Review

Autoantibody profiling for the study and treatment of autoimmune disease

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Received: 24 January 2002 Revisions received: 5 March 2002 Accepted: 11 March 2002 Published: 7 May 2002

Arthritis Res 2002, **4**:in press

Abstract

Proteomics technologies enable profiling of autoantibody responses using biological fluids derived from patients with autoimmune disease. They provide a powerful tool to characterize autoreactive B-cell responses in diseases including rheumatoid arthritis, multiple sclerosis, autoimmune diabetes, and systemic lupus erythematosus. Autoantibody profiling may serve purposes including classification of individual patients and subsets of patients based on their 'autoantibody fingerprint', examination of epitope spreading and antibody isotype usage, discovery and characterization of candidate autoantigens, and tailoring antigen-specific therapy. In the coming decades, proteomics technologies will broaden our understanding of the underlying mechanisms of and will further our ability to diagnose, prognosticate and treat autoimmune disease.

Keywords: autoantibodies, autoimmune disease, proteomics, protein arrays

Introduction

'Proteomics' is the large-scale study of expression, function and interactions of proteins [1]. Recent advances in the field spawned miniaturized proteomics technologies capable of parallel detection of thousands of different antigens using submicroliter quantities of biological fluids. This review will focus on proteomics technologies that enable characterization of autoantibody responses (Table 1).

Early immunoassays capable of multiplex analysis include: ELISAs, fluorescence-based immunoassays, and radioimmunoassays performed in microtiter plates; arrays of peptides synthesized on plastic pins [1,2]; western blot analysis; and genetic plaque-based and colony-based assays. All of these technologies are limited by requirements for relatively large quantities of reagents and of clinical samples. Genetic plaque-based and colony-based assays are further limited by incomplete addressability; DNA sequence analysis is required to determine the identity of the antigens at each location on the array.

Ekins as well as Fodor *et al*. proposed, in the late 1980s, the use of miniaturized and addressable immunoassays, including 'multianalyte microspot immunoassays' and photolithography-generated peptide arrays [3,4]. Another major advance was the development of robotic printing devices by Patrick Brown and colleagues for precise deposition of cDNA to fabricate DNA microarrays [5]. These devices are inexpensive and widely available, and several groups recently extended their use to generate ordered arrays of proteins [6,7]. Major advances have been made in the past 2 years towards development and application of miniaturized, addressable arrays of proteins, peptides and other biomolecules.

Miniaturized proteomics technologies for autoantibody profiling

Although proteomics is in its infancy, a diverse and powerful set of proteomics technologies is under rapid development (Table 1). Planar surface arrays currently offer the greatest per-array complexities, but are limited by their

EAE = experimental autoimmune encephalomyelitis; ELISA = enzyme-linked immunosorbent assay; hnRNP = heterogeneous nuclear ribonucleoproteins; IDDM = insulin-dependent diabetes mellitus; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus; Sm/RNP = Smith ribonucleoproteinsTh = T helper cell.

