Parallel Detection of Autoantibodies with Microarrays in Rheumatoid Diseases

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Background: Clinical needs often dictate testing for several autoantibodies in a single patient with evidence of autoimmune disease. We developed a microarray containing 15 autoantigens for the detection of autoantibodies in rheumatoid autoimmune diseases.

Methods: We synthesized recombinant centromere protein B, cytokeratin 19, SSA 52-kDa antigen, SSA 60-kDa antigen, SSB antigen, and Jo-1 antigen and prepared anti-nuclear antibody antigens. Cyclic citrullinated peptide, histone, goat IgG for detection of rheumatoid factor, double-stranded DNA, and single-stranded DNA were purchased, as were recombinant small nuclear ribonucleoprotein U1, topoisomerase I, and Smith antigen (Sm). All 15 antigens were of human origin except calf thymus Sm. Proteins were printed on polystyrene. The arrays were incubated with serum samples and then with horseradish peroxidase-conjugated secondary antibodies and chemiluminescent substrates, and light signals were captured by a charge-coupled device camera-based chip reader. Antibodies were quantified by use of calibration curves. Positive samples were confirmed by commercially available methods.

Results: The detection limit of the microarray system was 20 pg of IgG printed on the polystyrene support. More than 85% of the confirmed positive sera were detected as positive with the microarray system based on cutoff values established with the microarray system. The imprecision (CV) of the microarrays was <15% for all 15 autoantibody assays, with the exception of single-stranded DNA (18% and 23%) within and between batches. Characteristic autoantibody patterns were seen in patients with clinical diagnoses of rheumatoid arthritis (n = 83), systemic lupus erythematosus (n = 71), systemic sclerosis (n = 36), polymyositis (n = 38), and Sjogren syndrome (n = 20).

Conclusions: This microarray system provides results similar to those by conventional methods. Assessment of the diagnostic accuracy of the system remains to be done.

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Autoimmune diseases affect an estimated 3–5% of the total population *(1)*, with the highest prevalence in the elderly *(2, 3)*. The common feature in this group of diseases is the presence of autoantibodies, and the pattern of antibodies present is used for distinguishing among the disorders in this group *(4)*. Rheumatoid disorders are difficult to recognize in the early stage, but in late stages of the diseases, multiple organs can be affected and damage can be irreversible. Thus, sensitive and comprehensive testing is needed.

Commonly used laboratory methods for the detection of autoantibodies in the serum include indirect immunofluorescence microscopy and immunochemical assays *(5)*. Indirect immunofluorescence microscopy traditionally uses various rodent tissues for reaction with the serum samples. A major drawback of this approach is that it does not define the specific antigen and thus is unable to provide information for the design of more specific treatment strategies. Advances in molecular biology have contributed to the identification of autoantigens and allowed their production, and thus have made assays for them feasible. However, to date each autoantibody has been measured with a separate assay, and results obtained from different assay systems are not interchangeable.

In recent years, biochip/microarray technology has become a powerful tool for parallel analysis. Nucleic acid-based arrays have been highly effective in studies of gene expression and human disease *(6–8)*. Protein, peptide, and carbohydrate arrays have also been fabricated *(9–15)*. Microarrays have been reported for the ToRCH infectious antigens *(16)* and for allergens *(17, 18)*, 18

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autoantigens *(19)*, and for 196 putative autoantigens relevant to autoimmune diseases *(20)*.

We describe here a microarray system for parallel detection of autoantibodies in rheumatoid diseases, and preliminary studies of the patterns of autoantibody positivities seen in patients who carried clinical diagnoses of autoimmune diseases.

