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Protein arrays for studying blood cells and their secreted products

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Summary: Protein microarrays have been developed and partially validated for studying blood cells, which play a role in many human diseases. Arrays of capture antibodies are commercially available for analyzing cytokines and intracellular signaling proteins. Several academic laboratories have developed antigen microarrays for characterizing autoimmune and allergic diseases, with a goal toward using such arrays to profile antibodies found in blood or other biological fluids. Arrays composed of major histocompatibility complex tetramers have been constructed and validated for analysis of immune responses in mice, paving the way toward studying antigen-specific T-lymphocyte responses. Finally, reverse-phase protein lysate microarray technology, first developed for analyzing cancer cells from tissue sections, has now been demonstrated for studying living cells, including knockout cells, cells treated with drugs such as kinase inhibitors, and rare populations of lymphocytes such as regulatory T cells. The goal of this review is to focus on advances in and future uses of arrays of proteins that can be printed on glass microscope slides using traditional microarray robots that are commonly found at academic medical centers. Dissemination of protein array technology will occur in the next decade and will markedly change how immunology research, particularly in the fields of autoimmunity and inflammation, is conducted.

Introduction

Many systemic autoimmune diseases are characterized by the production of autoantibodies that serve as useful diagnostic markers, surrogate markers, and prognosticators (1). T lymphocytes and plasma cells, the cells that secrete the antibodies that react against self-molecules, have risen to the fore as important cells in the immune system that play critical roles in disease pathogenesis. B cells are now considered important even for diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), and autoimmune diabetes [type I diabetes (T1D)], diseases traditionally thought to be 'T-cell-mediated diseases'. Antibodies have been shown to be not only useful diagnostic markers but also in some cases have been shown to be directly pathogenic (2, 3). The last 40 years have seen enormous strides in the discovery of the autoantigens that are targeted as well as the linear epitopes that are recognized by the antibodies. It is only in the last decade that significant strides have been taken that have led to the development of novel technologies that allow large-scale studies of cells within the immune system. Perhaps the best example is large-scale

DNA microarray analysis, which allows transcript profiling of many different cell types, including peripheral blood mononuclear cells (4, 5) and diseased organs (6–9, reviewed by Dr Peter Gregersen in this issue). Interested readers are referred to several other excellent reviews of this subject that have been published that have identified useful transcription profiles for diseases that include MS (10), RA (11), polymyositis (7), and systemic lupus erythematosus (SLE) (12, 13).

In the current article, I review multiplexed protein array assay platforms that allow one to analyze the proteome of various immune cells and cell products, with a focus on autoimmune diseases. Much of this treatise focuses on work that has been done in the Utz, Steinman, and Robinson laboratories at Stanford (principally by W.H. Robinson and S. Chan), as well as several other Stanford laboratories (P.O. Brown, M. Davis, G. Nolan, R. Tibshirani, G. Chu, and C.G. Fathman). Focus is on technologies that can be performed at most academic research centers, which do not require complicated protocols, sophisticated machinery, and other materials that are not readily available outside of specialized academic centers or companies. Many of these novel technologies have opened an unprecedented window that will allow us to take snapshots of the immune response in health and also the abnormal immune response that is associated with autoimmune and inflammatory diseases. While many other technologies remain to be developed, more than enough already exist to allow us to solve some of the most interesting yet complicated diseases of mankind (14).

Proteomics technologies that are currently available can be divided into two different groups. The first are assays that are considered to be 'unbiased' and are best suited for discovery. The proteomics assay that best fits this mold is mass spectrometry. There are currently many different protocols, instruments, and data analysis tools within the mass spectrometry field (15). The main idea behind this approach is to measure minute quantities of thousands of analytes and to compare samples between diseased patients, controls, or between subtypes of disease (e.g. relapsing-remitting MS and chronic progressive MS). Approaches are then undertaken to determine how the proteome changes in response to various therapeutic interventions.

The second approach could be termed the 'biased' proteomics approach. Biased proteomics platforms are less suited to discovery of new analytes but yet have yielded several exciting discoveries. These approaches can be broken into three categories. The first category is that of protein microarrays (16–19). A large portion of this review is focused on protein microarray technology that has been developed at Stanford and has been preceded by important discoveries and inventions by others, both in the field of autoimmunity and in other scientific disciplines. Protein arrays can be used to study molecules that are secreted by lymphocytes, including autoantibodies (see below), cytokines, and chemokines (20–23), as well as lymphocytes themselves, including cell-surface proteins, components of signaling pathways, apoptosis-specific proteins, and inducible factors. A variety of formats have been invented including planar array-based methodology, bead-based assays, and others (24). A critical point to understand about all of these technologies is that they are 'non-hypothesis driven'. In other words, an investigator makes no assumptions about the disease sample except a conjecture that the sample contains an analyte (or panel of analytes) in a disease that is present in abnormal amounts in the disease state versus a sample obtained from a healthy control patient. The goals of these discoveries are to shed light on the pathogenesis of the disease and to identify useful biomarkers or surrogate markers for the clinic.

Few validated proteomics discoveries have been published in immunology. For the most part, I describe here proof of concept experiments performed by our laboratory and the Steinman and Robinson laboratories, and others that suggest that these technologies may soon be useful in the clinic. However, this application remains to be seen and will be dependant in large part on future development of reproducible assay formats, novel reagents, novel methods of detection that do not depend on fluorescence (25, 26), and especially tools that remain to be invented.

Protein microarray technology

The relative success of transcriptional profiling using arrays of cDNAs or oligonucleotides deposited on the surface of glass microscope slides suggested that similar technology could be developed for studying proteins and peptides. The origin of protein and peptide array technology is controversial, although early studies by Ekins and colleagues (27–29) in England in the late 1980s demonstrated that one could use miniaturized and spatially addressable immunoassays, including what Ekins described as 'multianalyte microspot immunoassays'. These early studies were aimed at reducing to practice the use of small amounts of analytes for measuring, in a multiplexed manner, biologically relevant proteins. Fodor and colleagues (30) then went on to develop methods for synthesizing peptides at spatially addressable positions using photolithography, a forerunner to the methodology used to

produce oligonucleotide arrays by Affymetrix. Because it would require many more masks to synthesize comprehensive libraries of peptides using photolithography, this methodology has generally not been used for large-scale synthesis of peptides on slide surfaces. It is well-suited, however, for synthesis of oligonucleotides, which are composed of only four bases and therefore require orders of magnitude fewer masks in order to synthesize useful arrays.

Arrays are most commonly produced by spotting biomolecules onto planar surfaces using a variety of different techniques. This includes contact printing using solid pins, ink jet technology, stamping, and as described above, *in situ* synthesis of peptides (31, 32). Dozens of different solid supports have been described, most of which employ various chemistries to the surface of glass microscope slides (32, 33). Many of these chemistries use derivatized glass microscope slides, in which a chemical is activated and allows for proteins or peptides to bind specifically to the surface of the slide, usually through covalent bonds with free amine groups (34). More recently, hydrogel surfaces have been developed which mimic a biological fluid and allow for the protein to remain in a more native conformation, preventing denaturation which could therefore inhibit protein–protein interactions during the bioassay. A large portion of the time that is spent by laboratories that use protein microarrays for their studies is spent on technology development and testing of various arrayers, printing methodology, surface chemistry, and technique optimization for individual purposes (35).

There are generally four different planar array platforms in use (36, 37), and each of these methods is best suited for asking certain biological questions. First, arrays of antibodies can be synthesized in which each individual antibody recognizes a unique analyte in solution (22, 23, 38–40). The array is then probed with biological specimens, which could include a cell lysate, a supernatant prepared from tissue culture cells, or some other complex biological mixture such as serum. The antibodies that have been deposited on the surface of the array capture the analytes. Bound analytes are detected either by having previously labeled all proteins in the complex mixture with a fluorescent label or by using a second antibody that is fluorescently labeled. The second method is to deposit antigens or other analytes on the surface of slides and to use these to identify antibodies that recognize each of the individual antigens (34, 36, 38, 41–44). As discussed below, this approach is perfectly suited for the study of autoantibodies or antibodies that are made in response to an infection or vaccination. A third method is to spot cell lysates on slides and to use specific monoclonal antibodies to measure the amount of a particular analyte that is present in that lysate. This technology, referred to as reverse-phase protein lysate microarray (RPPL microarrays) technology, was first described for the study of cancer and has more recently been expanded for use in autoimmune disease by our laboratory (45–47). Finally, several laboratories have spotted reagents that specifically detect cell-surface molecules, such as major histocompatibility complex (MHC) molecules or other cell-surface receptors (48). In this case, the array is probed with whole cells rather than with biological fluids, and the bound cells can be quantitated using a microscope.

