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PAPER

Multiplexed assays for identification of biomarkers and surrogate markers in systemic lupus erythematosus

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Validated biomarkers and surrogate markers are badly needed for monitoring patients with systemic lupus erythematosus (SLE), both for routine clinical care and for clinical trials research. SLE is difficult to study in clinical trials, in part because the disease is so heterogeneous. Very few useful markers have been identified, and even those that historically have been thought to be valid have been recently questioned. This report will focus on the use of emerging multiplexed assay formats that enable analysis of hundreds or even thousands of analytes simultaneously. Their potential and pitfalls for monitoring patients with SLE, particularly those enrolled in clinical trials testing novel therapeutics, will be discussed. *Lupus* (2004) **13,** 304–311.

Key words: biomarkers; clinical trials; multiplexed assays; SLE; surrogate markers

Introduction

Systemic lupus erythematosus (SLE) is one of the most fascinating and heterogeneous of all human diseases, affecting as many as 1 500 000 Americans.[1] The disease is particularly devastating because most of its victims are women in their child bearing years. Many organs can be involved in varying combinations, including kidneys, blood elements such as platelets and red blood cells, the central nervous system, skin, cardiovascular system, joints and lungs.[2] SLE manifestations can also occur as components of other connective tissue diseases such as mixed connective tissue disease (MCTD), systemic sclerosis, Sjögren's disease, overlap syndromes, and rheumatoid arthritis (RA). While many of the pathophysiologic mechanisms contributing to this family of autoimmune arthritides are likely to be shared, mechanisms that are unique to each disease may in part explain the clinical differences that exist in individual patients. Identifying these mechanisms, and markers that can serve as unique identifiers of each of these diseases and disease subsets, is of critical importance for understanding human autoimmunity, and for defining useful endpoints that may aid in clinical trials design. The importance of this is underlined by the fact that no new therapeutic agent has been approved for SLE by the United States Food and Drug Administration (USFDA) in over 30 years. Identification of valid biomarkers and surrogate markers for use in clinical trials will almost certainly remove an important barrier for biotechnology and pharmaceutical companies who are contemplating the development of new drugs for connective tissue diseases, or pursuit of an indication in SLE for a therapeutic that is already approved for another disease.

A review of biomarkers and surrogate markers for SLE took place at the Biomarkers for the Assessment of Systemic Lupus Erythematosus Conference in Bethesda, MD in March 2003. A surprising theme that emerged from this meeting was that even traditional SLE markers such as complement levels, anti-double stranded DNA (dsDNA) antibody titers, and acute phase measures such as erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) have been challenged as valid markers for SLE. While there is certain to be debate about this in other featured articles in this issue of *Lupus*, it is clear that novel markers are sorely needed, both to better understand SLE and to expedite the development of the next generation of therapeutics for SLE.

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A non hypothesis driven approach to marker discovery: multiplexed assays

In an attempt to discover useful markers for SLE and other autoimmune diseases, several groups have chosen to perform large scale screens using emerging high throughput assays. It has taken nearly a decade for this approach, which initially lacks a scientific hypothesis to test, to be accepted by the scientific community. However, success in cancer biology[3] using DNA microarray technology has led to large scale screening efforts in autoimmune disease that have paid clear dividends. I will review recent advances in SLE and several other representative autoimmune diseases using four different multiplexed assay systems: 1) transcriptional profiling using cDNA and oligonucleotide array formats, as well as polymerase chain reaction (PCR) based assays; 2) autoantibody profiling using autoantigen microarrays and related technologies; 3) multiplexed cytokine and chemokine assays; and 4) multiplexed signaling protein assays. Examples of successful use of these assay platforms in other diseases, particularly autoimmune diseases such as myositis, multiple sclerosis (MS), and RA, will be highlighted whenever possible. A schematic describing what biomolecules can be studied using these multiplexed assays is described in Figure 1.