Materials and Methods

ANTIGENS, ANTIBODIES, AND REAGENTS

We selected 15 autoantigens for this study. Centromere protein B (CENP-B),[3] cytokeratin 19 (CK19), SSA 52-kDa antigen (SSA/52), SSA 60-kDa antigen (SSA/60), SSB antigen, and Jo-1 antigen were produced in this laboratory with recombinant techniques in a bacterial expression system. Anti-nuclear antibody antigens (ANAs) were prepared in this laboratory. Cyclic citrullinated peptide (CCP) was synthesized commercially. Histone, goat IgG for detection of rheumatoid factor (RF), double-stranded DNA (dsDNA), and single-stranded DNA (ssDNA) were purchased from Sigma. Small nuclear ribonucleoprotein U1 (U1snRNP), topoisomerase I (Scl-70), and Smith antigen (Sm) were bacterial recombinant proteins purchased from DiaRect AG. All antigens were of human origin except Sm, which was from calf thymus. Glutathione *S*-transferase (GST) was produced in this laboratory with recombinant techniques in a bacterial expression system and included in the microarray as a negative control.

Goat anti-human IgG polyclonal antibody was purchased from Sigma. Horseradish peroxidase (HRP) and the Super Signal ELISA chemiluminescent substrate were purchased from Pierce. Antibody conjugation was according to a previous report *(21)*. Assays for detection of various autoantibodies were purchased from EuroImmun AG, IMTEC Immundiagnostika, SCIMEDX, Aesku Lab Diagnostika, and BioSystems. Other reagents were all analytical grade. The charge-coupled device (CCD) camera was from Roper Scientific. Blood samples were collected from patients at local hospitals with consent for testing the sensitivity and specificity of the microarray. We used the patients' clinical diagnoses, which were based on presenting symptoms and laboratory tests performed by existing methods. We did not attempt to confirm the diagnoses according to international standards for this preliminary study of immunoreactivity by microarrays. All tests were done by physicians or trained technicians.

MICROARRAY FABRICATION

We arrayed 20 nL of recombinant GST (0.15 g/L); human IgG solutions at concentrations of 1, 2, 4, 8, and 16 mg/L; and each of the 15 autoantigens with concentrations ranging from 0.04 (CCP) to 0.3 g/L (dsDNA) in polystyrene wells in duplicate, using a Cartesian ink jet printer, the GT5000 Gantry System. After spotting, the wells (microarrays) were sealed and left at room temperature overnight, and blocked next day with Tris-buffered saline-Tween (TBST; containing, per liter, 8.5g of NaCl, 0.1 mol of Tris, and 1 mL of Tween 20, pH 7.6) containing 100 g/L fetal bovine serum and 40 g/L sucrose for 30 min at 37 °C. The microarrays were dried at room temperature and stored at 4 °C until use.

PROCESSING OF THE MICROARRAY

Each array was incubated with 200 μL of serum (diluted 1:50 with TBST) for 30 min at 37 °C. After being rinsed and washed three times for 2 min with TBST, the array was incubated with 200 μL of HRP-labeled goat anti-human IgG antibody for 30 min at 37 °C. After another rinse and three 2-min TBST washes, 140 μL of chemiluminescence substrate was added and allowed to react for 1 min. Light signals were captured with a in-house-constructed ChipReader based on a CCD camera and controlled with a computer system using in-house-developed software. Commercial assays with serum samples were performed according to the manufacturers' instructions.

DATA ANALYSIS

The human IgG internal calibration curve was fitted with a linear curve fit (Excel; Microsoft). The amounts of autoantibodies in the sera were obtained by interpolating the relative light units collected by the ChipReader with the IgG internal calibration curve on the chip. Because the HRP-labeled goat anti-human IgG antibody that was used in this study cross-reacted with IgM, the autoantibodies values obtained can be considered a combination of both IgG and IgM. Because results obtained from different current assay systems are not interchangeable, the cutoff values used in this study were determined with the microarray based on a 95th percentile obtained with 800 samples obtained from healthy individuals. Reactivities of patient samples were defined as positive when autoantibody values were above the cutoffs or negative when autoantibody values were below the cutoffs.

