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Technology Insight: can autoantibody profiling improve clinical practice?

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SUMMARY

A hallmark of autoimmune diseases is the production of high titers of highly specific autoantibodies, which are routinely measured to guide clinical decision-making. Multiplex antigen microarrays are powerful tools that can provide profiles of the autoantibodies found in blood and other biological fluids. This high-throughput technology allows for rapid identification of antibody and antigen biomarker sets, which is sorely needed in the clinic to improve diagnosis, predictions of prognosis, and selection of targeted therapies. In this article we will describe the antigen microarray technologies that are currently available, and those that are in development. We highlight recent applications for antibody profiling, as well as the challenges that need to be faced before such technologies enter the clinic.

KEYWORDS antigen array, autoantibody, autoimmunity, protein array, proteomics

REVIEW CRITERIA

The PubMed database was searched for articles published up to 1 March 2006, including electronic early release publications. The search terms we used were "protein array", "protein microarray", "antigen array", "antigen microarray", "autoantigen microarray", "autoantibody microarray", "lipid microarray", "protein biomarker" and "autoimmune profiling". The search was limited to English-language publications. Abstracts were reviewed and articles were chosen based on the relevance of the abstract's content. Full articles were obtained and their reference lists were searched for additional material when appropriate. Some unpublished experimental results were conveyed to the authors by personal communication.

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INTRODUCTION

Autoimmune diseases are characterized by the production of high-affinity, high-avidity autoantibodies.[1] As a consequence of an intricate interplay between T and B cells, and between natural killer T cells and B cells, the specificity of the B-cell autoantibody response reflects the overall specificity of the autoreactive T-cell response.[2] Autoreactive lymphocytes are rare (1 out of 10,000 lymphocytes or fewer). Individual autoreactive T lymphocytes are difficult to study because the reagents needed for their detection are highly specialized and tedious to produce.[3] B cells, however, produce large quantities of autoantibodies that are easily detectable in serum or other biological fluids, such as cerebrospinal fluid and synovial fluid.[4] These arguments provided a rationale for the development of traditional tools for autoantibody detection, including enzyme-linked immunosorbent assay (ELISA), western blot and immunoprecipitation.

The limitations of these assays (lengthy and laborious procedures, large sample volumes, and high cost) and the availability of simple technologies created for genome-based research facilitated the development of multiplex antigen array technology platforms.[5] Current technologies have proved to be invaluable as a means of expediting discovery of new biomarkers, and a number of inventions are on the horizon that will further increase the rate of progress. Multiplex antigen arrays have been used to identify signature autoantibody profiles in animal models of autoimmunity and in human diseases. This technology has a number of applications: prediction of disease onset; improved diagnosis; prediction of disease severity and classification of patients into subgroups; design of antigen-specific, individualized therapies; assessment of patients' responses to therapy and prediction of side effects; and discovery of new autoantigens. In this article, we describe the current uses of antigen array technologies and their potential future clinical applications. In addition, we highlight important technological advances in the field of antigen arrays.

ANTIGEN ARRAY TECHNOLOGY

Microarray technology was initially developed for gene expression profiling.[6] The first generation of DNA arrays used samples of complementary DNA (cDNA) deposited on glass microscope slides at discrete, known positions. RNA isolated from cells was used to generate (fluorescently or isotopically) labeled cDNA probes by reverse transcription, and these probes were incubated on the surface of the glass slide. The amount of labeled probe that had hybridized with an immobilized cDNA was then measured. The wide availability of automated microarray apparati at academic centers, and the realization that mRNA abundance is not highly predictive of protein levels in cells, prompted the adaptation of these tools for proteomic assays.[7] Protein arrays have been designed to detect protein abundance, function, and post-translational modifications such as phosphorylation and citrullination. Function-based microarrays are created by immobilizing proteins or other biomolecules on a solid surface (Figure 1), and then screening for interactions between the immobilized proteins and other molecules such as antibodies.[8] This technology has been used to characterize the autoantibody repertoire in autoimmune diseases,[9–15] IgE reactivity in allergy,[16,17] and the immune response to infections,[18–22] vaccination,[19,23,24] and cancer[25–27] (Table 1).