Transcriptional profiling in autoimmune diseases

Despite the advances made in analysing transcript profiles in closely related cancers, only recently have similar studies been published for autoimmune diseases. Lock *et al.* published one of the most interesting and well validated studies in human MS by analysing mRNA transcript profiles from cells derived from MS plaques, comparing acute/active lesions and chronic/silent lesions.[4] Dozens of surprising and unexpected molecules were identified in this screen, including histamine receptors, osteopontin, prostaglandin D synthase, and alpha B crystallin. When homozygous knockout mice for two of these molecules (immunoglobulin Fc gamma receptor and granulocyte colony stimulating factor) were analysed in the experimental autoimmune encephalomyelitis (EAE) model of MS, profound effects were observed, validating the use of DNA microarrays for identifying markers for autoimmune diseases. This has led to the preclinical development of therapeutics aimed at several identified targets, including osteopontin and histamine receptors. Similar studies have also been performed in which peripheral blood cells from MS patients were analysed by quantitative or semi-quantitative PCR.[5,6] In some cases, cells were isolated at different time points

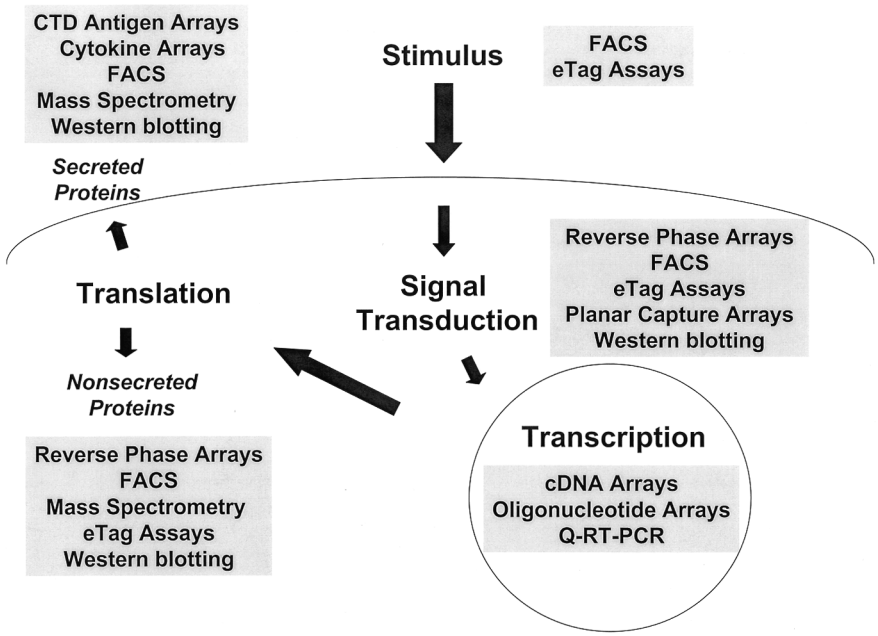

Figure 1 A schematic representation summarizing pathways to be analysed in lupus research, and multiplexed assay formats that can be employed for such studies. Many different cells, including lymphocytes, antigen presenting cells (APCs), and cells targeted by disease (e.g., kidney, skin) can be studied. Entire pathways connecting the cell surface, nucleus and ribosome can be analysed simultaneously, using combinations of complementary technologies. Abbreviations include the following: FACS, fluorescence activated cell sorting; Q-RT-PCR, quantitative reverse transcription polymerase chain reaction; cDNA, complementary DNA; CTD, connective tissue disease.

(e.g., comparing disease flares with periods of remission), or were treated *in vitro* with immunosuppressive agents such as corticosteroids. Several potential markers were described, although none of these has yet been validated in large studies or in clinical trials. It remains to be seen whether these markers will prove to be useful in clinical decision making, or in monitoring patients enrolled in clinical trials.