Results

DESIGN OF THE AUTOANTIGEN MICROARRAY

The microarray was designed to screen and detect antibodies present in the most common systemic rheumatoid autoimmune diseases, including rheumatoid arthritis (RA), system lupus erythematosus (SLE), Sjogren syndrome (SS), polymyosis (PM), and systemic sclerosis (SSc). The selection of some autoantigens was based on their ability to detect disease-specific autoantibodies (Ta-

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[3] Nonstandard abbreviations: CENP-B, centromere protein B; CK19, cytokeratin 19; SSA/52, SSA 52-kDa antigen; SSA/60, SSA 60-kDa antigen; ANA, anti-nuclear antibody antigen; CCP, cyclic citrullinated peptide; RF, rheumatoid factor; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; U1snRNP, small nuclear ribonucleoprotein U1; Scl-70, topoisomerase I; Sm, Smith antigen; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; CCD, charge-coupled device; TBST, Tris-buffered saline–Tween; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjogren syndrome; PM, polymyositis; and SSc, systemic sclerosis.

Table 1. Autoantibody tests most frequently requested for each rheumatoid disease.

Disease	Most frequently requested tests
SLE	dsDNA, Sm, ANAs
SS	SSA-52, SSA/60, SSB, ANAs
SSc	Scl-70, CENP-B, ANAs
RA	RF, CCP
PM/Dermatomyositis	Jo-1, ANAs

ble 1). For example, dsDNA and Sm were selected because of their specificities for the detection of SLE. Other autoantigens selected for their relative specificities were SSA and SSB for SS, Scl-70 and CENP-B for SSc, RF and CCP for RA, and Jo-1 for PM. Some less specific autoantigens were included to increase the sensitivity of the microarray. ANAs were also added to the matrix as nonspecific markers to increase the total detection rate and as a positive control because ANA autoantibodies are found in most cases of the above-mentioned diseases. In addition, CK19 autoantibody was found in certain rheumatoid diseases in our preliminary results and was thus

