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MILLENNIUM AWARD RECIPIENT CONTRIBUTION

Proteomics for the Development of DNA Tolerizing Vaccines to Treat Autoimmune Disease

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Autoimmune disease affects 3% of the world population, yet current therapies that globally suppress immune function are inadequate. Tremendous need exists for specific and curative therapies, and we describe a strategy for development of antigen-specific therapies that inactivate pathogenic lymphocytes causing tissue injury. Major barriers to development of antigen-specific therapies for T-cell-mediated autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, and autoimmune diabetes, include (i) lack of knowledge of the specificity of autoimmune responses, for which proteomic technologies represent powerful tools to identify the self-protein targets of the autoimmune response, and (ii) lack of methods to induce specific immune tolerance, for which DNA tolerizing vaccines represent a promising strategy. We termed our approach Reverse Genomics: use of the proteomics-determined specificity of the autoantibody response to develop and select DNA tolerizing vaccines. Studies performed using animal models for multiple sclerosis and autoimmune diabetes support our Reverse Genomics approach. Through integration of proteomics with specific tolerizing therapies, we are developing a comprehensive approach to treat human autoimmune disease.

Key Words: **autoimmune disease; autoantibodies; DNA vaccines; protein arrays; antigen-specific therapy.**

INTRODUCTION

Despite remarkable progress in the field of autoimmunity over the past half century, our understanding of and treatments for human autoimmune disease remain primitive. Current therapies, including corticosteroids, cytotoxics, TNFα antagonists, and interferon β, globally suppress or modulate immune function. Such therapies do not adequately control disease activity in the majority of patients with T-cell-mediated autoimmune diseases, including multiple sclerosis (MS), rheumatoid arthritis (RA), and insulin-dependent diabetes mellitus (IDDM) (1–3).

Tremendous need exists for fundamental and specific therapies, such as antigen-specific therapies that inactivate only the offending lymphocytes. In contrast to conventional use of DNA vaccines to induce immune responses against microbes (4), it was recently discovered that DNA vaccines can induce tolerance to the encoded autoantigens to treat autoimmunity (5, 6). We propose administration of DNA tolerizing vaccines defined by the binding specificity of patient autoantibodies and have termed our approach Reverse Genomics.

BIOLOGICAL AND TECHNICAL CHALLENGES IN DETERMINING THE SPECIFICITY OF AUTOIMMUNE RESPONSES

To develop and administer antigen-specific therapies one must have methods to determine the specificity of autoimmune responses. Autoimmune responses are coordinated by autoreactive $CD4^+$ T lymphocytes, and the specificity of the autoimmune response is defined by their antigen receptors.

Determination of the specificity of autoimmune responses is complicated by the heterogeneity of lymphocyte populations, technical difficulties in examining their antigen receptors, and the vast number of potential autoantigens. Autoreactive T and B cells exist at low frequencies, generally less than 1 in 50,000 lymphocytes in both peripheral blood and autoimmune lesions (7, 8). Due to their low frequencies, autoreactive lymphocytes are not amenable to study with flow cytometry using fluorescently conjugated antigen or MHC–peptide multimers. It is not technically feasible to generate arrays of MHC–peptide multimers. T cell proliferation assays require substantial numbers of peripheral blood mononuclear cells and are labor intensive. The polymerase chain reaction and DNA microarrays, capable of detecting RNA transcripts encoding specific receptors, are inadequate. Due to these biological and technical limitations, it is not currently

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possible to determine the specificity of the autoreactive T cell response in individuals at the level of the proteome.

Although direct study of autoreactive B cells is limited by analogous difficulties, B cells secrete high-affinity autoantibodies (9). In T-cell-mediated autoimmune diseases, some autoantibodies are involved in tissue injury, such as MOG-reactive autoantibodies in MS (10). For the majority of autoantibodies their pathogenetic roles are ill defined (9). Nevertheless, the specificity of the autoantibody response and linkage to its cognate $CD4^+$ T cell response make autoantibodies powerful tools for determining the specificity of autoimmune responses.

