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Antigen Microarray Profiling of Autoantibodies in Rheumatoid Arthritis

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Objective. **Because rheumatoid arthritis (RA) is a heterogeneous autoimmune disease in terms of disease manifestations, clinical outcomes, and therapeutic responses, we developed and applied a novel antigen microarray technology to identify distinct serum antibody profiles in patients with RA.**

Methods. **Synovial proteome microarrays, containing 225 peptides and proteins that represent candidate and control antigens, were developed. These arrays were used to profile autoantibodies in randomly selected sera from 2 different cohorts of patients: the Stanford Arthritis Center inception cohort, comprising 18 patients with established RA and 38 controls, and the Arthritis, Rheumatism, and Aging Medical Information System cohort, comprising 58 patients with a clinical diagnosis of RA of <6 months duration. Data were analyzed using the significance analysis of microarrays algorithm, the prediction analysis of microarrays algorithm, and Cluster software.**

Results. **Antigen microarrays demonstrated that autoreactive B cell responses targeting citrullinated epitopes were present in a subset of patients with early RA with features predictive of the development of severe RA. In contrast, autoimmune targeting of the native epitopes contained on synovial arrays, including several human cartilage gp39 peptides and type II collagen, were associated with features predictive of less severe RA.**

Conclusion. **Proteomic analysis of autoantibody reactivities provides diagnostic information and allows stratification of patients with early RA into clinically relevant disease subsets.**

Rheumatoid arthritis (RA) is a polysynovitis of presumed autoimmune etiology that affects 0.6% of the population. Despite decades of research, the autoantigen targets and the molecular basis of RA remain poorly understood. The observed heterogeneity of disease manifestations, clinical course, and treatment responses suggests that unappreciated subtypes of RA exist on the molecular level. For example, a subpopulation of RA patients develop autoantibodies against citrullinated epitopes such as those represented by cyclic citrullinated peptide (CCP), which is associated with erosive disease (1,2). Another example is the heterogeneity in responsiveness to tumor necrosis factor α antagonist therapy (3,4). The advent of proteomics technologies has enabled large-scale analysis of proteins to identify biomarkers that delineate disease subtypes of RA, and to gain insights into the mechanisms underlying these subtypes.

For T cell–mediated autoimmune diseases, in-

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cluding RA, type 1 diabetes (T1D), and multiple sclerosis (MS), the presence of serum autoantibodies can predate the onset and be predictive of the development of clinical symptoms (5,6). In asymptomatic patients and in patients with undifferentiated arthritis, the presence of anti-CCP antibodies is a predictor of progression to RA (2). Detection of anti-CCP antibodies has been shown to provide a sensitivity of 70% and a specificity of 98% for the diagnosis of established RA (1,7). The process of citrullination is the result of the posttranslational conversion of arginine to citrulline by a family of enzymes termed peptidyl arginine deiminases (PADs). Vimentin and fibrinogen are considered candidate autoantigens in RA, based on the presence of these proteins in rheumatoid joints and the presence of autoantibodies against the citrullinated forms of these proteins in subpopulations of RA patients (8,9).

Autoantibodies targeting native proteins have also been described in RA. These include reactivities against heat-shock proteins (including Hsp65, Hsp90, DnaJ, and BiP), heterogeneous nuclear RNPs (hnRNP) A2/B1 (RA33) and D, annexin V, calpastatin, type II collagen, glucose-6-phosphate isomerase (GPI), elongation factor, and human cartilage gp39 (10). Nevertheless, our understanding of the specificities of the autoimmune responses in RA remains limited, and therefore comprehensive, parallel analyses of the autoantibody reactivities against the candidate autoantigens present in RA patients are necessary.

We recently reported the development of connective tissue disease microarrays to profile autoantibodies in 8 human autoimmune diseases (11), myelin arrays to monitor B cell epitope spreading and to develop more effective tolerizing vaccines in murine autoimmune encephalomyelitis (12), and human immunodeficiency virus arrays to identify antiviral antibody responses associated with efficacious vaccination against an immunodeficiency virus in macaques (13). We describe herein the development of synovial arrays that contain panels of >200 peptides and proteins representing candidate RA autoantigens. We applied these synovial arrays to profile autoantibodies in sera derived from RA patients and control subjects, and identified autoantibody biosignatures associated with clinical features that are predictive of the development of more severe arthritis.

PATIENTS AND METHODS

Antigens. Many of the antigens were purchased from Sigma (St. Louis, MO), except for DnaJ and Hsp65, which were purchased from Stressgen, Victoria, British Columbia, Canada, and except for the following antigens, which were synthesized in our laboratories: recombinant hnRNP-B1 and hnRNP-D (from J. S. Smolen, Vienna Medical School, Vienna, Austria), recombinant BiP (14) (from G. Panayi, Guy's Hospital, London, UK), mouse and human recombinant GPI (15) (from C. Benoist and D. Mathis, Harvard Medical School, Boston, MA), linear and cyclic citrulline–modified filaggrin peptides (7) (from JWD and WJVV), overlapping peptides derived from human cartilage gp39 (16) (from GS), and overlapping peptides derived from hnRNP-A2 (17) (from S. Muller, Institut de Biologie Moleculaire et Cellulaire, Strasbourg, France). Additional native and citrulline-substituted 20-mer peptides derived from the fibrinogen α chain, vimentin, and filaggrin were synthesized (Sigma-Genosys, The Woodlands, TX).

