection systems are important for establishing diagnostic tools and standardization of AECAs measurement, (b) identification will enable thorough analysis of the pathogenicity of AECAs, and (c) AECA-autoantigen interactions may be good targets for specific therapeutic approaches against highly pathogenic autoantibodies.

3. Technologies for Identification of Autoantigens for AECAs

The prevalence of AECAs varies according to the type of ECs used for detection [44]. It was demonstrated that AECAs cross-react with human fibroblasts [45], and partial inhibition of AECA activity was documented by absorption of the AECA-containing sera with mononuclear cells [8]. It was also reported that a structure shared by platelets and ECs was recognized by a subset of AECAs [46]. These data suggested that the target antigens of AECAs may include not only EC-specific but also non-EC-specific molecules.

Target antigens of AECAs have been investigated intensively, but they are heterogeneous, and the following classification of target antigens was proposed: membrane component, ligand-receptor complex, and molecule adhering to the plasma membrane [8]. The EC autoantigens may be either constitutively expressed or translocated from intracellular compartment to membrane by cytokines, such as IL-1 and tumor necrosis factor α (TNFα), or physical effects [8, 47]. The reported autoantigens and their pathogenicities are summarized in Table 2 [7, 9, 22–24, 42, 43, 47–56].

Several molecules can bind to ECs and are called "planted antigens" for AECA presumably via charge-mediated mechanisms, a DNA-histone bridge, or a specific receptor. Myeloperoxidase, DNA, and β2-glycoprotein I (β2-GPI) are thought to adhere to ECs during incubation of ECs with sera from patients. Extracellular matrix components, such as vimentin, may also be target antigens for AECAs [57]. Proteinase 3 (PR3) could represent another potential cryptic target antigen [58]. PR3 has been maintained to migrate to the plasma membrane of ECs, following stimulation [8].

As methods for identification of target antigens of AECAs, immunoprecipitation and WB of glycoproteins from the EC membrane with AECA-positive sera have been used [8, 23]. Although numerous protein bands were reported as candidates for target antigens by this method, some of the bands were considered to be artifacts [8], and further identification of given bands was also sometimes difficult.

Alternative methods have been developed, such as proteomics analysis using two-dimensional electrophoresis followed by matrix-assisted laser desorption ionization time of flight mass spectrometry [8, 23] and expression libraries [8, 42, 56].

Proteomics analysis identified vimentin, Hsp60, voltage-dependent anion-selective channel 1 (VDAC-1), peroxiredoxin 2, and ATP synthase as targets for AECAs [41, 43, 48–50]. Expression libraries also identified tropomyosin, T-plastin, and RLIP76 [42, 56], and these technologies are therefore promising. The problem is that most of the molecules reported to date as targets for AECAs are intracellular proteins (Table 2) although AECAs must be directed against the cell surface. These two methods are not specific for detecting cell-surface molecules rather than intracellular molecules. In addition, extraction of some membrane proteins has been reported to be difficult in proteomics analysis, and this may make it difficult to identify such proteins as AECA targets [7].

To overcome this problem, we constructed a novel expression cloning system for specific identification of cell-surface antigens [9], which we call serological identification system for autoantigens using a retroviral vector and flow cytometry (SARF) (Figure 1), and we have confirmed that this system is useful to identify autoantigens expressed on the EC surface [9].

4. Strategy for Identification of Cell-Surface Autoantigens: SARF

4.1. Generation of HUVEC cDNA-Expressing Cells (Figure 1(a)). Our strategy to identify AECA target molecules involves use of a retroviral vector system and flow cytometry [9]. As described previously, antigen patterns of ECs differ among other ECs [27]. Because we used HUVECs as a substrate for AECAs measurement, we generated a HUVEC cDNA library using HUVECs grown in the same conditions as for AECAs measurement and ligated it into the retroviral vector, pMX [59]. Then, the HUVEC cDNA library in pMX was retrovirally transfected into the YB2/0 rat myeloma cell line [60]. As the localization of cellular molecules depends on their structures, only cell-surface molecules are expressed on the surface of YB2/0 cells transfected with the HUVEC cDNA library.

4.2. Sorting of Cells Expressing Cell-Surface Autoantigens (Figure 1(b)). AECAs can bind only to cell-surface molecules in flow cytometry. Therefore, sorting of IgG-binding cells can concentrate and isolate cells expressing target molecules for AECAs on the cell surface. After staining of HUVEC cDNA-expressing YB2/0 cells with AECA IgG and secondary antibody, cells with strong fluorescent signals are sorted by flow cytometry. This step of sorting is repeated for several rounds to concentrate AECA IgG-binding cells. After concentration,

Table 2: Reported target antigens of anti-endothelial cell antibodies.

Disease	Target antigen	Pathogenicity
Systemic lupus erythematosus	DNA-DNA-histone	
	Ribosomal P protein PO	
	Ribosomal protein L6	
	Elongation factor 1-alpha	
	Adenylyl cyclase-associated protein	
	Profilin 2	
	Plasminogen activator inhibitor	
	Fibronectin	
	Heparan sulfate	
	β2-glycoprotein I	
	Heat-shock protein 60 (Hsp 60)	Apoptosis
	Heat-shock protein 70 (Hsp 70)	
	Fibronectin leucine-rich transmembrane protein 2 (FLRT2)	Complement-dependent cytotoxicity
Mixed connective tissue disease	Voltage-dependent anion-selective channel 1 (VDAC-1)	
Systemic sclerosis	Topoisomerase I	
	Centromere protein B (CENP-B)	
Vasculitis	Proteinase 3	
	Myeloperoxidase	
	Peroxiredoxin 2	Cytokine secretion
	Adenosine triphosphate (ATP) synthase	Intracellular acidification
Microscopic polyangiitis	Human lysosomal-associated membrane protein 2	
Behçet's disease	Alpha-enolase	
	C-terminus of Ral-binding protein 1 (RLIP76)	Apoptosis
Kawasaki disease	Tropomyosin	
	T-plastin	
Transplantation	Vimentin	
	Keratin-like protein	
Thrombotic thrombocytopenic purpura	Glycoprotein CD36	
Heparin-induced thrombocytopenia	Platelet factor 4 (PF4)	
	Heparin sulfate	

several cell clones can be established from the AECA IgG-binding cell population by the limiting dilution method.

4.3. Identification of Novel Cell-Surface Autoantigens. After polymerase chain reaction (PCR) amplification and cloning of HUVEC cDNA inserted into the genomic DNA of cloned cells, DNA sequencing can be performed followed by BLAST analysis, which enables the identification of the inserted cDNA. In this step, microarray analysis is an alternative method to identify the inserted cDNA. Next, an expression vector of the identified cDNA is generated and transfected into a cell line that does not express the identified protein. Finally, it is necessary to confirm that AECA IgG shows binding activity to 7-amino-actinomycin D-(7-AAD-) negative identified protein-expressing cells. If the binding activity is confirmed, it can be concluded that the identified protein is a novel autoantigen.

5. Novel Autoantigens Identified by SARF

5.1. FLRT2. We reported the membrane protein FLRT2 as a novel autoantigen of AECAs in patients with SLE based on results obtained using SARF [9]. FLRT2 is type I transmembrane protein located on the plasma membrane [61]. FLRT2 was shown to be expressed in the pancreas, skeletal muscle, brain, and heart with Northern blotting [61], and we confirmed the expression of FLRT2 on HUVECs and other ECs by flow cytometry and IIF [9]. Anti-FLRT2 antibody activity accounted for 21.4% of AECAs in SLE, and anti-FLRT2 activity was significantly correlated with low levels of complement C3, C4, and CH50 [9]. Anti-FLRT2 antibody induced CDC against FLRT2-expressing cells including ECs, indicating that anti-FLRT2 autoantibody may exhibit direct pathogenicity [9].

5.2. ICAM-1. As AECAs can be detected in patients with collagen diseases, especially SLE, RA, and Takayasu arteritis [9], we further attempted to identify the autoantigens using SARF. One sample (X10-3) from an RA patient showed strong AECA activity (Figure 2(a)), and we selected this serum sample as the prototype of AECA for subsequent cell sorting. Using SARF, HUVEC cDNA-expressing YB2/0 cells were stained with X10-3 IgG and fluorescein isothiocyanate-(FITC-) conjugated secondary antibody, and cells with strong

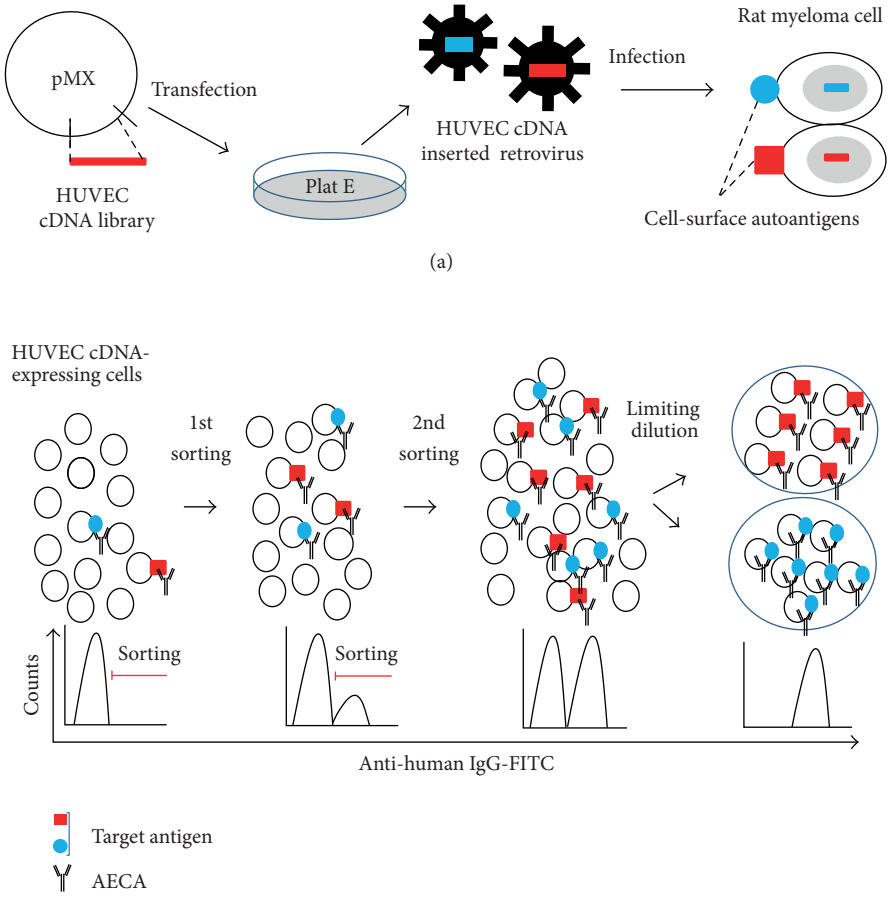

Figure 1: Serological identification system for autoantigens using a retroviral vector and flow cytometry (SARF). (a) Generation of human umbilical vein endothelial cell (HUVEC) cDNA-expressing cells. (b) Sorting of cells expressing cell-surface autoantigens.

FITC signals were sorted by flow cytometry (Figure 2(b)). After the 4th sorting, cells bound to X10-3 IgG were markedly increased (Figure 2(c), left), and the C5 clone was established from the X10-3 IgG-binding cell population by the limiting dilution method (Figure 2(c), right). Microarray analysis revealed that the signal of ICAM-1 was significantly increased ($2^{6.16}$-fold), and we confirmed that the ICAM-1 cDNA was inserted into the genomic DNA of X10-3-C5 clone (Figure 2(d)). We also confirmed the expression of ICAM-1 on the X10-3-C5 clone (Figure 2(e)). Next, we generated an expression vector for ICAM-1, which was transfected into YB2/0 cells. X10-3 IgG showed significant binding activity to 7-AAD-negative ICAM-1-expressing YB2/0 cells (Figure 2(f)), indicating that X10-3 IgG has anti-ICAM-1 activity. Thus, the membrane protein ICAM-1 was identified as a novel autoantigen of AECA in RA. ICAM-1 is an immunoglobulin-(Ig-) like cell adhesion molecule expressed by several cell types, including leukocytes and ECs. ICAM-1 plays an important role in both innate and adaptive immune responses. It is involved in the transendothelial migration of leukocytes to sites of inflammation, as well as in interactions between antigen presenting cells (APC) and T cells (immunological synapse formation) [62].

ICAM-1 was also confirmed to transduce signals "outside in" [63, 64]. The cross-linking of ICAM-1 with monoclonal antibodies was reported to activate the mitogen-activated protein kinase (MAPK) kinases ERK-1/2 and/or JNK [65–67]. The activation of ERK-1 lead to AP-1 activation [66], the ERK-dependent production and secretion of IL-8 and RANTES [67], and upregulation of VCAM-1 on the cell surface [66, 68]. ICAM-1 cross-linking can also upregulate tissue factor production [69] and proinflammatory cytokines, including IL-1 [70]. Lawson et al. reported production of anti-ICAM-1 IgM after cardiac transplantation, and the antibody induced robust activation of the ERK-2 MAPK pathway [71]. The use of anti-ICAM-1 antibody was examined for the treatment of RA, but the second course of therapy was associated with adverse effects suggestive of immune complex formation [72]. Identification of anti-ICAM-1 antibody in a patient with RA suggested that this autoantibody may exhibit such pathogenic roles.

5.3. Pk (Gb3/CD77). Using serum from an SLE patient who showed hemolytic anemia, SARF revealed that cDNA inserted into the cloned cells that were sorted with this AECA-IgG was alpha 1,4-galactosyltransferase (A4GALT).

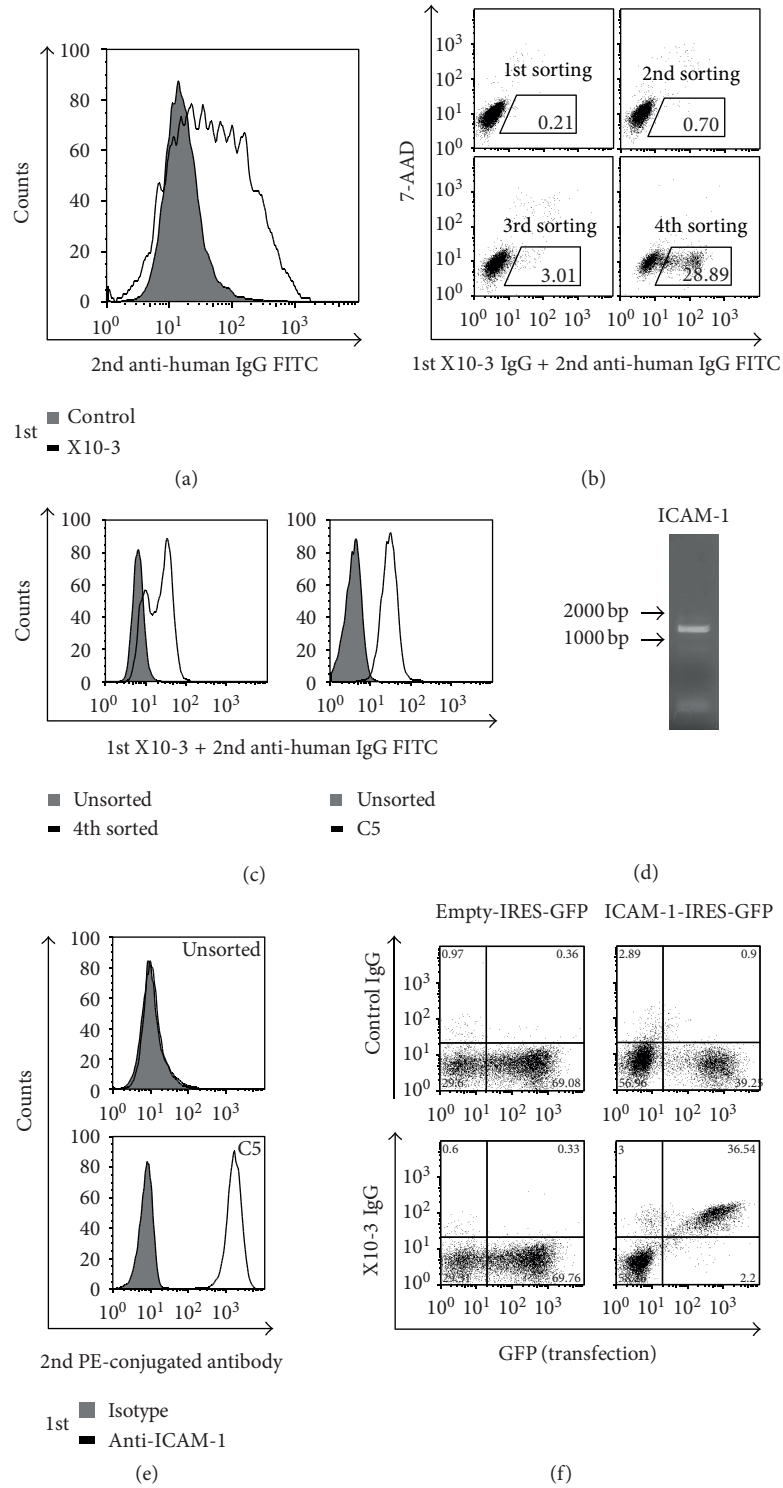

Figure 2: Identification of intercellular adhesion molecule 1 (ICAM-1) as a target antigen of anti-endothelial cell antibodies (AECAs). (a) Nonpermeabilized HUVECs were stained with 0.5 mg/mL of IgG of control or X10-3 from a patient with rheumatoid arthritis followed by secondary antibody and analyzed by flow cytometry. (b) HUVEC cDNA-expressing cells were stained with 0.5 mg/mL of X10-3 IgG followed by secondary antibody, and cells in the positive fraction were sorted (black box). (c) Unsorted and 4th sorted cells (left) and unsorted and cloned cells from 4th sorted cells, C5 (right), were stained with 0.5 mg/mL of X10-3 IgG followed by secondary antibody and analyzed by flow cytometry. (d) ICAM-1 cDNA fragments inserted into the genomic DNA of C5 were amplified, and PCR products were electrophoresed on an 0.8% agarose gel. (e) Unsorted and C5 were stained with isotype control or anti-ICAM-1 antibody, followed by secondary antibody and analyzed by flow cytometry. (f) Expression vector, empty-IRES-GFP, or ICAM-1-IRES-GFP were transfected into YB 2/0 cells, and these cells were stained with 0.5 mg/mL of control IgG or X10-3 IgG, followed by secondary antibody and analyzed by flow cytometry.

This AECA showed significant binding activity to 7-AAD-negative A4GALT-overexpressing YB2/0 cells. The A4GALT locus encodes a glycosyltransferase that synthesizes the terminal Galα1-4Gal of Pk (Gb3/CD77) glycosphingolipid [73, 74]. This means that synthesis of the terminal Galα1-4Gal is needed for the binding of this AECA-IgG.

Gb3 is the Pk blood group antigen and has been designated CD77 [74]. Monoclonal antibodies against Pk (Gb3/CD77) are used as markers for Burkitt's B-cell lymphoma and are able to initiate apoptosis [75]. Pk (Gb3/CD77) plays a direct role in the entry of Shiga toxin into the cell [76], and the presence of Pk (Gb3/CD77) in the ECs of the kidney accounts for the development of hemolytic uremic syndrome during bacterial infection with Shigella species that produce verotoxin [77]. The anti-Pk (Gb3/CD77) antibody was reported to cause acute intravascular hemolytic transfusion reactions and recurrent spontaneous abortions due to damage to the placenta [73, 78]. These data suggested that Pk (Gb3/CD77) is one of the target antigens of AECAs in SLE patients manifesting hemolytic anemia, and that anti-Pk (Gb3/CD77) antibody may exhibit some pathogenic roles.

Identification of A4GALT indicated the usefulness of SARF, which can be used to identify genes that encode not only the membrane protein itself, but also the transferase(s) responsible for modifying the membrane protein.

As described above, this system is very useful for identification of cell-surface autoantigens. Although this system seems to present difficulties in sorting cells at very low frequency, we could isolate and clone autoantigen-expressing cells by repeated sorting.

As AECAs are a heterogeneous group of autoantibodies that target ECs, it is predicted that there are different autoantigens. Thus, it is important to determine the clinical significance and potential pathogenicity of identified autoantibodies. If an autoantibody is specific for a disease or pathophysiology, it could be used as a marker for diagnosis or classification according to the underlying pathophysiology. At the same time, the pathogenic potential of the autoantibody should also be examined. Along with in vitro studies mentioned previously, experimental animal models of identified autoantibody should be constructed to determine the pathogenetic reactions in vivo.

6. Summary

AECAs are considered to be critical, especially for vascular lesions in collagen diseases, but most are directed against molecules localized within the cell and not expressed on the cell surface. In addition to conventional immunoprecipitation and WB, proteomics and expression library analyses have been performed to identify the targets for AECAs with some success. SARF was developed to identify autoantigens expressed on the EC surface with greater sensitivity. Using SARF, we successfully identified three different membrane proteins as targets for AECAs: FLRT2 from patients with SLE, ICAM-1 from a patient with RA, and Pk (Gb3/CD77) from an SLE patient with hemolytic anemia. Using this technology, it may be possible to determine cell-surface autoantigens of AECAs and achieve a comprehensive understanding of AECA-mediated vascular injury. Furthermore, SARF can be used when autoantibodies against cell-surface molecules are considered to take part in autoimmune diseases. The identification of such pathogenic autoantibodies may enable the development of more specific intervention strategies in autoimmune diseases.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

The authors thank the staff of the Department of Hematology and Rheumatology, Tohoku University, for help and discussion. This work was supported in part by Network Medicine Global-COE Program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and Biomedical Research Core of Tohoku University Graduate School of Medicine.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2013, Article ID 572815, 7 pages
http://dx.doi.org/10.1155/2013/572815

Research Article

Development of a Recombinant Cell-Based Indirect Immunofluorescence Assay for the Determination of Autoantibodies against Soluble Liver Antigen in Autoimmune Hepatitis

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Received 4 October 2012; Accepted 16 November 2012

Academic Editor: Pietro Invernizzi

Autoantibodies against soluble liver antigen (SLA) are specific markers for autoimmune hepatitis (AIH) type 1. In contrast to the determination of other AIH-associated autoantibodies by indirect immunofluorescence assay (IFA), detection of anti-SLA relied up to now on ELISA or immunoblot based on bacterially expressed recombinant protein. In order to develop a complementary IFA substrate, SLA isoform 1 was recombinantly produced in the human cell line HEK293 and controlled by a rabbit hyperimmune serum against SLA. The recombinant cells were used in IFA (RC-IFA) to analyze sera from 20 AIH patients with anti-SLA positivity predetermined by ELISA together with 80 controls (20 anti-SLA negative AIH, 15 primary biliary cirrhosis, 15 HCV, and 30 healthy blood donors). Using RC-IFA, anti-SLA was detected in all ELISA positive AIH sera but in none of the controls. Furthermore, a cytosolic fraction of HEK293 containing SLA was able to neutralize the autoantibodies in all positive sera in a dose-dependent manner. HEK293 cells expressing SLA are a valid substrate for the serodiagnosis of AIH relevant autoantibodies by IFA. In concert with cryosections of primate liver, rat kidney, rat liver, rat stomach, and HEp-2 cells, they enable the parallel determination of all autoantibodies associated with autoimmune liver diseases.

1. Introduction

Autoimmune hepatitis (AIH) is a serious chronic liver disease that affects both sexes and all ages and races [1]. Accurate diagnosis allows for early administration of immunosuppressive treatment which sharply decreases the disease activity in the great majority of the cases [2–4].

The clinical diagnosis of AIH is challenging at times, such that autoantibody markers are commonly used to assist clinicians in their decision [2, 5]. Antinuclear antibodies (ANA) and smooth muscle autoantibodies (SMA) define type 1 AIH (AIH-1), and anti-liver kidney microsomal type 1 (anti-LKM1) and anti-liver cytosol type 1 (anti-LC1) characterize type 2 (AIH-2). Additionally, antibodies against soluble liver antigen (SLA) were found to be highly specific markers of AIH [6–9]. All of these autoantibodies are routinely tested in the differential diagnosis of autoimmune liver diseases in general and AIH in particular [2, 5, 9].

Testing of anti-SLA is included in the recommendations of the International Autoimmune Hepatitis Group (IAIHG) [10]: the early revised criteria that were issued mainly for the better definition of AIH cases involved in research studies [11, 12] and the most recent simplified criteria for the diagnosis of AIH issued to help clinicians in the routine

clinical practice [13]. Despite its limited clinical sensitivity of 7–19% [14–16], dependent on the ethnical background as it has been estimated by commercially available test systems, the testing for anti-SLA antibodies seems to be justified because they can be considered pathognomonic markers of AIH, with specificity close to 100% [14].

Finally, both the IAIHG and the American Association for the Study of Liver Diseases (AASLD) guidelines advice routine testing of anti-SLA [2, 12, 13], as these autoantibodies appear to be the only ones for which a consensus has been reached regarding their clinical relevance and in particular their ability to identify AIH patients with more severe disease and worse outcome [17–23].

With the exception of anti-SLA, AIH-associated autoantibodies are routinely detected by indirect immunofluorescence assay (IFA)—still the gold—standard for the detection of ANA and SMA, with or without complementary enzyme-linked immunosorbent assays (ELISA) or line/dot blots based on the target antigens of the respective autoantibodies [5, 24]. For IFA, rodent liver, kidney and stomach tissues, and HEp-2 cells are in general used as test substrates. In contrast, widely available assays for anti-SLA have so far relied on bacterially expressed recombinant protein employed in ELISA or blot assays.

The current diagnostic approaches suffer from two severe limitations: first, there is no uniform assay platform such that laboratories either need to introduce an additional test for anti-SLA next to IFA or have to employ less competent ELISA and blot assays for ANA and SMA to avoid IFA, and second the nowadays available anti-SLA assays are only able to detect antibody reactivity to primary structures whilst missing responses against conformational epitopes. But comprehensive epitope mapping analyses demonstrated that SLA epitopes are mainly conformational [21]. Therefore, eukaryotically expressed SLA was proposed as a more competent target antigen due to the alleged presence of conformational epitopes that are not presented by the bacterially expressed antigen [6].

In the present study, an IFA based on eukaryotically overexpressed SLA was developed in order to complement the portfolio of commonly use substrates for the testing of autoantibodies associated with autoimmune liver diseases.

2. Material and Methods

2.1. Serum Samples. As positive controls, 20 serum samples containing anti-SLA, determined with commercially available ELISA and line blot (Euroimmun, Germany) based on the SLA variant first described by Wies et al. [6, 14]. The negative control group consisted of 20 patients with autoimmune hepatitis (AIH) type 1 before onset of treatment in which in the conventional anti-SLA antibodies were not detectable. AIH type 1 was defined using the criteria of the 2004 consensus statement of the international AIH group [12]. As further controls, sera from 15 patients with primary biliary cirrhosis and high-titre antimitochondrial antibodies, 15 patients with hepatitis C virus infection, and 30 healthy blood donors without any features suggestive of liver disease were used. In adherence to the Helsinki principles, informed consent from all patients was obtained whose material was used in this study.

2.1.1. Cloning, Expression of SLA in E. coli, and Purification. The coding DNA for SLA isoform 1 (Swiss-Prot acc. no. Q9HD40) was obtained by PCR on a cDNA (BioSource, Germany, Genbank acc. no. BX648976), with primers as in Table 1 introducing BsmBI restriction sites. PCR reaction and subsequent ligation with NcoI/XhoI digested pET24d were carried out as in Sitaru et al. [25] thereby adding a C-terminal hexahistidine-tag to the recombinant protein (SLA-His-coli). The final construct was verified by DNA sequencing (MWG Biotech).

SLA-His-coli was expressed in *E. coli Rosetta* (DE3) pLacI (Novagen) and purified under denaturing conditions by immobilized metal chelate affinity chromatography (IMAC) and cation exchange chromatography following the protocol as in Probst et al. [26].

2.1.2. Hyperimmune Serum against SLA. For the generation of a polyclonal serum, rabbits were immunized with SLA-His-coli following a standard 87-day programme (Eurogentec, Belgium). Two normal New Zealand white rabbits were each immunised subcutaneously with 200 μg of recombinant human SLA emulsified in Freund's complete adjuvant (FCA). The animals were boosted with 100 μg of antigen at days 14, 28, and 42 without FCA. At the end of the immunisation protocol, test bleedings were obtained and sera were separated by centrifugation and stored in aliquots at −20°C until used. Pre-immune rabbit sera were used as negative controls. Serum reactivity was controlled by westernblot using SLA-His-coli.

2.1.3. Cloning and Expression of SLA in HEK293. The coding DNA for SLA isoform 1 (Swiss-Prot acc. #Q9HD40) was obtained by PCR on a cDNA (BioSource, Germany, Genbank acc. #BX648976), with primers as in Table 1 introducing BsmBI restriction sites. The PCR reaction was carried out as in Sitaru et al. [25]. After digestion with BsmBI, the amplification product was ligated with NcoI/XhoI-linearized pTriEx-1 (Merck Biosciences, Germany). SLA without a tag was expressed in the human cell line HEK293 (SLA-HEK) after ExGen500-mediated transfection (Fermentas, Germany) according to the manufacturer's instructions. For the preparation of substrates for the indirect immunofluorescence test, HEK293 were grown on sterile glass slides, transfected, and allowed to express the recombinant protein for 48 hours. Slides were washed with PBS, fixed either with acetone or with 1% (w/v) formalin in acetone for 10 minutes at room temperature, air-dried, and stored at −20°C until use.

Alternatively, cells were transfected in standard T-flasks and harvested after 72 hours expression by removing the cell culture medium, scraping the cells off in PBS, washing three times in 20 mmol/L tris-HCl pH 7.4, 150 mmol/L sodium chloride, 5 mmol/L EDTA, 1 mmol/L PMSF and final resuspension in 10 μL/cm^2 culture surface 20 mmol/L tris-HCl pH 7.4, 10% (w/v) sucrose, 5 mmol/L EDTA, 1 mmol/L PMSF. Cells were frozen at −80°C until further use. For

Table 1: Primer sequences for PCR amplification of cDNA fragments of SLA. Primers were synthesized by MWG, Germany. F: forward primer; R: reverse primer.

Protein	Restriction sites	Primer sequences (5′–3′)
SLA-His-coli	BsmBI	F: ATTACGTCTCACATGAACCGCGAGAGCTTCGCGGCG
	BsmBI	R: ATTACGTCTCTTCGAGTGAAGAAGCATCCTGGTATGTGTC
SLA-HEK	BsmBI	F: ATTACGTCTCACATGAACCGCGAGAGCTTCGCGGCG
	BsmBI	R: ATTACGTCTCTTCGAGTCATGAAGAAGCATCCTGGTATGTG

the preparation of cell-free supernatants, the cells were thawed and diluted with 4 volumes of 20 mmol/L tris-HCl pH 7.4, 50 mmol/L potassium chloride, 5 mmol/L EDTA, and 1 mmol/L PMSF. Cell lysis was promoted by dounce homogenization. Cell nuclei were removed by centrifugation at 700 ×g, 4°C for 10 minutes. The supernatant was saved whereas the sediment was lysed for a second time. Finally, the combined supernatants were centrifuged at 100.000 ×g, 4°C for 60 minutes and the resulting supernatant was stored in aliquots at −80°C until further use.

2.2. Indirect Immunofluorescence Assay (IFA). IFA was conducted using slides with four types of substrates: HEK293-SLA and wild-type HEK293, each acetone and formalin fixed, according to the standard instructions for HEp-2 cells (Euroimmun). In some cases, rabbit hyperimmune sera were used in the first step of IFA followed by incubation with Cy3 anti-rabbit IgG (Jackson Research, United Kingdom). In all cases, the incubated slides were evaluated independently by two experts. In neutralization experiments, samples with antigen content were mixed with diluted sera 30 minutes prior to the IFA as described elsewhere [27].

2.3. ELISA for the Detection of Human Autoantibodies Against SLA. Microtiter plates (Nunc, Germany) were coated with SLA-His-coli (up to 10 μg/mL) in PBS for 2 hours at 25°C, washed three times with washing buffer (0.05% (w/v) Tween-20 in PBS), and blocked with blocking buffer (0.1% (w/v) casein in PBS) for 1 hour. Saturation of the plates was analyzed alternatively by incubation with a murine monoclonal anti-hexahistidine-tag antibody or polyclonal anti-SLA rabbit serum diluted 1 : 2,000 in sample buffer (0.05% (w/v) Tween-20, 1% (w/v) casein in PBS) for 30 minutes. After washing three times, bound antibodies were detected by incubation with anti-mouse IgG HRP conjugate (Dianova, Germany) or anti-rabbit IgG HRP conjugate (Sigma-Aldrich, Germany), respectively, diluted 1 : 2,000 in sample buffer, for 30 minutes, subsequent washing as described above, followed by addition of TMB substrate (Euroimmun) for 15 minutes. Reactivities of human sera were analyzed using the same procedure, except for a different dilution of the sera (1 : 200) and the use of appropriate conjugates (Euroimmun). All incubation steps were carried out at room temperature. The OD was read at 450 nm using an automated spectrophotometer (Tecan, Germany).

2.3.1. SDS-PAGE and Westernblots. Proteins were analyzed following SDS-PAGE using the NuPAGE system (Invitrogen, Germany) according to the manufacturer's instructions. In some cases, proteins were electrotransferred to nitrocellulose membranes and then used in westernblots. In the first immunological reaction human sera diluted 1 : 200, rabbit sera diluted 1 : 2,000 or a murine monoclonal antibody against hexa-histidine diluted 1 : 2.000 in universal buffer plus (Euroimmun) were applied. Bound antibodies were visualized by anti-IgG conjugated to alkaline phosphatase and NBT/BCIP (Euroimmun) as described earlier [28]. Some proteins were also analyzed by MALDI-ToF fingerprinting and MALDI-ToF tandem mass spectrometry after SDS-PAGE and tryptic cleavage [29]. Protein concentrations were determined by bicinchoninic acid assay (Sigma, Germany).

3. Results

3.1. Preparation of Recombinant SLA Proteins and a Corresponding Rabbit Serum. Coding DNA for human full-length SLA isoform 1 was ligated with a prokaryotic expression vector and expressed in *E. coli* (SLA-His-coli). The protein, purified by metal chelate affinity chromatography, migrated according to its calculated mass of 52 kDa when separated by SDS-PAGE (Figure 1(a)). Additionally, two minor bands corresponding to smaller masses were visible. Identities of the proteins as full-length SLA and fragments thereof were verified by MALDI-ToF fingerprinting and reactivity of a murine monoclonal antibody against hexa-histidine. Its use for the immunization of New Zealand White rabbits provoked high-titer reactivity against itself (Figure 1(b)). Anti-SLA rabbit serum reactivity in turn verified the presence of recombinant SLA in HEK293 transfected with a eukaryotic expression vector containing its coding sequence by westernblot (Figure 1(c)) and its accessibility in the indirect immunofluorescence assay. In contrast, wild-type HEK293 as well as HEK293 transfected with the same vector backbone containing an unrelated coding sequence did not produce similar results.

When SLA-expressing HEK293 were homogenized in hypotonic buffers, the recombinant protein (SLA-HEK) could be released in a soluble form as verified by presence of >80% SLA-HEK in the supernatant after ultra-centrifugation. In contrast, SLA-His-coli could only by solubilised using high concentrations of chaotropic agents.

3.2. Denatured SLA for Autoantibody Determination. When either SLA-His-coli or SLA-HEK were used in westernblot, all 20 sera from anti-SLA positive patients with AIH produced a band corresponding to the position of the anti-SLA rabbit

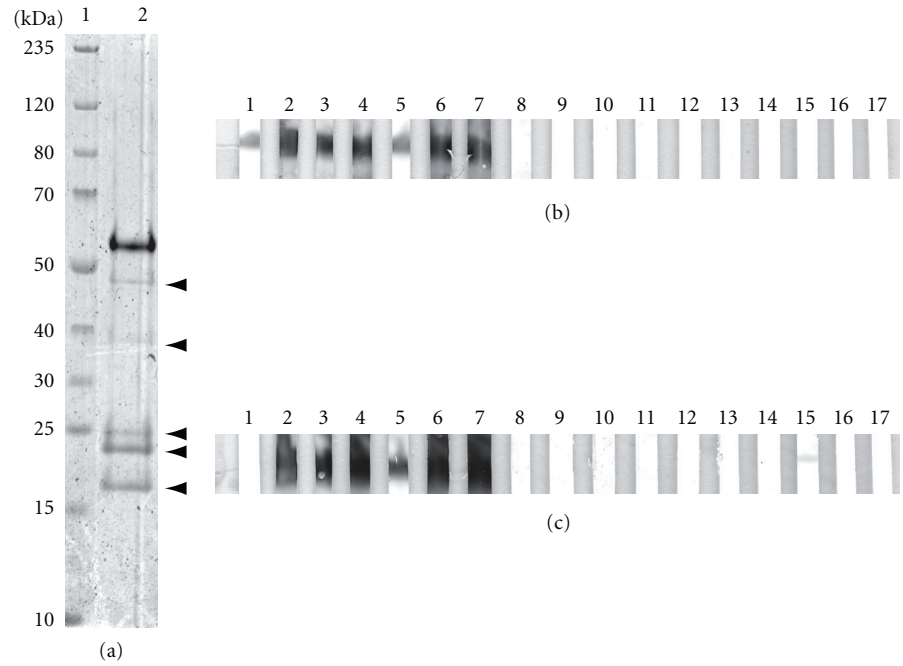

Figure 1: Characterization of recombinant SLA proteins by SDS-PAGE and westernblot. (a) Bacterially expressed SLA was analyzed by SDS-PAGE with coomassie staining. Lane 1: molecular mass markers, kDa indicated; lane 2: 2 μg recombinant SLA, arrow heads indicate the presence of anti-His-Tag antibody reactive SLA-fragments verified by mass spectrometry, (b) 1 μg/lane recombinant SLA purified from *E. coli,* (c) or cell-free supernatant from HEK293 expressing SLA; lane 1: murine monoclonal antibody against hexahistidine; lanes 2: rabbit polyclonal serum against SLA; lanes 3–7: anti-SLA positive sera from patients with autoimmune hepatitis (AIH); lanes 8–12: anti-SLA negative sera from patients with AIH; lanes 13–17: sera from healthy blood donors; the band in c, lane 15 does not correspond to SLA.

serum reactivity. None of the sera from 20 anti-SLA negative AIH patients, 15 PBC patients, 15 patients with HCV infection or from 30 healthy blood donors generated bands at identical positions (Figure 1(b)).

SLA-His-coli could be immobilized to microplates and saturated the surfaces at 200 ng per well as verified by a high maximum ELISA signals (E_{450} > 4) and a sigmoidal saturation curve obtained after incubation of anti-hexahistidine antibody and anti-SLA rabbit serum, respectively. Following ROC analysis, a usable cut-off value of E_{450} = 0.25 was defined. Similar to the westernblot, signals above the cut-off value were reached in 20 AIH sera but in none of the controls (Figure 2).

In neutralization experiments, SLA-His-coli and cytoplasmic supernatants of HEK293 expressing SLA but not similar fractions of wild-type HEK293 were able to inhibit the anti-SLA reactivities of all 20 human sera and the anti-SLA rabbit serum with SLA-His-coli in ELISA in a dose-dependent manner. Maximum inhibition rates were equal for SLA-His-coli and SLA-HEK.

3.3. Recombinant Cell-Based Indirect Immunofluorescence for Autoantibody Determination. When HEK293 expressing SLA, either fixed with acetone or 1% (w/v) formalin in acetone, were used as substrates in indirect immunofluorescence (IFA), all 20 anti-SLA positive AIH sera produced a cytoplasmic staining pattern (Figure 3(a)) that

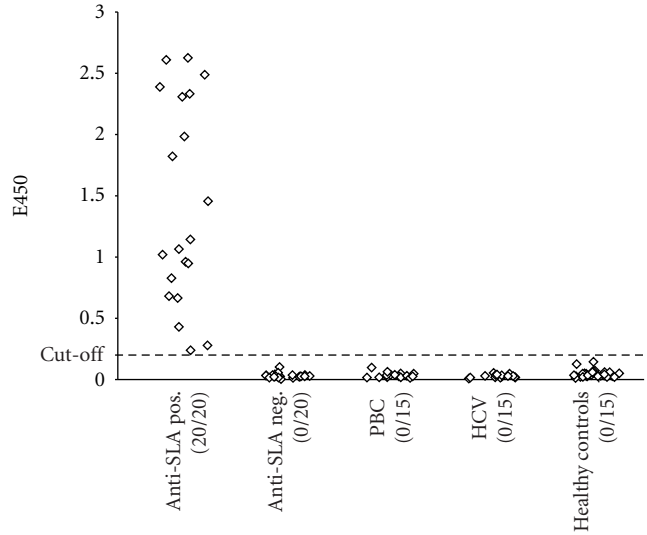

Figure 2: Autoantibodies against SLA detected with ELISA based on bacterially expressed protein. Bacterially expressed SLA (SLA-His-coli) was used to form the solid phase in an indirect ELISA for the determination of human IgG antibodies in 20 anti-SLA positive autoimmune hepatitis sera, 20 anti-SLA negative autoimmune hepatitis sera, 15 primary biliary cirrhosis sera, 15 HCV sera, and 30 sera from healthy blood donors. Positive and total numbers of sera are given below the diagrams. The cut-off value is presented by a dotted line.

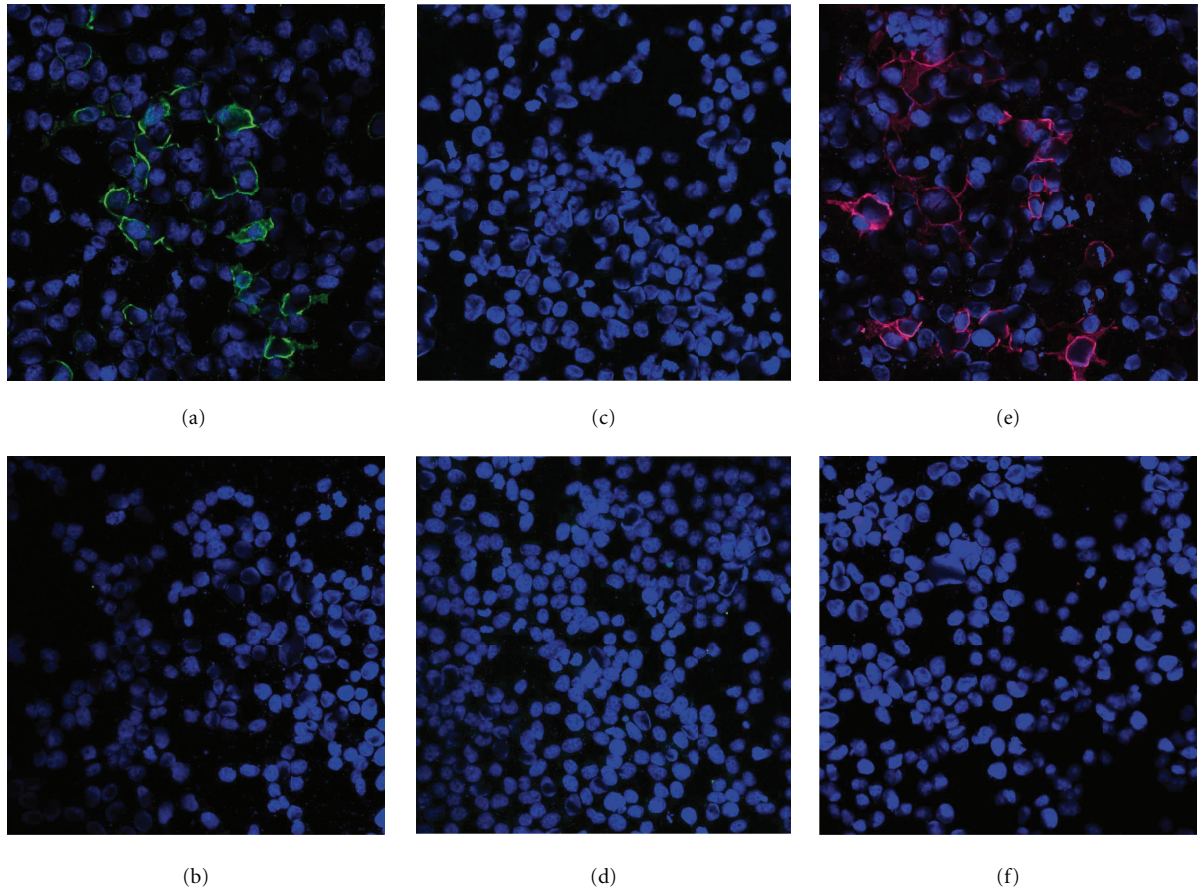

Figure 3: Immunofluorescence staining patterns of HEK293 expressing soluble liver antigen. HEK293 expressing soluble liver antigen (SLA) and wild-type HEK293 were incubated with either 1 : 100 diluted human serum or 1 : 100 diluted anti-SLA rabbit serum and bound antibodies were visualized with either anti-human IgG FITC (green) or anti-rabbit IgG Cy3 (red) conjugates. Nuclear DNA was stained with TO-PRO-3 (blue). (a, c, e) HEK293-SLA; (b, d, f) untransfected HEK293; (a & b) representative anti-SLA positive serum from a patient with autoimmune hepatitis; (c & d) representative anti-SLA negative serum from a healthy blood donor; (e & f) anti-SLA rabbit serum.

was absent in the wild-type HEK293 (Figure 3(b)). Similar staining patterns were produced by the anti-SLA rabbit serum (Figures 3(c) and 3(d)) whereas none of the control sera reacted, independent of the fixation. Staining patterns were in all cases better defined when cells were fixed with formalin (Supplementary Figure 1 available online at http://dx.doi.org/10.1155/2013/572815) which eased the interpretation of the results considerably, especially in the presence of antinuclear or anti-mitochondrial antibodies.

In neutralization experiments, cytoplasmic supernatants of HEK293 expressing SLA but neither similar fractions of wild-type HEK293 nor SLA-His-coli were able to abolish the reactivities of all 20 human sera with SLA-HEK in IFA in a dose-dependent manner whereas co-incubation of sera with SLA-His-coli only reduced the anti-SLA titers in the human sera but was not able to abolish the reactivities. In contrast, the reactivity of the anti-SLA rabbit serum could be neutralized by both, SLA-His-coli and SLA-HEK.

In an immunoprecipitation assay with subsequent detection of SLA by westernblot (see Supplementary Materials and Methods and Supplementary Figure 2) anti-SLA positive AIH sera precipitated SLA-HEK whereas none of the 20 anti-SLA negative AIH sera was able to pull down recognizable amounts of the eukaryotically expressed protein.

4. Discussion

The present study is the first to report the development of an IFA which allows the detection of anti-SLA autoantibodies, an autoantibody marker of AIH. These autoantibodies do not produce a recognizable immunofluorescent pattern by conventional indirect immunofluorescence, regardless of whether tissue or known cell line are used as antigenic substrates [5]. The reason for this is unclear but it can be speculated that either the concentration of the target antigen is too low in the respective IFA substrates to produce a staining *per se* or is significantly reduced after fixation due to its solubility, similar to the effect of easily soluble lactoferrin and myeloperoxidase after ethanol fixation of neutrophils [30, 31]. The latter view is in line with the recent identification of SLA as the *O*-phosphoseryl-tRNA(Sec)

selenium transferase [7], which converts *O*-phosphoseryl-tRNA(Sec) to selenocysteinyl-tRNA(Sec). The enzyme is essential for selenoprotein biosynthesis and, as such, is primarily expressed in liver where it is located as an easily soluble protein within the cytosolic fraction [32, 33].

The recombinant HEK293 cell-based IFA (RC-IFA) is based on overexpressed SLA that is immobilized in the cytoplasmic region of the cells due the use of formalin in the fixation step. The cells show an easily visible and strong staining when incubated with anti-SLA antibodies of either human or rabbit origin (Figure 3). At the same time, the results are highly specific because non-anti-SLA reactivities can be easily distinguished from reactions with wild-type HEK293 incubated in parallel. The new anti-SLA RC-IFA will greatly assist routine laboratories which use IFA for hepatitis-related autoantibody screening. A combination of conventional IFA testing based on rodent tissue and HEp-2 and the SLA RC-IFA can detect the whole spectrum of autoantibodies that are diagnostically relevant for AIH, as they have been described by the IAIHG and the American Association for the Study of Liver Diseases (AASLD) practise guidelines [2, 12, 13]. This comprehensiveness of IFA, together with its high proficiency, may be attractive to laboratories that have previously turned away from IFA [12, 24].

Our results also indicate the presence of conformational epitopes in the HEK293-expressed SLA, confirming earlier speculations [7, 18, 21]: a cytosolic fraction of the recombinant cells was able to abolish the reactivity of human and rabbit anti-SLA antibody positive sera in RC-IFA. In contrast, the bacterially expressed homologue only showed an incomplete inhibition of human sera. On the other hand, anti-SLA antibody positive sera raised in a rabbit by immunization with SLA produced in *E. coli* could be neutralized. We speculate that this behaviour mirrors the presence of exclusively linear epitopes in the recombinant protein purified from *E. coli* inclusion bodies under denaturing conditions.

When designing the study, we expected that the developed RC-IFA would offer a much higher sensitivity than ELISA or blot assays based on prokaryotically expressed protein [14–16]. The studies done by Ma et al. [21] and Vitozzi et al. [18] had postulated that some AIH-1 and AIH-2 sera have reactivity to recombinant SLA restricted to conformational epitopes. Our results do not confirm this assumption. All sera found positive for anti-SLA by the ELISA were also positive by the RC-IFA SLA and *vice versa*. Obviously, all anti-SLA positive sera contain simultaneous reactivity to both linear and conformational epitopes. However, the AIH patient cohort was small and the study had a retrospective design based on the serological characterization of anti-SLA with established assays such that the outcome might be biased. Also, we cannot exclude the possibility that the epitopes presented by the recombinant cells are not the same with those recognised by the previously reported radioligand assays [7, 18, 21]. A large prospective study using all the available assays and well-defined sera in parallel, potentially conducted under the auspices of the IAIHG, would be needed to establish the validity of the assays.

Provided that the present findings are confirmed on larger cohorts and in other laboratories, the anti-SLA RC-IFA is able to serve as an equal supplement for ELISA or blot assays in routine laboratories and can as such be implemented in daily liver-associated autoantibody testing.

Abbreviations

AP:	Alkaline phosphatase;
BSA:	Bovine serum albumin;
FITC:	FLuorescein isothiocyanate;
HCV:	Hepatitis C virus;
HRP:	Horse raddish peroxidase;
IFA:	Indirect immunofluorescence assay
MALDI-ToF:	Matrix-assisted laser desorption/ionization trap-time of flight mass spectrometry;
PAGE:	Polyacrylamide gel electrophoresis;
RC-IFA:	recombinant cell-based indirect immunofluorescence assay;
SLA:	soluble liver antigen;
AASLD:	American Association for the Study of Liver Diseases;
IAIHG:	International AIH Group;
AIH:	autoimmune hepatitis;
PBC:	primary biliary cirrhosis.

Disclosure

Christiane Radzimski, Christian Probst, Bianca Teegen, Kristin Rentzsch, Inga Madeleine Blöcker, Cornelia Dähnrich, and Lars Komorowski are employees of Euroimmun AG, Germany; WSch and WSt are board members of Euroimmun AG, Germany; CP, CD, WSch, and WSt are shareholders of Euroimmun AG, Germany.

Acknowledgments

The authors thank Beatrice Schneider, Sabrina Voigt, Beatrice Ziegler, and Stephan Zitzewitz for their excellent technical assistance.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 370107, 8 pages
doi:10.1155/2012/370107

Research Article

BPI-ANCA and Long-Term Prognosis among 46 Adult CF Patients: A Prospective 10-Year Follow-Up Study

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Received 27 September 2012; Accepted 22 October 2012

Academic Editor: Dimitrios P. Bogdanos

Introduction. Anti-neutrophil cytoplasmic antibodies specific for bactericidal/permeability-increasing protein (BPI-ANCA) are frequent in CF patients and mainly develop in response to infection with *Pseudomonas aeruginosa*. It is not known to what extent BPI-ANCA correlates to prognosis. *Objectives*. To evaluate the prognostic value of IgA-BPI-ANCA, measured at the beginning of the study, for transplantation-free survival. *Methods*. A cohort of 46 adult, nontransplanted CF patients was generated, 1995–1998, and characterized using Leeds criteria, lung function, and IgA-BPI-ANCA levels measured by ELISA. The cohort was followed until December 2009, using the combined endpoint of death or lung transplantation. *Results*. Lung function and IgA-BPI-ANCA, but not Leeds criteria, were significantly associated with adverse outcome. No patient with normal lung function at baseline reached endpoint. Within 10 years 8/11 with high BPI-ANCA reached an endpoint compared to 3/17 ANCA-negative patients. A similar result was seen within the Leeds I group where 7 out of 9 BPI-ANCA-positive patients reached endpoint, compared to none of the 5 patients without BPI-ANCA. *Conclusions*. IgA-BPI-ANCA is associated with adverse outcome among *Pseudomonas aeruginosa* infected CF patients, suggesting that BPI-ANCA is a biomarker of an unfavourable host-pathogen interaction.

1. Introduction

Cystic fibrosis (CF) is a disease with multiple clinical manifestations, where the prognosis for the individual often is difficult to foresee. The lack of reliable predictors of the disease course is a well-recognized problem in CF patients [1]. Pulmonary disease is the main determinant of morbidity and mortality in CF [2] and hence it is important to identify factors that can explain and predict variations in lung function.

The genetic correlation between pancreatic sufficiency and milder disease has been established since it became possible to genotype CF patients [3]. In a longitudinal study including the whole Swedish CF population it was shown that CFTR genotypes found in conjunction with long-term pancreatic sufficiency phenotype were associated with a better pulmonary function [1].

ΔF508 is the most common mutation, with an allele frequency of 70% in Swedish CF patients. Homozygote patients generally have a more severe clinical phenotype than ΔF508 heterozygotes and patients with no F508 allele, although substantial phenotypic variability is seen [2, 4]. McKone et al. [2] studied CFTR genotype as a predictor of prognosis in CF and found that patients with a high risk CFTR genotype had a greater than two fold risk of death compared to patients with a low risk genotype.

Pseudomonas aeruginosa (*PsA*) colonization is a well-established risk factor in CF. In patients who subsequently died or became subjects for lung transplantation, chronic *PsA* colonization was significantly more frequent [5]. FEV1 is the variable of lung function that best reflects the progression of lung disease in CF; impaired vital capacity (VC) is seen only in late stages of the disease [6].

Bactericidal/permeability-increasing (BPI) protein is a protein found in the azurophilic granules of neutrophil granulocytes. BPI has a potent antimicrobial activity against Gram-negative bacteria, such as *PsA*, by neutralising the endotoxin and by playing a part in opsonization of the bacteria. [7–13]. Anti-neutrophil cytoplasmic antibodies (ANCA) with BPI specificity have been identified in different diseases associated with Gram-negative bacteria, such as inflammatory bowel diseases (IBD) and primary sclerosing cholangitis [14], and are frequently present in CF patients [11, 15, 16].

BPI-ANCA seems to develop in response to *PsA* colonization, but there are also patients colonized with *PsA* who do not develop BPI-ANCA [17]. After eradication of *PsA* colonization by lung transplantation a significant decrease in BPI-ANCA levels has been seen [17] and in a recently published study [18] it was shown that BPI-ANCA levels significantly decreased after sinus surgery. Exactly why BPI-ANCA is produced is a so far poorly understood process. One theory is that chronic infections stimulate extensive release of BPI triggering autoantibody production [16]. Schultz suggested that chronic Gram-negative infection accompanied by local neutrophil accumulation results in the delivery of BPI-coated particles to immature dendritic cells thereby inducing autoimmunity to BPI [19].