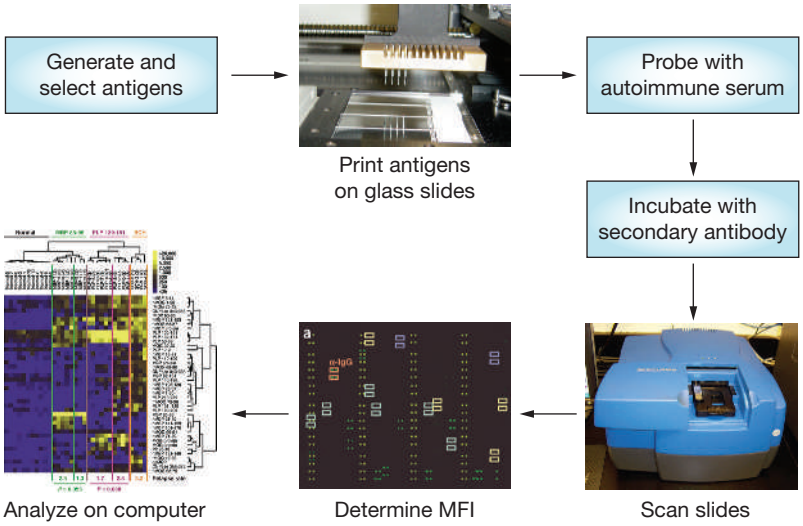

Figure 1 A schematic diagram that illustrates the steps involved in content generation, processing and data analysis for antigen microarrays. Antigen content is generated from known autoantigens or from candidate autoantigens produced by tissue fractionation or mass spectrometry, or identified by screening of gene expression libraries. Selected antigens are printed onto glass microscope slides as distinct microspots, by highly automated printers. Slides are incubated with sera or other biological fluids from patients or animals with autoimmune diseases. Bound autoantibodies are detected by fluorescently labeled secondary antibodies. The slides are then scanned using an automated scanner. The mean fluorescence intensity of the secondary antibody is determined using a computer program. The fluorescence intensity of each spot correlates with the abundance of a given autoantibody in the sample. Bioinformatics tools are used to cluster the data points into groups. Modified with permission from Macmillan Publishers Ltd *Nat Biotechnol* © (2003).[13] Abbreviation: MFI, mean fluorescence intensity.

USE OF ANTIGEN MICROARRAYS TO MONITOR AUTOIMMUNE DISEASE

Autoantigen arrays that detect rheumatic diseases

The first antigen array designed to detect and characterize autoantibodies was developed by Joos *et al.*[28] This array contained 18 prominent protein and nucleic acid antigens; when probed (i.e. tested) with well-characterized human autoimmune sera, the array had a sensitivity and specificity for the detection of autoantibodies that was comparable to that of ELISA. Next, a large-scale protein antigen array was developed by Robinson and colleagues to study connective tissue diseases and other arthritides (Figure 2).[9] This array comprised 196 different biomolecules, including proteins, peptides, nucleic acids, ribonucleoprotein complexes and post-translationally modified antigens. When probed using well-characterized sera from patients with autoimmune diseases including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), this array showed superior sensitivity and specificity to ELISA in the detection of autoantibodies over a 2–3-log linear range. The results were concordant with those obtained with conventional assays (e.g. ELISA, western blot and immunoprecipitation).[9] Hueber and colleagues extended this concept by designing a 1,536-feature synovial proteome array for RA.[10] The authors used this array to define two distinct subpopulations of RA patients: patients with autoreactive B-cell responses directed against citrullinated epitopes, who developed features of severe RA; and a second group of patients who had developed autoreactivity against native epitopes, and who developed less-severe clinical disease.

Antigen arrays for prediction of disease onset and activity

Quintana *et al.*[15] examined the IgG autoantibody repertoire associated with cyclophosphamide-induced diabetes in male nonobese diabetic mice. Their array used 266 candidate antigens. They

Table 1 Selected studies that utilized antigen arrays for antibody profiling.