Polystyrene is widely used as the support material in immunochemical assays such as ELISA. The suitability of polystyrene as a support for the fabrication of the microarray in this study was first tested with human IgG, which was used to generate the internal calibration curve. Human IgG was spotted on polystyrene wells at increasing amounts and reacted with a second goat anti-human IgG antibody, which was labeled with HRP. Chemiluminescence was generated after substrates were added, and images were captured with a CCD camera (Fig. 1A). The light signals generated increased proportionally with increasing amounts of each spotted antibody, as shown by the linear correlation between the signal and the antibody concentration (Fig. 1, B–D). Serum did not appear to interfere with the use of IgG to generate calibration curves because reactions in the absence of serum and in the presence of positive and negative sera all suggested high reproducibility and a dose–response relationship, as indicated by the r^2 values being >0.98. The proper internal
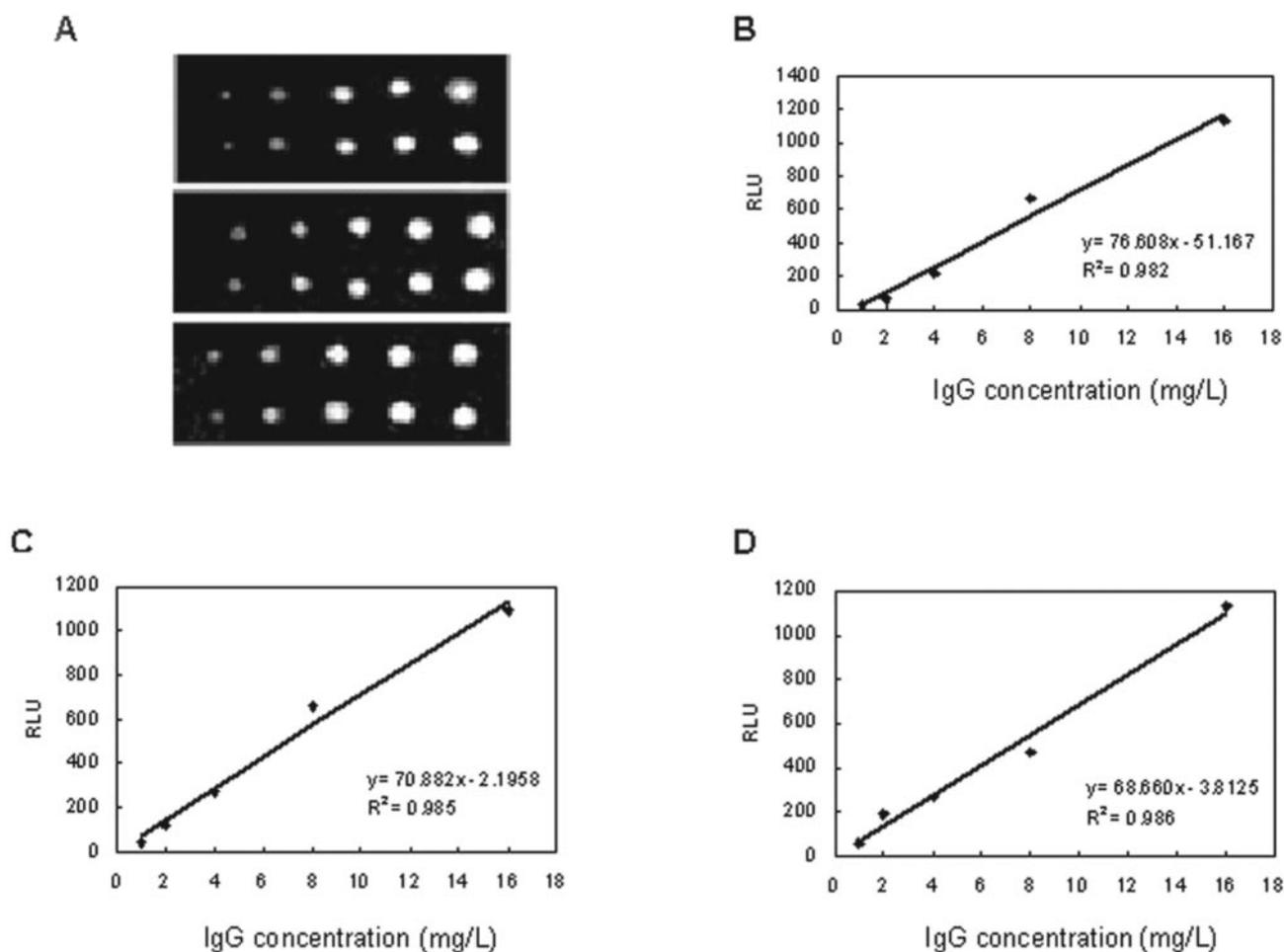

Fig. 1. Calibration curves for spotted human IgG.

RLU, relative light units. Images were captured by CCD for a sample containing the HRP-labeled detection antibody diluted with blocking buffer (*B* and *top panel* in *A*), for an autoantibody-negative serum (*C* and *middle panel* in *A*), and for an autoantibody-positive serum (*D* and *bottom panel* in *A*).
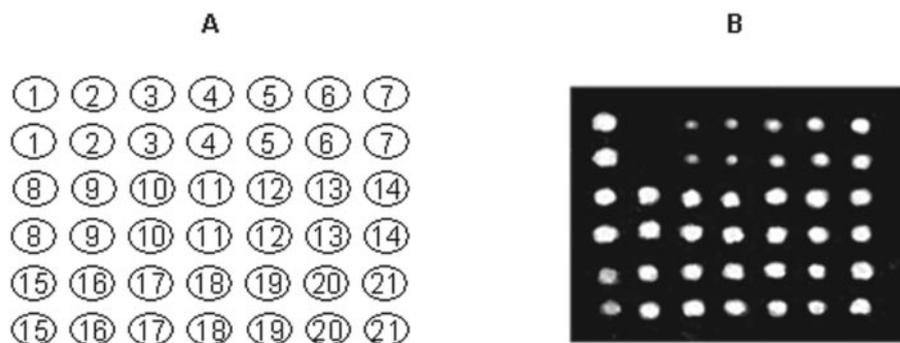
controls and autoantigens were spotted (Fig. 2A) to generate a full microarray.