Several groups have published analyses of transcript profiles of synovial tissue derived from human patients with RA or osteoarthritis (OA).[7–10] As with the MS study described above, a limitation of this type of analysis is that synovial tissue is composed of many different cell types, including T and B lymphocytes, synoviocytes, antigen presenting cells, platelets and monocytes. This makes analysis of the material inherently more difficult than when homogeneous tissue (e.g., leukemic blast cells from blood or lymphoma cells isolated from lymph nodes) is used. Nevertheless, their results provided some interesting insights into RA pathogenesis, including the discovery that chemokine receptors and transcription factors such as STATs and NF-κB are important for disease perpetuation. Undoubtedly, similar studies are under way for analysing blood cells and synovial tissue taken at different time points following therapeutic interventions for RA.

One of the earliest studies of transcriptional profiling in connective tissue diseases was reported in 2001 by Tezak *et al.*[11] In this study, biopsy specimens from pediatric patients with dermatomyositis served as the source of mRNA. The most striking finding was that many of the transcripts that were upregulated encoded proteins that were inducible by Type I (alpha and beta) interferons. This includes the Ro60 protein, an autoantigen that is commonly targeted by autoantibodies in patients with SLE and Sjögren's disease, and also in a large subset of Jo-1 positive myositis patients. A similar interferon biosignature was observed in an *in vitro* anti-viral model (NF90). These results represent an early demonstration of the potential importance of interferons in connective tissue diseases, and have led to similar studies in SLE.

Two important reports of transcriptional profiling in SLE were published in 2003 that in many ways mirrored the pediatric myositis experiments by identifying an important role for interferons in SLE pathogenesis.[12,13] Both papers built on previous SLE microarray studies,[14,15] as well as the studies of Kotzin and colleagues, who used oligonucleotide arrays to identify differences in splenocyte transcripts between a congenic strain of SLE prone animals and control mice.[16] Amazingly, Rozzo and colleagues discovered that only two transcripts were significantly upregulated in SLE B cells, and both encoded interferon inducible (Ifi) proteins (termed Ifi 202 and Ifi 203). The human homologue of one of these proteins (p16) was previously shown nearly a decade ago to be an interferon inducible autoantigen, suggesting the intriguing possibility that a subset of autoantigens may be 'cryptic', induced only in the presence of cytokines. The more recent Bennett and Baechler papers have been the subject of considerable discussion and have been reviewed in detail elsewhere.[17,18] Briefly, both studies identified lupus biosignatures in peripheral blood cells from SLE patients, including a 'neutrophil' and an 'interferon' biosignature. A correlation was found with the lupus biosignatures and disease severity, and in one study the signature was altered when patients were treated with steroids.[13] Neither study has yet been validated using stringent confirmatory assays, but the fact that similar families of genes were identified suggests that this approach is worth pursuing. While important first steps, these studies also underscore the difficulties in correlating results from multiplexed assays with heterogeneous clinical manifestations such as those found in SLE patients.

Autoantibody profiling technologies

A serologic hallmark of SLE and other connective tissue diseases is the production of high affinity autoantibodies directed against intracellular biomolecules.[19] For the most part, most autoantibody titers have not proven to be particularly useful biomarkers or surrogate markers in clinical trials, with the possible exception of anti-dsDNA antibodies. Studies of antibodies directed against components of the Ro/La complex, U1-small nuclear ribonucleoprotein (U1-snRNP) particle, and phospholipids have shown that titers do not correlate with disease activity.[19]

What have not yet been performed are large scale, longitudinal profiling studies of many different antigens, as well as many epitopes from individual antigens. It remains possible, and one could argue likely, that analysis of only whole antigens such as the intact U1-snRNP is likely to miss important dominant epitopes that could correlate with certain disease manifestations or clinical profiles. Such a detailed analysis in pemphigus foliaceus (Fogo Selvagem) has recently revealed that aymptomatic family members of affected patients possess antibodies to the COOH terminal EC5 domain of the skin protein syndesmoglein, and that disease manifestations develop only when autoantibody epitope spreading occurs and targets the NH_2 terminal EC1 and EC2 domains over time.[20] By studying many epitopes at one time in SLE patients, a similar pattern may be identified that correlates with disease flares or new disease manifestations, a prediction that is supported by a recent retrospective study of SLE patients.[21]