Disease	Reference	Human or animal	Human cohort size	Number of antigens screened
Autoimmune disease				
CTD	Robinson WH *et al.* (2005)[9]	Human	50	196
RA	Hueber W *et al.* (2005)[10]	Human	76	225
SLE	Kattah MG *et al*. (2006)[31]	Animal	N/A	60
SLE	Graham KL *et al*. (2006)[36]	Animal	N/A	140
SLE	Sekine H *et al*. (2006)[32]	Animal	N/A	36
SLE	Balboni I and Utz PJ[a]	Human	Ongoing	60
SLE	Zhen QL *et al.* (2005)[11]	Both	37	30
MS	Robinson WH *et al.* (2003)[13]	Animal	N/A	232
MS	Kanter JL *et al.* (2006)[14]	Both	16	50
Vasculitis	Alemi G and Utz PJ[a]	Both	Ongoing	50
DM type 1	Quintana FJ *et al.* (2004)[15]	Animal	N/A	266
Oncology				
Prostate cancer	Wang D *et al.* (2002)[25]	Human	60	22
Ovarian cancer	Chatterjee M *et al.* (2006)[27]	Human	69	65
Microbiology				
HIV	Neuman de Vegvar HE *et al.* (2003)[19]	Animal	N/A	430
Allergy				
IgE-mediated hypersensitivity	Hiller R *et al.* (2002)[16]	Human	20	96

Studies that used large-scale arrays of at least 20 antigens were chosen. [a]Unpublished data. Abbreviations: CTD, connective tissue disease; DM, diabetes mellitus; MS, multiple sclerosis; N/A, not applicable; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

identified a panel of 27 antigens that distinguished mice that were susceptible to diabetes from those that were diabetes-resistant. Interestingly, this panel did not distinguish between diabetic and nondiabetic mice after the induction of diabetes, and a new set of antigens seemed to be important later in disease progression. A glomerular proteome array was subsequently used to identify autoantibody clusters that could predict disease activity in a mouse model of SLE, as well as a cohort of patients with SLE.[11] Although neither of these two studies confirmed their positive results by comparison with other methods, they clearly demonstrate that the content of the arrays and the statistical methods used to analyze the data are critically important. Currently, the SAM (Significance Analysis of Microarrays) software developed at Stanford University is the standard method for identifying antigens with significant differences in array reactivity between different groups. Cluster analysis is used to arrange SAM results into relationships.[10]

Other researchers have used recombinant proteins derived from expression libraries to create disease-specific microarrays to analyze the autoantibody profiles associated with alopecia areata, cardiomyopathy and murine SLE.[12,29,30] In our laboratory, the autoantibody repertoire of mice with pristane-induced lupus has been extensively analyzed using arrays containing approximately 50 antigens.[31,32] Although use of these arrays was predicted to improve the detection of disease, the pathophysiological significance of most antigens identified in the above studies remains unclear.

Epitope mapping

By displaying representative peptide epitopes (single antigenic sites that are targeted by autoantibodies) of a known autoantigen at individual

positions (spots) on arrays, it is possible to distinguish epitopes that are targets of the autoimmune response from those that are not. Robinson *et al.*[13] used epitope mapping to investigate experimental autoimmune encephalomyelitis (EAE), a mouse model of human multiple sclerosis. SJL/J mice were immunized with various encephalitogenic antigens to induce EAE. Despite being genetically identical, mice immunized with the same peptide developed reactivity not only to shared peptides but also to distinct autoantigenic peptides. This heterogeneity of response was also characteristic of epitope spreading as the disease progressed. Epitope spreading describes the phenomenon by which autoreactive B-cell responses broaden, first to encompass different antigenic sites in the same biomolecule (intramolecular spreading), then to different molecules altogether (intermolecular spreading). Epitope spreading has been observed in humans with SLE[33] and in other disorders such as pemphigus foliaceus.[34] Moreover, reactivity against a wide spectrum of myelin epitopes after immunization, and extensive intramolecular and intermolecular epitope spreading over time, was associated with a severe disease course in EAE mice. Fine-specificity epitope mapping provides important insights into the pathogenesis of autoimmunity, and might provide the basis for classification and prediction of prognosis in human patients.

