Autoantibodies in early arthritis: Advances in diagnosis and prognostication

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ABSTRACT

Several excellent reviews have recently been published on the significance of autoantibodies in rheumatoid arthritis (RA) (1-4). Here we: (i) review selected longitudinal studies examining the predictive utility of autoantibodies in early arthritis and early RA cohorts; (ii) assess the relevance of autoantibodies as an independent parameter for prediction and prognostication of RA; and (iii) describe the potential of multiplex autoantibody assays, including miniaturized, high-throughput microarray technology, to improve diagnosis and prognostication in recent-onset synovitis/early arthritis patients.

Diagnosis and prognostication in recent-onset arthritis

Early diagnosis of RA and reliable outcome prediction are issues of paramount importance in early arthritis clinics (5). A number of novel treatment modalities have been introduced over the past 5 years, and rheumatologists are now attempting to institute optimal treatment in recent-onset arthritis. It is recognized today that the 1987 ACR classification criteria (6) are frequently insufficient for the diagnosis of early RA, particularly in population-based cohorts of patients with recent-onset arthritis (7). A great need exists to classify accurately and stratify patients with recent-onset arthritis to guide therapeutic decisions.

Significant progress has been made in the development of better prediction models (8), elucidating the role of potential predictors including acute phase reactants (9),and identifying additional biomarkers with predictive value. Such biomarkers include the shared epitope (10) and urinary type II collagen C-telopeptide (11). Yet, an unmet need remains for the development of diagnostic tools to further improve prediction of RA and prognostication of future health outcome (12).

One promising approach is proteomic profiling of autoantibody responses in human serum and other biological fluids. Proteomic technologies enable the parallel, high-throughput detection of autoantibodies using small quantities of valuable biologic samples. To this end, we used a split-pin robotic arrayer [http://cmgm.Stanford.edu/pbrown] to generate high-density autoantigen microarrays on glass microscope slides (13). Preliminary observations from our arthritis antigen microarray project (Table I) are outlined below. Beyond the scope of this review, and discussed by us in detail elsewhere (14), this and other high-throughput proteomics technologies for autoantibody profiling enable: (i) large-scale characterization of the evolution of humoral immune responses in patients and in animal models of autoimmune disease; (ii) selection of antigen targets for induction of antigen-specific tolerance; and (iii) discovery of novel autoantigens.

Autoantibodies as predictors in RA

Autoantibodies are useful laboratory markers for the diagnosis and classification of a variety of autoimmune diseases. For certain diseases they are predictive of organ involvement and disease severity (12, 15). For decades, the determination of rheumatoid factor (RF) has been the central autoimmune laboratory test performed in early-onset arthritis, playing a critical role for both diagnosis and to a lesser extent outcome prediction in RA (9, 16). Studies of serum samples stored in large serum banks indicated that RF may be identified years prior to the onset of RA in certain seropositive patients (17). However, it is widely recognized that RF testing is too non-specific to be used as a wide-scale screening tool to identify RA patients in the primary care setting (12, 18, 19).

Several additional autoantibodies have recently demonstrated better perfor-

mance than RF and have been proposed as diagnostic and prognostic markers for RA. Most prominently, a class of autoantibodies recently shown to recognize deiminated peptide epitopes, first described for the epidermal protein (pro)filaggrin by Schellekens *et al.* (20) and Girbal-Neuhauser *et al*. (21), may represent sensitive and specific markers for RA. This finding was bolstered by the recent observation that autoantibodies from RA serum also recognize fibrinogen that has undergone *in vitro* deimination using purified peptidyl arginine deiminase (PAS) (22).

Deiminated fibrin represents an excellent candidate antigen for RA since this protein is detected in the synovium of RA patients (23). Moreover, autoantibodies against other candidate antigens in RA such as vimentin (anti-Sa reactivity) may also target deiminated epitopes (24). Together, these reports pave the road for a new paradigm in RA autoimmunity: Deimination is a crucial post-translational modification for the generation of immunogenic B cell epitopes in RA. Conversion of the amino acid L-arginine to L-citrulline is catalyzed by the enzyme peptidyl arginine deiminase (PAD). *In vitro* deiminated recombinant filaggrin (25), a synthetic three-dimensional cyclic citrulline-substituted filaggrin peptide (CCP) (26), and most recently *in vitro* deiminated fibrinogen (27) were used to develop assays now broadly validated for the detection of serum autoantibodies against deiminated epitopes.