Earlier publications from our study group have shown a correlation between the presence of BPI-ANCA and reduced lung function in CF [17, 20, 21]. A high level of BPI-ANCA was associated with more severe lung disease both when measured with radiology and spirometry [16, 20, 22]. The presence of BPI-ANCA has also been associated with a higher number of antibiotic courses, low body mass index, the presence of resistant *PsA*, CF related liver disease, hypergammaglobulinemia, male sex, and inflammatory syndrome [23]. In a previous study we also found indications that the presence of BPI-ANCA predicted outcome [17].

We have now extended this followup to more than 10 years for all patients. The aim of this prospective study was to follow the progress of lung disease in 46 adult CF patients to elucidate the significance of a positive IgA-BPI-ANCA as a prognostic factor, in relationship to lung function and pseudomonas colonization status.

2. Patients and Methods

A cohort was generated in 1995–1998, as described earlier by Carlsson et al. [21]. Forty-six patients, all regular adult nontransplanted patients at the Lund CF Center, were included in the cohort. All patients at the centre were eligible for the study and 46 out of the 54 patients who were registered during the inclusion period were included. Reasons for not participating were trivial, such as not seeing a nurse assigned to the study during the inclusion period. The mean age of the patients at inclusion was 24.6 years (range 18.4–44.6); twenty were female, twenty-six male (Table 1). The study was approved by the Ethical Committee at Lund University and all participants gave their written informed consent.

Table 1: Characteristics of the adult CF cohort at the time of BPI-ANCA sampling (1995–1998).

Number of patients: (*n*)	
Total	46
Males	26
Females	20
Age: (years)	
Mean	26.2
Range	18.4–44.6
CFTR mutation: (*n*)	
ΔF508del/ΔF508del	24
Others	22
FEV1.0 % predicted: (*n*)	
>80%	16
50–80%	17
<50%	13
IgA BPI-ANCA:	
Negative (≤67 U)	
(*n*)	17
(mean age, years)	27.5
Positive (>67–200 U)	
(*n*)	18
(mean age, years)	26.3
High (>200 U)	
(*n*)	11
(mean age, years)	24.6
Leeds classification of *PsA* colonization: (*n*)	
I (chronic)	24
II (intermittent)	2
III (free of *PsA*)	8
IV (never *PsA*)	12
Diabetes mellitus: (*n*)	
Yes	5
No	34
NA	1

The CF diagnosis was confirmed genetically in all cases as part of the clinical routine and the results of mutation analyses and all other clinical data were obtained from patient records. When the patients were subdivided into groups according to CFTR genotype (A, homozygosity for ΔF508; B, severe/severe mutation, i.e., one class I, class II, or class III mutation on each allele including heterozygosity for ΔF508; C, one or two missense mutations, i.e., class IV or V mutations; D, one or two unknown mutations), no significant differences in lung function, bacterial colonization, or BPI-ANCA levels were seen between the genotype groups at inclusion [24].

The patients were followed prospectively until December 31, 2009. Endpoint was death or lung transplantation, treated as equal.

2.1. Statistical Analysis. Statistical calculations were performed using SPSS for Windows version 19. Survival curves

Table 2: *PsA* colonization, BPI-ANCA, and outcome. Adult CF-patients divided into groups based on Leeds classification and IgA BPI-ANCA levels at baseline and subsequent endpoints during followup.

Leeds classification	Leeds I + II			Leeds III			Leeds IV		
BPI-ANCA level	BPI−	BPI+	BPI++	BPI−	BPI+	BPI++	BPI−	BPI+	BPI++
Inclusion (*n*)	5	12	9	6**	1	1	6	5*	1
5 years % with endpoint	0	16.7%	33.3%	16.7%	0	0	0	20%	0
(*n* with endpoint)		(2)	(3)	(1)				(1*)	
10 years % with endpoint	0	33.3%	77.8%	33.3%	0	100%	0	20%	0
(*n* with endpoint)		(4)	(7)	(2**)		(1)		(1*)	
Last followup, % with endpoint	20%	41.7%	88.9%	33.3%	0	100%	0	40%	0
(*n* with endpoint)	(1)	(5)	(8)	(2**)		(1)		(2*)	

*Patient with colon cancer.
**Patient with *Burkholderia cepacia*.

were estimated using Kaplan-Meyer method. Log rank tests were used to compare survival between subgroups. Cox proportional hazard regression was used to estimate hazard ratios.

2.2. Lung Function. FEV1 was measured by spirometry at the Department of Clinical Physiology, Lund University Hospital, on a yearly basis, following the guidelines from the American Thoracic Society [25]. The results were expressed as proportion of predicted values (FEV1% pred) calculated according to Quanjer et al. [26] from the patients' height, age, and sex. The lung function was categorized into three groups based on the spirometry results: normal lung function, FEV1 >80% pred; moderate lung damage, FEV1 50–80% pred; and severe lung damage, FEV1 <50% pred.

2.3. BPI-ANCA. IgA BPI-ANCA was analyzed by ELISA and measured at the time of inclusion. IgA-BPI-ANCA was measured again after 5 and 10 years in those patients who until that date had not reached an endpoint. Purified BPI was obtained from Wieslab AB (Lund, Sweden) and direct binding was performed [27]. In short, antigens were coated onto microtiter plates at a concentration of 1 microliter/mL in a bicarbonate buffer. Serum samples were diluted 1/80 and incubated for one hour. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-human IgA (Eurodiagnostica, Malmö, Sweden). IgA-BPI-ANCA was quantified from a calibrator curve that was serially diluted and the results expressed as arbitrary units (U). The cut-off level for IgA BPI-ANCA was determined to be > or = 67 arbitrary units (U/L) from the mean absorbance value of 42 normal paediatric sera + 3SD [17] and levels over 200 (three times the positive level) were arbitrarily considered as high. The level 200 was chosen independently of results and was created to separate moderate BPI-ANCA levels from high levels.

2.4. Bacterial Colonization. Samples for respiratory secretion cultures were taken when the patient attended a routine outpatient visit. Sampling, transport, and culture were performed according to routine procedures. History of bacterial colonization was obtained from patient records as far back as possible, and colonization was defined according to the Leeds criteria [28].

3. Results

The cohort of 46 adult CF patients was followed prospectively from inclusion 1995–1998 up until December 31, 2009. Death and lung transplantation were defined as endpoints. In general, the entire Swedish CF population is doing better for each decade, and life expectancy for CF patients, as a group, increases over time. Consistent with this notion the present cohort had, as a group, a fairly well preserved lung function, with a mean FEV1% pred of 70% at inclusion. Only thirteen of the patients had, at the beginning of the study, a lung function of less than 50% FEV1% pred. Twenty-six of the patients were chronically colonized with *PsA* at the start of the study. Even though the follow-up time in this study was over ten years, in some cases even as long as 14 years, 27 patients were still alive and not transplanted, at the final followup (59%).

In total seven patients reached an endpoint within five years after inclusion and 15 within ten years (Table 2). The ten year result includes three patients who died and 12 patients who received a lung transplant. One of the patients who died acquired colon cancer when he had a relatively good lung function. He was included in the study in 1995 (FEV1 78% pred), but in connection to his operation and treatment for colon cancer, he became *PsA* colonized and his lung function deteriorated rapidly. There is no doubt that his CF lung disease contributed to his death, but the main cause of death was the colon cancer.

In the cohort there was only one patient colonized with *Burkholderia cepacia* at inclusion, and she was known to carry the bacteria from 1995. This patient had moderate lung damage for a very long time (FEV1 55% pred), which after pregnancy worsened and she was lung transplanted shortly after. None of the patients in the study was carrying MRSA.

In the cohort there were 26 male and 20 female patients. After five years three male and four female patients had reached an endpoint. After ten years five males and ten females were either dead or had received a lung transplant, and at the final followup nine males and ten females had experienced an endpoint.

Eleven patients had insulin treated diabetes mellitus when the study began. 34 were not diabetic and for one patient information about diabetes was not available. At the time of final followup five of the diabetic patients (45,4%) and fourteen of the nondiabetic patients (41,2%) had reached endpoint.

3.1. Leeds Classification and Long-Term Outcome. The well-known association between *PsA* colonization in CF patients and adverse clinical outcome can be seen also in this study (Table 2 and Figure 1(a)), but bacterial colonisation categorized by the Leeds classification was statistically not a significant determinant of outcome ($P = 0.113$). After ten years eleven (42%) out of the 26 patients belonging to Leeds I or II had experienced an endpoint, and at December 31, 2009, 54% were either dead or had received a lung transplant. Compared to this the patients who were free of earlier *PsA* (Leeds III) or who had never been infected with *PsA* (Leeds IV) did better. At the time of final follow-up only five of these patients (20%) had reached endpoint. The group of five patients includes the man with colon cancer and the woman with *Burkholderia cepacia*. These two patients are being marked by $*$ and $**$, respectively, in all tables.

3.2. BPI-ANCA Level Is More Informative Than Leeds Classification. The association between IgA-BPI-ANCA level at inclusion and an adverse outcome is evident from Table 2. After ten years 15 patients had reached an endpoint; out of these only two (13%) were IgA-BPI-ANCA negative at inclusion. The median IgA-BPI-ANCA level of all patients reaching an endpoint within ten years was 251 ELISA units as compared to 69 for the 31 patients who did not experience such an event.

In total, 16 out of the 29 (55%) BPI-positive patients were either dead ($n = 4$) or had been lung transplanted ($n = 12$) on the date for the final followup. Out of the 17 IgA-BPI-negative patients only one patient died and two received a lung transplant; one of them is the woman with *Burkholderia cepacia.*

In contrast to Leeds groups, IgA-BPI-ANCA level was significantly correlated to outcome. The hazard ratio for one standard deviation of BPI-ANCA, used as a continuous variable, was calculated to 1.76 (95%; CI: 1.25–2.48; $P \leq 0.001$). Also when comparing survival between subgroups based on IgA-BPI-ANCA as depicted in Figure 1(b) a significant result was obtained (log rank test, $P = 0.002$). Of particular interest is the finding that also when the analysis is confined to chronically colonized patients (Leeds I group), there is a significant hazard ratio for one standard deviation of IgA-BPI-ANCA (1.69; CI: 1.13–2.53; $P = 0.011$).

Chronic colonization with *PsA* is known to eventually lead to severe lung damage, as shown in earlier studies. Five patients in this cohort were colonized with *PsA* but had not developed IgA-BPI-ANCA at the time of inclusion (Table 2). None of these five patients reached endpoint within ten years, but one did before December 31, 2009. Their mean FEV1 at inclusion was 95% of predicted and after ten years their lung function was still surprisingly well preserved, mean FEV1 79% pred, considering that they are chronically colonized with *PsA,* a greater degree of deterioration in lung function would have been expected. After ten years of followup four of the patients still had not developed IgA-BPI-ANCA (one unknown), although they were all still *PsA* colonized. Four of these patients were pancreatic insufficient, one sufficient. As a comparison the mean FEV1 at inclusion among the chronically *PsA* colonized BPI-ANCA-positive patients was only 50% of predicted.

BPI-ANCA is associated with *PsA* colonization and in this cohort, out of the 26 patients who are categorized as belonging to Leeds I and II, 21 were IgA-BPI-ANCA positive. However, we also found six patients who were IgA-BPI-ANCA positive belonging to Leeds group IV which means that they had developed BPI-ANCA without being known to have harboured *PsA* (Table 2). One was the patient with colon cancer mentioned above. When studying the course of the remaining five we found that all remained in Leeds class IV after five years of followup, indicating that IgA-BPI-ANCA did not predict colonization in these cases. However, ANCA levels fell in all these individuals and were negative in three after 5 years. The patient in this group with the highest titre at inclusion (244 units) did actually acquire a *PsA* infection between 5 and 10 years of followup. Overall these five patients followed a favourable course; only one developed severe pulmonary insufficiency and received a lung-transplant 10.5 years after inclusion in the study.

3.3. Lung Function and Long-Term Outcome. Lung function at inclusion was a very important predictor for the long-term prognosis. As shown in Figure 1(c), none of the patients with a normal FEV1 at inclusion reached endpoint during the followup. Patients with a severe lung damage at inclusion reached endpoint to a very high degree, 11 out of 13 patients (Table 3). The hazard ratio for an increase in FEV1% pred with one standard deviation was 0.334 (0.18–0.60; $P \leq 0.001$).

A positive IgA-BPI-ANCA was associated with low lung function at inclusion (Table 3). Mean FEV1 in the whole BPI-ANCA+ group was 58% pred (median 60%) and in the IgA-BPI-ANCA-negative group 87% pred (median 93%). The moderate sample size and the association between low lung function and adverse outcome in this cohort make it difficult to analyze whether IgA-BPI-ANCA provides any additional information when FEV% pred is known. But it is of interest to note, however, that among patients with severe lung damage all patients with high ANCA levels reached end point within ten year as compared to 3 out 7 with lower values (Table 3). When looking at patients with moderate lung function impairment, disregarding the patients reaching endpoint from non-*PsA* associated causes, two out of five patients with high ANCA reached an endpoint

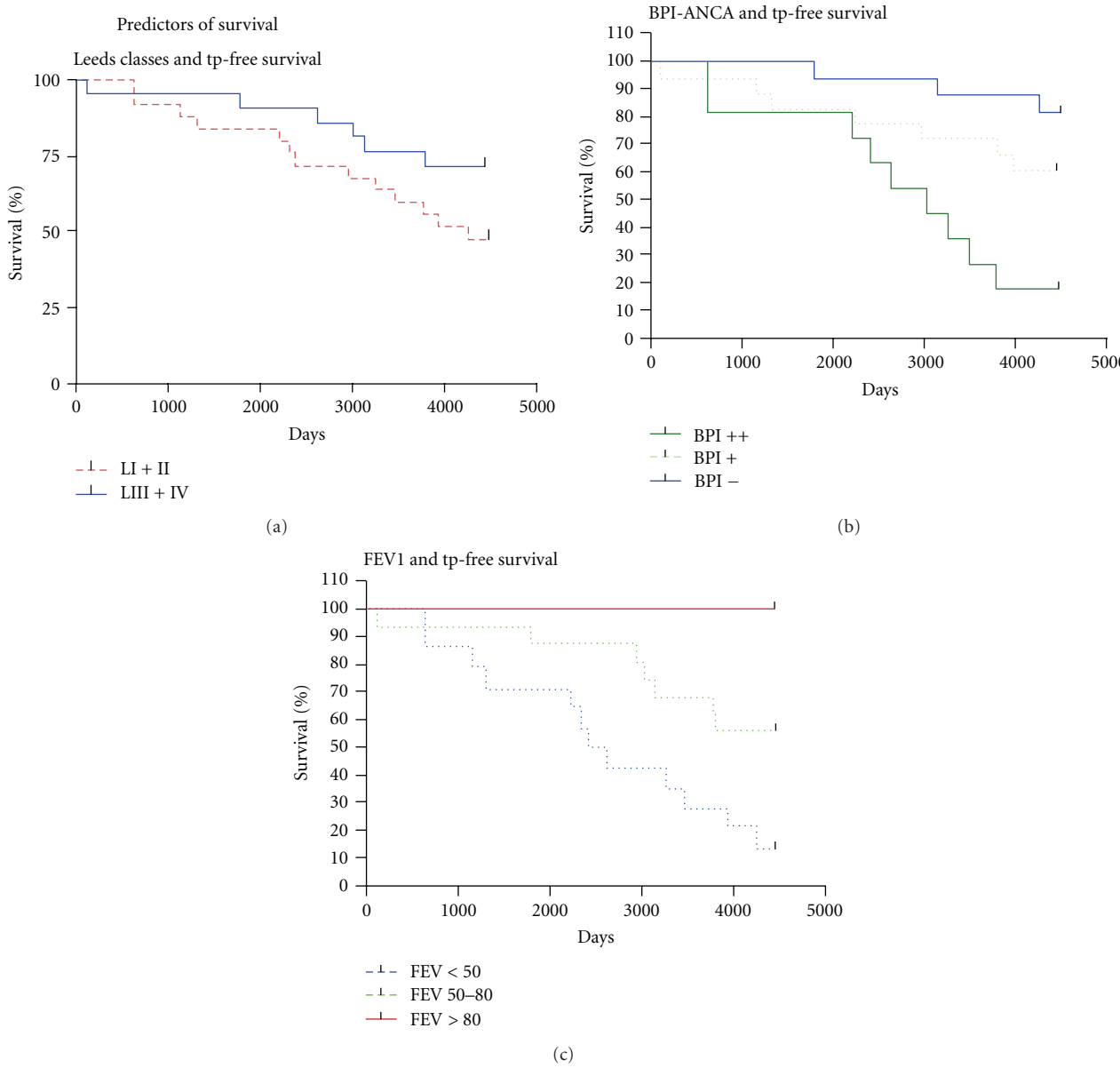

Figure 1: (a) PsA colonization and transplantation free survival. The blue line shows patients who are not chronically colonized with *PsA* (never had *PsA* or free from earlier colonization, Leeds class IV or III). The red line shows patients who are chronically or intermittently colonized with *PsA* (Leeds class I or II) (log rank test $P = 0.13$). (b) BPI-ANCA and days of transplantation free survival. The blue line indicates patients without IgA-BPI-ANCA, the dotted line patients with IgA-BPI-ANCA level of 67–200, and the green line patients with a BPI-ANCA above 200 at inclusion. There is a clear difference in transplantation free survival for BPI-negative patients compared to patients with a high BPI-ANCA (log rank test $P = 0.002$). (c) Lung function and transplantation free survival. Patients with FEV1 >80% pred, FEV1 between 50 and 80% pred, and FEV1 below 50% pred are indicated by red line, green dotted line, and blue dotted, respectively. None of the patients with a normal lung function at inclusion die or receive a lung transplant during over ten years of followup. The patients who have a low lung function at the start of the study experience a negative outcome to a very high degree (log rank test $P < 0.001$).

within ten years compared to two out of 10 patients with lower ANCA levels.

4. Discussion

In a disease as complex as CF tools to predict the prognosis would be very useful for the clinician, trying to find out which patient who best needs the most active therapy. In a world where the bacteria are getting more and more resistant and effective antibiotics are few it is important to use the pharmacological options we have at hand as effectively as possible and in an environmentally correct way.

This study shows that presence of autoantibodies to the neutrophil granule constituent bactericidal/permeability-increasing protein (BPI-ANCA) of IgA class is strongly associated with an adverse outcome. End-stage lung disease,

Table 3: Lung function, BPI-ANCA, and outcome. Patients divided into groups based lung function and IgA BPI-ANCA levels at baseline, and subsequent end-points during followup.

Lung function	FEV1 pred > 80%			FEV1 pred 50–80%			FEV1 pred < 50%		
BPI-ANCA group	BPI−	BPI+	BPI++	BPI−	BPI+	BPI++	BPI−	BPI+	BPI++
Inclusion (*n*)	11	5	0	5**	7*	5	1	6	6
5 years % with endpoint	0	0	—	20%	14.3%	20%	0	33%	33%
(*n* with endpoint)				(1)	(1*)	(1)		(2)	(2)
10 years % with endpoint	0	0	—	40%	28.6%	40%	0	50%	100%
(*n* with endpoint)				(2**)	(2*)	(2)		(3)	(6)
Last followup, % with endpoint	0	0	—	40%	42.8%	60%	100%	66.7%	100%
(*n* with endpoint)				(2**)	(3*)	(3)	(1)	(4)	(6)

*Patient with colon cancer.
**Patient with *Burkholderia cepacia*.

as indicated by death or lung transplantation, had after 10 years developed in 8 of 11 patients with high IgA-BPI-ANCA levels as compared to 2 of out 17 BPI-ANCA-negative patients. Overall there is a very strong correlation between levels of IgG and IgA-ANCA, even though some patients exhibit divergent results. For the sake simplicity we present in this study data only on IgA-BPI-ANCA, because our earlier studies have suggested that IgA-BPI-ANCA show a slightly better correlation with decreased lung-function.

Leeds classification is an established tool to categorize *PsA* colonisation and thus to predict outcome [28]. When comparing the predictive capacity of Leeds classification with BPI-ANCA we found that a BPI-ANCA test taken at a single occasion was significantly correlated to outcome while the classification based on series of sputum cultivations was not. The main reason for this difference was the fact that Leeds I patients with negative ANCA tests had a very good prognosis. A probable explanation for this is that presence of ANCA heralds that an unfavourable host-pathogen interaction has taken place.

Even though *Pseudomonas aeruginosa* infection is believed to be one main culprit for the progression of CF lung disease [29] a multitude of other factors influence the course. The complexity of the disease and the variability in phenotype make it difficult to foresee which patient is going to deteriorate in lung function. The *Burkholderia cepacia* complex is a well-known aggressive pathogen that can cause very fast deterioration in CF patients [30] and there are other pathogens where we are unsure about their capability of causing lung damage, for instance *Stenotrophomonas maltophilia* and different fungal infections. Complications to CF as diabetes mellitus and malnutrition play important roles in the progress of the disease and influence the deterioration of lung function [31–33]. Compliance to treatment is most probably another factor of importance. Even if BPI-ANCA heralds an unfavourable host-pathogen interaction with *PsA*, there are always a multitude of factors affecting outcome, deflating the statistical value of the predication based on the test. The number of patients in this study is unfortunately too low to do a multivariant analysis where all known factors could be compared, which is a shortcoming of this study.

In this cohort of 46 patients, IgA-BPI-ANCA, lung function, and *PsA* colonization have been followed over time, and we see patients who at the beginning of the study belong to one colonization group, but who over the time acquire a different status. The timely relation of when a patient acquires *PsA* and possibly thereafter IgA-BPI-ANCA would be interesting to study. It has been shown possible to influence the BPI-ANCA level by lung transplantation and sinus surgery [18] and the possible BPI-ANCA change after PsA treatment would be another issue to study.

PsA serologies are used to detect early infection in order to allow eradication of the bacteria before it becomes a chronic habitant in the lungs. Titres are also used to classify patients as chronic or not [24, 34, 35]. The relation between BPI-ANCA and different *PsA* serologies has so far not been studied. It is not known if BPI-ANCA yields the same set of information as available *PsA* serology tests or not. The fact that most ANCA-positive patients in the Leeds IV group remained free of PsA hints that BPI-ANCA is not a reliable tool to find early colonization. The finding that some patients in Leeds group I remained ANCA negative over a decade also suggests that BPI-ANCA is something else than *PsA* serology test. The relationship between BPI-ANCA and *PsA* serologies needs to be more thoroughly addressed.

5. Conclusion

The results of this study show that BPI-ANCA, a single serological test at one occasion, is a prognostic biomarker for the long-term outcome among adult CF patients and suggest that it has better ability to predict prognosis than a classification based on multiple sputum cultures. However, it is not clear if BPI-ANCA (or Leeds criteria) adds additional prognostic information regarding development of end-stage lung disease when the degree of lung function impairment is known. Larger studies are needed to establish the usefulness of BPI-ANCA in different clinical situations.

Acknowledgments

This work was funded by Bengt Andreassons foundation and the Swedish Heart Lung foundation.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 369546, 9 pages
doi:10.1155/2012/369546

Review Article

Clinical Relevance of Autoantibodies in Patients with Autoimmune Bullous Dermatosis

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Received 4 November 2012; Accepted 10 December 2012

Academic Editor: Dimitrios P. Bogdanos

The authors present their experience related to the diagnosis, treatment, and followup of 431 patients with bullous pemphigoid, 14 patients with juvenile bullous pemphigoid, and 273 patients with pemphigus. The detection of autoantibodies plays an outstanding role in the diagnosis and differential diagnosis. Paraneoplastic pemphigoid is suggested to be a distinct entity from the group of bullous pemphigoid in view of the linear C3 deposits along the basement membrane of the perilesional skin and the "ladder" configuration of autoantibodies demonstrated by western blot analysis. It is proposed that IgA pemphigoid should be differentiated from the linear IgA dermatoses. Immunosuppressive therapy is recommended in which the maintenance dose of corticosteroid is administered every second day, thereby reducing the side effects of the corticosteroids. Following the detection of IgA antibodies (IgA pemphigoid, linear IgA bullous dermatosis, and IgA pemphigus), diamino diphenyl sulfone (dapsone) therapy is preferred alone or in combination. The clinical relevance of autoantibodies in patients with autoimmune bullous dermatosis is stressed.

1. Introduction

The most frequent autoimmune bullous skin disorders are bullous pemphigoid (BP) and pemphigus vulgaris (PV). The diagnosis of both diseases relies not only on the clinical features but also on the detection of skin- or membrane-bound and circulating autoantibodies. We first diagnosed subepidermal bullous dermatosis in 1970 [1] by means of a direct immunofluorescence technique (DIF). We have subsequently examined, diagnosed, treated, and followed up several hundred patients with bullous skin diseases, and in this paper we present our experience in comparison with the literature findings.

2. Patients and Methods

Since 1970, we have diagnosed and treated 431 patients with BP (age range 38–102 years, mean 71.6 years), 14 children with juvenile BP (age range 3–14 years, mean 7.5 years), and 273 patients with pemphigus (age range 21–83 years, mean 53.9 years). All clinical investigations were conducted with the understanding and the consent of the patients. We are currently treating 47 patients with pemphigus and 45 with BP. The diagnoses were based on the clinical features and routine histological and immunohistological examinations [2]. For DIF tests, we used the intact skin adjacent to the bulla as substrate and antihuman IgG, IgA, IgM, and C3 conjugates labeled with FITC for antibody detection. For indirect immunofluorescence (IIF) examinations, we used esophagus samples from monkey and rabbit, and normal human skin; and for the salt split skin (SSS) tests, we applied normal human skin digested in 1.0 M NaCl solution [3]. Antibody detection was carried out with the same antihuman immunoglobulin (Ig) conjugates as for the DIF tests. The dilution of the sera was routinely 1 : 32. Western blot studies were performed according to Hashimoto et al., with slight modifications [4, 5]. The normal human skin pieces were incubated in 1.0 M NaCl at 4°C for 72 hours. The epidermis was then easily separated from the dermis and epidermis pieces were homogenized in a solution containing 31.2 mM Tris-HCl, 2% SDS, 1 mM PMSF, 2 mM EDTA, and 0.1 M dithiothreitol, and incubated for 24 hours at 4°C. The homogenizate was next centrifuged at 15000 g and the supernatants were stored at −70°C until use.

The constituent proteins of the epidermal or dermal extracts were separated by SDS-PAGE (with 6% separating gel) and then transferred to nitrocellulose before probing with the test sera. All sera were used to probe immunoblots at a dilution of 1 : 40. Specific binding by the sera was detected by using peroxidase-linked class-specific second antibodies (goat anti-human IgG and IgA) and visualized with diaminobenzidine. For ELISA studies, antigenic epitopes of BP antigens were predicted by Peptide Structure and Plot Structure software, and the predicted peptides were chemically synthetized and screened with the use of serum from BP patients. The best antigenic epitopes were inserted as monomer and homo- and hetero-oligomer forms into fusion-expression plasmids inframe to the C-terminus of glutathione-S-transferase. Fusion products were expressed in *E. coli* cells and purified by affinity chromatography. The recombinant proteins were used [6, 7] for the detection of antibodies in the sera of BP subjects and controls (healthy persons or patients with PV or other bullous dermatoses). More recently, we have applied commercially available ELISA tests for the detection of the main autoantibody entities (MESACUP BP180 and BP230 tests desmoglein 1 (Dsg1) and desmoglein 3 (Dsg3) tests; MBL Medical and Biological Laboratories, Nagoya, Japan).

3. Results and Discussion

3.1. Autoantibodies in Pemphigoid. The diseases of the pemphigoid group are associated with tissue-bound and circulating autoantibodies against the protein components of hemidesmosomes. BP230 is an intracellular protein of 230-kDa that belongs to the plakin family of cytolinkers [8, 9]. The protein takes part in the linkage of the keratin filament network to the basal cell side [8–10]. The other autoantigen, BP180, is a transmembrane glycoprotein with extra- and intracellular parts. The extracellular portion consists of 15 collagenous and 16 noncollagenous subdomains and is also called collagen XVII [11]. The 16th noncollagenous domain (NC16A), which is localized just outside the cell membrane of the basal keratinocyte, is the immunodominant component of BP180. It serves as a cell-surface receptor, contributing to the maintenance of dermoepidermal cohesion by binding to laminin 5 [8]. BP180 is probably also involved in epidermal differentiation by facilitating the detachment of keratinocytes from the basal cell layer [12]. Autoantibodies against BP180 and BP230 mainly belong to subclasses IgG1, IgG4, and IgE. Antibodies against BP180 are responsible for the initial blisters, and anti-BP230 antibodies enhance the inflammatory reaction.

Autoantibodies in sera from epidermolysis bullosa acquisita (EBA) patients will bind to a 290-kDa band, the alfa chain of collagen VII, whereas this is not the case with sera from all other primary blistering diseases. A second band, of 145 kDa, will often be labeled with EBA antibodies. In most patients with linear IgA dermatoses (LABD), the recognized target antigens are constituted by 97-kDa or 120-kDa proteins, two fractions of BP180. LABD is characterized by circulating IgA antibodies against a 230-kDa antigen of dermal extracts, while in patients with IgA pemphigoid there is no reactivity against this antigen [13].

In mucous membrane pemphigoid (MMP), the target antigens vary; subsets of patients affected exclusively by oral and ocular mucosal diseases have autoantibodies targeting BP180, laminin332, $\alpha 6\beta 4$ integrin, laminin311, and BP230 [14, 15]. Our investigations have demonstrated that $\alpha 6$ integrin antibodies can be identified in patients with BP who do not display mucous membrane symptoms in the oral cavity [16]. In herpes gestationis (HG), the target antigen is BP180 NC16A and antibody against BP230 can also be demonstrated.

3.2. Autoantibodies in Pemphigus. Most types of pemphigus antibodies are directed against the desmosomal cadherins known as Dsg1 and Dsg3. The PV antigen is a protein complex of 230 kDa which contains Dsg3 with a molecular weight of 130 kDa and plakoglobin with a molecular weight of 80 kDa. These two proteins are colligated by a disulfide bridge. Dsg3 is expressed primarily in the basal epidermal layers throughout the mucosa, while Dsg1 is expressed primarily in the upper levels of both the mucosa and the epidermis. Dsg3 alone is therefore able to maintain the mucosal integrity, and the impairment of Dsg1 does not cause mucosal lesions. If the patient has only mucocutaneous symptoms, anti-Dsg3 antibodies can be detected. In the event of skin and mucous membrane involvement, antibodies can be found against both Dsg1 and Dsg3. In PV there are always antibodies against Dsg3, while in pemphigus foliaceus (PF) only Dsg1 can be detected. In cases of paraneoplastic pemphigus, there are autoantibodies against some other desmosomal proteins, such as desmoplakin1, desmoplakin2, envoplakin, periplakin, plectin, BP230, BP180, and a not-further-characterized 170 kDa protein [17].

3.3. Diagnostic Possibilities in Patients with Autoimmune Blistering Diseases. The diagnosis of autoimmune blistering diseases can be confirmed by histological and immunopathological studies [18]. The exact diagnosis and the differential diagnosis rely on the cooperative work of the clinician and the histopathologist, but up-to-date laboratory tests are also indispensable. The localization of the bulla formation plays an important role in the diagnosis of these diseases.

BP is most commonly seen in the elderly [19]. The lesions are tense blisters that occur either on healthy skin or on an erythematous base. The histological features of BP include a subepidermal blister with an inflammatory infiltrate that is often rich in eosinophils, but also contains lymphocytes, histiocytes, or neutrophils (Figure 1(b)). These can also be observed in several other related conditions, and therefore further diagnostic testing is essential. DIF investigations should be performed on healthy or erythematous nonbullous perilesional skin where linear basement membrane zone deposits of Ig-s (mainly IgG, and more rarely IgA, IgM, and IgE) and C3 are visible (Figure 1(c)). Similar DIF findings can also be seen in several other autoimmune blistering diseases, including EBA, cicatricial pemphigoid (CP), HG, and bullous eruption of systemic lupus erythematosus (VBSLE).

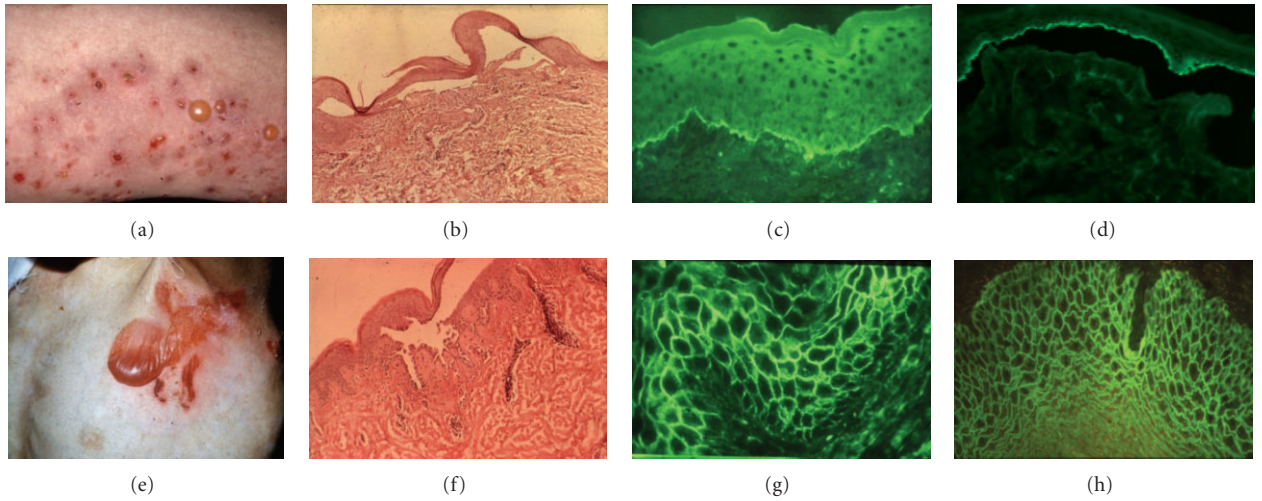


Figure 1: Bullous pemphigoid and pemphigus vulgaris. (a) The clinical picture of generalized pemphigoid with tense blisters, crusts, and erythematous plaques. (b) The histological picture of BP; subepidermal blister. (c) Linear basement zone deposits of IgG in BP. (d) Circulating IgG antibodies bind to the epidermal site of SSS in a patient with BP. (e) The flaccid blister in PV. (f) The histological picture of PV with intraepidermal blister. (g) DIF analysis demonstrates binding of C3 to the intercellular cement substance (ICS) in the upper stratum Malpighii. (h) An IIF study with monkey esophagus as substrate demonstrates the presence of anti-ICS antibody with antihuman IgG conjugate.

IIF studies using SSS are therefore necessary for an appropriately complete evaluation of these patients [19]. SSS studies reveal that patients with BP and HG have circulating IgG antibodies attached to the epidermal side of the SSS (lamina lucida), while patients with EBA and VBSLE have circulating IgG antibodies that bind to the dermal side of the SSS (lamina densa and the anchoring fibril zone) (Figures 1(d) and 4(d)). Some patients with CP exhibit circulating IgG antibodies that bind to both the epidermal and the dermal side of the SSS preparation; these patients have the antilaminin-5 variant. An entity of the pemphigoid group is the lamina lucida type of linear IgA disease (IgA pemphigoid), characterized by IgA autoantibodies against BP180. Csorba et al. [20] strongly suggest that IgA pemphigoid and IgG BP are the two endpoints of the clinical spectrum of an immunological loss of tolerance against the components of hemidesmosomes, mediated by both IgG and IgA autoantibodies [20]. Previous studies have demonstrated that BP autoantibodies react predominantly with two distinct proteins of the hemidesmosomes, BP230, and BP180 [6]. Through western blotting, the presence of these two autoantibodies can be demonstrated. Western blot analysis can be useful in establishing the diagnosis of EBA and LABD. cDNAs of both BP autoantigens have been isolated and the amino acid sequences for these antigens have been deduced. From this point, several attempts have been made to diagnose bullous dermatosis with ELISA technology by the use of recombinant proteins [6, 7, 17, 21]. The sensitivity of our technique with ELISA assays of the serum of BP patients was 90% [6]. Other investigators who utilized different antigens and commercial kits reported very similar results [21]. However, the sensitivity can be increased up to 100% when various ELISA assays are applied to the NC16A domain and other extracellular portions of BP180 or BP230 or both. ELISA tests have nowadays largely replaced immunoblotting and immunoprecipitation techniques which are technically much more demanding.

PV, the most common form of pemphigus, affects a younger population. PV can involve the skin and/or mucous membranes. Histological studies on cutaneous and mucosal biopsies reveal acantholysis in the suprabasilar part of the epidermis (Figure 1(f)). In PF, the blister formation is subcorneal. DIF studies typically demonstrate the binding of IgG and/or C3 to the intercellular cement substance (ICS) in the upper stratum Malpighii (Figure 1(g)). Both clinically and immunopathologically, IgA pemphigus is a unique entity. DIF studies have identified ICS reactions with IgA and C3. IIF studies with the use of epidermal substrate (monkey or rabbit esophagus) as substrate allow the detection of the presence of anti-ICS antibody with IgG and C3 in PV and PF, but with IgA and C3 in IgA pemphigus (Figure 1(h)).

The sera of patients with PV typically bind to a 130-kDa protein (Dsg3). The binding of sera to a 160-kDa protein (Dsg1) is seen in patients with PV, but to desmocollin 1 and to Dsg3 in patients with IgA pemphigus. Autoantibodies in paraneoplastic pemphigus typically target Dsg3 and proteins of the plakin family, including envoplakin, periplakin, plectin, desmoplakin, BP180, BP230, and a not-further-characterized 170-kDa protein [17]. In Western blot analysis, therefore we can see a "ladder" configuration (Figure 2(a)).

The recombinant ectodomains of Dsg1 and Dsg3 have been utilized to develop highly sensitive and specific ELISA assays. While the Dsg ectodomains are expressed in insect cells in two of the ELISA systems (MBI, Nagoya, Japan), the ectodomains in the other two available ELISA tests are generated in human HEK293 cells (Euroimmun, Lübeck, Germany) with a high potential of correct expression of

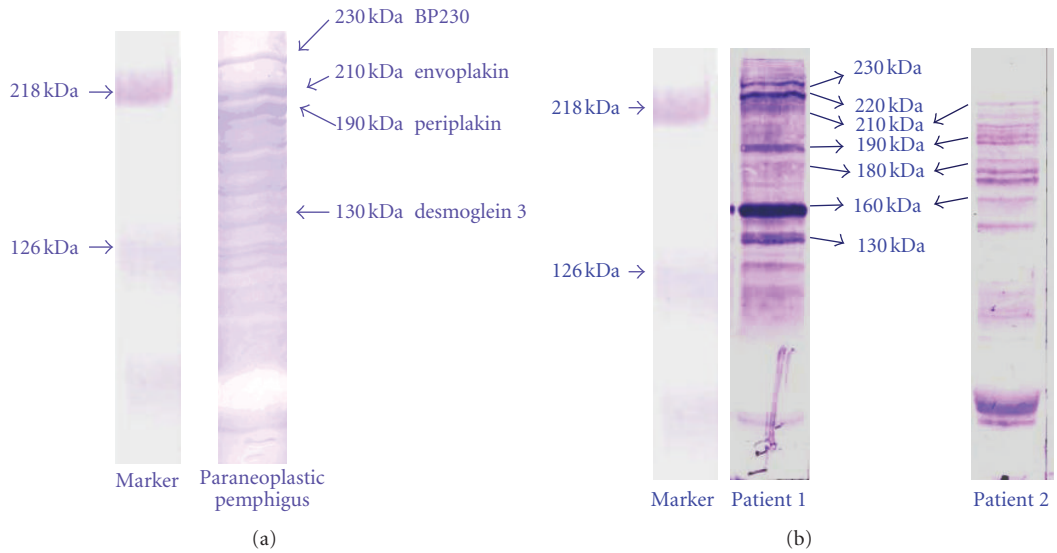


Figure 2: Western blot investigations. (a) A "ladder" configuration can be seen in paraneoplastic pemphigus. (b) A "ladder" configuration of paraneoplastic pemphigoid on western blot analysis.

conformational epitopes and the advantage that only the mature protein forms are expressed [17]. The ELISA systems are not only suitable for the diagnosis of the pemphigus group, but also useful in monitoring serum autoantibody levels during the course of the disease. In paraneoplastic pemphigus, an ELISA test based on the recombinant N-terminus of envoplakin and periplakin has been developed. It has been shown that the ELISA reactivity correlates with the disease activity of pemphigus patients.

3.4. Clinical Aspects of BP. BP diseases are subepidermal blistering illnesses with an incidence of 10 new cases per 1 million persons per year [19]. BP is characterized clinically by generalized, pruritic tense blisters, and crusts, usually on erythematous or apparently normal skin, together with infiltrated and urticarial plaques, papules, or eczematous lesions. The symptoms are most often symmetric and are located predominantly on the trunk and proximal extremities. Involvement of the oral cavity has been described in 10–30% of the cases [21]. BP sometimes begins with a nonbullous phase and nonspecific symptoms such as intense pruritus, excoriations, and urticarial or papular lesions, when several other diseases may be suspected, for example, eczema, a drug reaction, or chronic prurigo.

BP can be classified into two main groups: typical and atypical pemphigoid. In the typical type, generalized, localized, seborrheic, mucous membrane and paraneoplastic variants can be distinguished**.** Generalized pemphigoid is the most common form of the disease, with dozens to hundreds of blisters, usually affecting the elderly; the average age is 65 years (Figure 1(a)).

In most cases in the BP group we found the linear basement membrane zone deposition of IgG and C3 (365 patients), but in 20 patients linear basement membrane zone reactions with IgA and also with C3 were visible, and in 33 patients only C3, the third component of the complement could be observed. In 13 patients there was linear basement fluorescence with all Ig's and also with C3.

The localized form is characterized by some solitary eruptions on the head or on the extensor surface of the extremities, without causing complaints

In certain cases of localized pemphigoid, it is not necessary to use systemic corticosteroids; topical corticosteroid therapy can lead to total recovery.

Seborrheic pemphigoid generally presents in older women with erythema and crusting in the seborrheic area [22].

In MMP, also known as CP, the oral cavity, conjunctiva, and laryngeal, nasal, or genital mucosa may be involved. With the exception of the oral mucosa, it usually heals with scars. In the mouth, desquamative gingivitis is the most frequent form. In cases of eye involvement, blindness can be the most serious complication, due to scarring.

Since the 1970s, we have had 37 patients with tumors (gastrointestinal, gynecological, urological, pulmonary, or endocrine) in BP. In some of these cases, the "ladder" configuration was seen on immunoblot analysis, between the typical 180 and 230 kDA bands of BP; the linear basement zone deposition of C3 was detected only with DIF (Figure 2(b)). The presence of a paraneoplastic type has been questioned in the literature in recent years. Some reports have indicated an increased frequency of certain cancers (such as digestive tract, lung) and lymphoproliferative disorders [23–26]. In contrast, other authors have concluded that there is no such connection [1, 27, 28].

In some of our patients, we observed linear basement zone deposits of IgA together with deposits of C3; in these cases, antibodies against BP180 and BP230 could be detected. We consider that this type should be distinguished from LABD, which is a unique entity both clinically and by western blot analysis.

The atypical group of pemphigoid includes LABD, linear IgM dermatosis, HG, VBSLE, EBA, and juvenile BP or drug-induced pemphigoid [29].

LABD is predilected on the face, especially periorally, on the scalp, and around the ears. Initially, pruritic, erythematous plaques and papules occur, which later display an annular or herpetiform pattern. New blisters may appear around an older lesion, forming a rosette-like shape [13]. We have encountered 2 patients with LABD.

HG, which occurs most commonly in the third trimester of pregnancy and rarely in the postpartum period, causes intense pruritus. Many patients exhibit urticarial plaques, papules, or multiform lesions on the abdomen, but small herpetiform vesicles are rare, and differential diagnostic problems can therefore arise.

In EBA, inflammation predominates at the beginning, after which scarring and fragility of the skin can be seen. The clinical signs are located at the pressure-exposed areas (Figures 3(a), 3(b), 3(c), and 3(d)). We have found 3 patients with EBA during the past 40 years. This is caused by IgG antibodies against type VII collagen [30, 31].

Infants and children can also be affected; the signs of juvenile BP are similar to those of bullous impetigo (Figures 4(a) and 4(b)). We have treated 14 children with juvenile BP, most of whom exhibited linear basement deposits of IgA. The cause is unknown, though vaccination and drug intake may be assumed in some cases [32, 33].

Subjects with VBSLE have small grouped blisters on the light-exposed skin (Figure 4(c)), with antibodies against type VII collagen or laminin 5. Three of our patients also have SLE [34, 35] and in 1 patient Sjögren's syndrome is associated with bullous symptoms [36].

3.5. Clinical Aspects of Pemphigus Diseases. Diseases in the pemphigus group are rare, but often life threatening; they manifest intraepidermal blistering with suprabasal acantholysis. The various forms of pemphigus are differentiated on the basis of their clinical, immunopathological, and molecular biological features [2, 18]. They can be divided into two groups: typical and atypical pemphigus. Within the typical group, PV, PF, pemphigus seborrheicus (PS), pemphigus vegetans, and pemphigus erythematous (PE) can be distinguished.

The most common form is PV, which is characterized by extensive flaccid blisters, mucocutaneous erosions, and a hemorrhagic crust (Figure 1(e)). The mucocutaneous signs usually appear earlier than the skin problems; the content of the bulla is straw colored. The site of predilection is the face; while the areas of mechanical irritation such as the intertriginous regions, shoulders, elbows, buttocks, and back are also affected. The blisters tend to spread peripherally. PV occurs most often in the middle aged, generally starting with easily rupturing blister formation in an uninflamed skin area. The provoked Nikolsky' sign is positive. The skin lesions are less pruritic and often painful.

Pemphigus vegetans is suspected of being the infectious form of PV [37], where purulent plaques and granulomatous vegetation appear. Typical locations of pemphigus vegetans are the axilla, the perianal region, the genital tract, the nasolabial folds, or the scalp. Most of our cases proved to be PV, but we have had 9 patients with PF, 8 patients with PS, 2 patients with pemphigus herpetiformis (PH), 2 patients with paraneoplastic pemphigus, and 2 patients with pemphigus vegetans.

PF is the superficial and less severe variant of PV [38]. The symptoms can be seen primarily on the scalp, the face, and the chest, as flaccid blisters which easily rupture and evolve into fine sheets of scales.

PE, also called Senear-Usher disease, is similar to PF, but characterized by an additional "lupus band" of granular deposits of IgG and complement along the epidermal basement membrane zone. The clinical symptoms begin with erythematous patches on sun-exposed areas.

The atypical pemphigus group comprises drug-induced, paraneoplastic, IgA pemphigus and PH.

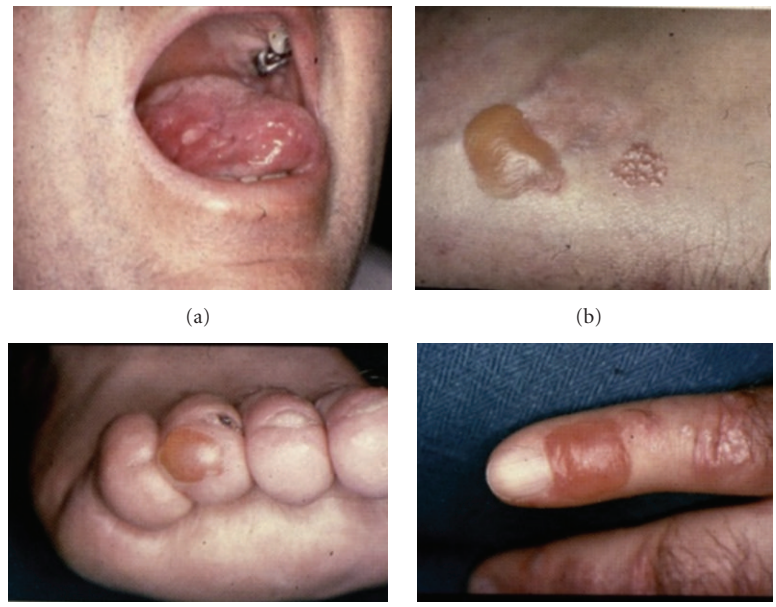
Drug-induced pemphigus can be caused by penicillamine, angiotensin-converting enzyme inhibitors, and pyrazolone derivatives. It displays the symptoms of PF or PE.

In paraneoplastic pemphigus, there are polymorphic cutaneous lesions ranging from blisters to erosions, and even denudation on the trunk and the extremities, but also on the palms and soles. Severe, hemorrhagic, painful oral erosions are typical. This form tends to be associated with hematologic neoplasms. We have diagnosed 2 patients with this disease.

IgA pemphigus has two variants. The subcorneal pustular type is characterized by flaccid pustules which coalesce, leading to annular or polycyclic scaled lesions. The disease which is often associated with IgA gammopathy has a good prognosis. The second intraepidermal neutrophilic type is localized to the intertriginous regions and trunk.

PH combines the clinical features of dermatitis herpetiformis with the immunopathological features of PV. The patients have small grouped vesicle and experience intense pruritus.

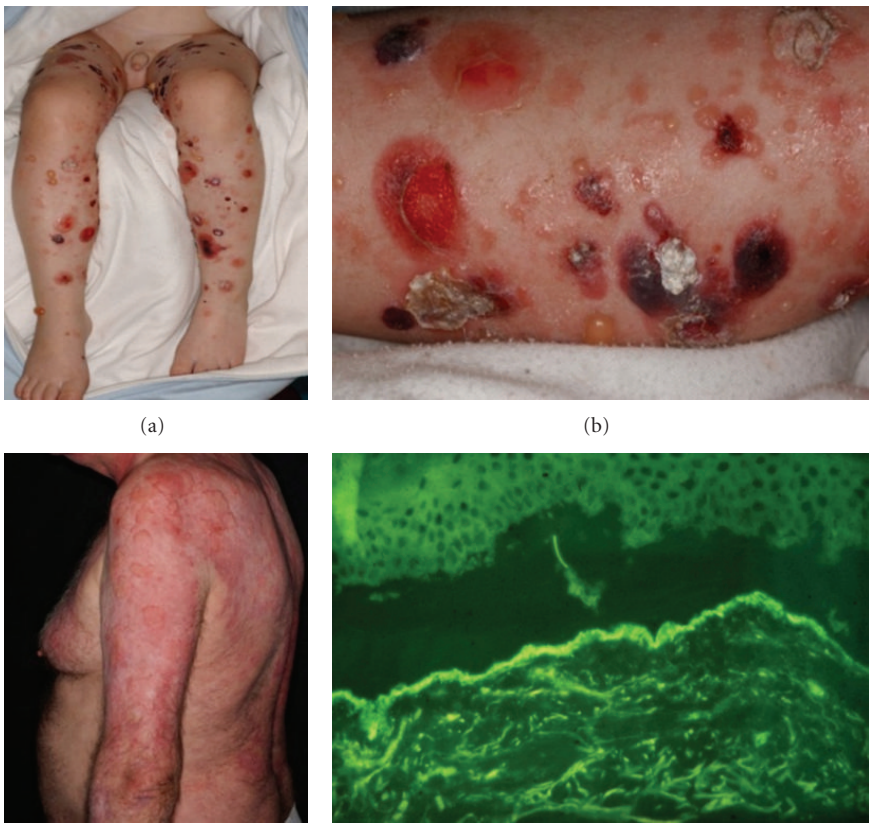
3.6. Therapy. For the treatment of autoimmune bullous diseases, we use immunosuppressive therapy and systemic corticosteroid as recommended in the literature [39]. In pemphigus we prescribe a higher dose (1.2 mg/body weight/day), and in pemphigoid a lower dose (0.75 mg/body weight/day) of corticosteroid, if necessary supplemented with azathioprine. Gastroprotective medication and potassium and calcium replacement are also regular. We gradually reduce the dosage of the immunosuppressants. In both pemphigus and bullous pemphigoid, when progression stops (usually after 2 weeks, when new blisters no longer appear), we reduce the corticosteroid dose, with alternation of administration of the starting dose and half of the starting dose every second day. Then, in line with the patient's clinical symptoms, we continue the reduction of the corticosteroid dose, usually at weekly intervals, maintaining the situation that the higher dose is always alternated with a dose that is 50% lower. When the higher dose reaches 30 mg/day, we reduce the lower dose to zero. The rate of dose reduction



(a) (b)

(c) (d)

Figure 3: Epidermolysis bullosa aquisita. (a) Erosions in the oral cavity in EBA. (b) Blisters on the wrist in EBA. ((c) and (d)) Blisters on the fingertips in EBA.



(a) (b)

(c) (d)

Figure 4: Juvenile bullous pemphigoid and bullous eruption of systemic lupus erythematosus. ((a) and (b)) Tense blisters and hemorrhagic crusts on the extremities of a 5-year- old boy with juvenile BP. (c) Erythematous plaques and some blisters in a VBSLE patient. (d) Circulating IgG antibodies bind to the dermal site of SSS in VBSLE.

and the final dose can depend on the patient's status: faster in pemphigoid, and slower in pemphigus, during a period of some months or even years, until the definitive maintenance dose is reached. Finally, the maintenance corticosteroid dose (in pemphigoid generally 15–20 mg, and in pemphigus 25–30 mg corticosteroid) is administered only every second day. Thus the difference in the mode of reduction of the doses from the literature recommendations is that we reduce the corticosteroid dose intermittently by alternating between a lower and a higher corticosteroid dose, together with progressive dose reductions. Because of this, we observe fewer patients with severe corticosteroid side effects, the clinical signs can be kept in equilibrium, and even a symptom-free status can be achieved.

It may occur that the corticosteroid therapy alone is not sufficient for the attainment of the complete remission. In these cases, and especially in pemphigus, we supplement the therapy with 2.5 mg/body weight/day of azathioprine. In severe or certain special cases, other medicaments and therapies may be suggested, in accordance with the literature (cytostatic drugs, intravenous Ig, and plasmapheresis). Novel targeted treatments such as immunoadsorption or rituximab, an anti-CD20 monoclonal antibody, have recently proven to be highly effective in severe and refractory autoimmune blistering diseases [40]. In patients with IgA autoantibodies we use diamino diphenyl sulfone therapy, alone or in combination.

4. Conclusions

Our experience of more than 40 years with several hundred patients with autoimmune bullous disease may be summarized as follows.

(1) In the BP group, DIF most frequently reveals linear deposits of IgG and C3 at the dermoepidermal junction, but sometimes linear basal membrane zone deposits of IgA and C3. These cases correspond to classical BP both clinically and by western blot assay, and in some of them circulating antibodies are detected with antihuman IgA conjugate on IIF analysis. We regard these cases as IgA pemphigoid. LABD, which also manifests the linear deposition of IgA, is a separate, unique entity with very low prevalence. Via the typical clinical symptoms and western blotting it can be well differentiated from IgA pemphigoid. We consider it is reasonable to list IgA pemphigoid in another group. Almost all of our juvenile BP cases were IgA pemphigoid. Diamino diphenyl sulfone has a role in the treatment of both diseases.

(2) Regarding the literature argument as to whether BP is a paraneoplastic disease [1, 23–26, 28], we agree with those [27] who reject its paraneoplastic nature, because BP is a disease of the elderly, in whom malignant tumors appear more frequently irrespectively of BP. However, it is a fact and our cases also prove that systemic or local malignant tumors do occur in numerous cases. Immunological examination of these cases revealed that in most cases only linear C3 deposits were visible on DIF, with a "ladder-like" configuration on western blotting in some cases similarly as in paraneoplastic pemphigus. Following such an immunological result, a tumor search always yielded a positive result. Removal of the tumor influenced the prognosis of BP significantly, and the maintenance dose could be attained earlier. For these cases, we use the expression paraneoplastic pemphigoid.

(3) The ELISA technique has practical importance in the primary diagnosis of both BP and pemphigus. Our investigations have indicated that the methodically chosen antigenic epitope or a combination of epitopes can substitute the total antigen structure immunologically and their production can be solved with the recombinant fusion technique [6, 7]. The usefulness of these antigenic epitopes is proved by the systematic provocation of BP-like symptom in mice [41]. Useful commercial antigens are currently available for the ELISA test.

