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Review

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Protein arrays for autoantibody profiling and fine-specificity mapping

Protein arrays provide a powerful approach to study autoimmune disease. Autoimmune responses activate B cells to produce autoantibodies that recognize self-molecules termed autoantigens, many of which are proteins or protein complexes. Protein arrays enable profiling of the specificity of autoantibody responses against panels of peptides and proteins representing known autoantigens as well as candidate autoantigens. In addition to identifying autoantigens and mapping immunodominant epitopes, proteomic analysis of autoantibody responses will further enable diagnosis, prognosis, and tailoring of antigen-specific tolerizing therapy.

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Contents

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Abbreviations: CTD, connective tissue disease; **EAE**, experiment autoimmune encephalomyelitis; **Ig**, immunoglobbulin; **MS**, multible sclerosis; **PBC**, primary biliary cirrhosis; **RA**, rheumatoid arthritis; **SLE**, systemic lupus erythematosus

1 Introduction

Autoimmune diseases result from aberrant activation of T and B lymphocytes which attack self-molecules (termed autoantigens). Most but not all autoantigens are proteins or protein complexes. Aberrant autoimmune responses destroy cells and tissues containing these self-proteins, thereby causing the clinical syndromes classified as autoimmune diseases. Autoimmune responses are coordinated by autoreactive $CD4^+$ T lymphocytes. These autoreactive T cells reciprocally activate B cells, which then differentiate into memory B cells as well as plasma cells. The sole purpose of plasma cells is to produce high-affinity, high-avidity antibodies directed against the original activating antigen. In the case of autoreactive plasma cells, large amounts of autoantibodies are produced and secreted into the blood where they circulate and can deposit in tissues and organs, generating acute and chronic inflammation.

2 Autoantibody specificity reflects the specificity of autoimmune responses

The specificity of B cell autoantibody responses reflects the overall specificity of the autoimmune response. B cells are professional antigen presenting cells that provide and receive help from $CD4^+$ T cells. B cells bind, internalize, process, and present major histocompatibility (MHC)-bound peptides derived from macromolecular antigens that are specifically recognized by their rearranged cell surface immunoglobulin (Ig) receptors. B cells can only provide help to, and receive help from, T cells

that recognize these MHC-bound peptide epitopes. The reciprocal nature of T and B cell activation results in the activation of autoreactive T and B cells that recognize epitopes derived from the same macromolecular complex. This system likely evolved to insure that B and T cells could coordinate their attack against invading pathogens without also damaging the host. The specificity of the B cell autoantibody response therefore reflects the overall specificity of the autoreactive T cell response. This provides the rationale for the use of protein array profiling of autoantibody responses to gain insights into the overall immune response.

Autoreactive B and T cells are very rare. For example, of the order of 1 out of 10 000 or fewer lymphocytes from a diseased patient is autoreactive, based on limiting dilution and ELISPOT experiments [1, 2]. The detection of individual autoreactive T lymphocytes requires highly specialized reagents such as tetramers [3], which are tedious to produce, are specific for only a single epitope, and are not amenable for detecting rare populations of autoreactive T cells. Because B cells produce and secrete large quantities of soluble antibodies which are readily detectable in the serum, it is a simpler task to study autoantibodies using a variety of different techniques, including enzyme linked immunosorbent assays (ELISAs), Western blot analysis, immunoprecipitation analysis, and flow-based assays.

Protein arrays are well suited for the study of autoantibody responses for a number of reasons. Our labs as well as several other laboratories in academia and in industry have taken advantage of the abundant, high-affinity autoantibodies that are present in the serum of patients with rheumatic diseases to develop a specific protein array technology that can be applied directly to studying human disease. In this review we will summarize advances in protein array technology that have catalyzed our ability to analyze the "serum autoantibody proteome." There are five main areas in which we and others are currently developing or employing protein array technology for the study of autoantibodies: (i) to improve the diagnosis of autoimmune diseases; (ii) to study the natural progression of the immune response, both in autoimmunity and following vaccinations and infections; (iii) to identify serum autoantibody biosignatures that might identify subsets of patients with certain clinical features, prognostic outcomes, or who might be expected to respond well or have an adverse event related to a therapeutic intervention; (iv) to develop an "antigen-specific tolerizing therapy" based on the presence or absence of serum autoantibodies; and (v) to discover unique, novel autoantigens. Each of these uses will be described in more detail below.