Several additional autoantibody specificities have been studied in early rheumatoid arthritis, including anti-hnRNP A2/RA-33 (28), anti-alpha-enolase (29), anti-Sa (30, 31) and anti-calpastatin (32). Early autoimmune responses directed against other candidate antigens, including the glycolytic enzyme glucose 6-phosphate isomerase (GPI) (33, 34) and the endoplasmic reticulum molecular chaperone BiP (35, 36), are under active investigation. Moreover, studies are underway to determine if multiparameter assays provide improved diagnostic and prognostic value over individual autoantibody testing in recent-onset arthritis.

Bläss *et al*. screened for 6 different autoantibodies, and using computer-assisted analysis identified several reactivity patterns associated with RA (37). Line immunoassays allow for parallel detection of autoantibodies directed against a panel of up to 15 antigens on nitrocellulose strips (38). We developed high-density antigen microarrays that provide the capacity to detect autoantibody reactivity against hundreds or thousands of antigens simultaneously (13) (see below).

Autoantibodies to predict diagnosis of RA in early arthritis patients

Autoantibody reactivities directed against RF, keratin, perinuclear factor, hnRNPA2/RA33, Sa, citrulline-substituted filaggrin peptides, deiminated fibrinogen peptides, calpastatin and alpha-enolase have been investigated for their occurrence in early arthritis (Table I). Their association with RF is considerable, and the prevalence of individual antibody specificities in seronegative RA was disappointingly low in some cohorts (30). Based on these studies it was argued that certain single-parameter diagnostic tests, even when highly specific, may contribute only marginally in distinguishing RA from non-RA patients in early arthritis clinics (1).

Promising recent data, obtained from longitudinally-studied large inception cohorts, report remarkable sensitivity of ELISAs that detect autoantibodies specific for deiminated peptides derived from filaggrin (39) or fibrinogen (40). At the 2003 European Workshop for Rheumatology Research (Marseille, France), L. Nogueira and colleagues presented results on the performance of an *in vitro* deiminated fibrinogen peptide (hFibA) ELISA. In an inception cohort of 352 patients with recent-onset arthritis of less than one year's duration, 175 patients progressed to RA. At a 98% specificity level, the sensitivity of anti-hFibA ELISA was 65%, compared with sensitivities of 54% for the commercially available anti-CCP ELISA and 26% for RF detection by nephelometry (40). Although the above reports await confirmation in additional cohorts, these studies have ignited a debate as to whether testing for serum autoantibodies directed against deiminated or citrulline-substituted antigens should replace RF testing.

In a smaller cohort of 96 Austrian patients with very recent-onset arthritis (< 3 months), CCP reactivity was observed in 30% of the 61 patients that developed RA within the following year (41). These results are similar to the findings in the NIH early synovitis cohort (30). We also observe similar results using antigen microarray technology and samples from patients with less than 6 months disease duration from the Arthritis, Rheumatism and Aging Medical Information System,

Table I. Selected antigens contained on current synovial proteome microarrays ('arthritis chips').

RA candidate antigens	Citrulline-substituted cyclic and linear filaggrin peptides (12 peptides), overlapping collagen type II peptides (~400 peptides), overlapping HCgp39 peptides (~70 peptides), hnRNP A2 peptides (14 peptides) Ro60/52, La, HSP 60, 70, 65, 90, dnaJ, human recombinant BiP, keratin,vimentin, fibrinogen,native and citrullinated, fibrinogen peptide A and B. Collagen type I-V, acetyl-calpastatin, annexin V, recombinant hnRNP B1 and D, GPI
Other antigens	dsDNA, RNA, rRNA, PDH, aldolase, topoisomerase I, Jo-1, snRNP proteins, Sm-complex, Scl-70, Scl-100, PARP, cardiolipin
Controls	Candida antigen, Hepatitis A and B vaccine, Pneumococcal vaccine, Influenca vaccine Human IgG/IgM.

HCgp39: human cartilage glycoprotein 39; HSP: heat shock protein; BiP: endoplasmic molecular chaperone; hnRNP:heterogeneous nuclear ribonucleprotein; PDH: pyruvate dehydrogenase; GPI:glucose-6-phosphate isomerase; rRNA: ribosomal RNA; snRNP:small nuclear RNP; Sm complex:Smith complex; PARP: poly (ADP-ribose) polymerase.

Table II. Autoantibodies for the diagnosis of RA in early arthritis – longitudinal studies. Epidemiological characteristics.