(4) With a new method for the treatment of both BP and pemphigus, the corticosteroid side effects were greatly reduced, without loss of the therapeutic effect. We suggest administration of the maintenance corticosteroid dose only every second day in both bullous diseases.

(5) The autoantibodies have an outstanding role in both the diagnosis and the differential diagnosis. The detection of tissue-bound autoantibodies by DIF remains of great value in the diagnosis of bullous dermatoses; the ELISA technique is now playing a major part. The detection of circulating autoantibodies (IIF, western blotting, and SSS) is important from the aspect of the differential diagnosis of certain special disease forms.

(6) In brief, the technique developed by Lever [42], and Jordon et al. [43, 44] in the mid-20th century for the diagnosis of autoimmune bullous diseases basically remains in place. With the subsequent refinements of that technique, all of the special forms of the various autoimmune bullous diseases can be unambiguously diagnosed.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 432176, 5 pages
doi:10.1155/2012/432176

Research Article

Atrioventricular Conduction Delay in Fetuses Exposed to Anti-SSA/Ro and Anti-SSB/La Antibodies: A Magnetocardiography Study

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Received 1 October 2012; Revised 27 November 2012; Accepted 27 November 2012

Academic Editor: Dimitrios P. Bogdanos

Background. The presence of anti-SSA/Ro and anti-SSB/La antibodies during pregnancy is associated with fetal congenital heart block (CHB), which is primarily diagnosed through fetal echocardiography. Conclusive information about the complete electrophysiology of the fetal cardiac conducting system is still lacking. In addition to echocardiography, fetal magnetocardiography (fMCG) can be used. fMCG is the magnetic analogue of the fetal electrocardiogram (ECG). *Patients and Methods*. Forty-eight pregnant women were enrolled in an observational study; 16 of them tested positive for anti-SSA/Ro and anti-SSB/La antibodies. In addition to routine fetal echocardiography, fMCG was used. Fetal cardiac time intervals (fCTIs) were extracted from the magnetic recordings by predefined procedures. ECGs in the neonates of the study group were performed within the first month after delivery. *Results*. The PQ segment of the fCTI was significantly prolonged in the study group ($P = 0.007$), representing a delay of the electrical impulse in the atrioventricular (AV) node. Other fCTIs were within normal range. None of the anti-SSA/Ro and/or anti-SSB/La fetuses progressed to a more advanced heart block during pregnancy or after birth. *Conclusion*. The study identified a low-risk population within antibody positive mothers, where PQ segment prolongation is associated with a lack of progression of the disease.

1. Introduction

Fetal exposure to anti-SSA/Ro and anti-SSB/La antibodies is associated with the development of congenital heart block (CHB). The incidence is about 2% in primigravid mothers. The risk is five to tenfold higher in women who previously had an affected child with either CHB or a neonatal lupus rash [1, 2]. Fetuses with CHB carry high rates of mortality (20%) and morbidity (>60% of the surviving children require a permanent pacemaker in adulthood) [3, 4].

Risk factors associated with a poor outcome are gestation <20 weeks, ventricular rate ≤50 bpm, hydrops, and impaired left ventricular function [4, 5]. In addition to the expected course of the disease, life-threatening cardiomyopathy is also found in the offspring in 10%–15% of cases and can occur in utero or postnatally [6].

The pathogenesis of the disease is presumed to involve transplacental maternal IgG autoantibodies, which cause an atrioventricular (AV) delay. However, the first manifestation may also be sinus node dysfunction, atrial or ventricular

ectopies or bundle branch blocks, but also junctional ectopic tachycardia or ventricular tachycardia [7]. Regarding the pathogenesis of these variable expressions of immune-mediated fetal cardiac disease, the spectrum is diverse. Alterations in the selective expression of calcium ion channels as well as accumulation of apoptotic cells were discussed [7–9].

Current methods to monitor fetal heart function are based on echocardiography, Doppler, and tissue Doppler techniques, which provide indirectly through the mechanical assessment information of the fetal heart rhythm. Arrhythmias can be roughly classified, but details of the cardiac electrophysiology are incomplete.

Zhao and colleagues [10] reported underdetection in approximately 30% of cases with paroxysmal brief arrhythmias (junctional or ventricular tachycardia) associated with isoimmune CHBs using echocardiography.

To improve the complete electrophysiological assessment, further approaches to cardiac monitoring in these fetuses are needed. Fetal magnetocardiography (fMCG) is a new, noninvasive, and preclinical method, which can be used in addition to echocardiography. The groundwork for MCG analysis was built on different MCG devices primarily constructed for adults. Cardiac time intervals (CTIs) can be determined in an ECG-like fashion by recording magnetic fields generated by electric currents in the fetal heart. In addition, this method allows the detection of heart rhythm, heart rate trends, signal amplitudes, and unsuspected arrhythmias [11, 12].

Therefore, the aim of our study was to evaluate whether fMCG can be used to detect *early* electrophysiological signs of atrioventricular delay in antibody-exposed fetuses. The primary endpoint of the study was the prolongation of atrioventricular conduction.

2. Patients and Methods

2.1. Patient Population. Data collection for this controlled observational study at the fMEG Center Tübingen was completed in July 2012. Baseline characteristics of all 48 patients were evaluated with regard to medical history, previous pregnancy outcomes, and medication intake.

Sixteen fetuses of pregnant women were included in the study group. These patients were measured up to four times. Therefore, each measurement of the study group was matched by gestational age to one of the control group.

At study entry, all patients of the study group fulfilled the following inclusion criteria: presence of anti-SSA/Ro and/or anti-SSB/La antibodies tested by an enzyme linked immunosorbent assay (ELISA) and/or an immunofluorescence test, an immunodiffusion test and dot blots by a commercial laboratory. Rheumatologic disease was diagnosed by a rheumatologist. There was no limit concerning the duration of medication intake. Pregnancies >20 weeks of gestation with a normal heart beat and a structural normal heart were included.

Healthy women with uncomplicated pregnancies and normally developing fetuses served as controls. Neonatal outcome including normal fetal heart rate was assessed by a pediatrician.

Exclusion criteria for all neonates were chromosomal abnormalities, malformations, congenital infections, and/or acidosis at birth (umbilical artery cord gas pH < 7.0 or APGAR score after 5 minutes <5).

The study was approved by the ethics review board of the University Hospital Tübingen. Informed written consent was obtained from each subject.

2.2. Methods. At the beginning of the study, conventional echocardiography was performed on the study group to evaluate structural cardiac abnormalities, myocardial function, and fetal heart rate, in addition to the regular ultrasound check.

fMCG measurements were also performed on the study group and on the control group. Each measurement was matched to one from a healthy fetus based on the gestational age (GA). fMCG analysis was conducted by three blinded observers.

Prior to the beginning of each fMCG measurement, ultrasound was performed in all patients to check the fetal position and localise the fetal heart. Furthermore, cardiotocography (CTG) was performed over a 20-minute period to obtain complete information about the health of the fetus.

2.3. Measurement Technique. fMCG is a noninvasive method for recording magnetic fields generated by the electric currents of the fetal heart [12]. It records magnetic fields generated by electrical currents in the fetal heart with highly sensitive sensors, so-called superconducting quantum interference devices (SQUID). SQUID sensors enable the display of fetal CTIs and provide detailed beat-to-beat analysis. The fMCG recordings were acquired using a 156-channel biomagnetic system (SARA system, VSM Med Tech Ltd. Port Coquitlam, Canada) for 15–45 minutes at a sampling rate of 1220.7 Hz. The data were analysed afterwards according to a recently implemented procedure for the fMEG-system: a bandpass filter was used between 1 and 100 Hz. The maternal MCG signal was detected and removed by signal space projection [13–15]. The fetal heart signal was detected, and the R peak was marked using the same technique. The marked fetal signals were averaged with a pre- and post-trigger-interval to extract the fMCG trace.

The time points identified were used to calculate the duration of the CTIs as follows:

P wave = $P_{\text{end}} - P_{\text{onset}}$, QRS complex = $\text{QRS}_{\text{end}} - \text{QRS}_{\text{onset}}$, T wave = $T_{\text{end}} - T_{\text{onset}}$. The QT interval was defined as QRS complex + ST segment + T wave. The PR interval was determined as P wave + PQ segment.

2.4. Laboratory Analysis. Anti-SSA/Ro and anti-SSB/La antibodies were detected using an ELISA test and/or an immunofluorescence test, an immunodiffusion test and dot blots. The ELISA test (Laboratory Seelig, Karlsruhe, Germany) is a very sensitive test and has a reference value <50 U/mL for anti-SSA/Ro and anti-SSB/La antibodies. The immunofluorescence test (Laboratory Klein, Tübingen,

Germany) has a high specificity but less sensitivity. This titre information was available in positive and negative categories. Patients who tested positive for elevated levels of anti-SSA/Ro and anti-SSB/LA antibodies on the ELISA and/or by the immunofluorescence test, the immunodiffusion tests and dot blots were included in the study.

2.5. Statistical Analysis. Statistical analysis was performed using SPSS 20.0 (IBM) for Windows. All items were tested for a normal distribution using the Kolmogorov-Smirnov Test. As the data were normally distributed, the t-test was used for all CTIs. $P < 0.01$ was regarded as statistically significant.

3. Results

3.1. Patient Population

3.1.1. Study Group. Sixteen mothers were included in the study group. The median age of the mothers with systemic lupus erythematosus ($n = 11$) or Sjögren's syndrome ($n = 5$) was 32 years (range 21–46 years). Anti-SSA/Ro antibodies were found in eleven patients (>3000 U/mL, $n = 1$, >225 U/mL, $n = 6$, <225 U/mL, $n = 4$). Anti-SSB/La antibodies yield in five patients (>700 U/mL, $n = 1$, >225 U/mL, $n = 4$). Five patients had both antibody types. Patients with detected antibodies had an obvious clinical disease. The maternal suppressive therapies in these 16 patients were high-dose prednisolone ($n = 2$), low-dose prednisolone ($n = 10$), hydroxychloroquine ($n = 9$), cyclosporine ($n = 1$), and azathioprine ($n = 4$). One patient completely refused therapy. Most of the patients received more than one medication.

Sixteen fetuses were measured with a median gestational age of 31 weeks (range 24–38 weeks). The neonatal outcomes revealed eleven term newborns. Five neonates were premature (32–37 weeks GA). Fourteen neonates were healthy, whereas two fetuses showed a thrombopenia, but no further treatment was necessary.

3.1.2. Control Group. The median age of the 32 mothers in the control group was 32 years (range 23–40 years). All of these pregnant women were healthy. Pregnancy was not influenced by a previous disease. Thirty-two healthy fetuses were measured with a median GA of 31 weeks (range 24–38 weeks). One fetus was measured twice. The neonatal outcomes included 32 healthy fetuses without any abnormalities. All fetuses were born at term, except for one, who was born at 33 weeks gestation.

Baseline characteristics of the patients are shown in Table 1.

3.2. Fetal Cardiac Time Intervals. Cardiac time intervals were calculated in 16 patients of the study group and 32 patients of the control group. Altogether, 66 measurements in 48 patients were included in the complete analysis.

Five patients in the study group were measured once, six patients were measured twice, four patients were measured three times, and one patient was measured four times with at least two weeks between consecutive measurements. Fetuses in the control group were measured once, except for one, who was measured twice.

Therefore, each measurement was matched based on the gestational age of the fetus to a measurement from a healthy fetus.

The average heart rates of both cohorts were 135 ± 10 beats per minute (bpm). The heart rates were within normal limits [16].

The cardiac time intervals for all patients are shown in Table 2. The PQ segment (isoelectric segment between the end of the P wave and the beginning of the QRS complex) was significantly prolonged ($P = 0.007$) in the study group compared to the control group. Other CTIs did not differ significantly. The T wave and QT interval yielded a low identification, especially in early gestational ages.

Table 1: Baseline characteristics of the patients.

	Study group ($n = 16$ pts)	Control group ($n = 32$ pts)
Median age of mothers (years; range)	32 (21–46)	32 (23–40)
Height (meters; SD)	1.67 (±0.08)	1.68 (±0.07)
Body mass index before pregnancy	26 (±6)	24 (±4)
Body mass index during pregnancy	28 (±7)	27 (±4)
Prednisolone ≤10 mg (n)	10	0
Prednisolone >10 mg (n)	2	0
Dexamethasone (n)	0	0
Hydroxychloroquine (n)	9	0
Azathioprine (n)	4	0
Cyclosporine (n)	1	0
Male newborns (n)	8	19
Female newborns (n)	8	13
Mean birth weight (g; SD)	2902 (±510)	3489 (±550)
Mean birth length (cm; SD)	49 (±2)	51 (±2.5)

3.3. Postnatal Electrocardiograms. Thirteen postnatal electrocardiograms in the study group were analysed. All neonates had a normal sinus rhythm, and none had a congenital heart block. The median PR interval was in the normal range (100 ms) with reference to a mean heart rate (129 ± 13 bpm). The results were comparable with the widely used norm values of newborns reported by Park and Gunteroth (PR interval = 100–110 ms within 120–140 bpm) [17]. The median PQ segment and the P wave were within the normal range (50/50 ms). The prolongation of the PQ segment observed in the fetuses was not obvious in the neonatal ECG.

4. Discussion

The main finding in this study was that the PQ segment (PR interval—P wave), measured by fMCG, was significantly

Table 2: Fetal cardiac time intervals.

Characteristics	Study group $n = 33$ measurements mean ± SD (ms)	Control group $n = 33$ measurements mean ± SD (ms)	Statistical significance (t-test)
Fetal heart rate (beats per minute)	135 ± 10	135 ± 10	ns
P wave	53 ± 15	58 ± 13	ns
PQ segment	56 ± 10	49 ± 10	$P = 0.007$
PR interval	109 ± 17	107 ± 15	ns
QRS complex	53 ± 7	52 ± 7	ns
T wave	168 ± 52*	140 ± 28#	ns
QT interval	261 ± 49*	232 ± 35#	ns

*$n = 21$ measurements, #$n = 18$ measurements.

prolonged in the study group whereas all other cardiac time intervals were within normal range.

Van Leeuwen et al. [12] reported on PQ segment duration in healthy fetuses. Notably, this parameter was dependent on fetal heart rate and gestational age. Based on the careful matching of gestational age and no observed differences in heart rate, our results were not influenced by either effect.

For clinical decisions, PQ segment greater than the normal mean but within two standard deviations is not classified as first-degree AV block. The PQ segment prolongation might more reflect an IgG antibody effect in the development of atrioventricular node damage.

The molecular mechanisms leading to complete heart block are still unclear, but maternal antibody deposits were found in the heart of fetuses dying of congenital heart block and were thought to contribute to an inflammatory reaction that eventually induces fibrosis and calcification of the AV node [18].

Boutjdir and colleagues [19] confirmed this in fetal cardiac preparations. Anti-SSA/Ro antibodies caused reversible blockade of L-type calcium channels. The authors proposed an initially and reversibly inhibition of inward calcium flux through L-type calcium channels. This effect can cause a delay or interruption of the atrioventricular conduction. First-degree congenital heart block in a fetus without progression suggests that L-type calcium channels can be dynamically altered in fetuses [7, 20, 21]. It is still unclear when the point of transition to irreversibility occurs.

Therefore the PQ segment prolongation in this study might reflect the early antibody effect, as these findings could not be confirmed by neonatal ECG.

Several limitations have to be considered, most of which are related to the small number of patients and the multiple measurements in the study.

In conclusion, the morbidity and mortality associated with complete congenital heart block suggest the need for effective cardiac monitoring to avoid pre- and antenatal complications. This study population represents a "low-risk population" within antibody positive mothers, where PQ segment prolongation is associated with a lack of progression of the disease. The results might represent an early antibody effect.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

The authors thank J. F. Strasburger, MD, Professor of Pediatrics, Division of Cardiology, Children's Hospital of Wisconsin-Milwaukee and Fox Valley, USA for her great advice and review of the paper. They received funding from Deutsche Stiftung für Herzforschung" (F02/11) and DFG (Bi 195/50).

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 284740, 8 pages
doi:10.1155/2012/284740

Review Article

New Platform Technology for Comprehensive Serological Diagnostics of Autoimmune Diseases

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Received 22 September 2012; Accepted 16 November 2012

Academic Editor: Pietro Invernizzi

Antibody assessment is an essential part in the serological diagnosis of autoimmune diseases. However, different diagnostic strategies have been proposed for the work up of sera in particular from patients with systemic autoimmune rheumatic disease (SARD). In general, screening for SARD-associated antibodies by indirect immunofluorescence (IIF) is followed by confirmatory testing covering different assay techniques. Due to lacking automation, standardization, modern data management, and human bias in IIF screening, this two-stage approach has recently been challenged by multiplex techniques particularly in laboratories with high workload. However, detection of antinuclear antibodies by IIF is still recommended to be the gold standard method for antibody screening in sera from patients with suspected SARD. To address the limitations of IIF and to meet the demand for cost-efficient autoantibody screening, automated IIF methods employing novel pattern recognition algorithms for image analysis have been introduced recently. In this respect, the AKLIDES technology has been the first commercially available platform for automated interpretation of cell-based IIF testing and provides multiplexing by addressable microbead immunoassays for confirmatory testing. This paper gives an overview of recently published studies demonstrating the advantages of this new technology for SARD serology.

1. Introduction

Systemic autoimmune rheumatic diseases (SARDs), such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), idiopathic inflammatory myopathies (IIM), Sjögren's syndrome (SjS), and antineutrophil cytoplasmic antibody (ANCA) associated systemic vasculitis (AASV), are often accompanied by the occurrence of nonorgan-specific autoantibodies (AAb) [1–4]. Especially, antinuclear antibodies (ANA) and anticytoplasmatic autoantibodies (ACyA) have been proven to be useful markers in the serological diagnosis of SARD and may also assist in the prognosis, subclassification as well as monitoring of disease activity. Indirect immunofluorescence (IIF) on HEp-2 (human epidermoid laryngeal carcinoma) cells has become the most established method for the screening of antibodies within the two-stage diagnostic strategy for SARD [4–6]. The unsurpassed high sensitivity of ANA assessment by IIF renders this method an ideal tool for the screening stage followed by confirmatory testing with different immunological assay technologies [4, 7, 8]. However, interpretation of IIF staining patterns is rather time consuming due to lacking

automation and also highly subjective, making appropriate standardization difficult [4, 9]. Therefore, IIF has been increasingly replaced by novel techniques based on solid-phase immunoassays (e.g., ELISA, dot/line immunoassay, and addressable bead/microarray assays) [9–13]. These methods can be automated and are more cost efficient in particular in terms of the rising diagnostic demand due to the growing clinical impact of autoimmune diseases. However, high rates of false-negative findings have been reported for these techniques [10, 14]. Addressing this issue, the respective American College of Rheumatology (ACR) task force confirmed IIF as the gold standard for ANA testing [10].

Nevertheless, shortcomings of ANA assessment by IIF need to be overcome to employ this technique in a modern laboratory environment for SARD-associated antibody testing successfully. In the past decade, increasing standardization and automation efforts have been made to diminish the high intra- and interlaboratory variability and to render this method more accessible to high throughput screening [12, 15–18]. Apart from system solutions for automatic sample preparation, diagnostic companies have started to introduce new technologies for automated IIF pattern interpretation. These commercially available systems are generally based on digital acquisition and analysis of IIF images by pattern recognition algorithms. Some of these systems only distinguish between positive and negative screening results (Helios, Aesku.Diagnostics, Wendelsheim, Germany; Image Navigator, Immuno Concepts, Sacramento, USA; Cytospot, Autoimmun Diagnostika, Straßberg, Germany), whereas other systems are also able to classify basic staining patterns (AKLIDES, Medipan, Dahlewitz/Berlin, Germany; Nova View, Inova, San Diego, USA; Zenit G Sight, A. Menarini Diagnostics, Grassina-Firenze, Italy; Europattern, Euroimmun, Lübeck, Germany) [8, 19].

The fully automated interpretation system AKLIDES developed in the framework of the VideoScan technology is the first commercially available platform which has been evaluated in clinical studies [20, 21]. Based on fluorescence microscopy with different fluorochromes, the system is able to quantify fluorescence intensity and interpret basic staining patterns of HEp-2 cell IIF [22]. Recently, the application range of the AKLIDES platform has been expanded to ANCA and anti-double stranded DNA (dsDNA) AAb assessment employing fixed human neutrophils and *Crithidia luciliae* as substrates, respectively [23–25]. Furthermore, the AKLIDES system is now able to perform cell-based IIF assessment of γH2AX foci used for individual biodosimetric evaluation of DNA double-strand breaks (DSBs) [26]. Remarkably, multiplex addressable microbead-based immunoassays (MIAs) have been developed for confirmatory testing of SARD-associated antibodies on the AKLIDES platform thereby creating the first combined diagnostic solution for IIF screening and confirmatory testing in autoimmune serology.

The present paper provides an overview of recently published studies comparing the AKLIDES system with methods used in routine diagnostics referring to standardization, automation, and reliability of this new technology.

2. AKLIDES Platform

2.1. Technical System and Composition. The AKLIDES system is based on a novel composition of different hardware modules combined with innovative mathematical pattern recognition software algorithms, enabling fully automated image acquisition, analysis, and evaluation of immunofluorescence tests.

The backbone of the AKLIDES system is formed by a motorized inverse fluorescence microscope (Olympus IX81, Olympus Corp., Tokyo, Japan) including different objectives and dualband filtersets, which can be switched automatically. A movable scanning stage (IM120, Märzhäuser, Wetzlar, Germany) with exchangeable inlays allows precise selection of the requested xy position and measurement of slides as well as microtiter plates. Fluorescence excitation is achieved through light emitting diodes (LEDs) (precisExcite, CoolLED, Hampshire, UK). A gray level camera (PS4, Kappa opto-electronics, Göttingen, Germany) is used for image acquisition. All devices are connected to a computer to run necessary software applications and to provide sufficient data storage (Figure 1(a)). The software contains modules to control hardware equipment, the software autofocus, image analyzing algorithms, and data analysis. All algorithms were implemented using programming language C++ (Visual Studio 2008; Microsoft, Redmond, USA) and OpenMP for parallelization of tasks. For automatic image acquisition a novel software-based autofocus was developed based on Haralick's characterization of image texture by analyzing occurrences of gray scale transition. Additional nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) within cell based assays was used for autofocusing, quality evaluation, and object recognition [22, 27].

2.2. Software Concept. Automatic IIF evaluation by AKLIDES comprises a sequential, multistage process including image acquisition, quality control, object segmentation, object description, and object classification (Figure 1(b)). After selecting the requested xy position on the slide, dynamic autofocusing is performed in DAPI channel, starting with coarse focusing to find the approximate position of the substrate, subsequently followed by fine focusing with narrow z steps to determine the exact focal plane [28]. To exclude images that are not suitable for further evaluation due to over- or underexposure, artifacts, air entrapments, or inhomogeneities in fluorescence staining, a qualitative image analysis was implemented. Therefore, every image is divided into tiles of equal size. Comparison of subsequently calculated tile sharpness, and homogeneity was used for quality evaluation [22]. In order to select circular-shaped elements in the images (cell nuclei, beads), object segmentation was accomplished by using a histogram-based mixture model threshold algorithm to model the background intensity, followed by watershed transformation. Segmented objects were characterized by different boundary, regional, topological, and texture/surface descriptors. For pattern recognition, 200 attributes were implemented, leading to a variety of approximately 1,400 object describing criteria after appropriate combination [22].

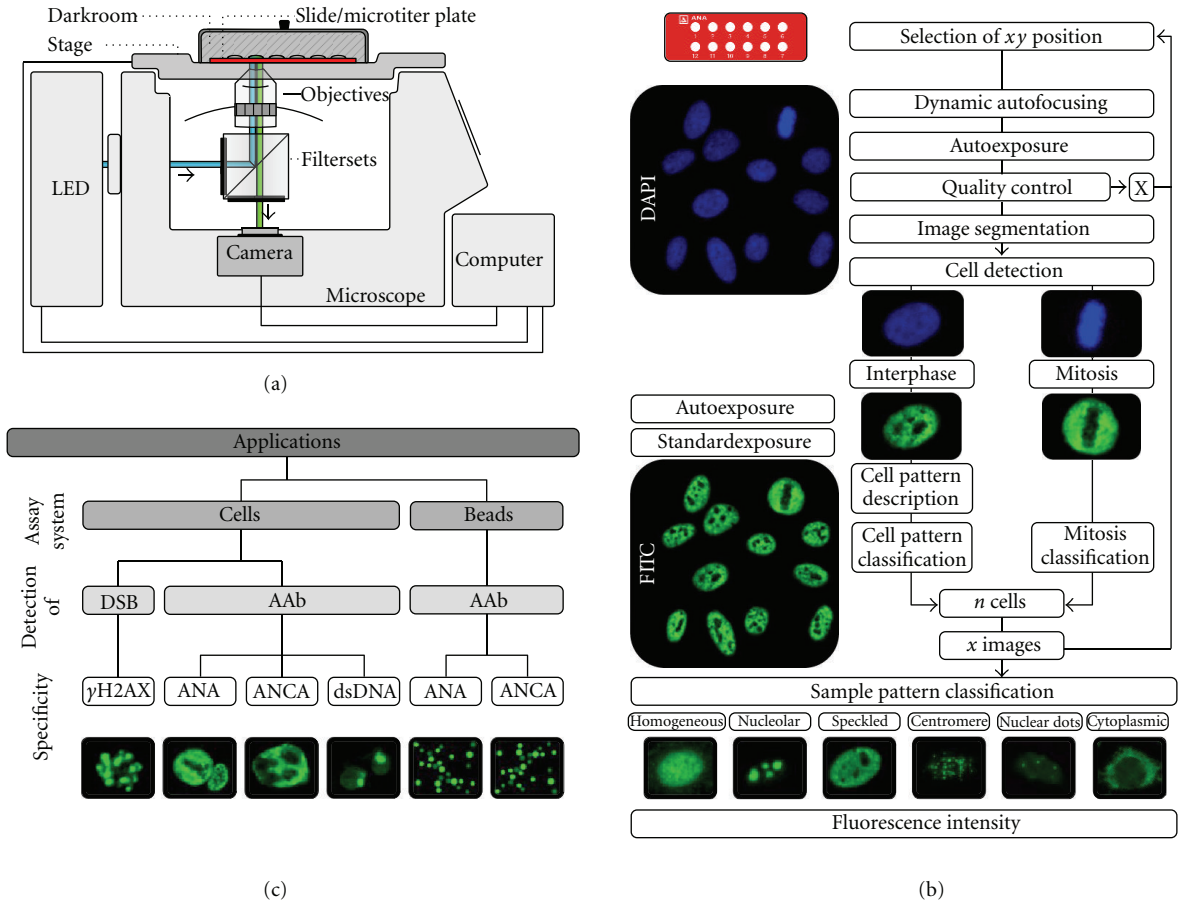

Figure 1: AKLIDES platform for automated evaluation of cell-based IIF testing and multiplex addressable microbead-based immunoassays analysis. (a) Schematic drawing of the main hardware components of the AKLIDES system based on a fluorescence microscope combined with different filtersets and objectives, a LED unit, a gray level camera, a movable scanning stage, and a controlling computer. (b) Flowchart of measurement and evaluation of automated HEp-2 IIF assay interpretation by the AKLIDES system including a sequential, multistage process of image acquisition, quality control, object segmentation, object description, and object classification [27]. (c) Application range of the AKLIDES system divided into evaluation of cell-based IIF testing for DSB and AAb detection as well as AAb analysis using bead-based multiplex assays.

DAPI staining was used to detect cell location and to identify mitotic cells, whereas pattern recognition was performed in FITC channel mode. Object classification of immunofluorescence patterns was accomplished by combining structural and textural features following a sequence of three hierarchical decision steps: (i) positive/negative discrimination, (ii) location assessment of nucleus, cytoplasm, and chromatin of mitotic cells, and (iii) fluorescence pattern recognition [27]. With the current approach six basic HEp-2 cell staining patterns can be distinguished: cytoplasmic, homogeneous, speckled, nucleolar, centromere, and multiple nuclear dots [29]. Automatic pattern recognition can be achieved by two different strategies: self-learning algorithms and those using static classificators. AKLIDES is based on static classificators in order to standardize interpretation of HEp-2 assays, whereas self-learning methods would increase interlaboratory variance [29].

For image intensity evaluation, the AKLIDES software calculates a reactivity index (RI), combining several parameters including image intensity, image contrast, and number of gray level occurring in the entire image. The RI is also influenced by the exposure time. To ensure an ideal exposure, an automatic computing method was implemented to correct the exposure time of the scene depending on the highest gray level value in the image (single artifacts excluded). This even allows the detection of patterns with weak absolute image intensity (e.g., centriole, multiple nuclear dots). On the basis of the RI value of 200 blood donor samples cut-offs for borderline and positive reactivities were determined [27, 29].

Evaluation of multiplex addressable MIA is comparable to already described cell assay except for DAPI counter staining and detection of subcellular locations. Essential autofocusing and quality control are directly performed with polymerized microbeads, and substructuring of microbead surface is not required.

2.3. Applications. To overcome the drawbacks of IIF and to address the increasing demand for SARD-associated antibody detection, different technical approaches for automated IIF testing have been developed [8, 15, 16, 18, 21, 29].

Based on fluorescence intensity quantification and pattern recognition of IIF images, commercially available IIF assays for ANA and ACyA have been introduced on the fully automated IIF interpretation system AKLIDES [20, 23, 25, 30]. Antibody detection and fluorescence staining are performed using fluorescein-isothiocyanate-coupled sheep anti-human IgG conjugate and DAPI-containing mounting medium. After adaptation of pattern recognition algorithms, the application spectrum of the AKLIDES system was expanded to ANCA detection on formalin and ethanol fixed human neutrophil granulocytes as well as detection of AAb against dsDNA using *Crithidia luciliae* immunofluorescence tests (CLIFTs).

By incorporating addressable MIA for multiplexing, the application range of the AKLIDES platform presents a unique system solution for SARD serology and can be divided into two major groups, respectively, (i) screening of antibodies by cell-based IIF assays and (ii) analyzing of multiplexed microbead-based immunoassays as confirmatory testing for AAb detection. A further novel application of the AKLIDES system is the measurement of dsDNA DSBs by evaluating γH2AX foci (Figure 1(c)).

3. Screening of AAb by Cell-Based Assays

3.1. ANA Detection on HEp-2 Cells. In 2010, Egerer et al. reported the first clinical evaluation of an automated IIF interpretation system by using AKLIDES technology for ANA assessment in the routine laboratory environment of both a university and a private referral laboratory [20]. Comparing positive and negative findings of 1,222 sera obtained from patients with suspected SARD, an agreement of 93.0% (859/924) and of 90.6% (270/298) between AKLIDES interpretation and visual reading of ANA in the university and the private laboratory was achieved, respectively. Further pattern evaluation comparing visually and automatically defined fluorescence patterns of AKLIDES positive samples revealed an agreement of 90.1% and 92.7% for the university and private laboratory, respectively. Discrepancies in image recognition were mainly seen for sera demonstrating mixed patterns, AAb against nuclear membrane or cytoplasmic staining [20].

Recent studies by Kivity et al. and Melegari et al. showed similar results [30, 31]. In the report by Kivity et al., ANA and ACyA assessment by AKLIDES was compared to manual screening on HEp-2 cells and to an ANA immunodot assay. A total of 397 sera samples were investigated, including 137 apparently healthy donors, 34 patients with SLE, 111 patients with DM or PM, 74 patients with SSc, and in addition 41 samples with rare AAb pattern (e.g., nuclear dots, Golgi apparatus, lysosomal like). There was 100% agreement among the 34 SLE samples, which were all tested positive in the three methods applied. The AKLIDES system detected more positive results in DM/PM (95%) compared to manual HEp-2 cell interpretation (74%) and immunodot assay (64%). Out of 74 sera from patients with SSc 97% were tested positive by AKLIDES, whereas manual reading of HEp-2 cells and immunodot analysis only showed positive findings in 92% and 86% of these cases, respectively. According to ROC curve analysis for the interpretation of DM/PM and SSc sera using the AKLIDES system, sensitivities between 97-98% and specificities between 91-92% could be calculated. Regarding the analysis of SSc sera, pattern recognition was found to be correct in 82% of anti-CENP-B-positive samples and in 72% of anti-Scl-70 positives. Analysis of 41 sera with rare AAb by the AKLIDES system demonstrated positive findings in 95% of the cases investigated and showed a good correlation between manual and automated pattern recognition in terms of distinguishing between nuclear and cytoplasmic staining. A further study reported by Melegari et al. also confirmed the excellent agreement of positive and negative findings by visual and AKLIDES reading of IIF on HEp-2 cells [30]. Analyzing 66 routine samples and 116 selected samples with known AAb levels, only two discordant findings were obtained with the AKLIDES system, resulting in a remarkable total agreement for ANA screening of 98.9%.

3.2. ANCA Detection on Human Neutrophil Granulocytes. Besides ANA testing also ANCA assessment by the AKLIDES system was evaluated by Melegari et al. [30]. In this study, 46 samples were analyzed by IIF on the AKLIDES system, by two experts on a fluorescence microscope as well as by anti-MPO and anti-PR3 ELISA. The agreement between manual and automated IIF reading was reported to be 89.1% (41/46).

In a different study by Knuetter et al., ANCA testing was performed in 293 sera from patients with AASV and other SARDs on ethanol- and formalin-fixed neutrophils [23]. Comparison of positive and negative findings between manual and automatic reading revealed a very good agreement for ethanol- ($\kappa = 0.871$) and formalin-fixed neutrophils, ($\kappa = 0.866$). Furthermore, differentiation of cANCA and pANCA pattern by the AKLIDES system showed a good agreement for ethanol- ($\kappa = 0.739$) and formalin-fixed neutrophils ($\kappa = 0.742$) [23].

In a recent report by Damoiseaux et al. comparing visual and automated ANCA evaluation of ethanol- and formalin-fixed neutrophils, sera from patients with AASV tested positive for MPO- ($n = 40$) or PR3-ANCA ($n = 39$), and different groups of control sera were analyzed [24]. Visual IIF testing of PR3-ANCA-positive patients showed a cANCA pattern in 92% of the cases on ethanol- and in 97% on formalin-fixed slides, whereas AKLIDES reported positive cANCA findings in 74% and 95% of the samples, respectively. Concerning ethanol-fixed neutrophils, 90% of sera from MPO-ANCA-positive patients revealed a pANCA pattern using routine microscopy, whereas AKLIDES detected pANCA staining in 80% of the samples [24].

3.3. Anti-dsDNA AAb Detection on Crithidia luciliae. Anti-dsDNA antibodies can be readily detected by IIF employing CLIFT [32, 33]. Automated interpretation of CLIFT by AKLIDES provides the basis for the standardized detection of highly disease-specific anti-dsDNA antibodies. In a recent study including 44 serum samples of SLE patients, Melegari et al. compared automated and visual analysis of IIF approaches for anti-dsDNA antibody detection on *Crithidia*

luciliae as well as corresponding ELISA data. The agreement between the results obtained by expert reading, and the AKLIDES system was 91.0% [30].

4. Confirmatory Testing of AAb by Multiplexed Addressable Microbead-Based Assays

Positive antibody findings in the screening for SARD serology are recommended to be confirmed by molecular immunoassays in a second diagnostic stage. In order to create a combined platform allowing both high-sensitive antibody screening by IIF and analysis of specific AAb, MIAs were developed for the AKLIDES system. These addressable MIAs utilize multiple carboxylated polymethylmethacrylate bead populations differing in size and/or concentrations of fluorescent dye for multiplexing. Each population was covalently labeled with a specific antigen, and beads were immobilized onto 96-well microtiter plates. By suspending different bead populations within one well, a multiplex assay can be performed. Bound AAb were detected with secondary fluorophore-coupled anti-human-IgG antibodies. Classification of bead populations and measurement of corresponding ligand fluorescence intensity can be readily performed by AKLIDES [34].

4.1. ANA Multiplex Assay. A multiplex MIA for detection of six different antinuclear AAb on the AKLIDES system was developed and evaluated by Grossmann et al. [34]. In total, seven microbead populations differing in size and/or ratio of fluorescent dyes were covalently labeled with either Scl-70, Sm, SS-A (Ro60), SS-B (La), CENP-B, dsDNA or human IgG. Bead classification and measurement of mean fluorescence intensity of 72 sera from patients with autoimmune diseases were performed by the AKLIDES systems. For comparison of results obtained by MIA and ELISA, Cohen's kappa was determined, showing perfect agreement concerning Scl-70, Sm, CENP-B, and SS-B AAb ($\kappa = 1.000$). A very good agreement was found for dsDNA ($\kappa = 0.961$) and good agreement for SS-A ($\kappa = 0.783$) AAb, respectively [34].

4.2. ANCA Multiplex Assay. In order to accomplish specific antibody assessment for the serological diagnosis of AASV, a multiplex addressable MIA was developed, enabling detection of AAb against MPO, PR3, and the noncollagen region of the alpha-3 subunit of collagen IV (GBM).

Addressable MIA was performed of 265 sera, including 51 patients with active granulomatosis with polyangiitis (Wegener's), 41 patients with microscopic polyangiitis, and 10 patients with Goodpasture syndrome (GPS) or anti-GBM nephritis as well as 108 control sera from donors with ANA-associated systemic rheumatic disease and 55 donors with rheumatoid arthritis. Results of MIA were compared with results achieved by IIF and ELISA. Comparison of MIA findings by AKLIDES with results of conventional assays showed a very good interrater agreement based on Cohen's kappa calculation for anti-PR3 ($\kappa = 0.927$), anti-MPO ($\kappa = 0.868$), and anti-GBM testing ($\kappa = 0.938$). Discrepancies detected between the different methods were mainly found in sera with low reactivities [34].

5. Detection of Double-Strand Breaks by Automated Indirect Immunofluorescence Testing

The image processing capabilities and fluorescence pattern recognition algorithms of the AKLIDES system represent a platform beyond antibody detection by IIF. One further application is the quantification of dsDNA DSB. After DSB formation, large numbers of histone H2AX molecules in the vicinity of the DSB become phosphorylated at serin139 (γH2AX), leading to complex formation including different molecules responsible for DNA repair and chromatin remodeling. The number of fluorescent foci revealed by specific anti-γH2AX staining of these particular complexes has been found to correlate with the number of DSB [35, 36]. Quantification can be performed by fluorescence microscopy, determining the average number of fluorescent γH2AX found in the nuclei of 100 cells [26]. Since visual interpretation is time consuming and heavily influenced by subjective reading, the AKLIDES system was adapted for fully automated assessment of γH2AX foci.

Results of automated interpretation of radiation induced γH2AX foci were reported by Runge et al. in 2012 [26]. In this study, γH2AX analysis was performed by manual reading of three laboratories and by fully automated AKLIDES measurement investigating PCCl3 cells (thyroid rat cell line) after exposure to different doses (0–5 Gy) of ^{188}Re. Data confirmed a high interlaboratory variability of 38.4% for manual reading of γH2AX foci assessment in different laboratories. In contrast, a good agreement of automated and manual γH2AX foci analysis was revealed between AKLIDES and the laboratory which collaborated in the adaption of the pattern recognition algorithms for AKLIDES ($R^2 = 0.931$) [26].

6. Conclusion

Testing for AAb is an essential step in the serological diagnosis of autoimmune diseases in particular SARD [3, 4, 37]. As a matter of fact, one of the first techniques available in routine laboratories and still the recommended method for ANA screening is IIF testing currently preferably on HEp-2 cells [38]. However, the IIF technique is characterized by time-consuming and subjective evaluation, insufficient automation as well as poor standardization [11, 39]. Furthermore, inconsistencies in description and classification of staining patterns have been reported to hamper standardization efforts [9, 20, 31, 40]. To respond to the growing number of particularly ANA tests for SARD serology, new methods based on solid phase immunoassays, like ELISA or multiplexing technologies have been developed [13, 41–46]. However, ANA testing by IIF is still recommended to be used as the gold standard method due to its high sensitivity and may outperform microbead technology under optimal conditions [10, 47]. Employing a limited

number of autoantigens, ANA ELISA or ANA multiplex assays revealed up to 35% false-negative results compared with ANA IIF testing on HEp-2 cells [10]. In order to automate and standardize ANA detection on HEp-2 cells, different commercially available platforms were developed, and preliminary data show high agreement between visual and automated ANA interpretation [8, 17, 19, 27, 48, 49]. In particular, progress in automated interpretation of subcellular patterns and location determination as well as novel pattern recognition algorithms for IIF has pioneered the commercialization of this new technology [50–52].

Recently, several studies have been published about the performances of the first fully automated interpretation system AKLIDES, combining automatic image acquisition and pattern recognition. The AKLIDES platform has been developed to become a multipurpose bioanalytical tool, which is able to analyze different kinds of cell- and bead-based fluorescence assays [22, 27, 28]. Reported data showed a high agreement between manual and automated interpretation of cell-based IIF testing for ANA, ANCA, and anti-dsDNA assessment. Not only automated discrimination between positive and negative results but also pattern recognition of fluorescence images showed a good correlation with visual IIF reading. Although the number of patterns recognized and pattern recognition accuracy needs to be further improved, automatic interpretation of cell-based IIF assays by AKLIDES may be used in autoimmune laboratories and can be a helpful screening tool in routine diagnostics especially for exclusion of negative samples [31]. Nevertheless, positive findings provided by the system should always be confirmed by an expert [30].

Most clinical immunology laboratories apply a two-stage approach for ANA testing, starting with an initial screening on HEp-2 cells, which is followed by confirmatory testing [3, 4, 11]. Expanding the AKLIDES systems to assess addressable MIA created a unique platform, which for the first time allows fully automated evaluation of cell-based screening tests and antigen-specific multiplex assays on one system. Detecting multiple SARD-associated AAb simultaneously in one run, multiplex ANA testing is more efficient compared to the conventional ELISA technique. With regard to most of the investigated autoantigens, studies demonstrated very good agreements of results achieved with either ANA or ANCA multiplex assay and corresponding single ELISAs. Thus, multiplexing provides the basis for antibody profiling as an efficient and promising approach in the serology of autoimmune disorders [53–57].

The technology of digital fluorescence image acquisition and automatic pattern recognition can be extended to other currently established cell-based IIF assays. Such a different cell-based IIF technique is the quantification of γH2AX foci, which is very time consuming, subjective, and not suitable for high-throughput screening in its present version [26]. Besides satisfying performances regarding SARD-associated antibody assessment, the AKLIDES system demonstrated adequate results for the automated assessment of γH2AX foci in irradiated cells.

The need for both standardized SARD-associated antibody detection as well as evaluation of γH2AX foci is growing [26, 40, 58]. The AKLIDES technology can help to improve inter- and intralaboratory variations and may enable high-throughput diagnostics. Furthermore, cost-efficient analysis of large volumes of samples in routine laboratories may support the finding of new relevant antibodies for improved antibody profiling in SARD serology [3].

Abbreviations

AAb: Autoantibody
ANA: Antinuclear antibody
ACyA: Anticytoplasmic antibody
ANCA: Antineutrophil cytoplasmic antibody
AASV: ANCA-associated systemic vasculitis
cANCA: Cytoplasmic ANCA
CLIFT: *Crithidia luciliae* immunofluorescence test
DSB: Double-strand break
dsDNA: Double-stranded DNA
ELISA: Enzyme-linked immunosorbent assays
GBM: Antiglomerular basement membrane
GPS: Goodpasture syndrome
IIF: Indirect immunofluorescence
IIM: Idiopathic inflammatory myopathy
LED: Light-emitting diode
MIA: Microbead-based immunoassay
MPO: Myeloperoxidase;
pANCA: Perinuclear ANCA
PR3: Proteinase 3
RA: Rheumatoid arthritis
SARD: Systemic autoimmune rheumatic disease
SLE: Systemic lupus erythematosus
SjS: Sjögren's syndrome
SSc: Systemic sclerosis.

Authors' Contribution

D. Reinhold and D. Roggenbuck shared senior authorship.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 606195, 12 pages
doi:10.1155/2012/606195

Review Article

Clinical and Pathological Roles of Ro/SSA Autoantibody System

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Received 4 October 2012; Accepted 19 November 2012

Academic Editor: Dimitrios P. Bogdanos

Anti-Ro/SSA antibodies are among the most frequently detected autoantibodies against extractable nuclear antigens and have been associated with systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS). Although the presence of these autoantibodies is one of the criteria for the diagnosis and classification of SS, they are also sometimes seen in other systemic autoimmune diseases. In the last few decades, the knowledge of the prevalence of anti-Ro/SSA antibodies in various autoimmune diseases and symptoms has been expanded, and the clinical importance of these antibodies is increasing. Nonetheless, the pathological role of the antibodies is still poorly understood. In this paper, we summarize the milestones of the anti-Ro/SSA autoantibody system and provide new insights into the association between the autoantibodies and the pathogenesis of autoimmune diseases.

1. Introduction

Systemic autoimmune diseases, including systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS), are a category of medical conditions that affects multiple organs and are related to autoimmune responses. These are commonly characterized by the development of autoantibodies against intracellular autoantigens. In fact, diagnosis, classification, and prognosis often rely on specificity and levels of the autoantibodies, in addition to clinical symptoms and other laboratory evaluations. Among autoantigens, extractable nuclear antigens (ENA) are soluble cytoplasmic and nuclear components with over 100 different antigens described. The main antigens used in immunological laboratories for detection are Ro, La, Sm, RNP, Scl-70, and Jo1 [1]. Anti-Ro/SSA and anti-La/SSB antibodies are among the most frequently detected autoantibodies against ENA and have traditionally been associated with SLE, SS, subacute cutaneous lupus erythematosus (SCLE), and neonatal lupus erythematosus (NLE) [2–5]. Anti-Ro/SSA and anti-La/SSB can be detected in 70–100% and 40–90%, respectively, of patients with SS [6], and the presence of these autoantibodies is one of the criteria for the diagnosis and classification of SS [7, 8].

Anti-Ro/SSA and anti-La/SSB antibodies were originally described in 1961 as two precipitating antibodies reacting with antigens contained in extracts from salivary and lacrimal glands of patients with SS, termed SjD, and SjT, respectively [9]. SjD antigen was reported to be insensitive to trypsin or heat, while SjT antigen could be destroyed by the same treatment. In 1969, Clark et al. described the presence of antibodies in the sera of patients with SLE that reacted with ribonucleoprotein (RNP) antigens present in extracts of rabbit and human spleen [10]. The authors named the antibody "anti-Ro antibody" after the original patient in whom the antibodies were identified. The same group also found antibodies to another soluble cytoplasmic RNA protein antigen, "La" [11]. At about the same time, Alspaugh and Tan noted the existence of autoantibodies in the sera of many SS patients, which react with antigens termed "SSA" and "SSB," [12]. SSB antigen was described also as "Ha", an antigen targeted by sera from patients with SLE and SS

[13]. Later, Ro and La were demonstrated to be antigenically identical to SSA and SSB [14].

While anti-Ro/SSA antibodies are primarily found in patients with SLE and SS, they are also sometimes seen in other systemic autoimmune diseases, such as systemic sclerosis (SSc), polymyositis/dermatomyositis (PM/DM), mixed connective tissue disease (MCTD), and rheumatoid arthritis (RA) [15, 16].

Although these anti-Ro/SSA antibodies have been used as a useful diagnostic marker for SLE and SS for decades, the pathological significance of the antibodies still remains to be clarified. In this paper, we summarize the milestones of the anti-Ro/SSA autoantibodies and provide new insights into the association between the autoantibodies and autoimmune diseases.

2. Two Autoantigens to SSA Autoantibodies, Ro52 and Ro60

In 1981, Lerner et al. showed that the Ro antigens associate with small cytoplasmic RNAs and form Ro-ribonucleoproteins (Ro-RNP) particles [45]. Later, Ro antigens were found to consist of two different proteins, Ro60 and Ro52. The target antigen for anti-Ro autoantibodies was first identified as a 60 kDa protein, which exists as RNP complexes with small cytoplasmic RNAs (hY-RNA) in 1984 [46]. Subsequently, complementary DNA (cDNA) of Ro60 was cloned [47, 48]. Ben-Chetrit et al. first discovered that a 52 kDa protein, named Ro52, was a part of the Ro antigens in 1988 [49], and three years later cDNA of human Ro52 was cloned [50, 51]. In humans, the *Ro60* gene is approximately 32 kb in size, located on chromosome 19, while the *Ro52* gene is 8.8 kb in size, located on chromosome 11. Although the Ro52 protein was initially suggested to be part of the Ro-hY-RNA complex with Ro60 [49, 52, 53], subsequent studies failed to confirm a direct interaction of the proteins [54, 55]. Recent studies provided evidence that Ro52 and Ro60 are localized to different cell compartments and that anti-Ro52 and anti-Ro60 antibodies have different clinical associations [15]. Thus it still remains unclear why autoantibodies to these two proteins are so closely linked.

Ro52 is an interferon (IFN)-inducible protein [56–64], and it is also induced by viral infection or Toll-like receptor (TLR) engagement via type I IFN induction [59, 62, 65, 66]. Following the first demonstration of Ro52 ubiquitin E3 ligase activity by Wada et al. several reports supporting the conclusion have been published by other groups [57, 67–70]. Recent studies, including *Ro52* gene disruption studies, suggest that Ro52 is a negative regulator for proinflammatory cytokine production. Yoshimi et al. noted an increase in production of NF-κB-dependent cytokines, such as IL-1β, TNFα, and IL-6, that was observed in *Ro*52$^{-/-}$ fibroblasts as compared to wild-type fibroblasts [62]. Data consistent with this report were published by another group [71, 72]. Another group reported that Ro52-deficient mice develop uncontrolled inflammation and systemic autoimmunity as a consequence of minor tissue injury caused by ear tagging [63]. In these mice, bone marrow-derived macrophages and splenocytes from the mutant mice released more inflammatory cytokines, IL-6, TNFα, type I IFN, and IL-23, upon TLR activation as compared to wild type.

Several studies suggest possible associations between allelic polymorphisms of *Ro52* and the disease susceptibility and increased anti-Ro52 antibodies in SLE and SS [73–76]. Furthermore, about a twofold increase in the expression of Ro52 transcripts in peripheral blood mononuclear cells (PBMC) of patients with SLE and SS as compared with healthy controls has been reported [68].

On the other hand, Ro60 antigen binds to ~100 nt noncoding RNAs called hY-RNA [45–48, 77]. It was recently reported that the Ro60 protein, having a ring shape, binds to misfolded noncoding RNAs in vertebrate nuclei and acts as a quality checkpoint for RNA misfolding with molecular chaperones for defective RNAs [78, 79]. The misfolded RNAs are targeted by Ro60 for degradation [80–82]. The mice lacking the Ro60 protein develop an autoimmune syndrome characterized by autoantibody production, glomerulonephritis, and increased sensitivity to irradiation with ultraviolet light [83]. Thus, Ro60 may have a role to protect against autoimmune response.

3. Epitopes on Ro Autoantigens

Several studies showed that epitopes of Ro52 and Ro60 proteins have different conformational dependence [84–87]. On the Ro52 protein, most sera recognize linear epitopes in the denatured molecule, generally located in the leucine zipper site and not expressed on the surface of the native protein. In contrast, the epitopes recognized by anti-Ro60 antibodies are highly conformational and the antibodies largely lose the binding activity to the denatured protein.

Dörner et al. showed that the central region, amino acid (aa) 153–245, is the main immunogenic region of Ro52 antigen, and the strongest antigenic epitopes are located within aa 197–245 region including the leucine zipper motif [88]. Antibody responses are directed against this major antigenic region regardless of the underlying autoimmune diseases, although the strikingly different levels of antibodies and the recognition of epitopes on aa 153–196 may be related to different disease expressions. Subsequently, sera from patients with SS were found to react heterogeneously to polyubiquitylated Ro52, probably due to their different antigenic epitopes [89].

McClain et al. described that the initial epitope on Ro60, prior to clinical disease onset, includes a peptide, aa 169–180 [90]. This epitope directly cross-reacts with a peptide, aa 58–72, of the Epstein-Barr virus nuclear antigen-1 (EBNA-1). The data support the hypothesis that the Epstein-Barr virus infection had a putative triggering effect by enhancing the development of autoantibodies to Ro60 through molecular mimicry [91, 92].

Polyclonal class-switched anti-Ro and anti-La responses can be elicited by immunization of normal mice with recombinant La protein [93]. In this process, the production of autoantibodies to different nonoverlapping regions of La was induced. Moreover, the same immunization rapidly

induces the production of specific Ro60 antibodies. Reciprocally, mice immunized with Ro60 protein produced anti-La antibodies. This intra- and intermolecular spreading of autoantibody response suggests that the development of autoantibodies to multiple components of the Ro/La RNP complex may follow the initial response to a single epitope and suggest as a general explanation for the appearance of mixed autoantibody patterns in different systemic autoimmune diseases.

The accessibility of Ro/La complex for the immune system is still unknown. Based on the antigen-driven immune response hypothesis, several works suggest that it could be related to an abnormal expression on the cell surface as a consequence of UV irradiation [94–96], oxidative stress [97], TNFα treatment [98], viral infection [99], or estradiol treatment [100]. Another mechanism for anti-Ro and anti-La antibodies' production could be related to antigen-containing apoptotic debris during programmed cell death [101, 102].

4. Anti-Ro Antibody and Demographic Feature

Anti-Ro antibodies can be detected alone in many sera while anti-La antibodies are usually accompanied by anti-Ro antibodies. HLA class II phenotype might support epitope spreading. The presence of anti-Ro and/or anti-La antibodies is more strikingly associated with HLA-DR3 and/or HLA-DR2 [103, 104]. HLA-DR3 is associated with both anti-Ro and anti-La antibody production while HLA-DR2 favors anti-SSA antibody synthesis [105]. HLA-DQ alleles are also linked to anti-Ro and anti-La antibody responses. Both DQ1 and DQ2 alleles are associated with high concentrations of these autoantibodies [106]. The data from restriction fragment length polymorphism (RFLP) analysis also indicated that HLA-DQ alleles are related to anti-Ro antibody response [107]. In this study, all patients with anti-Ro antibodies had a glutamine residue at position 34 of the outermost domain of the DQA1 chain and/or a leucine at position 26 of the outermost domain of the DQB1 chain. Patients with both anti-Ro and -La antibodies were more likely to have all four of their DQA1/DQB1 chains containing these amino acid residues than either anti-Ro-negative SLE patients or controls. These data implicate specific amino acid residues on both DQA1 and DQB1 chains located on the floor of the antigen-binding cleft of the HLA-DQA1:B1 heterodimer.

Ro52 is an immunologically independent autoantibody system, and anti-Ro52 antibodies can exist without the presence of concomitant anti-Ro60 antibodies in systemic autoimmune diseases. Peene et al. found that anti-Ro52 antibodies are precipitin negative, not picked up by Ro enzyme-linked immunosorbent assays (ELISA) based on natural Ro proteins, and have no specific antinuclear antibody (ANA) fluorescence staining pattern [108]. As a consequence, anti-Ro52 antibodies are frequently not detected by classical Ro detection methods, which have a bias towards anti-Ro60 reactivity. Moreover, Schulte-Pelkum et al. showed that anti-Ro52 and anti-Ro60 reactivities can mask each other and that more than 20% of Ro positive sera can go undetected in assays that utilize blended antigens [15]. Therefore, the authors recommended that anti-Ro52 and anti-Ro60 antibodies should be tested separately.

