Efficacy of Autoantigen Microarrays for Detecting Autoantibodies in Patients with Systemic Lupus Erythematosus

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Purpose. To compare the performance characteristics of autoantigen microarrays with those of commercial enzyme-linked immunosorbent assays (ELISAs) in detecting five common autoantibodies (anti-dsDNA, anti-Sm, anti-RNP, anti-Ro, and anti-La) in the sera of systemic lupus erythematosus (SLE) patients.

Methods. Serum samples were obtained from 80 SLE patients and 20 sera from healthy subjects were selected as controls in this study. Autoantigens were spotted onto poly-L-lysine microscope slides by a robotic microarrayer. The slides were incubated, first with serum samples and subsequently with Cy-5-conjugated secondary antibodies. Both microarrays and commercial ELISAs were utilized to detect the five autoantibodies in the sera of 80 SLE patients and 20 normal controls. The results were compared to assess the correlation, sensitivity and specificity between the two immunoassays.

Results. The specificity of the microarray assay for each of the five autoantibodies was good (≥ 90%). The sensitivity of microarray assay for detecting anti-dsDNA, anti-Ro, and anti-La autoantibodies was more than 90%. In general, there was good agreement between the microarray assay and commercial ELISAs.

Conclusions. The microarray is a suitable assay format for diagnosing autoimmune diseases. It may have potentially important advantages in terms of high-throughput applicability, expansibility, convenience, and cost. However, continued efforts to optimize the performance conditions are still needed. **(Mid Taiwan J Med 2005;10:131-7)**

Key words

autoantibodies, autoantigen microarray, systemic lupus erythematosus

INTRODUCTION

Autoimmune diseases affect 3% of the world population [1]. Injury to organs and tissues is mediated primarily through autoreactive antibodies or autoreactive T cells. The best examples of systemic autoimmune diseases are rheumatic variants such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjogren's syndrome, systemic sclerosis, and dermatomyositis. SLE is considered to be the prototypic systemic autoimmune disease, and IgG autoantibodies directed to different autoantigens are primarily responsible for the multitude of possible clinical manifestations. The hallmark of this disease is elevated serum levels of antibodies to nuclear constituents (antinuclear antibodies; ANAs) [2]. These include native and denatured

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DNA, histones, and some soluble nuclear RNA proteins, termed Sm and nRNP. More than 95% of active untreated SLE patients are found to have positive antinuclear antibody test results using the indirect immunofluorescence technique. However, a positive ANA result usually warrants further investigation with specialized assays to determine ANA specificity because a positive ANA test is no longer considered a specific test for SLE. Moreover, some specific ANAs possess diagnostic and prognostic implications and are closely related to certain clinical manifestations of SLE. ANA specificities are traditionally detected using several assays, including double immunodiffusion, counterimmunoelectrophoresis, enzyme-linked immunosorbent assay (ELISA), radioimmunoprecipitation and immunoblot [3,4]. However, none of these technologies are conducive to miniaturization on the micrometer scale, and all are limited by the requirement for relatively large quantities of reagents and clinical samples.

The advent of automated, robust microdeposition technologies has allowed the development of high-density ordered arrays of molecules (microarrays) [5]. The microarray format, which incorporates true parallelism and miniaturization, is crucial to large-scale and high-throughput biological analysis [6,7]. In recent years, protein microarray technology has shown great potential for basic research, drug and drug-target identification, as well as diagnostics [8-10]. Microarray-based immunoassays are useful for all diagnostic applications in which several parameters of one sample have to be analyzed in parallel. The microarray requires a very small sample volume which is of great importance for applications when only minimal amounts of sample are available. The most common form of analytical arrays are antibody microarrays in which antibodies (or similar reagents) that bind specific antigens are arrayed on a glass slide at high density and used to measure the presence and concentration of proteins in a complex mixture [10,11]. In addition to antibody microarrays, the past year has also seen progress in antigen-printed microarrays that are used for the detection of circulating antibodies in clinical specimens [12,13]. Joos et al has described the construction of autoantigen microarrays containing 18 prominent autoantigens spotted on membranes or derivatized glass slides for determination of the autoantibody titer using chemiluminescence-based systems [12]. Robinson et al constructed miniaturized autoantigens arrays containing 196 distinct biomolecules to perform large-scale multiplex characterization of autoantibody responses and identify and define relevant autoantigens in human autoimmune diseases [14].