RATIONALE FOR USE OF AUTOANTIBODY RESPONSES TO MONITOR T-CELL-MEDIATED AUTOIMMUNITY

Immune responses evolved to focus B and T cell responses on the same antigenic targets. B cells expressing a rearranged immunoglobulin specific for a particular antigen take-up, process and present linear epitopes of that antigen to activate and receive help from autoreactive $CD4^+$ T cells. The reciprocal nature of B and T cell activation selects for autoreactive B and T lymphocytes with antigen receptors directed against the same macromolecular antigens.

Reverse Genomics is based on the hypothesis that the specificity of the autoantibody response correlates with that of the autoreactive helper T cell response. Although there are examples of concordance and discordance between the fine specificity of autoreactive B and T cell responses, autoreactive B and T cell responses are directed against the same macromolecular autoantigen targets (11–13). We believe that autoantibodies can be used to identify the targets of autoimmune responses at the whole protein or macromolecular level. The ability to use the specificity of the autoantibody response to identify the specific self-protein(s) or macromolecule(s) against which an individual is autoreacting is enabling for evaluating candidate autoantigens and selecting antigen-specific therapy.

Studies in IDDM demonstrate the utility of autoantibody profiling. The presence of a combination of serum autoantibodies against GAD, insulin, and IA-2 are diagnostic or predictive of future development of IDDM (14, 15). The utility of autoantibody profiling in IDDM suggests that multiplex autoantibody determination could also have prognostic and diagnostic utility for other T-cell-mediated diseases, such as MS and RA.

PROTEOMIC TECHNOLOGIES TO DETERMINE THE SPECIFICITY OF AUTOANTIBODY RESPONSES

Proteomics is the large-scale study of protein expression, function, and interactions. Conventional methods to determine the specificity of autoreactive B cell responses include enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) analysis. These methods are hindered by the vast numbers of potential autoantigens and requirements for relatively large quantities of each antigen and human sample. Miniaturized proteomic technologies now enable multiplex analysis of the specificity of autoantibody responses in individual patients using nanogram quantities of antigen and submicroliter quantities of patient samples.

A variety of proteomic technologies enable profiling of autoantibody responses (16, 17). We refined the methods of Haab and colleagues (18) to develop antigen arrays. A robot is used to attach putative peptide and protein autoantigens to the surface of derivitized microscope slides in an ordered array (Fig. 1) (19). Büssow and colleagues describe methods to use cDNA expression libraries to generate spacially addressable arrays for autoantigen discovery (20). Other proteomics technologies include *in situ*-generated spatially addressable arrays of antigens, such as photolithography-generated peptide arrays, production of polypeptides by genetically transformed cells, and synthesis of peptides on arrays of pins (21–23); spatially addressable arrays of living cells expressing antigens (24, 25); arrays of antigens attached to fluorescently addressable beads (26), such as Luminex, Inc.'s LabMAP system (www.luminex.com and www.bd.com); arrays of nanoparticles, including addressable multimetal microrods (27), being developed by SurroMed, Inc. (www.surromed.com); arrays of antigens attached to nonparticle fluorescent tags, such as Aclara, Inc.'s eTAG system that utilizes cleavable fluorescent labels with unique electrophoretic mobilities (www.aclara.com); and microfluidics systems for multiplexed characterization of autoantibody–antigen interactions (28) (see microfluidics.stanford.edu, www.calipertech.com and www.aclara.com).

Specialized Proteomes for Characterizing Autoimmune Disease

We developed specialized proteome arrays, containing a spectrum of potential autoantigens derived from autoimmune tissue targets, to study specific diseases. We are developing "myelin proteome" arrays to study MS and EAE (described below); "connective tissue disease" arrays that contain nuclear and other autoantigens targeted in autoimmune rheumatic diseases (19); "synovial proteome" arrays containing candidate RA autoantigens, including BiP, hnRNP A2/B1, GP-39, glucose-6-phosphate isomerase, collagens, as well as native and deiminated fibrinogen, vimentin, and filaggrin to study RA and its animal model collagen-induced arthritis (CIA); and "islet cell proteome" arrays containing IA-2, glutamic acid decarboxylase, insulin,