Although cDNA microarray technology for transcript profiling was developed in the early 1990s and fairly rapidly disseminated, it has taken almost a full decade before protein microarray technology has begun to see more widespread use, particularly in studies of autoimmunity. One of the first studies to look at protein–protein interactions on the surface of glass microscope slides was by MacBeath and Schreiber in 2001 (34). In this seminal article, the authors created simple microarrays using a robotic microarrayer that was similar to the one used initially for making cDNA transcript arrays. A variety of different proteins were deposited on the surface of slides including monoclonal antibodies, intracellular proteins such as the 50-kilodalton component of nuclear factor-κB (NF-κB), and other antigens. They went on to demonstrate that antigen–antibody interactions could be detected at a level that was approximately equivalent to that seen with standard techniques, such as enzyme-linked immunosorbent assays (ELISA) or with Western blotting. Protein microarrays were also shown in this article to be useful for detecting protein–protein interactions that required a drug, the rapamycin-binding protein FK-BP12. Finally, to demonstrate that the enzymatic functions of proteins could also be assayed, they immobilized candidate substrates for a kinase, and demonstrated that only a limited number of substrates that had been deposited could be phosphorylated *in vitro* by the kinase in the presence of cofactors and γ-adenosine triphosphate. Overall, this elegant demonstration of protein microarray technology opened the door for many others in the field. The development of protein microarray technology allowed investigators to leverage the advances in cDNA microarray technology that had taken place during the previous decade. This technology included using the same equipment, such as DNA microarray robots, scanners, protocols for conjugation techniques, and also data analysis using many of the same statistical and computer algorithms that had been developed for analyzing transcript profiles.

The MacBeath and Schreiber article (34) was soon followed by an article by Haab and colleagues (38), who analyzed in greater

detail antigens and antibodies that were spotted individually on the surface of glass microscope slides. They characterized 115 antigen-antibody pairs in this study. The idea was to determine whether antigens and antibodies denatured upon binding to the surface of a glass microscope slide when they dried, because this would have a major impact on whether this technology could move forward for asking important biological questions. They demonstrated, somewhat surprisingly, that approximately 50% of antigens could be deposited on the surface of a slide and then later detected using a commercially available monoclonal antibody that had been conjugated to a fluorophore to probe the array. In most cases, the binding was sensitive to the point that it was comparable to what one would find using ELISA or Western blot, and was also fairly specific. In most cases, the dynamic range was over at least two logs, suggesting that this technique could be useful in biological systems where several orders of magnitude difference could be observed in a biological specimen. One would have predicted that spotting of antibodies rather than antigens would have led to an even more robust result, because antibodies have a compact globular structure and are relatively stable. However, it was very disappointing in their study to note that only 20% of commercially available monoclonal antibodies could be spotted onto a glass microscope slide and remain in a form that could then capture an analyte that was present in solution. For those antibodies that did work, it was clear that this assay format could be used for measuring multiple analytes at one time. Haab *et al.* (38) were able to detect markers of acute ischemic injury (for example, creatine kinase MB and troponin, both of which are released following a myocardial infarction), cytokines [for example, tumor necrosis factor α (TNFα) and interleukin-1 (IL-1)], and malignancy [prostate-specific antigen (PSA)]. All of these markers were present at relatively elevated concentrations, but nevertheless physiological, in the range of ng/ml quantities. This important study demonstrated the utility of protein arrays for measuring biologically relevant molecules and also pointed out limitations and areas for development. In parallel, Robinson and colleagues moved forward with development of autoantigen microarrays for the study of human autoimmune disease and mouse models of autoimmunity.

Antigen microarray technology for identifying serum antibodies

Serum autoantibodies are a hallmark of most autoimmune diseases (1). When a clinician sees a patient in the clinic, a variety of tests is ordered, some of which are very nonspecific, such as anti-nuclear antibody tests, and some of which are very specific, such as antibodies directed against Smith and double-stranded DNA (dsDNA). Each of these assays is ordered individually by the clinician and is performed in a diagnostic laboratory as a separate ELISA for each antigen. The clinician then receives the results back, generally in days to weeks. The clinical findings from the patient are correlated with a constellation of laboratory findings in order to try to establish a diagnosis. Relatively little effort has gone into trying to understand patterns of autoantibody production within individual patients, for example to subset patients into prognostic groups or to better define which therapies the patient should receive. Therefore, several methods have been developed in order to get a 'wide angle picture' of the autoantigens that are targeted in an individual patient, with a goal to improve diagnosis and therapy in these patients.

The first such article was that of Joos *et al.* (43). In the study, Joos and colleagues (43) used a robotic microarrayer to print arrays of 18 prominent autoantigens onto the surface of microscope slides. The slides were coated with nitrocellulose membranes in one part of the experiment, and in the other, silane-treated slides were employed. The arrays were then probed using highly defined serum samples obtained from human patients with systemic rheumatic diseases. Bound antibodies were detected using a secondary antibody that had been conjugated to the enzyme horseradish peroxidase (HRP), and they were revealed using a chemiluminescent substrate and imaging using a commercially available charge-coupled device chemiluminescence camera. Unlike the results obtained by Haab *et al.* (38), the vast majority of antigens were recognized by serum antibodies found in the blood of these patients. There appeared to be very little cross reactivity, and the amount of antibody that could be detected was similar to that achieved using a standard clinical ELISA. This result is perhaps not so surprising given that the autoantibodies that are produced in SLE and other connective tissue diseases (CTDs) are generally of very high affinity and relatively high titer. Thus, autoimmune sera are perfectly suited for development of a protein microarray platform, given their physical properties.

In 2002, our laboratory constructed the first large-scale autoantigen microarray designed specifically for large-scale multiplexed characterization of autoantibody responses (49). Unlike the array constructed by Joos *et al.* (43), our array was much more comprehensive and included linear peptides that had been synthesized off-chip. The array was composed of 1152 features that contained 196 individual biomolecules. The array represented the main autoantigens that were targeted in all of the CTDs. Virtually all of the antigens were provided by collaborators within the field who had been studying the antigens for the previous decade, although a subset of

the antigens were purchased from commercial vendors or synthesized in the laboratory directly. Arrays were probed with a panel of previously characterized serum samples that had been studied extensively in a series of studies in Paul Anderson's laboratory at Harvard (50, 51). We also studied almost 100 additional samples obtained from various investigators or through the Stanford Arthritis Clinic. The overall scheme for producing the arrays is surprisingly simple and is presented in *Fig. 1*.

In this study, we used poly L-lysine-coated glass microscope slides, which were placed on the bed of a robotic microarrayer. Autoantigens were diluted in phosphate-buffered saline or glycerol-containing solutions and placed individually into wells of a 384 well plate. A robotic microarrayer using split pin capillary tips was then used to deposit each of the antigens directly on the surface of glass microscope slides at reproducible, spatially-addressable positions. In our initial studies, approximately 120 slides were printed at a time, and the slides were refrigerated until used. The features were approximately 100 microns in diameter, although there was some variability depending on the solvent in which particular antigens were diluted and the type of pin that was used. Arrays were blocked in a manner very similar to that which would be used for blocking a nitrocellulose membrane for a Western blot, then probed with diluted serum samples obtained from CTD patients. Bound antibody was detected using secondary antibodies specific for human immunoglobulin G (IgG) and IgM that had been conjugated to a fluorophore such as Cy3 or Cy5. In some experiments, we also directly labeled the serum samples with fluorophores and did a comparative analysis. For example, serum sample A was conjugated to Cy3, and serum sample B was conjugated to Cy5. Equal amounts of each serum sample were then mixed together and used to probe the array. This idea is reminiscent of what is usually done for transcript profiling experiments, allowing one to potentially standardize the assay. We generally do not use this technique for most of our studies, due to the expense of having to label individual samples and the difficulty with obtaining reproducible covalent binding of fluorophores to the samples. Finally, the arrays are washed, and the images are scanned and analyzed. The bound, fluorescently conjugated antibodies are quantitated using standard software.

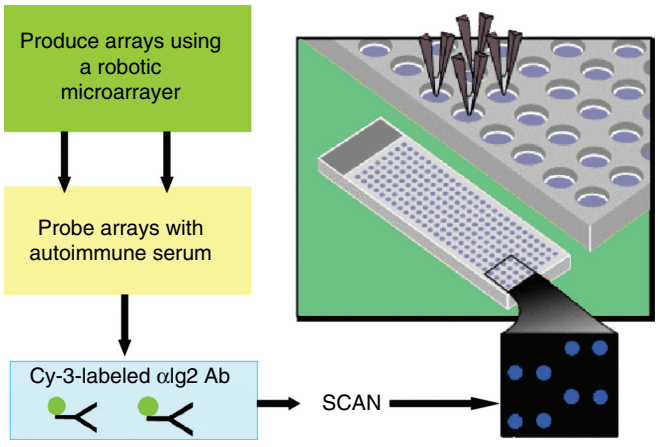

Fig. 1. Workflow for antigen microarray platform technology. Recombinant or synthetic autoantigens, including peptides, mutant or wildtype proteins, modified antigens, or intact autoantigenic complexes are placed into wells of a microtiter plate prior to printing on derivatized glass microscope slides with a robotic microarrayer. Arrays are blocked and probed using serum or other source of autoantibody. Bound antibodies are visualized using secondary antibodies conjugated to spectrally resolvable fluorophores and scanning slides with a fluorescence scanner.

