

The Challenge of Analyzing the Proteome in Humans with Autoimmune Diseases

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ABSTRACT: Analysis of blood samples from patients suffering from autoimmune diseases remains a mainstay in the clinic for initial diagnosis, prognostication, and clinical decision making. In particular, testing for the presence of serum autoantibodies has proved to be one of the most useful confirmatory assays for many different diseases. Recent genomic and transcript profiling studies have implicated certain cytokines, surface receptors, signaling pathways, and cell types in the pathogenesis of inflammatory diseases. The next obvious step is to delve into the much more complex level that follows the genome and transcriptome—the expressed proteome. This review focuses on several proteomics technologies being applied and/or developed by our laboratory for the study of autoimmunity, cancer, and cardiovascular disease, all of which are known to be associated with defects in immunity and inflammation. The findings of other participants in the recent Human Immunology Conference hosted by the Dana Foundation and the New York Academy of Sciences (May 17 & 18, 2005) are included. In particular, major pitfalls in the study of the human proteome are pointed out, and important areas for immediate investigation to move the field forward as rapidly as possible are proposed.

KEYWORDS: proteomics; protein arrays; autoimmunity; signaling; autoantibodies

INTRODUCTION

Proteomics is the comprehensive study of the proteins expressed in a cell, tissue, biological fluid, or organism. We and others hypothesize that comparison of the proteome in healthy and diseased blood cells or biological fluids will provide insights into autoimmune disease pathogenesis, and will also identify useful biomarkers and, ultimately, surrogate markers that can be used in the clinic. Relatively few methods have emerged that appear promising for this purpose, in particular mass spectrometry, cytokine profiling using a variety of techniques, MHC-tetramer arrays, lysate and autoantigen microarray profiling, and polychromatic phospho-flow cytometry.¹ Initial studies using these techniques in inbred animal models of autoimmunity have been very successful, and have argued strongly for similar studies in human beings. Unfortunately, human patients are genetically diverse, are exposed to different envi-

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ronmental and dietary conditions, have relatively long disease courses that make them difficult to study over time, are highly mobile, and are treated with different therapeutic regimens at different medical centers. Moreover, current proteomics assay platforms are not standardized, nor do reference samples exist with which to disseminate many of these assays. This has meant that few centers are capable of properly analyzing samples from patients with rare autoimmune diseases and patients enrolled in clinical trials.

In this chapter we describe three techniques presented at the Human Immunology Conference that are being used by our lab—autoantigen microarrays for antibody profiling, lysate microarrays for profiling of signaling pathways, and phospho-flow cytometry for studying signaling events in single cells—focusing on pitfalls associated with each platform. We conclude by identifying areas for future investigation that need to be addressed for proteomics to succeed in the coming decade.

AUTOANTIGEN MICROARRAY TECHNOLOGY FOR ANTIBODY PROFILING

Protein microarrays have generated much excitement over the past five years because of their potential to profile protein levels and their posttranslational modifications at a scale that has never before been possible. It has been the hope of the research community that protein microarrays will impact the field of proteomics in much the same way that DNA microarrays have impacted genomics. Although the concept of using microarrays to profile proteins quantitatively is straightforward, progress in protein profiling microarrays has been slower than originally anticipated. A dearth of high-quality capture reagents (e.g., monoclonal antibodies) of high affinity and low cross-reactivity that bind analytes in their native conformation in solution is the major obstacle that currently faces the field.^{2,3} In contrast, “protein function” microarrays have made significant strides in recent years and will likely become the dominant protein microarray platform in the near future. Protein function microarrays essentially consist of a large number of purified proteins and peptides arrayed on a planar surface. A seminal paper by MacBeath and Schreiber in 2000 described a series of elegant proof-of-principle experiments demonstrating the potential of protein function microarrays to screen for novel protein–protein interactions, kinase substrates, and protein targets of small molecules.⁴

Joos, Robinson, and others adapted protein microarray technology for the study of autoimmunity with the construction of a connective tissue–disease autoantigen microarray using a conventional DNA microarrayer.^{5–7} Autoantigen microarrays consist of purified proteins, synthetic peptides, nucleic acids, carbohydrates, and lipids that are deposited in a spatially addressable manner on chemically derivatized glass microscope slides. The arrays are probed with serum or other biological fluids (e.g., synovial fluid, cerebrospinal fluid) from autoimmune patients or animals. Bound antibodies are subsequently detected using fluorescently tagged secondary antibodies. The fluorescence intensity of each spot thus corresponds to the titer of the specific autoantibody present in the original biological sample. Because potentially thousands of antigens can be printed on a single slide, antigen microarrays represent a powerful platform for high-throughput profiling of serum autoantibodies.