In vitro citrullination. Keratin, fibrinogen, and vimentin were citrullinated using rabbit muscle PAD (Sigma) as described previously (18). Successful citrullination was confirmed by Western blotting using rabbit anticitrulline antibodies (Upstate Biotechnology, Lake Placid, NY).

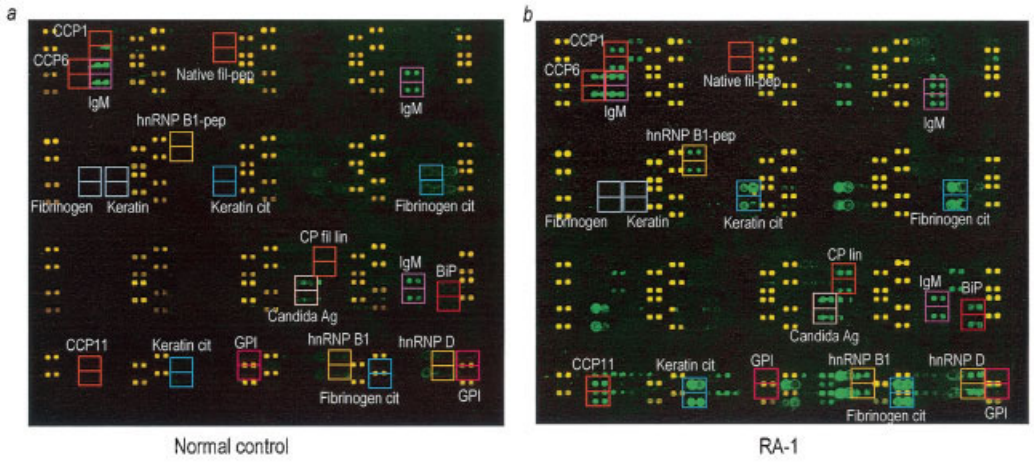
Antibodies. Monoclonal antibodies were purchased from Sigma (anti-Hsp65 and anti-Hsp70) or were generated (by WJVV) in one of our laboratories (anti-La).

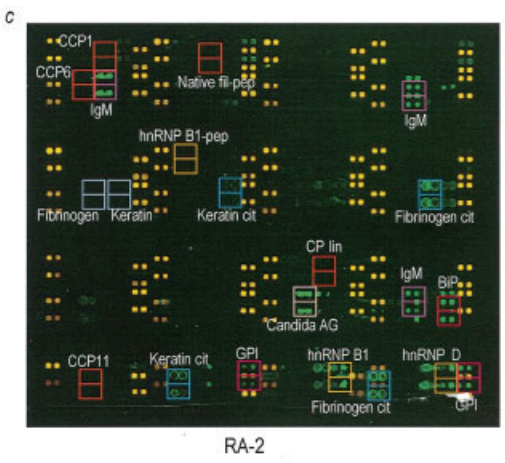
Sera. All sera were collected under institutional review board–approved protocols and after provision of informed consent from the study subjects. The Stanford Arthritis Center samples were derived from 18 patients with established RA according to the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) revised criteria for the classification of RA (19), 27 patients with arthritis in the setting of other autoimmune and nonautoimmune conditions, including systemic lupus erythematosus (SLE), ankylosing spondylitis, psoriatic arthritis, gout, and osteoarthritis, and 11 healthy controls. The Arthritis, Rheumatism, and Aging Medical Information System (ARAMIS) cohort comprised 58 randomly selected serum samples from the 793 patients in the ARAMIS early RA inception cohort (20). These samples were obtained from patients with a clinical diagnosis of RA of <6 months duration. Reference sera were provided for anti-CCP reactivity (by WJVV) and for anti–hnRNP-B1, anti–hnRNP-D, and anti-Ro52/La reactivity (by J. S. Smolen and G. Steiner).

Production of antigen microarrays. Antigens were diluted to 0.2 mg/ml in phosphate buffered saline (PBS) or water and robotically attached in ordered arrays on derivatized poly-L-lysine–coated glass slides (CEL Associates, Pearland, TX) or ArrayIt SuperEpoxy slides (TeleChem International, Sunnyvale, CA) as described previously (21). Individual antigen features had an average diameter of 200 μm.

Probing and scanning of autoantigen arrays. Arrays were circumscribed with a hydrophobic marker pap pen, blocked overnight with PBS containing 3% fetal calf serum and 0.05% Tween 20 (Sigma), and probed with 300 μl of 1:150 dilutions of RA or control patient serum, followed by washing and incubation with a 1:4,000 dilution of Cy3-conjugated goat anti-human IgG/IgM secondary antibody (Jackson ImmunoResearch, West Grove, PA). Arrays were scanned using the GenePix 4000 scanner, and the median pixel intensities of the features and background values were determined using GenePix Pro version 3.0 software (Molecular Devices, Union City, CA).

