

Antigen Microarrays for the Study of Autoimmune Diseases

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BACKGROUND: The immune response involves the activation of heterogeneous populations of T cells and B cells that show different degrees of affinity and specificity for target antigens. Although several techniques have been developed to study the molecular pathways that control immunity, there is a need for high-throughput assays to monitor the specificity of the immune response.

CONTENT: Antigen microarrays provide a new tool to study the immune response. We reviewed the literature on antigen microarrays and their advantages and limitations, and we evaluated their use for the study of autoimmune diseases. Antigen arrays have been successfully used for several purposes in the investigation of autoimmune disorders: for disease diagnosis, to monitor disease progression and response to therapy, to discover mechanisms of pathogenesis, and to tailor antigen-specific therapies to the autoimmune response of individual patients. In this review we discuss the use of antigen microarrays for the study of 4 common autoimmune diseases and their animal models: type 1 diabetes, systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis.

CONCLUSIONS: Antigen microarrays constitute a new tool for the investigation of the immune response in autoimmune disorders and also in other conditions such as tumors and allergies. Once current limitations are overcome, antigen microarrays have the potential to revolutionize the investigation and management of autoimmune diseases.

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The immune response results from complex networks of genes and proteins acting in a concerted manner. Although several techniques have been developed to analyze these gene and protein interactions, the analysis of adaptive immunity presents an additional chal-

lenge: its output is the activation of heterogeneous populations of T cells and B cells with diverse affinity and specificity for target antigens. In addition, the immune responses associated with infections or autoimmune diseases target not one but several antigens. Thus, there is a need for the development of high-throughput assays to monitor immune reactivity.

Numerous strategies have been developed to study T-cell and B-cell functions. Assays aimed at characterizing T cells are limited by the low frequency of antigen-specific T cells and usually require relatively large numbers of cells. The measurement of antigen-specific T-cell responses has been greatly improved with the development of fluorescence-activated cell sorting assays using tetramers of recombinant major histocompatibility complex (MHC)² molecules loaded with specific peptides (1, 2). These tetramers are not easily synthesized and can be used only to detect T cells that react with a specific peptide in a specific MHC allele. Thus, although MHC-peptide tetramers are invaluable tools for the study of peptide-specific T cells, their use is limited in the study of the T-cell response directed against a large number of antigens in human populations with diverse MHC alleles.

B cells produce large quantities of antibodies detectable in serum, plasma, and other clinically relevant fluids like synovial fluid (SF) and cerebrospinal fluid (CSF) (3, 4). Thus, circulating antibodies allow the analysis of the specificity of the B-cell response, even in the context of low frequencies of antigen-specific B cells. Moreover, sustained antibody production requires the support of T helper cells, and indeed B cells and T cells have been found to share target specificities, suggesting that the antibody response can be used as a surrogate for the study of T-cell immunity (5, 6). Thus, the analysis of antibody reactivity offers an opportunity to overcome some of the limitations associated with the study of the T-cell response in health and disease. Moreover, the analysis of antibody repertoires can en-

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² Nonstandard abbreviations: MHC, major histocompatibility complex; SF, synovial fluid; CSF, cerebrospinal fluid; HSP, heat shock proteins; T1D, type 1 diabetes; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; MS, multiple sclerosis; RRMS, relapsing–remitting course; SPMS, secondary progressive MS; PPMS, primary progressive course MS; CNS, central nervous system; HSP, heat shock protein.

able us to capitalize on existing specimen collections (serum, plasma, CSF, and SF) from well-characterized patient cohorts to study clinical problems of interest.

The interaction of antibodies with their target antigen does not require the participation of additional molecules such as the MHC, facilitating the development of assays for the measurement of antibody reactivity. Examples of these techniques are ELISA, Western blot, immunoprecipitation, flow-based assays, fluorescent-based immunoassays, and RIA. However, these techniques require relatively large sample volumes and are not compatible with high-throughput multiplex analysis. Early attempts at multiplex studies of the antibody response were based on quantitative Western blots with cell and tissue lysates as the source of antigens (7, 8). However, the main limitation of these Western blot–based assays is the unknown identity of the targeted antigens once positive hits have been found.