2.1 Autoantibodies for the diagnosis of autoimmune disease: Why develop protein arrays?

Autoantibodies are a hallmark of many autoimmune diseases. For certain autoimmune diseases, the detection and quantification of autoantibodies provide diagnostic utility, and are routinely used in the clinic for diagnosis (Table 1). Routine assays for detection of autoantibodies are generally performed by ELISAs and fluorescence immunoassays. Individual assays are performed in microtiter plates, with each well representing a single antigen. For many clinical entities, clinicians order a host of individual ELISAs or fluorescence immunoassays to establish the diagnosis and to provide prognostic data to assist with clinical decision making. These tests are performed one-at-a-time, are laborious, and can be expensive. As will be discussed below, protein arrays to characterize autoantibodies have tremendous potential to improve the quantity, and perhaps the quality, of serologic information that is made available to the practicing clinician.

While there is little debate that detection of autoantibodies can be of tremendous importance to clinicians, there is great debate within the literature regarding the

Table 1. Autoimmune diseases characterized by the presence of diagnostic serum autoantibodies

Disease	Prominent autoantigen(s)
Systemic lupus erythematosus	DNA, histones, U1-snRNP, Ro/La particle
Sjögren's disease	Ro/La autoantigen complex
Antiphospholipid antibody syndrome	Phospholipids, β2-glycoprotein 1
Mixed connective tissue disease	U1-snRNP
Systemic sclerosis	Topoisomerase I, centromere proteins
Myositis	SRP, tRNA synthetases
Rheumatoid arthritis	Citrullinated antigens, immunoglobulin
Wegener's granulomatosis	Antineutrophil cytoplasmic antigens (ANCA)
Autoimmune diabetes	Insulin, IA-2, glutamic acid decarboxylase
Primary biliary cirrhosis	Pyruvate dehydrogenase and AKGDH complexes
Autoimmune thyroid diseases	Thyroperoxidase, thyroglobulin
Coeliac disease	Tissue transglutaminase
Bullous skin disease	Desmogleins
Myasthenia gravis	Acetylcholine receptor

significance of autoantibodies as mediators of disease [4, 5]. Table 1 lists select autoimmune diseases for which determination of autoantibody specificities provides important diagnostic utility. Examples of autoimmune diseases in which the pathophysiology is mediated by autoantibodies include Grave's disease (a destructive, inflammatory disease of the thyroid gland), myasthenia gravis (an autoimmune disease in which autoantibodies are produced against the acetylcholine receptor, leading to muscle weakness and its complications), antiphospholipid antibodies (in which antibodies bind to cell surface lipids, causing spontaneous abortions and clot formation in both the venous and arterial circulation) [5], and Wegener's granulomatosus (in which antineutrophil cytoplasmic antibodies can be transferred in animal models, producing inflammatory kidney and lung disease) [6]. Detection of specific serum autoantibodies is an important component of the diagnostic criteria for such diseases.

The first group to have described development of antigen arrays for the specific purpose of analyzing autoantibodies was Joos *et al*. [7]. This group described antigen arrays generated by robotic attachment of 18 prominent antigens in ordered arrays on nitrocellulose membranes and silane-treated microscope slides. Following incubation with human sera, arrays were incubated with a secondary antibody conjugated to horseradish peroxidase. A luminescent substrate was then added and arrays imaged with a charge-coupled device (CCD) chemiluminescence camera. Joos *et al*. [7] demonstrated sensitive and specific detection of autoantibodies specific for many of the 18 antigens contained on the arrays.

We adapted the methods of others [7–9] to develop antigen arrays to profile autoantibody responses in biological samples using fluorescence-based detection methods [10]. Antigen arrays are produced by attaching a spectrum of putative protein and peptide autoantigens to planar surfaces in an ordered array using a robotic microarrayer. In our initial paper [10], we developed specialized arrays to study connective tissue diseases (CTDs), such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), mixed connective tissue disease (MCTD), scleroderma, polymyositis, and Sjögren's syndrome. We used a robotic arrayer to print 1152-feature arrays containing 196 distinct proteins and peptides that represent prominent antigens in autoimmune connective tissue diseases. Arrays were incubated with human sera followed by anti-human secondary antibody conjugated to the Cy-3 fluorophore. Arrays were scanned and images analyzed to determine the level of autoantibody binding. We demonstrated that our antigen arrays were four to eight-fold more sensitive than conventional ELISAs, and that detection of autoantibody concentration was linear over a 3-log range.