Although the technology is still in its developmental stages, and many technical hurdles must be overcome before it is sufficiently reliable for routine clinical and research applications, our group and others have generated promising data demonstrating its utility in autoimmunity research. In the following sections, we review recent work on antigen microarrays and speculate on potential applications of this technology.

APPLICATIONS OF ANTIGEN MICROARRAYS

Although the precise role of autoantibodies in the pathogenesis of many autoimmune diseases is not clear, and in some cases is controversial, it has been known for many years that serum autoantibodies have important diagnostic value, especially for connective tissue diseases. The main problem with relying on autoantibodies alone for establishing a diagnosis is that most autoantibodies have shown poor sensitivity and/or specificity for their associated diseases. It is our hypothesis that it is the profile of multiple autoantibody reactivities, not reactivity to a single autoantigen, that will provide a diagnostic test with the highest sensitivity and specificity. Autoantigen microarrays can be used to test this hypothesis based on the pattern of reactivity to potentially thousands of unique whole antigens, as well as epitopes contained within individual antigens. More important, we believe that it may be possible to screen for patients who are likely to develop disease by monitoring their autoantibody profiles before clinical manifestation of symptoms. This is important because interventions can be administered to those patients to prevent or delay the disease. A recent study by Quintana *et al.* showed that the profile of autoantibody reactivity using a diabetes autoantigen microarray could distinguish male nonobese diabetic (NOD) mice that were susceptible to cyclophosphamide-induced diabetes from those that were resistant.⁸ Importantly, the autoantibody profiles were obtained from mice before the appearance of disease manifestations, indicating that microarray profiling may be valuable as a screening tool for patients with an increased likelihood of disease development. Indeed, autoantibodies are frequently found in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and type 1 diabetes (T1D) patients many years before the onset of clinical symptoms.⁹⁻¹² Whether microarray profiling of autoantibodies has a higher positive predictive value than any single autoantibody test alone remains to be determined. This line of reasoning can be extended to prognostication in human patients with an established autoimmune diagnosis. Let us take SLE as an example. Although up to 75% of SLE patients develop some form of renal disease, the severity of renal involvement varies widely between patients, ranging from minimal deposition of immune complexes in the mesangium to severe glomerulonephritis (GN) leading to nephrotic syndrome and/or renal insufficiency. Although antibodies to C1q and autoantibodies of certain isotypes (e.g., IgG3) have been linked to lupus nephritis, there is currently no prognostic marker that is used clinically to predict renal disease in an individual SLE patient.^{13,14} Can autoantibody microarray profiling aid in the prognostic classification of lupus patients? Precisely this question is being addressed in our laboratory using lupus autoantigen microarray analysis in pediatric lupus patients, and in adult patients enrolled in clinical trials testing novel therapeutics.

Recent work from our laboratory and the Robinson laboratory has expanded further the potential application of antigen microarrays for guiding therapy of autoimmune diseases and infectious diseases such as human immunodeficiency virus infection (HIV).¹⁵ Our lab is interested in the development of DNA vaccines for antigen-specific tolerizing therapy.¹⁶ However, the diversity of autoantigens being targeted in any given autoimmune disease poses a challenge to selecting the appropriate antigens for inclusion in the DNA vaccine. We hypothesize that autoantigen microarrays can help guide this selection process by profiling the autoantibody response. To test this hypothesis, a “myelin proteome” microarray was constructed to profile the evolution of autoantibody responses in experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS). Based on the autoantibody profile generated, autoantigen targets were selected and included in expression vectors for immunization. The resulting DNA vaccine was successful in treating established EAE. Furthermore, using the myelin proteome microarray, we were able to show reduced epitope spreading in vaccinated mice.¹⁶ This study highlights the potential use of autoantigen microarrays to aid the design of patient-tailored therapies and to monitor patient responses to therapies. Studies currently are being performed in our laboratory to determine whether this new therapeutic paradigm will work in human patients. This work has led to an ongoing phase I/II trial (Bayhill Therapeutics, Palo Alto, CA) of a tolerizing DNA plasmid-encoding myelin basic protein (MBP) in human MS patients, who are being monitored using antigen microarray technology for profiling autoantibodies. Similar monitoring studies in SLE, primary biliary cirrhosis (PBC), and vasculitis patients treated with Rituximab and other investigational drugs also are underway.