There exists a paper showing that the prevalence of isolated anti-Ro52 antibodies was approximately 0.5%, and that detection did not lead to any significant clinical benefit as it was never the only explanation of symptoms [109]. On the other hand; several groups demonstrated the importance of separate detection of anti-Ro52 and anti-Ro60 antibodies when considering the diagnosis and, in particular, of patients with myositis [15, 41]. In a recent study, the frequency of anti-Ro52 antibodies was similar to that of anti-Ro60 in all groups but the myositis (35.4% versus 0.0%, $P < 0.001$) and SSc (19.0% versus 6.0%, $P < 0.005$) cohorts using the consensus of three different laboratory methods [15]. In the same study, the percentages of anti-Ro52 antibodies detected without anti-Ro60 antibodies also varied from 5.4% in childhood SLE to 35.4% in the myositis group. In the SS group, 63.2% of anti-Ro52 antibody-positive sera also had autoantibodies to Ro60.

Since Rutjes et al. found anti-Ro52 reactivity in 58% of anti-Jo-1 antibody-positive myositis sera in 1997 [41]; several groups confirmed the data in subsequent studies [15, 110–112]. The average coincidence of reactivity against Ro52 and Jo-1 was 70% ($P = 0.0002$, odds ratio = 14.17, $\kappa = 0.54$) in anti-Jo-1 antibody-positive sera of myositis patients in a recent study [15]. These observations also suggest previous conclusions that anti-Ro52 antibody is indeed an independent autoantibody for myositis [108].

Anti-Ro52 antibodies are frequently coexpressed with antibodies to soluble liver antigen (SLA) [40, 113]. The presence of anti-Ro52 antibodies has been reported in 77–96% of patients with anti-SLA antibodies, and patients with dual antibodies had a higher frequency of HLA DRB1*03 and lower occurrence of HLA DRB1*04 than patients with anti-Ro52 antibodies alone.

5. Anti-Ro Antibodies and Autoimmune Diseases

Anti-Ro antibodies are the most prevalent autoantibodies among many autoimmune diseases, although their pathological role is still controversial [114]. Clinical manifestations related to anti-Ro antibodies are summarized in Table 1.

5.1. SLE and SS. Anti-Ro antibodies are frequently observed in association with SLE [115–117], SS/SLE overlap syndrome [118], SCLE [19], and NLE [25, 36, 119–122]. In contrast, anti-La antibody is more closely associated with SS. Anti-Ro antibodies can be detected in 70–100% and 40–90% of patients with SS and SLE, respectively, while anti-La antibodies can be detected in 35–70% and 45% of patients with SS and SLE, respectively [6]. SLE patients with C2 and C4 deficiency tend to have anti-Ro antibodies with cutaneous manifestations and polyarthritis, without renal or CNS features [116, 117, 123].

Anti-Ro and anti-La antibodies are found earlier than other SLE-related autoantibodies, such as anti-dsDNA, anti-ribonucleoprotein (RNP), and anti-Sm antibodies, and are

Table 1: Clinical manifestations related to anti-Ro antibodies.

Clinical manifestation	Disease	Ro52 specificity	Reference
Cutaneous manifestation			
Photosensitivity	SLE		[17]
	RA		[18]
Subacute cutaneous lesion	SLE/SCLE		[19, 20]
Purpura	SLE		[21, 22]
	SS		[23, 24]
	RA		[18]
Cutaneous NLE	NLE		[25]
Sicca symptom	SS		[26]
	SSc		[27]
	RA		[18, 28, 29]
Scleritis	RA		[28]
Interstitial lung disease	SLE	+	[16, 30, 31]
	SSc		[32, 33]
	PM/DM		[34, 35]
Congenital heart disease			
Complete heart block	NLE		[25, 36–38]
Prolonged QT interval	NLE		[25]
Liver dysfunction			
Liver function test abnormality	NLE		[25]
High serum bilirubin level	PBC	+	[39]
Advanced histological stage	PBC	+	[39]
	AIH-1	+	[40]
Musculoskeletal involvement			
Myositis	PM/DM	+	[15, 41, 42]
Arthritis	SLE		[43]
Hematological disorder			
Leukopenia	SS		[23]
	RA		[18]
Lymphopenia	SS		[23]
Neutropenia	SLE		[44]
	NLE		[25]
Anemia	NLE		[25]
	RA		[29]
Thrombocytopenia	NLE		[25]
Immunological disorder			
Hypocomplementemia	RA		[18]
High serum IgG level	SS		[23]
	RA		[28]
High serum IgM level	PBC	+	[39]

SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; SCLE: subacute cutaneous lupus erythematosus; SS: Sjögren's syndrome; NLE: neonatal lupus erythematosus; SSc: systemic sclerosis; PM: polymyositis; DM: dermatomyositis; PBC: primary biliary cirrhosis; AIH-1: type 1 autoimmune hepatitis.

present, on average, 3.4 years before the diagnosis of SLE [124]. Another paper also shows that the autoantibody type that appears first before the onset of symptoms is anti-Ro antibodies that appear at a mean of 6.6 years [125]. Some groups suggest a close association between anti-Ro antibodies and late onset SLE, with the onset of symptoms after the age of 50 [20, 126]. There are conflicting data as to the correlation of anti-Ro antibody titers with disease activity during the course of SLE and SS [127–131].

Anti-Ro antibodies have been reported to be associated with photosensitivity, SCLE, cutaneous vasculitis (palpable purpura), and hematological disorder (anemia, leukopenia,

and thrombocytopenia) [17, 19, 21, 22, 44, 132]. Interstitial pneumonitis has been also closely associated with anti-Ro antibodies in patients with SLE, but there is so far no evidence of a direct involvement of the antibodies in the pathogenesis of the pulmonary disease [16, 20, 30, 31]. The relationship between anti-Ro antibodies and nonerosive deforming arthritis, called Jaccoud's arthropathy, has been reported [43, 133].

In SS, anti-Ro, and anti-La antibodies are present in the lacrimal fluid of some patients and their presence in serum or lacrimal fluid is associated with the severity of keratoconjunctivitis sicca [26]. High titers of anti-Ro and anti-La antibodies also have been shown to be associated with a greater incidence of extraglandular manifestations, especially purpura, leukopenia, and lymphopenia [23, 24].

5.2. NLE. NLE is a passively transferred autoimmune disease that occurs in some babies born to mothers with anti-Ro and/or anti-La antibodies [120, 134]. The most serious complication in the neonate is congenital complete heart block (CHB), which occurs in 1–5% of such pregnancies and 6–25% of subsequent pregnancies with a previously affected child with CHB [37].

Since the 1950s, it was recognized that maternal autoantibodies can cross the placenta and that fetuses of mothers with an autoimmune disease may develop isolated congenital complete atrioventricular block that has been already recognized as a distinct clinical entity [38, 135]. In the early 1980s, a close association between maternal anti-Ro and anti-La antibodies and CHB was shown [36, 136, 137].

Other features of NLE are frequently observed after birth and include cutaneous rash, hematological disorder (thrombocytopenia, leukopenia, and anemia), and liver dysfunction [25]. Unlike CHB, these symptoms of NLE usually resolve within 6 months after birth, coincident with the time of the clearance of the maternal antibodies from the infants' circulation.

A recent paper described that all cardiac complications seen in neonates were associated with moderate to high ($\geq$50 U/mL) maternal anti-Ro antibody levels, independently of anti-La antibody titers [38]. The event rate of CHB was 5% for prospectively screened fetuses with high anti-Ro antibody levels ($\geq$50 U/mL; odds ratio: 7.8) and 0% for those exposed to lower titers. On the other hand, infants with prenatal exposure to high titers of anti-La antibodies ($\geq$100 U/mL) were most likely to have noncardiac manifestations of NLE, with an event rate of 57% (odds ratio: 4.7). These findings suggest that the concentration of maternal autoantibodies, rather than their presence, is associated with the development of NLE. Thus fetal echocardiography should be reserved for women with high anti-Ro antibody titers [38].

As most mothers of neonates with NLE do not have any connective tissue disease, a previous suggestion that all pregnant women should be screened for anti-Ro antibodies irrespective of their symptoms or clinical status may be considered reasonable [138].

5.3. Other Autoimmune Diseases. Anti-Ro antibodies are found also in 3–11% of patients with SSc [15, 27, 32, 139] and associated with sicca symptoms and severe pulmonary involvement [27, 32, 33]. Anti-Ro antibodies are detectable in 5–15% of patients affected by idiopathic inflammatory myopathy, including polymyositis (PM) and dermatomyositis (DM). PM/DM patients with anti-Ro antibodies frequently showed a specific reactivity to Ro52 without Ro60 [15, 41, 42, 140]. The presence of anti-Ro52 antibodies is associated with anti-Jo-1 antibodies or other anti-aminoacyl transfer RNA synthetase (ARS) antibodies [15, 41, 140–142]. The coexistence of anti-Ro and anti-Jo-1 antibodies seems to be related to a more severe interstitial lung disease compared with the patients with anti-Jo-1 antibodies alone [34, 35].

Anti-Ro antibodies are detected in 3–15% of patients with RA [28, 29, 143]. Most RA patients with anti-Ro antibodies share the same extra-articular features, such as sicca, photosensitivity, purpura, leukopenia, anemia, and hypocomplementemia [18, 28, 29]. Some authors also have mentioned a strong association between anti-Ro antibodies and the development of side effects by treatment with gold salts or D-penicillamine [144–146]. In a recent report anti-Ro antibodies are suggested to be an independent factor associated with an insufficient response to tumor necrosis factor (TNF) inhibitors in patients with RA [147].

Anti-Ro52 antibodies have a high specificity in primary biliary cirrhosis (PBC), an autoimmune liver disease. They are found in 28% of patients with PBC and in a more advanced histological stage [39]. Higher serum bilirubin and IgM levels at the time of diagnosis are related to anti-Ro52 antibodies. Antibodies to Ro52 are also detected in 38% of patients with type 1 autoimmune hepatitis, and they, together with anti-SLA antibodies, are independently associated with the development of cirrhosis and hepatic death or liver transplantation [40].

6. Pathogenic Role of Anti-Ro Antibodies

Although the pathogenic role of autoantibodies in autoimmune disease has not yet been clarified, hypotheses have been put forward indicating that anti-Ro antibodies might have a direct role in damaging tissues. UV irradiation induces de novo synthesis of the Ro antigens in both the cytoplasm and the nucleus in keratinocytes [96]. Besides, UV irradiation increases the expression of the antigens on the cell surface [94, 96], enhancing the possibility of direct injury of keratinocytes by anti-Ro antibodies [95]. Based on the data, Norris developed a hypothetical model of the pathogenesis of photosensitivity [148]. (1) UV exposure leads to an increased synthesis and expression of Ro antigen on the surface of epidermal keratinocytes; (2) anti-Ro antibodies from the circulation bind to the antigens on the cell surface; (3) the Fc domains of the bound anti-Ro antibodies are recognized by lymphocytes, leading to keratinocyte death. This hypothesis was consistent with the following study showing that photosensitivity and titer of circulating anti-Ro/anti-La antibodies were directly correlated with the expression of Ro and La antigens in skin specimens of

patients with SLE [149]. However, patients with SS and SLE with anti-Ro and/or anti-La antibodies only infrequently show photosensitivity [150].

Additional evidence for a direct pathogenic role of anti-Ro and anti-La can be found in studies of NLE. The cardiac damage is related to the expression of Ro and La antigens in fetal cardiac tissue from the 18th to 24th week, particularly located on the surface of cardiac myocytes [151–153]. Previous studies demonstrated that binding of anti-Ro and/or anti-La antibodies to apoptotic cardiocytes impairs their removal by healthy cardiocytes during the physiological cell deletion process in embryogenesis [154]. It also increases urokinase plasminogen activator- (uPA-)/uPA receptor- (uPAR-) dependent plasminogen and TGF-β activation [155, 156]. Again, it is still unclear why NLE develops in only some but not all antibody-exposed fetuses.

Interestingly, some anti-Ro antibody-positive adult patients with connective tissue disease may have disturbances in cardiac repolarization. Significantly increased mean corrected QT (QTc) intervals were present among anti-Ro antibody-positive patients when compared to anti-Ro antibody-negative individuals [157, 158]. The prolonged QTc interval seems to be directly attributable to the electrophysiological interference of anti-Ro antibodies with the inhibition of the Iκr current in cardiac myocytes [159]. Ventricular arrhythmias may also be more prevalent in those with anti-Ro antibodies [160].

Is there any possibility for anti-Ro antibodies to meet with Ro antigens inside the cell? Recently, it has been reported that IgG can enter the cytoplasm of nonimmune cells through the cell membrane together with virus [161]. In this paper, Ro52 acts as a cytosolic IgG receptor; it rapidly recruits incoming antibody-bound virus and targets it to the proteasomal degradation via its E3 ubiquitin ligase activity. This suggests the possibility of intracellular autoantigen-autoantibody interaction. A recent report shows that anti-Ro52 antibodies inhibit the E3 ligase activity of Ro52 by sterically blocking the E2/E3 interaction between Ro52 and UBE2E1 [162]. Although it still remains to be investigated whether enough anti-Ro52 autoantibodies can enter cells to sufficiently inhibit Ro52 function as a negative proinflammatory cytokine regulator, this inhibition may contribute to the pathogenesis of SLE and SS by inhibiting Ro52-mediated ubiquitylation.

7. Conclusions

Although anti-Ro antibodies have been used as a useful diagnostic marker for SLE and SS, they are the most prevalent autoantibodies among various autoimmune diseases. Above all, anti-Ro52 antibodies are specifically associated with myositis, SSc, and PBC. Furthermore, anti-Ro52 antibodies are related to a variety of symptoms in autoimmune diseases. Thus, separate measurement of anti-Ro52 and anti-Ro60 antibodies should be clinically useful. Anti-Ro52 antibodies may have pathological roles not only by damaging tissues directly but also by inhibiting the activity of Ro52 antigens. Further investigations into the Ro autoantigen-autoantibody system may offer a new strategy for treating autoimmune diseases.

Conflict of Interests

The authors have no financial conflict of interests.

Acknowledgments

The authors thank Mr. Tom Kiper for his review of the paper. This work was supported by research Grants from Japan Society for the Promotion of Science KAKENHI (no. 23791118) (R. Yoshimi) and Kanagawa Nanbyo Study Foundation (R. Yoshimi). This work was also supported partly by the Intramural Program of NICHD, National Institutes of Health, USA.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 494356, 6 pages
doi:10.1155/2012/494356

Review Article

The Clinical Significance of the Dense Fine Speckled Immunofluorescence Pattern on HEp-2 Cells for the Diagnosis of Systemic Autoimmune Diseases

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Received 12 October 2012; Accepted 1 November 2012

Academic Editor: Dimitrios P. Bogdanos

Antinuclear antibodies (ANAs) are a serological hallmark in the diagnosis of systemic autoimmune rheumatic diseases (SARD). The indirect immunofluorescence (IIF) assay on HEp-2 cells is a commonly used test for the detection of ANA and has been recently recommended as the screening test of choice by a task force of the American College of Rheumatology. However, up to 20% of apparently healthy individuals (HI) have been reported to have a positive IIF ANA test, primarily related to autoantibodies that target the dense fine speckles 70 (DFS70) antigen. Even more important, the DFS IIF pattern has been reported in up to 33% of ANA positive HI, but not in ANA positive SARD sera. Since the intended use of the ANA HEp-2 test is to aid in the diagnosis and classification of SARD, the detection and reporting of anti-DFS70 antibodies and their associated pattern (DFS) as a positive test significantly reduce the specificity and the positive likelihood of the ANA test. This has significant implications for medical management and diagnostic algorithms involving the detection of ANA. Recently, a novel immunoadsorption method has been developed that specifically blocks anti-DFS70 antibodies and, therefore, significantly increases the specificity of the ANA test for SARD. This immunoadsorption method has the potential to overcome a significant limitation of the ANA HEp-2 assay. The present paper summarizes the current knowledge about anti-DFS70 antibodies and their clinical impact on ANA testing.

1. History of ANA Testing

The presence of autoantibodies directed against intracellular antigens, especially antinuclear antibodies (ANAs), is a serological hallmark of systemic autoimmune rheumatic diseases (SARD) [1]. In 1958, Friou first described an indirect immunofluorescence (IIF) assay for the detection of antinuclear antibodies (ANA)—which is a historic landmark in the continuing long history of ANA testing in clinical medicine [2]. In most diagnostic laboratories, the ANA test uses HEp-2 tissue culture cells, a cell line which was established in 1952 by Alice E. Moore et al. and derived from tumors that had been produced in irradiated and cortisone treated weanling rats after injection with epidermoid carcinoma tissue obtained from the larynx of a 56-year-old male [3]. The HEp-2 cell—a virtual native protein and nucleic acid array comprised of hundreds if not thousands of potential autoantigens, has been an ideal substrate for the detection of ANA [4]. Over forty years ago and then during the following decades when HEp-2 cells were introduced and used as the IIF substrate of choice, the ANA IIF test using these cells revolutionized the diagnosis of SARD, especially of systemic lupus erythematosus (SLE) and systemic sclerosis.

In recent years, the IIF assay on HEp-2 cells has been replaced in many laboratories by high throughput and economical screening immunoassays, which incorporate the key SARD autoantibody target antigens into a single assay, on platforms such as ELISA and multiplex assays based on addressable laser bead technology [5]. However, due to a perceived high prevalence of "false negative" results and lack of standardization of innovative test algorithms (i.e., reflex testing) that attended these newer approaches, the American

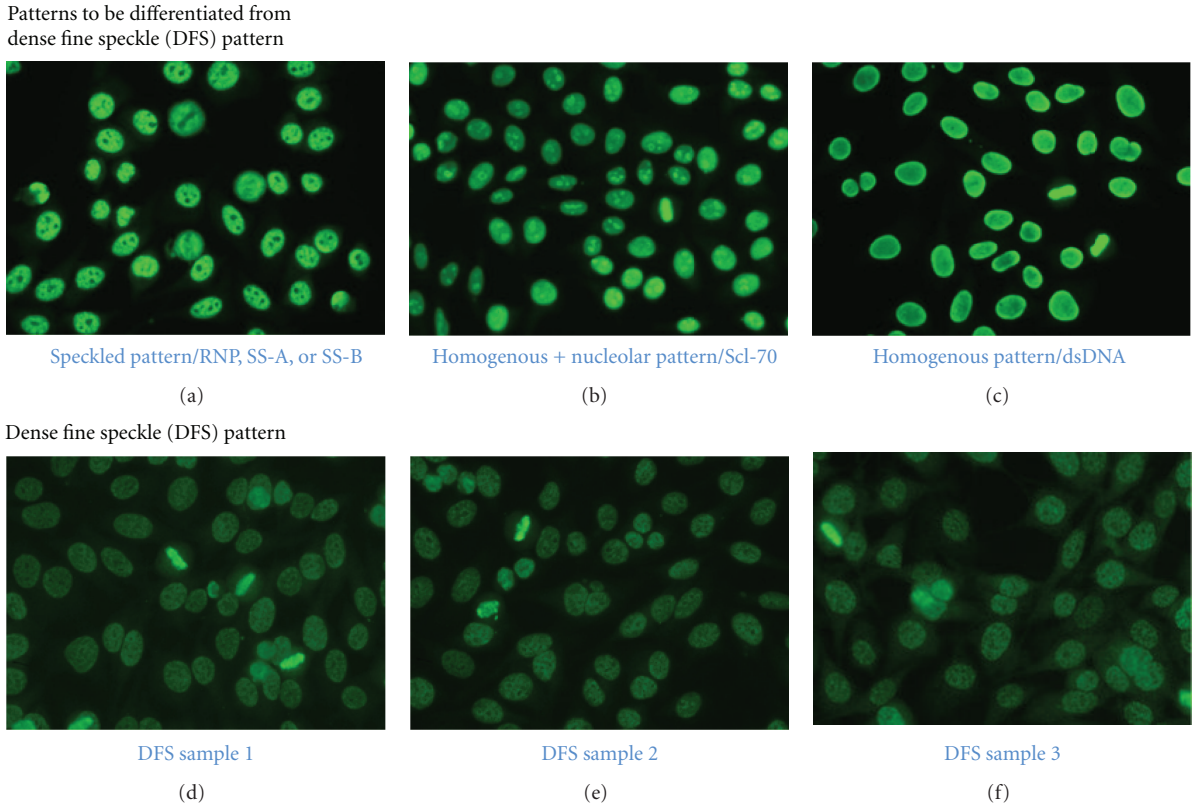

Figure 1: Identification of the dense fine speckled pattern is not always an easy task. The DFS pattern has to be differentiated from (a) speckled patterns generated by autoantibodies against RNP, Ro60 and SS-B (La), (b) from homogeneous patterns generated by anti-Scl-70 antibodies, and from (c) homogenous patterns generated by anti-dsDNA antibodies. DFS patterns generated by three different samples are shown in (d)–(f). Samples (d) and (e) are monospecific anti-DFS70 samples, and sample (f) also contains low titers of antibodies against extractable nuclear antigens (ENA).

College of Rheumatology (ACR) formed a task force who recommended that the traditional IIF ANA method on HEp-2 cells should remain the screening test of choice [4]. This has resulted in many laboratories moving back to the traditional HEp-2 cell based IIF method as screening test for ANA.

Coincident with these events, the first digital imaging systems were developed [6, 7] with an advantage of reducing two of the major drawbacks of the ANA IIF method, namely, the subjectivity of human interpretation of IIF results, and the lack of high throughput and automated reading technologies. Nevertheless, several challenges attending the HEp-2 IIF methodology persist [8, 9] and other technologies for ANA detection continue to evolve [10, 11].

One of the most important drawbacks of the HEp-2 IIF assay as a screening test is its limited specificity for SARD [9, 12]. Approximately 20% of serum samples from healthy individuals (HI) have been reported to have a positive ANA test [13], the majority of which are reported to be directed to the dense fine speckles 70 (DFS70) antigen [13].

2. History and Clinical Association of Anti-DFS70 Antibodies

Anti-DFS70 antibodies were initially identified in a patient with interstitial cystitis [14] but were later associated with various conditions, especially atopic dermatitis [15]. Since their first description, anti-DFS70 antibodies have been found in the sera of patients with a variety of conditions including cancer [16], and even more interestingly in HI [13, 17]. Dellavance et al. evaluated over 10,000 ANA positive samples by HEp-2 IIF followed by a confirmatory immunoblot and reported that anti-DFS70 antibodies were common among ANA-positive individuals with no evidence of SARD and that among autoimmune patients with this autoantibody over a half had evidence of autoimmune thyroiditis [18]. Although the spectrum of clinical associations and the mechanisms of anti-DFS70 induction are still unclear, different research teams have confirmed that anti-DFS70 antibodies are curiously more prevalent in apparently HI than in SARD patients [13, 15]. In addressing the prognostic and long-term outcome of individuals that have anti-DFS70 antibodies, it was recently reported that none of the 40 anti-DFS70 positive HI developed SARD over an average of 4-years of clinical followup [12]. Based on these observations, it has been suggested that the presence of isolated anti-DFS70 antibodies could be used as a biomarker to exclude the diagnosis of SARD, such as SLE [12, 13, 19]. Explanations for the decreased prevalence of anti-DFS70 autoantibodies in SARD patients continue to be unclear, but may relate to concurrent therapeutic,

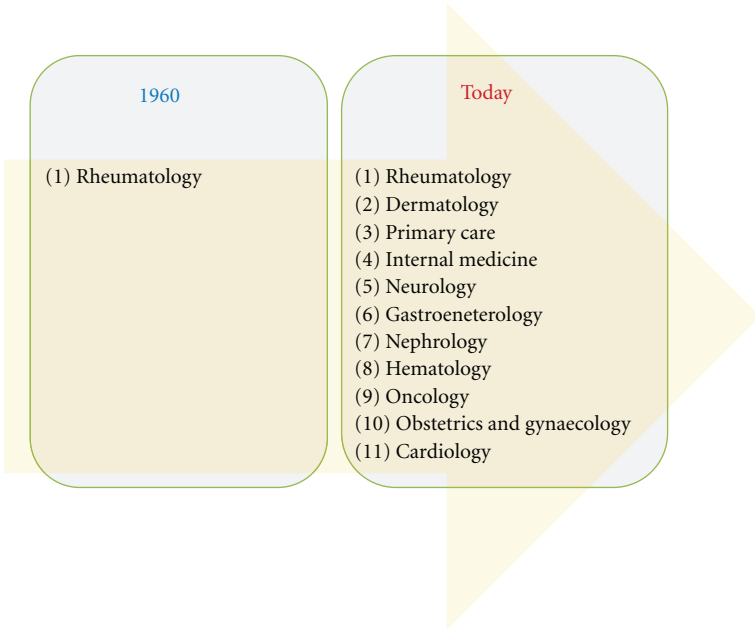

Figure 2: Change in ANA test referral patterns. Historically, when the ANA HEp-2 test became available in the 1960s exclusively rheumatologist ordered the ANA test. With the emerging recognition that many other diseases are associated with ANAs, a broad range of clinical disciplines order the ANA test.

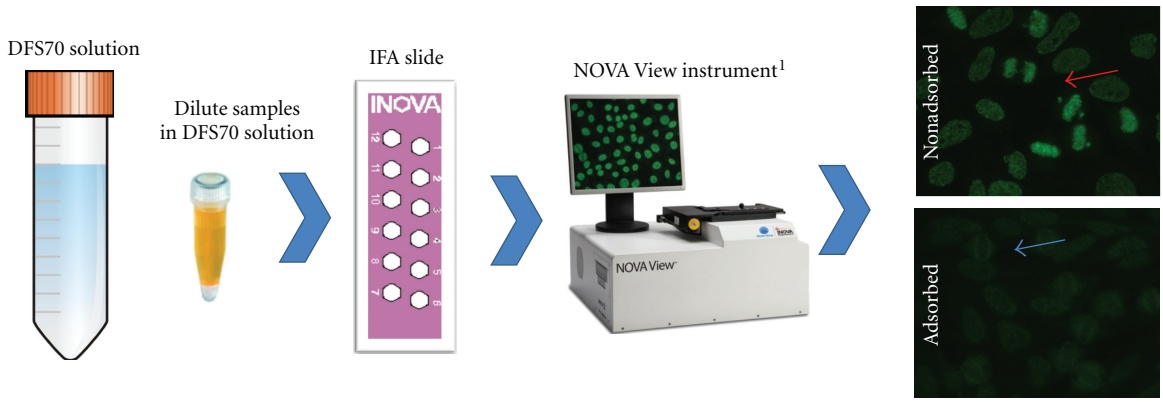

Figure 3: Immunoadsorption of anti-DFS70 antibodies. Serum samples are diluted in sample buffer containing recombinant DFS70 antigen and applied to HEp-2 cells on slides. Following immunodetection using a secondary antibody, indirect immunofluorescence is detected using NOVA View or a conventional microscope. Anti-DFS70 antibodies are specifically blocked.

demographic, genetic [20], racial, and/or technological variables.

3. IIF Pattern and Cellular Function of DFS70

The typical IIF staining pattern has been described as DFS that are rather uniformly distributed throughout the interphase nucleus and, most notably, are also localized on metaphase chromosomes (see Figures 1(d)-1(e)) [21]. As with other patterns, the typical DFS pattern can vary depending on the manufacturer source of the HEp-2 slides used as substrate [22]. Since a 70-kDa protein was recognized by immunoblotting, the antigen was initially termed DFS70 but eventually the primary target autoantigen was identified as the lens epithelium derived growth factor (LEDGF) [23] and/or DNA binding transcription coactivator p75 (reviewed in [15]). This protein is highly expressed in prostate tumor tissue [16] and has a number of physiological functions including serving as a cofactor for human immunodeficiency virus replication through interaction with the viral integrase [24].

4. Change in ANA Test Referral Pattern

Historically, when the ANA HEp-2 test became available in the 1960s, predominantly rheumatologists and clinical immunologists ordered the ANA test. With the emerging recognition that many other diseases with autoimmune

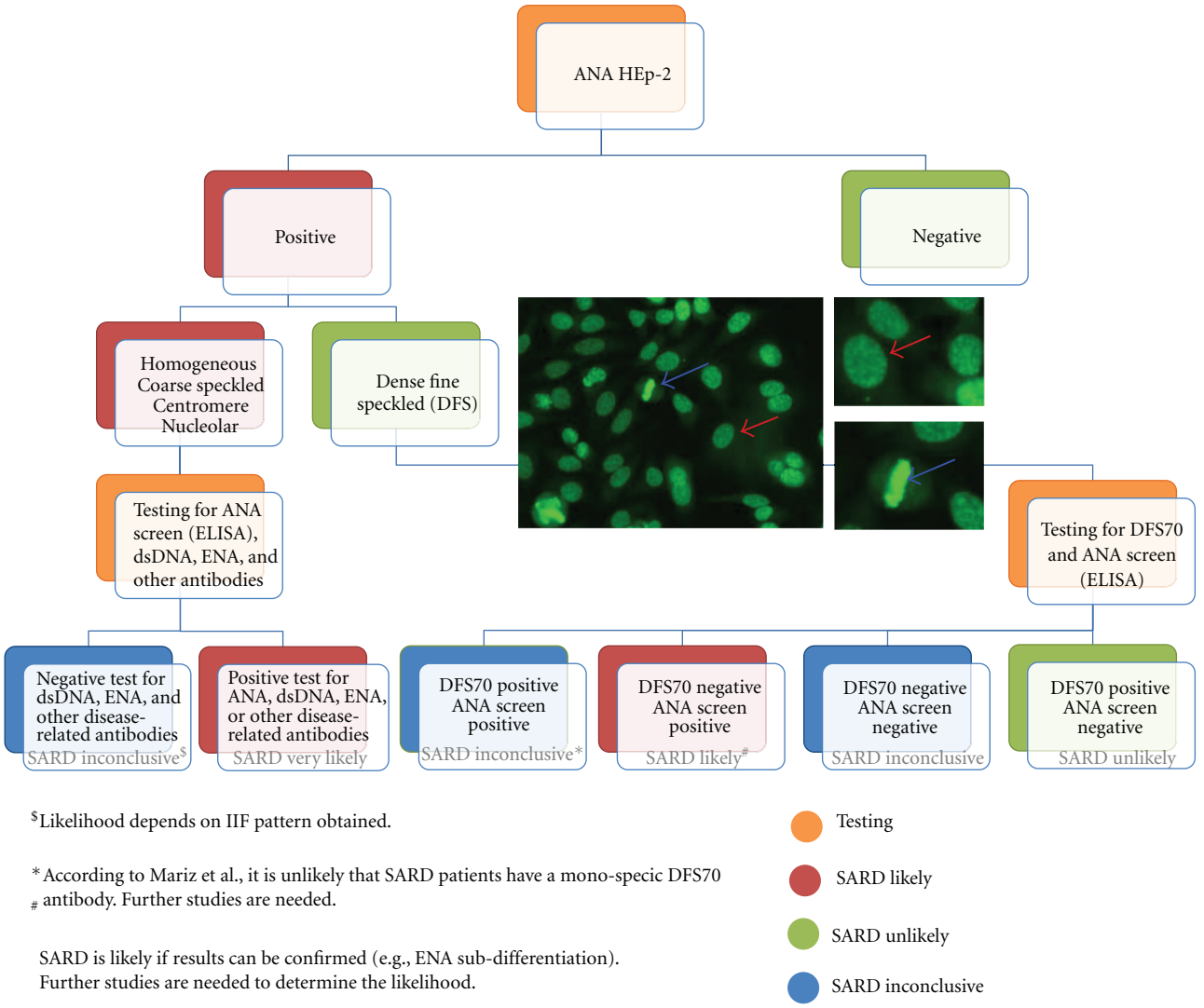

Figure 4: Characteristic staining pattern and suggested test algorithm considering anti-DFS70 antibodies. The characteristic dense fine speckled (DFS) staining pattern of interphase cells is indicated by the red arrow and the strong chromosome staining of metaphase cells by the blue arrow. Samples with a DFS pattern should be tested for anti-DFS70 antibodies by a confirmatory test and by ANA Screen ELISA (QUANTA Lite ANA Screen ELISA) containing various autoantigens. Patients with negative ANA Screen ELISA and positive DFS70 result have a low likelihood for having SARD. Patients with a positive ANA Screen ELISA either in combination with a positive or a negative DFS70 test result have an increase likelihood of having SARD.

features are also associated with ANAs, a broader range of clinical disciplines (i.e., primary care, dermatology, nephrology, gastroenterology, neurology, oncology, hematology, obstetrics, gynaecology, as well as cardiology) currently order the ANA test (Figure 2). This change in test referral patterns has tremendous consequences for the posttest probability of disease since screening tests with limited specificity (such as IIF ANA) are strongly affected when the pretest probability in a given population decreases.

5. Detection of Anti-DFS70 Antibodies

Anti-DFS70 antibodies can be detected by various technologies including IIF [12], immunoblot [12], ELISA [22], addressable laser bead assay (ALBIA, unpublished data), and a novel chemiluminescent assay [25]. Most likely other technologies will be successfully employed such as line immunoassays and lateral flow point of care diagnostics containing purified human DFS70 as one of the antigens. A recent strategy that was developed to assist in the detection of anti-DFS70 antibodies is an immunoadsorption IIF assay [25] as described in the following section.

6. Immunoadsorption of Anti-DFS70 Antibodies

In a recent study, the DFS IIF pattern was found in 33.1% of ANA positive HI compared to 0.0% of ANA positive

patients with SARD ($P < 0.0001$), a result that significantly affects the diagnostic power and efficiency of the IIF assay [12]. Thus, accurate IIF pattern recognition, interpretation, and reporting of results to clinicians are of high importance because it could decrease the necessity of urgent referral of patients with a positive ANA for tertiary care consultation and evaluation [9]. Since the definitive identification of the DFS IIF pattern might be challenging for routine diagnostic laboratories [22] and inaccurate interpretation can have significant consequences, a method that can prevent anti-DFS70 antibodies from binding to their cognate target and producing the DFS pattern was postulated to significantly improve the performance characteristics of ANA by IIF on HEp-2 substrates. Consequently, a novel method allowing for the immunoadsorption of anti-DFS70 antibodies was developed that was meant to yield considerable costsavings by eliminating or reducing unnecessary additional tests (i.e., extractable nuclear antigen profiles or SARD specific autoantibody arrays) [25]. In this approach, patient serum samples are diluted in a sample buffer containing recombinant DFS70 antigen and pipetted onto the HEp-2 cell substrate in wells on glass slides. Following a washing step to remove unbound components, FITC-conjugated antihuman IgG secondary antibody is added. Subsequently, after removing unbound FITC conjugate, the IIF pattern is analyzed under a standard fluorescence microscope or with the NOVA View (INOVA Diagnostics) digital imaging system (see Figure 3). The NOVA View is an inverted microscope that takes pictures of each well, reads common patterns, semiquantifies ANA titers, and through a proprietary algorithm suggests an interpretation of the result.

7. Consequences for ANA Testing: A New Algorithm

In a previous study, 172/21,512 (0.8%) of consecutive serum samples tested for ANA by IIF showed the typical DFS pattern [26] and this was one of the most common IIF patterns observed in the routine clinical diagnostic laboratory. Since the presence of ANA is considered a reliable screening clinical indicators for SARD and are included in the classification criteria for SLE [27], ANA-HEp-2 testing outside a proper clinical framework may yield a sizable portion of ANA-positive individuals with no consistent evidence of SARD. This has the potential to cause undue concern and anxiety in patients, their families, and physicians alike [12], or even lead to unwarranted therapies [28]. This becomes even more crucial with the compelling evidence that autoantibodies appearing in the serum may precede the clinical onset of SARD by many years [29]. As pointed out in a recent article, not all sera demonstrating the DFS pattern are from HI and it remains unclear whether this IIF staining pattern is universally recognized in clinical diagnostic laboratories. The discrimination between DFS and the so-called "quasihomogeneous pattern" might especially be a challenge for routine diagnostic laboratories [9]. This underlines the importance of a better understanding of anti-DFS70 antibodies and the inclusion of testing for anti-DFS70 antibodies into diagnostic algorithms (see Figure 4). A suggestion is that samples with a DFS staining pattern identified by IIF should be tested for anti-DFS70 antibodies using a specific immunoassay and then the test results and the significance of the findings need to be clearly explained to clinicians.

Abbreviations

ANA: Antinuclear antibody
DFS: Dense fine speckled
HI: Healthy individuals
IIF: Indirect immunofluorescence
SARD: Systemic autoimmune rheumatic diseases
SLE: Systemic lupus erythematosus.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 167132, 7 pages
doi:10.1155/2012/167132

Review Article

Macroprolactinemia: Diagnostic, Clinical, and Pathogenic Significance

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Received 12 September 2012; Revised 2 November 2012; Accepted 8 November 2012

Academic Editor: Xavier Bossuyt

Macroprolactinemia is characterized by a large molecular mass of PRL (macroprolactin) as the main molecular form of PRL in sera, the frequent elevation of serum PRL (hyperprolactinemia), and the lack of symptoms. Macroprolactin is largely a complex of PRL with immunoglobulin G (IgG), especially anti-PRL autoantibodies. The prevalence of macroprolactinemia is 10–25% in patients with hyperprolactinemia and 3.7% in general population. There is no gender difference and a long-term followup demonstrates that macroprolactinemia develops before middle age and is likely a chronic condition. Polyethylene-glycol- (PEG-) precipitation method is widely used for screening macroprolactinemia, and gel filtration chromatography, protein A/G column, and ^{125}I-PRL binding studies are performed to confirm and clarify its nature. The cross-reactivity of macroprolactin varies widely according to the immunoassay systems. The epitope on PRL molecule recognized by the autoantibodies is located close to the binding site for PRL receptors, which may explain that macroprolactin has a lower biological activity. Hyperprolactinemia frequently seen in macroprolactinemic patients is due to the delayed clearance of autoantibody-bound PRL. When rats are immunized with rat pituitary PRL, anti-PRL autoantibodies are produced and hyperprolactinemia develops, mimicking macroprolactinemia in humans. Screening of macroprolactinemia is important for the differential diagnosis of hyperprolactinemia to avoid unnecessary examinations and treatments.

1. Introduction

Prolactin (PRL) is an anterior pituitary hormone that plays an important role in lactation during pregnancy but has many other biological functions such as osmoregulation, angiogenesis, and immunoregulation [1]. PRL facilitates the maturation of T cells via IL-2 receptor expression, impairs B cell tolerance to self-antigens through the anti-apoptotic effect, develops antigen-presenting cells, and enhances immunoglobulin production [2]. The increase in serum PRL concentrations (hyperprolactinemia) often develops symptoms such as amenorrhea and galactorrhea in women and impotence in men. It is caused physiologically by pregnancy and pathologically by PRL secreting pituitary adenoma (prolactinoma), hypothalamic and pituitary diseases compressing pituitary stalk, antidopaminergic drugs, hypothyroidism, chest wall diseases, and hepatorenal disorders [3]. However, 29% of hyperprolactinemia has been classified as "idiopathic" because the causes are unknown [4]. Microadenomas in the pituitary gland that cannot be detected by computed tomography (CT) or magnetic resonance imaging (MRI) have been postulated to enter this category.

Anti-PRL autoantibody was found to be one of the major causes of "idiopathic" hyperprolactinemia [5]. It binds to PRL (molecular mass of 23 kDa) forming a large immune complex of PRL with IgG (macroprolactin) and tends to increase serum PRL concentrations. Macroprolactinemia is defined as having macroprolactin (molecular mass greater than 150 kDa) in the predominant molecular form of PRL in sera.

There are reportedly several autoantibodies against hormones other than PRL: insulin [6], glucagon [7], thyroid

hormone [8], parathyroid hormone [9], anterior pituitary hormones such as adrenocorticotropic hormone (ACTH) [10], luteinizing hormone (LH), follicle stimulating hormone (FSH) [11], growth hormone (GH) [12] and thyroid stimulating hormone (TSH) [13], and posterior pituitary hormones such as vasopressin and oxytocin [14]. This paper focuses on the diagnostic, clinical, and pathogenic features of macroprolactinemia.

2. Diagnosis of Macroprolactinemia

Macroprolactin is mostly a complex of PRL with IgG, especially anti-PRL autoantibodies (Figure 1). The screening of macroprolactinemia is performed by polyethyleneglycol- (PEG-) precipitation method, and the confirmative and qualitative examinations include gel chromatography, protein A/G column, and ^{125}I-PRL binding studies [15]. Advantage and disadvantage of these methods are summarized in Table 1.

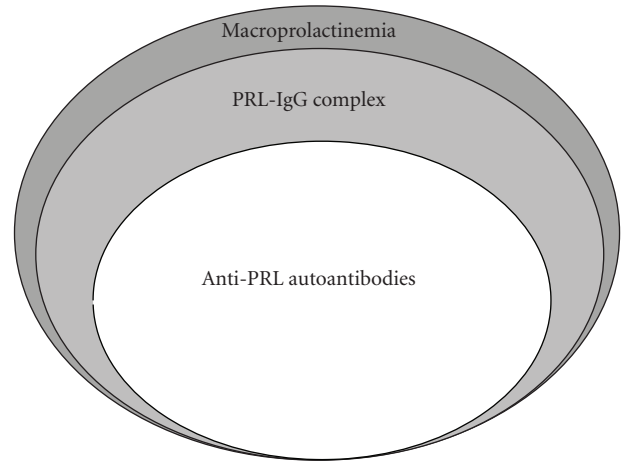

Figure 1: Macroprolactinemia, IgG-bound PRL, and anti-PRL autoantibodies. Macroprolactinemia is a heterogeneous condition with different etiologies; 87% of macroprolactin was PRL-IgG complex and 67% of macroprolactin was autoantibody-bound PRL [15]. Although anti-PRL autoantibody-bound PRL is a major form of PRL-IgG complex and PRL-IgG complex is a major form of macroprolactin, there may be PRL-IgG complex other than autoantibodies and macroprolactin other than PRL-IgG complex as shown in grey area. The diagnosis of macroprolactinemia is made based on the results of PEG-precipitation method or gel filtration chromatography, IgG-bound PRL by protein A or protein G column method, and anti-PRL autoantibody-bound PRL by ^{125}I-PRL binding study.

2.1. Polyethylene-Glycol- (PEG-) Precipitation. Since we first applied PEG precipitation method, which had been used to detect anti-insulin autoantibodies, to diagnose macroprolactinemia due to anti-PRL autoantibodies [5], this method has been used for the screening of macroprolactinemia because of its simplicity [19–28]. The method was validated against gel filtration chromatography, a gold standard for the diagnosis of macroprolactinemia [19]. To determine free PRL concentrations, serum samples (50 μL) are mixed vigorously with 50 μL of cold PEG (molecular weight 6000, 25% in water) and centrifuged at 9,100 $\times$g for 10 min to remove macroprolactin. Serum samples are treated identically, but with water instead of PEG, to determine total PRL concentrations. The PEG-precipitable PRL (%), which represents the amount of macroprolactin, is calculated as follows: (total PRL-free PRL)/total PRL $\times$ 100. PEG-precipitation ratio greater than 60% (recovery less than 40%) is used as the cut-off value for the diagnosis of macroprolactinaemia. Gibney et al. [23] proposed an alternative presentation, that is, showing absolute values of free PRL in the supernatant after PEG precipitation. When PRL levels after PEG precipitation fall within a reference range derived from similarly treated normal sera, this is considered a normal result. From a clinical point of view, this is reasonable because such presentation can disclose the patients who need further examinations and treatments for hyperprolactinemia. Both presentations (PEG-precipitation ratio and free PRL value) may be desirable until the time when it is clarified that anti-PRL autoantibodies in macroprolactinemia do not affect PRL actions and macroprolactinemia is totally a benign condition.

As to the prevalence of macroprolactinemia screened by PEG-precipitation method, it is noteworthy that the detectability of macroprolactin varies a lot according to the PRL assay systems [28]. It is possible that some reagent antibodies against PRL in assay kits can recognize anti-PRL autoantibody-bound PRL and others not. Moreover, it may be attributable to the heterogeneity of macroprolactinemia [17, 29].

2.2. Gel Filtration Chromatography. Traditionally, gel filtration chromatography has been used to separate various molecular forms of PRL: little (monomeric) PRL (molecular size: 23 kDa), big PRL (45–50 kDa), and big-big PRL (more than 100 kDa) [30]. Little PRL is a major form of pituitary and serum PRL, big PRL is a dimer of little (monomeric) PRL, and big-big PRL has been believed to be an aggregate of monomeric PRL. Macroprolactin is defined as a large molecular-sized PRL greater than 100 kDa that is included in big-big PRL, and the state in which the ratio of macroprolactin is substantially increased in sera is called macroprolactinemia (Figure 2). There is no a clear-cut value of "substantial increase", but conventionally a diagnosis of macroprolactinemia is made when more than 30–60% of PRL is in the macroprolactin form of gel filtration chromatography [27]. Although gel filtration chromatography was regarded as the gold standard for the diagnosis of macroprolactinemia, it is time, labor, and cost consuming. Thus this method is used to confirm the diagnosis of macroprolactinemia.

2.3. Protein A/G Column. Protein A binds to the Fc portion of immunoglobulin molecules without interfering with the antigen-binding site. Protein G binds only to IgG and its subclasses, separating out IgA, IgM, IgD, and albumin,

Table 1: Advantage and disadvantage of methods for the diagnosis of macroprolactinemia.

	Advantage	Disadvantage
Polyethylene glycol (PEG)	Simple and inexpensive Screening for macroprolactinemia	Not highly specific
Gel chromatography	Accurate Confirming macroprolactinemia	Time consuming and expensive
Protein A/G	Identifying IgG-bound PRL (common cause of macroprolactin)	Expensive
^{125}I-PRL binding	Identifying anti-PRL autoantibodies (common cause of IgG-bound PRL)	Time consuming and hazardous Needs radio isotope facilities

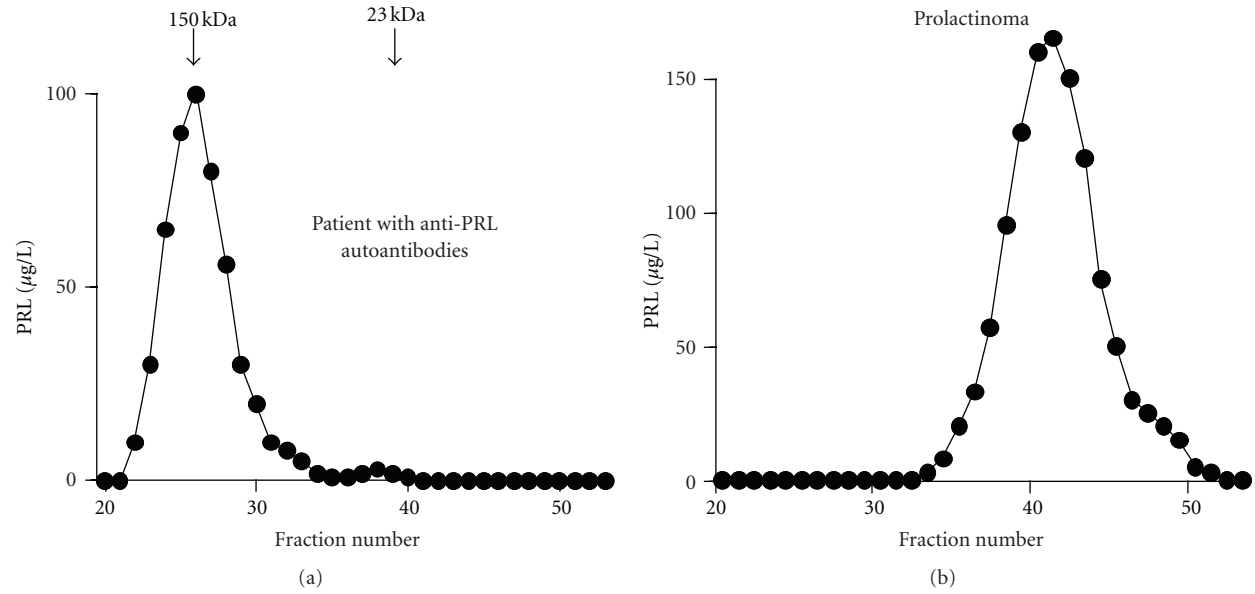

Figure 2: Gel filtration chromatography of macroprolactin and prolactin. Representative gel filtration chromatography of PRL in serum samples of macroprolactinemia (a) and prolactinoma (b). (Reproduced from [16]).

which may bind to protein A [31]. These reagents are used to identify macroprolactin due to PRL-IgG complexes, and most patients with macroprolactinemia belong to this category [15, 17].

2.4. ^{125}I-PRL Binding Study. Serum samples (100 μL) and ^{125}I-PRL (20000 cpm/50 μL sodium phosphate buffer containing 0.1% bovine serum albumin and 0.1 mol/L NaCl) were incubated for 1 h at room temperature. After incubation, 200 μL of cold 25% (wt/wt) PEG 6000 (final concentration 12.5%) was added, and the mixture was mixed vigorously and centrifuged at 3000 rpm for 20 min. The sediment was washed once with 12.5% PEG, and the radioactivity was measured with γ-counter [5, 15, 17].

3. Clinical Significance

3.1. Prevalence of Macroprolactinemia. Figure 3 shows the prevalence of macroprolactinemia and its gender and age dependency in a large group of hospital workers ($n = 1330$) [17]. Macroprolactinemia is present in 3.68% in general population. The prevalence is not different between women and men, and it tends to increase in elderly people. In patients with hyperprolactinemia, the prevalence of macroprolactinemia is reportedly 10–25% [20–23].

3.2. Symptoms. Hyperprolactinemia tends to develop in patients with macroprolactinemia [17] because of the delayed clearance of macroprolactin. Although hyperprolactinemia frequently causes menstrual irregularities and galactorrhea in women and loss of libido in men, patients with macroprolactinemia often lack such clinical symptoms of hyperprolactinemia [18]. *In vitro* experiment using T47D human breast cancer cells, the bioactivity of autoantibody-bound PRL was found to be low [32], compatible with the clinical characteristics. However, controversial results are also reported [22] probably because the prevalence of macroprolactinemia is so high that the patients with macroprolactinemia might have other causes of hyperprolactinemia as well [27]. Women with macroprolactinemia can get pregnant and deliver normal babies without any treatments of hyperprolactinemia [33]. Long-term follow-up studies of macroprolactinemia show that macroprolactinemia persisted but no symptomatic progression was

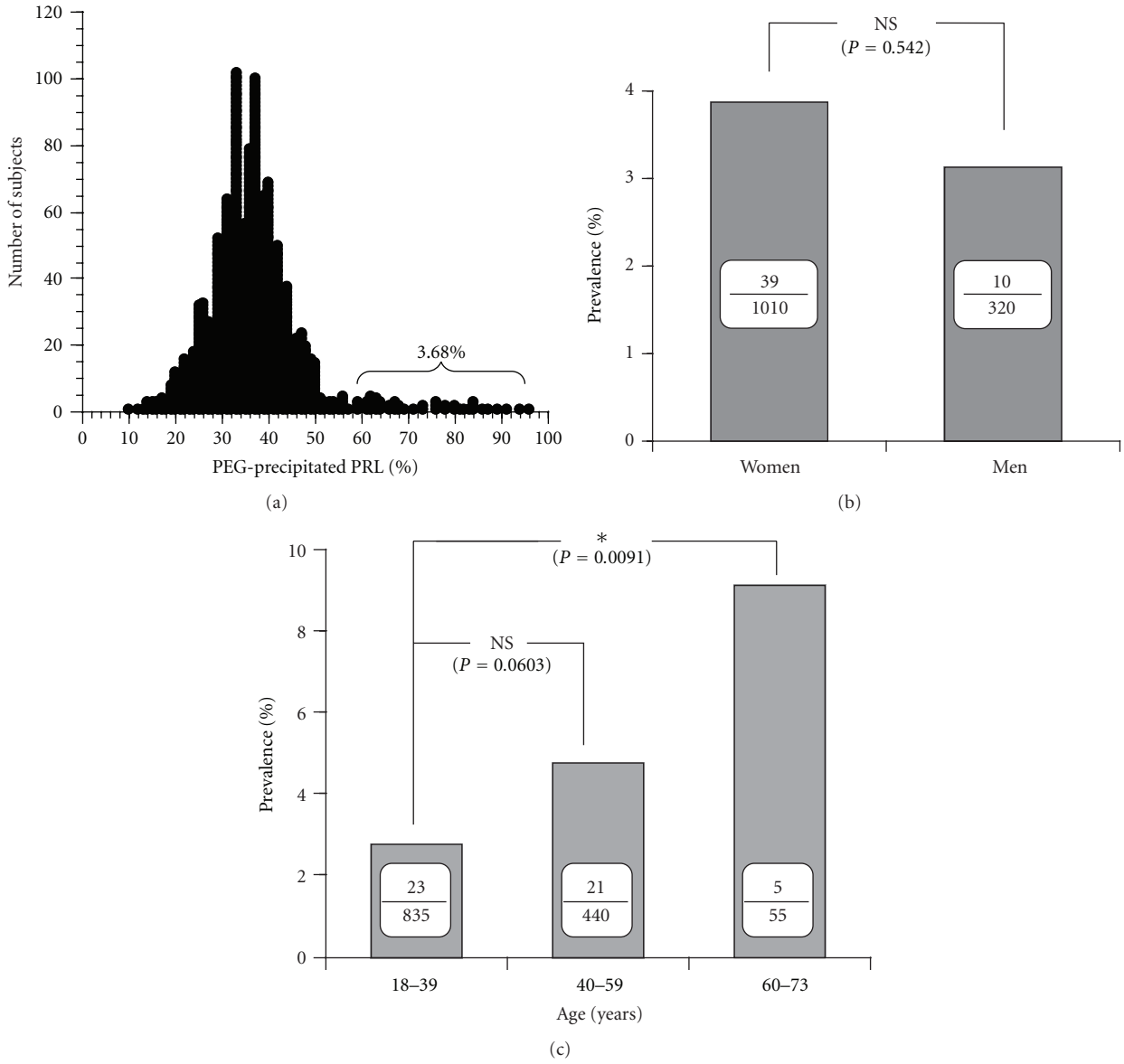

Figure 3: The prevalence of macroprolactinemia and its gender and age dependency in normal subjects. (a) The circle represents the value of PEG-precipitation ratio in each individual and the vertical line shows the number of subjects. The graph shows almost a normal distribution except for a subgroup (macroprolactinemia) having the ratio greater than 60% (mean + 2SD). (b) The prevalence of macroprolactinemia in women and men. (c) The prevalence of macroprolactinemia in different age groups. (Reproduced from [17]).

noted [15, 34]. Thus it is strongly recommended that all patients with hyperprolactinemia should take the screening test of macroprolactinemia in order to avoid unnecessary examinations and treatments.

3.3. Association with Other Autoimmune Disorders. Anti-PRL autoantibodies are a major cause of macroprolactinemia. Thus it is possible that some autoimmune disorders might be accompanied with macroprolactinemia. There were some case reports showing the association between macroprolactinemia and autoimmune thyroid disorders such as Graves' disease and Hashimoto's thyroiditis [35, 36]. Hyperprolactinemic SLE patients reportedly had a higher frequency of macroprolactinemia (14/43, 32.6%) [37]. However, other studies examining a large number of patients revealed no specific association between macroprolactinemia and autoimmune disorders [22, 38]. It is likely that autoimmune mechanisms may be directed mainly toward prolactin molecule in macroprolactinemia.