We applied our CTD arrays to profile autoantibody responses in over 50 well characterized autoimmune serum samples, and found our array results to be highly concordant with results from conventional assays including ELISAs, Western blot and immunoprecipitation analysis. Examples of conventional assay-validated, array-determined reactivities included detection of autoantibodies specific for: Ro52 and La in serum from a patient with Sjögren's disease; DNA, histone protein, U1–70 kDa and serine-arginine-rich (SR) proteins in serum from an SLE patient; Ro52 and Jo-2 in serum from a polymyositis patient; pyruvate dehydrogenase complex in serum from a primary biliary cirrhosis (PBC) patient; topoisomerase I in serum from a diffuse scleroderma patient; centromere binding protein B in serum from a limited scleroderma patient; and hnRNP-B1 in serum from an RA patient [10]. Thus, antigen arrays demonstrated sensitive and specific detection of autoantibody reactivities that are in part diagnostic for eight different autoimmune connective tissue diseases. These studies will serve as a strong foundation for future studies, some of which are outlined below, employing autoantigen arrays.

Valenta and colleagues [11, 12] developed allergen arrays containing 94 purified allergen molecules to monitor IgE reactivity profiles for diagnosis, and for tailoring therapy. Array-based IgE profiling reflected the clinical sensitization patterns of patients for allergens including birch, grass pollen, animal dander, mites and molds. However, an imperfect association existed between the magnitude of the wheal in skin testing and microarray-determined allergen-specific IgE levels. In the clinic, allergen skin testing for reactivity against 25–100 allergens is used to guide selection of allergen desensitization therapy. In allergen desensitization therapy allergen extracts are delivered to patients at a regular interval, such as weekly, to attempt to divert allergy-promoting IgE responses to nonallergy promoting responses including the production of IgG antibody isotypes. This provides an established model for delivery of tailored allergen-specific therapy. In Section 2.4 below we present an analogous strategy to use proteomic determination of autoantibody specificities to develop and select the most appropriate antigen-specific tolerizing vaccine.

2.2 Examination of the natural progression of autoimmune and antimicrobial B cell responses: Epitope spreading

Epitope spreading is the process by which immune responses diversify their specificity to target additional epitopes on the initially-targeted molecule (termed intramolecular spreading) as well as on other polypeptides (termed intermolecular spreading) within the tissue under

attack [13, 14]. Epitope spreading likely evolved as a mechanism to prevent mutagenic escape of microbes from host immunity [15]. In autoimmune disease this protective mechanism results in the detrimental expansion of the autoimmune response to encompass many different self molecules. The role of epitope spreading in autoimmune disease initiation and progression is controversial.

In murine and human SLE and autoimmune diabetes, spreading of autoreactive B cell responses is associated with progression to clinically symptomatic disease [16–18]. Epitope spreading likely reflects the entrenchment of autoimmune responses, and such diversification of autoimmune responses poses significant challenges to the implementation of antigen-specific tolerative therapy, as described below [19]. Attempts to study epitope spreading of autoantibodies have been made by several groups studying SLE and bullous skin diseases. For example, Hirata *et al*. [20] used Western blotting to study the natural history of autoantibody profiles in the NZB/NZW F1 and MRL/lpr mouse models of SLE, demonstrating that the initiating antigen in the MRL/lpr mouse is the histone and nucleic acid binding protein nucleolin. A major problem with such an analysis is that the bands observed by Western blotting do not identify the target antigen, and laborious down-stream experiments must then be done to definitively determine the identity of each protein to which the antibodies are bound. Other assays such as ELISA could be performed; however, it is impractical to perform hundreds of individual ELISAs on each serum sample. Protein microarrays are perfectly suited for such studies since the position of each deposited antigen on the array surface is known. We are currently employing our $\sim$300 feature CTD protein and peptide antigen arrays to analyze serum derived from four different animals models of SLE, as well as cohorts of pediatric and adult patients with this disease. Analogous arrays have also been developed by our labs for identical studies in multiple sclerosis, autoimmune diabetes, primary biliary cirrhosis, and RA (our unpublished data).