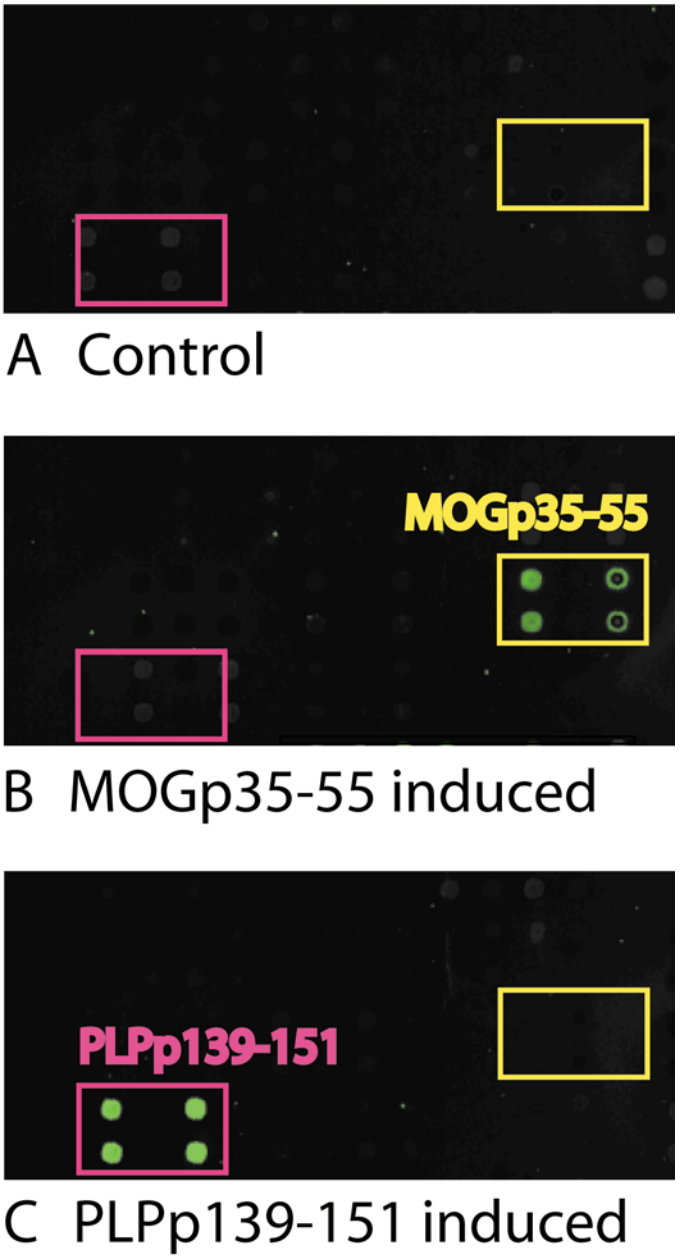

FIG. 1. "Myelin proteome" array determination of the specificity of the autoantibody response in EAE. Myelin proteome arrays were probed with serum from a control C57BL/6 mouse (A), obtained at peak disease (approximately day 18) from a C57BL/6 mouse induced with MOGp35-55 (B) or obtained at peak disease (approximately day 17) from an SJL mouse induced with PLPp139-151 (C). Arrays were generated using a robotic microarrayer to attach myelin peptides and proteins in an ordered array on poly-L-lysine-coated microscope slides. Printed arrays were blocked overnight, incubated with 1:150 dilutions of serum, followed by Cy-3-conjugated goat anti-mouse IgG/M antibody, and scanned. Images represent less than 1/50th of the overall myelin proteome array. Comprehensive protocols are presented in our paper (19) and on the World Wide Web at: http://www. stanford.edu/group/antigenarrays/.

and heat shock proteins for study of IDDM and nonobese diabetic (NOD) mice.

Myelin Proteome Arrays Detect Specific Autoantibodies in EAE and MS

Myelin proteome arrays contain myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), oligodendrocyte-specific protein (OSP), and αb crystallin, as well as overlapping peptides derived from these and additional myelin proteins. Using myelin proteome arrays we detect autoantibodies directed against MOGp35-55 at the time of peak disease in mice induced to develop EAE with MOGp35-55 (Fig. 1B) and against PLPp139-151 in mice induced with PLPp139-151 (Fig. 1C).