4. Pathogenic Significance

4.1. Characteristics of Anti-PRL Autoantibodies. When IgG was purified from sera using protein G column, a significant amount of PRL was copurified with IgG in patients with macroprolactinemia. When this IgG fraction was analyzed

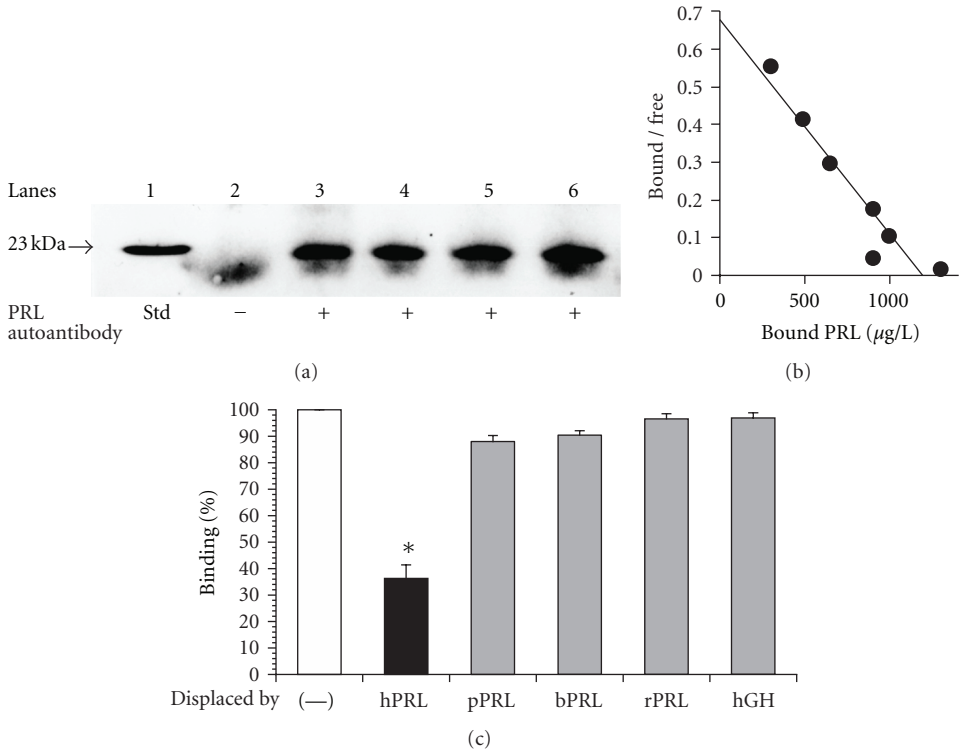

Figure 4: Electrophoresis of macroprolactin and Scatchard analysis of anti-PRL autoantibodies. (a) IgG was purified from the sera of the patients with macroprolactinemia (lanes 3–6) using a protein G column and run on SDS-PAGE under a nonreducing condition. PRL, which bound to the autoantibodies, was dissociated and immunostained at the same position as the 23 kDa human PRL standard (lane 1). The 23 kDa PRL band was not observed when IgG from a patient with prolactinoma was used (lane 2). The fuzzy bands migrating faster than PRL may be nonspecific staining of IgG light chain. (b) Scatchard analysis revealed low-affinity and high-capacity autoantibodies. (c) Displacement of ^{125}I-hPRL-autoantibody complex by human PRL and other related peptides; hPRL: human PRL, pPRL: porcine PRL, bPRL: bovine PRL, rPRL:r at PRL, and hGH: human GH. Only hPRL could displace the binding of ^{125}I-PRL with the autoantibodies. (Reproduced from [5, 15, 18]).

on SDS-PAGE under nonreducing condition, the IgG-bound PRL was dissociated and immunostained at the same position as the 23 kDa human PRL standard as shown in Figure 4(a), suggesting that PRL is noncovalently bound to IgG [18]. Scatchard analysis revealed low-affinity and high-capacity autoantibodies as shown in Figure 4(b) [5, 33] and only human PRL could displace the binding of ^{125}I-PRL and anti-PRL autoantibodies as shown in Figure 4(c) [15, 39], suggestive of the specificity of the autoantibody to human PRL. The subclass of anti-PRL autoantibodies was mainly IgG4, suggesting that chronic antigen stimulation may be involved [40].

4.2. Mechanisms of Hyperprolactinemia. Dopamine or bromocriptine, dopamine D_2 receptor agonist, decreases serum PRL concentrations [1]. Administration of these drugs to the patients with macroprolactinemia did not decrease serum PRL concentrations so much as in prolactinoma, suggesting that the clearance of PRL is delayed [33]. This was confirmed by the animal experiments showing that anti-PRL autoantibody-bound PRL was cleared from the rat circulation more slowly than monomeric PRL [18] and that hyperprolactinemia developed in animal model of macroprolactinemia [41]. Moreover, a significant positive correlation was present between anti-PRL autoantibody titers and serum PRL concentrations in humans [33], suggesting that anti-PRL autoantibody is a cause of hyperprolactinemia. It is assumed that the hypothalamic negative feedback mechanism by autoantibody-bound PRL does not work because the complex cannot access to the hypothalamus and/or anti-PRL autoantibodies interfere with the binding of PRL to the receptor. Thus, serum PRL concentrations may increase until free PRL concentrations exceed normal PRL concentrations, when negative feedback mechanisms begin to operate to make free PRL levels down. Actually, free PRL concentrations in sera from most patients with macroprolactinemia are within normal range [17, 27].

4.3. Mechanisms of Low Bioactivity. Macroprolactinemia is characterized by a lack of clinical symptoms of hyperprolactinemia and several studies using different PRL bioassay systems showed that the bioactivity of macroprolactin is low [32, 42, 43]. The epitope mapping using deleted PRL fragments revealed that the binding sites of PRL molecule

to anti-PRL autoantibodies reside in N and C terminals of PRL [39]. The core human PRL structure is made up of four major α-helices. The α-helix groups are in two antiparallel pairs, helix 1/helix 4 and helix 2/helix 3, each pair being packed more closely together. Therefore, several N- and C-terminal amino acid residues are located closely in the three-dimensional structure forming a part of binding site 1 to human PRL receptors [44]. Thus, anti-PRL autoantibodies and PRL receptors bind to the similar regions on PRL molecule, raising a possibility that the autoantibodies may compete the binding of PRL molecule to its receptors, resulting in the low biological activity.

4.4. Possible Causes of Anti-PRL Autoantibody Production. Mechanisms involved in the development of anti-PRL autoantibodies are unknown. Genetic susceptibility and environmental factors may alter the immune response in hosts as postulated in other autoimmune disorders [45]. On the other hand, some changes in PRL molecule may increase the antigenicity leading to the production of anti-PRL autoantibodies. The finding that IgG4 was a predominant subtype of anti-PRL autoantibodies supports the latter possibility because IgG4 response occurs in chronic antigen response. Switch from IgG1 to IgG4 response is driven by the repeated exposure to allergens, and IgG4 is a predominant subclass in other autoantibodies [46, 47]. Posttranslational modifications such as phosphorylation of PRL might be involved in the possible altered antigenicity [40].

5. Conclusion

Macroprolactinemia should be examined in all serum samples with hyperprolactinemia for the differential diagnosis because it is one of the major causes of hyperprolactinemia. Neither medications nor further examinations are recommended if free PRL concentrations after precipitating macroprolactin with PEG are normal, because the biological activity of macroprolactin is low and pregnancy is possible without any treatment for hyperprolactinemia. Macroprolactinemia is a heterogeneous condition with different etiologies and further study is necessary to glean it in its entirety.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 265868, 7 pages
doi:10.1155/2012/265868

Research Article

Correlation of Circulating Glucocorticoid-Induced TNFR-Related Protein Ligand Levels with Disease Activity in Patients with Systemic Lupus Erythematosus

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Received 30 September 2012; Accepted 3 November 2012

Academic Editor: Pietro Invernizzi

The aim of this paper is to investigate the correlation of glucocorticoid-induced tumor necrosis factor receptor- (TNFR-) related protein ligand (GITRL) with disease activity and organ involvement in patients with systemic lupus erythematosus (SLE). Serum GITRL levels were measured in 58 patients with SLE and 30 healthy controls matched for age and sex. Patients were assessed for clinical and laboratory variables. Correlations of serum GITRL levels with SLEDAI, laboratory values, and clinical manifestations were assessed. Serum GITRL levels were determined by ELISA. Serum GITRL levels were markedly increased in patients with SLE compared with healthy controls (mean 401.3 ng/mL and 36.59 ng/mL, resp.; $P < 0.0001$). SLE patients with active disease showed higher serum GITRL levels compared to those with inactive disease (mean 403.3 ng/mL and 136.3 ng/mL, resp; $P = 0.0043$) as well as normal controls (36.59 ng/mL; $P < 0.0001$). Serum GITRL levels were positively correlated with SLEDAI, titers of anti-dsDNA antibody, erythrocyte sedimentation rate (ESR), and IgM and negatively correlated with complement3 (C3). Serum GITRL levels were higher in SLE patients with renal involvement and vasculitis compared with patients without the above-mentioned manifestations.

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder characterized by the production of various autoantibodies that cause damage to multiple organs involving the skin, joints, heart, lungs, kidneys, and central nervous system (CNS) [1]. However, the precise etiology remains unclear. SLE is characterized by hyperreactivity of B lymphocytes, hyper-gammaglobulinemia, circulating immune complexes, and production of organ-specific and non-organ-specific autoantibodies. Moreover, dysregulated cellular immune responses are at times featured as lymphopenia and monocytosis. Numerous studies have shown that both T-cell activation and proinflammatory cytokine production are critically involved in SLE pathogenesis.

Glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) is a type I transmembrane protein belonging to the TNFR superfamily, and its cytoplasmic domain shares strong homology with a subgroup of the TNFR superfamily lacking the death domain, including CD27, CD134 (OX40), and CD137 (4-1BB). GITR is expressed predominantly on $CD4^+CD25^+$ regulatory T cells at high levels [2–4]. Moreover, other cells with regulatory activity, such as $CD4^+CD25^-$, $CD8^+CD25^+$, and $CD8^+CD28^-$ cells, express GITR at high levels [5]. However, its expression has also been detected on many cell types of both innate and adaptive immunity including monocytes, macrophages, neutrophils, dendritic cells (DCs), B cells, NK cells, and mast cells, and its expression level is increased after activation and during inflammatory or autoimmune processes [6–9]. GITR is activated by its ligand GITRL

(TNFSF18), a type II transmembrane protein belonging to the TNF superfamily. GITRL is expressed on a subpopulation of T cells and monocytes [10, 11]. Notably, antigen-presenting cells and endothelial cells are found to express high levels of GITRL [12, 13]. The GITR/GITRL pathway has been shown to modulate DC function and promote T-cell-mediated immunity [14]. Recent studies have also indicated that the functional interaction of GITR with its cognate ligand GITRL delivers a potent costimulatory signal to enhance T-cell activation and cytokine production with significant implications for cancer immunotherapy [15–17]. Moreover, GITRL has been shown to modulate cytokine release and NK cell reactivity in chronic lymphocytic leukemia [18].

As a costimulatory molecule for $CD4^+$ effector T-cell activation, GITR has been implicated in the development of autoimmune disease as revealed by recent studies on the murine model of collagen-induced arthritis (CIA) [19, 20]. Wang et al. showed that treatment of CIA mice with GITRL resulted in an earlier onset of arthritis with markedly increased severity of arthritic symptoms and joint damage, accompanied by significantly increased Th17 cells [21]. Furthermore, it was found that GITRL protein levels in the serum samples of rheumatoid arthritis (RA) patients were significantly higher than those in samples from healthy control subjects [21]. Notably, the increased levels of GITRL in RA patients were positively correlated with the DAS-28 scores of these patients [21]. However, it is currently unclear whether dysregulated GITRL expression is also involved in the development of other autoimmune diseases.

In this study, we sought to determine the possible involvement of GITRL expression in the development of SLE by examining the correlation of serum GITRL levels with disease activity and clinical manifestations in SLE patients.

Table 1: Characteristics of SLE patients and the control subjects.

	SLE ($n = 58$)	Control ($n = 30$)
Age (years)	30.6 ± 11.5	29.7 ± 6.8
Sex (female/male)	54/4	27/3
Disease duration (months)	24.9 ± 40.9	—
Alopecia *n* (%)	13 (22.4)	—
Rash *n* (%)	34 (58.6)	—
Arthritis *n* (%)	11 (19.0)	—
Fever *n* (%)	15 (25.9)	—
Pleuritis *n* (%)	9 (15.5)	—
Anemia *n* (%)	28 (48.3)	—
Leukopenia *n* (%)	23 (39.7)	—
Thrombocytopenia *n* (%)	5 (8.6)	—
Current renal disease *n* (%)	33 (56.9)	—
Vasculitis *n* (%)	24 (41.4)	—
Anti-ds-DNA *n* (%)	34 (58.6)	—
Low C3 *n* (%)	35 (60.3)	—
Low C4 *n* (%)	30 (51.7)	—
ESR *n* (%)	41 (70.7)	—
CRP *n* (%)	19 (32.7)	—
24-hour urine protein *n* (%)	28 (48.3)	—
SLEDAI	0 to 34 (22.5 ± 12.02)	—

Abbreviations—SLE: systemic lupus erythematosus; SLEDAI: systemic lupus erythematosus disease activity index; C3: complement 3; C4: complement 4; CRP: C-reactive protein; anti-ds-DNA: anti-double stranded DNA antibody; ESR: erythrocyte sedimentation rate; SLE: systemic lupus erythematosus; values are expressed as mean ± standard deviation.

2. Materials and Methods

2.1. Patients and Serum Samples. The study group comprised 58 patients (54 women and 4 men) with a mean age of 30.6 ± 11.5 years. All patients were recruited from the Department of Rheumatology, The First Affiliated Hospital of Nanjing Medical University, China, between December 2011 and June 2012 and fulfilled the revised American College of Rheumatology criteria for SLE [22], and individuals with other rheumatic diseases, infections, or malignant tumors were excluded from the study. Sera were also collected from 30 healthy controls at the same hospital, and all recruited healthy controls were excluded from having any autoimmune diseases. There were no significant differences in the ages or sex ratios between the two groups. Clinical and laboratory information obtained at the time of serum sampling included age, gender, antinuclear antibodies (ANA), the titers of antidouble stranded (ds) DNA antibody, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and the levels of IgG, IgM, and IgA, complement 3 (C3) and C4 and 24-hour urine protein. The clinical data from the patients were recorded in Table 1. Antinuclear antibodies (ANA) were detected by indirect immune fluorescence, and the titers of antidsDNA were detected by enzyme-linked immunosorbent assay (ELISA). Disease activity was assessed using the SLE Disease Activity Index (SLEDAI) and ranged from 0 to 34 (mean ± SD = 22.5 ± 12.02). The study was approved by the Research Ethics Committee of Jiangsu Province Hospital with informed consent from both patients and control group subjects.

A volume of 5 mL peripheral venous blood was collected from each patient and control subject and allowed to clot at room temperature for 2 hours. Samples were then centrifuged for 10 min at 800 g. The serum samples were separated and frozen at −80°C until further analysis.

2.2. Determination of Serum GITRL Levels. Serum GITRL levels were measured using an ELISA kit (RayBiotech Inc.) according to the manufacturer's instructions. Briefly, serum samples (1 : 200 dilution) and standards were added to the wells. After incubation and washing, biotinylated antihuman GITRL antibodies were added, followed by incubation with HRP-conjugated streptavidin and color development with TMB substrate solution. The intensity of the color reaction was measured using a microplate reader (Bio-Rad, Beijing, China) at a wavelength of 450 nm. Concentrations of GITRL were determined by a standard curve according to the manufacturer's instructions.

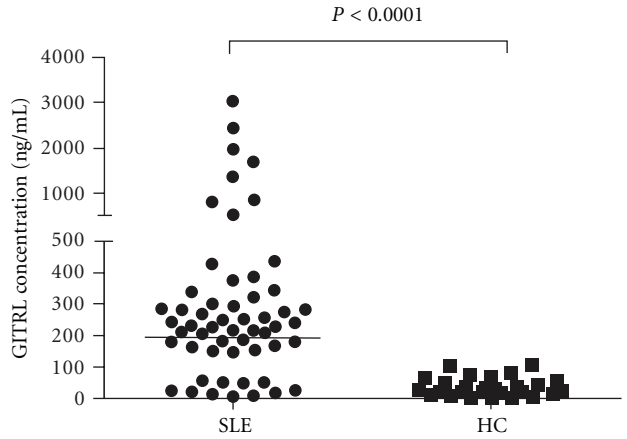

Figure 1: Comparison of serum GITRL levels between SLE and HC. Serum GITRL levels were significantly elevated in SLE patients versus HC. Each symbol represents an individual patient and healthy donor. Horizontal lines indicate median values. HC: healthy control; SLE: systemic lupus erythematosus.

2.3. Statistical Analysis. Data were presented as mean ± standard deviation unless specified otherwise. Statistical analysis was performed using SPSS for Windows (version 11.5). The Mann-Whitney rank sum test or Kruskal-Wallis tests were used to compare GITRL levels. The correlation between GITRL levels and various values were analyzed by Spearman's rank correlation coefficient. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Serum GITRL Levels Were Significantly Higher in Patients with SLE Than Normal Controls. In this study, 58 SLE patients and 30 healthy controls were recruited. There were no significant differences in both mean age and sex distribution between SLE patients and normal controls (Table 1).

To determine whether GITRL expression was dysregulated in SLE patients, we examined serum GITRL levels by ELISA. As shown in Figure 1, serum levels of GITRL were significantly higher in SLE patients than in healthy subjects (401.3 ± 79.96 ng/mL versus 36.59 ± 8.50 ng/mL; $P < 0.0001$), indicating that GITRL overexpression is probably involved in the pathogenesis of SLE.

3.2. Serum GITRL Levels Were Markedly Higher in SLE Patients with Active Disease. We further grouped the SLE patients by active or inactive phase according to their SLEDAI scores. SLE patients were divided into the active group (SLEDAI score $\geqslant 8$) and the inactive group (SLEDAI score < 8; $n = 15$) [23]. As shown in Figure 2, serum GITRL levels in SLE patients with active disease were significantly higher than those with inactive disease (403.3 ± 81.23 ng/mL versus 136.3 ± 34.41 ng/mL; $P = 0.0043$), furthermore serum GITRL levels were positively correlated with SLEDAI ($r = 0.317$; $P = 0.018$), indicating a close correlation of increased GITRL expression with SLE disease activity.

Table 2: Correlation between serum GITRL levels laboratory values.

Parameter	Correlation coefficient	P value
Anti-ds-DNA	0.467	0.021
ESR	0.284	0.048
CRP	−0.164	0.271
C3	−0.423	0.001
C4	−0.256	0.059
IgG	0.161	0.24
IgA	−0.099	0.484
IgM	0.548	0.0001
ANA	0.224	0.093
24-hour urine protein	0.057	0.713

SLEDAI: SLE disease activity index; anti-ds-DNA: anti-double stranded DNA antibody; C3: complement 3; C4: complement 4; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IgG: immunoglobin G; IgA: immunoglobin A; IgM: immunoglobin M; ANA: antinuclear antibody.

3.3. Correlation of Serum GITRL Levels with Laboratory Values. To further determine the relationship between serum GITRL levels and laboratory test results including the titers of anti-dsDNA, ESR, CRP, C3, C4, and Ig levels, it was found that serum GITRL levels were positively correlated with the titers of anti-dsDNA antibody, ESR, and IgM ($r = 0.467$, $P < 0.021$; $r = 0.284$, $P = 0.048$; $r = 0.548$, $P < 0.0001$, resp.; Figure 3). Interestingly, there was a negative correlation between serum GITRL levels and C3 ($r = -0.423$; $P = 0.001$, Figure 3). However, no significant correlations were found between serum GITRL levels and CRP, C4, and ANA (Table 2).

3.4. Correlation of Serum GITRL Levels with Clinical Features in SLE. Serum GITRL levels were further compared among SLE patients with and without certain clinical features to assess the associations between serum GITRL levels and clinical manifestations. We did not detect any significant differences in serum GITRL levels between patients in the presence of fever, alopecia, arthritis, chest affection, anemia, thrombocytopenia, and leucopenia and those lacking the above-mentioned clinical manifestations (Table 3).

However, we found that serum GITRL levels were significantly higher in patients with lupus nephritis and vasculitis compared with those patients without these manifestations as well as with normal controls ($P = 0.0273$; $P = 0.0493$, resp.; Figure 4), indicating a correlation of increased serum GITRL with renal damage and vasculitis in SLE patients.

4. Discussion

The pathogenesis of SLE involves complex interactions among genetic and environmental factors as well as the immune systems. SLE represents the classical prototype of systemic autoimmune disease in which loss of immune

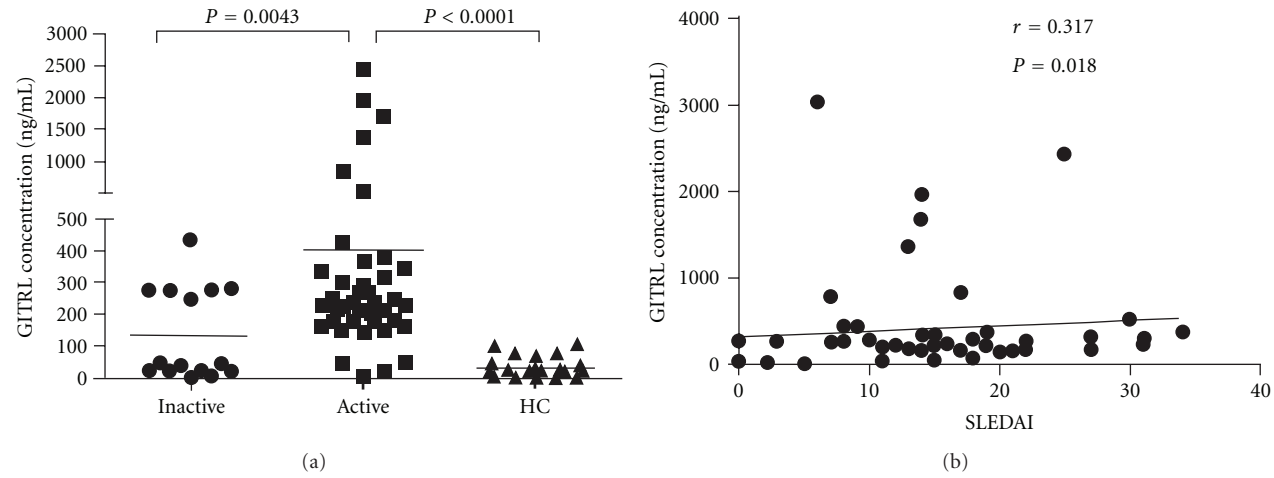

Figure 2: Comparison of serum GITRL levels among SLE patients with active disease and inactive disease as well as HC. (a) Serum GITRL levels were significantly elevated in SLE patients with active disease compared with those with inactive disease together with HC. (b) Serum GITRL levels were positively correlated with SLEDAI. Each symbol represents an individual patient and healthy donor; horizontal lines indicate median values. HC: healthy control; SLE: systemic lupus erythematosus.

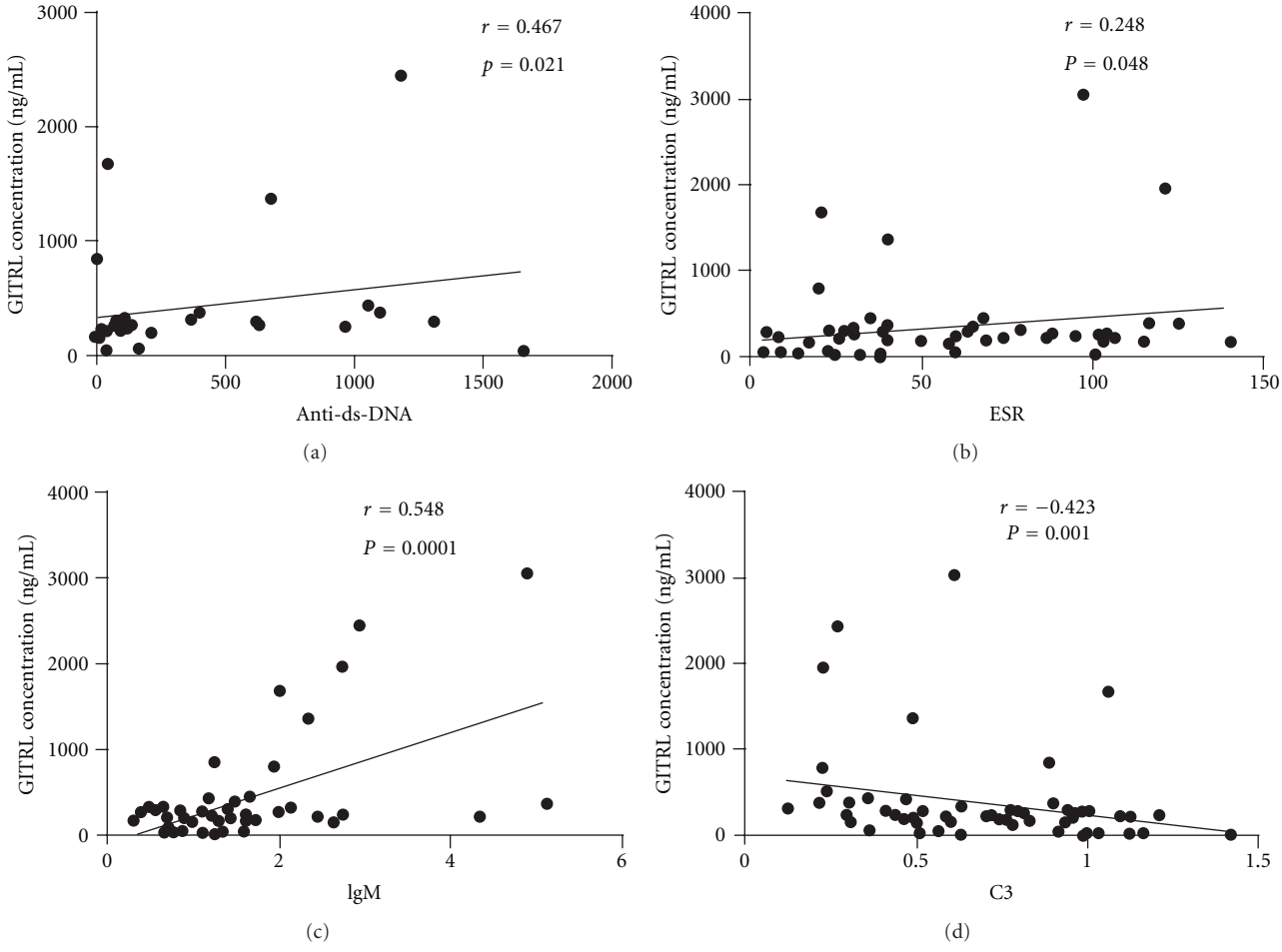

Figure 3: Correlation of serum GITRL levels with laboratory values. Each symbol represents an individual patient. (a) Positive correlation was observed between serum GITRL levels and the titers of antidsDNA antibody. (b) Positive correlation was also seen between serum GITRL levels and ESR. (c) Positive correlation was also observed between serum GITRL levels and IgM. (d) Negative relationship was observed between serum GITRL levels and C3. Anti-dsDNA antibody, antidouble stranded DNA antibody. C3: complement 3; ESR: erythrocyte sedimentation rate; IgM: immunoglobulin M.

Table 3: Serum GITRL level in SLE patients with or without clinical characteristics.

Clinical characteristic	Present (n) Mean ± SD	Absent (n) Mean ± SD	P value
Alopecia	(13) 437.2 ± 136.6	(45) 371.7 ± 90.76	0.6933
Rash	(34) 481.8 ± 116.3	(24) 251.2 ± 77.81	0.1052
Arthritis	(11) 503.9 ± 201.3	(47) 355.7 ± 81.38	0.5061
Fever	(15) 506.8 ± 162.8	(43) 327.8 ± 81.28	0.3339
Chest affection	(20) 516.9 ± 183.9	(38) 317.7 ± 64.74	0.3176
Anemia	(28) 472.2 ± 138.5	(30) 306.3 ± 70.83	0.2926
Leukopenia	(23) 273.7 ± 22.82	(35) 404.4 ± 88.34	0.1576
Thrombocytopenia	(5) 361.3 ± 134.3	(53) 388.8 ± 82.81	0.8669
Renal disease	(33) 418 ± 95.09	(25) 183.3 ± 38.32	0.0273*
Vasculitis	(24) 431.4 ± 106.7	(34) 201.6 ± 29.92	0.0494*

*$P < 0.05$, means significant difference.

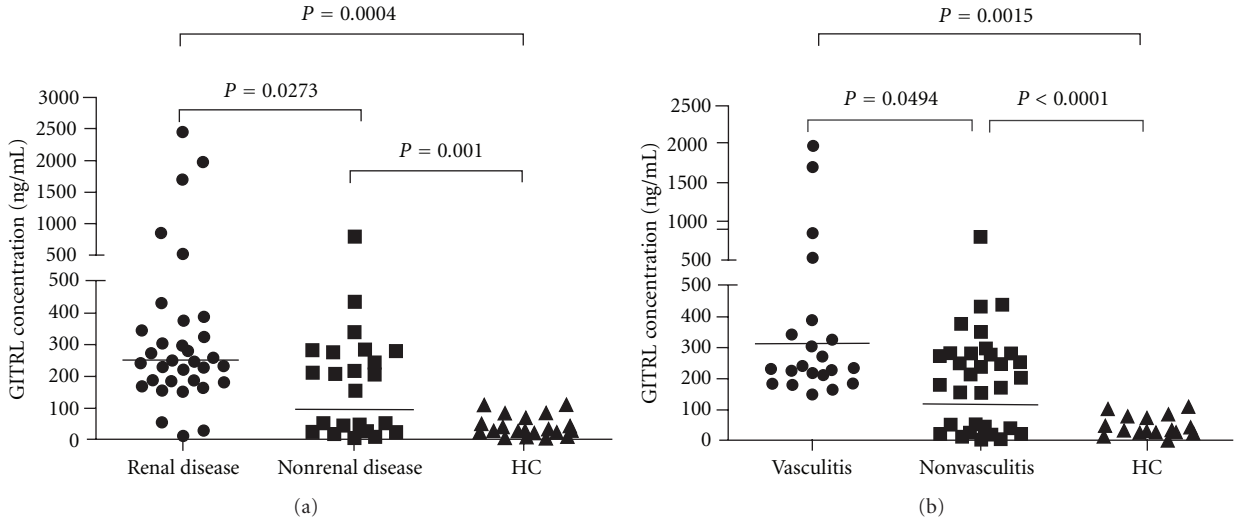

Figure 4: Elevated serum GITRL levels in SLE patients with organ damage. (a) Serum GITRL levels exhibited elevation in SLE patients with renal involvement ($n = 33$) relative to patients without renal involvement ($n = 25$) as well as HC. (b) Serum GITRL levels were higher in SLE patients with vasculitis ($n = 24$) than patients in the absence of vasculitis ($n = 35$) as well as HC. Each symbol represents an individual patient; horizontal lines indicate median values. GITRL: glucocorticoid-induced TNFR-related protein ligand; HC: healthy control; SLE: systemic lupus erythematosus.

tolerance to self-antigens leads to activation and expansion of autoreactive lymphocytes, uncontrolled production of several autoantibodies, and release of inflammatory mediators that ultimately damage multiple organs. Numerous cytokines and costimulatory molecules are implicated in immune dysregulations, leading to autoimmune pathogenesis. In this study, we have revealed a close correlation of circulating GITRL levels with the disease activity in SLE patients. Our data have clearly shown that serum levels of GITRL are positively correlated with SLEDAI, the titers of anti-dsDNA antibody, renal involvement, and vasculitis in SLE. Here, we provide new evidence indicating the possible involvement of GITRL overexpression in the disease progression of SLE.

Many studies have showed the insufficiency or dysfunction of Tregs closely correlates with the development of autoimmune and inflammatory diseases as well as the interruption of immune homeostasis [24–26]. Pedroza-Gonzalez et al. showed that tumor Tregs upregulated the expression of glucocorticoid-induced GITR compared to Tregs in tumor-free liver tissue and blood. Meanwhile, treatment with soluble GITRL induced a decrease in the suppression mediated by the activated tumor-infiltrating Tregs and restores the proliferative capacity and cytokine production of $CD4^+CD25^-$ T cells [27]. A previous study by Crispin et al. reported a significant reduction of $CD4^+CD25^+$ Tregs in active SLE patients when compared to healthy controls and inactive patients [28]. Recent studies have demonstrated that the activation of GITR/GITRL pathway could stimulate the proliferation of effector T-lymphocytes and partially reverse the immunosuppressive function of $CD4^+CD25^+$ Tregs [29]. These results suggest an important role of GITR/GITRL activation in the loss of immune tolerance, especially in the dysfunction of Tregs. Future

studies are needed to investigate whether and how GITR-GITRL pathway modulates the homeostatic regulation of Treg/Th17 cells during the pathogenesis of autoimmune diseases such as SLE. Our results from ELISA analysis have found higher levels of serum GITRL in the patients with active disease, consistent with previous findings that significantly elevated levels of GITRL are detected in RA patients with active disease [21]. Together, these results indicate a proinflammatory role of GITR/GITRL pathway in driving autoimmune progression. Although the source of the levels of serotype GITRL is currently unclear in this work, we speculate that activated dendritic cells could be one of the major cell types for GITRL overproduction in SLE patients, as indicated by recent findings that significantly increased expression of GITRL was detected in $CD11c^+$ DCs during the development of experimental arthritis in mice [21]. Future studies are needed to examine whether DCs from SLE patients express increased levels of GITRL and to identify the signals that trigger the shedding of membrane GITRL from these cells. Furthermore, studies are required to verify whether treatment with glucocorticoids or immunosuppressive agents might exert their therapeutic effect on SLE patients by inhibiting the overexpression of GITR and GITRL proteins.

In the current study, SLE patients are divided into active and inactive groups according to their SLEDAI scores [23]. We have found that serum GITRL levels in patients with active disease are significantly higher than those in patients with inactive disease as well as normal controls. It has been reported that treatment with GITR specific antibody or removal of T cells with high GITR expression can induce organ-specific autoimmune diseases in normal mice [30]. Recent evidence indicates that the costimulation of T cells through GITR signaling pathway may function *via* the induction of MAPK and NF-κB activation [13]. Since SLE is an autoimmune disease characterized by massive abnormal immune response, it is generally assumed that dysregulation of immune T-cell tolerance occurs in both human and murine SLE. It is conceivable that elevated serum GITRL levels in SLE patients may be one of the possible factors which lead to aberrant immune response. Thus, our current findings indicate the involvement of GITR-GITRL in the disease activity of SLE, a notion further supported by our results on the correlation between serum GITRL levels with several laboratory values, such as the titers of anti-dsDNA antibody and ESR, IgM in SLE patients. Our data also reveal a negative relationship between serum GITRL levels and C3. Since C3 is one of complement components, participating in elimination of immune complexes through combination with immunoglobulins to disturb interaction of crystallizable fragment in space, its reduction and the elevation of anti-dsDNA antibody titers indicate the disease activity of SLE.

In patients with SLE, most common clinical manifestations include alopecia, mucosal ulcer, arthritis, malar rash, anemia, thrombocytopenia, leucopenia, nephritis, and vasculitis [31]. Although we have not observed any correlation of serum GITRL levels with rash, alopecia, fever, chest affection, arthritis, anemia, thrombocytopenia, and leucopenia, we find that serum GITRL levels are significantly higher in patients with renal involvement and vasculitis when compared to the patients without the above-mentioned disease manifestations. La Cava et al. demonstrated that the production of autoantibodies by B cells in SLE patients could be interrupted *via* induction of Tregs since Tregs could inhibit the production of dsDNA antibodies by B cells *via* cell contact inhibition induced by membrane bound TGF-β and GITR molecules [32]. Benjamin et al. have also reported that $CD4^+CD25^+$ effector memory T cells expressing CD134 and GITR are closely associated with disease activity and their participation in Wegener's granulomatosis [33]. Thus, these findings also suggest that activation of GITR by GITRL may be involved in the development of nephritis and vasculitis in SLE.

5. Conclusions

Our current data on the correlation of elevated serum GITRL with disease activity in SLE suggest that GITR-GITRL may participate in the pathogenesis of SLE. These findings provide new insights in understanding the disease pathophysiology of SLE. Further studies are needed to validate GITRL as a new biomarker to assess the disease activity of SLE.

Authors' Contribution

L. Gu and L. Xu contributed equally to this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (NSFC no. 81273294 and NSFC no. 30971319), Chinese National High Technology Research and Development Program, Ministry of Science and Technology (SS2012AA020830).

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 520970, 5 pages
doi:10.1155/2012/520970

Clinical Study

Clinical Usefulness of the Serological Gastric Biopsy for the Diagnosis of Chronic Autoimmune Gastritis

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Received 14 September 2012; Accepted 18 October 2012

Academic Editor: Dimitrios P. Bogdanos

Aim. To assess the predictive value for chronic autoimmune gastritis (AIG) of the combined assay of anti-parietal-cell antibodies (PCA), anti-intrinsic-factor antibodies (IFA), anti-*Helicobacter pylori* (Hp) antibodies, and measurement of blood gastrin. *Methods*. We studied 181 consecutive patients with anemia, due to iron deficiency resistant to oral replacement therapy or to vitamin B12 deficiency. *Results*. 83 patients (45.8%) tested positive for PCA and underwent gastroscopy with multiple gastric biopsies. On the basis of the histological diagnosis, PCA-positive patients were divided into 4 groups: (1) 30 patients with chronic atrophic gastritis; they had high concentrations of PCA and gastrin and no detectable IFA; (2) 14 subjects with metaplastic gastric atrophy; they had high PCA, IFA, and gastrin; (3) 18 patients with nonspecific lymphocytic inflammation with increased PCA, normal gastrin levels, and absence of IFA; (4) 21 patients with multifocal atrophic gastritis with "borderline" PCA, normal gastrin, absence of IFA and presence of anti-Hp in 100% of the cases. *Conclusions*. The assay of four serological markers proved particularly effective in the diagnostic classification of gastritis and highly correlated with the histological profile. As such, this laboratory diagnostic profile may be considered an authentic "serological biopsy."

1. Introduction

Chronic autoimmune gastritis (AIG), also known as type A chronic atrophic gastritis, is an organ-specific disease that causes malabsorption of essential elements and pernicious or microcytic anemia, and it is a predisposing factor to carcinoid tumour and gastric adenocarcinoma. It is generally asymptomatic up to an advanced stage of atrophy and/or dysplasia of the mucosa [1, 2]. Histologically, it is characterised by a chronic inflammatory disorder of the gastric body and the fundus, sustained by a cell-mediated aggression by $CD4^+CD25^-$ Th1 lymphocyte effectors. The main target of immunological injury is the H^+/K^+-adenosine-triphosphate enzyme (ATPase), a protein of the membrane that coats the secretory canaliculi of the parietal cells and is responsible for the secretion of the hydrogen ions in exchange for the potassium ions (proton pump) [3, 4]. Induced by a triggering factor not yet entirely identified, the $CD4^+CD25^-$ T-cells, together with macrophages and B lymphocytes, infiltrate the submucosa, the lamina propria, and the gastric glands causing the loss of parietal, principal, and ghrelin-producing cells or P/D1 cells [5, 6]. The damage of the body and fundus mucosa results in [3, 7]

(a) hypo/achlorhydria, as a result of destruction of the parietal cells;

(b) hypergastrinemia, as a result of alteration of the negative feedback mechanism modulated by gastric acidity and governed by somatostatin via paracrine mechanism;

(c) malabsorption, which causes iron-deficiency anemia resistant to oral treatment or vitamin B12-deficiency anemia;

(d) presence of circulating autoimmune antibodies directed against the proton pump and the intrinsic factor, a consequence of the effector cytokine action of the T lymphocytes on humoral immunity;

(e) reduction of serum levels of pepsinogen and ghrelin, the principal and P/D1 cells being destroyed as bystanders of the parietal cells.

The presence of immunological markers and/or the change in the biochemical markers may be indicative of autoimmune gastritis, even in a clinically asymptomatic subject. Moreover, since these markers appear long before the clinical symptoms in the natural history of the disease, their assay ought to make it possible to identify AIG in an earlier phase [8, 9].

The purpose of our study was to assess the predictive value (VP) for AIG of the combined testing for anti-parietal-cell antibodies (PCA), anti-intrinsic-factor antibodies (IFA), anti-*Helicobacter pylori* (Hp) antibodies and gastrin, in patients suffering from iron-deficiency or vitamin B12 deficiency anemia, and to analyse the diagnostic efficacy of this laboratory profile in the selection of the subjects candidate to endoscopy.

2. Patients and Methods

Over the span of 14 months, 181 consecutive patients were recruited (19 males and 162 females; age: 25–81 years), referred by their GPs with diagnosis of microcytic iron-deficiency anemia resistant to oral treatment (39.8%) or vitamin B12 deficiency macrocytic anemia (60.2%), clinical conditions that are correlated to AIG [3]. None of the patients had bleeding of the gastroenteric tract nor, in the case of the female sex, menometrorrhagia, or liver disease, chronic inflammatory gut disease, or other diseases causing malabsorption. In all patients, blood cell count and the microscopic analysis of the peripheral blood smear showed moderate anisopoikilocytosis of the erythrocytes and, in 67% of the cases of macrocytic anemia, hypersegmentation of the neutrophils.

The assay of serum iron and vitamin B12 was carried out using enzymatic and immunological methods, respectively, on automated analysers with detection by modular structure enhanced chemiluminescence (Modular P and Modular E 170, Roche Diagnostics, Basle, Switzerland).

In the 181 selected patients the following markers were assayed:

(a) anti-parietal-cell antibodies (PCA), using quantitative immunoenzymatic method (ELISA) (Aesku.Diagnostics, Wendelsheim, Germany (cutoff value: 30 U/mL));

(b) anti-intrinsic-factor antibodies (IFA), using quantitative ELISA method (Aesku.Diagnostics—cutoff value: 20 U/mL);

(c) gastrin, using automated quantitative immun-chemiluminescent method (Immulite 2000, Siemens HealthCare Diagnostics, Flanders, NY, USA—reference interval: 35–115 pg/mL);

(d) anti-*Helicobacter pylori* antibodies (anti-Hp) using quantitative ELISA method (Orgentec Diagnostika, Mainz, Germany—cutoff value: 6 U/mL).

The cut-off values adopted were those suggested by the manufacturers.

The study protocol scheduled that the recruited subjects that presented positivity for PCA and/or IFA should, after informed consent, be subjected to esophagogastroduodenoscopy (EGDS), irrespective of the levels of gastrinemia. The site and number of the biopsy samples were decided in accordance with recent recommendations [1, 10, 11]: one biopsy from the greater curvature of the fundus, two biopsies from the greater curvature of the body, and two biopsies from the lesser curvature of the body, that is, from the sites involved in the chronic inflammation process typical of AIG. The statistical analysis was carried out using Student's *t*-test for nonpaired data and the statistics software SPSS 13.0 (Chicago, IL, USA).

3. Results

Eighty-three out of 181 patients (45.8%) tested positive for PCA, 14 of them (16.8%) had also positive IFA. Among the 69 patients who were positive for PCA only, 36 (52.2%) had microcytic, and 33 (47.8%) had macrocytic anemia. In the 14 patients with positivity for both PCA and IFA, microcytic anemia was present in 4 cases (28.6%) and macrocytic anemia in 10 cases (71.4%). Forty-four patients out of 83 (53%) had raised gastrin, whereas gastrin was normal in all the seronegative subjects. Anti-Hp antibodies were present in 33 (39.7%) patients. All 83 PCA-positive subjects and, as control, 11 PCA-negative subjects with gastrin within the normal range, referred by the GP to the gastroenterologist for recurrent dyspeptic disturbances, underwent EGDS with multiple gastric biopsies. On the basis of the histological diagnosis it was possible to divide the patients into four groups (Table 1): Group 1: 30 (36%) patients (mean age: 53 ± 20) with a histological profile of chronic atrophic gastritis of body and fundus; Group 2: 14 (17%) subjects (mean age: 70 ± 16) with metaplasic gastric atrophy of body and fundus, histological lesion more severe and extensive than that observed in Group 1; Group 3: 18 (22%) patients (mean age: 46 ± 12) had nonspecific lymphocytic gastritis; Group 4: 21 patients (25%) (mean age: 71 ± 12) had multifocal atrophic gastritis.

Serological analysis showed that Group 1 patients had high concentrations of PCA (mean 65 U/mL), high gastrin (mean 1048 pg/mL), absence of IFA, and presence of anti-Hp antibodies in 26% of the cases; Group 2 patients had an average concentration of PCA of 59 U/mL, gastrin of 1523 pg/mL, positivity for IFA, and absence of anti-Hp; Group 3 patients had an average concentration of PCA of 52 U/mL, normal gastrin, absence of IFA, and presence of anti-Hp in 21.1% of the cases; Group 4 patients had

Table 1: Analytic data of the four serological markers in relation to histological diagnosis.

Histological diagnosis	Number of patients	Age (years) (mean ± SD)	PCA (U/mL) (mean ± SD) (cutoff 30)	IFA (U/mL) (mean ± SD) (cutoff 20)	Gastrin (pg/mL) (mean ± SD) (cutoff 115)	Anti-Hp positivity (%)
Group 1: autoimmune gastritis	30	53.5 ± 20.0	65 ± 29	12 ± 2	**1048** ± 956	26.0
Group 2: gastric atrophy	14	70.4 ± 16.5*	59 ± 23	67 ± 8*	**1523** ± 713*	0*
Group 3: non-specific lymphocytic gastritis	18	45.7 ± 12.0	52 ± 17	5 ± 4	38 ± 8*	21.1
Group 4: multifocal atrophic gastritis	21	70.6 ± 11.9*	18 ± 8*	6 ± 6	56 ± 11*	100*

PCA: anti-parietal-cell antibodies; IFA: anti-intrinsic-factor antibodies; anti-Hp: anti-*Helicobacter pylori* antibodies; *statistical significance ($P < 0.05$).

an average concentration of PCA of 18 U/mL (SD ± 8), normal IFA and gastrin, and all were anti-Hp positive (Table 1).

The 11 sero-negative subjects showed normal levels of gastrin and a histological profile of superficial gastritis.

4. Discussion

In the natural history of many autoimmune diseases, specific autoantibodies may be present in the preclinical or subclinical phase of the disease, when the functions of the target organ are conserved or offset by homeostatic mechanisms [12, 13]. In AIG too, it is possible to hypothesise that the appearance of specific antibodies may precede by many years the onset of clinical symptoms, the increase of serum gastrin, the decrease of A and C pepsinogens and ghrelin, and the deficiency of iron and vitamin B12. However, observations on the predictive role of PCA and IFA in the natural history of autoimmune gastritis are relatively rare and discordant [14–16]. Nonetheless, a recent prospective study carried out on patients with autoimmune thyroid diseases demonstrated that, after 5 years, 24% of the subjects who were PCA and IFA positive at the time of enrolment developed histologically diagnosed AIG [17]. The same study also showed that the concentration of PCA increases progressively up to a peak, followed by a decrease as a result of ongoing destruction of the gastric mucosa. Our results show that PCA and IFA assays can be predictive of AIG and are useful in the selection of patients to be referred for diagnostic procedures. We consecutively enrolled 181 patients with microcytic iron-deficiency anemia resistant to oral treatment or macrocytic anemia due to vitamin B12 deficiency (known as frequently related to AIG in otherwise asymptomatic individuals), referring over the span of 14 months to our Laboratory Medicine Service. On patients' sera we measured PCA, IFA, and gastrin, as markers of gastric mucosa damage. We also included anti-Hp antibodies since, in addition to being considered as predictive of mucosa damage, they also correlate with the presence of PCA in patients with AIG, possibly due to molecular mimicry between gastric ATPase and bacterial urease [18–21].

The histological diagnosis after EGDS allowed to classify the patients in 4 subgroups.

In Group 1, histology showed chronic atrophic gastritis of the body and fundus. The lamina propria and the glandular tissue were infiltrated by mononuclear cells (T and B lymphocytes and macrophages). The T-cell-mediated epithelial lesion, mainly at the level of parietal cells, results in reduction of gastric acid secretion and consequently impairment of the control mechanism inhibiting gastrin secretion by G-cells of the antrum. In fact, these patients have PCA positivity and raised gastrin; this serological profile, in subjects with iron- or vitamin B12-deficiency anemia, is highly predictive of autoimmune lesion of the stomach mucosa.

In Group 2, histology showed metaplastic atrophy of body and fundus, more severe than that observed in Group 1, with extensive lymphocyte, monocyte, and neutrophil mucosa infiltration. The lesions were extended to over 50% of glands in the total area of the biopsy material: areas of pseudopyloric metaplasia were also present. As shown in Table 1, the quantitative assay of PCA and gastrin was unable to discriminate AIG from gastric atrophy. However, PCA values were slightly lower in atrophy, presumably because of a more extensive parietal cell destruction [17, 22]. Instead, the presence of IFA in this group of patients correlated with a more serious histological lesion and therefore has likely a greater predictive value for gastric atrophy. None of these subjects was positive for anti-Hp; the destruction of gastric epithelium, which is necessary for the survival of Hp, prevents the bacteria from colonising and proliferating in the stomach [23]. Negativity for anti-Hp associated with positivity for PCA, IFA and raised gastrin in patients with anemia increases the positive predictive value for the diagnosis of gastric atrophy.

Group 3 patients showed lymphocytic gastric inflammation, nonspecific histological lesion characterised by infiltration of the gastric mucosa by mononuclear cells without glandular damage. They showed presence of PCA, normal gastrin, absence of IFA, and in some cases presence of anti-Hp. Group 4 patients had atrophic gastritis of the antrum and body, with focal intestinal metaplasia of the glands, caused by Hp, and showed absence of PCA and IFA, normal gastrin, and presence of anti-Hp.

Therefore, based on our data, normal serum gastrin and the absence of IFA have a fair negative predictive value for autoimmune lesion of the mucosa of the stomach, as demonstrated by correlating the biopsy profile with the laboratory results in the 39 subjects of Groups 3 and 4; they tested positive for PCA only (notably, at very low values in those with gastritis due to Hp), but showed no lesions of gastric epithelium. On the contrary, the rise of gastrin and the presence of IFA, in association with PCA, are highly predictive for AIG and atrophy, as apparent from the data obtained in the first two groups of patients.

Table 2: Biochemical-antibody results in relation to the histological diagnosis of gastritis in the studied population.

Histological diagnosis	PCA	IFA	Gastrin	Anti-Hp
Autoimmune gastritis	+	−	+	±
Gastric atrophy	+	+	+	−
Non-specific lymphocytic gastritis	+	−	−	±
Multifocal atrophic astritis	−	−	−	+

Thus, although these results should be confirmed in several larger studies, the determination of the 4 laboratory markers (PCA, IFA, anti-Hp, and gastrin) could be regarded as a "serological biopsy" useful in selecting patients candidate to EGDS (Table 2).

Testing for PCA and IFA using ELISA method features elevated analytical and diagnostic accuracy, provides continuous quantitative results, and improves standardisation of the analytic procedure, compared to discrete quantitative or qualitative tests (such as indirect immunofluorescence and immunoblotting). Moreover, it makes it possible to monitor the disease, with special reference to mucosal atrophy development [22]. The anti-Hp assay increases the diagnostic value of the "serological biopsy." It should be noted that patients with nonspecific gastritis were younger than those with AIG, but with comparable PCA and anti-Hp positivity; this suggests the need to monitor the patients showing such histological profile by serology, for example, once a year, since AIG may occur in some of them, especially if they are genetically predisposed, such as first-degree relatives of patients with AIG or subjects with genetic variations at the *ABO* gene locus [24, 25].

In conclusion, autoimmune gastritis is a frequently asymptomatic disease that can cause atrophy of the gastric mucosa and in approximately 10% of cases evolve into a carcinoid tumour or adenocarcinoma [1, 26]. In the absence of clinical symptoms, only the reasoned use of laboratory tests makes it possible to clearly classify the patient with suspected AIG to be subjected to endoscopic examination. The presence of risk factors and/or of therapy-resistant B12 or iron-deficient anemia, in the absence of aetiological agents that can be directly correlated, is clinical criteria that suggest to test for PCA, IFA, and gastrin as immunological and biochemical markers of AIG. This profile can be considered as an authentic "serological biopsy" [27–29] considering that the hypergastrinemia identifies the damage to the mucosa of the body and fundus of the stomach, and the presence of the antibodies proves the autoimmune origin of the aggression of the mucosa itself. Indeed, the data obtained in our study demonstrate that the positivity for PCA and elevated gastrin correlate with damage to the mucosa of the body and of the fundus typical of AIG. The IFA positivity indicates the presence of a more serious histological profile (atrophy). Significant values of PCA, in the absence of IFA and an increase in gastrin, are associated with nonspecific lymphocytic inflammation rather than with autoimmune damage to the gastric mucosa. Finally, the elevated predictive value and the clinical specificity of the "serological biopsy" are confirmed by the evidence that none of the patients with normal gastrin and absence of IFA revealed lesions of the gastric mucosa of an autoimmune type.

Conflict of Interests

The authors declare no conflict of interests.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 854795, 4 pages
doi:10.1155/2012/854795

Research Article

Association of Autoantibodies to BP180 with Disease Activity in Greek Patients with Bullous Pemphigoid

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Received 7 September 2012; Revised 24 October 2012; Accepted 25 October 2012

Academic Editor: Dimitrios P. Bogdanos

39 bullous pemphigoid (BP) patients were studied to assess the clinical significance of anti-BP180 and anti-BP230 circulating autoantibodies of BP and correlate their titers with the clinical scores of the BP Disease Area Index (BPDAI) and the Autoimmune Bullous Skin Disorder Intensity Score (ABSIS) as well as with the intensity of pruritus measured by the BPDAI pruritus component. All parameters were evaluated by the time of diagnosis (baseline), month 3, and month 6. Titers of anti-BP180 autoantibodies were strongly correlated with BPDAI ($r = 0.557$, P value < 0.0001) and ABSIS ($r = 0.570$, P value < 0.0001) values, as well as with BPDAI component for the intensity of pruritus (rho = 0.530, P value = 0.001) at baseline. At month 3, titers of anti-BP180 autoantibodies were strongly correlated with BPDAI (rho = 0.626, P value = 0.000) and ABSIS (rho = 0.625, P value = 0.000) values, as well as with the BPDAI component for the intensity of pruritus (rho = 0.625, P value = 0.000). At month 6, titers of anti-BP180 autoantibodies were strongly correlated with BPDAI (rho = 0.527, P value = 0.001) and ABSIS (rho = 0.526, P value = 0.001) values, as well as with the BPDAI component for the intensity of pruritus (rho = 0.525, P value = 0.001). There was no statistically significant correlation between titers of anti-BP230 autoantibodies and the BPDAI, ABSIS, and BPDAI component for the intensity of pruritus at the same time points.

1. Introduction

Bullous pemphigoid (BP) is the most common subepidermal autoimmune bullous disease. Detection of anti-BP180 and/or anti-BP230 serum autoantibodies by commercially available ELISA kits has almost become nowadays a routine method for the diagnosis and followup of BP patients [1–3].

During the last decade, efforts to evaluate the clinical extent and severity of autoimmune bullous diseases, generally, have led to the establishment of scoring systems. Up to today there are two already validated scoring systems for pemphigus, the Autoimmune Bullous Skin Disorder Intensity Score (ABSIS) and the Pemphigus Disease Area Index (PDAI) [4].

ABSIS has been introduced in 2007, in order to achieve an improved evaluation and monitoring of the status of both oral and cutaneous lesions in patients with pemphigus. It has been used, though, in other autoimmune bullous diseases, such as BP and epidermolysis bullosa acquisita [5]. The Pemphigus Disease Area Index (PDAI) was developed by the International Pemphigus Definitions Committee in 2008, to evaluate both mucosal and cutaneous lesions [6].

In 2011, the International Pemphigoid Committee proposed the BP Disease Area Index (BPDAI), in order to achieve a consistent reporting of the outcomes in BP reports and studies [7]. BPDAI has small differences from the PDAI scoring sheet, as there is more emphasis given to the lesions on the extremities, than face, scalp, and mucosa. There are separate columns for the extent of blistering and for the urticarial/eczematous lesions that may be more extensive in BP [7].

In the present study, we correlated two clinical scores, not yet validated for bullous pemphigoid (BPDAI, ABSIS), and

the BPDAI component for the intensity of pruritus, with the titers of circulating autoantibodies (anti-BP180, anti-BP230), in a group of Greek BP patients, in order to assess the disease activity during the first six months after diagnosis.