We are also developing viral antigen arrays to monitor the evolution of antiviral antibody responses following vaccination and infectious challenge. We developed a simian-human immunodeficiency virus (SHIV) array, containing proteins and overlapping peptides representing the virus. Using these arrays we demonstrated that SHIV vaccines accelerate the generation of antiviral antibody responses in macaques (Neuman de Vegvar and Robinson, manuscript submitted). We further demonstrate that following SHIV challenge, antiviral antibody responses ultimately converge to target a set of dominant epitopes independent of the vaccine regimen, host MHC alleles expressed, and divergent antiviral T cell responses.

2.3 Proteomics technologies for multiplex analysis of autoantibody responses: Identification of biosignatures for diagnosis, prognosis, and selecting therapy

Proteomic autoantibody profiling provides the potential to define serum autoantibody biosignatures that identify subsets of patients with different clinical subtypes of disease, prognostic outcomes, or who are likely to respond to or experience an adverse event associated with a therapeutic intervention.

Recent data suggests that the T cell-mediated diseases juvenile onset autoimmune diabetes and RA can be diagnosed, or future development predicted by, the presence of combinations of autoantibody reactivities. Juvenile onset autoimmune diabetes (also termed Type I diabetes, or insulin dependent diabetes) is predicted or diagnosed by the presence of combinations of serum autoantibodies directed against insulin, glutamic acid decarboxylase and IA-2 [16]. Detection of autoantibody reactivity against insulin, glutamic acid decarboxylase or IA-2 in isolation does not provide predictive value. Asymptomatic family members whose serum autoantibodies recognize two or more of these molecules have a 68% likelihood of going on to develop clinical diabetes within 5 years [16]. If all antibodies specific for all three molecules are present, then the likelihood approaches 100%. In RA, combinations of autoantibody reactivities against 30 citrulline-modified peptides demonstrated higher sensitivity for the diagnosis compared to analysis of reactivities against individual peptides [21].

The diagnostic utility of combinations of autoantibodies for autoimmune diabetes and rheumatoid arthritis suggests that autoantibody profiles could possess diagnostic and predictive value for other T cell-mediated autoimmune diseases. This provides the rationale for multiplex analysis of autoantibody specificities in autoimmune diseases using proteomics technologies.

We recently developed and validated a 2304-feature array containing 232 peptide and protein antigens derived from the myelin sheath to study multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). MS and EAE are autoimmune demyelinating diseases in which T cells attack the myelin sheath of oligodendrocytes in the central nervous system. Direct immunofluorescence analysis and elution studies both demonstrated the presence of tissue-bound autoantibodies in central nervous system lesions (termed plaques) in MS patient brains obtained at autopsy [22]. The putative targets of the autoimmune response in MS include myelin basic protein (MBP) and myelin oligodendrocytic glycoprotein, and these myelin proteins are also targeted in

EAE. Many studies have demonstrated T cell epitope spreading to target multiple epitopes on proteolipid protein and MBP in EAE [14, 23–25]. We applied our myelin arrays to profile autoantibody responses in acute and chronic EAE (Robinson *et al*., manuscript submitted). In acute EAE, the diversity of antimyelin B cell responses predicted subsequent disease activity. Animals with persistent EAE activity demonstrated previously unrecognized extensive intra- and inter-molecular epitope spreading of their autoreactive B cell responses to simultaneously target multiple different myelin proteins.

Our data demonstrate extensive epitope spreading and diversification of autoreactive B cell responses in EAE. If such extensive epitope spreading occurs in human autoimmunity, one would predict that antigen-specific tolerative therapies that deliver an individual epitope or protein will provide marginal efficacy. It may prove necessary to utilize 'cocktail' tolerative vaccines, in which more than one dominant epitope or protein is delivered to turn off the immune response, in order to tolerate such diverse autoimmune responses. Taken together, our studies in EAE and SLE represent the first large-scale characterization of B cell epitope spreading to be described, and will provide an invaluable roadmap for future experiments in these and other diseases listed in Table 1.

2.4 Antigen arrays guide development and selection of antigen-specific therapy

One of our central goals is to use proteomic analysis of autoimmune responses to develop and select antigen-specific tolerizing therapies. Antigen-specific therapies specifically inactivate the autoreactive lymphocytes mediating tissue injury, preserving global immune function to fight infection. In order to develop antigen-specific therapies, one must know the specificity of the autoimmune response. Proteomics technologies can determine the specificity of such responses in cohorts of patients with a specific autoimmune disease, thereby enabling development of disease-specific tolerizing therapies. Proteomics technologies also enable determination of the specificity of the autoimmune response in individual patients, thereby enabling tailored therapy.