SERUM REACTIVITY OF THE ANTIGEN MICROARRAY
Antibody concentrations in the sera were calculated by use of the IgG calibration curve. However, antibody concentrations calculated this way might not reflect the actual concentrations in the sera because they are different IgG species that could have different affinities for the HRP-labeled goat anti-human IgG antibody. It is also possible that autoantibodies from different patients may vary in their affinities to the same antigen, giving rise to the possibility that calculated autoantibody concentrations from different samples were not comparable. Therefore, cutoff values for the 15 autoantibodies were determined based on the 95th percentiles for sera from a healthy population. Because values obtained from different systems are not comparable, categorization of reactivities were simplified into positives and negatives only. Reactivities were defined as positive when IgG values were above the cutoffs or negative when IgG values were below the cutoffs.

Sera with confirmed positive autoantibody reactivities were mixed to produce a pooled serum sample that contained autoantibodies against all 15 antigens. This pooled serum sample was then used to investigate the ability of the array to detect autoantibodies in the sera of patients with autoimmune diseases. Shown in Fig. 2B is an example of the reaction, which showed that the microarray was able to detect every autoantibody species in the serum pool. Furthermore, various positive sera confirmed with other immunochemical methods were also used separately to test the ability of the array to detect autoantibodies. Comparison of results obtained with microarrays and other methods was based on simple positive/negative classification because actual amounts of antibodies measured with different methods are not comparable. Overall, at least 85% of the confirmed positive sera were detected as positive based on cutoff values established with the microarray system. The CV for antibody concentrations calculated with the microarrays

were <15%, with the exception of ssDNA (18% and 23%, respectively), for the autoantibodies within and between batches. All tests were performed in triplicate.

DETECTION OF AUTOANTIBODIES IN RHEUMATOID PATIENTS
The autoantigen microarray was then used for serodiagnosis of patients with autoimmune rheumatoid diseases. Blood samples from a total of 248 clinically confirmed rheumatoid patients were collected from January to June 2003 and analyzed at the end of collection period (Table 2). These patients, 16–75 years of age, had a F:M gender ratio of 60:40, showed mild to severe clinical symptoms, and received no treatment before the collection of blood samples. Shown in Fig. 3 are representative reactions of the autoantigen microarray with sera from patients diagnosed with five common autoimmune rheumatoid diseases: SLE, SS, SSc, RA, and PM. Each disease had an autoantibody pattern distinct from the others. For example, the serum from an RA patient showed a RF autoantibody that was not detected in the other samples. The SS serum shared several autoantibodies, such as SSA/60, SSA/52, and Jo-1, with the PM serum but did not have autoantibodies against U1snRNP or Scl-70. The SSc serum shared autoantibodies against SSA/52 and SSB with sera from patients with SS or PM but did not have autoantibodies against Jo-1 or SSA/60. Finally, the sera from the SLE patients also showed a pattern of autoantibodies that was distinct from the other four, none of which had Sm autoantibody.

The detection rates in the autoantigen microarray for the autoantibodies associated with the above-mentioned rheumatoid diseases, based on the cutoffs established in this study to define positive and negative diagnosis, are summarized in Table 2. The results are mostly consistent with reported detection rates and specificities (*22*). For example, a good percentage (42%) of SLE sera had autoantibodies against dsDNA as well as against SSA/52 (54%), SSA/60 (48%), and ssDNA (49%). SLE was also the only disease condition in which autoantibodies against Sm (25%) and U1snRNP (27%) were detected. SS patients

Table 2. Microarray detection of autoantibodies in sera from patients with rheumatoid diseases.