The objectives of this study were to detect the presence of anti-dsDNA, anti-Sm, anti-RNP, anti-SSA, and anti-SSB antibodies using autoantigen microarrays in sera from 80 SLE patients, and to compare the results with those obtained from commercially available ELISAs (EliA™ ANA Test; Pharmacia Diagnostics, Germany).

MATERIALS AND METHODS

Patients

Serum samples were obtained from 80 SLE patients who fulfilled the 1982 revision of the American College of Rheumatology criteria [15]. The consensual consecutive patients included 13 males and 67 females (age range, 14 to 72 yr; mean age, 38 yr). Serum samples from 20 healthy individuals were included as normal controls. Informed consent was obtained from all patients and volunteers. The study was approved by the Institutional Review Board of the China Medical University Hospital. Serum aliquots were stored at –20°C until assayed.

Preparation of autoantigen proteins and antibodies

Reagents were obtained from a number of sources. The autoantigens included: Ro/SSA, La/SSB, Sm, U1 small nuclear ribonucleoprotein complex (U1snRNP), dsDNA, human-IgG; and Cy-5-conjugated goat-anti-human IgG antisera (Jackson Immunoresearch; West Grove, Pennsylvania). Rabbit muscle myosin served as the negative control. The autoantigen powder was suspended in Tween-20 and phosphate-buffered

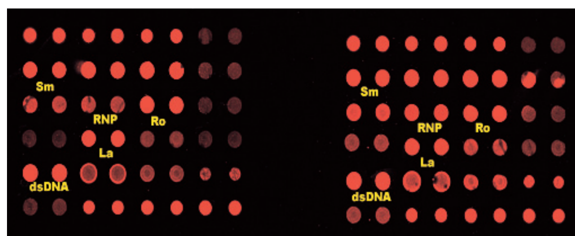

Figure. The ordered autoantigen arrays were generated by spotting autoantigens in 4-replicate sets using a robotic microarrayer. Bound antibodies were detected using cyanine (Cy)-5-conjugated goat-anti-human IgG before scanning. A GenePix 4000 Scanner, with GenePix Pro 4.0 software (Axon Laboratories; Foster City, California) was used to determine the median fluorescence intensities of feature generated by cyanine (Cy)5.

saline (PBS) at 1:1 w/v, mixed for several minutes, and then shaken for 30 minutes at 4°C. The insoluble residues were removed by centrifugation at 12,000 rpm. The suspension was then stored at –20°C until used.

Autoantigen microarray fabrication

The arrays were provided by Taimont Biotech Incorporation. Autoantigens were diluted with phosphate-buffered saline solution (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 [pH7.4]), and transferred into 96 well-plates. A robotic microarrayer (Array07) spotted the antigens onto poly-L lysine-coated microscope slides (Schott, Germany) (Figure). The spotted microarrays were sealed in slide boxes and stored at 4°C.

Reaction

The arrays were blocked for 30 minutes at 37°C in blocking buffer (PBS with 0.1% Tween-20 and 2.5% non-fatty milk), rinsed, washed twice with 1X PBS, and then incubated for 20 minutes at 37°C with 100 μL of 1:100 dilutions of each patient's serum sample in blocking buffer. The arrays were rinsed and washed twice with 1X PBS and then incubated with 100 μL of a 1:400 dilution of Cy™5-conjugated goat-anti-human IgG secondary antibody for 20 minutes at 37°C, washed twice for 7 minutes in 1X PBS which contained 0.1% SDS, and then rinsed twice with water. The arrays were blow-dried by nitrogen gas.

Fluorescence Detection

The reacted autoantigen-arrayed chips were scanned by a GenePix 4000 Scanner, and the median fluorescence intensities generated by cyanine (Cy)5 and background pixels were determined by GenePix Pro 4.0 software (Axon Laboratories; Foster City, California) (Figure). For the figure, intensity values represent median values from quadriplicate antigen features using the formula (median feature pixel intensity–median background pixel intensity) for each individual feature on each array. Fluorescence values of more than 1200 for anti-dsDNA, 400 for anti-Sm, 400 for anti-nRNP, 450 for anti-Ro, and 800 for anti-La antibodies were considered positive.