Before applying this concept to human patients enrolled in clinical trials, we tested the hypothesis that autoantibody profiles could be used to design a tolerizing therapeutic cocktail, using a DNA vaccine-based strategy in EAE, a rodent model of human MS. We demonstrated that myelin antigen arrays are useful adjuncts for the development and tailoring of antigen-specific tolerizing vaccines in EAE (Robinson *et al*., submitted). We developed tolerizing DNA vaccines [26] based on the specificity of antimyelin autoreactive B cell responses, and provided data suggesting that tolerative vaccines encoding a greater number of array-determined targets possessed greater efficacy. We termed this strategy 'reverse genomics'. These data suggest that proteomic monitoring of autoantibody profiles can be used to guide development of more efficacious tolerizing DNA vaccines. We anticipate that this strategy can also be applied to facilitate development and selection of other antigen-specific therapies, including peptide- and protein-based tolerizing therapies.

3 Antigen array monitoring of the response to therapy

We have also applied our myelin arrays to monitor responses to tolerizing vaccines in the EAE model, and we observed modulations of autoantibody profiles associated with efficacious tolerative therapy (Robinson *et al*., manuscript submitted). Tolerizing therapies that prevented EAE progression also prevented epitope spreading of autoreactive B cell responses. These data suggest that proteomic monitoring of autoantibody responses may provide an important surrogate for responses to tolerative therapy, both in clinical practice and in clinical trials. Clinical trials for autoimmune diseases frequently require 6–24 m study periods in order to demonstrate efficacy based on clinical parameters. Our protein array technology could provide a valuable surrogate in early phase clinical trials to facilitate optimization of dosing regimens and identification of efficacious therapies. We are currently in the process of analyzing biological samples derived from patients enrolled in clinical trials in which antigen-specific interventions are being tested, including injectable peptides (for treatment of RA and MS) and DNA vaccines (for treatment of MS). Other human trials in infectious disease (*e.g.*, vaccination protocols for anthrax, smallpox, and other bioterrorism agents) and autoimmunity (*e.g.*, SLE) will almost certainly enlist antigen array technology as one of their secondary endpoints. Finally, we have initiated preclinical studies using DNA vaccines in rodent models of SLE and PBC, with a major goal to determine what happens to autoantibody profiles following our interventions (our unpublished data).

3.1 Autoantigen discovery

For many other autoimmune diseases the specificity of the autoimmune response remains unknown (Table 2) and no specific autoantibody reactivities have been identified. For many of the diseases listed in Table 2, it is likely that serum autoantibodies exist but that they cannot be

Table 2. Putative autoimmune diseases with unidentified autoantigens

Alopecia areata
Ankylosing spondylitis
Autoimmune hemolytic anemia
Autoimmune thrombocytopenic purpura
Chronic inflammatory demyelinating polyneuropathy
Crohn's disease
Dermatitis herpetiformis
Eosinophilic pneumonia
Epidermolysis bullosa acquisita
Essential mixed cryoglobulinemia
Glomerulonephritis
Guillain-Barré syndrome
Idiopathic pulmonary fibrosis
IgA nephropathy
IgA linear dermatosis
Immune-mediated infertility
Inflammatory bowel disease
Juvenile arthritis
Lichen planus
Ménière's disease
Multiple sclerosis
Myocarditis
Neutrophilic dermatoses (Sweet's)
Polychondritis
Polyglandular syndromes
Polymyalgia rheumatica
Psoriasis
Psoriatic arthritis
Reiter's syndrome
Rheumatic fever
Rheumatoid arthritis
Sclerosing cholangitis
Ulcerative colitis
Vasculitidies
ANCA positive vasculitis
Giant cell arteritis
Polyarteritis nodosa
Takayasu's arteritis
Henoch Schoenlein purpura
Churg-Strauss vasculitis
Kawasaki's disease
Hypersensitivity vasculitis
Behçet's disease
Thromboangiitis obliterans

detected because the target autoantigen has not yet been found. As will be discussed below, protein arrays provide a powerful approach to profile autoantibody responses in autoimmune disease for autoantigen discovery.

Several distinct areas of research are necessary to identify and define the relevant autoantigens in particular autoimmune diseases, including: (i) discovery of candidate autoantigens; (ii) characterization of the sensitivity and specificity of reactivity against individual or combinations of candidate autoantigens for disease; and (iii) demonstration of the relevance of autoimmune responses directed against the putative autoantigen(s) in mediating the pathophysiology of disease.