REVERSE-PHASE PROTEIN LYSATE MICROARRAYS

Reverse-phase protein (RPP) microarrays were described originally by the Petricoin and Liotta groups in a paper showing activation of prosurvival pathways (e.g., phosphorylation of Akt) in the transition from normal prostate epithelium to prostate intraepithelial neoplasia, and then to invasive prostate cancer.¹⁷ Before we delve into the technical aspects of RPP microarrays, it is helpful to distinguish first between “forward-phase” and “reverse-phase” approaches, and to discuss why RPP arrays have unique advantages over other array formats for protein profiling, especially for the study of signaling networks, which often are dysregulated in cells involved in the initiation and maintenance of autoimmunity.

As noted earlier, the lack of specific monoclonal antibodies for binding native proteins in solution poses a challenge to the development of conventional protein profiling arrays, in which the capture reagents are immobilized on the array surface. One can view the forward-phase approach as an array of miniaturized immunoprecipitation (IP) reactions that occur proximal to the array surface. Based on our own experience, past literature, and information supplied by commercial vendors, we estimate that only about 20% of commercially available antibodies that target intracellular proteins can be used for IP experiments. It is important to note that this estimate applies only to conventional antibodies. We estimate that the fraction of phosphorylation-specific antibodies that can be used for IP reactions is less than 10%. In contrast, close to 100% of these antibodies should bind to their antigen with

high affinity when the antigen is denatured and immobilized on a membrane, as in Western blotting. The forward-phase approach is complicated further by the issue of detection. Direct labeling of the protein sample with fluorescent “tags” is one approach that has been tried by several groups. However, the tags can interfere sterically with antibody binding if they are located within binding epitopes. Furthermore, the efficiency of protein labeling reactions is notoriously variable, making quantitative comparisons between samples difficult. Currently, the “sandwich immunoassay” approach appears to be the best option for forward-phase microarrays. However, antibody pairs are not available for many intracellular proteins. This led us to explore reverse-phase protein microarrays, in which the samples are immobilized on the array surface. The arrays are then probed with specific antibodies that are either phosphorylation-state-dependent or -independent. Bound antibodies are detected using secondary antibodies conjugated to horseradish peroxidase (HRP), followed by a tyramide-based amplification step that deposits biotin molecules adjacent to the HRP signal. A fluorescent signal is generated by incubating the arrays with fluorophore-conjugated streptavidin. The main advantage of this technique is that thousands of samples can be analyzed simultaneously on the same platform, greatly increasing throughput and simplifying quantitative analysis between samples. Furthermore, an exceedingly small amount of sample is required for printing the arrays, thus permitting the analysis of rare and valuable patient samples. Importantly, most if not all commercially available antibodies should work in this format, unlike forward-phase approaches. A clear limitation to this approach is that only one analyte can be measured on a single array. To partially overcome this limitation, arrays can be printed in a multisector format (arrays of arrays), allowing for up to 16 analytes to be analyzed on a single slide. Although this number is still orders of magnitude smaller than the number of probes on a DNA microarray, it is not as limiting as one might think if the analysis is focused on one functional class of proteins (e.g., signal transduction proteins or apoptosis-related proteins). A particularly successful application of RPP microarrays has been the profiling of phosphorylation states of signaling proteins with the use of phospho-antibodies.¹⁸ This has been possible only within the last few years with the availability of an expanding collection of well-characterized phospho-specific antibodies. Indeed, the use of RPP microarrays for the analysis of phospho-signaling networks in immune-related cells has been the focus of our research.^{18,19}

POLYCHROMATIC FLOW CYTOMETRY FOR STUDYING SIGNALING PATHWAYS IN SINGLE CELLS

Fluorescence-activated cell sorting (FACS) has revolutionized the study of immunology. FACS has allowed the discovery and characterization of antigen-specific T and B lymphocytes, and has identified subsets of cells that have important functional roles in the immune system. In 2002, Perez and Nolan reported a comprehensive demonstration of intracellular staining of cells with phospho-specific antibodies.²⁰ In this platform, cell populations are identified by using monoclonal antibodies specific for various cell-surface proteins. Each of the antibodies is conjugated to a spectrally resolvable fluorophore. A complex mixture of cells can be analyzed, as one

can gate on different cell-surface markers. Cells are then fixed and permeabilized using protocols developed in the Nolan lab. The cells are stained using antibodies that recognize intracellular proteins, including cytokines, chemokines, structural proteins, apoptosis-specific proteins, and, more important, signaling molecules such as kinases and their substrates. Monoclonal antibodies recognizing phospho-specific epitopes in particular were used in their original study. Perez and Nolan showed the simultaneous detection of several different kinase families, including mitogen-activated protein kinases (P38 MAPK, P44/42 MAPK, and JNK), kinases associated with cell survival (AKT/PKB), and a T-cell activation kinase (TYK2). Signaling was measured in peripheral blood mononuclear cells (PBMCs) following a variety of different physiologic stimuli. Human memory cells as well as naive lymphocytes were exposed to cytokines, and the signaling pathways were studied using five different differentiation markers to analyze the cell populations. Finally, hierarchical signaling nodes were studied in an attempt to order the activation of each of the kinases. This important paper showed that polychromatic flow-cytometric analysis of signaling pathways could be performed at a single-cell level, with obvious implications for the study of human samples, particularly from patients with autoimmune disease.