To determine the sensitivity, specificity, and dynamic range of this approach, we spotted several different autoantigens, including pyruvate dehydrogenase complex and a ribonucleoprotein particle (RNP) component (La) on the surface of slides and probed them using purified human polyclonal antisera. In this case, the antibodies had been purified using protein A/G sepharose chromatography. Known quantities of the polyclonal antibody were then used to probe the arrays. We demonstrated linear detection of the antibodies ranging from a span of 1–900 ng/ml. When compared directly with standard ELISA that is used in clinical practice, this technique was four to eightfold more sensitive and also could be extrapolated to five other autoantigens that were tested, including Ro-52, topoisomerase (topo 1 or SCL70), and dsDNA. The detection limit in some cases was as low as one nanogram per ml, far below what is necessary for use in routine clinical practice. A representative image demonstrating prominent autoantigens that can be detected in eight different autoimmune diseases, is shown in *Fig. 2*.

To determine the specificity of the binding, we performed a series of experiments in which recombinant antigens were conjugated to beads and first used to preclear antibodies from serum before probing arrays. We focused on a panel of well-characterized serum samples from a previous study, samples which were known to have reactivity to the proteins Ro-52, La, Scl-70, and Jo-1. This specificity determination is actually a very important question for such an assay format, because human autoimmune sera are known to be cross-reactive in some cases. This area is somewhat controversial within the field (52, 53). Using this approach, we were able to reproducibly eliminate reactivity against one antigen without affecting the reactivity of antibodies to other antigens on the array. However, careful examination of arrays from those

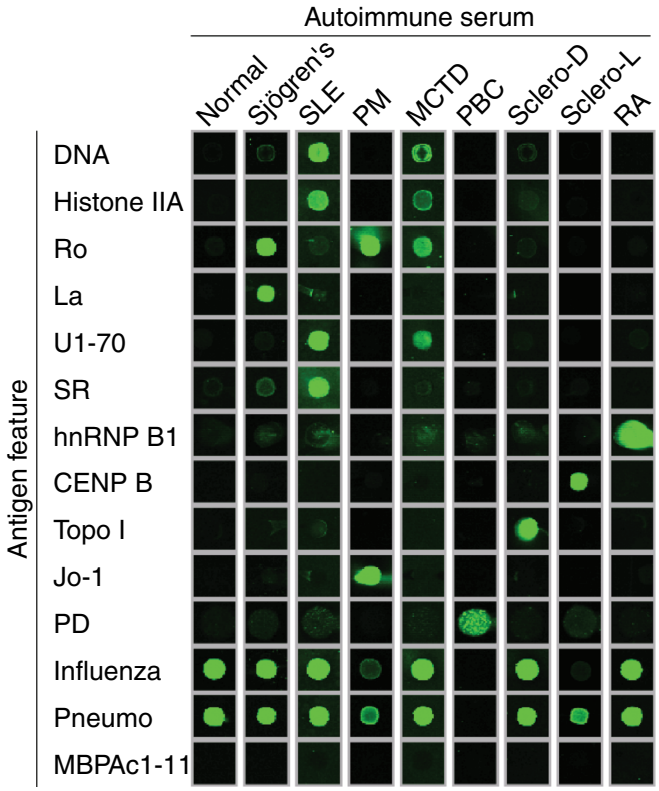

***Fig.* 2. Identification of disease-specific autoantibodies in human serum.** Autoantigen arrays were incubated with diluted patient serum samples from (a) a healthy individual (normal) for which no specific autoantibody reactivities were detected; (b) Sjögren's syndrome demonstrating autoantibody reactivity against Ro52 and La; (c) Systemic lupus erythematosus (SLE) demonstrating reactivity against DNA, histone H2A, U1–70 kDa, and serine arginine splicing factors (SR proteins); (d) Polymyositis (PM) demonstrating reactivity against Jo-1 and Ro52; (e) Mixed connective tissue disease (MCTD) demonstrating reactivity against DNA, histone H2A, Ro52, and U1–70 kDa; (f) Primary biliary cirrhosis (PBC) demonstrating reactivity against pyruvate dehydrogenase complex (PDH); (g) Diffuse scleroderma (sclero-D) demonstrating reactivity against topoisomerase I (topo I); (h) Limited scleroderms (sclero-L) demonstrating reactivity against centromere protein B (CENP B); and (i) RA demonstrating reactivity against heterogeneous RNP (hnRNP-B1). The autoimmune disease serum used to probe each array is indicated along the top of the figure, and each column of cut and pasted antigen features contained within a gray box are representative antigen features from a single array. A myelin basic protein peptide recognized on arrays by autoantibodies in serum from rodents with experimental autoimmune encephalomyelitis (EAE) was included as a representative negative control (MBP^{68-86}). Figure and legend reprinted with permission from (49) Robinson WH *et al.* Autoantigen microarrays for multiplex characterization of auto antibody reponses. Nat Med 2002;9:295–301.

experiments demonstrated that there were other cross-reactive epitopes elsewhere on the array that were removed by preclearing with bound antigen.

We next asked whether we could detect linear epitopes that were recognized by autoantibodies in the same serum sample. The majority of the molecules that were spotted on our arrays were in fact linear peptides. The idea behind this line of experimentation was to demonstrate that it was possible to detect a particular protein or RNP complex that was targeted in a patient, to then go on to determine which proteins in the complex were recognized, and ultimately which linear epitopes within these proteins were recognized. Thus, it should be possible to obtain all of this information from a single experiment, using approximately 1 μl of starting serum. We demonstrated that several important histone-specific linear epitopes were targeted in the human serum samples, including histone $1^{111-127}$ and histone $2B^{1-25}$. These results were confirmed in a blinded manner by standard ELISA in the laboratory of one of our collaborators, Sylviane Muller. As discussed later, the ability to look at many linear epitopes at one time has very important uses in the study of autoimmune disease, in particular in determining whether an animal or human has alterations in epitope spreading that occur over time, either as part of the natural history of the disease or in response to a therapeutic intervention.

In the final part of our initial description of this technology, we performed a series of additional experiments further aimed at uses in autoantibody profiling. First, we demonstrated using monoclonal antibodies specific for phosphorylation-specific epitopes that we could detect such altered proteins on the surface of slides. This finding is of particular importance, because a number of autoantigens have been shown to be recognized in a post-translationally modified form (54, 55). These antibodies include those directed against RNA polymerase in lupus (56), serine-arginine splicing factors (SR proteins) in SLE (54), and citrullinated antigens, which are the main targets of autoantibodies in patients with RA (57). We also demonstrated that one could use autoantigen microarray technology for profiling isotype-specific antibodies. This application is useful because certain isotypes are associated with different types of immune responses. For example, in mice, IgG1 and IgE subtypes are associated with T-helper type 2 (Th2) responses, and IgG2a is associated with Th1 responses. We demonstrated that using highly specific secondary antibodies that recognized only one of the antibody isotypes, we could use these to obtain ratios of antibodies that had been bound to specific antigen features. Several large-scale studies of multiplex isotyping are nearing completion in my laboratory and Bill Robinson's laboratory, focused on animal models of SLE and MS, respectively.

In summary, autoantibody profiling, using equipment that is standard on virtually all academic campuses and is widespread in industry, can be used to profile many different antibodies at one time in biological specimens derived from patients with autoimmune disease. The stage was now set to

use this technology to actually discover something, as opposed to just demonstrating that one could detect on the surface of a slide what one expected to detect.

Autoantigen microarrays for analyzing vaccines in animal models

At the time of the publication of the article described above (49), virtually every article published on protein arrays was either a review article touting the potential of protein arrays (many authored or coauthored by myself) or a relatively small-scale demonstration of spotting analytes onto the surface of slides and detecting them using monoclonal antibodies or in rare cases serum. We chose to move to the next step in protein array technology, which was to use arrays for discovery. We constructed an array specific for another autoimmune disease, namely experimental autoimmune encephalomyelitis (EAE), a mouse model for MS (58). An array was constructed of approximately 230 distinct antigens, composing an array of 2304 features (59). The antigens included purified or recombinant proteins as well as synthetic peptides. Virtually all of these antigens were derived from the myelin sheath or were overlapping peptides derived from proteins that were found within the sheath. There were also a number of molecular mimics of such proteins, including viral and bacterial proteins that the literature had suggested might serve to trigger the disease. Recombinant antigens, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), and many others were used. Various modified forms of MBP were also employed, because MBP undergoes a number of post-translational modifications including phosphorylation and citrullination on over a dozen different residues.

The first step in construction of any array is to perform simple validation experiments. In the case of the CTD array described above, this test was performed using highly characterized human sera and in some cases monoclonal antibodies or polyclonal antibodies that were known to be specific for a subset of the antigens on the array. We performed a similar series of experiments to validate the EAE/MS array. Antibodies specific for linear epitopes within some of the molecules including $PLP^{139-151}$, $PLP^{178-199}$, and MOG^{35-55} were used. The results, as expected, showed that there was very specific detection of the cognate antigens by their respective antibodies. These results were confirmed using standard ELISA, and the sensitivity and specificity as well as dynamic range were similar to that recorded for CTD arrays (59). An important point to make here is that with all protein array technology, it is nearly impossible to validate every antigen or feature on the array. We generally validate a subset of the antigens before using arrays, and then validate any interesting or important results derived from probing arrays with biological serum, focusing on positive peptide reactivities identified in the screen.