2. Materials and Methods

39 patients suffering from BP were consecutively selected to participate in this prospective cohort study. Diagnosis of BP was confirmed, before the initiation of any treatment, by histology, direct immunofluorescence, and detection of circulating anti-BP180 and anti-BP230 autoantibodies by ELISA (commercially available MBL kits, Japan). The cut-off value for the MBL assays was that of 9.0 U/mL and the upper detection range was 150.0 U/mL.

Titers of circulating autoantibodies were measured by the time of diagnosis (baseline) and every three months thereafter until month 6 (month 3, month 6). At the same time points (baseline, month 3, and month 6), the clinical severity was evaluated using the BPDAI and ABSIS scores, as well as the BPDAI component for the intensity of pruritus. BPDAI has a score of 0–120 for three distinct parameters (total 360): number and size of bullous lesions, number and size of erythematous nonbullous lesions, and number and size of mucosal lesions. In our group of patients, there were no mucosal lesions. ABSIS has a range from 0 to 206. It measures the extent and quality of skin lesions (0–150), the extent (0–11) and severity of mucosal lesions (0–45). BPDAI pruritus component ranges from 0 to 30. It measures the severity of itch during the past 24 hours (0–10), the past week (0–10), and the past month (0–10).

Administration of systemic prednisolone at a dose of 0.5 mg per kg BW per day and tapering upon response was the induction therapeutic scheme, which was applied to all patients.

The ethics board approval and patients' informed consent were provided.

We did not include an age- and gender-matched control group in our study as the diagnostic ELISA procedures (anti-BP180 and anti BP 230) are well established and the specificity and sensitivity is proven up to now in many studies. We aimed to focus on the assessment of disease activity and on the use of the newly introduced clinical scoring systems.

2.1. Statistical Analysis. Descriptive statistics including the mean, the standard deviation (SD), the median, the minimum, and the maximum values were used in order to present continuous variables, while frequency distributions and percentages were used for categorical data. The normality of the continuous variables was tested with the Shapiro-Wilk test. Pearson's correlation coefficient and Spearman's rank test were also used to explore relationships between continuous variables. Preliminary analyses were performed to ensure no violation of the assumptions of normality, linearity, and homoscedasticity. Wilcoxon's signed rank test was performed to evaluate significant differences in the BPDAI, ABSIS, BPDAI components for the intensity of pruritus, anti-BP180 autoantibodies, and anti-BP230 autoantibodies between baseline and month 3, as well as between month 3 and month 6. All tests were two sided, and the significance level was chosen to be $\alpha = 0.05$. Data analysis was performed using the statistical package for social sciences SPSS 15.0.

3. Results and Discussion

In total, 39 patients participated in the study. All patients remained under followup throughout the 6-month period. There was a female preponderance in our sample with 56.4% ($n = 22$) female patients and 43.6% ($n = 17$) male patients. The median patients' age was 76.0 (range: 28.0–91.0). Patients' clinical characteristics at baseline, month 3, and month 6 are summarized in Table 1. The number of patients with clinically active BP, positive anti BP 180 autoantibodies (≥9.0 U/mL), and positive anti BP 230 autoantibodies (≥9.0 U/mL) at all different time points are presented in Table 2.

In our BP patients during the first 6 months after diagnosis, there was a small number of recurrences (Table 2). After management of the extended eruption, most patients remained clear of lesions during the next follow-up visits, being under a maintenance dose of 5 mg prednisolone. Only 6 and 4 patients presented with a relapse of the disease at month 3 and 6, respectively (Table 3).

At baseline, a large, positive, statistically significant correlation was detected between anti-BP180 autoantibodies and (a) BPDAI ($r = 0.557$, P value < 0.0001), (b) ABSIS ($r = 0.570$, P value < 0.0001), and (c) BPDAI component for the intensity of pruritus (rho = 0.530, P value = 0.001). Therefore, high levels of anti-BP180 autoantibodies are associated with higher BPDAI, ABSIS, and BPDAI component for the intensity of pruritus. On the contrary, there was no statistically significant correlation between anti-BP230 autoantibodies and (a) BPDAI (rho = 0.206, P value = 0.208), (b) ABSIS (rho = 0.245, P value = 0 anti-BP180), and (c) BPDAI component for the intensity of pruritus (rho = 0.192, P value = 0.242), suggesting that high levels of anti-BP230 autoantibodies are not associated with higher BPDAI, ABSIS, and BPDAI component for the intensity of pruritus.

At month 3, a large, positive, statistically significant correlation was detected between anti-BP180 autoantibodies and (a) BPDAI (rho = 0.626, P value = 0.000), (b) ABSIS (rho = 0.625, P value = 0.000), and (c) BPDAI component for the intensity of pruritus (rho = 0.625, P value = 0.000), whereas there was no statistically significant correlation between anti-BP230 autoantibodies and (a) BPDAI (rho = 0.135, P value = 0.411), (b) ABSIS (rho = 0.129, P value = 0.434), and (c) BPDAI component for the intensity of pruritus (rho = 0.105, P value = 0.525).

At month 6, a large, positive, statistically significant correlation was detected between anti-BP180 autoantibodies and (a) BPDAI (rho = 0.527, P value = 0.001), (b) ABSIS (rho = 0.526, P value = 0.001), and (c) BPDAI component for the intensity of pruritus (rho = 0.525, P value = 0.001). On the other hand, no statistically significant correlation was detected between anti-BP230 autoantibodies and (a) BPDAI

Table 1: Patients' clinical characteristics at baseline, month 3, and month 6.

Clinical characteristics	Statistics	Values ($n = 39$) at baseline	Values ($n = 39$) at month 3	Values ($n = 39$) at month 6
BPDAI (0–360)	P value		0.000*	0.838
	Mean ± SD	52.5 ± 24.7#	1.8 ± 4.5	1.5 ± 4.6
	Median (min–max)	50.0 (2.0–104.0)	0.0 (0.0–15.5)	0.0 (0.0–17.1)
ABSIS (0–206)	P value		0.000*	0.646
	Mean ± SD	44.7 ± 21.7#	1.7 ± 4.1	1.4 ± 4.3
	Median (min–max)	43.5 (1.5–92.5)	0.0 (0.0–14.1)	0.0 (0.0–15.7)
BPDAI pruritus index (0–30)	P value		0.000*	0.682
	Mean ± SD	23.1 ± 7.3	0.5 ± 1.3	0.5 ± 1.7
	Median (min–max)	24.0 (0.0–30.0)	0.0 (0.0–5.0)	0.0 (0.0–7.0)
BP180 (U/mL)	P value		0.000*	0.001*
	Mean ± SD	87.9 ± 46.6#	11.1 ± 12.1	8.7 ± 9.7
	Median (min–max)	88.4 (10.0–190.8)	7.2 (2.3–49.1)	6.6 (1.9–41.1)
BP230 (U/mL)	P value		0.000*	0.115
	Mean ± SD	36.5 ± 36.5	18.2 ± 12.9	15.7 ± 13.8
	Median (min–max)	26.4 (1.5–151.2)	16.9 (1.4–56.2)	9.0 (2.1–59.2)

* Wilcoxon's signed rank test statistically significant; #variable normally distributed.

Table 2: Number of patients presenting clinically active BP, positive anti BP180 autoantibodies, and positive anti BP230 autoantibodies at all different time points.

	Baseline	Month 3	Month 6
Clinically active BP	39	6	4
Anti BP180 autoantibodies	39	7*	4
Anti BP230 autoantibodies	26	26	21

* One case had no signs of clinically active BP, but his anti BP180 autoantibodies had the borderline value of 9.2.

(rho = 0.308, P value = 0.057), (b) ABSIS (rho = 0.307, P value = 0.057), and (c) BPDAI component for the intensity of pruritus (rho = 0.313, P value = 0.052).

The NC16A domain of BP180, located at the extracellular portion of BP180 or BPAg2, is the main targeted antigen in most cases of BP [1–3]. BP230 is an intracytoplasmic protein, and as autoantibodies are not considered to access into the intact keratinocytes, production of anti-BP230 autoantibodies may represent an epiphenomenon. To our knowledge, there is no study indicating the correlation between the titers of anti-BP180 and anti-BP230 autoantibodies with each other. The role of BP230 in BP pathogenesis remains unclear [2].

In many patients there are anti-BP230 autoantibodies by the time of diagnosis, and in some they are detected later in the course of the disease. Several studies suggest that anti-BP230 autoantibodies may play an important role in the onset of clinical symptoms and the formation of blisters [8], while others suggest that anti-BP230 autoantibodies may bind to the target antigen in injured keratinocytes or even in intact ones by penetrating the cells and possibly contribute to the perpetuation of disease [9].

There is a limited number of BP patients in which there are only anti-BP230 circulating autoantibodies, and thus, it is suggested to perform the detection of both, anti-BP180 and anti-BP230 autoantibodies, in all cases [1, 10, 11].

Still, according to a most recent study, there is about 8% of BP patients whose sera react to regions of BP180 exclusively outside of the NC16A domain, and thus, BP may not be identified using the currently available BP180 ELISA. Use of the BP230 ELISA in the above study would only have detected 1 of the 4 patients with non-NC16A BP [12].

In a number of studies, the diagnostic sensitivity and specificity of the commercially available assays has been elucidated, and moreover, it has been associated with the course of disease [13]. Titers of anti-BP180 autoantibodies have been reported to correlate with disease activity, pruritus severity, peripheral blood eosinophil counts, and disease duration [13–17].

Titers of anti-BP230 autoantibodies have been repeatedly reported not to parallel with the course and severity of BP [10, 13–18].

In accordance with the published experience, in our group of patients, titers of anti-BP180 autoantibodies were strongly correlated with the clinical scores BPDAI and ABSIS, as well as with the BPDAI component for the intensity of pruritus in three consecutive measurements, by the time of diagnosis (baseline), at month 3, and at month 6. On the other hand, there was no statistically significant correlation between titers of anti-BP230 autoantibodies and BPDAI, ABSIS, and BPDAI component for the intensity of pruritus. An additional observation was that the rate of reduction of anti-BP230 autoantibodies titers, in our small sample of BP patients, seemed to be slower from this of anti-BP180 autoantibodies titers after initiation of therapy.

4. Conclusions

Detection of both anti-BP180 and anti-BP230 autoantibodies is needed for the establishment of diagnosis in all cases of

Table 3: BPDAI score, anti BP180 autoantibody titres, and anti BP230 autoantibody titres in patients who revealed a clinical relapse at different time points.

Case	Baseline			Month 3			Month 6		
	BPDAI	BP180	BP230	BPDAI	BP180	BP230	BPDAI	BP180	BP230
1	2.0	27.0	5.0	0.0	5.3	4.6	14.4	36.1	21.0
2	36.0	141.4	5.5	6.0	35.6	6.1	0.0	6.9	7.1
3	76.0	77.2	11.0	10.9	32.1	13.0	0.0	6.9	9.0
4	2.0	10.6	6.1	0.0	2.4	6.0	16.1	38.9	15.1
5	84.0	100.8	19.6	13.3	34.9	20.1	0.0	4.4	8.9
6	2.0	10.0	3.6	0.0	3.1	2.9	17.1	41.1	17.7
7	50.0	98.2	34.4	15.5	36.7	32.2	0.0	8.6	13.2
8	56.0	120.3	37.9	0.0	8.8	16.9	12.2	29.9	25.9
9	64.0	93.2	31.7	11.2	41.9	30.3	0.0	7.7	24.1
10	82.0	118.2	17.8	14.4	49.1	21.2	0.0	6.7	15.6

suspected BP. Followup of only anti-BP180 titers correlates with disease activity. Our first experience of the combined use of detection of circulating BP autoantibodies every three months with the completion of BPDAI and ABSIS scoring sheets was positive. We strongly believe that this combination offers an easy option for close followup of patients with BP.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 868251, 7 pages
doi:10.1155/2012/868251

Review Article

Autoantibodies to Apolipoprotein A-1 in Cardiovascular Diseases: Current Perspectives

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Received 2 October 2012; Accepted 19 October 2012

Academic Editor: Dimitrios P. Bogdanos

Immune-mediated inflammation plays a major role in atherosclerosis and atherothrombosis, two essential features for cardiovascular disease (CVD) development, currently considered as the leading cause of death in the western world. There is accumulating evidence showing that humoral autoimmunity might play an important role in CVD and that some autoantibodies could represent emerging cardiovascular risk factors. Recent studies demonstrate that IgG autoantibodies against apolipoprotein A-1 (apoA-1) are raised in many diseases associated with a high cardiovascular risk, such as systemic lupus erythematosus, acute coronary syndrome, rheumatoid arthritis, severe carotid stenosis, and end-stage renal disease. In this work, we aimed at reviewing current data in the literature pointing to anti-apolipoprotein A-1 antibodies (anti-apoA-1 IgG) as a possible prognostic and diagnostic biomarker of cardiovascular risk and appraising their potential role as active mediators of atherogenesis.

1. Introduction

Immune-mediated inflammation plays a major role in atherosclerosis and atherothrombosis, two essential features for cardiovascular disease (CVD) development, currently considered as the leading cause of death in the Western world [1]. Although initially related to a lipid metabolism abnormality, atherosclerosis is now considered as a chronic multifactorial immune-mediated inflammation of the arterial wall, where transendothelial migration of circulating immuno-competent cells within the artery is the key step [1]. By fulfilling the "Koch's postulates", recent work suggested that atherosclerotic low-grade inflammation might be even considered as an autoimmune disease [2]. This hypothesis is supported by two main pieces of evidence. Firstly, patients suffering from an autoimmune disease, such as systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS), and rheumatoid arthritis (RA), display an increased cardiovascular (CV) risk, independently of the Framingham risk factors [3–5]. Secondly, in patients with overt CVD, but without concomitant autoimmune diseases, many different autoantibodies have been shown to predict poor CV outcome [6]. Some of these autoantibodies might directly influence atherosclerotic processes triggering innate immune receptors signaling either toward a pro- or an anti-inflammatory response, as reviewed elsewhere [6]. Lately, humoral autoimmunity to apolipoprotein A1 (apoA-1)—the major protein fraction of high-density lipoproteins (HDL), conferring to the latter most of its atheroprotective role [7–9]—has gained considerable interest, mostly by displaying intriguing CV prognostic and diagnostic properties in different diseases. The aim of this manuscript is to review the existing data in the literature pointing to anti-apolipoprotein A-1 antibodies (anti-apoA-1 IgG) as an emergent prognostic and diagnostic biomarker of cardiovascular risk in clinically overt autoimmune settings, as well as in nonautoimmune conditions, and to appraise their potential role as active

mediators of atherogenesis according to available data in the literature.

2. Anti-apoA-1 IgG in Autoimmune Diseases

2.1. Anti-apoA-1 IgG in SLE and in APS. Anti-apoA-1 IgG were first identified in 1998 by Dinu and colleagues who demonstrated that high levels of those autoantibodies were found in a significant subset of SLE (32.5%) and primary APS patients (22.9%) and displayed a high affinity to nascent and mature HDL molecules [10]. Three years later in 2001, the group from Abe and colleagues characterized six different monoclonal anti-apoA-1 antibodies derived from two SLE patients [11]. Those autoantibodies showed a functional heterogeneity in their cross-reactivity, reacting preferentially with oxidized apoA-1 and with other self-antigens, such as cardiolipin (CL), single-strand DNA, and thrombin [11]. The relative selectivity of anti-apoA-1 IgG for its presupposed target was further confirmed two years later by Delgado Alves and colleagues, who demonstrated that anti-apoA-1 IgG antibodies cross-reacted with anti-HDL and with anti-cardiolipin antibodies [12]. Nevertheless, if 58% of SLE sera containing high levels of anti-HDL cross-reacted with CL, only 25% of those sera were reactive to apoA-1, suggesting that anti-apoA-1 IgG could represent a distinct and specific subclass of anti-HDL antibodies [12]. By demonstrating that anti-HDL IgG were inversely correlated with paraoxonase-1 (PON-1) activity and with the total antioxidant capacity of the corresponding sera [13], Batuca and colleagues were the first in 2003 to suggest that anti-HDL, and later anti-apoA-1 IgG [14], could be involved in atherogenesis, and more specifically to be related to the occurrence of dysfunctional HDL [14, 15], whose pathophysiological importance in atherogenesis started to be recognized [16]. Lately, the same group demonstrated that this effect was due to a decrease in PON-1 activity, leading to an increase of proinflammatory reactive oxygen species [17, 18], but the causal nature of this association is still under investigation.

2.2. Anti-apoA-1 IgG in Rheumatoid Arthritis. The existence of anti-apoA-1 in RA patients was initially described by Vuilleumier and colleagues, in 2010, who demonstrated a high titre of those autoantibodies in 17% of RA patients [17]. Furthermore, in this single-center prospective study, of 133 subjects, the authors demonstrated that RA patients with high levels of anti-apoA-1 IgG had much worse cardiovascular event-free survival (median followup period of 9 years) when compared to patients tested negative for those autoantibodies (43% versus 9%, $P = 0.001$) [17]. In this study, being positive for anti-apoA-1 IgG (with anti-apoA-1 IgG values above the 97.5th centile of anti-apoA-1 IgG values obtained on 140 healthy blood donors) increased the risk of major adverse cardiovascular events (MACE, defined by the occurrence of fatal nonfatal acute coronary syndrome or stroke) by 4-fold (adjusted hazard ratio 4.2; 95% confidence interval (95% CI): 1.5–12.1). This was independent of traditional cardiovascular risk factors and RA disease duration [17]. Finally, patients tested positive for those autoantibodies were shown to have higher plasma levels of inflammatory mediators associated with atherosclerotic plaque vulnerability in humans, such as matrix metalloproteinase 9 (MMP-9) and oxidized low-density lipoproteins (oxLDL) [18, 19].

These results were corroborated in a smaller case-control Swedish study which confirmed the aforementioned association with oxLDLs and demonstrated that anti-apoA-1 IgG levels were higher in RA patients than in control subjects and were higher in RA patients with a history of MACE when compared to those without [20]. Finally, a recent study demonstrated that when compared to 6 different CV prognostic biomarkers, anti-apoA-1 IgG was the only one found to provide incremental predictive ability over the 10-year global Framingham risk score, improving the area under the curve from 0.72 to 0.81, and it improved reclassification statistics using the integrated discrimination index (IDI) by 175% ($P < 0.001$) [21]. Thus, in this study, anti-apoA-1 IgG was found to be the only candidate to provide better and complementary prognostic information to traditional cardiovascular risk factors.

Taken together, those results suggest that anti-apoA-1 IgG have a CV prognostic value mostly in RA patients, where they are associated with proinflammatory cytokine profile and appear to provide incremental predictive ability over Framingham risk factors. Nevertheless, whether this CV prognostic feature also applies to SLE and APS patients is currently elusive and warrants further studies. Finally, knowing how and if assessing anti-apoA-1 IgG could impact the actual CV risk stratification and subsequent therapeutic management of RA patients remains to be demonstrated.

3. Anti-apoA-1 IgG in Nonautoimmune Conditions

3.1. Anti-apoA-1 IgG in Acute Coronary Syndromes. The existence of anti-apoA-1 IgG in nonautoimmune settings was initially reported in a retrospective study published in 2004, which demonstrated that the prevalence of high levels of anti-apoA-1 IgG was higher in myocardial infarction (MI) patients than in healthy blood donors (21% versus 1%; $P = 0.001$) [22]. This prevalence was of the same order of magnitude as previously reported in SLE and APS patients [10–13], and in this work, the authors retrieved a significant association between anti-apoA-1 IgG and serum amyloid A (SAA) protein, a multifunctional protein located at the crossroad of inflammation, cholesterol homeostasis, and atherogenesis [23, 24]. The findings from this study were confirmed and extended by a case-control study from the same group involving 127 patients with acute coronary syndrome (ACS), 140 healthy blood donors, 34 patients with stroke, and 58 patients with acute pulmonary embolism [25]. In this study, the prevalence of high anti-apoA-1 IgG levels was 11% in the ACS group, 2% in the healthy blood donor group, and 0% in the acute pulmonary embolism group. In ACS patients, those considered as positive for anti-apoA-1 IgG (with anti-apoA-1 IgG values above the

97.5th centile of anti-apoA-1 IgG values obtained from 140 healthy blood donors) had significantly higher median levels of oxLDL when compared to ACS patients tested negative for those autoantibodies ($P < 0.0001$) [25]. In this study, the presence of any autoimmune disease was excluded based upon the study exclusion criteria, and all the patients tested positive for anti-apoA-1 IgG were found to be negative for anti-*beta2* glycoprotein (anti-β2GPI), rheumatoid factor (RF), anti-nucleosome, anti-nucleoprotein-specific, and for ANCA-specific anti-MPO (myeloperoxidase) and anti-PR3 (proteinase 3) antibodies [25].

Two years later, the same group demonstrated the prognostic value of anti-apoA-1 IgG on a single center prospective study, including 221 patients hospitalized for acute MI, who all completed a one-year followup [26]. In this study, patients which had high levels of anti-apoA-1 IgG upon admission had a worse cardiovascular event-free survival at one year than patients tested negative for those autoantibodies (63.2% versus 88.5%, $P = 0.001$). Risk analyses indicated that being positive for anti-apoA-1 antibodies increased the subsequent risk of major adverse cardiovascular events (MACE, consisting of fatal or nonfatal ACS or stroke and hospitalization for acute heart failure) by 4-fold, independently of traditional CV risk factors (OR, 4.3; 95% CI, 1.46–12.6; $P = 0.007$). Furthermore, when compared to patients with low anti-apoA-1 IgG levels, those tested positive for anti-apoA-1 IgG were found to have: (i) higher basal heart rate upon discharge, a well-established cardiovascular prognostic feature after MI [27–29], and (ii) a higher proinflammatory cytokine profile in plasma, possibly associated with atherosclerotic plaque vulnerability [30].

Because many different autoantibodies have been shown to yield significant CV prognostic information, as extensively reviewed in [6], the same investigators undertook an ancillary study on the same cohort of patients comparing in a "head-to-head" manner the prognostic accuracies of autoantibodies to β2GPI domains I and IV, cardiolipin, apolipoproteinA-1 (anti-apoA-1 IgG), heat-shock protein 60 (anti-HSP-60), and phosphorylcholine (anti-PC IgM) [31]. Based upon ROC curve analyses, anti-apoA-1 IgG was found to be the only autoantibody to significantly predict the occurrence of subsequent MACE, and a trend was observed for anti-cardiolipin and anti-HSP60 antibodies ($P = 0.05$ and $P = 0.07$, resp.). Although significant, the area under the curve (AUC) was rather modest (AUC: 0.65, $P = 0.007$) but was found to be identical to that from the 10-year global Framingham risk score, used to determine patient therapeutic management [32]. Finally, Cox regression analyses demonstrated that being positive for anti-apoA-1 IgG increased the 1-year MACE risk by 4-fold, independent of the 10-year global Framingham risk score (hazard ratio: 3.8, $P = 0.002$) [31]. Those preliminary results indicate that in secondary prevention patients, anti-apoA-1 IgG could well be the most promising humoral autoimmune candidate for MACE prediction in nonautoimmune settings. Larger multicentre randomized control trials are now needed to determine whether an anti-apoA-1 IgG-based CV risk stratification algorithm could reach clinical decision making. Until then, no clinical recommendations can be proposed.

Finally, a recent publication by Keller and colleagues suggests that anti-apoA-1 IgG could be of diagnostic utility in patients presenting to the emergency room for acute chest pain [33]. In this single-centre prospective study involving 138 patients, the investigators demonstrated that anti-apoA-1 IgG values assessed on the first sample available had a relatively good diagnostic accuracy for non-ST elevation myocardial infarction (NSTEMI), with an AUC of 0.75 ($P < 0.0001$) that could be increased up to 0.88 when combined with anti-PC IgM and the NSTEMI-TIMI score to generate the clinical antibody ratio (CABR) score. Also, anti-apoA-1 IgG was found to be a good predictor (AUC 0.80, $P < 0.0001$) of subsequent troponin I elevation when the first sample was tested negative, which was the secondary endpoint of this study. Risk analyses indicated that in the presence of high anti-apoA-1 IgG levels the risk of subsequent NSTEMI diagnosis was increased by 6-fold after the adjustment for NSTEMI-TIMI score (OR: 6.4, 95% CI: 1.72–24.2). At the prespecified cut-off, this test displayed interesting negative predictive values of 88% and 95% for the primary and secondary study endpoints, respectively. However, the positive predictive values were too low to be clinically meaningful [33]. One important limitation of this work is the lack of a suitable contemporary comparator, such as a sensitive cardiac troponin assay, known to overcome the current limitations of conventional troponin assays [34]. Whether anti-apoA-1 IgG or the CABR score could provide incremental diagnostic value over those highly sensitive assays is far from obvious and need to be challenged in further studies. Also, cost-effectiveness studies are required to determine the impact of anti-apoA-1 IgG or CABR score assessment on both patient management and its related costs.

3.2. Anti-apoA-1 IgG in Patients with Severe Carotid Stenosis. Montecucco and colleagues investigated whether anti-apoA-1 IgG antibodies could be retrieved in a prospective cohort of 102 Italian patients who underwent carotid endarterectomy for severe carotid stenosis and retrieved a prevalence of high anti-apoA-1 IgG levels of 20% [35]. The patients included were devoid of any known CV events of atrial fibrillation and did not display any concomitant inflammatory diseases [35]. Conventional histochemistry analyses were performed on carotid biopsies of every patient and revealed that those tested positive for anti-apoA-1 IgG (in the serum) were found to have higher intraplaque infiltration of macrophages (11% versus 7%, $P = 0.04$), neutrophils (10% versus 3%), a higher expression of MMP-9 (19% versus 5%, $P < 0.0001$), and a lower total collagen content (18% versus 21%, $P = 0.002$) when compared to patients tested negative for those autoantibodies [35]. Although there is currently no strict consensus about the exact definition of vulnerable plaque in humans [36], this phenotype fulfills some of the proposed characteristics of a vulnerable atherosclerotic plaque [37]. The possible causal nature of this association has been proposed by the investigators based upon relevant *in vitro* and animals experimental results described in detail later on. Those results suggest that anti-ApoA-1 IgG assessment could be an interesting option to identify carotid atherosclerotic

plaque vulnerability, avoiding the use of unstandardized and resource demanding imaging modalities.

As atherosclerotic plaque rupture is one of the major determinants of myocardial infarction and stroke occurrence, those observations should be accompanied by a MACE increase in patients tested positive for those autoantibodies. To test this hypothesis, a one-year followup extension of this study is currently ongoing and will include a total of 178 patients.

3.3. Anti-apoA-1 IgG in Patients under Chronic Dialysis. Chronic kidney disease (CKD) is well known to be associated with an increased CV risk and gave rise to the so-called cardio-renal syndrome [38]. The premature CVD observed in CKD and maintenance hemodialysis (MHD) patients has been attributed to the superimposition of both traditional CV and nontraditional CV risk factors [38, 39]. Among the nontraditional CV risk factors, humoral immune dysfunction could contribute to ESRD-related CVD [40]. To this respect, low levels of anti-atherogenic antibodies, such as anti-PC IgM, were shown to independently predict all cause mortality in MHD patients [41], whereas high levels of proatherogenic autoantibodies directed against oxLDL are increased in hemodialysis patients [40] and possibly related to CVD in those patients [42].

In a cross-sectional study involving 66 MHD patients, Pruijm and colleagues lately reported a prevalence of anti-apoA-1 IgG positivity of 20% [43]. Significant associations were retrieved between circulating levels of anti-ApoA-1 IgG and dialysis vintage, a major determinant of vascular calcification known to negatively impact MHD patient prognosis [44]. Whether the presence of anti-apoA-1 IgG could explain the occurrence of dysfunctional and proinflammatory HDL reported in CKD patients [45] remains elusive and devoid of any experimental and clinical evidences. Nevertheless, their existence in the MHD illustrates the potential role of humoral autoimmunity in CKD-related atherogenesis. Whether anti-apoA-1 IgG could yield some prognostic information in CKD or MHD patients is currently unknown and clearly warrants further studies.

4. Anti-apoA-1 IgG and Cardiovascular Risk: A Causal Relationship?

Clinically driven *in vitro* and animal studies tend to support a causal role between anti-apoA-1 IgG and CV risk through different mechanisms.

First of all, when exposed to human monocytes-derived macrophages, anti-apoA-1 IgG were found to induce a dose-dependent production of different proinflammatory molecules, such as IL-8, MMP-9, IL-6, TNF-α, and MCP-1 [21, 30, 35]. In those studies, the exact mechanisms were not reported but the potential confounder of lipopolysaccharide contamination has been systematically addressed and reasonably excluded by Limulus assays. Further mechanistic insights were brought by Pagano and colleagues who demonstrated that the direct anti-apoA-1 IgG proinflammatory effect was mediated by their interaction with the toll-like receptor (TLR)-2/CD14 complex. Supported by bioinformatics modeling and *in vitro* results, this surprising finding appears to be related to structural homology between apoA-1 and TLR-2 [30], supporting the molecular mimicry hypothesis to account for this cross-reactivity. Those findings are in accordance with previous reports supporting the general concept that autoantibodies, such as antiphospholipid and anti-HSP antibodies, can promote sterile inflammation through their interaction with different TLRs [46–48].

Another potential proinflammatory mechanism inferred to anti-apoA-1 IgG antibodies is their ability to promote neutrophil chemotaxis toward IL-8 and TNF-α (both expressed within atherosclerotic plaques) when compared with CTL medium or CTL IgG treatments, a phenomenon not observed for monocytes [35]. The exact mechanisms accounting for this phenomenon are currently unknown.

Secondly, mirroring the clinical finding showing a positive association between anti-apoA-1 IgG levels and resting heart rate [26], a well-established cardiovascular prognostic feature after MI [27–29], *in vitro* studies demonstrated that in presence of aldosterone, anti-apoA-1 IgG can elicit a dose-dependent increase of the spontaneous contraction rate of neonatal rat ventricular cardiomyocytes [26, 49]. Although still fragmentary, this positive chronotropic effect observed *in vitro* is mediated by L-type calcium channel activation induced by the nongenomic downstream activation of mineralocorticoid receptor, involving phosphatidyl 3-kinase pathways [49]. Interestingly, this chronotropic effect was abrogated by eplerenone but not with aldactone, as well as by intravenous immunoglobulins [26, 49]. Nevertheless, the anti-apoA-1 IgG specific receptor on cardiomyocytes has not been described yet and is under active investigation in our laboratory.

Animal studies appear to provide convergent results with the aforementioned findings. Indeed, Montecucco and colleagues demonstrated that passive immunization of apoE −/− mice under normal chow diet (a validated model of atherosclerosis) increased the size of atherosclerotic lesions and induced a vulnerable phenotype, mirroring the one retrieved in humans consisting in higher intraplaque MMP-9 expression, higher neutrophil content, and lower collagen content when compared to control IgG [35]. In a lupus-prone mice model, the presence of anti-apoA-1 antibodies has been shown to be associated with a decrease in the antioxidant properties of HDL related to a decrease in PON-1 activity leading to an increase of proinflammatory reactive oxygen species [50], corroborating the clinical observations reported earlier in humans [13–15, 17, 18].

5. Conclusions and Future Perspectives

To summarize, recent studies demonstrate that IgG autoantibodies against apolipoprotein A-1 (apoA-1) are raised in many diseases associated with a high cardiovascular risk, such as SLE, ACS, RA, severe carotid stenosis, and end-stage renal disease. So far, high levels of anti-apoA-1 IgG were shown to be an independent prognostic marker of poor CV outcome in MI and in RA patients, to display clinically

relevant properties for NSTEMI diagnosis in acute chest pain patients and to be associated with atherosclerotic plaque vulnerability in patients with severe carotid stenosis. In many studies, high levels of anti-apoA-1 IgG are associated with a proinflammatory cytokine profile, and in SLE/APS, those autoantibodies have been shown to be associated with the presence of dysfunctional HDLs.

Concomitantly, *in vitro* data tend to indicate that anti-apoA-1 IgG are active modulators of atherogenesis by (i) promoting sterile inflammation through TLR2/CD14 complex and by (ii) eliciting specific neutrophil chemotaxis. Furthermore, intriguing *in vitro* experiments suggests that those autoantibodies could act as a proarythmogenic molecule through an aldosterone-dependent L-type calcium channels activation that can be reverted by existing therapeutic compounds. In parallel, mice models demonstrate that passive immunization with anti-apoA-1 IgG increases atherogenesis, as well as atherosclerotic plaque vulnerability, and decreases the antioxidant properties of HDL by inhibiting PON-1 activity. Taken together, those preliminary results need to be replicated in larger multicentre cohorts, and a better understanding of their physiopathological involvement in atherogenesis is required. Nevertheless, the current converging *in vitro* and animal observations lend weight to the hypothesis that anti-apoA-1 IgG are active mediators of atherogenesis rather than an innocent bystander. If true, those autoantibodies could in the future represent a new possible therapeutic target.

Currently, approximately 20% of patients with patent CVD do not display any Framingham risk factors [51], baring out the importance of identifying new and reversible emergent CVD risk factors. Because antibody-mediated diseases and some cardiovascular conditions can be treated by specific immunologic therapeutic strategies, such as passive immunization [52], anti-apoA-1 IgG could represent an innovative theranostic candidate allowing the identification of subset of CVD that could benefit from this kind of therapy in the future and substantially contribute to personalized medicine in the field of CVD.

Acknowledgments

This work was supported by Swiss National Science Foundation Grant no. 310030_140736 to Dr. N. Vuilleumier and by Valentine Gerbex-Bourget Foundation.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 651058, 7 pages
doi:10.1155/2012/651058

Research Article

Automated Indirect Immunofluorescence Evaluation of Antinuclear Autoantibodies on HEp-2 Cells

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Received 5 October 2012; Accepted 18 October 2012

Academic Editor: Dimitrios P. Bogdanos

Indirect immunofluorescence (IIF) on human epithelial (HEp-2) cells is considered as the gold standard screening method for the detection of antinuclear autoantibodies (ANA). However, in terms of automation and standardization, it has not been able to keep pace with most other analytical techniques used in diagnostic laboratories. Although there are already some automation solutions for IIF incubation in the market, the automation of result evaluation is still in its infancy. Therefore, the EUROPattern Suite has been developed as a comprehensive automated processing and interpretation system for standardized and efficient ANA detection by HEp-2 cell-based IIF. In this study, the automated pattern recognition was compared to conventional visual interpretation in a total of 351 sera. In the discrimination of positive from negative samples, concordant results between visual and automated evaluation were obtained for 349 sera (99.4%, kappa = 0.984). The system missed out none of the 272 antibody-positive samples and identified 77 out of 79 visually negative samples (analytical sensitivity/specificity: 100%/97.5%). Moreover, 94.0% of all main antibody patterns were recognized correctly by the software. Owing to its performance characteristics, EUROPattern enables fast, objective, and economic IIF ANA analysis and has the potential to reduce intra- and interlaboratory variability.

1. Introduction

The detection of autoantibodies against the cell nuclei (ANA) and cytoplasmic components plays an important role in the diagnosis of many autoimmune diseases, such as systemic lupus erythematosus, mixed connective tissue disease, rheumatoid arthritis, progressive systemic sclerosis, dermato-/polymyositis, Sjögren's syndrome, and chronic active autoimmune hepatitis. The prevalence of ANA varies between 20 and 100%, depending on the disease and type of antibody [1–4].

The gold standard for ANA screening is indirect immunofluorescence (IIF) on human epithelial (HEp-2) cells [5–7]. Displaying a multitude of authentic autoantigens, this antigenic substrate enables highly sensitive preidentification of autoantibodies by their characteristic fluorescence patterns [8], and the determination of their titers. In addition, the confirmation of positive screening results and the identification of single ANA specificities by monospecific immunoassays (e.g., enzyme-linked immunosorbent assay (ELISA) or immunoblot) are recommended to support differential diagnosis, disease monitoring, and prognostic assessment. This two-step strategy has been challenged by automated ELISA and multiplex approaches promising easy, cost-effective high-throughput performance and standardization [9, 10]. However, these assays may produce inaccurate (false negative) screening results, mainly because the number of displayed purified or recombinant antigens is limited, or, when using nuclear homogenates as substrate, relevant epitopes may be altered or lost during the process of solid-phase coating [5, 6, 11–15].

As mentioned before, HEp-2-cell-based IIF is the method of choice for ANA screening. Although there are some automation solutions for IIF incubation about to be launched

on the market, the evaluation is still carried out visually by laboratory technicians, thus being time consuming, subjective, error prone, and contributive to inter-observer variability. This, together with the growing demand for ANA testing, reinforces the need for automation and standardization of IIF evaluation. So far, only a few more or less advanced commercial platforms based on automated motorized camera-microscopes and digital image analysis software have been introduced [16–22].

In the current study, we evaluated a novel system (EUROPattern Suite) for largely automated processing of IIF slides, and the recording and interpretation of immunofluorescence images of HEp-2 cells. The performance of this novel system was compared to visual IIF interpretation, focusing on positive/negative classification and pattern recognition.

2. Materials and Methods

2.1. Human Sera. Two sample collectives were examined. Collective A consisted of 200 consecutive serum samples submitted to a reference laboratory (Lübeck, Germany) for routine ANA testing. Empirically, the majority of these samples tend to show complicated mixed patterns, whereas only a few of them are negative. Collective B comprised 151 serum samples originating from different referral laboratories, including 44 samples from patients with systemic rheumatic disease (10 systemic lupus erythematosus, 10 systemic sclerosis, 16 Sjögren's syndrome, 8 dermato-/polymyositis), 12 samples with specific ANA or anticytoplasmatic autoantibodies, 47 samples from disease controls, and 48 samples from healthy blood donors. The samples were blinded for analysis. All study procedures were approved by the local ethics committee.

2.2. Indirect Immunofluorescence (IIF) Assay. ANA detection was performed by IIF using HEp-2 cells (Euroimmun, Lübeck, Germany). The cells were coated onto cover slips, fixed with acetone, cut into fragments (biochips), and glued onto microscope slides. The complete incubation process was carried out manually: serum samples diluted 1 : 100 were incubated with the HEp-2 cell substrate for 30 minutes at room temperature. After washing with PBS-Tween, the slides were incubated for another 30 minutes with goat anti-human IgG conjugated with fluorescein isothiocyanate plus propidium iodide for counterstaining (Euroimmun) to label specifically bound antibodies. After a second washing step and embedding, the slides were evaluated.

2.3. Evaluation of Antinuclear Autoantibodies. IIF slides were subjected to automated immunofluorescence microscopy (as described below), with the system taking focused images of all reaction fields. Subsequently, using the same images, the fluorescence patterns were evaluated in two ways: (i) by the EUROPattern software (Euroimmun) and (ii) visually by two laboratory technicians who worked independently without reference to the other's and the software's readings. Sera with an antibody titer equal to or greater than 1 : 100 were considered as positive. Based on HEp-2 cells, the following patterns were reported: homogenous, speckled, nucleolar, nuclear dots, centromeres, cytoplasmic, and negative.

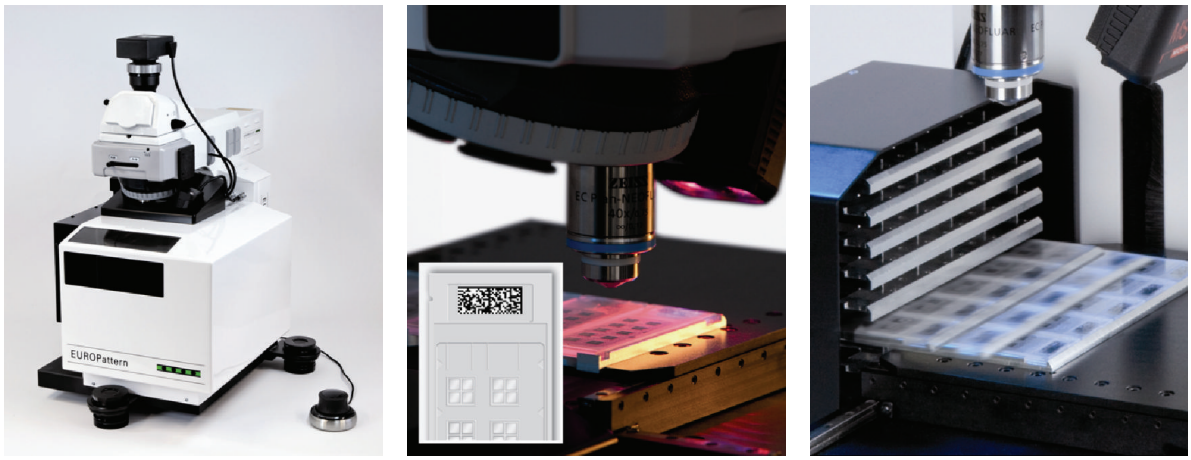
2.4. Automated Processing. The EUROPattern Suite (Euroimmun) consists of an automated EUROPattern Microscope, the laboratory management software EUROLabOffice, and the pattern recognition software EUROPattern.

The specialized EUROPattern Microscope has been tailored to the requirements of automated IIF. As a motorized camera-microscope, it provides automated acquisition of high-resolution immunofluorescence images. It contains a slide magazine with a capacity for 500 reaction fields and a matrix code scanner enabling slide identification. Instead of conventional illumination fittings it has a controlled LED, which maintains a constant light flux (>50,000 hrs), ensuring highly reproducible results (Figure 1).

EUROLabOffice supports IIF processing by data exchange with a central Laboratory Information System (LIS), automatic protocol generation, interconnection with further laboratory devices (e.g., dilution/incubation systems), and data connection with EUROPattern for archiving of IIF images and automatic image interpretation. The software consolidates all the results from IIF and other analytical techniques (ELISA, immunoblot, and radioimmunoprecipitation assay) into one report per patient and provides a validation process.

EUROPattern is a fast and comprehensive IIF pattern recognition system providing objective test results. As a closed system, EUROPattern requires specific HEp-2 or HEp-20-10 cell-based test kits (Euroimmun) with a particular anti-human IgG conjugate enabling image segmentation by counterstaining. The software identifies the cells, calibrates the image, classifies the image as negative or positive, and, in case of a positive result, extracts 179 features and identifies the pattern(s). The classification is based on k-nearest neighbour algorithm with a reference database of more than 5,000 images (115,000 cell references) and rule-based synthesizing of cell results to one result per dilution. Single as well as mixed patterns can be identified. If a sample has been incubated in different dilutions, EUROPattern additionally merges the results of the different images into one patient report containing the patterns and estimated antibody titer.

EUROPattern is a computer-aided diagnostic system, meaning that all automatically retrieved results have to be validated by the laboratory staff in the Graphical User Interface (GUI), which is plugged into EUROLabOffice. For an efficient laboratory process, all images with a negative result can be displayed in a list, ordered by a normalized image fluorescence intensity. If deemed necessary, the positive/negative cutoff may be corrected by mouse click and all remaining negatives can be validated as well in one step. All positive results can be reviewed patient by patient. For each patient, the EUROPattern GUI displays the images of different sample dilutions and the consolidated results, including the identified patterns with the corresponding estimated titer and the calculated confidence value. The automatically generated result can be further detailed using a readily displayed list of antibody patterns (Figure 2).



(a) (b) (c)

Figure 1: EUROPattern Microscope for automated acquisition of high-resolution immunofluorescence images. (a) Motorized microscope including camera, controlled LED, microscope control device, optional eyepieces, (b) matrix code reader, and (c) slide magazine. The microscope stage and the magazine are surrounded by a casing keeping out the sunlight and protecting the substrate fluorescence from fading. The microscope is part of the EUROPattern Suite, which additionally contains a laboratory management software (EUROLabOffice) and an automatic pattern recognition software (EUROPattern).

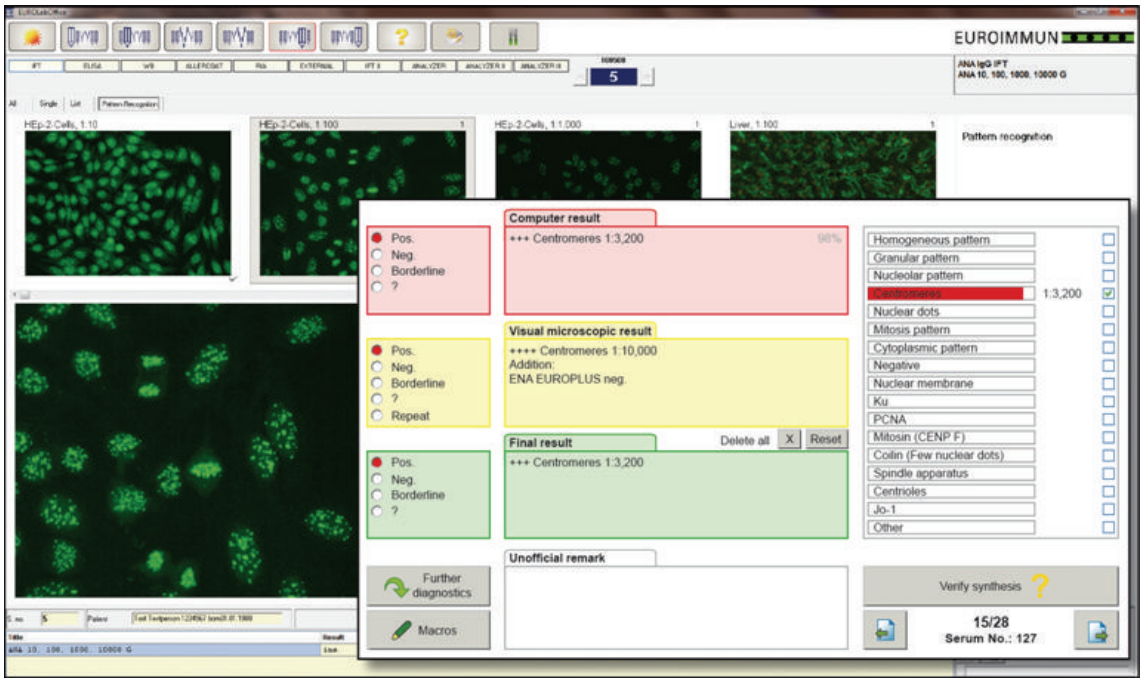


Figure 2: Graphical user interface of EUROPattern. For each patient sample, EUROPattern displays the images of different sample dilutions, preliminary or additional results, and the software-generated result (recognized pattern, antibody titer, and calculated confidence value) on one report form. The software-generated result can be confirmed by mouse click or, if necessary, modified and specified using a readily displayed list of fluorescence patterns.

2.5. Statistics. The degree of interrater agreement between visual and automated antibody pattern interpretation was assessed by the percentage of concordance and by kappa coefficients. According to Altman [23], kappa (κ) values were interpreted as follows: $\leq$0.20 poor, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 good, and 0.81–1.00 very good agreement. Statistical analyses were carried out using GraphPad QuickCalcs (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Positive/Negative Classification. The efficient usage of any automated ANA detection system requires first of all

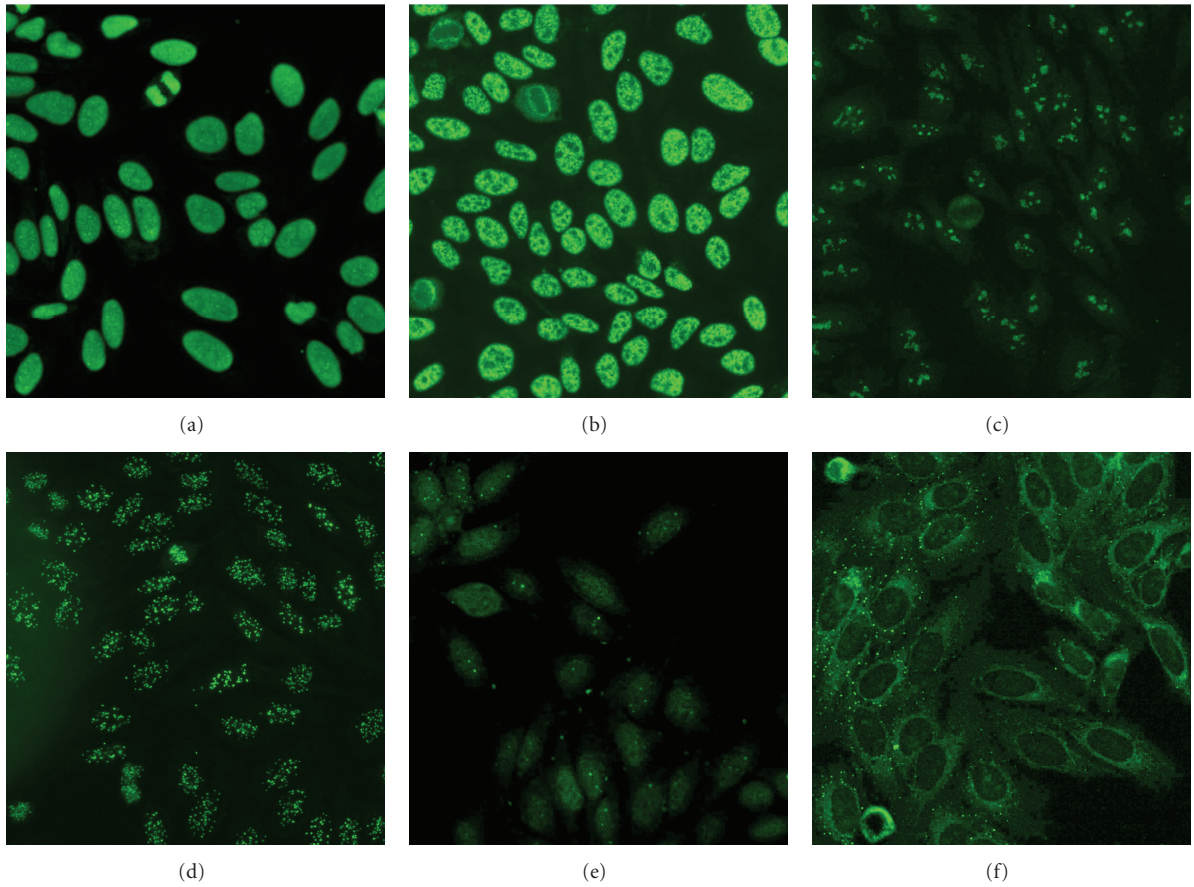


Figure 3: Representative indirect immunofluorescence patterns of antinuclear and anticytoplasmic autoantibodies on HEp-2 cells: (a) homogenous, (b) speckled, (c) nucleolar, (d) centromeres, (e) nuclear dots, and (f) cytoplasmic.

a reliable identification of negatives. Therefore, the capability of the EUROPattern system to differentiate negative from positive samples was analyzed.

About 40 installations of EUROPattern in different laboratories worldwide have revealed that visual IIF analysis remains partially subjective, resulting in the requirement to keep the fluorescence intensity cutoff configurable. This classificator setting is part of an optimization process during the introduction in immunologic laboratories to set the relation of sensitivity to specificity. As an approach to standardization, a recommended basic setup is available for EUROPattern that has been chosen for the evaluation of the EUROPattern classificator.

Out of a total of 200 sera sent to a reference laboratory for routine ANA testing (collective A), 193 sera were classified as antibody positive both by visual and automated evaluation. Out of 7 sera tested negative by visual examination, 6 were negative in EUROPattern, whereas one sample was reported positive with cytoplasmic fluorescence.

Out of 151 sera from rheumatic patients and controls (collective B), 79 sera were assessed as positive and 71 as negative both by visual and automated examination. There was one discrepant serum that was negative by visual evaluation, but demonstrated faint positive fluorescence (low probability rate) according to EUROPattern.

Referring to the total of 351 samples, there was an agreement of 99.4% ($\kappa = 0.984$) between the visual and automated approach regarding positive/negative discrimination. The analytical sensitivity and specificity of EUROPattern amounted to 100% and 97.5%, respectively, while the positive and negative predictive value were 99.3% and 100%, respectively (Table 1).

3.2. Pattern Recognition. In daily routine with EUROPattern, after the one-step validation of the samples classified as negative, all remaining positive samples have to be validated by the laboratory staff patient by patient. We analyzed the ability of EUROPattern for recognition of homogenous, speckled, nucleolar, centromere, nuclear dotted, cytoplasmic, and negative patterns (Figure 3).

In collective A, correct and complete pattern recognition (including mixed patterns) was observed in 49.0% of the samples (47.7% of positive samples). In 93.0% of the samples (93.3% of positive samples) at least the main pattern was recognized correctly.

In collective B, correct and complete pattern recognition (including mixed patterns) was observed in 74.2% of the samples (51.9% of positive samples). In 96.7% of the samples (94.9% of positive samples) at least the main pattern was recognized correctly.

Table 1: Comparison of software-based and visual positive/negative classification.

	Visual evaluation					
	Collective A ($n = 200$)		Collective B ($n = 151$)		Total ($n = 351$)	
	Positive	Negative	Positive	Negative	Positive	Negative
EUROPattern						
Positive	193	1	79	1	272	2
Negative	0	6	0	71	0	77
Concordance	99.5%		99.3%		99.4%	
κ-value	0.921		0.987		0.984	
Sensitivity	100%		100%		100%	
Specificity	85.7%		98.6%		97.5%	
PPV	99.5%		98.8%		99.3%	
NPV	100%		100%		100%	

κ: kappa-value indicating interrater agreement, PPV: positive predictive value, NPV: negative predictive value.

Table 2: Main pattern recognition by EUROPattern.

	EUROPattern performance					
Main ANA pattern	Collective A ($n = 200$)		Collective B ($n = 151$)		Total ($n = 351$)	
	No. of samples	Pattern recognized	No. of samples	Pattern recognized	No. of samples	Pattern recognized
Homogenous	24	20 (83.3%)	9	7 (77.8%)	33	27 (81.8%)
Speckled	94	90 (95.7%)	36	33 (91.7%)	130	123 (94.6%)
Nucleolar	18	17 (94.4%)	27	26 (96.3%)	45	43 (95.6%)
Centromeres	3	3 (100%)	1	1 (100%)	4	4 (100%)
Nuclear dots	1	1 (100%)	1	1 (100%)	2	2 (100%)
Cytoplasmic	53	49 (92.5%)	5	5 (100%)	58	54 (93.1%)
Negative	7	6 (85.7%)	72	71 (98.6%)	79	77 (97.5%)
Total	200	186 (93.0%)	151	144 (95.4%)	351	330 (94.0%)

Referring to the total of 351 serum samples, the automatically retrieved results were correct and complete (including mixed patterns) in 59.8% of all samples (48.9% of positive samples). The overall efficiency of automated main pattern recognition was 94.0% and varied for the different patterns, declining in the following order: centromeres, nuclear dots (100%) > negative (97.5%) > nucleolar (95.6%) > speckled (94.6%) > cytoplasmic (93.1%) > homogenous (81.8%). In 21 out of 351 (6.0%) sera, the main pattern was not recognized (Table 2).

4. Discussion

The automation of IIF processing and evaluation is a major step towards standardization of the method. Standardization is a crucial concern because of intra- and interlaboratory variations, which may have methodological causes (e.g., microscope, type and running time of the microscope bulb, test kits, reagents, and incubation method/device) but can also be influenced by subjective image interpretation, individual expertise, and experience of the laboratory staff. Systems for automated IIF evaluation may contribute to the reduction of errors and pave the way to standardized ANA testing [16, 21].

In this study, we compared the results of classical visual reading with automated pattern recognition by EUROPattern for 351 samples. None of the 272 positive patient samples were missed out, and 77 out of 79 negatives were identified as negative by the software. Based on a 99.4% agreement with visual interpretation, EUROPattern proved highly capable of performing reliable positive/negative discrimination of IIF results. In comparison, for the AKLIDES software (Medipan, Berlin, Germany) an agreement with visual positive/negative discrimination of 90.0 to 98.9% was reported [17, 18, 20, 21]. The agreement rate of the NOVA View system (Inova, San Diego, USA) amounted to 92.2% [22].