An elegant extension of this study was performed looking at cancer cells. Irish and colleagues applied the same technology to the study of acute myeloid leukemia (AML) samples.²¹ The hypothesis being tested was that differences in signaling could segregate patients into those who may or may not respond to therapy, and also might correlate with clinical outcome regardless of therapy. Cancer cells were obtained from patients through collaboration with investigators in Norway. Cells were frozen and then analyzed using phospho-specific FACS. Cells were thawed and exposed to various cytokines before staining with antibodies directed against phosphorylated STAT molecules, as well as other signaling proteins. The results obtained through genetic analysis as well as disease outcome correlated well with unique cancer network profiles that were obtained by FACS.

The Nolan lab has now initiated studies in collaboration with our lab and the Robinson lab as part of our NHLBI proteomics contract. We are studying the natural history of the autoimmune response in three different mouse models of lupus, and in the collagen-induced arthritis (CIA) model of rheumatoid arthritis. We are analyzing subsets of B cells, T cells, and dendritic cells obtained from mice at different ages, and correlating results with pathologic specimens obtained at the same time points. Autoantibody profiling also is being studied in these animals. Samples are being obtained after stimulation with various cytokines and chemokines, and ligation of cell-surface receptors. The initial goal is to replicate published data demonstrating defects in T-cell receptor and other signaling pathways in these autoimmune diseases. Ultimately, we hope to identify novel alterations in signaling pathways. The second stage of the study is to identify changes in the signaling proteome in response to therapeutic interventions, using mice treated with standard therapies that are used currently in clinical practice in humans. These regimens include treatment with oral steroids and cyclophosphamide, exposure to a lipid-lowering class of drugs (statins), and treatment with a variety of proprietary inhibitors of LFA-1. DNA vaccine studies also are being analyzed using this approach, although MHC-tetramers that would allow the study of antigen-specific immune responses do not yet exist for lupus or rheumatoid arthritis. These studies have been extended to the study of human samples, described in more detail in this issue by Perez.²²

CRITICAL PROBLEMS THAT MUST BE SOLVED

Proteomics studies in animal models of autoimmunity form the basis for the next step—the study of human patients. However, animal models, particularly rodent models, are highly biased, as the animals are inbred, and in some cases complete Freund's adjuvant (CFA) is used to initiate disease, markedly enhancing antibody production and T-cell activation above physiologic levels. A number of important limitations in using proteomics to study autoimmunity were identified by attendees at the Dana Foundation meeting: (1) Handling and storage of clinical samples need to be optimized and standardized. Freeze/thaw protocols need to be validated fully, particularly for the study of human blood cells. (2) The enormous volume of data generated by proteomics platforms such as mass spectrometry and protein arrays will require creative advances in bioinformatics. (3) Inter- and intra-assay normalization for autoantibody profiling remains to be resolved, a task that is of the highest priority for the future. (4) Improvements in technologies, particularly more reproducible methods for printing proteins, and better methods for performing and quantitating mass spectrometry, are required. (5) The generation of high-affinity, non-cross-reactive antibodies is perhaps the biggest challenge facing future development of reverse-phase protein microarrays. Without prior protein separation, the accuracy of the results depends solely on the specificity of the antibodies that are used. (6) Newly developed detection technologies using alternative fluorophores need to be validated further. Fluorophores that emit in the infrared range permit scanning at higher wavelengths. (7) Finally, the development of reagents such as MHC-tetramers for studying human antigen-specific immune responses may fundamentally alter our understanding of autoimmune disease pathogenesis.

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[*Competing interests:* P.J. Utz states the following conflict of interest disclosures, all directly relevant to proteomics technology described in this manuscript: In the past three years he has served as a consultant to Becton Dickinson Biosciences (San Jose, CA) and Genentech, Inc. (South San Francisco, CA). He is a member of the Scientific Advisory Board of Monogram Biosciences (South San Francisco, CA), XDX, Inc. (South San Francisco, CA), Centocor (Horsham, PA), MedImmune (Gaithersburg, MD), and Biogen/IDEC (Cambridge, MA). He is a cofounder and consultant at Bayhill Therapeutics (Palo Alto, CA), which is using antibody microarray profiling in a human MS trial.]

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