Several laboratories are developing arrays of polypeptides, peptides and tissue fractions in an attempt to discover novel autoantigens. Walter and colleagues [27–30] are arraying polypeptides expressed and purified from cDNA expression libraries and incubating the resulting arrays with sera derived from patients with autoimmune disease. These investigators generated cDNA expression libraries from human fetal brain and other tissues. cDNA expression constructs are expressed in liquid bacterial cultures, the recombinant polypeptides are purified using nickel chromatography, and then ink-jet technology is used to generate ordered arrays of the recombinant polypeptides on PVDF membranes. Arrays containing 27 648 human fetal brain cDNA products have been produced, although due to redundancy it is estimated that only 1000–5000 distinct polypeptides are represented [30, 31]. These arrays have been probed with serum from patients with autoimmune diseases, including inflammatory bowel disease, and reactive cDNA products are being characterized.

Other investigators are developing arrays of recombinant proteins or synthetic peptides using a variety of methodologies and technologies. Examples include arrays of mammalian cells [32] or yeast [33] expressing defined cDNAs, and arrays produced using *in situ* cell-free transcription and translation of cDNA [34]. Arrays of synthetic peptides are being generated using standard photolithography [35], maskless photolithographic fabrication using digital micromirrors [36, 37], and synthesis of peptides on pins [38, 39].

Once candidate autoantigens are identified, proteomics technologies can be used to screen candidate antigens to determine the sensitivity and specificity of individual, and combinations of, autoantibody reactivities in cohorts of autoimmune and control patients.

4 Multiplex antibody isotype analysis

Array-based multiplex antibody isotype analysis could provide insights into events that lead to development of autoimmunity and help identify offending autoantigens. Warren *et al*. [40] demonstrated that antibody isotype subclass switching was associated with progression to clinical disease in endemic pemphigus foliaceus. They observed that normal subjects possessed a desmoglein-1-specific IgG1 and IgG4 response, and that patients with

pemphigus foliaceus possessed 20-fold higher levels of IgG4. Patients with active disease demonstrated a 75-fold higher level of IgG4-specific antibodies directed against desmoglein-1. Their data suggests that acquisition of increased anti-desmoglein-1 IgG4 responses may be a critical step in the development of clinical disease. These data provide the rationale for multiplex analysis of autoantibody isotypes.

Antigen arrays enable multiplex analysis of autoantibody isotypes. Th1 immune responses mediate autoimmune tissue destruction and are associated with the production of complement-fixing antibody isotypes – IgG2a and IgG2b in mice and IgG1 and IgG3 in humans [41]. In contrast, Th2 responses generally protect against autoimmune tissue injury and are associated with allergy, asthma and atopy. In Th2 diseases, production of non-complement fixing antibody isotypes – IgG1 and IgE in mice and IgG2 and IgG4 in humans – results. Using isotype-specific secondary antibodies and a comparative method in which spectrally-resolvable fluorophores are conjugated to each isotype-specific secondary antibody (analagous to methods used for RNA transcript profiling), antigen arrays can be utilized for multiplex analysis of the isotype usage of autoantibodies [10]. Proteomic analysis of autoantibody isotypes could help identify the autoantigens driving autoimmunity and autoimmune tissue injury.

One might predict that pathogenic $CD4^+$ and B cell responses would result in the production of complement-fixing antibody isotypes, while nonpathogenic responses would be associated with the noncomplement fixing isotypes. Isotype analysis will prove critical to understanding the mechanisms governing autoimmunity, and will be useful for analyzing animals and humans enrolled in clinical trials of antigen-specific interventions.

5 Fluid-phase proteomics technologies

To circumvent potential limitations of planar array systems, including drying and alteration of immunologic determinants that can result from attachment to solid supports, fluid-phase multiplex assay systems are being developed. In such systems antigens are labeled with addressable beads [42], tags [43], nanoparticles [44] or other molecules to enable identification of reactive species. Efforts in the lab of one of the authors (PJU) are underway to develop microfluidic systems for multiplex analysis of protein-protein interactions, and to use novel methods of detection such as carbon nanotubes to improve protein array technology [45, 46]. We refer readers to several recent reviews in which we described these proteomics technologies in detail [47–49].