Antigen Microarrays

The construction of a multiplex ELISA using a 96-well microarray format was the first step toward the development of high-throughput methods for the analysis of antibody reactivity (9). The combination of these miniaturized assays with techniques developed to spot proteins onto chemically derivatized glass slides at extremely high spatial densities (10) led to the development of antigen microarrays (11, 12). Those arrays initially included protein and peptide antigens, but later on incorporated other antigens such as lipids (13, 14).

In addition to their ability to measure hundreds of antigen–antibody interactions in parallel using a small amount of sample, antigen microarrays are analytically more sensitive than conventional ELISAs, producing a signal that shows a linear correlation with the concentration of antigen-specific antibodies over several orders of magnitude (11, 14). Some limitations, however, are associated with the use of antigen microarrays for the analysis of antibody responses. As in any other solid-phase assay, the 3-dimensional structure of the antigens can be affected by their adsorption or covalent linkage to the slide surface, or by the drying of the microarrays. These changes in antigen structure can destruct conformational epitopes of clinical relevance and can also expose epitopes that otherwise are not accessible to antibodies. Nevertheless, antibodies against conformational epitopes have been quantified with antigen microarrays, suggesting that this technique can detect antibodies to linear and at least some conformational epitopes.

An additional obvious limitation is that antigen microarrays can detect only antibodies reactive with the antigens used in their construction, ruling out the detection of antibodies against unexpected target antigens in

screening experiments. However, 3 different approaches have been used to overcome these limitations.

WHOLE PROTEOME EXPRESSION IN VITRO

Felgner and coworkers developed a PCR-based approach that allows the expression of hundreds of open reading frames in parallel using a cell-free system (15). This novel approach was used to construct protein microarrays containing pathogen proteomes, which were later used to analyze the antibody response in serum samples from infected mice and humans. These studies led to the identification of immunodominant antigens of diagnostic value (16, 17) and also identified antibodies associated with protective immunity in response to vaccination (18).

SYNTHETIC PROTEOMES

Larman et al. recently described phage immunoprecipitation sequencing in which synthetic oligonucleotides encoding 36–amino-acid peptides that cover all open reading frames in the human genome are packaged in a phage for display on the phage surface (19). In this technique phages expressing candidate autoantigens are immunoprecipitated with antibodies in the patient sample and identified by high-throughput DNA sequencing (19). Although in its current form this approach cannot be easily used in the clinic, it might identify disease-linked antigens that could then be used to prepare antigen microarrays for clinical applications.

PEPTOIDS

Peptoid microarrays provide an alternative approach to overcome the limitations imposed by antigen selection during the construction of antigen microarrays for biomarker discovery. Reddy and coworkers used a microarray of 4068 peptoids to generate a collection of molecular shapes available for interaction with antibodies (20). Using this platform the authors identified antibody–peptoid interactions of biomarker value. Although in this approach the identity of the antigen targeted *in vivo* by the antibodies with biomarker value is unknown, this method provides a broad epitope landscape to interrogate the entirety of the antibody repertoire.

Finally, a more challenging limitation for the clinical use of antigen microarrays is the existence of batch effects that make difficult the comparison of results obtained in different experiments and at different locations. Those batch effects have also been detected and dealt with in cDNA microarray studies (21, 22), and thus this limitation will likely be overcome.