Synovial array data analysis. Results of synovial arrays were expressed as normalized median net digital fluorescence





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Quantitative analysis of array reactivities

Antigen	Normal	RA-1	RA-2
CCP1 (cyc 112-15)	10	4,575	215
Native filaggrin-pep	10	10	91
CP lin (lin 0139-32)	10	1,389	10
CCP6 (Ala-6)	264	3,183	126
CCP11 (Ala-11)	64	4,271	32
BiP	905	1,334	5,268
Candida Ag	1,308	3,341	31,809
hnRNP B1	760	2,335	2,887
hnRNP D	855	4,253	3,316
hnRNP B1-pep	171	4,253	220
GPI	245	256	4,912
Fibrinogen	10	40	10
Fibrinogen cit	41	8,109	1,044
Keratin	20	14	77
Keratin cit	287	3,874	880
IgM	15,000	15,000	15,000

Figure 1. Representative results of synovial arrays. A robotic microarrayer was used to print peptides and proteins representing candidate rheumatoid arthritis (RA) autoantigens in ordered arrays on derivatized microscope slides. Arrays were probed with 1:150 dilutions of patient or control sera followed by Cy3-labeled anti-human IgG/IgM detection antibody prior to scanning. Green fluorescence indicates reactive antibodies, while yellow fluorescence indicates marker features used to orient the arrays containing Cy3- and Cy5-prelabeled bovine serum albumin **(a–c)**. Individual arrays were probed with normal control serum **(a)** or serum from 2 RA patients **(b** and **c)**. Quantitative analysis of the array reactivities was carried out in these same samples (positive values, in median net digital fluorescence units, are highlighted) **(d)**. CCP = cyclic citrullinated peptide; cit = citrullinated; fil = filaggrin; pep = peptide; Ag = antigen; GPI = glucose-6-phosphate isomerase; CP lin = citrullinated linear peptide; hnRNP = heterogeneous nuclear RNP.

units, representing the median values from 4–8 identical antigen features on each array normalized to the median intensity of 12–20 anti-IgM features. Significance analysis of microarrays (SAM) (22) was used to identify antigens with statistically significant differences in array reactivity between groups of patients with different diagnoses and between subgroups of patients with early RA. Using SAM, each antigen was ranked on the basis of differences in mean array reactivity between the groups, divided by a function of the standard deviation, and then repeated measurements between groups were permuted to estimate a false discovery rate (FDR) for each antigen. Normalized median array values were mathematically adjusted and inputed into SAM, with selection of results on the basis of various criteria for the respective experiments. SAM results were arranged into relationships using Cluster software, and the results from the Cluster analysis were displayed using TreeView software (23).

For diagnostic class prediction, the prediction analysis

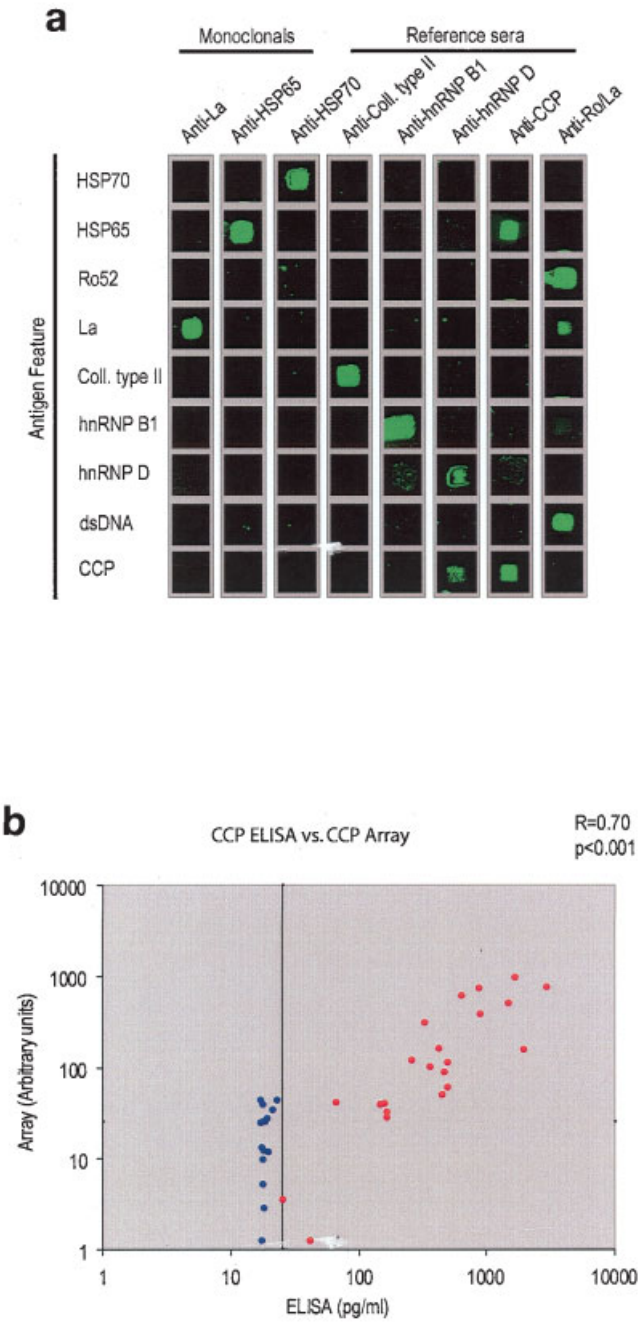

Figure 2. Validation of synovial arrays. Individual arrays were incubated with monoclonal antibodies specific for La, Hsp65, or Hsp70, a polyclonal antibody specific for type II collagen (coll.), or reference sera specific for heterogeneous nuclear RNP (hnRNP) B1, hnRNP-D, cyclic citrullinated peptide (CCP), or Ro/La. Antigen features from individual arrays were cut and pasted into columns to facilitate visual analysis (**a**). Array and commercial CCP-2 enzyme-linked immunosorbent assay (ELISA) results for detection of anti-CCP antibodies in rheumatoid arthritis patients derived from the Arthritis, Rheumatism, and Aging Medical Information System inception cohort were compared (**b**). Blue dots represent samples negative by CCP-2 ELISA, and red dots represent samples positive by CCP-2 ELISA. dsDNA = double-stranded DNA.