Many fine epitope mapping studies have been performed in SLE, generally using enzyme linked immunosorbent assays (ELISAs), western blots, or multiplexed studies employing peptides synthesized on pins. This latter technology has proven to be simple and reproducible, but requires large amounts of serum and is not currently amenable to high throughput formats.[22] The first large scale protein array using whole antigens spotted onto planar surfaces was reported by Joos and colleagues.[23] These studies built on advances in technology by many groups.[24] Joos immobilized a large panel of common autoantigens such as Ro, La, Smith, Jo-1, and topoisomerase onto the surface of glass microscope slides, then probed the slides using serum from patients with systemic rheumatic diseases. This methodology proved to be sensitive and simple to perform. A more detailed history of protein arrays can be found in several recent reviews.[24–26]

Robinson and colleagues constructed an array of 196 different biomolecules that were deposited on the surface of derivatized glass microscope slides.[27] Unlike earlier studies, many different classes of biomolecules were spotted, including proteins, peptides, enzymatic complexes, nucleic acids, ribonucleoprotein complexes and post-translationally modified antigens. Using over 100 different serum samples from patients with eight different autoimmune diseases, we demonstrated that autoantigen microarrays could be used to profile antibodies for all of these diseases. For most antigens, results correlated well with gold standard assays such as ELISA, western blot, and immunoprecipitation analysis. Sensitivity was noted to exceed that obtained by standard ELISA by four- to eight-fold, a finding that is certain to improve with newer detection methods and second generation fluorophores. Specificity was demonstrated in a series of preclearing experiments using epitope tagged antigens conjugated to beads. Preliminary experiments were also performed demonstrating that several different autoantibody isotypes could be detected simultaneously by using isotype specific secondary antibodies conjugated to spectrally resolvable fluorophores such as Cy3 and Cy5. The main conclusions from this early study were that: 1) large scale antibody profiling could be performed on hundreds of antigens simultaneously, and on $\sim$100 blood samples per week by a single scientist in an academic lab; 2) the methodology takes advantage of equipment that exists in every medical center or company that is performing DNA array studies, and therefore can be performed at many institutions worldwide using simple protocols; and 3) for the first time in a single assay format it became possible to identify not just, for example, the ribonucleoprotein complex targeted by the immune response of an individual SLE patient, but also which components of the complex, and which linear epitopes within each component, were targeted.

The stage was set for studies designed to analyse how the autoantibody response changes over time as part of the natural evolution of disease progression, and also in response to therapeutic intervention(s). Robinson *et al.* chose to use the EAE model for MS for this analysis.[28] A myelin proteome was constructed that contained 232 proteins and peptides that compose the myelin sheath, or are infectious disease molecular mimics of myelin proteins. Arrays were printed and probed as described earlier, and serum from different EAE models [e.g., disease induced using a proteolipid protein (PLP) peptide, a myelin basic protein (MBP) peptide, or a spinal cord homogenate (SCH)] was obtained at different time points and used to probe arrays. As expected, the peptide used to induce disease [delivered together with complete Freund's adjuvant (CFA)] was the initial target of autoantibodies, and the pattern of reactivity was distinct for each of the models. Surprisingly, autoantibody production began within 17 to 25 days and led to extensive epitope spreading, long before T cell autoreactivity and spreading was observed.[29] In the same study, a DNA vaccine composed of plasmids encoding autoantigens that had been identified by protein array analysis was delivered to animals in an effort to tolerize, and animals were followed for clinical improvement and for changes in their autoantibody profiles. Animals receiving a cocktail of autoantigens had a very significant decrease in their relapse rates, and this correlated with a marked reduction of epitope spreading to antigens encoded in the vaccine, as well as to other antigens present in the myelin sheath. This result differed from a similar study by deVegvar and colleagues in macaques who were immunized with a DNA vaccine encoding viral antigens prior to challenge with live simian–human immunodeficiency virus (SHIV) in that the antibody response did not diversify (as in EAE) but rather focused on a restricted panel of SHIV epitopes.[30] Taken together, these results demonstrate that antibody profiling can be used to identify self molecules for use in antigen specific tolerizing regimens, for analysing the evolution of the immune response in an animal model of autoimmunity, and for following the response to a therapeutic intervention. Identical studies to characterize the temporal steps in the autoantibody response in mouse models of SLE, insulin dependent diabetes mellitus (IDDM), RA, and primary biliary cirrhosis (PBC) are ongoing in several labs including mine, as are efforts to determine whether antibody profiling can be a useful surrogate for monitoring responses to novel therapies. While it is critically important to perform these studies in animal models, I will return at the end of this essay