Author/year (Ref)	Study population	Prediction of	Disease duration	N	Evolving to RA	AB/parameters ested	Results (key findings)	Comment
Saraux *et al.*, 2002 (52)	*Inception cohort, recent-onset arthritis, Brittany, France	Diagnosis	< 12 months	270	98	RF isotypes, AKA, APF, anti-RA33, ANA. Shared epitope	*3-test combination (IgM-RF + RF Latex + IgG-AKA) had sensitivity of 75% and specificity of 82%. Slightly better performance than single parameter test or 2-test combination. *PPV of two or 3 positive tests: 82%. *Sensitivity RF + AKA 33% when specificity set at 99%.	*Excellent study cohort, prospective, median follow-up 2 years, *AKA and ARF testing tedious
Goldbach-Mansky *et al.*, 2000 (30)	*NIH early synovitis cohort	Diagnosis	< 12 months	238	106	RF, AFA, anti-Sa, anti-CCP, AKA, anti-RA33	*Strong association of anti-CCP, anti-AFA, anti-AKA and anti-Sa with RF *In seropositive RA sensitivities ranging from 19% (ant-Sa) to 33% (anti-CCP) to 53% (RF); in seronegative RA 14% (anti-Sa and anti-CCP). *Anti-Sa predictive of more severe disease in male patients.	*Population-based cohort *Prospective, follow up 1 year *Multiple AB tested
Kroot *et al.*, 2000 (44)	*Dutch inception cohort *RA fulfilling ACR criteria	*Radiographic damage *Disability by HAQ score	< 12 months	273	n.a.	RF, anti-CCP	*Multiple regressiom analysis revealed significant prediction of radiograph. damage, but not disability, by anti-CCP ELISA *RF predictive of both outcome parameters.	*Prospective, follow-up 6 years. *Consider treatment bias (see text)
Saulot *et al.* 2002 (29)	*French inception cohort	*Diagnosis, *Radiographic damage	< 4 months	255	145	RF, anti-alpha enolase	*Novel antigen/antibody system *Specificity for RA 97.1%, sensitivity low	*Excellent patient cohort. *Only 2 AB tested. May be a useful complementary AB
Bukhari *et al* 2002 (43)	*Norfolk Arthritis Register Study cohort	*Radiographic damage	5 months (median)	439	413	RF Shared epitope	*High titer of RF is an independent predictor of radiographic deterioration *SE not predictive of erosiveness	*Large-population based cohort *No AB other than RF were determined
Meyer *et al.*, 2003 (45)	*French cohort, *RA fulfilling ACR criteria	*Radiographic damage	< 12 months	191	n.a.	APF, anti-CCP, AKA	*Anti-AKA: no prediction *Anti-CCP better prediction than RF	*5-year follow-up *Consider potential treatment bias (see text)
Nogueira *et al.*, 2003 (abstr) (40)	*French inception cohort	Diagnosis	< 12 months	352	175	RF, anti-CCP, anti-FibA	**Anti-FibA ELISA had highest sensitivity (64%), followed by anti-CCP ELISA (54%), and RF (27%) at 98% sensitivity level.	*Large cohort *New ELISA with high sensitivity for RA.
Bergholz *et al.*, 2003 (abstr) (37)	*German inception cohort	Diagnosis	< 12 months	?	n.a.	RF, anri-CCP, anti-RA33, anti-BiP, anti-calpastatin, anti-calreticulin	*Several AB profiles identify RA. *Sensitivity up to 59%	*6-parameter assay *Computer-assisted analysis of AB profiles *Details study cohort not published yet
Nell *et al.*, 2003 (abstr) (41)	*Austrian inception cohort	Diagnosis	< 3 months	94	61	Anti-CCP RF, anti-RA33	*PPV: anti-CCP 95%, RF 85%, anti-RA33 84%	*Very early arthritis cohort *Follow-up at least 1 year.

AB: autoantibody; AKA: anti-keratine antibodies; APF: anti-perinuclear factor (AKA and APF are directed against deiminated epitopes of filaggrin); CCP: cyclic citrullinated pepties; PPV: positive predictive value; NPV: negative predictive value; FibA: fibrinogen peptide A; SE: shared epitope.

National Inception Cohort of Rheumatoid Arthritis Patients (ARAMIS) (unpublished observations). Several recently published early arthritis/early RA studies are to be commended for their rigor in study design, including sample size, entry criteria and data analysis. An outline of these studies and their findings are summarized in Table II.