Antigen Microarrays in the Study of Autoimmune Diseases

cDNA microarrays (23) revolutionized the analysis of the transcriptional response, saving materials and re-

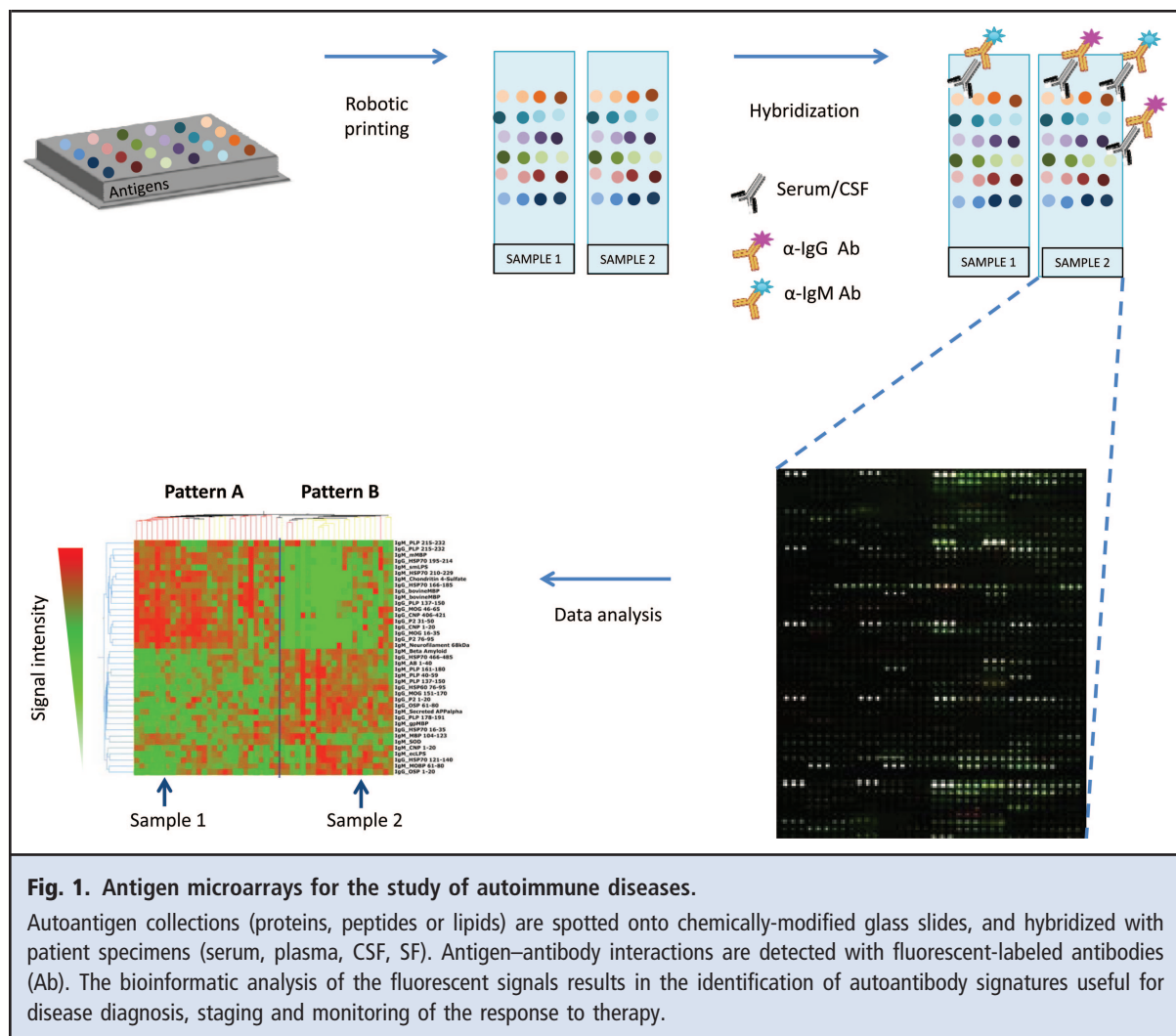


Fig. 1. Antigen microarrays for the study of autoimmune diseases.