to important issues and challenges to analysing humans with SLE and other connective tissue diseases.

Cytokine capture arrays

Important classes of secreted proteins, and ones that may prove to be useful biomarkers for SLE, are cytokines and chemokines. Cytokines can be quantified in biological fluids including serum and synovial fluid. A limitation of studying these fluids is that cytokines and chemokines are designed to act locally, and measurement of serum levels of most cytokines has not proven to be particularly useful. What may be more beneficial is to perform multiplex analysis of cytokines that are secreted from cultured cells from blood, synovium, or other disease tissues, either in the presence or absence of various stimuli such as antigen specific stimulation of T cells or B cells, or stimulation with other cytokines. Many different assay formats have been described and validated for simultaneous measurement of secreted molecules. These include fluorescence activated cell sorting (FACS) by staining with antibodies that recognize cytokines or chemokines that have not yet been released or have been captured soon after release;[31] planar arrays of cytokine specific monoclonal antibodies that serve as capture agents in sandwich assays;[32,33] bead based assays using coded beads to which capture antibodies have been bound;[34] and proximity assays such as eTag technology, in which electrophoretically resolvable fluorophores bound to cytokine specific monoclonal antibodies are released upon binding of analyte, followed by separation by capillary electrophoresis and quantitation by comparison with well characterized standards.[35,36] While advantages and disadvantages exist for all of these techniques, it is clear that dozens of cytokines and chemokines can be accurately quantified from submicroliter quantities of biological materials.

Signaling assays

Autoimmune diseases such as SLE and RA are driven by autoreactive lymphocytes and their products. Signals that emanate from cell surface receptors such as the B and T cell receptors are mediated by membrane associated protein complexes, adaptor proteins, kinases and other molecules. Many other cell surface receptors also utilize distinct signaling pathways that ultimately lead to activation of transcription factors and alterations in gene expression patterns. The ability to analyse signaling components and pathways can be harnessed to identify pathways that are activated in an aberrant manner in disease states, and can also be used to identify alterations in signaling pathways that occur as a result of therapeutic interventions.

Many defects in signaling by T and B cells have been described and will serve as a useful starting point for validating some of the emerging technologies that will be described below. Alterations in Th_1 and Th_2 balances, overproduction of autoantibodies, dysregulation of cytokine production and inappropriate responsiveness of T cells and B cells in general are hallmarks of SLE.[37,38] Signals through the B cell receptor (BcR) involving the Lyn kinase and the tyrosine phosphatase CD45 have been shown to be abnormal in SLE patients.[39] In SLE, T cell expression of the T cell receptor zeta chain is downregulated, although activation of costimulatory receptors leads to unusually high global phosphorylation.[40] Other studies have demonstrated that Lyn expression is deficient in B cells from SLE patients.[41] Lymphocytes from $\sim$80% of patients with SLE are profoundly deficient in the RI subunit of protein kinase A.[42] Downstream kinases such as PKR are also involved and might be related to impaired translational efficiency in T cells.[43] It is important to note that in all of these studies, individual kinases were studied (i.e., one kinase at a time). Most certainly, many other defects could be revealed by analysing many kinases, adaptors, and substrates simultaneously.

A number of different technologies have been developed that allow the multiplexed analysis of dozens of different signaling molecules. All take advantage of commercially available banks of monoclonal and polyclonal antibodies that specifically recognize phosphospecific epitopes on kinase substrates, some of which are themselves kinases. Five different platforms have emerged in the last year for multiplexed signaling protein assays.