Autoantibodies to predict severity and outcome of RA in early arthritis patients

Autoantibodies predict disease outcome at early stages of certain autoimmune diseases. For example, detection of autoantibodies against the E2 component of the pyruvate dehydrogenase complex in asymptomatic patients is highly predictive of subsequent development of primary biliary cirrhosis (PBC) (42). Autoantibodies directed against DNA topoisomerase I can precede severe pulmonary involvement in patients with Raynaud's syndrome who progress to develop systemic sclerosis (15). In contrast, in RA strong evidence for the utility of autoantibodies to predict more severe disease and unfavorable health outcome is not as well established.

Multiple new studies have examined the predictive role for autoantibodies in RA. RF has been demonstrated repeatedly to have value in predicting more progression of radiographic damage (9, 43). Well-designed longitudinal studies of community-recruited inception cohorts are necessary to investigate relationships of other autoantibodies as well as autoantibody profiles with disease outcomes. Most studies use 'worse radiographic damage' to assess the predictive value of autoantibodies, since this surrogate marker is most consistently associated with severe outcome (43).

Two recent investigations demonstrated that anti-CCP antibodies predicted worse radiographic damage in longitudinally-studied early RA cohorts with follow-up periods of 5 to 6 years (Table II) (44, 45). Other autoantibodies demonstrated to possess some predictive value for erosive joint disease include anti-alpha-enolase (29) and anti-Sa (30). The potential of additional markers, such as anti-hnRNP/RA33, anti-BiP, anti-GPI and the recently-described ACAST (antibodies to the C-terminal amino acids of calpastatin) (32), to predict more severe disease outcomes has not been fully explored. Importantly, treatment may strongly influence associations of autoantibodies, or other potential predictors, with surrogate markers of outcome including radiographic damage. The magnitude of this treatment bias may be more pronounced with longer disease duration and multiple treatments (46, 47).

Limitations of present autoantibody screening

The frequencies of autoantibodies (for example anti-CCP, anti-Sa) observed in the seronegative subgroup of recent-onset arthritis/early RA was relatively low in some studies (30), whereas others observe anti-CCP antibodies twice as frequently in a similar cohort (40). Confirmation of sensitivities and positive predictive values in additional recent-onset arthritis cohorts with different geographic, ethnic, genetic and socioeconomic compositions should be sought. The population of arthritis patients in the studies described herein may not be representative of populations in other clinical settings or countries, thus accounting for the discrepancies in single autoantibody reactivities. Moreover, variability in the performance of autoantibody assays may impact the predictive value. Thus far, only a few longitudinal studies have examined the prevalence of autoantibodies in early arthritis, and only certain studies determined autoantibody specificities simultaneously (Table II).

Large-scale longitudinal studies in well-defined inception cohorts are needed to assess multiple autoantibody reactivities head-to-head. Such studies will enable the full realization of the potential of autoantibody determination for outcome prediction in RA. Prediction may further improve with the discovery of novel autoantibody specificities, by optimizing assay performance, and by testing simultaneously for multiple autoantibody reactivities. Proteomics technologies represent a powerful approach to perform multiplex autoantibody profiling. Antigen microarray technology provides a simple and cost-effective tool to address these issues, and may help establish evidence-based guidelines for autoantibody testing in early arthritis patients.

Table III. Specificity and sensitivity of single autoantibodies for RA in early arthritis cohorts.

Antibody	Specificity	Sensitivity	References
AKA	0.93-0.99	0.26-0.33	(30), (52)
AFA	0.93	0.33	(30)
Anti-RA33	0.98	0.02-0.27	(28), (30), (41)
Anti-Sa	0.98	0.22-0.40	(30), (31)
Anti-CCP	0.91-0.98	0.30-0.70	(30), (40), (41), (44), (45)
Anti-hFibA	0.98	0.64	(40)
Anti-α-enolase	0.97	0.33	(29)

AKA, anti-keratin antibodies; AFA, anti-filaggrin antibodies; CCP, cyclic citrullinated peptide; hFibA, anti human fibrinogen peptide A.