Autoantigen collections (proteins, peptides or lipids) are spotted onto chemically-modified glass slides, and hybridized with patient specimens (serum, plasma, CSF, SF). Antigen–antibody interactions are detected with fluorescent-labeled antibodies (Ab). The bioinformatic analysis of the fluorescent signals results in the identification of autoantibody signatures useful for disease diagnosis, staging and monitoring of the response to therapy.

ducing costs to enable the high-throughput characterization of hundreds of genes in parallel. Similarly, the analysis of the antibody repertoire with antigen microarrays led to large-scale analyses of the immune response in autoimmune diseases (24–27), allergies (28, 29), response to tumors (30, 31), vaccinations (32), and infections (33). These studies identified antibody signatures of clinical value (Fig. 1) and also new mechanisms of disease pathogenesis. In this review we focus on the use of antigen microarrays for the study of autoimmune diseases.

Autoimmune diseases are characterized by the dysregulated activity of the immune system against self-antigens, resulting in chronic inflammation, tissue damage, and disability (34). The study of the immune response has important implications for the diagnosis, prognosis, treatment, and monitoring of autoimmune disease patients. In addition, it can also lead to the iden-

tification of new targets for therapeutic intervention. In the following sections we discuss how antigen microarrays have been used for the investigation of the immune response in 4 common autoimmune diseases: type 1 diabetes (T1D), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis (MS) (Table 1).

TYPE 1 DIABETES

T1D results from the selective destruction of insulin-producing β cells in the pancreas by autoreactive T cells (35). Although T1D is considered a T-cell-mediated disease, antibodies reactive with the pancreatic islets can be detected in patients with T1D, but the pathogenic role of these antibodies is still unknown. Regardless of their role in disease pathogenesis, islet-reactive antibodies precede the onset of hyperglycemia and are considered useful biomarkers for T1D (36, 37). Of

Table 1. Antigen microarrays in the study of autoimmune diseases.

Disease	Findings	References
T1D	a. Islet-reactive antibodies precede the onset of hyperglycemia.	a. Lehuen et al. (35); Bingly (36)
	b. Increasing the number of antigens tested improves the prediction of future disease development.	b. Lehuen et al. (35); Bingly (36)
	c. Specific antibody signatures differentiate T1D patients from healthy controls or patients affected by other diseases.	c. Hueber et al. (26); Nanto-Salonen et al. (37); Goldschmidt et al. (38)
	d. Serum antibodies predict the development of diabetes in the cyclophosphamide-accelerated diabetes model, before administration of cyclophosphamide.	d. Quintana and Cohen (39)
SLE	a. More than 100 autoantibodies may be present in SLE patients.	a. Liu and Davidson (40)
	b. Specific clusters of antibody reactivity are associated to glomerulonephritis and overall disease activity.	b. Quintana et al. (24); Liang et al. (44)
	c. First-degree relatives and patients suffering from incomplete LE have altered antibody profiles detectable with antigen microarrays.	c. Hueber and Robinson (45)
RA	a. Antibody patterns distinguish RA patients from controls.	a. Bizzaro et al. (52)
	b. Antibody signatures stratify patients in RA subgroups.	b. Bizzaro et al. (52)
	c. Antibodies to citrullinated peptides associated to disease severity.	c. Chandra et al. (53)
	d. Epitope spreading to citrullinated antigens characterizes the chronic phases of RA.	d. Hueber et al. (56)
	e. Increased blood levels of cytokines associated to antibody targeting of citrullinated autoantigens.	e. Li et al. (25); Zhao et al. (55)
MS	a. Antibody patterns distinguish MS from other diseases.	a. Quintana et al. (14)
	b. Serum antibodies discriminate different clinical forms of MS and pathological subtypes.	b. Quintana et al. (14)
	c. RRMS is characterized by antibodies to HSP.	c. Quintana et al. (14)
	d. Antibody response to lipids detected in MS, lipids might have pro- or antiinflammatory activities in MS.	d. Kanter et al. (13); Quintana et al. (14); Garren et al. (68); Farez et al. (72)