of microarrays (PAM) (2005, version 2.0 [22]) algorithm was applied to synovial array results from the Stanford Arthritis Center cohort. PAM was used to identify a panel of antibody reactivities that characterized the diagnostic class of RA, and errors were estimated via crossvalidation. In a second analysis, we trained the PAM on array results from CCP-2–positive versus CCP-2–negative (by enzyme-linked immunosorbent assay [ELISA]) RA patients from one-half of the ARAMIS early RA sample set (n = 29), and then used the second half of the samples (n = 29) as a test set for subset class prediction. (More technical details on sample classification by PAM can be found at the Web site http://www-stat.stanford.edu/~tibs/PAM/.)

ELISA. An ELISA kit (Immunoscan RA Mark 2; Euro-Diagnostica, Malmoe, Sweden) was used to detect CCP-2, carried out in accordance with the specifications of the manufacturer.

RESULTS

Synovial arrays for multiplex analysis of autoantibodies in RA. The 1,536-feature synovial antigen arrays contained 225 peptides and proteins that represent candidate autoantigens in RA. Printed antigens included native and in vitro–citrullinated keratin, filaggrin (7), vimentin (18), and fibrinogen (24), as well as Hsp60, Hsp65, Hsp70, Hsp90 (25), BiP (14), GPI (15), types I, II, III, IV, and V collagen (26), hnRNP-A2/B1 (RA33) (27), and human cartilage gp39 (28). Arrays also included peptides representing native human cartilage gp39, hnRNP-B1, and native and citrullinated epitopes derived from filaggrin, vimentin, and fibrinogen. These candidate antigens were robotically attached in ordered arrays to the surface of derivatized microscope slides, on which the binding of serum autoantibodies was detected.

We used synovial array technology to profile autoantibody responses in serum derived from RA patients and control subjects; digital images of representative arrays are presented in Figures 1a–c. Arrays incubated with sera derived from 2 RA patients revealed heterogeneity in the specificities of their autoreactive B cell responses. Autoantibodies from one of the RA patients recognized citrullinated, but not native, keratin and fibrinogen and several citrulline-substituted filaggrin peptides (CCPs 1 and 11, and a citrullinated linear peptide; no reactivity was observed against a corresponding unmodified [native] linear filaggrin peptide) (Figures 1b and d). Reactivities against the endoplasmic reticulum molecular chaperone BiP and the nuclear spliceosome proteins hnRNP-B1 and hnRNP-D were also observed. In contrast, the second RA patient exhibited minimal reactivity against citrullinated epitopes (low-level reactivity detected against citrullinated fibrinogen only), but did exhibit reactivity against BiP, hnRNP-B1 and hnRNP-D, and GPI (Figures 1c and d). Serum from a healthy control subject did not exhibit

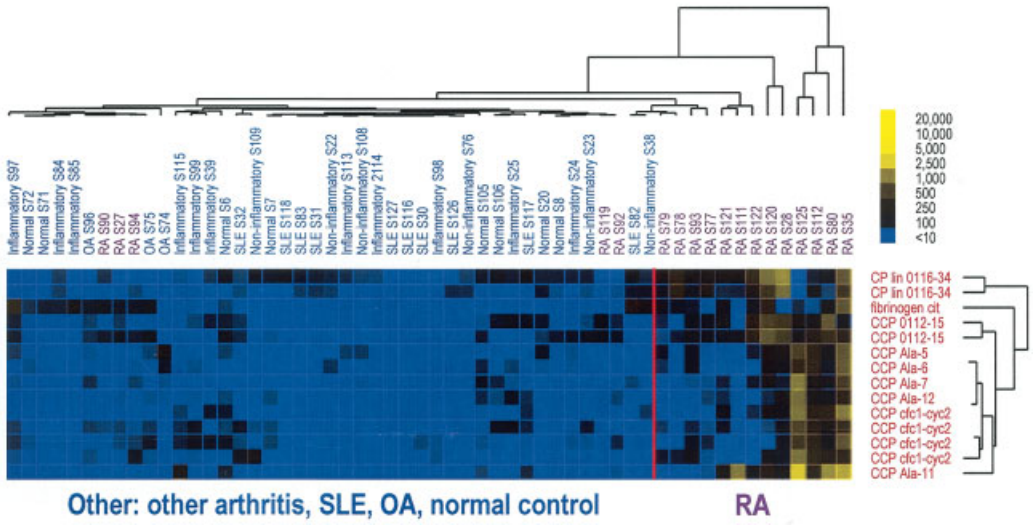

Figure 3. Delineation of a subpopulation of patients with rheumatoid arthritis (RA) by autoantibody targeting of citrullinated epitopes. Autoantibody reactivity was determined by synovial arrays in 18 RA and 38 control serum samples from the Stanford Arthritis Center. Significance analysis of microarrays followed by a hierarchical clustering algorithm were used to distinguish and order patient samples into cluster relationships based on similarities in their antigen reactivities (right dendrograms) and similarities in reactivities in the individual patient samples (top dendrograms) (false discovery rate <0.035, numerator threshold 1.35). Branch lengths on the dendograms represent the extent of similarities in array reactivity, and the legend bar in the upper right indicates the general locations of the clusters of RA and control patients. SLE = systemic lupus erythematosus; OA = osteoarthritis; CP lin = citrullinated linear peptide; fibrinogen cit = citrullinated fibrinogen; CCP = cyclic citrullinated peptide.

reactivity against these candidate autoantigens (Figures 1a and d).