Table 1

Proteomics technologies for autoantibody profiling: selected published studies

System	Assay format	Detection	Antigens tested in citation(s)	Estimated capacity per array	Comments	Reference
Antigen microarrays	Robotic attachment of antigens in ordered arrays on membranes and derivatized microscope slides	Secondary antibody; chemiluminescence	18	5000+	Demonstrate sensitive and specific detection of autoantibodies in serum on planar arrays	[16]
Protein microarrays	Robotic attachment of antigens in ordered arrays on derivatized microscope slides	Direct labeling of samples with fluorescent markers for comparative analysis	115	10,000+	Comparative analysis requires fluorescent labeling of individual samples; 50% of antigens detected	[7]
Antigen microarrays	Robotic attachment of antigens on derivatized microscope slides	Secondary antibody; fluorescence; comparative analysis with direct fluoresecent labeling of samples	196	10,000+	Detection of autoantibodies characteristic of eight autoimmune rheumatic diseases, including autoantibodies against proteins, peptides, nucleic acids, and macromolecular complexes	[17]
Bead microarrays (LabMAP™; cytometric bead array)	Antigens conjugated to sets of spectrally resolvable fluorescent beads	Fluorescence; analysis of individual beads using a flow cytometer	16	64 per well; 5000+ per 96-well plate	Fluid-phase; commercial development by Luminex, and Becton–Dickinson	[35]
Nanobarcodes™ particle technology	Attachment of antigens to addressable multimetal microrods encoded with submicrometer metal stripes	Light microscopy; fluorescence; mass spectrometry	2	80,000 using three distinct metals	Fluid-phase; Commercial development by SurroMed	[8]
Arrayed proteins from cDNA expression libraries	Expression and purification of polypeptides encoded in a cDNA expression library in microtiter plates, followed by robotic attachment to PVDF filters	Chemiluminescence	4800	10,000+	Performing autoantigen discovery; bacterial expression of autoantigens does not confer post-translational modifications	[13,14]
Protein *in situ* array	Protein array generated *in situ* using PCR and a cell-free transcription/ translation expression system	Colorimetric	15	96 per plate	Probably less robust than other systems	[36]
Photolithography-generated peptide arrays	*In situ* synthesis of peptides by photolithography	Fluorescence		10,000+	Linear peptide epitopes only; not under active development	[4]
Microarrays of cells expressing defined cDNAs	Robotic printing of cDNA in expression vectors on slides followed by incubation with adherent mammalian cells	Fluorescence	192	10,000+	Mammalian expression system confers certain post-translational modifications	[9]
Protein arrays of living transformants; modified yeast two-hybrid screen	Robotic delivery of yeast transformants expressing yeast open reading frames fused to an activating domain	Colorimetric	6000	Performed in 384-well microtiter plates	Arrays of yeast expressing fusion proteins	[10]
'Line immunoassay'	Electrophoresis of antigens and transfer to nitrocellulose membranes (western blot of purified antigens)	Chemiluminescense	15	< 50	Not high-throughput; commercial development by Innogenetics	[12]
'Universal protein array'	Dot-blots of purified antigens on nitrocellulose membranes	Secondary antibody; radioactivity	48	< 200	Requires large quantities of purified antigen and serum samples	[11]
'Lab-on-a-chip', microfluidics	Microchannels etched in solid supports; electrokinetic, electro-osmotic, electrophoretic, or pressure-driven flow	Fluorescence; UV light absorption	Limited	N/A	Fluid-phase assay; low-affinity binding detectable; kinetics can be calculated; commercial development by Caliper, Aclara, and Fluidigm	[37]
Peptides on pins (Multipin™)	*In situ* synthesis of peptides on polyethylene pins	Colorimeteric	96	96 per plate	Linear epitopes only; strip and re-use peptides on pins for subsequent experiments	[1,2]

N/A, not applicable; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride. For manufacturer details, please see text.

methods of binding autoantigens and of drying at the time of array production, which can distort and/or sterically interfere with immunologic epitopes. A variety of fluid-phase bead, tag, nanoparticle, and microfluidic systems, which generally utilize minimally disruptive methods to label antigens, are under development.

Arrays of addressable beads

Bead arrays enable multiplexed analysis of biomolecular interactions. The LabMAP™ system of Luminex (Austin, Texas, USA) utilizes 64 sets of spectrally resolvable fluorescent beads. Each set can be conjugated to a distinct antigen (or antibody or oligonucleotide). Following incubation with the test sample, analysis is performed using a flow cytometer. Further multiplexing is achieved by analysis of multiple wells in microtiter plates, each with beads conjugated to different sets of antigens.