Proteomics: Autoantibody profiling using microarrays

Proteomics technologies for miniaturized, multiplexed immunoassays for sensitive and specific detection of autoantibodies in biological samples are in their infancy (14). In the mid-1990s, Brown and colleagues introduced a split-pin robotic arrayer for high-throughput contact printing of ordered arrays of cDNA and oligonucleotides (48). We and others refined this technology for the production of protein and peptide antigen microarrays (13, 49, 50). We further optimized application of this platform for large-scale characterization of autoantibody responses in human autoimmune diseases (13). We subsequently generated syn-

ovial proteome antigen microarrays ("arthritis chips") containing ~650 known candidate antigens, including proteins, peptides, protein complexes, nucleic acids and enzymes (see Table I), attached to the surface of poly-L-lysine coated glass microscope slides.

Individual arrays are probed with serum from patients with autoimmune disease and controls, and autoantibody binding is detected using fluorophore-coupled anti-human antibodies. At 1:150 serum dilutions, only 2 ml of serum is required to probe an individual array. Use of coverslips further reduces the volume of serum needed. Arrays are scanned, and false-colored images analyzed. A detailed description of these microarray technologies and information regarding protocols, software and statistical tools can be found at the following Stanford University websites: [http://cmgm.Stanford.edu/pbrown], [http://www.Stanford.edu/group/antigenarrays] and http://www-stat.Stanford.edu/~tibs/].

We are applying our synovial antigen microarrays to detect serum autoantibody reactivity against a panel of citrulline-substituted filaggrin peptides (a gift from Dr. W.J. van Venrooij, University of Nijmegen, The Netherlands), including the cyclic citrullinated peptide (CCP) used in the commercially available anti-CCP ELISA. ELISA-validated preliminary results indicate that the sensitivity of anti-CCP reactivity in the Stanford ARAMIS recent-onset RA cohort of less than 6 months disease duration is about 50% (manuscript in preparation, W.H., P.J.U., and W.H.R.). Additionally, we observe reactivity against a variety of candidate antigens, including, hnRNP-A2/RA33 and hnRNP-D (a gift from Dr. G. Steiner, University of Vienna, Austria), BiP (a gift from Dr.G. Panayi, Guy's Hospital, London, UK), GPI (a gift from Dr. D. Mathis, Harvard Medical School, Boston, MA), Collagen type II, Ro, La, and heat shock proteins (HSPs) 65, 70 and 90, and several peptides derived from hnRNP-A2 (a gift from Dr. S. Muller, University of Strasbourg, France) and human cartilage glycoprotein 39 (HCgp39, a gift from Dr. G. Sønderstrup, Stanford University, CA). Four hundred overlapping peptides derived from collagen type II (a gift from Dr. L. Meyers, University of Tennessee, TN) are also spotted, as well as *in vitro* deiminated and native preparations of keratin, fibrinogen and vimentin.

Although we observe overlap of reactivities, our preliminary results corroborate the hypothesis that detection of panels of autoantibodies, as compared with individual autoantibody reactivities, increases the sensitivity and specificity for the diagnosis of RA (manuscript in preparation, W.H., P.J.U., and W.H.R.). Moreover, a larger panel of synthetic citrulline-modified peptides will become available for deposition on arrays in the near future, potentially enabling even higher degrees of sensitivity and specificity. This might be expected based on the results of Schellekens *et al*. who demonstrated higher sensitivity for RA when a panel of 9 different citrulline-substituted peptide variants were used for autoantibody detection, rather than single citrulline-substituted peptides (20). Linear and cyclic peptides may be recognized differentially by sera from different subsets of patients, suggesting heterogeneity of autoreactive B cell responses directed against deiminated epitopes (manuscript in preparation, W.H., P.J.U. and W.H.R.). Additional antigens are being added to our synovial proteome microarrays on an ongoing basis. Statistical algorithms including significance analysis of microarrays (SAM) and prediction analysis of microarrays (PAM) are being applied to define autoantibody profiles with greater diagnostic and prognostic utility in RA.

Summary and outlook

Significant progress has been made in recent years towards understanding the specificity of autoimmune responses in RA, and the utility of autoantibodies for diagnosis and outcome prediction in recent-onset arthritis. As proteomic technologies are developed and applied for autoantibody profiling, we anticipate that multiparameter testing will significantly improve the sensitivity and specificity of diagnosis and prediction in early RA. Similar to autoantibody screening in individuals at risk for autoimmune diabetes (51), autoantibody screening in early arthritis cohorts may also prove useful for recruitment and selection of patients for clinical trials. The advent of powerful high-throughput technologies in miniaturized formats ("lab-on-a-chip") will likely revolutionize how early autoimmune arthritis will be diagnosed and classified, enabling tailored and specific therapy for patients with early RA.

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