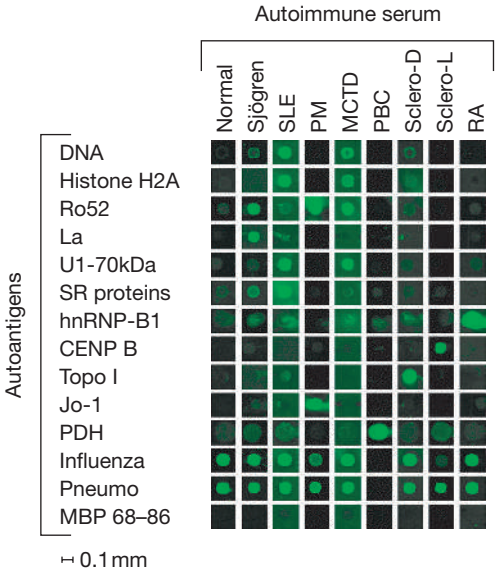

Figure 2 Protein microarray for connective tissue diseases. This array contained the autoantigens listed along the vertical axis, which were incubated with diluted serum samples from healthy (normal) individuals or from patients with the autoimmune diseases listed along the horizontal axis. Bound autoantibodies were detected by fluorescently labeled secondary antibodies. A myelin basic protein peptide that is recognized by sera from rodents with experimental autoimmune encephalomyelitis was included as a negative control. Reproduced with permission from Macmillan Publishers Ltd *Nat Med* © (2002).[9] Abbreviations: CENP B, centromere protein B; hnRNP, heterogeneous nuclear ribonucleoprotein; Jo-1, Jo-1 antigen; La, La antigen; MBP, myelin basic protein peptide; MCTD, mixed connective tissue disease; PBC, primary biliary cirrhosis; PDH, pyruvate dehydrogenase; PM, polymyositis; Pneumo, pneumococcal vaccine; RA, rheumatoid arthritis; Ro52, Ro52 antigen; Sclero-D, diffuse scleroderma; Sclero-L, limited scleroderma; Sjögren, Sjögren's syndrome; SLE; systemic lupus erythematosus; SR proteins, serine-rich and arginine-rich proteins; Topo I, DNA topoisomerase I; U1, U1 small nuclear riboprotein complex.

Immunoglobulin subclass analysis

Another important area with clinical implications is the measurement of relative amounts of immunoglobulin subclasses. The class of immunoglobulin produced by a plasma cell determines the effector function of the antibody (e.g. the ability to fix complement, or to engage activating or inhibitory Fc receptors), and also provides valuable clues in relation to the type 1 or type 2 polarization of the T cell or natural killer T cell that stimulated the autoreactive B cell earlier in the disease process.[35] Robinson and colleagues demonstrated that antigen arrays enable the identification of multiple isotype subclasses of antigen-specific autoantibodies, which could link antibody reactivity to effector function.[9] Immunoglobulin isotypes have been examined using antigen arrays in a mouse model of SLE and in human SLE.[36]

Nonprotein antigen arrays

Autoimmune responses are not only directed against proteins or nucleic acids, but also against lipids and carbohydrates.[14,25] Kanter and colleagues employed an array of lipids that are present in the myelin sheath to detect autoantibodies in cerebrospinal fluid obtained from patients with multiple sclerosis, and in serum obtained from mice with EAE.[14] Arrays that contain lipids and carbohydrates have been used to analyze antibody responses to *Mycobacterium tuberculosis*, Gram-negative bacteria,[18,21] and tumors.[26]