We next sought to perform an observation experiment in mice that were developing EAE following immunization with a myelin peptide together with complete Freund's adjuvant (CFA). SJL/J mice were immunized with $PLP^{139-151}$, MBP^{85-99}, or spinal cord homogenate (SCH). Serum was then obtained longitudinally in these mice over a 90-day period. Serum was used to probe arrays, and bound antibody was revealed using a secondary antibody specific for mouse IgG and IgM. Fluorescence intensity was then measured for each of the features, and the data were subjected to statistical analysis using a program developed by Gilbert Chu and Robert Tibshirani at Stanford called 'Significance Analysis of Microarrays' (60). One of the most striking findings of this study was that the autoantibody response was detectable early on during the course of the immunization, in fact preceding by weeks identical reactivity seen with T cells. Importantly, the autoantibody response was first directed against the initiating antigen, then spread to other parts of that antigen (termed intramolecular epitope spreading), and ultimately spread to other components of the myelin sheath (termed intermolecular epitope spreading) (61, 62). Epitope spreading of autoreactive T cells in this disease had been shown by several groups previously to occur between 3 and 8 weeks after disease induction, demonstrating that the B-cell response could be detected much earlier than a T-cell response (62).

The results obtained using microarrays were also compared with the clinical phenotype of the animals. EAE disease severity was scored over a 90-day period, and the number of relapses was tabulated. Hierarchical clustering of the antigen features demonstrated statistically significant differences between the groups and further demonstrated that mice that had less autoantibody reactivity had significantly fewer relapses compared with animals that had more autoantibody reactivity, as demonstrated on the arrays. This finding was particularly the case with animals immunized with MBP^{85-99} and with animals immunized with $PLP^{139-151}$. This result represented the first study demonstrating that early inter- and intramolecular epitope spreading occurred in chronic EAE and that simultaneous targeting of multiple myelin antigens occurred in this disease. Of interest was the random variation that was noted in many of the mice, which were in fact genetically identical and in many cases were housed in the same cage as their littermates. Presumably this variation has to do

with stochastic events that occur during the formation of the B- and the T-lymphocyte repertoire during development.

Based on this discovery that there is early epitope spreading within the B-cell response in EAE, we asked whether we could take advantage of this information in designing antigen-specific therapy for EAE. This step is the first in what could be considered patient-specific, antigen-specific tolerizing therapy in humans. In reviewing the array data, it was clear that four dominant antigens were reproducibly observed as important targets of antibodies. These included PLP, MBP, MOG, and myelin-associated glycoprotein (MAG). A significant amount of data had already been published on all of these antigens, in particular the role played by autoantibodies in MOG-mediated disease (63). A critical requirement for the development of any antigen-specific therapy is to have knowledge of the antigen or antigens that are driving the disease. It is widely accepted that MS and many other diseases are T-cell mediated (64), and thus we were relying on a surrogate marker (in this case B-cell autoantibody production) for identifying the target antigen.

To test this hypothesis, we took advantage of prior work from the Steinman laboratory (65–67) at Stanford, which demonstrated that tolerizing DNA vaccines appeared to be useful for downregulating the immune response in EAE. This observation was somewhat serendipitous, in that attempts to induce disease using DNA vaccines had failed, leading to the hypothesis that perhaps the DNA vaccines could in fact be promoting tolerance. Many other antigen-specific therapeutic interventions could also be tested in this model, including recombinant proteins, peptides, and molecules that may be targeted by the immune response. A DNA vaccine cocktail was therefore constructed and tested in the EAE model. Individual plasmids encoding PLP, MBP, MOG, and MAG were constructed in the pTARGET vector. This vector contains a cytomegalovirus (CMV) promoter, which drives the expression of the protein once taken up by cells. The cocktail was injected in a saline solution containing calcium and phosphate into the muscle of mice that had already developed their first relapse of EAE. The vaccine was then given periodically during the course of disease. Animals were monitored for relapse rate, and serum was obtained at varying times during the course of the experiment. The overall results of the experiment were quite striking. Animals receiving a DNA plasmid encoding a single epitope from PLP alone ($PLP^{139-151}$) had a small decrease in their mean relapse rate that was not significant, a result that varied slightly from a previous study demonstrating that this particular epitope alone could induce a very modest but statistically significant reduction in the relapse rate. When a cocktail of plasmids encoding all four antigens was used, there was a striking decrease in the mean relapse rate from 2.6 relapses over a 90-day period to 1.5 relapses (P-value $= 0.026$). When the cocktail was combined with a DNA plasmid encoding the Th2 cytokine IL-4, the relapse rate decreased even further to 0.9 (P-value $= 0.001$). This study demonstrates that the DNA vaccines have the potential to markedly decrease the number of relapses in this model, in particular when combined with IL-4 or other immunomodulatory agents.

The next experiment was to ask what happens to the autoantibody profiles in animals that had been treated with these DNA vaccines. One would predict that epitope spreading would be markedly reduced in the animals receiving the vaccines, as this correlated with a decrease in the mean relapse rate. This outcome is precisely what was observed. Again, using samples obtained at 10 weeks, serum autoantibody profiling using arrays demonstrated that there was a marked decrease in epitope spreading in animals receiving the cocktail plus IL-4 as well as the cocktail alone versus control animals receiving vehicle alone. Antibodies directed against the inciting antigen (in this case $PLP^{139-151}$) could still be detected, although epitope spreading within PLP and the antibody titers decreased in response to the antigen-specific DNA vaccine. This finding further suggested that using whole antigens encoded in plasmids, particularly when multiple antigens were given at one time, had the potential to tolerize more broadly to many different epitopes within the antigen. This result has clear implications for antigen-specific therapies that are already in the clinic for humans in which small epitopes, as opposed to entire proteins, are being employed in an attempt to induce immunological tolerance. This result also clearly demonstrates that autoantibody profiling has the potential to be used as a marker in monitoring therapeutic intervention trials (68).

An important series of experiments that has yet to be performed in this model is to test the hypothesis that animal-specific autoantibody profiling can be used to design a custom DNA vaccine or other antigen-specific vaccine for that particular animal. The experiment is quite simple, and it could be done in a relatively short period of time. In this experiment, animals would be induced to undergo EAE by immunizing with a peptide or SCH together with CFA. Serum would be obtained at an early time point following the first relapse, and autoantibody profiles would be obtained. Custom DNA vaccines or vaccines containing whole purified antigens would then be given to the animals based on their autoantibody profiles. For example, an animal making antibodies to

PLP or MBP would be immunized only with those antigens, while an animal making antibodies against MOG and MAG would receive a customized DNA vaccine just for those antigens. The animals would then be monitored for relapse rate, T-cell reactivity against all of the target antigens, and autoantibody profiles. This approach is virtually identical to the current approach used in allergy clinics, whereby an allergist determines allergen sensitivity in individual patients by skin testing, then designs a custom vaccine for that patient. Experiments have yet to be reported by any of the laboratories working in this area, and they are sorely needed.

A second study in an animal model of disease, a model of human immunodeficiency virus (HIV) infection, was performed by de Vegvar and colleagues (69) in Bill Robinson's laboratory at Stanford. A related viral infection, that caused by simian-human immunodeficiency virus (SHIV) in macaques, was studied. SHIVs are chimeric viruses made up of a number of genes from HIV-1 combined with genes such as gag, pol, nef, vif, and vpr from simian immunodeficiency virus (SIV). SHIVs have the ability to infect macaques and have proven to be a useful model for vaccine trials and drug trials for HIV. For both HIV and SHIV, $CD8^+$ T cells are the main players responsible for eliminating or suppressing viral replication, interrupting the life cycle of the virus. It has been well documented that protective antibodies cannot be raised in response to vaccines, nor is it thought that antibodies play a significant role in either disease pathogenesis or prevention. Nevertheless, the Robinson laboratory reasoned that studying antibodies in macaques that had been infected with virus, either as part of the natural history of infection or as part of a vaccine trial, would be useful, particularly because a number of different vaccines have now entered human clinic trials. To undertake these studies, arrays containing whole proteins as well as peptides derived from SHIV were constructed. As above, they were printed on the surface of derivitized microscope slides where they could be analyzed for their ability to be bound by serum antibodies. An array of 430 peptides and proteins from SHIV was constructed. Arrays were again validated with specific antibodies, using identical methodology to the validation of the CTD and EAE/MS arrays described earlier. This study benefited by large banks of peptides that are freely available through the Center for Disease Control (CDC) as well as monoclonal antibodies and other sera that have been banked and made available to any investigator interested in working on HIV or SHIV. As expected, there was good correlation between array reactivity and ELISA.