ELISA

The presence and levels of anti-dsDNA, anti-Sm, anti-nRNP, anti-Ro, and anti-La antibodies were determined by a commercially available IgG isotype-specific ELISA method (EliA™ ANA Test; Pharmacia Diagnostics, Germany), according to the manufacturer's instructions.

Specificity and sensitivity of autoantigen arrays

In this study, the specificity and sensitivity of the autoantigen microarray were calculated using the commercial ELISA result as a reference. The specificity of the microarray assay for each autoantibody was calculated using control sera; 20 healthy subjects were tested as normal controls. The negative sera for each autoantibody from the 80 SLE patients, as determined by commercial ELISA (EliA™ ANA Test), were selected as the autoimmune controls. The sensitivity of the microarray immunoassay for detecting each of the five autoantibodies was calculated in comparison to that of the positive sera assayed by commercial ELISA method.

Statistical analysis

The association between the autoantigen microarrays and the ELISAs was evaluated by Pearson product-moment correlation. A value of $p \leq 0.001$ was considered significant.

Table 1. Comparison of autoantigen microarrays with commercial ELISAs

Autoantibody	No. of blood samples	Postive autoantigen microarrays		Negative autoantigen microarrays		Sensitivity (%)	Specificity (%)
		Positive ELISAs	Negative ELISAs	Positive ELISAs	Negative ELISAs		
Anti-dsDNA	80	36	4	2	38	95	90
Anti-Sm	80	9	7	2	62	82	90
Anti-nRNP	80	17	2	5	56	77	97
Anti-Ro	80	48	3	4	25	92	93
Anti-La	80	17	2	1	60	94	97

Table 2. Correlation coefficients for autoantigen microarrays and commercial ELISAs

Autoantibody	No. of patients	Correlation coefficient
Anti-dsDNA	80	0.622**
Anti-Sm	80	0.360**
Anti-nRNP	80	0.772**
Anti-Ro	80	0.802**
Anti-La	80	0.900**

** $p \leq 0.001$.

RESULTS

The specificity was 100% for the 20 normal controls, 90% for anti-dsDNA, 90% for anti-Sm, 97% for anti-nRNP, 93% for anti-Ro, and 97% for anti-La in SLE patients. The sensitivity was 95% for anti-dsDNA, 82% for anti-Sm, 77% for anti-nRNP, 92% for anti-Ro, and 94% for anti-La in SLE patients (Table 1).

Correlation between the autoantigen arrays and commercial ELISAs

The association between the results obtained using the microarray system and those from the commercial ELISA were analyzed by Pearson product-moment correlation. The results are presented in Table 2. The correlation coefficients were as follows: 0.622 (anti-dsDNA), 0.360 (anti-Sm), 0.772 (anti-nRNP), 0.802 (anti-Ro), and 0.900 (anti-La). These results indicate general agreement between these two immunoassays ($p \leq 0.001$.).

DISCUSSION

Although proteomics is in its infancy, the greatest immediate impact of proteomics technology will be in the study of autoantibody profiling in the field of rheumatology. During the past few years, several techniques have been employed to detect various autoantibodies. The characteristics of each assay and comparisons between them have been discussed in the literature [3,4]. It has been shown that the various tests can vary greatly in specificity and sensitivity. ELISA of biochemically purified or recombinant antigens has gained wide acceptance among researchers for routine analysis of ANA specificities because of its high sensitivity and improved specificity [4]. However, conventional ELISA which uses limited quantities of biological fluids, with each analyte (e.g., for autoantibodies against ds-DNA, Ro, or Sm) determined in a separate assay, thereby increasing time and cost is not conducive to large-scale autoantibody profiling. Antigen microarrays using < 1 μL of serum per array will enable rapid and simultaneous detection of hundreds of autoantibody reactivities that will most likely replace the conventional methods. Although Joos et al [12], and Robinson et al [14] have demonstrated sensitive and specific autoantigen microarrays, extensive validation of array results will be essential prior to entry into routine clinical practice. Our data indicate that a protein microarray assay with indirect fluorescence detection can be used to determine the presence or absence of specific antibodies directed against various autoantigens in human sera. The availability of the internal dose-response IgG calibration curve, which provides more rigorous standardization of results, allowed us to demonstrate that antibodies from different serum samples bound to printed antigens can be quantified with high reproducibility. In contrast, the derivation of the calibration curves and processing of the samples in conventional