Validation of synovial arrays. To validate the synovial arrays, individual arrays were incubated with monoclonal antibodies, polyclonal antibodies, or reference sera specific for 8 candidate autoantigens, including La, Hsp65, Hsp70, type II collagen, hnRNP-B1, hnRNP-D, CCP, and Ro/La. These antibodies demonstrated specific detection of their corresponding antigen features (Figure 2a). Antibodies in the anti-CCP reference serum derived from a patient with RA also recognized Hsp65, and antibodies in the anti-Ro/La reference serum (derived from a patient with SLE) also recognized double-stranded DNA.

To further validate the synovial arrays, we compared array-determined anti-CCP antibody reactivity with ELISA-determined anti-CCP antibody reactivity in serum samples derived from the ARAMIS early arthritis cohort (Figure 2b). The correlation coefficient between the array (CCP-1 antigen feature) and the gold-standard commercial ELISA (CCP-2 peptide, which is not available for printing on arrays) was 0.70 ($P < 0.001$).

Delineation of a subpopulation of RA patients by autoantibody targeting of citrullinated epitopes. We used synovial arrays to profile autoantibody reactivity in samples derived from RA patients (n = 18) and control subjects (n = 38) in the Stanford Arthritis Center sample bank. The diagnosis of RA was based on the ACR revised classification criteria (19). The control group comprised samples from patients with a variety of autoimmune and nonautoimmune arthritic diseases and 11 healthy controls. The SAM algorithm (22) was applied to determine the antigen features with statistically significant differences in array reactivity between the RA patients and controls. Among the 225 antigens on synovial arrays, SAM identified 14 antigens, including citrullinated fibrinogen and 13 citrulline-substituted peptides, that detected statistically increased antibody reactivity in RA patients. A hierarchical cluster algorithm using a pairwise similarity function (23) was used to order patients and these SAM-identified antigen "hits" on the basis of the degree of similarity of their autoantibody reactivity profiles (Figure 3).

A subset of RA patients who demonstrated antibody reactivity against the citrullinated peptide and fibrinogen array "hits" were identified in clusters (Figure 3). Serum autoantibodies from a different subset of RA patients and the control subjects did not recognize these citrulline-modified antigens; these are interspersed over the remainder of the cluster image in Figure 3. Several other autoantibody specificities were detected on arrays, albeit at lower frequencies and