The 2304 feature SHIV proteome array was then used to follow animals that had been enrolled in a trial of an HIV vaccine. Separate trials were studied. In the first, macaques had been primed at 0 and 8 weeks with a DNA plasmid expressing SHIV 89.6 gag-pol-env or SHIV 89.6 gag-pol and then followed with a boost at 24 weeks with modified vaccinia virus Ankara (RMVA) expressing identical SHIV proteins as with the primes. For the third trial, macaques were inoculated at 0, 8, and 24 weeks with RMVA expressing SHIV 89.6 gag-pol-env. The animals were compared with macaques receiving DNA plasmid vectors that lacked inserts. With vaccination, all animals were challenged at 7 or 8 months with a pathogenic version of SHIV 89.6 called SHIV 89.6 P. All animals in the gag-pol-env plasmid plus RMVA or RMVA only groups successfully controlled virus upon challenge. In the animals receiving the DNA vaccine encoding gag and pol only with MVA, two of the six animals succumbed to challenge infection. In the controls, five of six animals died of acquired immunodeficiency syndrome by 28 weeks. Serum was obtained from these animals at serial time points during the vaccine trial period. The antibody profiling results represented arguably the most detailed analysis of B-cell responses to viral antigens that have been published in HIV research. The main finding of the study was that there was a strong convergence of antibody specificities to viruses to a restricted set of linear epitopes within the protein env. Unlike the results obtained in the mouse models of MS, these results were obtained in an outbred population of macaques who received vaccines that encoded different SHIV proteins. While the T-cell responses were divergent during this period of time, the B-cell responses were strongly convergent. This result is strikingly different than that observed in the EAE animals (59, 70), where the B-cell response was divergent, spreading to multiple different epitopes over time as the disease progressed. Although largely an observational study in a disease in which antibodies do not play a critical role, the studies of de Vegvar (69) raise the possibility that arrays could be developed for studying other infectious diseases in which antibodies do play an important role, including respiratory syncytial virus and hepatitis B virus.

In a pair of articles, arrays were constructed to study IgE-specific antibodies that mediate allergic diseases (71, 72). The first study used rolling-circle amplification (72), whereas the second used traditional array technology (71). An array containing 94 purified allergens was constructed, using similar methodology to that used for producing protein arrays for studying autoimmune diseases. Allergic diseases are characterized by the production of IgE antibodies that are present in very low titer in serum, because the bulk of the antibodies are already bound to the surface of mast cells and other cells that express Fcε receptors. The array

contained many important allergens that are routinely assayed in clinical practice including dust mites, molds, animal dander, and tree pollen. Another major component was grass pollen, which is one of the major allergens on the west coast of the United States. Arrays were probed using serum from patients with known allergic diseases. Results were compared with those obtained by skin testing in which the magnitude of a wheal that was raised by injecting small amounts of the allergen into the skin was measured. For the most part, there was a poor correlation between skin test results and serum results. The reason for this is not clear although the level of IgE antibody present in the serum is so low that it may be difficult to measure on a routine basis. Nevertheless, this study demonstrates that protein and allergen array studies are feasible for analyzing clinical samples. This result is particularly interesting as well, because allergen desensitization therapy using extracts of purified allergens is commonly used in allergy clinics. Patients are generally tested using skin tests to anywhere from 25 to 50 allergens. Based on the results of the test, the allergist then puts together a cocktail of allergens that is given to the patient in escalating doses, with the goal to desensitize (and ultimately cure) the patient. A variety of mechanisms have been proposed, and it has generally been observed that the isotype of the antibody is altered from an IgE to IgG isotype, which does not bind to the Fcϵ receptor present on mast cells. This concept underlies attempts being made in our and in many other laboratories to develop antigen-specific, patient-specific therapy for autoimmune diseases.

Protein microarrays for discovery

It should be clear from the discussion so far that protein microarrays as described are ill-suited for discovering new antigens. In virtually all of the cases that have been published to date, antigens that have been deposited on the surface of glass microscope slides or in other formats are 'known'. While it is true that patterns of reactivity could be obtained that could provide useful clinical information, this has yet to be realized in humans. Methods need to be developed that allow large-scale protein arrays in which proteins that are not known, target antigens are deposited on the surface of the arrays. Moreover, identifying functional changes within cells in such a format would also be a useful endeavor. There have been two articles published recently in this area that suggest that such an approach could be feasible and, in fact, will be a clear area in the future for advances in proteomics.

Snyder and colleagues (73) have developed a high-throughput platform for producing mammalian proteins in insect cells. cDNAs encoding individual proteins are cloned in-frame such that they express an epitope tag, such as 6x HIS or glutathione-S transferase (GST). cDNAs are expanded and expressed in bacteria prior to purification. The purified DNA is subcloned into a baculovirus expression system, which is used to infect insect cells. The insect cells express the protein under defined conditions. The cells are then lysed and the protein is purified using nickel chromatography. The purified proteins are placed in microtiter plates and spotted onto the surface of nitrocellulose-coded glass microscope slides. The deposited proteins are available for studies in which one might be looking at protein–protein interactions or, in the case of autoantigen discovery, for probing using serum or other biological fluids derived from patients with autoimmune disease. This work is now being carried out on a large scale by a Connecticut-based company, Protometrix, Inc. All aspects of the technology are performed in a robotic high-throughput manner and in most cases are done entirely in temperature-controlled rooms. This allows one to take advantage of already available cDNA libraries, and it also allows for stable expression and rapid batch purification of the proteins. At the time of this writing, several thousand proteins have been expressed using this system, an amazing accomplishment given the difficulty in working with proteins versus cDNAs. There are several advantages to working in a baculovirus system. First, proteins that are expressed often contain many of the important post-translational modifications that would be seen in mammalian cells. Second, the cells are easy to work with, and baculoviral vectors have been developed to improve the throughput of this approach. Third, if genome DNA fragments are used, then they contain alternative splice variants that can be spliced out by the insect cells; this approach should allow multiple splice variants of proteins to be produced. Although methods have not yet been developed to isolate these different isoforms to separately spot them on the arrays, the large-scale arrays produced by Snyder and colleagues (73) represent the largest collection to date.

An important problem with protein array methodology is that in virtually every platform used to date, proteins are prepared off chip or are purchased, then are deposited on the surface of the chip using a robotic microarrayer. While it is possible to produce peptides using photolithography, as described earlier, this technology is better suited for oligonucleotide synthesis. Labaer and colleagues (74) have attempted to tackle this problem by producing high-density protein microarrays on chip. cDNA microarrays are printed in which individual genes are cloned into a vector such that they express an in-frame GST tag. Rather than depositing cells on the

surface to serve as the machinery for making the protein, Labaer and colleagues use a cell-free system. A coupled transcription translation system derived from Hela cells is added to the surface of the slide. The Hela cell lysate contains all of the materials necessary for producing RNA as well as protein, including ribosomes and all of the necessary machinery. Following incubation for a set period of time, protein is synthesized and immediately deposited on the surface of the slide. To do so, at the time that the cDNA microarrays are printed, they are spiked with an anti-GST antibody, which serves to capture the newly synthesized protein as soon as it is made. Overall, density of their slides is still considered to be high density containing approximately 512 spots per slide. They demonstrated that they can produce up to 10 fmoles of protein at each discreet spot, with an overall coefficient of variation of sevenfold. They went on to demonstrate that they could use these arrays to study protein interactions between 29 individual replication proteins, identifying 63 previously unidentified interactions between these proteins. The major advantage of this technique is that is can be performed using standard cDNA microarrays that have been developed that contain any one of a number of different tags, thus taking advantage of many commercially available monoclonal antibodies specific for these tags. Nevertheless, there are some disadvantages to this approach. First, it is difficult to envision that each protein will be made at a relatively equivalent rate, and it will require a number of pilot experiments to try to standardize how much protein is synthesized at each spot. Secondly, there is the theoretical risk that as the protein is being transcribed and translated, it can diffuse from one feature to another. The main issue with such an approach is standardization. Nevertheless, standardization is an overall problem for all protein array studies, and this platform is truly unique with outstanding possibilities for advancing proteomics research in the near future.

RPPL microarrays for analysis of signaling pathways

Relatively early on, several members of my laboratory noted that autoantibody profiling was a useful technique but was one that did not allow us to look directly at the B lymphocytes that were synthesizing the autoantibodies. It became increasingly clear that new methods needed to be developed to study not the products of the lymphocytes but rather the cells themselves. Because deposition of antibodies on the surface of glass microscope slide has not yet shown to be a robust method for detecting analyte concentrations in cell lysates (38), we reasoned that there must be other methods that should be developed in order to enable this detection to occur. Moreover, new methods needed to be developed that allowed one to look at living cells, that is cells freshly isolated from patients or from animal models of autoimmunity, allowing for stimulation of these cells with antigen, drugs, cytokines, or other reagents that could mediate biological effects.

In 2001, Paweletz (47) developed RPPL microarrays for studying signaling pathways within tumor cells. The methodology was simple and elegant. Tumor cells were isolated directly from pathologic specimens using laser capture microdissection (LCM). Their hypothesis was that cells that were close to the invasion front of the cancer might express particular signaling molecules at higher levels, or have signaling pathways activated such as stress-activated protein kinase pathways or mitogen-activated protein kinase (MAPK) pathways. Pathway components could ultimately serve as drug targets and could certainly help in understanding the biology of tumors. They went on to demonstrate that cells obtained using LCM from the tumor front could be lysed and spotted on the surface of glass microscope slides. The cell lysates were then probed using monoclonal antibodies directed against phospho-specific epitopes. Normalization was relatively simple in that one could use antibodies for the non-phosphorylated versions of the analyte being studied or could normalize against actin or other housekeeping proteins. The surprising result of this study was that proteins were not destroyed by LCM, and in fact they could still be studied following this procedure. The authors demonstrated upregulation of the extracellular-regulated kinase (ERK) pathway in tumor cells (47, 75), and they were able to detect incredibly small numbers of proteins, on the order of less than one cell equivalent. Like the other techniques described above for protein arrays, this technology again took advantage of routine robotic microarrayers, scanners, and traditional software and statistical tools for data analysis.