6 Next generation methodologies

A variety of methodologies and technologies being developed will likely augment and facilitate proteomic analysis of autoimmune disease. Examples of novel methods of detection include use of resonance light scattering particles [50, 51], carbon nanotubes [46], and nanocantilevers [52]. Advances in detection methodologies will provide enhanced sensitivity and specificity, increased array complexity, and further miniaturized proteomics assay formats. Other areas of advancement will include refinement of array surface technologies and methods to express, purify, and catalyze directed post-translational modifications to large numbers of recombinant polypeptides.

7 Future directions

Proteomics technologies provide powerful methods for autoantigen discovery and profiling autoantibody responses. These technologies dramatically enhance our ability to identify autoantigens from large numbers of candidate proteins and peptides. Identification of the autoantigens targeted in autoimmune disease is a critical step towards unraveling the underlying etiology of, and developing antigen-specific tolerative therapies to fundamentally treat, autoimmune diseases. There is growing evidence that profiles of autoantibody reactivity, and not individual specificities, provide the greatest diagnostic and prognostic utility. In the coming decades, proteomic analysis of autoantibody responses will revolutionize the diagnosis, monitoring and treatment of autoimmune disease.

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8 References

[1] Anderson, A. C., Nicholson, L. B., Legge, K. L., Turchin, V. *et al.*, *J. Exp. Med.* 2000, *191*, 761–770.

[2] Targoni, O. S., Baus, J., Hofstetter, H. H., Hesse, M. D. *et al.*, *J. Immunol.* 2001, *166*, 4757–4764.

[3] Altman, J. D., Moss, P. A., Goulder, P. J., Barouch, D. H. *et al.*, *Science* 1996, *274*, 94–96.

[4] Smolen, J. S., Steiner, G., *Curr. Opin. Rheumatol.* 1998, *10*, 201–206.

[5] von Mühlen, C. A., Tan, E. M., *Semin. Arthritis Rheum.* 1995, *24*, 323–358.

[6] Xiao, H., Heeringa, P., Hu, P., Liu, Z. *et al.*, *J. Clin. Invest.* 2002, *110*, 955–963.

[7] Joos, T. O., Schrenk, M., Hopfl, P., Kroger, K. *et al.*, *Electrophoresis* 2000, *21*, 2641–2650.

[8] MacBeath, G., Schreiber, S., *Science* 2000, *289*, 1760–1763.

[9] Haab, B. B., Dunham, M. J., Brown, P. O., *Genome Biol.* 2001, *2*, research0004.0001–0004.0013.

[10] Robinson, W. H., DiGennaro, C., Hueber, W., Haab, B. B. *et al.*, *Nat. Med.* 2002, *8*, 295–301.

[11] Hiller, R., Laffer, S., Harwanegg, C., Huber, M. *et al.*, *FASEB J.* 2002, *16*, 414–416.

[12] Harwanegg, C., Laffer, S., Hiller, R., Mueller, M. W. *et al.*, *Clin. Exp. Allergy* 2003, *33*, 7–13.

[13] Steinman, L., *J. Exp. Med.* 1999, *189*, 1021–1024.

[14] Lehmann, P. V., Forsthuber, T., Miller, A., Sercarz, E. E., *Nature* 1992, *358*, 155–157.

[15] Milich, D. R., McLachlan, A., Thornton, G. B., Hughes, J. L., *Nature* 1987, *329*, 547–549.

[16] Verge, C. F., Gianani, R., Kawasaki, E., Yu, L. *et al.*, *Diabetes* 1996, *45*, 926–933.

[17] Craft, J., Fatenejad, S., *Arthritis Rheum.* 1997, *40*, 1374–1382.

[18] James, J., Harley, J., *Immunol. Rev.* 1998, *164*, 185–200.

[19] Robinson, W. H., Garren, H., Utz, P. J., Steinman, L., *Clin. Immunol.* 2002, *103*, 7–12.

[20] Hirata, H., Takahashi, A., Kobayashi, S., Yonehara, S. *et al.*, *J. Exp. Med.* 1998, *187*, 587–600.

[21] Schellekens, G. A., de Jong, B. A., van den Hoogen, F. H., van de Putte, L. B. *et al.*, *J. Clin. Invest.* 1998, *101*, 273–281.

[22] Genain, C., Cannella, B., Hauser, S., Raine, C., *Nat. Med.* 1999, *5*, 170–175.