Arrays of addressable tags

The eTAG™ assay of Aclara (Mountain View, California, USA) utilizes eTAG™ reporters that are fluorescent labels with unique and well-defined electrophoretic mobilities. Each eTAG™ label is coupled to an antigen (or another biological probe) via cleavable linkages. When an autoantibody binds to an eTAG™ reporter-labeled antigen, the coupling linkage is cleaved and the eTAG™ is released. Mixtures of eTAGs™ are readily separated and analyzed by capillary electrophoresis.

Arrays of addressable nanoparticles

SurroMed (Mountain View, California, USA) is developing a system based on addressable multimetal microrods intrinsically encoded with submicrometer stripes [8], termed Nanobarcodes™ particle technology. Using three different metals, 80,000 distinctive striping patterns are possible [8]. This far exceeds the complexity of fluorescence-based bead and tag systems.

Microfluidics approaches

Microfluidics utilizes microchannels for analysis of antigen–autoantibody interactions. Small quantities of biomolecules are separately introduced into a network of microchannels and subjected to electrokinetic, electro-osmotic, electrophoretic or pressure-driven flow, mixing and separation. Binding events, reflected by changes in mobility, are measured by UV absorption or fluorescent detection. Real-time millisecond quantitation of binding kinetics and detection of low-affinity interactions are among the important advantages of this system.

Arrays of living cells

Several groups have described arrays of living cells expressing transformed or transfected cDNA [9,10]. Such systems could be easily adapted for autoantibody profiling.

Arrays on planar surfaces

Methods to fabricate arrays on planar surfaces include stamping, ink jetting, capillary spotting, contact printing, and *in situ* synthesis. Commonly used solid supports include: nitrocellulose, nylon and polyvinylidene difluoride membranes; poly-L-lysine-coated, silane-treated, and other derivatized glass microscope slides; and glass microscope slides coated with gelatin, acrylamide and other coatings.

Membrane-based systems include low-density dot blot arrays on nitrocellulose membranes [11], autoantigens electrophoretically separated prior to transfer to membranes [12], and spotting of cDNA expression-library-produced proteins onto polyvinylidene difluoride filters [13,14]. The generation of arrays of polypeptides derived from cDNA expression libraries by Büssow and colleagues provides an elegant system for autoantigen discovery [13,14]. cDNAs are expressed and their protein products purified *in vitro*, following which purified proteins are robotically arrayed. On identification of autoantibody targets, their corresponding cDNAs are readily sequenced to genetically identify autoantigens. Walter *et al*. describe use of one such cDNA library, a human fetal brain cDNA expression library, for autoantigen discovery in inflammatory bowel disease [15].

Other workers are developing protein arrays on derivatized microscope slides. Joos *et al*. have demonstrated sensitive and specific autoantibody detection using microarrays containing serial dilutions of 18 antigens [16]. Haab *et al*. generated protein arrays to characterize 115 purified antigen–antibody pairs, demonstrating that 50% of the arrayed antigens and 20% of the arrayed antibodies where detectable when immobilized [7]. Some cognate ligands were detected at concentrations as low as 1 ng/dl [7].

We have modified and refined the experimental protocol introduced by Haab *et al*. [7] to develop spotted antigen arrays for analysis of autoantibody responses [17]. We applied this technology to analyze the autoreactive B-cell response in patients with autoimmune diseases including systemic lupus erythematosus (SLE), scleroderma, and mixed connective tissue disease [17].

Our antigen array technology utilizes a robotic arrayer to attach proteins, protein complexes, peptides, nucleic acids, and other biomolecules in an ordered array on poly-L-lysine-coated microscopic slides (Fig. 1) [17]. Approximately 1 nl of solution containing 200 pg antigen is deposited on each array to produce antigen features measuring 100–200 μm in diameter. Individual arrays are incubated with serum from patients or controls, followed by fluorescently labeled secondary antibody. We typically use 1:150 dilutions of human or animal serum to probe arrays, requiring 2 μl serum per array under standard protocols and only 0.15 μl serum per array when employing cover

slips [17]. Other biological fluids such as cerebrospinal fluid, synovial fluid, and tissue eluates may also be used (our unpublished observations).