Our laboratory sought to build on these studies to apply RPPL microarrays to analyze lysates prepared from living lymphocytes that had been exposed to stimuli such as receptor cross-linking or drug treatment (76, 77). Initial experiments were proof of concept in nature, with the main goal to demonstrate that we could use RPPL microassays to study living cells (*Fig.* 3). Whole cell lysates were prepared using standard protocols used for Western blotting. We used a standard robotic microarrayer used for other protein array studies to deposit lysates on the surface of glass microscope slides coated with nitrocellulose. Solid pins were used to deliver the lysates, as opposed to the split pin capillary tips that were used for autoantigens. The slides were then blocked

using a variety of different blocking agents and probed with either monoclonal antibodies directed against inducible proteins, or antibodies that recognized phosphorylation-specific epitopes. Bound antibodies were then detected using HRP linked to a secondary antibody. The signal was amplified using tyramide signal amplification technology in which the HRP enzyme catalyzes the deposition of a conjugate between biotin and tyramide onto the slide. The bound biotin was then detected using streptavidin that had been conjugated to Cy3 or other fluorophores, and fluorescence intensity was measured using a standard microarray scanner. Samples were normalized either to actin or by using antibodies that were capable of recognizing both the phosphorylated and the non-phosphorylated forms of the kinase or substrate.

To determine the sensitivity, specificity, and the overall dynamic range of the arrays, we prepared lysates from a cell line (A431 cells) into which a T-cell-specific protein, ζ-associated protein of 70 kDa (ZAP70), had been spiked into the lysate. We performed this over a range of dilutions and then probed arrays using a specific antibody directed against ZAP70. The limit of detection was determined to be in the range of 1 in 10^5–10^6 per mol fraction of ZAP70 to total protein. We estimated that this represents a limit of detection of approximately 1500 copies of the analyte per cell, assuming that there are approximately 100 pg of total protein in each cell. For most of the monoclonal antibodies that were used, an approximately two-log linear response was observed.

We next asked whether we could identify proteins that were upregulated in response to a physiologic stress. Jurkat lymphocytes were subjected to a heat shock at 42 °C and compared with cells that had been kept at 37 °C. Thirty minutes later, lysates were prepared, and were spotted onto the surface of arrays. We detected specific induction of heat-shock protein 70 (HSP 70) in the shocked cells as opposed to the control cells, confirmed using Western blotting. We estimated that the total number of cell equivalents that had been spotted at each feature approached between 5 and 25 cells depending on the diameter of the solid pins that were used. This finding demonstrates that one can study very rare cell populations using this technique, particularly when using living cells that are difficult to isolate.

Many phosphorylation-specific monoclonal antibodies have now been described and are commercially available. We took advantage of this availability in studying signaling pathways in Jurkat T cells (*Fig.* 4). We initially chose to study T-cell receptor activation, because it is arguably the best-studied receptor activation pathway known in mammalian cells. Jurkat cells were treated with phorbol 12-myristate 13-acetate (PMA), and lysates were prepared at varying times. PMA activates protein kinase C (PKC) which ultimately leads to the rapid activation and phosphorylation of the kinases MAPK/ERK 1 and 2 (MEK1/2) and p44/42 MAPK. We were able to reproduce these findings using lysate microarrays, and we quantitatively demonstrated that the kinetics of activation were precisely the same as that observed using Western blots. These results were normalized both to total levels of MEK1/2 and MAP kinase as well as to actin. To more carefully look at signal transduction kinetics, we profiled the phosphorylation kinetics of phospholipase C-γ1 (PLC-γ1) on a particular tyrosine residue, tyrosine 783, in Jurkat cells that were activated by crosslinking both the T-cell receptor with an anti-CD3 antibody, and the CD28 coreceptor. Lysates were prepared at varying times and following varying combinations of the cross linking agents. Isotype control antibodies were also used in this experiment. We observed a rapid increase in the PLC-γ1 level of phosphorylation within 2.5 min of stimulation. Phosphorylation was not sustained and in fact decreased quickly back to a baseline level within approximately 10 min. When CD28 stimulation was also applied to the cells, PLC-γ phosphoryl-

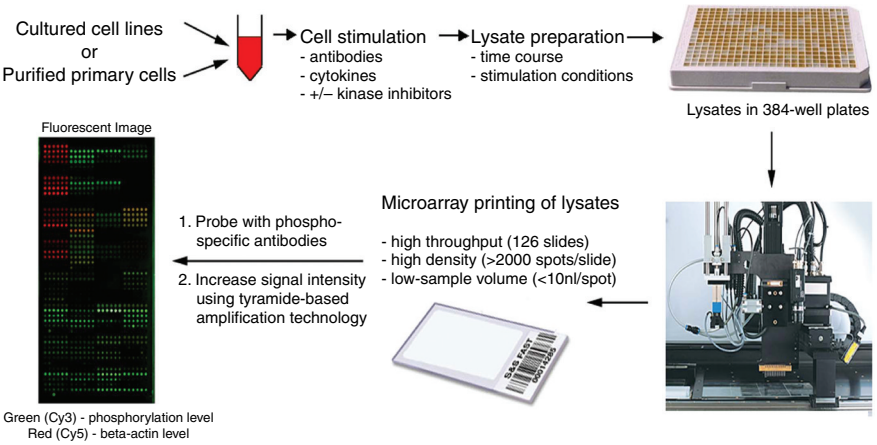

***Fig.* 3. Reverse-phase protein lysate microarray workflow.** Lysates from any cell type are prepared, placed into microtiter plates, and spotted onto nitrocellulose-coated slides using a robotic microarrayer. Arrays are then probed using antibodies, including monoclonal, polyclonal, or autoimmune serum antibodies prior to scanning and quantitation.

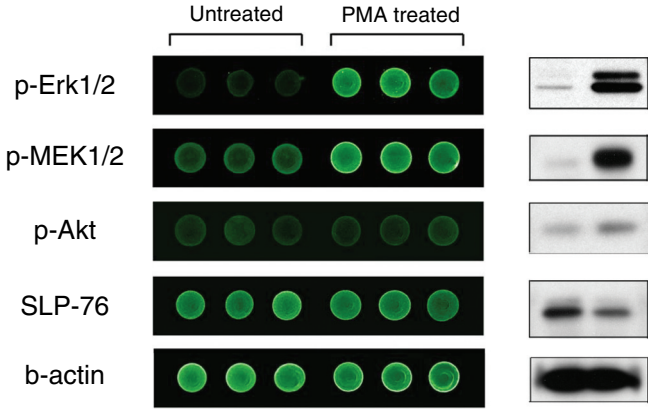

***Fig. 4.* Reverse-phase protein lysate microarray images and comparison with traditional western blots.** Jurkat T cells were cultured in the presence or absence of phorbol myristate acetate (PMA), and harvested 5 min later for lysate preparation. Corresponding western blots of the same samples are shown adjacent to the array figures. Antibodies specific for p44/42 MAPK, MEK1/2, and Akt are phosphorylation-state dependent, whereas antibodies directed against SLP-76 and β-actin are phosphorylation-state independent. Approximately 100 000 cell equivalents were loaded for each lane of the corresponding western blot, compared with 5–25 cell equivalents for the RPPLM features. Figure reprinted with permission from (76) Chan, *et al.* Protein microarrays for multiplex analysis of signaling pathways. Nat Med 2004;101:1390–1396.

ation was sustained for at least 30 min, suggesting that CD28 may play a role in altering the kinetics of activation of at least some of the kinases involved in T-cell activation. To further evaluate the role of PLC-γ1 in T-cell receptor stimulation, we took advantage of a cell line that was deficient in this signaling molecule. Wildtype Jurkat cells or PLC-γ1-deficient cells were exposed to CD3 and CD28 crosslinking antibodies, and lysates were prepared at varying times. As expected, we observed a very rapid phosphorylation of PLC-γ followed by the typical decrease in cells that expressed wildtype levels of PLC-γ. In cells that lacked PLC-γ, we were unable to detect the protein at all, confirming the lack of this protein in the mutant cell line. The dephosphorylation of two downstream molecules in this pathway, MEK 1/2 and p44/42 MAPK, was significantly faster in the PLC-γ1-deficient cells, with no appreciable change noted in the initial peak of phosphorylation. Taken together with the previous result, this finding demonstrates that PLC-γ1 does not play a particularly important role in determining the peak level of phosphorylation of certain kinases, but it likely plays a role in sustaining p44/42 MAPK activity.