note, the predictive power of islet-specific antibodies is strengthened by increasing the number of antigens tested, so serum autoantibodies directed against insulin, glutamic acid decarboxylase, and protein tyrosine phosphatase are used to determine the risk of developing T1D and have been incorporated to the design of clinical trials in T1D (36, 37). Note, however, that the risk of T1D development is calculated on the basis of the number of autoantibodies rather than the presence of any individual antibody. Thus, these data suggest that the investigation of the autoantibody repertoire can provide useful information on the future development of T1D.

The autoantibody repertoire in T1D was first investigated with an array of 87 autoantigens in a 96-well plate format. These studies found that specific antibody signatures targeting β -cell antigens, and also antigens not associated with pancreatic islets, differentiated patients with T1D from healthy controls or patients suffering from MS, Behçet disease, and type 2 diabetes (27, 38, 39). These findings suggest that the repertoire of self-reactive antibodies reflects the specific immune processes associated with different inflammatory diseases. However, the autoantibodies associated with each disease might not be directly

involved in pathogenesis and might result from the release of self-antigens that follows tissue destruction.

The predictive value of autoantibody repertoires in T1D was investigated with antigen microarrays to study nonobese diabetic mice in which diabetes was accelerated and synchronized with cyclophosphamide (12). Serum samples were taken from the mice before the treatment at 4 weeks of age and after the treatment, and IgG antibody reactivities were analyzed with microarrays containing 266 antigens. Reactivities against 27 antigens were found to distinguish mice susceptible to diabetes induction from resistant mice, before the administration of cyclophosphamide. Taken together, these studies demonstrated that antigen microarrays could be used to both diagnose and predict the future development of T1D.

SYSTEMIC LUPUS ERYTHEMATOSUS

SLE is a chronic and heterogeneous autoimmune disorder that affects several organs, including skin, kidneys, and brain, and is characterized by glomerulonephritis and the presence of antibodies directed to nuclear and cell-membrane components such as double-stranded DNA, histones, and α -elastin, among others (40). It has been postulated that more than 100

autoantibodies may be present in patients with SLE (41), suggesting that the relatively small number of autoantibodies that are routinely measured in the clinic does not properly capture all the available information regarding diagnosis, staging, and prognosis of patients with SLE. Moreover, adoptive transfer experiments revealed that antibodies to double-stranded DNA and glomerular antibodies may be pathogenic, whereas antibodies to histones or nucleosomes might not be, and thus the heterogeneity observed in the antibody response in SLE might have important clinical implications (42–44).

To provide a complete characterization of the antibody repertoire in SLE, Mohan and coworkers developed antigen microarrays comprising a collection of SLE-related antigens (25). Using these antigen microarrays, these investigators found clusters of antibody reactivity associated to glomerulonephritis and overall disease activity (25, 45). In follow-up studies, they also identified altered antibody profiles in first-degree relatives of patients with SLE and also in patients suffering from incomplete LE syndromes, defined as having at least 1 but less than 4 of the SLE diagnostic criteria and first-degree relatives with SLE. These studies suggested that antigen microarrays might provide a tool for the early diagnosis and identification of patients at risk of developing SLE (46). In combination with transcriptional profiling, these studies might also identify subsets of patients with SLE who have the highest risk for disease progression (47); these patients are candidates for more aggressive therapeutic interventions. Thus, antigen microarrays provide a platform to analyze the heterogeneous antibody response in SLE and its association with different pathologic processes.