Arrays are scanned using a fluorescence-based digital scanning device. Algorithms are available for nearest-neighbor (cluster) [18] and statistical analysis [19] of the data. Detailed protocols are presented both in our earlier work [17] and online [20]. Information for construction of robotic arrayers is also available [21].

Antigen arrays proved to be fourfold to eightfold more sensitive than conventional ELISA analysis for detection of autoantibodies specific for five recombinant autoantigens [17]. Moreover, antigen arrays demonstrated linear detection of antibody concentrations over a 3-log range [17].

Specialized proteomes for specific autoimmune diseases

We are developing specialized arrays representing the 'proteomes' of the tissue targets in various autoimmune diseases.

'Connective tissue disease' arrays

Our 'connective tissue disease' arrays contain 200 distinct proteins, peptides, nucleic acids, and protein complexes targeted in a host of autoimmune diseases, including SLE, polymyositis, limited and diffuse scleroderma, primary biliary sclerosis, and Sjögren's disease (Fig. 1) [17]. Specific antigens include Ro, La, histone proteins, Jo-1, heterogeneous nuclear ribonucleoproteins (hnRNPs), small nuclear ribonucleoproteins, Smith ribonucleoproteins (Sm/RNP), topoisomerase I, centromere protein B, thyroglobulin, thyroid peroxidase, RNA polymerase, cardiolipin, pyruvate dehydrogenase, serine–arginine splicing factors, and DNA.

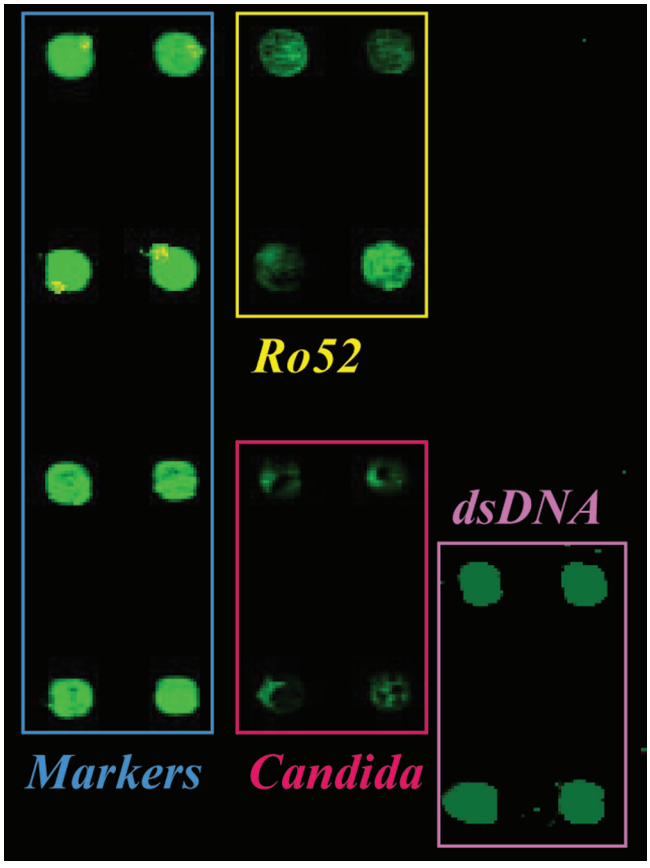
'Synovial proteome' arrays

We developed 'synovial proteome' arrays to study autoimmune arthritis involving synovial joints, including rheumatoid arthritis (RA) and its animal models. Our 'synovial proteome' arrays contain 650 candidate RA autoantigens, including deiminated fibrin, citrulline-modified filaggrin and fibrinogen peptides, vimentin, the endoplasmic chaperone BiP, glucose-6-phosphate isomerase, hnRNP A2/B1, collagens and overlapping peptides derived from several of these proteins.