In EUROPattern, the automatically determined patterns were correct and complete in 210 out of 351 cases and correct and meaningful but not complete ("main pattern") in another 120 cases, enabling main pattern recognition in 94.0% of cases. The lowest performance in pattern recognition was found for the homogenous pattern type (81.8%), while the performance rates for the other patterns ranged between 93.1 and 100%. This finding may be due to the fact that many investigators tend to interpret a dense granular pattern as a homogenous pattern, while the automated system reports a granular pattern. Moreover, the software-based recognition of a homogenous staining may be impaired by a superimposed granular nuclear or cytoplasmic fluorescence in samples with a mixed pattern.

Inadvertently, sera with an anticentromere or antinuclear dot pattern were underrepresented in the present study.

However, in preliminary studies, we evaluated the EUROPattern system by use of 23 samples demonstrating an anticentromere pattern and 32 samples demonstrating an antinuclear dot pattern by visual HEp-2 cell interpretation. EUROPattern identified the respective pattern in 19/23 and 26/32 sera, corresponding to recognition rates of 82.6% (anti-centromere) and 81.3% (anti-nuclear dot). Discrepant assessment was found for 10 samples in which additional staining patterns, such as strong cytoplasmic fluorescence, interfered with the automated assessment.

The recognition of mixed patterns is a critical point in IIF ANA detection, because dominant autoantibodies (or unspecific antibodies) may mask another diagnostically relevant autoantibody or complicate pattern differentiation. As also reported for the AKLIDES system [17, 21], distinction of patterns with two or more autoantibodies can be difficult, depending on their number, target, and titer. This point is also reflected by the present study, showing correct and complete pattern recognition (including mixed patterns) by EUROPattern in up to 74.2% of the samples (collective B). This rate appears moderate, but is fairly high considering the complex system requirements. To further improve the otherwise very good performance characteristics (e.g., sensitivity) of EUROPattern and other automated systems [18], the current deficiencies in differentiating mixed patterns and in identifying some particular antibody reactivities [17, 18, 21] have to be overcome. For this purpose, further software development will enable the classification of a larger variety of diagnostically relevant cell and tissue fluorescence patterns. Titering of the samples (at least two dilutions) is recommended to facilitate the interpretation of mixed patterns. Moreover, the concept of EUROPattern includes a short final step of approving positive results, in which the investigator can confirm, modify, or further detail the reported antibody patterns (if necessary). Based on this concept, the performance of the automated approach potentially increases to 100%, resulting in a system that provides highly efficient, fast, and standardized IIF ANA processing and evaluation. Accordingly, EUROPattern can be regarded as a powerful alternative to the conventional visual approach.

The results of the present study were obtained by manual assay incubation. Since the EUROPattern classificator is plugged into the Laboratory Management System EUROLabOffice, the available automated incubation systems can be integrated seamlessly into the IIF workflow process using the EUROPattern Suite.

For all of the 351 samples in this study, the EUROPattern Microscope delivered extremely sharp, high-resolution images which are a prerequisite for image processing and computer-aided diagnosis. Counterstaining not only provides solid nucleus finding and mitosis identification, but also ensures that a potentially failed focus will never lead to a false negative result.

Large laboratories with a high sample throughput tend to have a two-step IIF diagnostic process. Positive/negative screening is performed with a particular screening dilution (e.g., 1 : 80 or 1 : 100). Further dilutions are carried out for positive samples. EUROPattern merges the results from all available dilutions into one final result per patient, which is displayed together with all IIF images on a single patient-specific report form. The batchwise verification of negatives significantly shortens the analysis procedure.

Considering economic constraints and the growing demand for ANA detection in clinical practice, the system's unique capacity of slide accommodation (500 reaction fields) and high throughput (approximately 60 min for 100 reaction fields, depending on customer-specific settings) is of practical relevance, enabling the rapid processing of large sample quantities and overnight runs. Due to the casing around the magazine and microscope stage, the substrates are protected from bleaching and microscopy can be performed under normal room light conditions without need for a darkroom. The complete microscopic process, the acquisition of focused images, the management, processing, and archiving of data and images, is carried out by the system. EUROPattern recognizes most of the important ANA patterns, including mixed patterns, and calculates all corresponding titers. A diagnostic expert then performs the final validation of results at the office PC and may additionally access the system via a microscope control device. The EUROPattern Suite is in a continuous development process, which focusses on an even greater variety of fluorescence patterns and on several other features that will improve work processes, performance, and accuracy.

Certainly, the serodiagnosis of other autoantibody-associated diseases would also benefit from the implementation of automated IIF evaluation. For this purpose, the system will be applied to further antigenic substrates, such as neutrophil granulocytes in the detection of antineutrophil cytoplasmic antibodies in Wegener's granulomatosis, microscopic polyangiitis, and Churg-Strauss syndrome [24].

5. Conclusions

Compared to conventional visual IIF evaluation, EUROPattern proved to be very sensitive. This reliability is the basis for handing over the first step in ANA screening to an automated detection system. EUROPattern also proved to be highly efficient in sorting out negatives and providing good pattern recognition. The remaining process of validating positive results, which is carried out by qualified laboratory personnel patient by patient in the EUROPattern GUI, is now less time consuming and less error prone than direct visual reading. It can be expected that the intra- and inter-laboratory variation in IIF evaluation will be reduced efficiently by automation solutions, helping clinical laboratories to standardize IIF-based ANA diagnostics.

Conflict of Interests

J. Voigt, C. Krause, E. Rohwäder, S. Saschenbrecker, M. Hahn, M. Danckwardt, C. Feirer, K. Ens, and K. Fechner are employees of Euroimmun AG. W. Stöcker is board member of Euroimmun AG.

Acknowledgments

The authors are grateful to the members of the Institute for Neuro- and Bioinformatics at the University of Lübeck

(Germany) for their support. Moreover, the authors thank Sandy Simon for careful and critical reading of the paper.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 762874, 7 pages
doi:10.1155/2012/762874

Research Article

Automatic Reading of ANCA-Slides: Evaluation of the AKLIDES System

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Received 2 October 2012; Accepted 10 October 2012

Academic Editor: Dimitrios P. Bogdanos

The ANCA consensus prescribes screening by indirect immunofluorescence on neutrophils. We evaluated the first automated ANCA-pattern recognition system. C-ANCA ($n = 39$) and P-ANCA ($n = 40$) samples were selected from patients with ANCA-associated vasculitis (AAV). Non-AAV controls included sera from healthy controls ($n = 40$), sera with possible interfering antibodies ($n = 46$), or miscellaneous ANCA reactivity ($n = 31$). ANCA slides were analysed by AKLIDES and routine fluorescence microscopy. The C-ANCA pattern was recognized by routine microscopy in 92% and 97% on ethanol- and formalin-fixed slides, respectively. AKLIDES reported C-ANCA in 74% and 95%, respectively. P-ANCA was recognized by routine microscopy on ethanol-fixed neutrophils in 90%, while AKLIDES reported P-ANCA in 80%. Typically, only 65% and 33% of these samples showed the expected C-ANCA on formalin-fixed neutrophils by routine microscopy and AKLIDES, respectively. A C- or P-ANCA pattern was observed on ethanol-fixed neutrophils in 28% and 23% of the controls by routine microscopy and AKLIDES, respectively. Only 5% of the controls revealed C-ANCA on formalin-fixed neutrophils by routine microscopy and AKLIDES. Altogether, automated ANCA-pattern recognition by AKLIDES is promising. Distinction of C- and P-ANCA is good, but sensitivity on ethanol-fixed neutrophils needs improvement. When optimized, pattern recognition software may play an important role in AAV diagnostics.

1. Introduction

Detection of antineutrophil cytoplasmic antibodies (ANCAs) is relevant for the diagnosis of the ANCA-associated vasculitides (AAV), including granulomatosis with polyangiitis (GPA, previously referred to as Wegener's granulomatosis), eosinophilic granulomatosis with polyangiitis (EGPA; previously referred to as the Churg-Straus syndrome), microscopic polyangiitis (MPA), and renal-limited necrotizing crescentic glomerulonephritis (NCGN) [1]. Classification criteria for these diseases have been defined by the American college of rheumatology (ACR) [2] and the Chapel Hill consensus conference [3]. The presence of ANCA, however, is not part of these criteria which are primarily based on clinical manifestations and histopathology as observed in biopsies obtained from the affected tissues. More recently, a novel consensus methodology for the classification of AAV was developed and validated for epidemiological studies [4]. Importantly, the latter classification criteria incorporated the ANCA status of the patient.

The current international consensus on ANCA testing prescribes screening by indirect immunofluorescence (IIF) on ethanol-fixed neutrophils [5]. Four different patterns can be distinguished. First, the classical (C-)ANCA is characterized by a granular, cytoplasmic fluorescence with central or interlobular accentuation; second, a diffuse flat cytoplasmic fluorescence without interlobular accentuation may be referred to as atypical C-ANCA. In clinical practice, however, both patterns are difficult to distinguish and many clinical laboratories do label both these patterns as "C-ANCA." Third, the perinuclear (P-)ANCA is characterized by perinuclear staining, with or without nuclear extension. Reading of the P-ANCA pattern may be hampered by the presence of interfering antinuclear antibodies (ANAs). The perinuclear staining pattern actually is an artefact, since formalin-fixation results in a cytoplasmic staining

pattern, indistinguishable from C-ANCA on ethanol-fixed neutrophils. Finally, if a combination of cytoplasmic and perinuclear staining occurs, this is called atypical ANCA. Importantly, in AAV it is mandatory to establish with antigen-specific assays that ANCAs are directed either to serine protease 3 (PR3) or myeloperoxidase (MPO) for optimal diagnostic performance [1, 5, 6].

IIF is a labour-intensive technique, requires special expertise of the technician, and is hampered by the subjective reading of the slides [7]. The advent of microscope devices with integrated software for pattern recognition might overcome this problem [8]. The AKLIDES system is the first automated system for ANCA-pattern recognition based on the combination of ethanol- and formalin-fixed ANCA slides. In this study, we have evaluated the AKLIDES system using sera from AAV patients ($n = 79$) as well as distinct cohorts of relevant control sera ($n = 117$).

2. Materials and Methods

2.1. Patient Sera. Samples of AAV patients were selected based on the routine ANCA IIF analysis using ethanol-fixed ANCA slides (INOVA, San Diego, CA, USA) [9]. Samples with a C-ANCA pattern ($n = 39$) were selected from AAV patients (25 males and 14 females, median age 58 yrs, range 20–83 yrs) that were PR3-ANCA-positive at the time of diagnosis; titres varied from 1/32 up to >1/1024. Similarly, samples with a P-ANCA pattern ($n = 40$) were selected from AAV patients (25 males and 15 females, median age 60 yrs, range 19–78 yrs) that were MPO-ANCA-positive at the time of diagnosis; titres varied from 1/32 up to ≥1/1024. Sample selection was based on titres from our patient archive. Samples in this archive were stored from 2000 onward and were obtained from patients every time they visited the outpatient clinic (most patients visited the outpatient clinic at least 3-4 times/year). Antigen-specificity of ANCA was determined as described before [9]. In 34 of the selected C-ANCA samples ($n = 39$), PR3-ANCA were detectable, while in 25 of the selected P-ANCA samples ($n = 40$) MPO-ANCA were detectable.

Next to these AAV sera, 5 distinct series of control samples were included. First, sera of healthy controls ($n = 40$) were included. Second, sera with antinuclear antibodies (ANA) were included to examine ANA interference. ANA patterns and titres were determined by routine ANA IIF analysis using Hep-2000 cells as a substrate (Immuno Concepts, Sacramento, CA, USA). These ANA controls consisted of sera with a homogenous ANA in three distinct titres (1/80, $n = 6$; 1/320, $n = 7$; 1/1280, $n = 7$), and sera with a speckled pattern ($n = 5$), an atypic speckled pattern (SSA-pattern; $n = 4$), a centromere pattern ($n = 4$), and a nucleolar pattern ($n = 2$). The nonhomogenous ANA sera all had a titre of 1/1280. Third, sera ($n = 11$) with antimitochondrial antibodies (AMA) were included to examine AMA interference. AMA were originally detected by routine IIF on liver/kidney/stomach slides (Scimedx, Denville, NJ, USA) in a 1/20 dilution and confirmed as reactive with E2-component of the pyruvate dehydrogenase complex (Euroimmun, Lübeck, Germany). Fourth, sera ($n = 9$) with a C-ANCA pattern (titres 1/16–1/1024) on ethanol-fixed ANCA slides (INOVA) due to reactivity with bactericidal permeability-increasing protein (BPI) as determined by ELISA (Euro Diagnostica, Lund, Sweden). Fifth, sera ($n = 22$) with an atypical ANCA staining pattern on ethanol-fixed ANCA slides (INOVA). The sera of the fourth and fifth control cohorts were all negative for PR3- and MPO-ANCA and came from patients that did not have AAV.

Since for the analyses on patient material sera were obtained for diagnostic purposes and the rest-serum was used in an anonymous way, ethical approval and informed consent was not necessary according to the Dutch guidelines. All sera were stored at −30°C until analysis.

2.2. ANCA Detection. ANCAs were detected in parallel on ethanol- and formalin-fixed neutrophils (Medipan, Berlin, Germany) by IIF. For both substrates, the sera were diluted 1/20; no further titration was performed. The assays were performed according to the manufacturer's instructions. Importantly, these assays were specifically designed for automatic reading in the AKLIDES system. In particular, the mounting medium contained DAPI to enable automatic focussing.

ANCA detection was first performed by AKLIDES. The system consists of a combination of a fluorescence microscope with an LED light source, a scan stage, a camera, and a personal computer containing the AKLIDES software (Medipan). The software, version AKLIDES 1.1 ANCA module: Build 47 (March 2011), automatically reads out images by controlling scan positions (x- and y-directions), focussing on the DAPI-staining (z-direction), calibration and recording of the fluorescence signal. For ANCA detection, 10 scan positions per well appeared optimal (data not shown). The AKLIDES software automatically analyses the intensity and structure of the fluorescence signal for each sample. Positive-negative differentiation, a nuclear-cytosolic localisation and assignment of ANCA fluorescence to C- or P-ANCA, or ANA-dots is provided. If a pattern could not be assigned to a positive sample, the score undetermined is given.

The reproducibility of the automatic pattern recognition was determined by the manufacturer (Medipan) by analysing one sample with PR3-ANCA and one sample with MPO-ANCA three times in three different dilutions on both ethanol- and formalin-fixed neutrophils. The PR3-ANCA revealed 8x a C-ANCA and 1x an undetermined pattern on ethanol-fixed neutrophils and 9x a C-ANCA on formalin-fixed neutrophils; the MPO-ANCA revealed 9x a P-ANCA and 9x a C-ANCA pattern on both substrates, respectively. Overall reproducibility is therefore >97%.

Second, ANCA fluorescence was judged by routine fluorescence microscopy using a Zeiss microscope with an LED light source (Zeiss, Oberkochen, Germany). All slides were evaluated by two observers blinded for the results obtained by routine analyses and AKLIDES. In case of a difference in opinion, a third observer was decisive.

3. Results

3.1. ANCA Pattern Recognition in Sera of Patients with AAV: Visual Scoring. The results of ANCA pattern recognition, presented according to the titres measured by routine ANCA IIF, are summarized in Figure 1. Visual scoring of the Medipan slides with ethanol-fixed neutrophils revealed that in 36/39 samples (92.4%) the expected C-ANCA was observed (Figures 1(a) and 2(a)), while in 36/40 samples (90%) the expected P-ANCA was observed (Figures 1(b) and 2(c)). One C-ANCA sample (titre >1/1024) and two P-ANCA samples (titre 1/32) revealed an atypical pattern. Only samples with a low titre (1/32-1/64) were negative in the visual scoring of the Medipan slides. Altogether, the concordance in terms of pattern recognition with the historically performed routine ANCA IIF was 91.1%.

Visual scoring of the formalin-fixed neutrophils revealed in 38/39 of the samples (97.4%) the expected C-ANCA pattern (Figures 1(c) and 2(b)); the missed sample (titre 1/32) scored negative. The expected switch from P-ANCA to C-ANCA on formalin-fixed neutrophils, however, was observed in only 25/40 samples (62.5%); this was not related with the titres measured by historically performed routine ANCA IIF (Figures 1(d) and 2(d)). The samples that did not show a C-ANCA pattern on formalin-fixed slides ($n = 14$) were predominantly MPO-ANCA-negative (64.3%), while the majority of C-ANCA-positive samples ($n = 26$) were MPO-ANCA-positive (76.9%) as detected by an antigen-specific assay.

3.2. ANCA Pattern Recognition by AKLIDES in Sera of Patients with AAV. The AKLIDES system scored a C-ANCA on ethanol-fixed neutrophils in 29/39 samples (74.4%). Negative results were restricted to the samples with low/median titres (1/32–1/128). The positive samples in these titre categories revealed only a weak intensity (Figure 1(a)). Only one apparent mismatch (P-ANCA) was observed in a sample with a high titre (>1/1024); this sample was visually also scored in the wrong pattern, that is, atypical ANCA (Figure 2(e)). The C-ANCA was better recognized by the AKLIDES system on the formalin-fixed slides: 37/39 samples (94.9%) were positive (Figure 1(c)). Again, weak-positive results were limited to samples with low/median titres.

A P-ANCA on ethanol-fixed neutrophils was scored by the AKLIDES system in 32/40 samples (80.0%); four samples were scored as undetermined and one sample (titre 1/512) was erroneously scored C-ANCA (Figures 1(b) and 2(g)). The three negative samples had low titres (1/32-1/64) on ethanol-fixed neutrophils. As compared to the visual scoring, the expected P-ANCA to C-ANCA switch on formalin-fixed neutrophils was not very well recognized by the AKLIDES system (Figure 1(d)). Only 13/40 samples (32.5%) revealed a P-ANCA to C-ANCA switch. All other samples were negative.

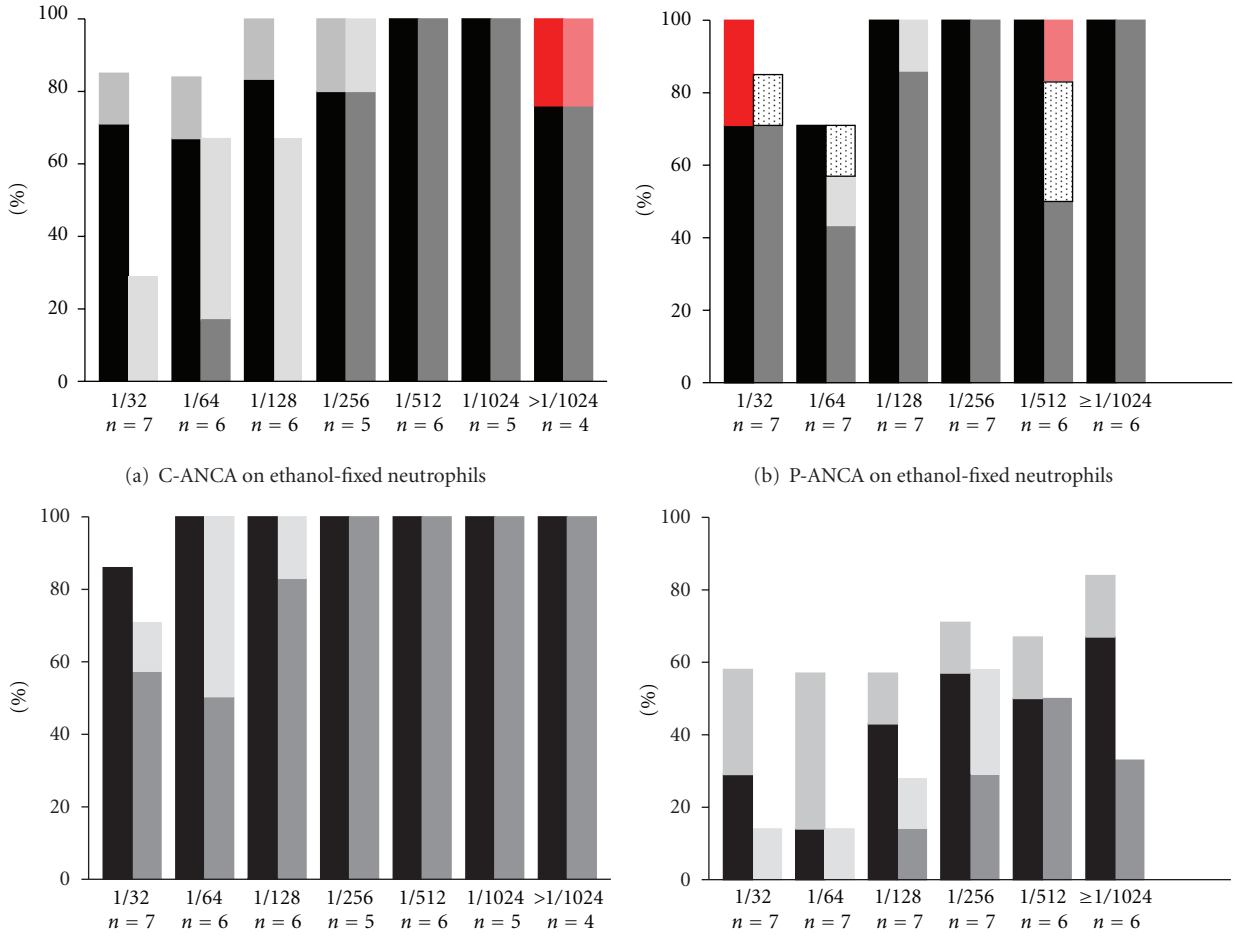
The concordance in pattern recognition on ethanol-fixed neutrophils between the AKLIDES system and the visual scoring was 79.7%. The discordant results were mainly due to the lower sensitivity of the AKLIDES system for the low titre C-ANCA samples ($n = 7$; Figure 1(a)) and the P-ANCA samples that were scored as undetermined ($n = 4$; Figure 1(b)). As mentioned, two apparent pattern mismatches were reported by the AKLIDES system (Figures 1(a), 1(b), 2(e), and 2(g).

3.3. ANCA Pattern Recognition in Sera of Healthy Controls. Visual scoring of the slides with ethanol-fixed neutrophils revealed negative results in 35/40 samples (87.5%). A P-ANCA ($n = 1$) and weak C-ANCA pattern ($n = 4$) was recognized in the positive samples. The AKLIDES system revealed negative results in 38/40 samples (95%). One sample scored as weak C-ANCA and another sample as undetermined. The concordance was 85%. All samples were reported negative on the formalin-fixed neutrophils by both the visual scoring and the AKLIDES system.

3.4. Interference of ANCA Pattern Recognition in Sera Containing ANA or AMA. ANA, and to a lesser extent AMA, are known to hamper ANCA pattern recognition on ethanol-fixed neutrophils. In particular, homogenous ANA may obscure the P-ANCA pattern. As summarized in Table 1, low-titre homogenous ANA could visually be recognized as such, but high-titre homogenous ANA may appear as P-ANCA when analysed in a low serum dilution. The AKLIDES system was not able to discriminate between a nuclear staining pattern and a P-ANCA pattern, independent of the ANA titre (Figure 2(i)). This resulted in an overall concordance of only 40%. Importantly, the majority of these samples were negative on formalin-fixed neutrophils (Figure 2(j)). Only 1 and 2 sample(s) gave a C-ANCA in the visual score and the AKLIDES system, respectively, (data not shown). Both samples had a 1/1280 homogenous ANA that was reported as P-ANCA by both the visual score and the AKLIDES system.

As can be concluded from Table 1, also samples with most other ANA patterns or AMA hampered ANCA pattern recognition on ethanol-fixed neutrophils, either visually (76.9%) or by the AKLIDES system (65.4%). Overall concordance in these samples was 57.7%. On formalin-fixed neutrophils the visual score revealed only a single C-ANCA in a sample with a 1/1280 speckled ANA, while the AKLIDES system reported a C-ANCA in 3 samples with AMA; two of these were also reported as P-ANCA on ethanol-fixed neutrophils. All other samples were negative on formalin-fixed neutrophils (data not shown).

3.5. ANCA Pattern Recognition in Sera Containing BPI-ANCA or Atypical ANCA. Sera with BPI-ANCA consistently revealed a C-ANCA in the visual score on ethanol-fixed neutrophils (Table 2 and Figure 2(k)); two of these samples also were weakly C-ANCA-positive on formalin-fixed neutrophils. Interestingly, the AKLIDES system reported negative results ($n = 6$) or ANA-dots ($n = 3$), but not C-ANCA on ethanol-fixed neutrophils (Table 2); again, two samples were weakly C-ANCA-positive on formalin-fixed neutrophils.



(a) C-ANCA on ethanol-fixed neutrophils

(b) P-ANCA on ethanol-fixed neutrophils

(c) C-ANCA on formalin-fixed neutrophils

(d) P-ANCA on formalin-fixed neutrophils

Figure 1: Pattern recognition by AKLIDES in sera of ANCA-associated vasculitis patients with C-ANCA/PR3-ANCA ($n = 39$; (a) and (c)) and P-ANCA/MPO-ANCA ($n = 40$; (b) and (d)) on ethanol-fixed neutrophils ((a) and (b)) and formalin-fixed neutrophils ((c) and (d)). Notably, P-ANCA samples are expected to reveal a C-ANCA pattern on formalin-fixed neutrophils (d). ANCA patterns were scored by routine fluorescence microscopy (black bars represent unequivocal, correct pattern recognition; intermediate grey bars on top of the black bars represent weak, but correct pattern recognition) and by AKLIDES (dark grey bars represent unequivocal, correct pattern recognition; light grey bars on top of the dark grey bars represent weak, but correct pattern recognition). Red and pink bars represent erroneous pattern recognition by routine fluorescence microscopy and AKLIDES, respectively, dotted bars indicate that the pattern was undetermined by AKLIDES. Data are expressed as percentage for each titre category; titres were historically measured during routine diagnostics.

Table 1: Interference of ANCA pattern recognition in sera containing ANA or AMA.

	Visual score	AKLIDES	Concordance
ANA homogenous			
1/80 ($n = 6$)	3 negative, 3 nuclear	3 negative, 3 P-ANCA	3/6 (50.0%)
1/320 ($n = 7$)	2 negative, 5 nuclear	2 negative, 5 P-ANCA	2/7 (28.6%)
1/1280 ($n = 7$)	4 nuclear, 3 P-ANCA	7 P-ANCA	3/7 (42.9%)
ANA speckled ($n = 5$)	2 negative, 2 nuclear, 1 atypic	2 negative, 2 P-ANCA, 1 ANA-dots	3/5 (60.0%)
Atypical speckled ($n = 4$)	1 negative, 3 P-ANCA	1 negative, 2 P-ANCA, 1 undetermined	3/4 (75.0%)
Centromere ($n = 4$)	3 nuclear, 1 atypic	4 ANA-dots	3/4 (75.0%)
Nucleolar ($n = 2$)	2 negative	2 negative	2/2 (100%)
AMA ($n = 11$)	1 negative, 2 P-ANCA, 2 C-ANCA, 4 atypic, 2 nuclear	4 negative, 3 P-ANCA, 1 C-ANCA, 3 ANA-dots	4/11 (36.4%)

AMA: antimitochondrial antibodies; ANA: antinuclear antibodies; ANCA: antineutrophil cytoplasmic antibodies.
NB: a nuclear pattern in the visual score is considered concordant with ANA-dots by the AKLIDES system.

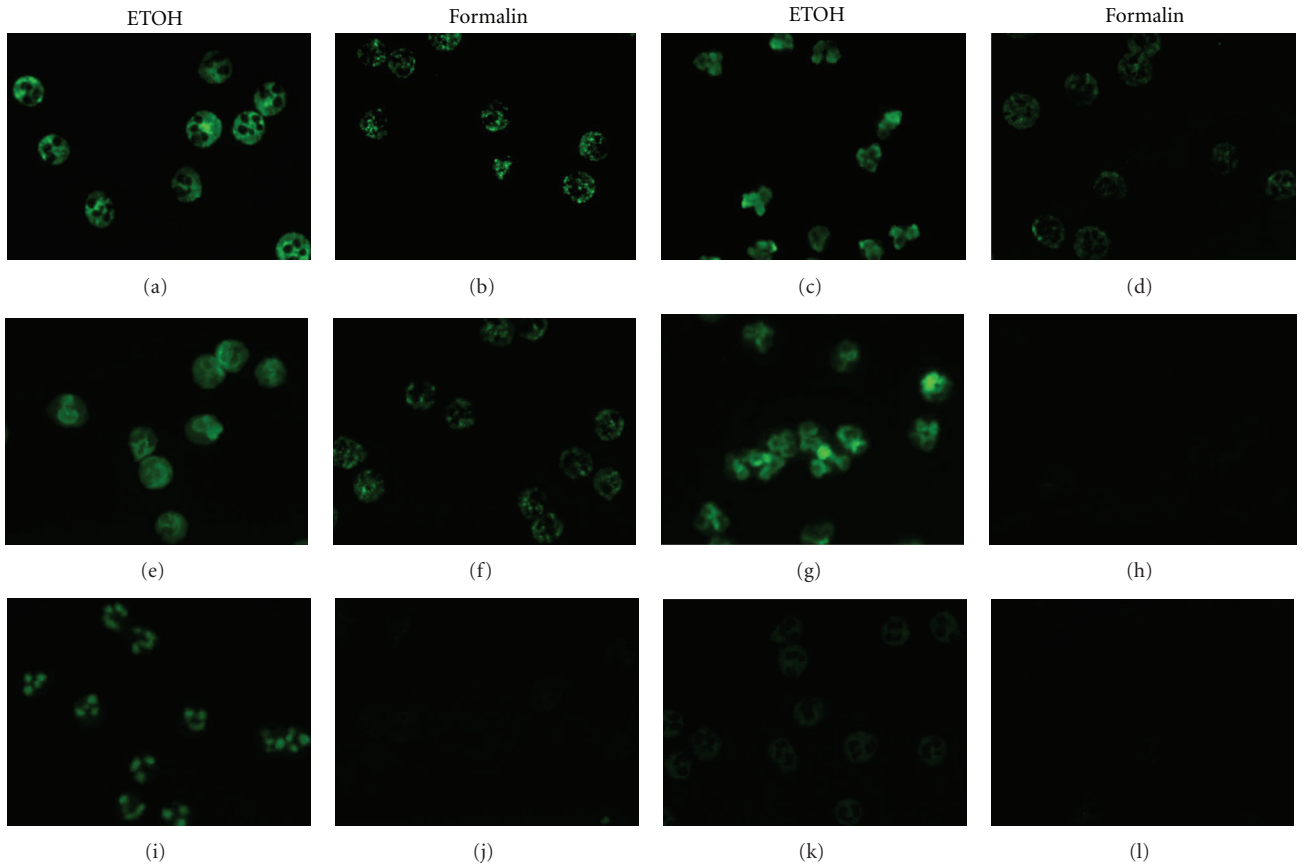


Figure 2: Images acquired by AKLIDES: typical C-ANCA on ethanol-fixed neutrophils (a) and formalin-fixed neutrophils (b); typical P-ANCA on ethanol-fixed neutrophils (c) and switch to C-ANCA on formalin-fixed neutrophils (d); expected C-ANCA revealing an atypical pattern by visual score on ethanol-fixed neutrophils and erroneously scored P-ANCA by AKLIDES (e), but consistent C-ANCA on formalin-fixed neutrophils (f); P-ANCA on ethanol-fixed neutrophils erroneously scored C-ANCA by AKLIDES (g) and consistent negative on formalin-fixed neutrophils (h); intermediate positive (1/320) homogenous ANA reported as P-ANCA on ethanol-fixed neutrophils by AKLIDES (i), being consistently negative on formalin-fixed neutrophils (j); BPI-ANCA revealing a weak C-ANCA pattern by visual score on ethanol-fixed neutrophils (k), scored negative by AKLIDES on both ethanol- and formalin-fixed neutrophils (l).

An atypical ANCA, as recognized in 22 samples by IIF on INOVA slides, was visually only found in 5 samples on Medipan slides containing ethanol-fixed neutrophils. The other samples were negative ($n = 8$) or revealed a C-ANCA ($n = 9$). Two samples with a C-ANCA also were weakly C-ANCA positive on the formalin-fixed neutrophils (data not shown). The AKLIDES system does not report atypical ANCA. Interestingly, the majority of these samples were scored negative ($n = 18$), while 4 samples were scored as P-ANCA ($n = 1$), C-ANCA ($n = 2$), or as undetermined ($n = 1$) (Table 2). All samples were reported negative by the AKLIDES system on formalin-fixed neutrophils (data not shown).

4. Discussion

In this study, we have evaluated the first automated system for ANCA pattern recognition on slides with ethanol- and formalin-fixed neutrophils. The data obtained in PR3-ANCA sera of AAV patients reveal that the AKLIDES system lacks sufficient sensitivity to ethanol-fixed neutrophils, but these sera are very well recognized as C-ANCA on formalin-fixed neutrophils. MPO-ANCA sera of AAV patients, however, are best recognized as P-ANCA on ethanol-fixed neutrophils, while they are poorly recognized as C-ANCA on formalin-fixed neutrophils. In control sera the most apparent interference was observed in sera with a homogenous ANA. In contrast to visual scoring, the AKLIDES system could not differentiate between P-ANCA and a nuclear pattern in most of these samples. On the other hand, the AKLIDES system can discriminate C-ANCA due to reactivity to PR3 (C-ANCA) or to BPI (negative), while this appeared visually impossible.

In the current study sample selection and interpretation of results were based on historically obtained data in routine diagnostics. This approach has two caveats. First, ANCA detection was historically performed on ethanol-fixed neutrophil slides of INOVA, while the AKLIDES system is restricted to Medipan slides. Differences between the

Table 2: ANCA pattern recognition in sera containing BPI-ANCA or atypical ANCA.

	Visual score	AKLIDES	Concordance
BPI-ANCA ($n = 9$)	9 C-ANCA	6 negative, 3 ANA-dots	0/9 (0.0%)
Atypical ANCA ($n = 22$)	8 negative, 9 C-ANCA, 5 atypical ANCA	18 negative, 2 C-ANCA, 1 P-ANCA, 1 undetermined	10/22 (45.5%)

ANA: antinuclear antibodies; ANCA: antineutrophil cytoplasmic antibodies; BPI: bactericidal permeability-increasing protein.

historically obtained routine results and the current visual score, therefore, can be attributed to the use of distinct substrates. The reduced sensitivity (~95%) of the Medipan slides for both C- and P-ANCA may, however, also be due to quenching of the fluorescent signal because the slides were first analysed by the AKLIDES system and next, visually by routine fluorescence microscopy. Second, selection of P-ANCA samples was based on MPO-reactivity at the time of diagnosis. The samples included, however, were P-ANCA-positive, but not necessarily MPO-ANCA-positive. This seems to be the explanation for many samples not giving a C-ANCA pattern on formalin-fixed neutrophils. The observed P-ANCA is most likely due to antibody reactivity to other minor granular components of neutrophils [10].

According to the international consensus on ANCA detection for AAV, the first step is to be performed by IIF [5]. As such, the IIF is primarily performed as a screening assay to select samples that require reflex testing for MPO- and PR3-ANCA. For this purpose, the sensitivity should be high, while the immunofluorescence pattern is irrelevant. Our current study shows that when positivity is defined as any reactivity on ethanol-fixed slides and/or formalin-fixed slides, the sensitivity for AAV of the AKLIDES system is 94%, while the sensitivity of the visual scoring is 96%. It should be stressed, however, that the samples included were not diagnostic samples and were not randomly selected. Therefore, this study is not appropriate to calculate either sensitivity or specificity for AAV, but at least reveals that the technical sensitivity of automatic reading equals visual scoring. A small study recently published revealed an overall agreement in positive and negative results between visual expert reading and automated AKLIDES interpretation of 87% [11]. Unfortunately, this study lacked any clinical information on the 46 samples included.

Like in ANA detection [12], the immunofluorescence pattern of ANCA has predictive value for the antigen specificity of the ANCA [13]. The international consensus prescribes that, whatever the ANCA pattern, testing for both MPO- and PR3-ANCA should be performed [5]. However, the specific combination of a C-ANCA/PR3-ANCA or P-ANCA/MPO-ANCA increases the clinical utility of ANCA diagnostics [6]. Therefore, correct pattern recognition is important and for this the reactivity on ethanol-fixed slides is leading. In this respect, it can be concluded from our study that the automatic recognition of the C-ANCA pattern on ethanol-fixed slides lacks sufficient sensitivity. This is accompanied by the possibility to discriminate the IIF pattern of BPI-ANCA from PR3-ANCA by the AKLIDES. For ANCA diagnostics, however, the first issue is more important than the latter. In a pilot study, recognition of the C-ANCA pattern on ethanol-fixed slides could be increased from 29/39 samples (74.4%) to 35/39 samples (89.7%). At the cost of the distinction between BPI- and PR3-ANCA, this software modification did not influence the reactivity in the healthy control cohort or the P-ANCA reactivity in the respective AAV cohort (data not shown). Apparently, changes in the software of the AKLIDES system enable to improve correct pattern recognition.

According to the international consensus, titration of ANCA IIF is optional [5]. Titration is recommended if serum samples are positive by IIF but negative for MPO- or PR3-ANCA, for followup of such patients, and to distinguish ANCA from interfering ANA. The latter might be the cause of the many P-ANCA reports by AKLIDES in samples with a homogeneous ANA. Titration of the samples might have enabled a better distinction of the nuclear staining pattern. The desire to quantitate the ANCA by titration can possibly be replaced by measuring fluorescence intensity. It has been reported that the higher the ANCA titre as measured by IIF, the higher the likelihood for having AAV [14]. It remains to be determined whether this also holds for fluorescence intensity as determined by the AKLIDES system. The clinical utility of ANCA quantification during followup is still a matter of discussion [1, 15, 16]. In the early days of image analysis, this technique performed slightly better in predicting relapses than routine IIF [17]. Data obtained by the novel systems are lacking.

5. Conclusion

Altogether it can be concluded that automatic reading of IIF by pattern recognition software has paved the way for a new discussion on the role of IIF in autoimmune diagnostics. The argument that IIF is hampered by subjective interpretation and poor interlaboratory reproducibility seems to be outdated. Although our data reveal that ANCA pattern recognition requires further improvements, the current achievements in combination with the possibility to adapt the software are very promising for the near future.

Acknowledgments

The authors thank the companies Medipan (Berlin, Germany) and Mediphos (Renkum, The Netherlands), representing Medipan in The Netherlands, for facilitating this study by providing the reagents and access to the AKLIDES system in their laboratory. They declare that the study was performed independently and that both companies were not involved in the design of the study or the interpretation of the results obtained and the conclusions drawn. Also,

the authors did not obtain financial support from these companies, besides the issues mentioned above, for this study or any other study. Therefore, they declare that there is no conflict of interests. They also thank Dr. C. Nieuwhof and Dr. P. van Paassen, clinical immunologists within the Maastricht University Medical Center, for state-of-the-art diagnosis of vasculitis patients.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 487521, 7 pages
doi:10.1155/2012/487521

Clinical Study

TRAF1 Gene Polymorphism Correlates with the Titre of Gp210 Antibody in Patients with Primary Biliary Cirrhosis

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Received 28 August 2012; Accepted 22 September 2012

Academic Editor: Pietro Invernizzi

Background. Polymorphisms of *TRAF1* (Tumor necrosis factor receptor-associated factor 1) are associated with rheumatoid arthritis (RA). Whether *TRAF1* polymorphisms confer increased risk for primary biliary cirrhosis (PBC), an autoimmune liver disease which can co-exist with RA, is unknown. *Aim of the Study*. To assess the frequency of the RA-conferring susceptibility *TRAF1* polymorphisms rs3761847 and rs2900180 in a cohort of PBC patients. The association of *TRAF1* polymorphisms with clinical features and autoantibody markers was also analyzed. *Methods*. We studied 179 PBC patients and 300 controls. Samples were genotyped for *TRAF1* gene polymorphisms by real-time PCR. Autoantibodies were tested by ELISA. *Results*. The frequency of rs3761847 and rs2900180 polymorphisms did not differ between patients and controls. Laboratory or clinical features were not associated with specific polymorphisms. Gp210 autoantibody titres were conspicuously higher among GG homozygotes of rs3761847 as compared with AA homozygotes ($P = 0.02$). In contrast, antichromatin titers were higher in AA compared to GG rs3761847 homozygotes ($P = 0.04$). Rheumatoid factor IgG titres were significantly higher in rs2900180 TT homozygotes than CC homozygotes ($P = 0.02$). *Conclusions*. *TRAF1* polymorphisms occur with the similar frequency in PBC patients and in the general population, but their presence is probably involved in the regulation of specific PBC-related autoantibodies.

1. Background

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease leading to cirrhosis and eventually liver failure and predominantly affects middle-aged women [1, 2]. Highly-specific antimitochondrial antibodies (AMA), as well as PBC-specific antinuclear antibodies such as those against sp100 and gp210, are serological hallmarks of the disease [3–10]. Significant proportion of patients with PBC suffer from concomitant autoimmune conditions such as Sjögren's syndrome, Hashimoto's thyroiditis, or systemic sclerosis (SS) [2, 11–15]. Several patients with PBC are found positive for IgG rheumatoid factor (RF), and up to 5% of patients with PBC may also have rheumatoid arthritis (RA) [16–18].

Although the etiology of PBC remains elusive, genetic factors are known to contribute to its pathogenesis [19–21]. Several studies in the past postulated major histocompatibility complex (MHC) class II polymorphisms as expressing the strongest association, but consensus has been reached mainly for genes exerting a reduced risk of developing PBC [22]. More recently, four genome wide association studies (GWAS) which included North American, Italian, predominantly North American, and British cohorts, respectively, identified a number of genes in non-HLA loci associated with PBC and highlighted the role of the IL-12 and tumor necrosis factor

(TNF) signaling pathways in the pathogenesis of PBC [23–26].

Polymorphisms of genes involved in TNF-receptor signaling such as TNF receptor-associated factor 1 (*TRAF1*) have been associated with RA [27] and other autoimmune conditions including systemic lupus erythematosus (SLE) [28], but not with giant cell arteritis [29]. *TRAF1* gene encodes an important protein which acts as a mediator of the TNF and CD40 transduction pathways [30, 31]. The presence of *TRAF1* polymorphisms seems to affect the natural history of RA, increasing the risk of erosions [32]. Their effect on mortality in RA remains controversial [33, 34].

The association of *TRAF1* polymorphisms with the risk of developing PBC has not been studied. In the present study, we assessed the prevalence of the *TRAF1* polymorphisms rs3761847 and rs2900180, both identified by a large GWAS as genetic risk factors for RA [27], in a homogenous cohort of Caucasian patients with PBC. As *TRAF1* is a potent immune modulator, we postulated that the presence of *TRAF1* polymorphisms may predispose to a distinctive autoantibody profile and performed a comprehensive analysis of PBC-specific and nonspecific autoantibodies detected in our patients with PBC with the presence of *TRAF1* polymorphisms.

2. Methods

2.1. Patients. A group of 179 patients with PBC were analyzed. All patients met the criteria for the diagnosis of PBC recently introduced by EASL guidelines, according to which PBC can be diagnosed if at least 2 out of the following 3 criteria are fulfilled: elevation of alkaline phosphatase, typical liver histology, and AMA seropositivity [35]. In 132 (74%) patients, the diagnosis of PBC was confirmed by a liver biopsy, and in 46 (35%) of these patients histological assessment showed liver cirrhosis. Patients with clear clinical and imaging features of liver cirrhosis were not subjected to liver biopsy on ethical grounds. In total, 61 (34%) patients had histological, clinical, and imaging features typical for liver cirrhosis. Demographic and laboratory data on analyzed subjects are summarized in Table 1. A cohort of 300 blood donors from the National Blood Services comprised a control group. Appropriate informed consent was obtained from each patient and blood donors included in the study. The study protocol was approved by the ethics committee of Pomeranian Medical University and conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008).

Table 1: Main demographic and laboratory data of 179 patients with primary biliary cirrhosis.

Feature	PBC ($n = 179$)
Age (median; range)	56 (22–80)
Gender (M/F)	21/158
Biopsy confirmed cirrhosis (Y/N)	46/86
AMA (pos/neg)	155/24
ALT (median; range), IU/L (N: 3–30)	84 (10–727)
ALP (median; range), IU/L (N: 40–120)	323 (37–2264)
GGT (median; range), IU/L (N: 3–30)	302 (11–2608)
Bilirubin (median; range), mg/dL (N: 0.2–1.0)	3.0 (0.2–40.5)
Albumin (median; range), g/dL (N: 3.8–4.4)	3.6 (2.1–5.2)
INR (median; range) (N: 0.8–1.2)	1.0 (0.8–2.3)
Cholesterol (median; range), mg/dL ($N < 200$)	238 (50–709)
Triglycerides (median; range), mg/dL ($N < 150$)	124 (42–334)

AMA: antimitochondrial antibody; ALT: alanine aminotransferase; ALP: alkaline phosphatase; GGT: gamma-glutamyl transpeptidase; INR: international normalized ratio.

3. Analysis of Autoantibodies

Autoantibody tests were performed at one site (INOVA Diagnostics, San Diego, CA, USA) on blinded serum specimens. A total of twelve autoantibody specificities (M2 EP (MIT3) IgG, gp210 IgG, sp100 IgG, chromatin IgG, centromere IgG, f-actin IgG, Scl-70 IgG, Jo-1 IgG, RNA polymerase III IgG, Ro52 IgG, CCP 3.0 IgG, and RF IgG) were analyzed by QUANTA Lite ELISA (INOVA Diagnostics, San Diego, CA, USA) as described elsewhere [4]. All QUANTA Lite ELISA tests were run and interpreted according to the manufacturer's instructions and are cleared for "*in vitro* diagnostic use" by United States Food and Drug Administration (FDA). Detailed performance data and instructions for the QUANTA Lite ELISA tests can be found online (http://www.inovadx.com).

4. *TRAF1* Genotyping

DNA from peripheral blood mononuclear cells was isolated using the DNeasy Blood & Tissue Kit (Qiagen). Oligonucleotide primers and TaqMan probes for two *TRAF1* polymorphisms (rs2900180 and rs3761847) were designed and synthesized by Applied Biosystems (assay ID: C_15849116_10 and C_2783640_10, resp.). The fluorescence data were analyzed with allelic discrimination 7500 software v.2.0.2.

5. Statistical Analysis

Data are shown as mean and standard error. All statistical analyses (chi square, odds ratios, confidence intervals) were performed using StatView software (Carry, NC, USA). The genotype and allelic frequencies were compared between patients and controls using Fisher's PLSD test. The analysis of genotype frequency within PBC patients with regards to the analyzed factors was performed using Fisher's PLSD test. P value < 0.05 was considered to be statistically significant.

6. Results

A summary of the obtained data is provided in Tables 2 and 3. No significant difference in genotype frequencies between patients with PBC and healthy controls was seen. The presence of these polymorphisms did not correlate with clinical features such as gender, age at presentation, pruritus, or cirrhosis at presentation (data not shown). Also they

Table 2: Distribution of *TRAF* polymorphisms (rs3761847 and rs2900180) in patients with primary biliary cirrhosis (PBC) and controls.

SNP	Allele/genotype	PBC (%) ($n = 179$)	Controls (%) ($n = 300$)	χ^2	P (Fisher exact)	OR
rs3761847	*A/G*	210/148	356/244	0.07	0.84	1.04 [0.79–1.35]
	AA	64 (35.8)	99 (33)	0.38	0.55	1.13 [0.77–1.67]
	AG	82 (45.8)	158 (52.7)	2.12	0.16	0.76 [0.52–1.10]
	GG	33 (18.4)	43 (14.3)	1.41	0.25	1.35 [0.82–2.22]
rs2900180	*C/T*	114/244	185/415	0.13	0.72	1.05 [0.79–1.39]
	CC	84 (46.9)	144 (48)	0.052	0.85	0.96 [0.66–1.39]
	CT	76 (42.5)	127 (42.3)	0.001	>0.99	1.02 [0.70–1.49]
	TT	19 (10.6)	29 (9.7)	0.11	0.75	1.11 [0.60–2.04]

Table 3: Laboratory features and *TRAF1* polymorphisms.

(a) rs3761847

Genotype	*AA*	*GG*	*P*
AST (IU/mL)	94.9 ± 18.1	67.5 ± 7.4	0.30
ALT (IU/mL)	91.5 ± 15.7	57.0 ± 5.3	0.16
AP (IU/mL)	342.1 ± 47.6	303.3 ± 36.8	0.58
GGT (IU/mL)	299.5 ± 45.4	367.1 ± 64.9	0.87
Bilirubin (mg/dL)	3.9 ± 0.9	3.1 ± 1.1	0.58
Albumin (g/dL)	3.9 ± 0.1	3.9 ± 0.1	0.83
INR	1.1 ± 0.1	1.1 ± 0.1	0.60
Cholesterol (mg/dL)	237.7 ± 16.9	225.7 ± 15.4	0.67

(b) rs2900180

Genotype	*CC*	*TT*	*P*
AST (IU/mL)	77.9 ± 12.2	71.8 ± 10.7	0.85
ALT (IU/mL)	76.1 ± 9.3	66.2 ± 7.8	0.74
AP (IU/mL)	343.5 ± 38.4	332.1 ± 56.4	0.89
GGT (IU/mL)	314.6 ± 40.3	355.8 ± 109.6	0.67
Bilirubin (mg/dL)	3.5 ± 0.8	3.3 ± 1.7	0.88
Albumin (g/dL)	3.8 ± 0.1	3.7 ± 0.2	0.54
INR	1.1 ± 0.1	1.2 ± 0.1	0.33
Cholesterol (mg/dL)	232.1 ± 13.5	227.6 ± 21.7	0.89

Abbreviations: AST: aspartate aminotransferase; ALT: alanine aminotransferase; AP: alkaline phosphatase; GGT: gamma-glutamyl transpeptidase; INR: international normalized ratio.

did not correlate with liver biochemistry at the diagnosis (Table 3). With regards to the rs3761847 polymorphism, analysis of autoantibodies revealed that the titres of gp210 were significantly higher among GG homozygotes as compared with AA homozygotes (42.6 ± 14.7 versus 10.8 ± 4.2; $P = 0.02$) and at the same time AA homozygoity was associated with higher titres of antichromatin autoantibodies (11.8 ± 2.8 versus 5.2 ± 1.2; AA versus GG $P = 0.04$). The analysis of the second polymorphism (rs 2900180) showed that TT homozygotes demonstrated significantly higher titres of rheumatoid factor IgG than CC homozygotes (16.7 ± 9.7 versus 7.6 ± 0.5, resp., $P = 0.02$). No statistically significant difference was found in terms of other autoantibodies. These data are summarized in Tables 4 and 5.

Table 4: Autoantibody data with regards to rs3761847 *TRAF1* polymorphism.

Autoantibodies	Genotype *AA*	Genotype *GG*	*P*
AMA	99.8 ± 8.0	111.4 ± 10.9	0.38
gp210	10.8 ± 4.2	42.6 ± 14.7	0.02
sp100	31.7 ± 7.8	42.2 ± 13.5	0.42
Actin	23.7 ± 3.3	17.2 ± 2.6	0.20
Centromere	20.7 ± 5.7	14.1 ± 6.1	0.46
Chromatin	11.8 ± 2.8	5.2 ± 1.2	0.04
RFIgG	7.4 ± 0.7	13.8 ± 6.4	0.06
CCP3	16.6 ± 6.2	23.2 ± 11.1	0.45
Scl-70	4.3 ± 0.4	3.5 ± 0.3	0.25
Jo-1	4.7 ± 0.9	3.6 ± 0.5	0.81
RNA-POLIII	6.7 ± 1.2	4.9 ± 2.3	0.43
Ro52	25.7 ± 5.5	33.4 ± 8.3	0.49

AMA: antimitochondrial antibody; RFIgG: rheumatoid factor IgG; RNA-POLIII: RNA polymerase III.

Table 5: Autoantibody titers in patients subgrouped according to rs2900180 *TRAF1* polymorphism.

Autoantibodies	Genotype *CC*	Genotype *TT*	*P*
AMA	97.3 ± 7.1	108.1 ± 13.4	0.48
gp210	18.6 ± 5.7	35.5 ± 18.6	0.29
sp100	33.5 ± 6.7	24.2 ± 10.9	0.53
Actin	20.9 ± 2.5	15.6 ± 2.3	0.36
Centromere	17.4 ± 4.4	13.6 ± 8.4	0.72
Chromatin	0.9 ± 2.2	4.6 ± 1.1	0.18
RFIgG	7.6 ± 0.5	16.7 ± 9.7	0.02
CCP3	14.7 ± 4.8	31.2 ± 16.8	0.09
Scl-70	4.3 ± 0.4	3.4 ± 0.4	0.25
Jo-1	4.9 ± 0.8	3.6 ± 0.7	0.81
RNA-POLIII	6.4 ± 1.0	6.4 ± 3.9	0.97
Ro52	28.0 ± 5.2	42.2 ± 13.2	0.28

7. Discussion

The *TRAF1* gene encodes a TNF receptor-associated factor 1, belonging to the TNF receptor (TNFR) associated factor (TRAF) protein family [36]. These proteins are responsible for mediation of signaling from various receptors of the TNFR superfamily. *TRAF1* together with *TRAF2* form

a heterodimeric protein complex which is required for TNF-alpha-stimulated activation of MAPK8/JNK and NF-kappaB [37, 38]. This complex also interacts with proteins responsible for inhibition of apoptosis, affecting the antiapoptotic signals from TNFRs [39]. TNFα has been found to play a critical role in the pathogenesis of various autoimmune conditions including RA and PBC [40].

Various polymorphisms of *TRAF1*/C5 have been studied and were found to occur more commonly in patients with RA of different origin [41–44]. In addition to their higher prevalence in patients with RA, *TRAF1*/C5 polymorphisms also seem to affect the natural history of the disease [45–47]. They have also been linked with SLE in some populations [48, 49], juvenile idiopathic arthritis [50] and alopecia areata [51], but not with giant cell arteritis [29] and pemphigus [52].

TRAF1 SNPs have never been investigated in PBC which is a chronic autoimmune liver condition. Since pathways involving TNF and IL-12 have been described [53–55] in the pathogenesis of PBC, seeking a potential relationship between *TRAF1* SNPs and PBC could be of interest. Additionally, PBC is associated with various autoimmune conditions including Sjögren's syndrome and systemic sclerosis, and the mechanisms responsible for this cooccurrence are the focus of ongoing research [56]. RA is found in up to 5% of patients with PBC, but the literature surrounding this association is scarce.

For this study, we selected two polymorphisms originally reported to confer increased risk for RA in GWAS [27]. The frequencies of the SNPs that we studied are comparable to those noted previously in European and other populations (displayed in http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?searchType=&rs=rs3761847 and http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?searchType=adhoc_search&type=rs&rs=rs2900180). They were also not significantly different from the ones seen in patients with PBC, suggesting that the *TRAF1* locus does not confer risk to PBC. This may also explain why RA is not highly prevalent in patients with PBC compared to other extrahepatic autoimmune manifestations such as sicca syndrome and Hashimoto's thyroiditis. These findings support the view that genes other than *TRAF1* which are related to the homeostasis of TNFα could be involved in the pathogenesis of PBC. Indeed, a recent GWAS by Mells et al. reported that 1q31 (*DENND1B*), 14q32 (*TNFAIP2*), and 12p13 (*TNFRSF1A*) confer susceptibility to PBC. These three loci relate to genes involved in TNF signaling pathways. Of those, TNFRSF1A belongs to the TNFR family, which also contains TNFRSF1B. Of interest, TNFRSF1A appears to interact with *TRAF2* but not with *TRAF1* [57–59]. Our analysis has found that the GG homozygotes had significantly higher titres of gp210 autoantibodies compared to AA homozygotes. Antibodies against the nuclear complex gp210 antigen are highly-specific for PBC [6, 9, 60] and their presence is strongly associated with a more rapid progression of PBC and worse outcome [61–64]. The fact that the presence of this polymorphism is not associated with other PBC-specific antinuclear antibodies, such as those against the sp100 nuclear body antigen, further underlines the unique association between *TRAF1* and gp210 autoantibody development. The mechanism that could explain this association needs to be explored. *TRAF1* has an inhibitory role in antigen-induced apoptosis of CD8+ T lymphocytes. It remains to be seen whether such an antiapoptotic role involves gp210-specific autoreactive lymphocytes present in PBC patients [65]. As *TRAF1* is also a negative regulator of TNF-receptor signalling, it may regulate the induction a cytokine milieu that promotes the persistence of gp210-specific autoreactive lymphocytes and the T-cell dependent production of antibodies against this nuclear pore complex protein. These speculations may serve as working hypotheses for future studies. However, our data need to be interpreted with caution, as there is no solid evidence to support the view that anti-gp210 seropositivity *per se* is a negative prognostic factor in patients with PBC. Nevertheless, the idea to correlate polymorphisms of immunoregulatory genes with humoral autoimmunity markers is not new, as previous researchers have attempted the same in diseases such as type 1 diabetes mellitus, systemic lupus erythematosus, and indeed RA [66–68]. In doing that, several studies identified relationships between HLA and non-HLA polymorphisms and the presence or the titres of disease-related autoantibodies. To date, no serious attempts have been made to correlate *TRAF1* polymorphisms and specific autoantibodies in autoimmune diseases such as RA and SLE.