There are a number of different methods available for disrupting kinase pathways in cells. One, described above, is to take advantage of cells that are deficient in the genes encoding various kinases or adapter molecules. Others include short interfering RNA (siRNA), which we currently have begun to use in the laboratory. We have also taken advantage of a variety of commercially available soluble drugs that are purported to block only specific molecules within certain pathways. We treated Jurkat T cells with three different inhibitors of T-cell activation (76). First, we used a phosphoinositide 3-kinase (PI3K) inhibitor, which specifically inhibits the activity of PI3K and prevents the phosphorylation of protein kinase B (PKB) on serine 473. Similarly, we used a MEK1/2 inhibitor which inhibits this kinase and prevents phosphorylation of p44/42 MAPK. We also used as a control rapamycin, which prevents the nuclear transport of nuclear factor of activated T cells and the subsequent activation of T-cell activation-specific genes such as IL-2. Lysates were prepared and spotted onto the surface of microscope slides and probed using phospho-specific antibodies, as described earlier. As expected, we observed that the PI3K inhibitor blocked phosphorylation of AKT but did not alter the phosphorylation of either MAPK, MEK1/2, or 3-phosphoinositide-dependent kinase 1 (PDK1). Conversely, treatment of cells with the MEK1/2 inhibitor specifically blocked the phosphorylation of MAPK in response to CD3 crosslinking but did not alter the phosphorylation of MEK1/2, AKT, or PDK1. This simple proof of concept experiment suggests that RPPL microarrays could be used for drug discovery and for teasing out subtle differences in kinase pathways that might result from drugs or other interventions.

We next employed RPPL microarrays to profile 62 different analytes, many of which are known to be involved in T-cell receptor signaling. Jurkat cells were treated either with anti-CD3, or with anti-CD3 plus anti-CD28. Lysates were then prepared and spotted on the surface of slides. Individual arrays were probed with monoclonal antibodies specific for 62 different signaling components. This preparation was done in such a way that six replicates were spotted onto each slide in an eight-pad subarray format, a format that is cheaper and better suited to studying small numbers of cell lysates. Global changes were noted in phosphorylation patterns of many proteins. As we had hoped, we identified many of the molecules that are already known to play a critical role in T-cell receptor stimulation, including ZAP70, MEK1/2, PLC-γ1, cAMP and cGMP-dependent protein kinases and protein kinase C family members (AGC) family kinases, and PKCμ. In total, 13 signaling proteins experienced statistically significant changes in phosphorylation when CD3 stimulation alone was applied to cells, and 14 proteins when both CD3 and CD28 costimulation was used. For the most part, the list overlapped precisely suggesting that costimulation by crosslinking of CD28 does not play a significant role in modulating signaling in the first 2.5 min following T-cell receptor engagement. All of these results were validated using standard Western blots.

Importantly, we also observed that several proteins were dephosphorylated in response to T-cell receptor signaling. One finding that was quite unexpected involved the protein Raf1, which was dephosphorylated on serine 259 shortly following T-cell receptor stimulation. It is known that Raf1 phosphorylation on this residue negatively regulates the kinase activity of the protein. This has been demonstrated by mutating this residue to alanine, generating a constitutively active kinase. Currently, it is not known how dephosphorylation occurs or which protein mediates this in T cells. These results were confirmed using peripheral blood lymphocytes isolated from a healthy donor, demonstrating that dephosphorylation of Raf1 occurs *in vivo* in non-transformed cells. Interestingly, the kinetics of dephosphorylation differ in primary cells, peaking at approximately 30 min as opposed to 2.5 min in tissue culture cells. We speculate that protein phosphatase 1 (PP1) and/or PP2A may play a role in catalyzing the dephosphorylation of RAF1 *in vivo*. Future experiments are now aimed at performing a much more comprehensive study of T-cell receptor activation, both by looking at much later time points and by expanding the number of antibodies by several hundred.

We believe that one of the most important uses of RPPL microarrays lies in its ability to analyze rare cell populations. For example, antigen-specific T cells and B cells are thought to exist as very rare populations of cells within humans. Tetramer technology may someday allow rare populations of these cells to be purified for further study using RPPL microarrays. It will be critical to develop methods that will allow a broad look at these cells without using the entire cell sample for an analysis of a single analyte. Many other cell populations can be defined specifically by their cell-surface receptors. One of the most important and best-studied cells within this context is $CD4^+CD25^+$ regulatory T cells. Several groups have demonstrated that this population of cells has the potential to modulate autoimmune responses and in transfer experiments can inhibit ongoing autoimmune disease (78, 79). Attempts are now being made to culture these cells *ex vivo*, expand them, and then to deliver them back to patients with the idea that they could then suppress an antigen-specific immune response. However, it is currently not clear whether cells that are freshly isolated from an animal that have a $CD4^+CD25^+$ surface phenotype will have the same physical properties and immunologic properties as would be cells that are stimulated *ex vivo* and expanded. As a relatively simple proof of concept experiment, we chose to study regulatory T cells that were expanded *ex vivo* as well as cells that were freshly isolated from mice. These cells constitute only approximately 5–10% of the total $CD4^+$ T-cell population in peripheral lymphoid tissues. However, it has been shown previously that the total numbers of T-regulatory cells is reduced in peripheral lymphoid tissue from mice lacking the IL-2 gene as compared with wildtype animals. We used RPPL microarrays to derive a snapshot of the phosphorylation state of a variety of different analytes that are present in these cells that had been stimulated with IL-2 and then compared them with a profile that we identified using activated $CD4^+$ T-cell blasts. Significant differences were noted in STAT phosphorylation between the two different cells types (76). Larger scale studies using antibodies directed against many different analytes are currently under way, with a goal to better understand regulatory T-cell biology. Such studies are almost certain to provide useful information about signaling pathways that are activated in regulatory T cells and can very clearly be applied to virtually any cell that can be purified, including dendritic cells, B lymphocytes, T lymphocytes, and other antigen-presenting cells.

RPPL microarrays represent an incredibly powerful and exciting tool that can be used to study many different biological processes. Unlike mass spectrometry approaches, a main limitation of this technology platform is the availability of monoclonal antibodies and other antisera for performing the experiments. Fortunately, the amount of material that is required is exceedingly small, on the order of less than one microgram of monoclonal antibody for each array. Moreover, the amount of lysate that is required for deposition is also exceedingly small, again representing approximately 5–25 cells per each feature. With the advent of newer printing tips and better spotting procedures and surfaces, it should be possible to get this number down well below five cells, perhaps approaching the single cell level. A main drawback to this approach is that one can only study proteins that have already been discovered and to which monoclonal antibodies have already been produced. Large-scale efforts are underway in the laboratory to screen monoclonal antibodies, both from commercial sources as well as from individual investigators, for their performance characteristics in this format. One goal of these studies will be to create a public database of monoclonal antibody performance, which should save other investigators working in this area from having to 'reinvent the wheel' in screening antibodies.

As described in our initial report, there are five main areas for which RPPL microarrays can be used and expanded in the future. (i) As already described in proof of concept experiments, this format should prove to be exceedingly useful for screening lead drug candidates, particularly drugs that target kinase pathways. Many kinase inhibitors are initially thought to be specific for a certain kinase, but as the drug continues

down a development pathway, it is frequently observed that the drug is not specific for that kinase. As part of toxicology efforts, it is known that activation of specific kinase pathways can be detrimental to cells. It may be possible to use RPPL microarrays to identify drugs that have undesired effects on signaling pathways and therefore to redesign the drugs so that these effects are eliminated. (ii) As described earlier, RPPL microarrays are perfectly suited for studying rare populations of cells. Petricoin and colleagues (75) have already demonstrated in a series of elegant experiments that this approach can be used to study tissues derived from patients with cancer. It should be possible to fractionate various cells using fluorescence-assisted cell sorting (FACS) methods or magnetic bead sorting platforms to derive relatively pure populations of cells from animals or from humans. The advent and expansion of LCM should also expedite the study of rare cell populations that can only be observed under a microscope. Examples of such cells that are already under study using this approach include cancer cells, β cells in the islets of Langerhans in diabetic animals, infiltrating T cells that can be found in close association with organs that are under attack as part of an autoimmune disease (P.J.U and C.G. Fathman, unpublished data), and cells found in primary and secondary lymphoid organs. Efforts are now also under way to try to better understand the inflammatory response that occurs in coronary artery disease, atherosclerotic diseases in general, and vasculitis, using a similar approach. This approach should also be useful for analyzing stem cells, and especially antigen-specific lymphocytes that have been isolated using tetramers. (iii) We have described studies using mouse samples and human samples. It should be clear that RPPL microarrays can be used to study essentially any organism for which large numbers of antibodies have already been generated. I would expect to see such studies being published in the near future in laboratories working on *Drosophila melanogaster*, *Caenorhabditis elegans*, yeast, and other organisms. In our hands, we have observed that most of the antibodies that are functional and recognize human proteins appear to crossreact and work well with mouse proteins (Steven Chan and P.J. Utz, unpublished observations). Whether they are capable of detecting homologs in other organisms remains to be determined. (iv) We are now poised to begin studying samples derived directly from patients. These samples would include biopsy material, blood cells, and cell lines obtained from patients. An elegant study by Gary Nolan and colleagues (80, 81) that was described in 2004 in studying acute myelogenous leukemia perfectly illustrates this point. In their study, samples from patients were obtained and frozen prior to institution of chemotherapy. Samples were later thawed, and the cells were stimulated with various cytokines prior to analyzing their phosphosignaling pathways using a FACS-based method. Perhaps not surprisingly, no differences were observed when cells that had not been stimulated with cytokines were used for the assay. So called 'cryptic' signaling pathways were then revealed when the cells were exposed to cytokines, demonstrating that defects in signaling pathways existed within these cells but could only be revealed by first treating the cells with a particular agent prior to performing the assay. This result strongly demonstrates that studying dead cells or tissue may be less fruitful than studying cells directly isolated from patients, which can then be manipulated prior to analysis. (v) RPPL microarrays can be used to identify other post-translational modifications in addition to phosphorylation. Many different modifications of proteins occur within cells including glycosylation, methylation, farnesylation, acetylation, ubiquitination, and proteolysis. Many antibodies have now been produced that allow one to look at specific post-translational modifications, particularly those that occur in response to a variety of stimuli. (vi) A particularly overlooked area within the biology of autoimmune cells is the movement of proteins and other biomolecules within a cell in response to a stimulus. Using RPPL microarrays, it should be possible to fractionate cells that have been treated with different stimuli and to deposit each of these cell fractions on the surface of a slide. In this way, it may be possible to determine where a particular protein goes in a cell and to correlate this with upstream phosphorylation states of proteins and kinase signaling pathways. In short, RPPL microarrays should prove to be one of the most important new tools in systems biology experimentation, allowing correlations to be made between transcriptional profiles from cells that are obtained using different technology (described elsewhere in this issue) with proteomic data.