'Myelin proteome' arrays

Our 'myelin proteome' arrays contain 500 proteins and peptides derived from the myelin sheath, the target of the autoimmune response in multiple sclerosis and in experimental autoimmune encephalomyelitis (EAE). These myelin antigens include myelin basic protein, proteolipid protein, myelin-associated glycoprotein, myelin oligodendrocytic glycoprotein, golli-myelin basic protein, oligodendrocyte-specific protein, cyclic nucleotide phosphodiesterase and overlapping peptides derived from these proteins. We are utilizing our 'myelin proteome' arrays to characterize the autoantibody response in EAE serum, multiple sclerosis patient serum and cerebral spinal fluid, and to guide selection of antigen-specific therapies in relapsing EAE [22].

Figure 1



The 'connective tissue disease' array. A 48-feature collage derived from a 1536-feature 'connective tissue disease' array probed with serum from a patient with systemic lupus erythematosus (SLE) is presented. This array demonstrates specific detection of two representative autoantibody reactivities, against Ro52 (upper center box) and double-stranded DNA (dsDNA, lower right box). Antibodies against *Candida* skin test antigens (lower center box) are also detected, and serve as a positive control. This collage contains four features representing the reactive antigens (boxed) and control antigens (not boxed). Arrays were produced using a robotic microarrayer to attach putative connective tissue disease autoantigens (listed in text) to poly-L-lysine-coated microscopic slides. The depicted array was incubated with a 1:150 dilution of serum derived from a patient with SLE and with ELISA-confirmed reactivity against Ro and DNA. Antibody binding was detected by incubation with Cy-3-labeled antihuman IgG/IgM secondary antibody. Marker spots (spotted Cy-3-labeled IgG, left box) are used to orient the arrays. Detailed protocols for production, probing, and scanning antigen arrays are presented in our earlier work [17] and online [21].

'Islet cell proteome' arrays

We are constructing 'islet cell proteome' arrays containing glutamic acid decarboxylase, IA-2, insulin and additional

candidate autoantigens in insulin-dependent diabetes mellitus (IDDM).

Applications for proteomics profiling of autoantibody responses

Autoantibody profiling for diagnosis

Autoantibodies have diagnostic utility for several autoimmune diseases. Such diseases include myasthenia gravis (antiacetylcholine receptor antibody), Grave's disease (antithyroid hormone receptor antibody), and SLE (combination of antinuclear antibodies, plus anti-DNA or anti-Sm antibodies). Furthermore, in T-cell-mediated IDDM, the presence of combinations of autoantibodies against at least two islet antigens, including insulin, glutamic acid decarboxylase, and IA-2, are diagnostic for or predictive of future development of IDDM [23]. The presence of autoantibodies against a single islet antigen has minimal clinical value. The clinical utility of autoantibodies in IDDM suggests that autoantibody profiles may have diagnostic utility for other T-cell-mediated diseases, such as RA and multiple sclerosis.

Monitoring epitope spreading: potential prognostic value

Intermolecular and intramolecular epitope spreading of the autoreactive B-cell response is associated with progression to overt clinical disease in human and murine SLE [24,25] and in IDDM [23]. Proteomics technologies are ideally suited to monitoring epitope spreading. Epitope spreading of the autoantibody response may represent a common harbinger of more severe and progressive autoimmunity, providing the clinician with valuable prognostic information to guide the use of nonspecific disease-modifying therapies.

Monitoring autoantibody isotype usage

Spotted antigen microarrays can identify antigen-specific autoantibody isotypes [17]. Th1-type immune responses, associated with production of interferon-γ and interleukin-12, generate antibodies of isotypes capable of fixing complement and causing tissue injury [26]. The ability to characterize isotype usage may facilitate the identification of offending autoantigens, based on determination of autoantigens against which autoantibodies of pathogenic isotypes are directed. Moreover, microarray isotype analysis may provide insight into both B-cell and T-cell autoimmunity because not only T cells, but also effector B cells, have been implicated in the reciprocal regulation of polarized Th1 versus Th2 cytokine production [27]. Therapeutic deviation of immune responses from Th1 to Th2 cytokine production has been associated with efficacious treatment of Th1-mediated immune disease [28,29].