RHEUMATOID ARTHRITIS

RA is characterized by the chronic inflammation of synovial joints and bone erosion, leading to joint destruction, pain, and disability (48). Patients with RA show heterogeneity in their clinical course and response to therapy, suggesting the existence of patient subsets in which the disease is driven by different molecular mechanisms. Current tests for RA diagnosis are not satisfactory because they are based on the detection of single biomarkers that are either not specific for RA or present only in a subset of patients (49, 50). In addition, these biomarkers are useful for the prediction of disease course and response to treatment (51, 52). Robinson and coworkers developed antigen microarrays containing synovial antigens to identify biomarker signatures for RA diagnosis and molecular stratification into clinically relevant subtypes (53). Comparing autoantibody reactivities in the serum of 120 patients with RA with <6 months of disease duration and pa-

tients with ankylosing spondylitis, psoriatic arthritis, and healthy controls, they found antibody patterns specifically associated with RA. Moreover, the antibody signatures also stratified the RA patients into different subgroups (53). Patients in 1 of those groups, characterized by antibodies to citrullinated peptides in which peptidylarginine has been converted to peptidyl-citrulline, were found to be more likely to develop severe RA. Conversely, patients with RA in whom the antibodies targeted native antigens developed less severe forms of the disease (54). These results were later extended by studies showing that circulating immunocomplexes contain citrullinated fibrinogen (55) and that increased blood concentrations of proinflammatory cytokines are associated with autoantibody targeting of citrullinated antigens (26). Indeed, the analysis of antibody repertoires with antigen microarrays in combination with the quantification of circulating cytokine concentrations identified a potential set of biomarkers that predicted the response to anti-tumor necrosis factor therapy (56). Thus, antigen microarrays provide a tool for patient stratification in terms of disease prognosis and response to therapy in RA.

In addition, the association between immunity to citrullinated antigens and worsening of RA led to new insights in disease pathogenesis. Studies performed in experimental models of RA found that epitope spreading to citrullinated antigens characterized the chronic phases of the disease (57). Moreover, following immunization with citrullinated fibrinogen, humanized transgenic mice expressing the RA-associated MHC class II molecule DRB1*0401 developed RA (58). This new experimental model of RA was characterized by T- and B-cell responses to the citrullinated immunizing antigen and also to additional citrullinated antigens (58). Thus, these data revealed an important role for citrullination in RA pathogenesis and highlighted the potential use of antigen microarrays for the identification of mechanisms of disease pathogenesis.

MULTIPLE SCLEROSIS

MS is the leading cause of neurological disability in young adults (59). In 85% of the patients, the disease initially follows the course of relapsing–remitting MS course (RRMS) in which acute attacks are followed by a complete recovery (60). The majority of patients with RRMS go on to develop secondary progressive MS (SPMS), characterized by the progressive and irreversible accumulation of neurological disability (60). In approximately 10% of patients, MS presents as primary progressive MS (PPMS). MS is also heterogeneous in its immunopathological patterns of active infiltrating inflammatory cells and demyelination (61, 62).

An adaptive immune response against the central nervous system (CNS) is linked to the pathogenesis of

RRMS. The progressive disease course observed in SPMS generally occurs in the absence of new inflammatory lesions, and therapies that target the adaptive immune response on SPMS show limited efficacy, suggesting that other mechanisms play a role in this stage of MS (63). Based on these observations, it has been proposed that although both adaptive and innate immune responses contribute to RRMS, sustained activation of CNS innate immunity drives SPMS (64).

Using microarrays containing a panel of 362 myelin- and inflammation-related antigens associated with neurological diseases, Quintana et al. found serum IgG and IgM antibodies that enabled them to discriminate between the different clinical forms of MS and pathologic subtypes, concomitant with the idea that different immune mechanisms are involved in each form of MS (14). Antibody patterns could also be used to discriminate RRMS, the most common form of MS, from other diseases such as SLE, adrenoleukodystrophy, and Alzheimer disease (14). The differences between RRMS and controls were also observed when intrathecal antibody production was measured with antigen microarrays (24). In addition to autoantibodies to myelin, RRMS was also characterized by autoantibodies to heat shock proteins (HSPs) that were not observed in PPMS or SPMS. HSPs have been linked to inflammation and its regulation (65), and thus it is possible that antibodies to HSPs indicate that these proteins play a role in the control of the immune response in MS. Indeed, α B-crystallin, a small HSP, is the most abundant gene transcript in active MS lesions (66) and has important antiinflammatory functions in T cells and macrophages and an antiapoptotic role in glia (67).