	Sera positive for antibodies, n (%)				
Autoantibody	SLE	SS	SSc	RA	PM
dsDNA	30/71 (42%)	6/20 (30%)	0/36 (0%)	18/83 (22%)	5/38 (13%)
SSA/60	34/71 (48%)	16/20 (80%)	6/36 (17%)	7/83 (8%)	25/38 (66%)
U1snRNP	19/71 (27%)	0/20 (0%)	0/36 (0%)	0/83 (0%)	0/38 (0%)
SSB	20/71 (28%)	6/20 (30%)	7/36 (19%)	2/83 (2%)	6/38 (15%)
Jo-1	0/71 (0%)	2/20 (10%)	1/36 (3%)	0/83 (0%)	27/38 (71%)
CK19	8/71 (11%)	6/20 (30%)	1/36 (3%)	9/83 (9%)	4/38 (11%)
Scl-70	18/71 (25%)	0/20 (0%)	15/36 (42%)	2/82 (2%)	0/38 (0%)
SSA/52	38/71 (54%)	8/20 (40%)	12/36 (33%)	1/83 (1%)	7/38 (18%)
Sm	18/71 (25%)	0/20 (0%)	0/36 (0%)	0/83 (0%)	0/38 (0%)
Histone	14/71 (20%)	4/20 (20%)	10/36 (28%)	4/83 (3%)	2/38 (5%)
ssDNA	35/71 (49%)	2/20 (10%)	6/36 (17%)	6/83 (7%)	4/38 (10%)
RF	2/71 (3%)	0/20 (0%)	2/36 (6%)	50/83 (60%)	0/38 (0%)
CENP-B	17/71 (24%)	10/20 (50%)	10/36 (28%)	6/83 (7%)	0/38 (0%)
CCP	10/71 (14%)	0/20 (0%)	0/36 (0%)	34/83 (41%)	0/38 (0%)
ANA	56/71 (79%)	20/20 (100%)	34/36 (94%)	22/83 (26%)	33/38 (87%)

usually have SSA or SSB autoantibodies or both, and the microarray system also detected those autoantibodies with high percentages (80% for SSA/60, 40% for SSA/52, and 30% for SSB) in SS patients. High (50%) and modest (30%) percentages of SS patients also had autoantibodies to CENP-B and CK19, respectively. For patients with SSc, Scl-70 autoantibody was detected in 42% of the cases. In addition, 33%, 28%, and 28% of the cases showed autoantibodies to SSA/52, histone, and CENP-B, respectively. RF antibodies were detected mainly in RA patients (60%). Modest percentages of autoantibodies to CCP (41%) and dsDNA (22%) were also detected in RA patients. Jo-1 autoantibody appeared highly specific for PM: it was detected in 71% of PM patients. SSA/60 autoantibody was also present in 66% of PM patients. ANA autoantibodies were present in most patients with SLE, SS, SSc, or PM (79%–100%), but in only 26% of RA patients. Only Sm and U1snRNP autoantibodies appeared specific for one disease (SLE). Others were present in two or more diseases with various frequencies.

Discussion

Detection of various autoantibodies in blood samples is a major part of the diagnosis of rheumatoid autoimmune diseases. Current methods used in clinical laboratories lack universal standards and include diverse techniques such as immunofluorescence microscopy, Western blotting, and ELISA, results of which are usually not comparable. In addition, such techniques require measurement of each autoantibody separately and thus are not practical for parallel, high-throughput analysis of multiple autoantibodies, which is necessary for cost-effective large-scale screening and diagnosis of rheumatoid diseases.

The goal of our investigations was to establish an affordable laboratory detection system for the screening and diagnosis of common rheumatic diseases with high throughput, high sensitivity and specificity, and reliable

results. The autoantigen microarray described here uses an inexpensive polystyrene support, minimum amounts of autoantigens and internal control reagents, limited amounts of blood samples, and a relatively inexpensive chemiluminescence detection protocol to detect the major autoantibodies associated with major systemic rheumatoid diseases with high throughput. This system integrates the most frequently requested tests in one miniaturized format with advantages in throughput and potential cost-effectiveness, but it gives results largely comparable to those obtained with techniques currently used in clinical laboratories (22) (Table 1). It has the potential to be more frequently requested in clinical laboratory testing and to enable earlier diagnosis and better patient care for the highly prevalent rheumatoid diseases.