Western blotting

Muller *et al.* have described a large scale western blotting approach to study $\sim$800 different molecules, many of which are associated with signaling or protein modification.[44] A pilot study comparing MRL/*lpr* splenocytes obtained at different time points with Balb/c splenocytes identified a panel of interesting molecules that are differentially regulated by the protein phosphatase PP2A. A similar analysis has been reported comparing human RA and OA synovial tissue lysates.[45]

eTag assays

Our lab is employing technology developed at ACLARA Biosciences (Mountain View, CA) to analyse lysates prepared from splenocytes from mouse models of SLE and from freshly isolated

peripheral blood lymphocytes (PBLs) from human SLE patients. In this assay format, fluorophores with different electrophoretic mobilities are conjugated to monoclonal antibodies specific for signaling molecules or phosphospecific epitopes from kinase substrates. Fluorophores are released upon antigen binding, and are then separated and quantitated by capillary electrophoresis. The utility of this assay format has already been validated in cancer biology systems.[35]

Fluorescence activated cell sorting (FACS) based signaling assays

Perez and Nolan have developed an elegant method that allows the investigator to study many different signaling molecules in a heterogeneous population of cells by first fixing and permeabilizing the cells prior to staining with signaling antibodies conjugated to spectrally resolvable fluorophores.[46] A key advantage of this technique is that one can define cell populations based on cell surface markers to identify subsets of cells that might be abnormally activated in SLE.

Capture arrays

A number of arrays are commercially available that employ capture antibodies that are spotted onto planar surfaces such as nitrocellulose or derivatized glass slides. These reagents are very expensive and the assay format has historically proven to be less than optimal for such studies.[47]

Reverse phase signaling arrays

Lysate arrays have been developed by Petricoin and colleagues for studying cancer biology.[48] Our lab has developed a modified assay in which cell lysates are spotted onto nitrocellulose coated slides, allowing the detection of over 60 different kinases and substrates using less than 30 cell equivalents per feature (Steven Chan and PJU, manuscript in preparation). Although the technique is currently in the validation phase, it could rapidly be employed to study dozens of signaling molecules and hundreds of SLE patient samples, in only a few days.

The future of multiplexed assays

It should be clear from this analysis that the technology to interrogate large numbers of biomolecules simultaneously already exists. Many other methods also exist or are being developed for application to the study of connective tissue diseases.[49] To identify biomarker or surrogate markers for SLE using multiplexed assays, there are four areas that need to be developed in the coming years. First, one of the major challenges that lies ahead is not in technology development, but rather in the availability of clinical materials for study. The formation of tissue, serum and blood cell banks of materials derived from SLE patients is critically important. Validated protocols for harvesting, processing and storing these reagents have been made publicly available through NIH sponsored resources such as the Immune Tolerance Network (ITN, http://www.immunetolerance.org). It will be especially useful to incorporate the collection of these materials directly into clinical trials protocols, both for early and late stage trials. Prospective collection of samples can be supplemented by greater accessibility to banked samples, which has already been shown to lead to exciting new discoveries in the area of SLE biomarkers.[21] Second, all of the novel technologies described herein need to be validated by other groups, and cross validated using gold standard assays, for example quantitative real time PCR, western blots and sandwich ELISAs. Third, a critical component to the success of multiplexed approaches is the development of robust bioinformatics and statistical tools. In many ways, this represents the 'new frontier' in biomedical research. Finally, multiplexed assays could provide insights into mechanisms of action of poorly characterized therapeutics, could identify patient subsets who might be predicted to have an adverse event or could identify patient subsets who might benefit from the study drug. Ultimately, individual biomarkers may turn out to be less important than patterns of markers that could serve as 'molecular biosignatures'.[48] The time has come to employ multiplexed assays as discovery tools in SLE, and to form a consortium of SLE centers to provide material for these studies. This would be an important first step toward more tailored therapeutics for SLE and other connective tissue diseases.

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