Antigen microarrays have also been used to monitor the effects of treatment in MS. The comparison of CSF samples from untreated patients with RRMS with samples from patients treated with methylprednisolone demonstrated that this antiinflammatory drug decreases the local production of antibodies within the CNS in RRMS patients (24). Moreover, antigen microarrays were also used to measure antimyelin immunity during a randomized placebo-controlled phase 2 trial designed to study the effects of a DNA vaccine encoding myelin basic protein on RRMS (68). The authors found that immune reactivity to myelin basic protein before the initiation of treatment was associated with a beneficial response to treatment. Taken together, these data indicate that antigen microarrays can be used to monitor the response to therapy in MS patients and potentially to identify patients that will benefit from a specific therapy.

Lipids are also important targets of the autoimmune response in MS (69). To investigate the immune response to lipids in MS, Kanter et al. developed mi-

croarrays of lipids present in the myelin sheath, including ganglioside, sulfatide, cerebroside, sphingomyelin, and total brain lipid fractions (13). The authors detected lipid-specific antibodies against sulfatide, sphingomyelin, and oxidized lipids in CSF samples from patients with MS. Similarly, lipid microarrays detected substantial reactivity of oligoclonal bands with myelin lipids (70), an important observation because lipid-reactive oligoclonal bands have been linked to disease progression in MS (71). Lipid-specific antibodies were also detected in serum samples from patients with MS (14). Collectively, these studies led to the identification of a pathogenic role for some lipids in MS. Kanter et al. also reported that sulfatide-specific antibodies boost disease development in a murine experimental model of MS (13). Moreover, Farez et al. identified a signaling pathway involving toll-like receptor 2 and poly[ADP-ribose] polymerase 1, which is activated by lipids in astrocytes, microglia, and infiltrating macrophages in the CNS, promoting neurodegeneration and the accumulation of clinical disability (72). Conversely, it has been recently reported that some autoantibodies in MS target a phosphate group in phosphatidylserine and oxidized phosphatidylcholine derivatives. These lipids ameliorated experimental MS in mice by suppressing T-cell activation. Thus, it has been proposed that the lipid-reactive antibodies detected in MS patients inactivate these natural antiinflammatory lipids (73). Taken together, these studies point to the importance of antigen microarrays for the study of lipid-specific immunity and the identification of mechanisms of disease pathogenesis.

Summary

Antigen microarrays provide a unique tool to interrogate the immune response in autoimmune disorders, with important potential applications for disease management. These applications include disease diagnosis, for the early diagnosis of individuals developing autoimmune disorders before the onset of symptoms and also for the identification of individuals at risk of developing a specific autoimmune disease; disease monitoring, to stage patients and monitor disease progression and response to therapy; and discovery of mechanisms of pathogenesis, leading to the identification of targets for therapeutic intervention. In the majority of the autoimmune diseases the immune response is extremely heterogeneous, i.e., the immune system in different patients targets different antigens. A corollary of this heterogeneity is that, to improve their efficacy, antigen-specific therapeutic approaches will need to be tailored to target the autoimmune response of each individual patient. Antigen arrays provide an opportunity to identify the antigens driving the autoimmune response in each pa-

tient and to develop personalized antigen-specific tolerogenic approaches, such as those based on DNA vaccines (74–80) or nanoparticles (81).

Antigen microarrays also provide a new tool for the investigation of the immune response in autoimmune disorders and other conditions such as allergies (82, 83). Once current technical limitations are overcome, antigen microarrays have the potential to revolutionize the diagnosis, monitoring, and therapy of autoimmune diseases.

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