One problem in the detection of autoantibodies, or any other antibodies, from patients is the lack of true quantitative calibration because of the different affinities of the antibodies to antigens or to the secondary, detection antibodies. In the case of autoantibodies, serum samples are often serially diluted for reactivity measurements, particularly when immunofluorescence microscopy is the chosen approach. Serial dilution has also been used for autoimmune diagnosis based on a microarray platform (19). Other ways of calibration include use of human IgG to quantify the antibodies, as in the case of serodiagnosis of ToRCH infectious diseases with antigen microarrays (16), or direct use of the signal, such as fluorescence intensity, to determine the reactivity of autoantibodies, as in the case of autoantigen microarrays (20). In any case, the cutoff value must be defined with each different assay system to determine positive reactivity.

We used human IgG to calibrate autoantibody concentrations in the patient samples and to establish the cutoff value for each autoantibody in the diseases studied at the 95th percentile for 800 healthy individuals. This approach
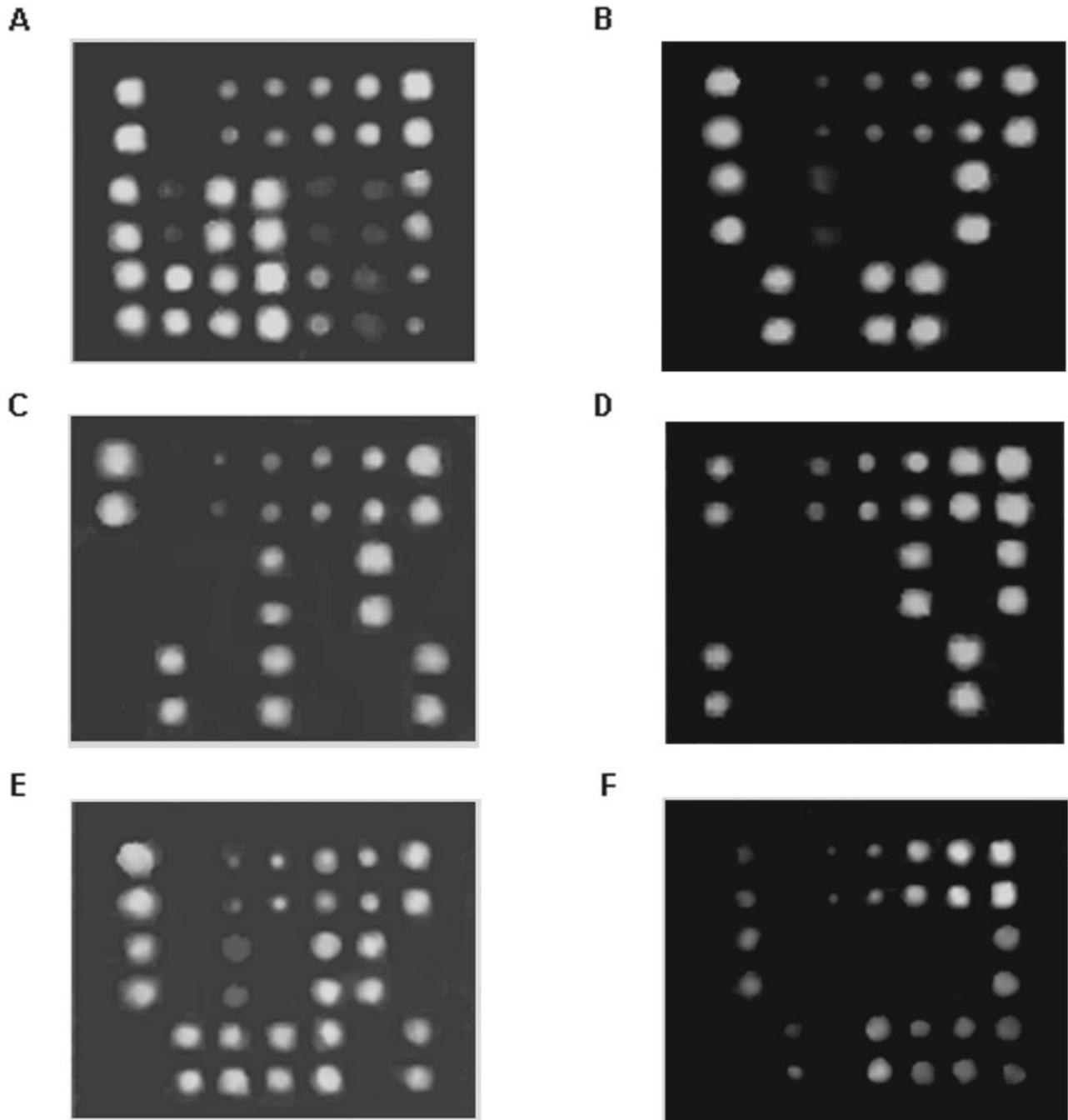
Fig. 3. CCD-captured images of arrays after reaction with sera from patients diagnosed with SLE (*A*), SS (*B*), SSc (*C*), RA (*D*), and PM (*E*) and a healthy control (*F*).