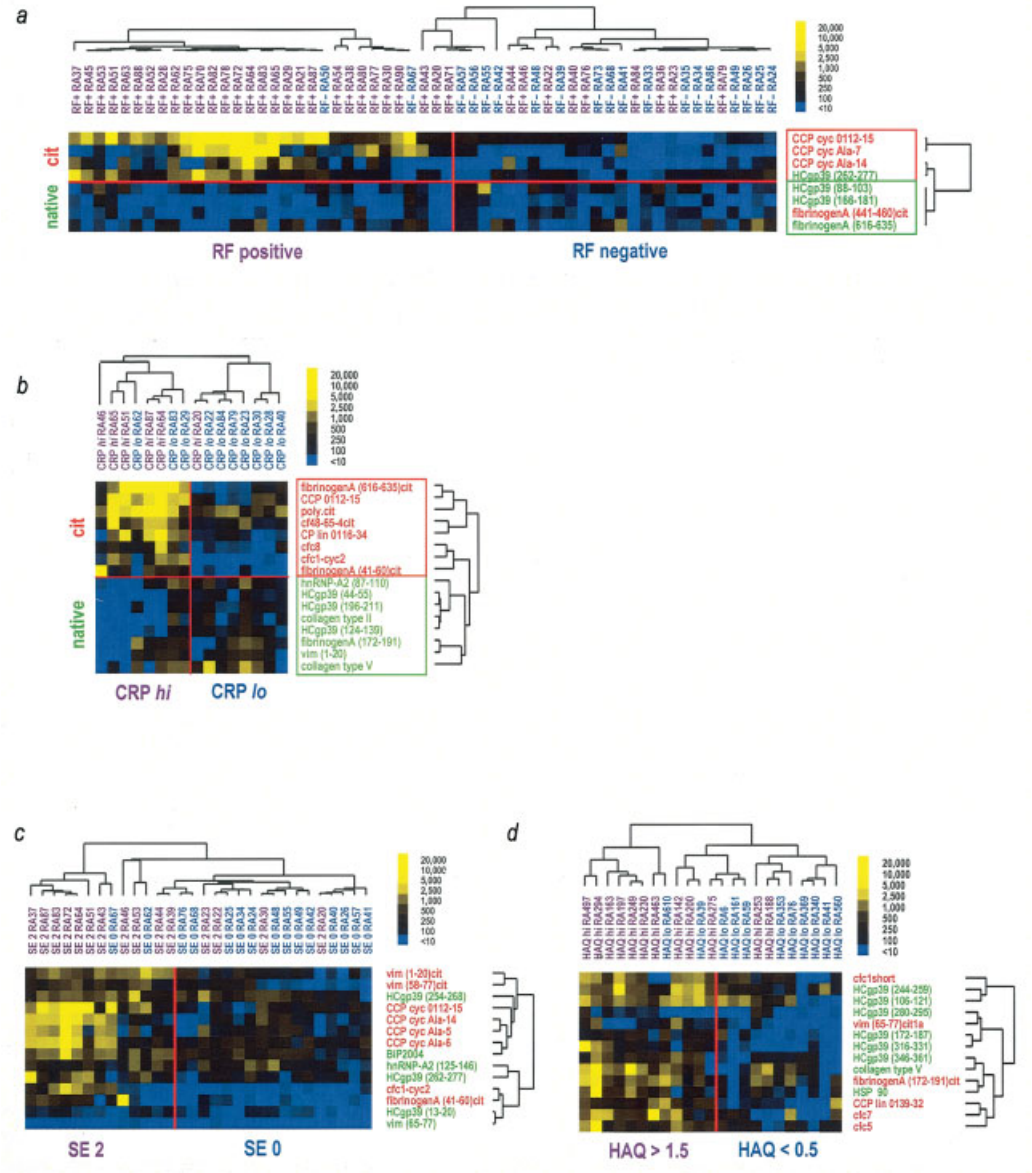

Figure 4. Association of more severe rheumatoid arthritis (RA) with autoantibody targeting of citrullinated epitopes. Pairwise significance analysis of microarrays (SAM) was performed to identify antigen features having statistically significant differences in synovial array reactivity that were associated with laboratory and clinical parameters previously identified to provide diagnostic and prognostic value. Specific analyses include comparisons of RA patients who were rheumatoid factor (RF) seropositive and RF seronegative **(a)**, female RF-seropositive RA patients with serum C-reactive protein (CRP) levels ≤0.5 mg/dl (lo) and ≥1.5 mg/dl (hi) **(b)**, female RA patients who possessed 2 copies of the shared epitope (SE) and no copies **(c)**, and female RA patients with baseline Health Assessment Questionnaire (HAQ) disability scores of ≤0.5 and ≥1.5 **(d)**. Hierarchical clustering was applied to arrange the patients and SAM-identified antigen features (dendrograms on the top and right, respectively), and the legend bar in the upper right of **a–d** indicates the general locations of the clusters of RA patients possessing the laboratory or clinical feature. Citrullinated antigens (cit) are shown in red type and native antigens are shown in green type (false discovery rate [FDR] <0.07, numerator threshold 1.0 in **a–c**; FDR <0.11, numerator threshold 2.0 in **d**). CCP = cyclic citrullinated peptide; HC = human cartilage; poly.cit = polycitrulline; CP lin = citrullinated linear peptide; hnRNP = heterogeneous nuclear RNP; vim = vimentin; HSP = heat-shock protein.

Table 1. Baseline characteristics of the ARAMIS patients with early rheumatoid arthritis (n = 58)*

Age, median (range) years	53.5 (19–78)
Female sex, no. (%)	45 (77)
RF positive, no. (%)	38 (65)
CRP, median (range) mg/dl	0.50 (0.09–15.7)
Median (range) disability score	1.125 (0–2.375)
Median (range) education level	12 (8–17)
DMARD treatment, no. (%)	24 (41)
Shared epitope present, no. (%)	40 (69)

* ARAMIS = Arthritis, Rheumatism, and Aging Medical Information System; RF = rheumatoid factor; CRP = C-reactive protein; DMARD = disease-modifying antirheumatic drug.

considerably lower specificity than the anticitrulline autoreactivity (results not shown).

We applied the PAM algorithm (22) for class prediction analysis within the Stanford Arthritis Center cohort of patients with a variety of rheumatic diseases. Based on the data sets obtained by synovial arrays, PAM identified a panel of 17 citrulline-substituted peptides whose antibody reactivities provided class prediction for the diagnosis of RA, at a sensitivity of 56% and a specificity of 97.5% in crossvalidation analysis.

Association of citrullinated epitope targeting with features predictive of severe arthritis. Combinations of 2 or 3 autoantibody reactivities provide greater predictive information than that provided by individual specificities in SLE, MS, T1D, and RA patients (5,29,30). To determine whether profiles of autoantibodies have greater utility in RA, we used synovial arrays to identify autoantibody profiles having an association with those clinical and laboratory parameters that have been linked to differences in disease severity and outcomes. Synovial array analysis was performed on serum samples from 58 randomly selected patients in the ARAMIS early arthritis inception cohort, from whom serum samples were obtained within 6 months of disease onset. The baseline characteristics of these patients (Table 1) were comparable with those of the overall cohort of 793 patients. Among the 58 randomly selected samples, 36 (62%) exhibited array reactivity against at least 1 citrullinated peptide or protein antigen, and 33 (57%) showed positive CCP-2 reactivity on ELISA. These results are consistent with the observations from other studies (2), showing that citrullinated epitopes are the targets of the autoimmune response in a substantial fraction of both patients with early RA and patients with established RA.

We performed pairwise comparisons of subgroups of RA patients to identify any associations of the autoantibody profiles with clinical and laboratory parameters that are predictive of a more severe disease course, including seropositivity for rheumatoid factor (RF) (31), elevations in the inflammation marker C-reactive protein (CRP) (31,32), presence of the HLA–DR4 shared epitope (SE) polymorphism (33), and increased baseline disability as assessed by the Health Assessment Questionnaire (HAQ) (34). Pairwise comparisons of synovial array results between RF-seropositive and RF-seronegative RA patients demonstrated increased targeting of citrullinated epitopes, including citrulline-substituted filaggrin peptides, in the RF-seropositive patients (Figure 4a).