immunoassays is carried out in separate tubes or wells, leading to frequent matrix problems which represent a known source of bias. The specificity of our microarray was good; however, no reactivity was observed among the 20 blood donor sera, and only a few low/equivocal positive values were obtained for each autoantibody on autoimmune controls. Furthermore, the sensitivity was good for anti-dsDNA, anti-Ro, and ant-La autoantibody detection. Fair sensitivity was demosntrated for anti-RNP and anti-Sm. In general, the results from the autoantigen microarray system correlated well with those from commercial ELISA. There were good correlations for anti-dsDNA, anti-RNP, anti-Ro, and anti-La for correlation coefficients over 0.60. The correlation for anti-Sm, however, was relatively weak. The sources of antigenic materials for our microarray assay and commercial ELISA were different, and this may, in part, be the cause of the differences in sensitivity. Moreover, certain autoantigens are not amenable to detection using poly-L lysine-coated glass slides [11,14], presumably due to loss of three-dimensional structures, electrostatic repulsion or steric interferences which may alter immunologic epitopes.

Although autoantigen arrays provide a practical means, in a low-cost and low-sample-volume format, of rapid screening of autoantibody specificities associated with various autoimmune diseases, which is conducive to early diagnosis and treatment, several aspects still need to be refined, including improvement of antigen production and purification, planar-surface chemistry for attachment of various autoantigens, and fluorescence detection and quantitation [14,16,17]. In addition to being applicable for autoimmune diagnosis, the autoantigen microarray has the advantage of expansibility. A variety of potential applications have been proposed, for example: characterization of the epitope-based propagation of autoantibody responses [18,19]; identification of isotype subclass for antigen-specific autoantibodies to study the pathophysiology of autoimmune diseases [14,16,17]; as a tool for novel autoantigen discovery [14,17]; and to guide the development and selection of antigen-specific therapies [20,21].

In conclusion, our findings demonstrate that not only can good analytical and clinical data be obtained using autoantigen microarrays, but also this format may have potentially important advantages in terms of convenience, cost and expansibility in comparison to conventional ELISA formats. Continued efforts are still needed, however, to optimize performance conditions in microarray systems. We believe that the fluorescence-based proteomics platform, utilizing simple protocols and widely available equipment, will be utilized routinely in clinical laboratories and have a great impact on the diagnosis, monitoring and treatment of autoimmune diseases in the near future.

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以自體抗原微陣列晶片偵測全身性紅斑性狼瘡病人血清中自體抗體之效力

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目的　臨床上要診斷病人是否爲全身性紅斑性狼瘡，除了依靠詳細的病史詢問及理學檢查之外，血清中一些特異性自體抗體的偵測也是診斷不可或缺。自體抗原微陣列晶片是近年新發展出的自體抗體測試方法，可以利用少量的血液樣本來偵測血液中的自體抗體。本研究嘗試用自體抗原微陣列晶片偵測紅斑性狼瘡病人血清中常出現的五種自體抗體，並評估其效力。

方法　以微陣列機將自體抗原點在經poly-L-lysine處理過的顯微鏡載玻片上，製成自體抗原微陣列晶片，採集經臨床診斷有紅斑性狼瘡病人共80人的血液樣本，分別用自體抗原微陣列晶片及ELISA兩種方法偵測血液中anti-dsDNA，anti-Sm，anti-RNP，anti-Ro，anti-La等五種自體抗體，再將兩者結果加以比較。

結果　用自體抗原微陣列晶片及ELISA兩者方法偵測血清中這五種自體抗體，兩者結果的相關性分別爲0.622、0.360、0.772、0.802 及0.900。與ELISA做比較，自體抗原微陣列晶片用以偵測血清中這五種自體抗體的敏感度分別爲95%、82%、77%、92%及94%，特異度分別爲90%、90%、97%、93%及97%。

結論　在臨床應用上，自體抗原微陣列晶片似乎可以提供與ELISA類似的結果，而自體抗原微陣列晶片在大量應用、擴充性、方便性及費用上具有優勢，可以利用極少量的血液樣本，進行大量而快速的檢驗。（中台灣醫誌 2005;10:131-7）

關鍵詞

自體抗體，自體抗原微陣列，全身性紅斑性狼瘡

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