ANTIGEN-SPECIFIC INDIVIDUALIZED THERAPY

Most immunosuppressive therapies for autoimmune diseases are nonspecific; they inhibit the immune response to all self and nonself antigens, which leads to increased rates of infections and malignancies. To circumvent these undesirable effects it would be advantageous to design therapies that inactivate only the autoreactive lymphocytes, and leave global immune function intact. Robinson *et al.*[13] used a myelin antigen array to identify autoantibody profiles in EAE mice, and to design antigen-specific, tolerizing DNA vaccines. A vaccine that contained a full-length pathogenic antigen (myelin basic protein) significantly decreased relapse rates and reduced epitope spreading. A similar but more pronounced effect was achieved with a DNA vaccine that combined multiple antigens identified by the array. Combination of this DNA vaccine with another DNA plasmid that encoded interleukin 4 (a type 2 helper T cell cytokine) further increased the efficacy of treatment. Phase I–II trials of tolerizing DNA vaccines have been completed in patients with multiple sclerosis, and a phase II trial of over 290 patients with this disease has now been fully enrolled.[37]

Antigen-specific tolerization with epitopes of oral type II collagen or cartilage glycoprotein 39 has been tested in animal models and in patients with RA, with disparate results. One possible reason for the variation in efficacy is that the putative antigen might undergo post-translational modification *in vivo*, which could alter T-cell recognition of the antigen and diminish the efficacy of tolerization.[38] Use of antigen arrays that contain modified epitopes of the therapeutic antigen could potentially generate autoantibody profiles that could be used to select appropriate patients for clinical trials of antigen-specific therapies.

An alternative and intriguing approach to therapeutic tolerization employs immunologically relevant antigens (such as heat-shock proteins) that are present or overexpressed under inflammatory conditions. One advantage of this approach is that there is no need to search for the inciting antigen(s), or to follow the spread or modification of their epitopes over time. DnaJ homolog subfamily A member 2 is a proinflammatory heat-shock protein that is the target of such a tolerization strategy. The peptide is delivered orally in very low doses to target mucosal immune cells, and has been tested in phase I–II trials in patients with RA, with favorable results.[37]

In summary, antigen arrays could emerge as essential tools in the design of clinical trials: they could be used to preselect patients who have reactivity to the putative antigens or epitopes used in immunotherapy, and to monitor immune responses to relevant epitopes as a surrogate marker of treatment efficacy and safety. Antigen arrays also have obvious applications in ongoing studies that use B-cell depleting agents (such as rituximab), both to analyze patients' autoantibody repertoires and to characterize the memory response over time to common antigens such as tetanus, pertussis, and other infectious agents that are vaccine targets.

ANTIGEN AND EPITOPE DISCOVERY

Analyses of antigen arrays are biased because only a limited number of candidate biomolecules are included in the assay.[39] To overcome this limitation, efforts continue to identify novel candidate biomolecules and to create high-density arrays. Biomolecules for inclusion in arrays can be generated using screening of genetic expression libraries,[12] tissue fractionation,[11] or mass spectrometry.[40] The advantages and disadvantages of each of these approaches were reviewed in 2006 by Robinson and colleagues.[41] Once new candidate antigens have been identified, antigen arrays can be used to test the sensitivity and specificity of antibody reactivity against the individual biomolecule or against combinations of biomolecules. In addition, representative linear peptides that are derived from the antigenic protein can be synthesized and displayed on an array. This approach led to the identification of the dominant linear epitopes of histone in SLE and to the demonstration of reactivity against citrullinated epitopes in a subgroup of RA patients.[9,10] A large-scale array that comprises over 8,000 recombinant human proteins is commercially available from Invitrogen® (Carlsbad, CA, USA).

TECHNOLOGICAL ADVANCES IN THE PIPELINE

The multiplexing capacity and significantly reduced procedure time, labor and sample-quantity requirement of antigen arrays makes them suitable for use in high-throughput research. Compared to studies that use gene

chips, however, studies that use protein arrays have had difficulty in obtaining reproducible results.[42] Many difficulties arise because of the wide variability in size and complexity of the different biomolecules (lipid, protein, nucleic acid, carbohydrate) that are spotted on arrays. Improvements in many areas are needed before protein arrays can aid the clinician, as described below.