To reduce heterogeneity within the subgroups of samples being compared, the CRP, SE, and HAQ analyses were performed in female subgroups or in female RF-seropositive subgroups of RA patients. Female RF-seropositive RA patients with CRP levels ≥1.5 mg/dl, as compared with female RF-seropositive patients with CRP levels ≤0.5 mg/dl, showed increased targeting of citrullinated epitopes (Figure 4b). In female RA patients possessing 2 copies of the SE polymorphism, as compared with female patients who did not possess the SE polymorphism, we observed autoantibody targeting of citrullinated epitopes derived from filaggrin, vimentin, and fibrinogen (Figure 4c). A few native antigens, including the heat-shock protein BiP, the hnRNP(125–146) peptide, and 3 native gp39 peptides, were also identified as autoimmune targets in the subgroup of patients with 2 copies of the SE (Figure 4c). Finally, synovial arrays demonstrated increased targeting of citrullinated epitopes in female RA patients who had high self-reported baseline disability (HAQ score ≥1.5), as compared with patients who had low self-reported baseline disability (HAQ score ≤0.5) (Figure 4d).

Association of differential targeting of citrullinated and native epitopes with predictors of disease severity. The SAM and cluster analyses performed to identify autoantibody profiles associated with RF-seropositive disease and elevated CRP levels also revealed differential targeting of the citrullinated and native epitopes contained on synovial arrays. Whereas in the RF-seropositive RA patients, citrullinated epitopes were preferentially targeted, in the RF-seronegative subgroup, low, but increased, reactivity against native epitopes derived from human cartilage gp39 and fibrinogen was evident (Figure 4a). In addition, whereas seropositive female patients with high CRP levels (≥1.5 mg/dl) possessed autoantibodies exclusively targeting citrullinated epitopes, the low-level CRP (≤0.5 mg/dl) subgroup possessed autoantibodies targeting native epitopes derived from human cartilage gp39, collagens, vimentin, and fibrinogen (Figure 4b). Within the antibody signatures that were inversely associated with a

high inflammation score, several autoantibody specificities were directed against native epitopes (vimentin[1–20], fibrinogen A[172–191], and fibrinogen A[616–635]) derived from antigens in which citrullinated epitopes are also targeted. On the basis of the antigens represented on these synovial arrays, our results suggest that citrullinated epitopes are predominantly targeted by the immune response of patients with high-grade joint inflammation, whereas reactivity against native peptides derived from the same and other proteins (type II collagen and human cartilage gp39) are associated with low-grade inflammation.

Correlation of autoantibodies against citrullinated peptides in patients with early RA. We calculated correlation coefficients for autoantibody reactivities against panels of citrullinated peptides within patients with early RA and found correlations to be highest for 7 cyclic peptides (R = 0.66, 95% confidence interval [95% CI] 0.59–0.72, $P < 0.0001$). Six strongly reactive peptides derived from vimentin, fibrinogen, and filaggrin showed a moderate correlation (R = 0.42, 95% CI 0.31–0.51, $P < 0.0001$), whereas the correlation among the complete panel of citrullinated peptides was the lowest (R = 0.30, 95% CI 0.25–0.35, $P < 0.0001$), indicating considerable individual heterogeneity in the reactivities against citrullinated epitopes among patients with early RA.

Subset prediction by PAM in patients with early RA. We also performed PAM analysis on the data set from synovial array analysis of patients with early RA in the ARAMIS cohort. After training on one-half of the sample set (n = 29) to identify a panel of array reactivities that distinguished the ELISA CCP-2–positive from the ELISA CCP-2–negative patients, the array results from the other half of the RA patients (n = 29) were used for prediction analysis. PAM correctly classified 88% of ELISA CCP-2–positive and 73% of ELISA CCP-2–negative RA patients in this test set. Array reactivities predictive of CCP-2–negative status included those against types I, II, and III collagen, hnRNP-A2(90–116), human cartilage gp39(166–181), and human cartilage gp39(284–301).

DISCUSSION

In this report, we describe the development of synovial antigen microarrays and its application to the profiling of sera derived from RA patients and control subjects. We identified patterns of differential antigen recognition that were associated with clinical subtypes of RA. Autoreactivity directed against human cartilage gp39, type II collagen, and other native autoantigens delineated a subpopulation of patients with early RA who had laboratory and clinical features predictive of mild arthritis. In contrast, citrullinated epitopes were preferentially targeted by autoantibodies in patients who possessed laboratory and clinical features predictive of severe arthritis. These data suggest that heterogeneity in the specificity of the autoreactive B cell responses among RA patients is associated with differences in disease manifestations and outcomes.

Synovial array analysis of autoantibodies demonstrated reactivity against multiple autoantigen candidates that have been previously described in the literature, including citrullinated fibrinogen, citrullinated vimentin, citrulline-substituted filaggrin peptides, hnRNP-A2/B1, BiP, type II collagen, and several heat-shock proteins. We also observed array reactivity against human cartilage gp39 peptides, against which B cell reactivity was not previously described. Reactivity against human cartilage gp39 peptides and native epitopes from fibrinogen, vimentin, and type II collagen was predominantly observed in patients possessing clinical and laboratory features predictive of less severe disease (Figures 4a–c). Human cartilage gp39 is a known target of T cell responses in RA (28,35), and this observation warrants further investigation of the role of human cartilage gp39 and other native antigens in the subpopulation of RA patients with features associated with less severe disease.