Autoantigen discovery and characterization

Proteomics technologies can be applied to discover novel autoantigens utilizing cDNA expression libraries [13,14], peptide libraries, or arrayed fractions of autoimmune-target tissues. Once candidate autoantigens are identified, proteomics technologies can rigorously characterize the sensitivity and specificity of autoantibodies directed against candidate antigens in cohorts of autoimmune and control patients. Of note, post-translational modifications of antigens are amenable to detection using our antigen arrays and other proteomics technologies. This is important because such modifications are strongly associated with autoimmune diseases including SLE and RA [30–32].

Guiding development and selection of antigen-specific therapy

In addition to proteomics monitoring of epitope spreading and isotype usage to gauge need for nonspecific disease-modifying therapies (already described), determination of the specificity of the autoantibody response may enable tailored antigen-specific therapy. Such antigen-specific therapies can be peptide-based or protein-based tolerizing therapies. Alternatively, they can be specific DNA tolerizing vaccines, a strategy we termed 'reverse genomics' [22]. We discuss use of the autoantibody response to drive antigen-specific therapy elsewhere [22,33].

Future directions: challenges and limitations

Although we have made significant progress developing proteomics technologies, major hurdles and significant work remain. Extensive validation of array results, using thousands of sera already characterized for antibody specificities by standard methods, will be essential for regulatory approval and entry into routine clinical practice.

A limitation of addressable microarray systems results from the attachment of antigens to surfaces, beads, nanoparticles, or tags, which may alter immunologic epitopes. Certain autoantigens are not amenable to detection using poly-L-lysine-coated glass slides [7,17]. We are addressing this disadvantage using alternative surface chemistries, and linkers to orient and to serve as spacers between antigens and the surface, particle, or tag. Bead and tag systems are currently limited by the relatively small numbers of addressable elements available.

Autoantibody profiling using antigen microarray technology does not provide direct information about the specificity of the T cells that mediate autoimmunity. Although there are examples of discordance of the fine peptide epitope specificity of the autoreactive T-cell and B-cell responses, there is a high degree of concordance between autoreactive B-cell and T-cell responses at the macromolecular level [23,34]. We believe the specificity of the autoantibody response is predictive of the specificity of the overall autoimmune response at the level of whole autoantigens. Further studies will be necessary to determine whether this powerful and enabling hypothesis is, in fact, valid.

Conclusion

The development of miniaturized proteomics technologies heralds the beginning of an era of multiplex, high-throughput analysis of autoantibody specificities and isotype usage. Spotted antigen arrays on derivatized microscope slides offer a fluorescence-based proteomics platform utilizing simple protocols and widely available equipment. In the future, fluid-phase arrays based on addressable particles and tags are likely to supplant planar arrays, due to their lower propensity to distort and to sterically interfere with immunologic epitopes. We anticipate that proteomics monitoring of autoantibody responses will have a major impact on the diagnosis, monitoring, and therapy of autoimmune disease.

Acknowledgements

The authors thank Dr H de Vegvar, J Tom and other members of the Utz and Steinman laboratories for scientific input. This work was supported by NIH K08 AR02133 and an Arthritis Foundation Chapter Grant to WHR, by NIH K08 AI01521, NIH U19 DK61934, an Arthritis Foundation Investigator Award, a Bio-X grant, and a Baxter Foundation Career Development Award to PJU, by NIH/NINDS 5R01NS18235 and NIH U19 DK61934 to LS, and by a James Klinenberg Memorial Fellowship from the Arthritis National Research Foundation to WH.

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