Peptide-MHC microarrays for studying antigen-specific T lymphocytes

The development of peptide-MHC tetramers has enabled many important studies to be performed regarding antigen-specific immune responses. The main use for tetramers to date has been in flow cytometric-based assays and more recently in *in situ* studies. Tetramers have proven useful for studying the response to microbes such as bacteria and viruses, autoimmune diseases, tumors, and seasonal allergens. The drawback of FACS-based methods is that it is time consuming, laborious, and can only address one antigen specificity at a time. Attempts have been made to construct libraries of peptides

analyzing T-cell receptor specificity, but this study has been limited to use in T-cell clones and is not particularly well suited for studying immune responses in heterogeneous T-cell populations. In 2003, Soen and colleagues (82) reported the development of peptide-MHC microarrays for studying T-cell responses. In this approach, different peptide-MHC molecules are spotted at spatially addressable positions onto glass microscope slides. Unlike arrays that are traditionally produced for autoantigens, allergens, and protein lysates, piezo-electric non-contact printers are used to deposit the tetramers and antibodies onto the surface of the slide. According to the authors, this preparation decreases the film disruption and allows control over the size of the spot and the precise amount of material that is ultimately deposited. The arrays are probed with T cells, which are then allowed to settle on the surface of the slide, where they are capable of interacting with the immobilized MHC tetramer complexes. The cells are washed prior to visualization under a microscope. Features are approximately 400 microns in diameter, thus allowing accommodation of approximately 1600 T cells if the entire area of the spot is covered with cells.

The initial validation experiments for the tetramer arrays were performed using two different peptide MHC complexes. The first was a peptide-MHC tetramer specific for the class I mouse antigen ovalbumin (OVA) conjugated to phycoerythrin. The second was an MHC class II-specific tetramer from the mouse antigen moth cytochrome c (MCC). Both of these tetramers had been previously well studied in FACS-based experiments and had been shown to be highly useful reagents for such validation studies. Tetramers were found to bind T cells as efficiently as spotting monoclonal antibodies specific for cell-surface markers that would be present on these T cells. Binding of tetramer could be achieved by spotting as little as 0.4–0.8 nanograms of tetramer at each feature. After printing, antigen-specific $CD4^+$ and $CD8^+$ T cells derived from transgenic mice were used. Cells were derived from OT-1 and 5C.C7 transgenic mice and were activated and expanded *in vitro* by culturing with specific peptides for each of the transgenes and in the presence of IL-2. Cells were then mixed in a one-to-one ratio and incubated on the surface of the arrays. Cells were allowed to bind for 10 min, and unbound cells were washed away. Each population of cells had been previously labeled with a spectrally resolvable fluorophore (either DiO or DiD lipophilic tracers). Antibodies specific for the cell-surface markers CD3 and CD28 were also spotted at spatially addressable positions on the same slide. Because both $CD4^+$ and $CD8^+$ cells expressed CD3 and CD28, both populations of cells bound to the monoclonal antibodies that had been printed on the surface of the array. In contrast, the MCC and OVA tetramers specifically captured only their specific cells. This experiment demonstrated the feasibility of this approach for studying antigen-specific T-cell responses.

Antigen-specific T cells exist as relatively rare populations, particularly in autoimmunity where they may be as rare as 1 in 100 000 cells. To demonstrate that relatively rare populations of T cells could be detected in a complex mixture of cells, lymphocytes and lymph node cells from syngeneic wildtype animals were prepared. Into this population were spiked T-cell receptor transgenic cells that were specific for MCC and OVA, respectively. Each of these specific T-cell receptor transgenic cell populations was first differentially labeled prior to mixing them with the other cells. Cell mixtures were incubated with arrays containing MHC peptides, as well as three antibodies specific for mouse CD8, mouse CD4, and mouse C28. Cells were incubated for slightly longer periods, up to 30 min, prior to washing and scanning of the slide. Specific binding was again observed, even in these rare cells, to the specific tetramers, with a detection limit of approximately 0.1% of antigen-specific T lymphocytes.

To study the use of these arrays using *in vivo*-generated material, the authors studied cells derived from C57BL/6 mice vaccinated with OVA. A very small fraction of cytotoxic T lymphocytes (CTLs), approximately 0.27% by FACS, were identified that specifically recognized OVA. Vaccination of another mouse demonstrated that there were no OVA-specific CTLs, as expected. These cells were also used to then probe an array of tetramers, this time using a lymphocytic choriomeningitis virus (LCMV)/K^d tetramer as a negative control. A significant number of cells from the mouse vaccinated with OVA bound to the OVA tetramer, while there was no binding observed to the unrelated tetramer. This result demonstrates that even weak immunization to an antigen can be detected using peptide-MHC arrays.

As a final part of their demonstration, the authors (82) asked whether they could detect a calcium flux by first taking OT-1 lymphocytes and loading them with a calcium-sensitive dye called Fura-2. The rationale behind this series of experiments was to ask whether the cells not only bound to the tetramer but also became activated. Calcium flux is considered to be an excellent marker for T-cell receptor stimulation. Arrays were kept at a constant temperature of 37 °C, and the levels of calcium were monitored by measuring the fluorescence intensity ratio at 340 nanometers and 380 nanometers at 30-second intervals. As expected, transient spikes in calcium flux were only observed when the cells were bound to the specific tetramer to which they were raised but not to tetramers to which they should not bind.

This elegant series of experiments demonstrates a novel and very useful tool for studying antigen-specific responses in T lymphocytes. Most investigators now are relegated to studying surrogate responses, often using autoantibodies as tools to determine the target antigen. This new methodology suggests that as the number of tetramers that are created increases, that arrays of tetramers can be used to profile antigen-specific responses in autoimmune diseases. Why is this particularly important? Antigen-specific therapies are being developed for several different autoimmune diseases including T1D (altered peptide ligand, HSP peptide, and recombinant glutamic acid decarboxylase), rheumatoid arthritis (type II collagen and oral therapy with HSP), bullous skin diseases (altered peptide derived from skin antigen), and multiple sclerosis (altered peptide ligand of MBP, peptide derived from MBP, and DNA vaccines encoding myelin components including MBP) (83). A problem for all of these studies is trying to determine the necessary dose that might be tolerogenic and to follow the antigen-specific response in patients. Methods that are currently used to do this are cumbersome, fraught with potential pitfalls, and can be difficult to perform in multicenter trials where specialized tests such as these are frequently done at only one of the centers. It is clear that if tetramers existed for any of the above antigens, or other potential autoantigens for these diseases, that vaccine trials could be better monitored in living patients. This monitoring would almost certainly improve our understanding of autoimmune diseases and could better predict which antigens may be dominant, and which doses of vaccine may be efficacious.

Future directions

As with most new technologies, the promise of things to come generally dominates over the reality of the present. Protein array-based platforms fall squarely in this group. That said, protein array platforms are better positioned than most other proteomics technologies for the next few years, as mass spectrometric approaches are perfected and perhaps replace protein microarray platforms in mainstream immunology research. The expanding number of identified autoantigens, the availability of large-scale commercial arrays composed of capture antibodies or recombinant proteins, and the creation of thousands of monoclonal antibodies by academic laboratories and companies will undoubtedly lead to important protein array-based discoveries in immunology. Most importantly, the next frontier will not take place *in vivo* or *in vitro* as is currently occurring, but rather '*in silico*' as bioinformatics tools are developed to mine enormous bodies of disparate datasets. If a conclusion were to be drawn from this review, it is that the biggest discoveries in the 'immunologic proteome' will not occur by individual scientists or individual laboratories, but rather by interdisciplinary teams of scientists from the disciplines of medicine, engineering, mathematics, and computer science.

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