Improved glass-slide coating techniques and detection systems

Glass slides are most commonly used as a flat (planar) surface support for antigen arrays, because they permit a high density of spots (features 100 μm in diameter can be resolved) and require small sample volumes (<1 μl). Different coatings for glass slides are being developed that encourage maximal preservation of antigen structure and antibody binding, while trying to minimize nonspecific (background) antibody binding.[43,44] For labeling and detection, most laboratories use fluorescent dyes that are simple to work with and nontoxic. Their disadvantages include poor reproducibility and relatively high interarray and intra-array variation. Multicolor detection enables relative instead of absolute quantification, so results from different samples can be directly compared without the confounding effect of interchip variation. In our laboratory, Kattah *et al.* have developed a novel, two-color, Fab-fragment labeling method, which allows for increased precision and reliability of detection of subtle variations in antibody reactivity. They identified both expected and previously unrecognized antibody reactivities in sera from mice with pristane-induced SLE.[31]

Development of nonplanar assay systems

Planar arrays are associated with inherent problems, including sample drying, which causes alterations in native protein conformation and potential loss of immunogenic epitopes. An alternative format to planar microarrays is xMAP technology (Luminex Corporation, Austin, TX, USA), which uses color-coded beads for protein immobilization and labeling.[42,45] Currently, bead-based, multiplexed assays are available in the US (and have been certified by the Clinical Laboratory Improvement Amendments program) to test for autoantibodies that are frequently observed in connective tissue diseases.

Improved data management and analysis

Management and interpretation of the huge data sets generated by high-throughput technologies such as antigen arrays is now possible, using computers with large memory capacity and rapid processing capability. Repositories have been generated that have defined minimum requirements for uniform data deposition. Despite these efforts, unification and comparison of data from different labs is difficult, because experimental conditions and clinical data are often not uniform and not represented digitally. One of the biggest challenges is to determine how to deposit all array experiments (proteomic and genomic) in a standard format that uses a common language for data integration. Computers could then analyze thousands of data points from different experiments and might identify underlying mechanisms that would otherwise not be discovered. One vision for the future is that scientists will perform virtual experiments '*in silico*' before going to the bench. This innovation would vastly reduce procedural time, reagent use and sample requirements, and ultimately speed up the pace of discovery.[46]

CONCLUSIONS AND FUTURE DIRECTIONS

The current standard procedure for immune diagnostic tests in the clinic is to use sera or other biological fluids in milliliter volumes for detection of a few autoantibodies. Antigen arrays would revolutionize clinical practice at several levels. Clinicians could acquire comprehensive panels of autoantibody profiles that could be integrated with clinical information and other biomarker data (i.e. cytokine profiles and intracellular signaling pathway activation profiles) to generate 'biosignatures' of their patients. Robinson and colleagues have already demonstrated that a combination of blood autoantibody and cytokine profiles can distinguish a 'high-inflammatory' and a 'low-inflammatory' subtype of RA.[41]

Data generated with antigen arrays need to be validated with conventional proteomic techniques, and different array platforms need to be individually validated using large human sample sets, and also cross-validated with each other. The discipline of bioinformatics faces perhaps the biggest challenge, that of managing the gigantic data sets generated by these different platforms. Generation of individual biomarker signatures will form the basis of antigen-specific

and patient-specific therapies. Clinical trials could be carried out in screened, selected patient populations, and the therapeutic and adverse-effect profiles of treatment could be correlated with patients' biosignatures. Finally, a multidisciplinary approach that combines the skills of physicians, statisticians, and bioinformaticians is needed to pave the way toward sophisticated, individualized medicine.

KEY POINTS

- Antigen arrays are available for rapid detection of multiple autoantibodies in microliter volumes of biological fluids
- Multiplex antigen arrays are invaluable tools for identifying disease-specific autoantibody signatures
- Autoantibody profiles can be followed over time as markers of disease remission or relapse
- Novel antigens can be identified by testing reactivity of autoimmune sera to candidate antigens
- Antigen-array technologies and the tools necessary for data interpretation are developing at an unprecedented rate
- Individual autoantibody signatures can form the basis for patient-specific therapies

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Competing interests
PJ Utz has declared associations with the following companies/ organizations: Avanir, Bayhill Therapeutics, Biogen Idec, Centocor, Genentech, Monogram Biosciences, XDx. See the article online for full details of the relationship. V Sharp declared she has no competing interests.