Clinical Applications for Autoantibody Profiling

Knowledge of the specificity of autoantibody responses can be utilized for: (i) diagnosis of autoimmunity or predisposition to develop autoimmunity (19); (ii) autoantigen discovery and characterization to develop novel antigen-specific therapies; (iii) selecting patients to receive tolerizing therapy for a clinical trial or in clinical practice; (iv) tailoring tolerizing therapy to individual patients; and (v) monitoring responses to tolerizing therapies, as represented by a reduction in epitope spreading or modulation toward antibody isotypes associated with protective Th2 immune responses.

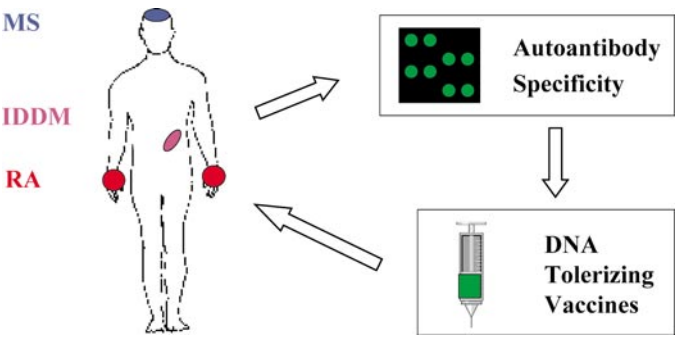

FIG. 2. Reverse genomics: Use of the proteomic-determined specificity of the autoantibody response to develop and select DNA tolerizing vaccines. Serum, synovial fluid, or CSF is obtained from patients with autoimmune disease. Antigen array or alternative proteomic analysis is performed to determine the specificity of the autoantibody response. Based on the consensus specificity of the autoantibody response in cohorts of autoimmune patients, novel DNA tolerizing vaccines encoding the targeted autoantigens can be developed. Knowledge of the specificity of the autoantibody response in individuals may identify patients likely to respond to an available DNA tolerizing therapy or enable patient-specific DNA tolerizing therapy.

DNA TOLERIZING VACCINES TREAT AUTOIMMUNITY

EAE is prevented and treated by DNA vaccines encoding myelin epitopes and proteins (5, 29). DNA vaccines encoding insulin (30, 31) or glutamic acid decarboxylase (GAD) (32) reduce the incidence of autoimmune diabetes in nonobese diabetic (NOD) mice. Genetic delivery of IL-4 in combination with DNA encoding myelin or islet proteins enhanced their protective effects (5, 32). DNA tolerizing vaccines encoding autoantigens alone reduce T cell proliferative responses (anergy) (29), while DNA tolerizing vaccines codelivering autoantigen plus IL-4 also induce protective Th2 responses (5).

Gene gun delivery of DNA encoding MBP coated on gold particles prevented induction of EAE in rats (33). Gene gun delivery of DNA is known to promote Th2 responses (34), and this method could have advantages in treating autoimmunity.

REVERSE GENOMICS: PROTEOMICS TO DRIVE DNA TOLERIZING VACCINES TO TREAT AUTOIMMUNITY

We are using antigen arrays to guide development and selection of antigen-specific DNA tolerizing vaccines, a strategy we termed Reverse Genomics (Fig. 2). Reverse Genomics entails (i) proteomic determination of the specificity of autoimmune responses and (ii) DNA tolerizing vaccines to induce specific tolerance.

Evidence for Reverse Genomics in Treating EAE and Autoimmune Diabetes in NOD Mice

Treatment of established EAE with DNA tolerizing vaccines encoding MOG, an array-confirmed target of the autoantibody response (Fig. 1), reduced disease severity (5). Treatment of prediabetic NOD mice, at an age at which they are known to have ongoing insulitis accompanied by anti-insulin and anti-GAD antibodies (35, 36), with DNA tolerizing therapies encoding insulin peptide or GAD reduced the incidence of diabetes (30, 32, 37). These data suggest efficacy of Reverse Genomics; treatment of EAE and NOD mice with DNA encoding their autoantibody targets treats autoimmunity.