[23] McRae, B. L., Vanderlugt, C. L., Dal Canto, M. C., Miller, S. D., *J. Exp. Med.* 1995, *182*, 75–85.

[24] Tuohy, V. K., Yu, M., Yin, L., Kawczak, J. A. *et al.*, *Immunol. Rev.* 1998, *164*, 93–100.

[25] Tuohy, V. K., Yu, M., Yin, L., Kawczak, J. A. *et al.*, *J. Exp. Med.* 1999, *189*, 1033–1042.

[26] Garren, H., Ruiz, P. J., Watkins, T. A., Fontoura, P. *et al.*, *Immunity* 2001, *15*, 15–22.

[27] Lueking, A., Horn, M., Eickhoff, H., Büssow, K. *et al.*, *Anal. Biochem.* 1999, *270*, 103–111.

[28] Walter, G., Büssow, K., Cahill, D., Lueking, A. *et al.*, *Curr. Opin. Microbiol.* 2000, *3*, 298–302.

[29] Cahill, D. J., *J. Immunol. Methods* 2001, *250*, 81–91.

[30] Büssow, K., Cahill, D., Nietfeld, W., Bancroft, D. *et al.*, *Nucleic Acids Res.* 1998, *26*, 5007–5008.

[31] Holt, L. J., Büssow, K., Walter, G., Tomlinson, I. M., *Nucleic Acids Res.* 2000, *28*, E72.

[32] Ziauddin, J., Sabatini, D. M., *Nature* 2001, *411*, 107–110.

[33] Zhu, H., Bilgin, M., Bangham, R., Hall, D. *et al.*, *Science* 2001, *293*, 2101–2105.

[34] He, M., Taussig, M. J., *Nucleic Acids Res.* 2001, *29*, E73–73.

[35] Fodor, S. P., Read, J. L., Pirrung, M. C., Stryer, L. *et al.*, *Science* 1991, *251*, 767–773.

[36] Singh-Gasson, S., Green, R. D., Yue, Y., Nelson, C. *et al.*, *Nat. Biotechnol.* 1999, *17*, 974–978.

[37] Blanchard, A. P., Friend, S. H., *Nat. Biotechnol.* 1999, *17*, 953.

[38] Geysen, H. M., Meloen, R. H., Barteling, S. J., *Proc. Natl. Acad. Sci. USA* 1984, *81*, 3998–4002.

[39] James, J. A., Harley, J. B., *J. Immunol.* 1992, *148*, 2074–2079.

[40] Warren, S. J., Arteaga, L. A., Rivitti, E. A., Aoki, V. *et al.*, *J. Invest. Dermatol.* 2003, *120*, 104–108.

[41] Abbas, A. K., Murphy, K. M., Sher, A., *Nature* 1996, *383*, 787–793.

[42] Fulton, R. J., McDade, R. L., Smith, P. L., Kienker, L. J. *et al.*, *Clin. Chem.* 1997, *43*, 1749–1756.

[43] Miller, J. P., *Anal. Res. Technol.* 2002, *3*, 16–17.

[44] Nicewarner-Pena, S. R., Freeman, R. G., Reiss, B. D., He, L.*et al.*, *Science* 2001, *294*, 137–141.

[45] Herr, A. E., Molho, J. I., Drouvalakis, K. A., Mikkelsen, J. C. *et al.*, *Anal. Chem.* 2003, *75*, 1180–1187.

[46] Chen, R. J., Bangsaruntip, S., Drouvalakis, K. A., Wong Shi Kam, N. *et al.*, *Proc. Natl. Acad. Sci. USA* 2003, *100*, 4984–4989.

[47] Hueber, W., Utz, P. J., Steinman, L., Robinson, W. H., *Arthritis Res.* 2002, *4*, 290–295.

[48] Robinson, W. H., Steinman, L., Utz, P. J., *Arthritis Rheum.* 2002, *46*, 885–893.

[49] Robinson, W. H., Steinman, L., Utz, P. J., *BioTechniques* 2002, *Suppl.*, 66–69.

[50] Yguerabide, J., Yguerabide, E. E., *Anal. Biochem.* 1998, *262*, 157–176.

[51] Yguerabide, J., Yguerabide, E. E., *Anal. Biochem.* 1998, *262*, 137–156.

[52] Fritz, J., Baller, M. K., Lang, H. P., Rothuizen, H. *et al.*, *Science* 2000, *288*, 316–318.