The most prominent reactivities in both serum sample sets were antibodies against citrullinated peptides and proteins. This observation was not surprising, since many publications have confirmed the original discovery by Schellekens et al (7) and Girbal-Neuhauser et al (36) that anticitrullinated epitope autoantibodies are a hallmark of RA. Many of the fibrinogen and vimentin epitopes (native and citrullinated) that we identified to be predominant targets of autoreactive B cell responses in RA were not described previously. Frequencies of anti-CCP antibodies as measured by commercial CCP-2 ELISA in the ARAMIS early RA inception cohort were 7% lower in comparison with the overall frequency of anticitrulline reactivity observed on arrays (Figure 2b).

In the ARAMIS early RA cohort, there was only moderate correlation of autoantibody reactivity against the spectrum of citrullinated epitopes represented on synovial arrays (R = 0.30, 95% CI 0.25–0.35, $P < 0.0001$). These data suggest that significant heterogeneity exists between patients with early RA in the specificity of their autoreactive B cell responses against citrullinated proteins.

Positivity for RF, possession of the SE polymorphism, high CRP values, and high HAQ disability scores have been associated with more severe RA (32,34,37). The SE polymorphism is also associated with RF seropositivity and anti-CCP antibodies (38). Synovial array analysis demonstrated that patients with 2 copies of the SE polymorphism had increased autoantibody reactivity against panels of citrulline-substituted peptides derived from fibrinogen, vimentin, and filaggrin as well as native epitopes derived from BiP, hnRNP-A2, and human cartilage gp39 (Figure 4c). We also observed distinct clusters of reactivity against citrullinated proteins and citrulline-substituted peptides in patients with high baseline CRP values (≥1.5 mg/dl), as compared with patients with low baseline CRP values (≤0.5 mg/dl) in whom native (noncitrullinated) peptides and collagens were targeted (Figure 4b).

An important limitation of the present study is the small number of antigens represented on the synovial arrays relative to the expressed proteome of a rheumatoid joint. It is likely that additional native antigens are targeted in RA, perhaps in both the low- and high-grade inflammation subgroups. Such reactivities may not have been detected due to the limited number of antigens represented on the synovial arrays used for these experiments.

Solid-phase immunoassays are prone to interference by total serum immunoglobulins and by the presence of RF. Total serum immunoglobulin and RF are factors relevant not only to antigen microarrays, but also to all solid-phase immunoassays, including CCP ELISA. We obtained the following data regarding anticitrulline peptide reactivity on arrays and anti-CCP reactivity in ELISA to suggest that the array reactivities observed are highly consistent with the results reported by other investigators in studies using the CCP ELISA. In our cohort of patients with early RA, there was considerable overlap between positivity for RF and CCP reactivities. Nevertheless, in our described data sets, 2 (11%) of 19 RF-negative patients tested strongly positive for a citrullinated filaggrin peptide that exhibited robust array performance (CCP 0112–15). These 2 patients and 1 additional patient (16%) tested positive by the commercial CCP2 ELISA (which uses a different peptide). In a seminal study in early RA, Goldbach-Mansky et al described significant overlap between anticitrulline autoantibodies and RF, and found that only 5 (14%) of 36 RF-negative RA patients tested positive by CCP ELISA (39). We also observed a lack of citrullinated peptide reactivity in a fraction of RF-positive patients (16% were CCP-2 negative by ELISA; 8% were citrullinated peptide negative by array). Together, these data demonstrate that at least a subset of the anticitrulline-modified peptide reactivity in RA is not due to RF, and that our antigen microarray results regarding antibody reactivity against citrulline-modified peptides are highly consistent with both the results from our laboratory and the findings of others using commercial CCP ELISAs.

Our results confirm and extend the recent observations reported by van Gaalen and colleagues (2) on the association of autoreactivity against citrullinated peptides and early RA. Whether citrullination of various proteins found in synovial tissue (i.e., vimentin, fibrinogen) plays a pathogenic role in subsets of RA patients or merely represents epiphenomena remains to be determined. In immunohistochemical studies, citrullinated proteins were found to be highly abundant in the joint tissue of patients with RA (40,41). However, increased citrullination is a posttranslational modification not specific to the inflamed joints of RA patients, since it is also observed in the brains of patients with MS (42). Hypothetical explanations for the loss of tolerance to citrullinated molecules in RA as compared with other inflammatory conditions include excessive or aberrant citrullination of synovial proteins, inability to maintain immune tolerance against citrullinated proteins, and generation of immune repertoires capable of targeting citrullinated epitopes (43).

Major challenges in the diagnosis and treatment of RA remain. Key issues include 1) the insufficient accuracy in the diagnosis of RA during its early stages, 2) the lack of adequate predictors of severity early in the disease, 3) the lack of predictors of differential responses to disease-modifying antirheumatic drug therapy, and 4) the safety and efficacy of expensive new biologic therapies (44). The findings presented herein suggest that autoantibody-based classification of early RA represents a promising approach for the elucidation of subtypes of RA that reflect differential disease mechanisms and activity, which could ultimately lead to differentiation of treatment requirements.

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