Our study is the first to assess the presence of these polymorphisms with the titres of twelve different autoantibodies, including not only closely-related and clinically significant autoantibody specificities but also others not immediately relevant to the diagnosis or prognosis of PBC. Hence, we showed that antichromatin autoantibodies occurred in significantly higher titres in AA homozygotes compared to GG homozygotes of rs3761847. The significance of antichromatin antibodies has not yet been studied in great detail in patients with PBC. In patients with autoimmune hepatitis, however, the presence of antichromatin antibodies is associated with an active disease and increased risk of relapse after steroid withdrawal [69, 70]. It has been speculated that they may define a subgroup of patients with AIH with worse outcome [71]. We also observed significantly higher titres of RF IgG in TT homozygotes of rs2900180. Again, a direct interpretation of this finding is difficult to be made. Rheumatoid factor is related to more aggressive articular destruction in patients with RA. RF-IgG can occur in 16–70% of patients with PBC, but its relevance in the natural history of this condition has not been studied. Further studies should define the association of these autoantibodies and *TRAF1* polymorphisms in patients with PBC. Our study has moved this process one step forward.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 640835, 8 pages
doi:10.1155/2012/640835

Clinical Study

Ileal Inflammation May Trigger the Development of GP2-Specific Pancreatic Autoantibodies in Patients with Crohn's Disease

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Received 22 June 2012; Accepted 7 September 2012

Academic Editor: Xavier Bossuyt

Why zymogen glycoprotein 2 (GP2), the Crohn's disease (CD)-specific pancreatic autoantigen, is the major target of humoral autoimmunity in inflammatory bowel diseases (IBD) is uknown. Recent evidence demonstrates that GP2 is also present on the apical surface of microfold (M) intestinal cells. As the colon lacks GP2-rich M cells, we assumed that patients with colonic CD are seronegative for anti-GP2. Anti-GP2 antibodies were tested in 225 CDs, including 45 patients with colonic location (L2), 45 with terminal ileum (L1) and 135 with ileocolonic involvement; 225 patients with ulcerative colitis (UC) were also tested. Anti-GP2 reactivity was detected in 59 (26.2%) CDs and 15 (6.7%) UCs ($P < 0.001$). Only 5 CDs with L2 had anti-GP2 antibodies, compared to 54/180 (30.0%, $P = 0.0128$) of the CDs with L1 and L3. Anti-GP2 antibody positive CD patients had higher ASCA titres compared to seronegative cases. Amongst the 128 CD patients with previous surgical intervention, 45 (35.0%) were anti-GP2 antibody positive compared to 14/97 (14.0%) without surgical ($P < 0.001$). Our data support the assumption that ileal inflammation is required for the development of anti-GP2 antibodies in CD, and suggest that the intestine rather than the pancreatic juice is the antigenic source required for the initiation of anti-GP2 antibodies.

1. Introduction

Pancreatic autoantibodies (PAB) detected by indirect immunofluorescence (IIF) are specific markers of Crohn's disease (CD), being present in approximately 27–39% of patients with this condition, but in fewer than 8% of patients with ulcerative colitis (UC) or other disorders unrelated to inflammatory bowel diseases (IBD) [1–7]. The major target antigen of PAB has recently been elucidated as a pancreatic glycosyl phosphoinositol (GPI) membrane-anchored protein, also known as zymogen glycoprotein 2 (GP2) [8].

It was previously believed that GP2 was exclusively expressed by pancreatic acinar cells [9, 10], but recent studies have clearly demonstrated that GP2 is also located in the microfold (M) cells of the follicle-associated epithelium (FAE) of intestinal Peyer's patches [11]. Thus, it appears that GP2 is located in the intestine, as well as the exocrine

pancreas, and this may explain its intriguing autoantigenicity in patients with CD [9–13].

Direct proof of the relationship between the autoantigenicity of GP2 and its peculiar location on the apical surface of the GP2-rich intestinal M cells has not yet been obtained [12]. PCR analysis of colonic biopsy material of anti-GP2 antibody positive patients with CD suggested that there is a CD-specific overexpression of GP2 in this disease [8], but the data are scarce and far from conclusive [12].

While M cells are found in abundance in the small intestine and in particular in the ileum, they are hardly detectable in the large intestine [14]. We assumed that the production of GP2 autoantibodies is triggered during ileal inflammation and that high expression of GP2 by M cells in the inflamed ileal environment is important for the release of this antigen and its continual exposure to the immune system [12]. If this holds true, it would be expected that patients with exclusively colonic CD would lack anti-GP2 antibodies as compared to patients with ileal or ileocolonic inflammation. Such information would also provide clues as to whether GP2 autoantibodies participate in the immunopathogenicity of CD or are just epiphenomena, secondary to ileal inflammation.

2. Patients and Methods

2.1. Patients. Serum samples of 450 patients from a cohort of 854 follow-up IBD patients seen in the outpatient clinics of one of the authors (A. Forbes) who runs a tertiary referral service in the UK (currently at University College Hospital, London) were tested.

The study population included 225 patients with CD (men/women: 98/127, 36.0 ± 14.3 years; disease duration 13.0 ± 10.1 years) and 225 UC patients (male/female: 113/112; age median: 51.0 ± 15.7; disease duration median: 14.0 ± 12.9, Table 1).

The diagnoses of CD and UC were based on current standard clinical, radiological, endoscopic, and histological criteria (Lennard-Jones criteria) [15]. The disease phenotype was determined based on the Montreal classification [16].

Disease location was the criterion for the selection of CD patients. All the patients with ileal (L1 = 45) and colonic (L2 = 45) involvement were included. A proportionally larger group of patients with extensive disease (ileal and colonic involvement, L3 = 135) was selected reflecting the higher prevalence in the original population. An equal number of patients with UC were randomly selected.

Follow-up samples were taken from 40 opportunistically selected patients (CD: 20, UC: 20) at various time points (median CD follow up of 3.0 ± 1.3 years; median UC follow up: 3.0 ± 1.0 years).

In addition, 75 serum samples from 50 healthy blood donors and 25 patients with irritable bowel syndrome have been included as normal and pathological controls, respectively. Laboratory, histological and clinical data recorded in an electronic database were used to analyse patients stratified into groups according to the presence or not of anti-GP2 antibodies.

The study was conducted in accordance with the Helsinki declaration and approved by the local ethics committees. Written informed consent was obtained from each individual. All sera had been stored at −20°C before analysis.

2.1.1. Detection of Anti-GP2 Antibodies by ELISA. IgG anti-GP2 antibodies were tested in serum samples of patients with IBD by a commercial ELISA (Generic Assays, Dahlewitz/Berlin, Germany) [17], according to the manufacturer's instructions. The assay is based on recombinant human GP2 expressed in *Spodoptera frugiperda* 9 cells as solid-phase antigen, as described previously [18]. Briefly, the plasmid pcDNA3.1 + GP2-trunc-Thrombin-His was used which codes the amino acid sequence of GP2 isoform BAA88166 (pancreatic GP2 alpha form) corresponding to the formal isoform 2 (NP_001493) missing the last 8 amino acids at the N-terminal end [19]. The cutoff for positivity was set to 20 AU/mL, as recommended by the manufacturer. The anti-GP2 IgG ELISA displayed an intra-assay variability of 4.3% and an interassay variability of 5.6% for samples giving 29 AU/mL and 27 AU/mL, respectively.

2.1.2. Detection of Antibodies to Saccharomyces cerevisiae (ASCA) by ELISA. In view of the high specificity of ASCA antibodies for CD and the frequent cooccurrence with pancreatic autoantibodies described in previous studies [8, 20], patients' serum samples were also tested for ASCA antibody reactivity. A commercially available ELISA (INOVA Diagnostics) kindly provided by Dr. Gary L. Norman was used for the quantitative determination of IgA and IgG ASCA antibodies, following the manufacturer's protocol. A cutoff for positivity was set to 25 AU/mL, as recommended by the manufacturer. The intra-assay coefficient of variation was 3.7% for a sample containing 65 AU/mL of ASCA IgA and 4.5% for a sample containing 45 AU/mL of ASCA IgG. The inter-assay coefficient of variation was 4.5% for a sample containing 52 AU/mL and 2.5% for a sample containing 52 AU/mL of ASCA IgA and ASCA IgG, respectively.

2.2. Statistics. All statistical tests were performed using the SPSS 15.0 statistical software package (SPSS Inc., Chicago, Illinois, USA). Prizm software (by GraphPad Software Inc., La Jolla, California, USA) was used for drawing the presented figures. An assumption of nonparametric variables was made and the comparisons were performed with Mann-Whitney, Fisher exact, and chi-square tests as appropriate. Wherever required a non-parametric Spearman correlation was performed. Results are presented as percentages and medians with standard deviation error and odd ratios with 95% confidence intervals (CI). All P values reported are for two-tailed analysis.

3. Results

3.1. IgG Anti-GP2 Antibodies in CD, UC, and Non-IBD Controls. IgG anti-GP2 reactivity was detected in 59 (26.2%) patients with CD and 15 (6.7%) patients with UC ($\chi^2 = 31.3$, df = 1, $P < 0.000$, odds ratio: 4.98, 95% CI 2.73 to 9.08).

Table 1: Main demographic and clinical characteristics of the 225 patients with Crohn's disease (CD) and the 225 patients with ulcerative colitis (UC) included in the present study.

	CD	UC
N	225	225
Sex (m/f)	98 (43.6%)/127 (56.4%)	113 (50.2%)/112 (49.8%)
Age (mean ± SD)	36 ± 14.3	51 ± 15.7
Age at diagnosis (mean ± SD)	23 ± 11.6	30 ± 14.6
Disease duration (mean ± SD)	13 ± 10.1	14 ± 12.9
Location *n* (%)	L1: 45 (20%) L2: 45 (20%) L3: 135 (60%)	E1: 28 (12.4%) E2: 66 (29.3%) E3: 131 (58.2%)
Behaviour *n* (%)	B1: 106 (47%) B2: 62 (28%) B3: 57 (25%) Perianal: 60 (27%)	
Age	A1: 46 (20%) A2: 156 (70%) A3: 23 (10%)	

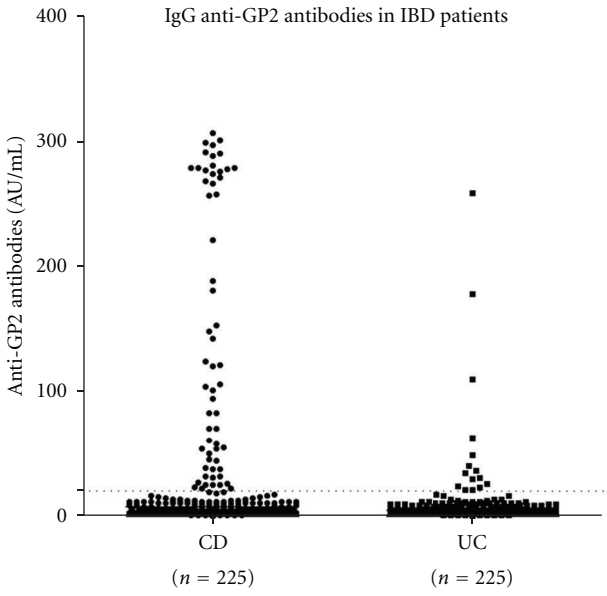

Figure 1: IgG anti-GP2 antibodies in 225 patients with Crohn's disease (CD) and 225 patients with ulcerative colitis (UC). A cutoff of 20 AU/mL established by the manufacturer of the commercial ELISA (Generic Assays) is indicated with a dot line.

The titres were significantly higher in CD in comparison to UC patients (U: 18920, $P < 0.0001$, Figure 1).

IgG antibodies were present in one (1/75, 1.33%; 47 AU/mL) non-IBD control tested (a 37-year-old female suffering from irritable bowel syndrome without family history of IBD).

The sensitivity of IgG anti-GP2 antibodies for IBD versus non-IBD controls was 16%, the specificity 99%, and the likehood ratio 12.33. When comparing CD versus UC then the sensitivity was 26%, the specificity 93%, and the likehood ratio 3.93.

3.2. IgG Anti-GP2 Antibodies in CD Patients according to Disease Location. CD patients with ileal (L1) or extensive disease (L3) presented higher prevalence of anti-GP2 IgG ($P = 0.0128$) with significantly higher titres as shown in Figure 2. Thus, anti-GP2 antibodies were present in 5/45 (11.1%) L2 CD, representing just 8.5% (5/59) of the total anti-GP2 positive CD cohort and 2.2% (5/225) of the total CD population included in the present study. This was statistically less prevalent compared to the 30% (54/180) anti-GP2 seropositivity seen in patients with L1 and L3, who represent the 91.5% of the total anti-GP2 seropositive CD patients and 24% (54/225) of the total CD population.

3.3. IgG Anti-GP2 Antibodies versus ASCA (IgA and IgG). A summary of the results is given as a Venn diagram in Figure 3. Amongst the 225 patients with CD, 141 (62.7%) and 99 (44.0%) had IgG and IgA ASCA, respectively. Overall, 153 (68.0%) CD patients had IgG and/or IgA ASCA compared to 28 (12.4%) UC patients ($P < 0.0001$). Among the ASCA (IgA and/or IgG) positive CD and UC patients, 50 (33%) and 5 (18%) were positive for IgG anti-GP2, respectively. Overall, 57 (40%) of the IgG and 38 (38%) of the IgA ASCA positive patients had anti-GP2 antibody reactivity, respectively. Only 35 (15.6% of the total 225) CD patients had simultaneous reactivity for ASCA (both IgG and IgA) and anti-GP2. These represented 59.3% of the total ($n = 59$) anti-GP2 antibody reactive cases. Among the 62 (28.0%) ASCA seronegative CD patients, 9 (15.0%) were positive for anti-GP2 IgG.

Although, there was no correlation between ASCA (IgA or IgG) and anti-GP2 titres in double positive patients, the titres of ASCA (IgA and IgG) were higher in patients positive for IgG anti-GP2 as shown in Figure 4.

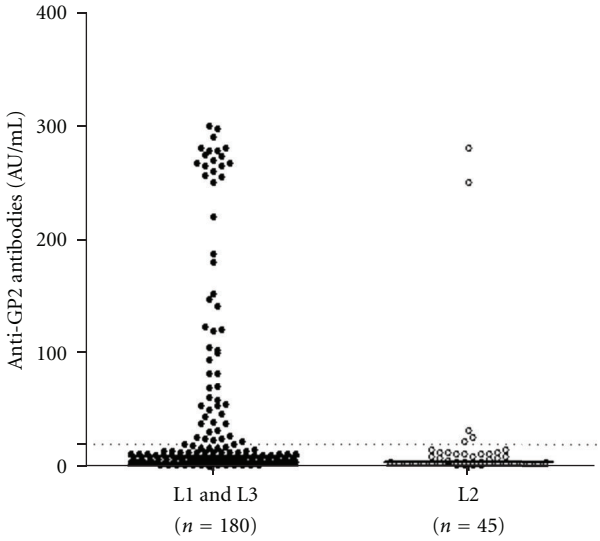

Figure 2: Anti-GP2 antibody titres in 225 patients with Crohn's disease (CD) stratified in two groups: patients with restricted colonic location (L2) and patients with ileal (ileal or ileocolonic) location (L1 and L3), according to Montreal classification.

There was no statistically significant difference in the prevalence of ASCA IgA in patients with different locations (L1+L3: 83/180 versus L2: 18/45, $P = 0.5056$). Patients with colonic disease (L2) though had lower prevalence of ASCA IgG (L1+L3: 123/180 versus L2: 19/45, $P < 0.0009$).

No difference was found in the prevalence of ASCA IgA in patients with different behaviour phenotypes (B1: 42/108, B2: 33/62 and B3: 24/57) but ASCA IgG were more prevalent in patients with stricturing disease (B1: 58/108, B2: 48/62, and B3: 35/57; $P = 0.0131$).

3.4. IgG Anti-GP2 Antibody Association with Other Clinical Parameters. There was no statistically significant difference between anti-GP2 IgG positive and negative CD patients in regards to age of disease onset and duration of disease (Figure 4). There was no correlation between anti-GP2 IgG titres and disease duration. The prevalence of IgG anti-GP2 antibodies differed when CD patients were stratified in subgroups according to their disease behaviour (Figure 5), but there was no difference in the titre medians between subgroups. IgG anti-GP2 antibodies were present in 29/106 (27%) CD patients with B1, 22/62 (35%) with B2, and 8/57 (14%) with B3 phenotype ($P = 0.0046$).

Family history of IBD was not associated with anti-GP2 antibody seropositivity. Among the 57 (25.3%) CD patients with family history of IBD, 13 (22.8%) were positive for anti-GP2 IgG compared to 43 out of 168 (25.6%) without family history of IBD ($P > 0.05$).

Amongst the 128 CD patients with previous surgical intervention, 45 (35.2%) were positive for anti-GP2 IgG compared to only 14/97 (14.4%) CD patients without surgical history ($\chi^2 = 12.25, \text{df} = 1, P < 0.000$, odds ratio: 3.214, 95% CI 1.641 to 6.298).

The prevalence of anti-GP2 antibodies did not differ amongst naive patients or patients at early stages of their disease (duration of disease less than two years) compared to patients with >2 years disease duration (4/22, 18% versus 54/203, 26%, $P > 0.05$). Also, the prevalence of anti-GP2 antibody reactivity did not differ amongst patients treated with or without infliximab (11/38 and 28.9% versus 48/187 and 25.7%; $P > 0.05$).

3.5. Behaviour of Anti-GP2 Antibodies over Time. An additional set of experiments was carried out to test the behaviour of anti-GP2 antibodies over time in 20 CD patients, including 8 (20%) who were anti-GP2 antibody positive at baseline. All 8 anti-GP2 antibody positive patients have shown a decline of their GP2 autoantibody titres at repeated testing. Of those, 5 retained their seropositivity and 3 became seronegative. Of the 12 anti-GP2 antibody negative cases at baseline, 2 became seropositive at relatively low titres during follow up (Figure 6). The single UC patient who was positive on initial sampling remained positive on the second test.

4. Discussion

Pancreatic antibodies directed against GP2 have been considered serological markers of CD, being present in approximately 20–36% of patients with IBD [12, 17–19, 21–23]. Why GP2 becomes an autoantigenic target in CD is unclear [12, 13, 24]. Also, why some but not all patients with CD develop humoral autoreactivity against this pancreatic autoantigen remains elusive [12]. Moreover, it is not clear whether these autoantibodies are secondary to intestinal destruction or participate in the induction of the disease.

In the present study, we assumed that patients showing a disease location restricted to the colon, and therefore without inflammation of the ileum, would lack antibody reactivity to GP2 [12]. We based our hypothesis on recent evidence indicating that there is GP2 expression in the intestine in addition to its former known pancreatic site of synthesis. It seems to be limited to the intestinal M cells [11]—the atypical epithelial cells that account for up to 10% of FAE [14, 25, 26]. The role of M cells is generally to phagocytose macromolecules and microbes and to transport them to the underlying mucosa-associated immune system for antigen presentation. Thus, M cells play a crucial role in maintaining the critical balance in terms of recognising and differentiating self and nonself. Intriguingly, M cells and Peyer's patches are particularly abundant in the distal part of the ileum [11, 27], which has been considered to be the most likely site of inflammation onset in newly diagnosed, adolescent patients with CD and is generally one of the most common sites for clinically apparent disease activity [28]. According to the above argument, intestinal inflammation sparing the ileum would not be able to release GP2 from the inflamed tissue. The release of GP2 would be a prerequisite for the activation of the immune system and the initiation of an autoimmune reaction that could lead to the induction of anti-GP2 antibodies [12].

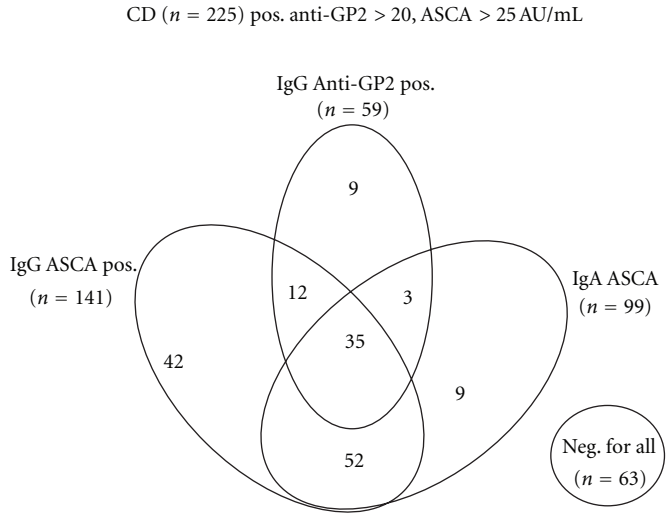

Figure 3: Venn Diagram showing IgA ASCA, IgG ASCA, and IgG anti-GP2 antibody reactivity of the 225 Crohn's disease (CD) patients.

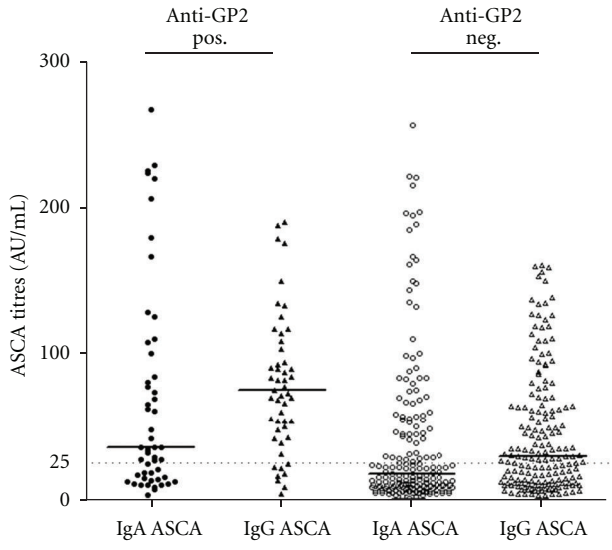

Figure 4: Comparison of IgA and IgG ASCA titres in IgG anti-GP2 antibody positive and negative patients with Crohn's disease (CD). Statistical analysis did not reveal significant differences amongst anti-GP2 antibody positive and anti-GP2 antibody negative CD patients in ASCA titres.

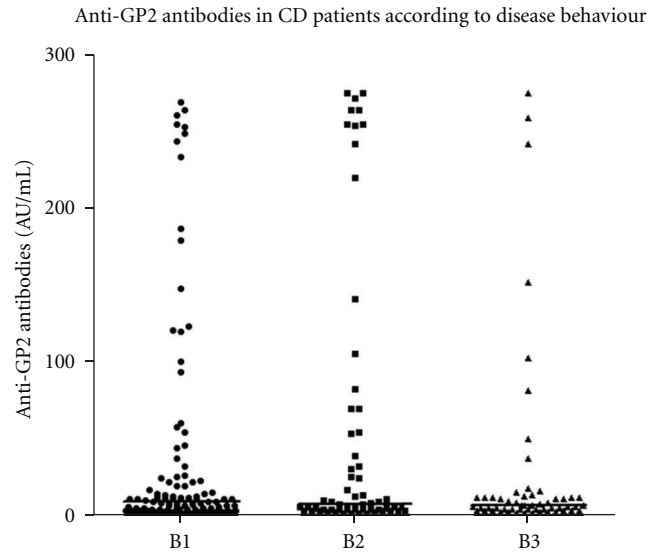

Figure 5: Comparison of IgG anti-GP2 antibodies in patients with Crohn's disease (CD) stratified in accordance to disease behaviour (Montreal Classification, B1, B2, and B3).

Indeed, our study clearly demonstrates that patients with the restricted colonic form of the disease do not show significant antibody reactivity against GP2 compared to those with a disease location that involves the ileum, the site of GP2-rich M cells. The necessity for ileal inflammation in order for anti-GP2 antibodies to be developed can also explain why only a minority of patients with UC have shown autoantibody reactivity against this antigen [12, 17–19, 21–23]. In our study, fewer than 7% of 225 patients with UC have shown anti-GP2 antibodies. This percentage is in agreement with most studies reporting on the prevalence of anti-GP2 antibodies in smaller cohorts of UC patients [8, 17–19].

A previous study reporting coexistence of ASCA and anti-GP2 in a significant proportion of CD patients [17] has been followed by other studies that were unable to replicate this finding [21, 22]. Also, comparison of various demographic and clinical parameters analysed in our cohorts was unable to show significant differences in terms of age of disease onset, as well as disease duration, in accordance with previous studies [21]. A lower prevalence was found in patients with penetrating disease B3 and this needs further investigation. Anti-GP2 antibodies were more prevalent in patients with previous surgical intervention than in those without (35% versus 14%, $P < 0.001$). The clinical relevance of this finding will remain uncertain until it is replicated in larger studies. Most studies conducted so far have been

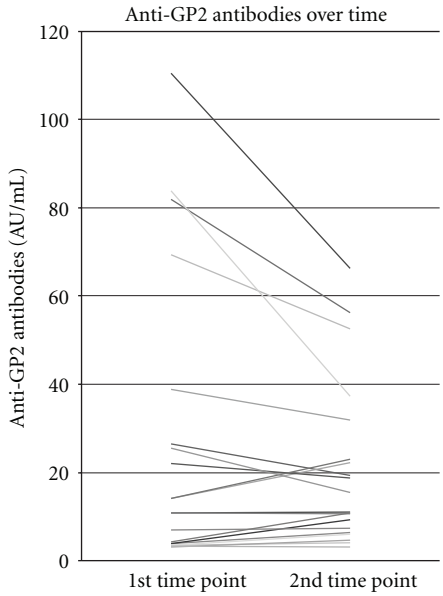

Figure 6: Behaviour of anti-GP2 antibodies during follow up in 20 randomly selected patients with Crohn's disease (CD), including 8 anti-GP2 antibody positive at baseline.

unable to provide a decisive outcome, and thus our data need to be interpreted with caution [8, 12, 17, 18, 21–23].

Our study cannot estimate accurately the prevalence of anti-GP2 in patients with CD, and this needs to be noted. Our cohort has an overrepresentation of patients with ileal involvement. Also, most serum samples originate from patients already treated. For an accurate estimation of the prevalence of anti-GP2 antibodies, a large cohort of naïve patients with Crohn's disease has to be tested. Taking into account that over the course of the disease, anti-GP2 antibodies decline, it would be logical to assume that the real prevalence of anti-GP2 antibodies may be higher than that so far reported in cohorts involving sera from already treated patients. Safer conclusions can only be reached if serial, large number of samples collected prospectively over a long-duration of follow up could be tested.

A few other points need to be made. The first point considers whether anti-GP2 antibodies contribute to the development of the disease or whether their existence is just an epiphenomenon following intestinal epithelial destruction [12]. Furthermore, it has been demonstrated that GP2 recognises FimH, which is a constituent of the type 1 pilus expressed on the outer membrane of some enterobacilli, such as *E. coli* and *Salmonella enterica* [11]. FimH have a lectin-like capability to bind certain glycoproteins in a mannose-dependent manner, and appear to be able to recognise GP2 [11, 13]. Specific interaction with FimH positive bacteria found in some but not all CD patients might explain the limited prevalence of anti-GP2 antibodies in IBD patients. Work on IBD animal models and GP2-deficient mice may shed a light on the pathogenic potential of GP2-specific immune responses. The fact that GP2 is expressed on the apical surface of M cells [11, 13] making it accessible to antibodies is of special interest, as it would support the notion that these cells may be the targets of antibody-dependent cytotoxicity [12]. On the other hand, a high proportion of individuals with CD appear seronegative for anti-GP2 antibodies, not only at baseline but also over time. This finding clearly indicates that the loss of immunological tolerance to this antigen is not an *a priori* condition for the development of the disease. There is no doubt that the pathogenesis of CD involves mechanisms other than those responsible for the induction of GP2 autoimmunity [29]. These mechanisms may be important for the establishment of the disease, acting in isolation or in combination with those leading to M cell-related induction of anti-GP2 antibodies, seen in over a quarter of patients with Crohn's disease. Why only those and not all patients develop these autoantibodies remains unclear. As anti-GP2 is absent in approximately 74% of patients with CD, the validity of the isolated detection of GP2-specific PAB is impaired. Most investigators agree that the routine use of isolated serological markers for diagnosis and especially for the follow up of patients with inflammatory diseases is limited by their inadequate performance in terms of the diagnosis and prognosis of CD [29–31]. ASCA, for example, are more prevalent in patients with CD and their participation in the routine testing of patients with CD is more than adequate. In conclusion, we share with others the notion that several serological markers must be used in combination to be more effective compared to isolated/single marker testing.

Nevertheless, anti-GP2 antibody testing appears to be one of those tests that can be added in the diagnostic workup of patients with CD. More work needs to be done over the next few years to understand the immunobiological role of this antigen and its relevance to IBD.

Emerging data indicating an important immunoregulatory role of GP2 for the emergence of innate and adaptive immunity (including the recruitment of regulatory T cells) in the intestine [32] may initiate an intense research in this field and elucidate the role of this interesting autoantigen.

Conflict of Interests

D. Roggenbuck is a shareholder of GA Generic Assays GmbH and Medipan GmbH. The remaining authors declare that they have no competing financial interests. A. Forbes and D. P. Bogdanos equally contributed to this work.

Acknowledgments

UCH receives financial support for its research from the UK Clinical Research Service, National Institute for Health Research. D. P. Bogdanos was supported by the Higher Education Funding Council for England. O. Romanidou, D. Smyk and M. Mytilinaiou were supported by the European Association for the Study of Liver (EASL). The authors thank Dr. Gary L. Norman (INOVA Diagnostics) for providing the ASCA ELISA kits. Part of this work was presented as a poster at the 8th International Meeting on Autoimmunity, Granada, Spain (May 2012).

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 354594, 6 pages
doi:10.1155/2012/354594

Clinical Study

Carbonic Anhydrases III and IV Autoantibodies in Rheumatoid Arthritis, Systemic Lupus Erythematosus, Diabetes, Hypertensive Renal Disease, and Heart Failure

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Received 8 July 2012; Accepted 14 August 2012

Academic Editor: Dimitrios Bogdanos

In the present study, the CA III and IV autoantibodies, CA activity, antioxidant enzymes and cytokines in rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), diabetes, hypertensive renal disease, and heart failure were investigated. The anti-CA III antibody titers in patients with RA, SLE, and type 1 diabetes (T1D) were significantly higher than that in control groups ($P < 0.05$). The anti-CA IV antibody titers in patients with RA, SLE, type 1 diabetic nephropathy (T1DN), and heart failure were significantly higher than that in control groups ($P < 0.05$) while anti-CA IV antibody could suppress the total CA activity. The SOD and GPx levels in patients with RA, SLE, and T1DN were significantly lower than that in control groups ($P < 0.05$). IL-6, IL-17, IFN-γ, and TNF-α levels were significantly higher in SLE group compared with the control group ($P < 0.05$). Weak but significant correlations were found between anti-CA III antibodies and ESR in RA ($r = 0.403$, $P = 0.013$) and SLE patients ($r = 0.397$, $P = 0.007$). These results suggested that the generation of CA III and IV autoantibodies, antioxidant enzymes, and cytokines might influence each other and CA autoantibodies might affect the normal physiology function of CA.

1. Introduction

Autoimmune diseases arise from an inappropriate immune response of the body against substances and tissues normally present in the body. If the immune system mistakes some parts of the body or some proteins free in body fluid as deleterious substances, the immune system will produce specific antibodies to attacks it. This may be restricted to certain organs or involve a particular tissue in different places. The mechanism of autoantibodies formation which is the most critical part of autoimmune diseases is still not fully clear [1]. The autoantibodies are found in some disease which is not usually defined as autoimmune disease such as heart failure [2]. Lots of autoantibodies are detected in many kinds of diseases, but only dozens of them are used in the clinical diagnosis and/or therapy monitoring. The relationship between the new-found autoantibodies and other clinical indicators needed to be evaluated systemically.

Carbonic anhydrases (CA, EC 4.2.1.1) are zinc-containing enzymes, which play a critical role in maintaining the intercellular/extracellular pH of most mammalian cells by catalyzing the interconversion between carbon dioxide and bicarbonate. CA is a group of widespread metalloenzymes and there are at least 15 different isoforms present in mammalian cells [3]. Some of these isozymes are membrane-bound enzymes (CA IV, CA IX, and CA XII CA XIV, etc.), whereas others are located in the cytosol (CA I, CA II, CA III, CA VII, CA XIII), CA V is mitochondrial and CA VI is secreted in the saliva and milk. Autoantibodies response to CA I, CA II, and CA IV have been found in the patients with rheumatoid arthritis (RA) and autoimmune pancreatitis (AIP) which are autoimmune diseases in the conventional sense [4–6].

CA III is a cytoplasmic enzyme that exhibits a relatively low carbon dioxide hydratase activity. It is expressed at

a very high level in skeletal muscle, where physical exercise has been shown to increase free radical production. CA III may play a role in scavenging oxygen radicals and thereby protecting cells from oxidative damage [7, 8]. In addition, CA III has been demonstrated to have a carboxyl esterase activity and phosphatase activity, which suggests that it is a tyrosine phosphatase [7]. In kidney, CA IV is present on the apical brush-border membrane and on the basolateral membrane of proximal tubule cells, which contributes to net transepithelial bicarbonate transport [9, 10]. Most cardiac CA appears to be bound to SR and sarcolemmal membranes while carbonic anhydrase IV is the predominant isozyme in the heart [11].

Circulating auto-antibodies have been critically linked to several kinds of diseases. Their prevalence, mode of action, and potential therapeutic modulation are intensively investigated. In the present study, we determined the antibodies response to the CA III and CA IV in the serum of Chinese patients with RA, systemic lupus erythematosus (SLE), type 1 diabetes (T1D), type 1 diabetic nephropathy (T1DN), type 2 diabetes (T2D), type 2 diabetic nephropathy (T2DN), hypertensive nephropathy, and heart failure using indirect enzyme-linked immunosorbent assay (ELISA) and investigated the possible associations between these antibodies and other indicators of these diseases.

2. Materials and Methods

2.1. Study Population. The design of this study was approved by the ethics committee of Xuanwu hospital of Capital Medical University, informed consent was obtained from all participants. 91 RA patients which did not receive immunosuppressive treatment for at least 1 year (57 females, 34 males, mean age 52.5 ± 11.8), 79 SLE patients which did not receive immunosuppressive treatment for at least 1 year (56 females, 23 males, mean age 32.5 ± 9.8), 157 T1D patients (90 females, 67 males, mean age 25.5 ± 8.7), 56 T1DN patients (30 females, 26 males, mean age 32.5 ± 11.8), 188 T2D patients (107 females, 81 males, mean age 62.5 ± 13.7), 65 T2DN patients (32 females, 33 males, mean age 65.5 ± 8.6), 165 hypertensive nephropathy patients (84 females, 81 males, mean age 68.4 ± 9.5), and 73 heart failure patients (25 females, 48 males, mean age 70.6 ± 7.4) were selected for this study. Age- and gender-matched control subjects were also included. Serum samples were stored at −80°C until analysis.

The following data of the subjects were determined using automatic analyzer: rheumatoid factor (RF) and ASO are measured by Dade Behring BN II using immunonephelometric method; erythrocyte sedimentation rate (ESR) was measured using Vacuette ESR System; high sensitive C-reactive protein (hsCRP) and SOD activity were measured using Hitachi 7600 biochemical analyzer. The quality control products were tested with specimen.

The activity of catalase (CAT) and GPx and the level of total antioxidant eaPaeity (TAC), CPx, and MDA in serum were determined following the kit instructions (BioVision). Anticyclic citrullinated peptide antibodies (anti-CCP) were measured by ELISA kit from Euroimmun Medical Laboratory Diagnostics. IL-6, IL-10, IL-17, were measured by ELISA kit from R&D Corporation. TNF-α and IFN-γ were measured by ELISA kit from Invitrogen Corporation.

2.2. Detection of IgG Anti-CA III and Anti-CA IV by ELISA. Specific antibodies to CA III and CA IV (Sigma) were identified in serum by an indirect ELISA method. To conduct the assay, 100 μL of the CA III (3.0 μg/mL) and CA IV (2.5 μg/mL) was incubated in an ELISA plate (Corning) at 4°C overnight. Microwells were then washed with phosphate-buffered saline (PBS: 0.01 M, pH 7.4) with 0.05% Tween-20 (PBST). Unbound sites were blocked by incubation with 200 μL 20% newborn calf serum (NCS) in PBS at 37°C for 1.5 h. Sera were diluted 1 : 200 in blocking buffer and aliquots of 100 μL were added to the wells. Wells coated with bovine serum albumin (BSA) were prepared for each sample, to assess nonspecific binding. After incubation at 37°C for 1 h, plates were washed 3 times with PBST. Subsequently, the captured antibodies were detected by a horseradish peroxidase- (HRP-) conjugated goat anti-human IgG (1 : 10000), Santa Cruz, which was diluted with 20% NCS in PBST (100 μL/well). After incubation at 37°C for 30 min, wells were washed 5 times with PBST. Color was developed by application of 100 μL of tetramethylbenzidine (Sigma) at 37°C for 20 min. The reaction was stopped by addition of 0.5 M sulfuric acid, and the optical density at 450 nm (OD_{450}), with 620 nm as the correction wavelength, was obtained using an ELISA plate reader (Thermo MK3). Each sample was assayed in duplicate. A positive serum sample was included in each assay and used to correct for interassay variations. Results were expressed as arbitrary units (AU) calculated as ([OD_{450} of sample − OD_{450} of the nonspecific binding of the sample]/[OD_{450} of the positive control − OD_{450} of the nonspecific binding of the positive control]) × 100 [12, 13].

2.3. Determination of CA Activity. Erythrocyte CA hydratase activity was determined using potentiometric method [13, 14]. 100 μL of serum was added to 2.4 mL of HEPES (pH = 8.80). The reaction was started by the addition of 2.5 mL ice-cold distilled water saturated with CO_2. The reaction tubes were kept in ice bath. The rate of fall in pH from 8.2 to 7.0 was monitored continually with pH meter (Orion 420C-81). Rates of uncatalyzed CO_2 hydration were subtracted from enzyme catalyzed ones. The enzyme unit (EU) of CO_2 hydratase activity was calculated by using the equation EU = $(to - tc)/tc$ where "to" and "tc" are the times for pH change of the nonenzymatic and enzymatic reactions, respectively. CA activity was expressed as EU/mL. For suppression experiment, the CA IV antibody (final concentration was 1 : 100) was added into the reaction system and then determined whether CA activity was suppressed ($n = 5$). Each sample was assayed in triplicate.

2.4. Statistical Analyses. Statistical analyses were performed using SPSS 13.0 for Windows. For normally distributed data, results are expressed as the mean and standard deviation (SD); differences between groups were assessed by t-tests. Differences between groups were analyzed using the Mann-Whitney U-test while correlations were determined by

Table 1: The anti-CA III, IV titers, and the CA activity of subjects (mean (SD)).

		RA	SLE	T1D	T1DN	T2D	T2DN	HTN	HT
anti-CA III (abs)	control	0.058 (0.015)	0.056 (0.021)	0.064 (0.015)	0.041 (0.009)	0.053 (0.012)	0.064 (0.013)	0.049 (0.010)	0.061 (0.012)
	patients	0.195* (0.079)	0.327* (0.644)	0.073 (0.022)	0.204* (0.083)	0.065 (0.023)	0.056 (0.019)	0.096 (0.057)	0.065 (0.025)
	positive	11.2%	14.8%	0.0%	6.7%	0.0%	0.0%	0.0%	0.0%
anti-CA IV (abs)	control	0.021 (0.013)	0.032 (0.013)	0.027 (0.006)	0.019 (0.007)	0.026 (0.009)	0.027 (0.011)	0.035 (0.012)	0.026 (0.009)
	patients	0.183* (0.063)	0.205* (0.688)	0.033 (0.013)	0.022* (0.105)	0.022 (0.006)	0.049 (0.018)	0.028 (0.011)	0.131* (0.045)
	positive	9.8%	10.6%	0.0%	4.2%	0.0%	0.0%	0.0%	5.3%
CA activity (EU/mL)†	control	8.65 (2.86)	7.96 (3.12)	8.12 (2.57)	8.56 (2.13)	8.63 (2.64)	8.47 (2.32)	7.98 (3.65)	8.12 (2.43)
	patients	10.37* (4.36)	12.48* (5.68)	8.66 (4.36)	13.07* (5.58)	9.32 (3.97)	12.65* (4.56)	9.65* (4.51)	13.47* (5.08)

*There were significant differences between patients and control ($P < 0.05$).
†$n = 10$ (5 males and 5 females).
HTN: hypertensive nephropathy; HT: heart failure.

computing Spearman rank correlation coefficients. P values of less than 0.05 were considered as significantly different.

3. Results

The anti-CA III antibody titers in patients with RA, SLE, and T1DN were significantly higher than that in control group ($P < 0.05$). The anti-CA III antibody titer of hypertensive nephropathy patients group was a little higher ($P > 0.05$) compared with control group.

The anti-CA IV antibody titers in patients with RA, SLE, T1DN, and heart failure were significantly higher than that in control group ($P < 0.05$). The anti-CA IV antibody titer in patients with T2DN was a little higher ($P > 0.05$) compared with control groups.

The CA activity of RA, SLE, T1DN, T2DN, heart failure, and hypertensive nephropathy was significantly higher than that in the control groups, respectively ($P < 0.05$, Table 1). The CA activity could be suppressed by 1 : 100 anti-CA IV antibodies ($P < 0.05$, Figure 1).

Table 2 showed that the sera RF, ASO, and ESR in RA, SLE, and T1DN groups were significantly higher than that in control group, respectively ($P < 0.05$). The positive rates of ESR in RA and SLE were significantly higher than other patients groups ($P < 0.05$). A few of these indicators were higher in T1D patients and T2DN patients group compare to the control group ($P < 0.05$) but still in the in biological reference interval range.

The SOD and GPx levels in the serum of patients with RA, SLE, and T1DN were significantly lower than that in control groups, respectively ($P < 0.05$). There was at least one of these antioxidant enzymes significantly reduced in the T1D, T2DN, and hypertensive nephropathy compared with control group ($P < 0.05$). There was significant difference of MDA level between RA, SLE, T1DN, and heart failure groups compared with control groups, respectively ($P < 0.05$). The MDA level in the T1D, T2DN, and hypertensive nephropathy groups were little higher than control groups but there was no significant difference ($P > 0.05$, Table 3).

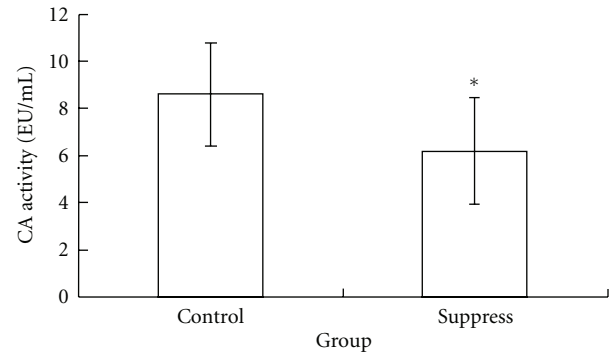

Figure 1: The total CA activity was suppressed by anti-CA IV antibody. *There was significant difference between suppress group and control group ($n = 5$) ($P < 0.05$).

IL-6, IL-17, and IFN-γ levels were significantly higher in RA group compared with the control group ($P < 0.05$). IL-6, IL-17, IFN-γ, and TNF-α levels were significantly higher in SLE group compared with the control group ($P < 0.05$). IL-6 levels were significantly higher in T1DN, T2D, and T2DN groups compared with the control groups ($P < 0.05$). There was no significant difference of these cytokine between heart failure and hypertensive nephropathy patients groups and control groups, respectively ($P > 0.05$, Table 4).

Weak but significant correlations were found between anti-CA III antibodies and ESR in RA ($r = 0.403$, $P = 0.013$) and SLE patients ($r = 0.397$, $P = 0.007$).

4. Discussion

CA III is abundantly expressed in fat, liver, and slow-twitch skeletal muscle fibers [15]. The activity of CA III in hydrating carbon dioxide is lower than CA I and CA II, while it

Table 2: The disease indicators of subjects (mean (SD)).

		RA	SLE	T1D	T1DN	T2D	T2DN	HTN	HT
RF (U/mL)	control	7.6 (5.2)	5.8 (3.5)	6.9 (4.3)	7.2 (4.3)	7.5 (3.4)	8.1 (3.9)	7.7 (3.1)	6.7 (4.1)
	patients	108.4* (51.9)	63.8* (7.9)	11.4 (5.6)	12.3* (5.3)	9.1 (4.8)	10.5* (6.7)	8.2 (4.1)	7.5 (3.2)
	positive	83.2%	50.3%	3.9%	5.1%	6.4%	5.8%	2.8%	2.1%
ASO (IU/mL)	control	133.1 (23.2)	118.5 (31.4)	115.8 (21.7)	113.6 (35.7)	129.5 (41.5)	123.4 (43.7)	141.3 (32.4)	135.4 (39.8)
	patients	151.6* (45.5)	135.4* (42.3)	125.6 (33.9)	154.2* (36.8)	123.8 (41.6)	139.8 (37.9)	133.1 (45.8)	128.3 (21.5)
	positive	7.8%	6.5%	6.3%	7.2%	4.1%	5.9%	3.8%	2.2%
Anti-CCP (U/mL)	control	2.6 (0.7)	3.1 (0.8)	ND	ND	ND	ND	ND	ND
	patients	63.7 (33.5)	17.0 (5.9)	ND	ND	ND	ND	ND	ND
	positive	61.2%	11.2%	ND	ND	ND	ND	ND	ND
ESR, (mm/h)	control	ND	ND	ND	ND	ND	ND	ND	ND
	patients	41 (15)	37 (9)	18 (7)	17 (9)	15 (6)	19 (5)	11 (3)	11 (4)
	elevated	79.2%†	65.1%†	5.5%	3.5%	1.7%	2.4%	1.3%	1.9%
hsCRP (mg/L)	control	1.8 (0.7)	2.1 (0.9)	2.3 (0.7)	1.8 (0.5)	2.4 (0.5)	3.2 (1.2)	2.9 (1.3)	2.8 (2.1)
	patients	12.8* (5.7)	16.5* (8.2)	3.1 (1.2)	6.3* (0.8)	2.3 (0.8)	3.7 (1.5)	4.4 (1.5)	5.1 (1.9)
	elevated	35.8%	42.5%	3.9%	4.8%	5.1%	5.5%	7.8%	6.7%

*There were significant differences between patients and control ($P < 0.05$).
†There were significant differences between T1D, T1DN, T2D, T2DN, HTN, and HT groups.
HTN: hypertensive nephropathy; HT: heart failure; ND: not determined.

Table 3: The antioxidant enzyme activity and MDA of subjects (mean (SD)).

		RA	SLE	T1D	T1DN	T2D	T2DN	HTN	HT
SOD (U/mL)	control	134.57 (13.13)	151.55 (14.97)	142.83 (13.98)	145.66 (17.97)	139.63 (22.80)	141.10 (21.57)	149.00 (18.92)	142.13 (16.30)
	patients	92.85* (36.87)	105.60* (33.55)	131.19 (23.98)	133.68* (31.36)	131.69 (43.67)	115.37* (38.56)	131.69* (42.36)	101.93* (36.34)
GPx (U/mL)	control	158.18 (34.41)	171.20 (45.60)	162.68 (42.02)	159.67 (37.94)	164.36 (41.25)	155.39 (44.98)	157.65 (39.86)	159.36 (41.23)
	patients	118.29* (40.30)	135.85* (41.97)	146.58* (39.23)	127.96* (43.63)	159.67 (34.76)	161.33 (45.68)	149.35 (48.64)	135.36* (45.39)
CAT (U/mL)	control	1.42 (0.45)	1.56 (0.37)	1.54 (0.55)	1.39 (0.36)	1.74 (0.45)	1.61 (0.47)	1.52 (0.51)	1.73 (0.41)
	patients	1.33 (0.56)	1.37 (0.53)	1.39 (0.59)	1.11* (0.42)	1.25* (0.51)	1.33* (0.51)	1.47 (0.31)	1.39* (0.63)
TAC (U/mL)	control	14.31 (5.13)	15.12 (3.35)	15.65 (3.63)	14.96 (4.32)	15.69 (3.36)	16.01 (4.51)	14.63 (3.39)	14.78 (4.13)
	patients	11.25* (4.56)	13.59 (6.65)	14.32 (4.13)	12.15* (5.30)	14.34 (4.69)	14.96 (6.66)	13.97 (4.63)	12.01* (5.22)
MDA (nmol/mL)	control	3.57 (1.01)	3.31 (0.94)	3.14 (1.02)	3.46 (1.32)	3.35 (0.85)	3.61 (0.98)	3.44 (1.13)	3.57 (1.20)
	patients	4.52* (1.25)	4.29* (1.37)	3.08 (1.33)	4.05* (1.25)	3.45 (1.09)	3.96 (1.23)	3.55 (1.15)	4.11* (0.87)

*There were significant differences between patients and control ($P < 0.05$).
HTN: hypertensive nephropathy; HT: heart failure.

has two reactive sulfhydryl groups, which can reversibly conjugate to GSH through a disulfide bond [16, 17]. This s-glutathionylation reaction is believed to be an important component of cellular defense mechanisms that prevent the protein oxidation which is irreversible. In the present study, we found that the anti-CA III antibody titers in patients with RA, SLE, and T1DN were significantly higher than that in control group, respectively, while at least one of the antioxidant enzyme activity levels of MDA level in the serum of RA, SLE, T1D, T1DN, and T2DN patients was significantly lower than that in control group. The results were similar with the researches which take RA and diabetes patients as subjects [18, 19]. It has been proven that anti-CA II antibody was 25% higher in T1D patients than the controls [13]. In the

Table 4: The cytokine levels of subjects (mean (SD)).

		RA	SLE	T1D	T1DN	T2D	T2DN	HTN	HT
IL-6 (ng/L)	control	2.31 (0.63)	2.28 (0.71)	2.39 (0.69)	2.25 (0.67)	2.01 (0.58)	1.97 (0.64)	2.33 (0.59)	2.18 (0.61)
	patients	5.02* (1.41)	4.38* (1.64)	2.47 (0.96)	3.05* (1.53)	2.78* (0.88)	2.85* (0.93)	2.58 (1.22)	2.35 (0.97)
IL-17 (ng/L)	control	1.24 (0.21)	1.26 (0.29)	1.36 (0.31)	1.38 (0.27)	1.25 (0.33)	1.33 (0.26)	1.39 (0.40)	1.28 (0.35)
	patients	1.96* (0.90)	2.13* (0.75)	1.55 (0.76)	1.97* (0.65)	1.41 (0.57)	1.48 (0.73)	1.27 (0.70)	1.35 (0.55)
TNF-α (ng/L)	control	12.37 (2.45)	11.97 (3.69)	12.39 (3.21)	13.17 (3.39)	12.02 (4.13)	13.39 (3.64)	12.47 (3.68)	12.63 (4.36)
	patients	14.65 (6.34)	16.97* (6.93)	13.23 (5.61)	14.37 (8.35)	15.23* (4.32)	16.37* (6.55)	11.95 (4.37)	12.96 (3.37)
IFN-γ (ng/L)	control	11.01	12.25	11.69	12.64	11.64	10.89	11.39	12.43
	patients	16.38* (6.36)	14.99* (4.08)	12.06* (4.11)	14.15* (5.62)	12.03 (4.21)	11.59 (3.03)	12.78 (4.32)	13.36 (5.69)

* There were significant differences between patients and control ($P < 0.05$).
HTN: hypertensive nephropathy; HT: heart failure.

present study, we found anti-CA III antibody and anti-CA IV antibody were positive in T1D and T2D patients.

Redox reactions are imperative to preserving cellular metabolism yet must be strictly regulated. Imbalances between reactive oxygen species (ROS) and antioxidants can initiate oxidative stress, which without proper resolve, can manifest into disease. Oxidative stress occurs when the generation of ROS overcomes the scavenging abilities of antioxidants. Such instances may be mediated by genetic lack of antioxidant enzymes as well as other triggers [20, 21]. T1D and T2D are frequently associated with increased oxidative stress [22]. The downregulation of antioxidant enzymes might be a trigger of autoimmune [13]. While the data from experiments using cell lines and animal models suggested that CA III might function to protect cells from oxidative damage [7, 22]. In this point of view, the anti-CA III antibody might be the trigger of the downregulation of antioxidant enzymes.

There are at least twenty autoantibodies associated with heart failure and cardiac dysfunction, including anti-Na pump, anti-SERCA, antilaminin and anti-hsp60 antibodies [2]. The injury to myocardium is believed to be a crucial trigger to the autoimmune of these two heart diseases. In this study, we found that the anti-CA IV antibody titer in the serum of heart failure patients was significantly higher than that of healthy control. The CA IV is the predominant CA isozyme in the heart, antigen exposure because of the long-time injury of heart cells might be the trigger of the generation of anti-CA IV antibody in heart failure patients [2, 11]. There was no significant change of the anti-CA IV antibody in the serum of patients with hypertensive nephropathy and T2DN, while total CA activity of these two diseases was higher than the control. It was suggested that the antigen exposure was not the trigger of the generation of anti-CA IV antibody in these diseases.

The cytokine including IL-6, IL-17, IFN-γ, and TNF-α were upregulated in these diseases. Overproduction of TNF-α supports and even amplifies the inflammatory process leading to insulin resistance [23]. TNF-α may activate both proapoptotic and antiapoptotic pathways. IL-17 plays an important role in the pathogenesis of several autoimmune diseases, and the importance of IL-17 has been demonstrated in various animal models [24, 25]. IL-6 has also been involved in metabolism, endocrine, and neoplastic disorders, and the endocrinopathy is also a motivator of autoimmune [2, 26].

Autoantibodies play important roles during physiological and pathological processes. Mouse models have shown that autoantibodies can activate the alternative pathway and induce in cell lysis and tissue damage [25]. In this study, we found that the anti-CA IV antibody could suppress the total CA activity. The mechanism of this phenomenon might be the active centre of CA IV was suppressed by the binding of CA IV and anti-CA IV antibody. This experiment was not done using anti-CA III antibody because the activity of CA III in hydrating carbon dioxide is very low (only about 2% of CA I and CA II [27]).

In conclusion, we found that there are unusually high titers of anti-CA III and/or anti-CA IV antibodies in the serum of RA, SLE, T1D, T1DN, T2D, T2DN, and heart failure patients with the abnormal level of cytokines and antioxidant enzymes. The generation of CA III and IV autoantibodies, the antioxidant enzymes, and cytokines might influence each other and the CA autoantibodies might affect the normal physiology function of CA.

Acknowledgment

This work was supported by the Natural Science Foundation of China (No. 81271924). The authors would like to thank Dr. Meng Shuang of the National Center for Clinical Laboratories, Beijing, People's Republic of China for the detection standardization of ELISA.

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