Reverse Genomics offers advantages over genomics and traditional drug discovery strategies that require complex, time-consuming, and expensive preclinical development processes. Utilizing the polymerase chain reaction, within weeks DNA encoding an array-identified autoantigen can be isolated and cloned into a DNA expression vector to generate a novel therapeutic for evaluation in clinical trails.

APPLYING REVERSE GENOMICS TO HUMAN AUTOIMMUNE DISEASE: TREATMENT OF ALLERGIC DISEASE PROVIDES A MODEL FOR PATIENT-SPECIFIC IMMUNOTHERAPY

In the allergy clinic, prick-puncture and intradermal skin tests that measure type I immediate hypersensitivity are used to assess allergic responses. Skin tests can be performed in an "ordered antigen array" on a patient's back to gauge allergic responses against 25–100 allergens simultaneously. Common test allergens are derived from pollens, house dust mites, mammals, insects, and foods. Based on the skin test results, the allergist selects extracts to administer as patient-specific immunotherapy.

In an analogous fashion to the use of skin testing to select allergen immunotherapy, proteomics could be applied to tailor tolerizing therapy to treat autoimmunity. Proteomic characterization of the autoantibody response could be used to select patients to be enrolled in clinical trials or to receive tolerizing therapies encoding a specific antigen. If delivery of the exact set of autoimmune targets proves superior, then proteomics could be applied to select customized therapies for individual patients.

COST-EFFECTIVENESS AND REGULATORY HURDLES FOR PATIENT-SPECIFIC THERAPY FOR AUTOIMMUNITY

Autoimmunity affects individuals of child-bearing age, during their peak productivity in the home and workplace. For the majority of autoimmune diseases, current therapies, including interferon β for MS and TNF antagonists for RA that cost approximately $10,000 per patient-year, are only marginally effective (1, 2). Without adequate treatment a significant fraction of patients become disabled, imposing great direct and indirect costs on society (38, 39). The high cost and marginal efficacy of current therapeutic regimens provide justification for tailored patient-specific therapy for autoimmunity. Effective tolerizing therapies have the potential to prevent disability and perhaps provide a life-long cure, which would be of great benefit to patients and society.

To date, no antigen-specific tolerizing therapies have demonstrated efficacy in late-stage clinical trials. It will take decades to demonstrate the safety and efficacy of DNA tolerizing and other antigen-specific therapies encoding multiple autoantigens to gain approval from regulatory agencies and enable tailored therapy for autoimmune disease.

PROTEOMICS TECHNOLOGIES FOR DEVELOPMENT AND SELECTION OF PEPTIDE, PROTEIN, AND OTHER BIOMOLECULE ANTIGEN-SPECIFIC THERAPIES TO TREAT AUTOIMMUNE DISEASE

In an analogous strategy to Reverse Genomics, knowledge of the specificity of the autoantibody re-

sponse could be applied to develop and select any antigen-specific tolerizing therapy. Although others have suggested use of the autoantibody specificity to guide peptide-based tolerizing therapies for B-cell-mediated autoimmune diseases (40), this strategy has not been described for the treatment of T-cell-mediated autoimmune diseases, such as MS, RA, and IDDM. Examples of non-polynucleotide-specific tolerizing therapies under development include (i) protein antigens (41); (ii) native peptides (42–44); (iii) altered peptide ligands (45, 46); (iv) other biomolecules, such as DNA, or proteins and peptides containing posttranslational modifications (47); and (v) antigens delivered orally to induce "oral tolerance" (48).

SUMMARY

With the advent of proteomics technologies it is now possible to profile the specificity of the autoantibody response in individuals and cohorts of patients. We propose use of the specificity of the autoantibody response to develop and select DNA tolerizing vaccines and other antigen-specific therapies for use in the clinic. Treatment of EAE and NOD mice with DNA encoding targets of the autoantibody response suggest the efficacy of Reverse Genomics. We hope to use Reverse Genomics to bring tailored and specific tolerizing therapy from the bench to the bedside to treat human autoimmune disease.

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