Serum autoantibodies were detected after the array was incubated with HRP-labeled secondary goat antibody against human IgG, followed by the addition of signal-generating substrates and signal acquisition with a CCD camera.

yielded mostly expected results for patients with autoimmune disorders. Among the exceptions were the autoantibodies against DNA, which have been widely accepted as highly specific for SLE. In our results, modest percentages of SS (30%) and RA (22%) patients had DNA autoantibodies. To examine whether reactive impurities in DNA might be the reason for the nonspecificity, we carefully extracted purchased DNA multiple times to eliminate possible impurities, but this did not materially improve the specificity in the assay. Thus, we do not have an explanation for this finding with our microarray system.

Most autoantibodies appeared to be disease-nonspecific (Table 2), which is consistent with most reported studies. Therefore, detection of multiple autoantibodies increases the sensitivity of disease detection. In addition,

detection of multiple autoantibodies may actually help differentiate rheumatoid diseases because each disease may have a unique autoantibody profile. Indeed, preliminary results obtained with limited samples using the Support Vector Machine (http://www.support-vector.net/software.html), an algorithm for machine learning, suggest that SLE and RA can be predicted accurately with this mathematical tool (data not shown). Further study with larger sample pools is needed to generate sufficient data to test this hypothesis, and certain mathematical tools, such as the Support Vector Machine or artificial neural networks, may be required to analyze such data.

The parallel detection of the microarray system may be particularly useful in combination with mathematical tools because it minimizes the matrix effects (23) that exist between individual assays, such as in ELISAs, because the calibrators and the autoantibodies were analyzed under exactly the same conditions; it therefore generates comparable results for the measurement of multiple analytes and thus is most feasible for generating data that can be analyzed by pattern-recognition artificial-intelligence software in differential disease diagnosis. Therefore, microarrays combined with artificial intelligence analysis may provide additional improvements in throughput, cost-effectiveness, and accuracy for molecular diagnosis of autoimmune diseases.

The microarrays are also suitable for the discovery and evaluation of novel autoantibodies, as reported previously (20) or as shown in this study, in which the autoantibody to CK19 was found in 30% of SS patients and in lower percentages in four other diseases, and CENP-B autoantibody was found to be increased in SS, SSc, and SLE. In addition, CCP autoantibody, which is not tested for as frequently in clinical laboratories, was also detected in 41% of RA patients.

In conclusion, our results together with previous reports demonstrate the feasibility of using microarrays as a tool for high-throughput, low-cost diagnosis of autoimmune diseases as well as other applications.

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