Lupus: Open Access

Cohen, Lupus Open Access 2016, 1:3

Review Article OMICS International

Antigen-Microarray Profiling of Antibodies in SLE: A Personal View of Translation from Basic Science to the Clinic

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Received date: September 23, 2016; **Accepted date:** October 18, 2016; **Published date:** October 24, 2016

Abstract

In October of 2015, the startup company ImmunArray announced the launch of a microarray platform – the iChip® – to profile repertoires of serum antibodies and autoantibodies. The first iChip® product – the SLE-key® RuleOut test – is designed to help the physician rule out a diagnosis of systemic lupus erythematosus (SLE) in suspected patients. The aim of this review is threefold: the first aim is to describe how basic observations and a philosophical notion led me to undertake the development of what has turned out to be a clinically useful aid in dealing with complex diseases; the second aim is to describe the role of a company in overcoming the technological and informatics challenges involved in translating basic research into patient welfare; the third aim is to discuss why SLE, like other complex medical problems, can be better managed using immune profiling. Basic scientist readers might learn here about the path to clinical application; clinician readers might learn here about the complicated origins of seemingly simple tests.

Keywords: Immunological homunculus; Antigen microarray; Autoantibody profiling; Antibody repertoires; Diagnosis; Lupus; Multiplex assays.

Part One - Basic Science

The immunological homunculus

My involvement in the iChip® originates from the theory of the Immunological Homunculus – a concept I first published in 1989 [1]. For some time I had been studying models of autoimmune diseases induced by immunization, such as experimental autoimmune encephalomyelitis (EAE) [2] or adjuvant arthritis (AA) [3]. Evidently, the adaptive immune system responded in a biased way to immunization to tissues of the central nervous system (CNS): antigens such as myelin basic protein (MBP) were more likely to induce antibody or T-cell responses than were other CNS antigens present in even greater amounts in a whole spinal cord immunization [4]. Moreover, foreign antigens cross-reactive with self-antigens often dominated the immune response to bacteria – for example, in AA, immunization to killed Mycobacteria induced a major T cell response to HSP60 cross-reactive with self [5]. Why would particular self- or self-like antigens attract a response that was greater than the response elicited by other self-antigens or even by foreign antigens in the immunogenic preparation? Was it possible that antigen receptors on B cells and T cells for these favored antigens were prevalent in immune repertoires even before immunization – as if the immune system had already been primed to seek out these antigens? These experimental questions raised a more fundamental question; what evolutionary advantage might there be in an adaptive immune system poised a priori to respond to certain body molecules over others?

A notable feature of the adaptive immune system is that it learns from somatic experience: immunization leads to the induction of long-lasting immunological memory. The other body system that, like the immune system, learns from somatic experience is the brain. The mammalian CNS deploys a neurological homunculus of dominant networks of neurons organized a priori to compute essential features of the individual self and the world. These functional networks are grouped into distinct regions in the sensory and motor cortices that together generate a functional representation of a little man – a neurological homunculus [6].

The bias to certain self-antigens suggests that the immune system, too, might express an immunological homunculus – an internal image of key body molecules encoded by positive selection of antigen receptors to generate skewed repertoires of T cells and B cells [7-9]. Obviously, any self-antigen biases would have to enhance fitness; the autoimmunity expressed by repertoires of T cells and B cells in healthy individuals must be doing more good than harm [10-14].

Autoantibody profiles disclose health and susceptibility to disease

We set out to document an aspect of the immunological homunculus by detecting the binding of autoantibodies to many different antigens in a single sample of serum. Living creatures are collective networks of interconnected reactivities [15]; hence, a systems immunology view requires multiplex profiling of repertoires. We began with an analysis of autoantibodies to multiple self-antigens in human type 1 diabetes (T1D) using an ELISA assay in 96-well microtitre plates; we found that autoantibodies to an array of 87 different antigens discriminated between T1D patients and healthy subjects [16]. It was clear, however, that a standard ELISA assay was not a feasible way to proceed – the assay was not precise, and the amounts of antigen and the volumes of serum needed were too costly and wasteful. We then adapted to serology a technology initially developed to study gene expression. We developed an antigen microarray chip based on precise robotic spotting of hundreds of candidate antigens on a glass slide; serum antibodies binding to these antigens were detected by laser

activation of fluorescence-labeled second antibodies binding to the bound test antibodies; the strength of the fluorescence signal could be used to rank relative degrees of antibody binding; finally, all this could be done using a few microliters of serum and nanograms of antigen.

The first study using the new microarray chip was challenging: might the autoantibody profile of an individual be able to inform us about susceptibility or resistance to a future bout of T1D. Fortunately, a mouse model of T1D suited the challenge. About 50% of male mice of the NOD strain develop T1D spontaneously over six or more months of observation, but the lag phase to overt T1D can be markedly accelerated by injecting the mice with cyclophosphamide [17]. Despite accelerated onset, still only about 50% of the treated mice develop T1D; the remaining 50% resist the disease [18]. In other words, a given male NOD mouse may or may not resist induction of T1D with about equal probability. Could autoantibody profiling of the mouse before cyclophosphamide induction predict its future response? Indeed, our informatics analysis revealed that IgG autoantibodies to 27 of 266 candidate antigens established a profile that predicted the future development of diabetes to a significant degree [19]. This multiplex microarray study in mice suggested that predictive diagnosis might also be feasible in human health management. It was worth applying the approach to humans.

The immunological homunculus at birth

To establish the baseline human homunculus, we studied autoantibody repertoires in the cord sera of human newborns and in the peripheral blood of their mothers. Maternal antibodies of the IgG isotype are known to be actively transported across the placenta to the developing fetus; maternal IgM and IgA isotypes, in contrast, do not cross into the fetus [20], so any antibodies of these isotypes in cord blood would have had to have been produced by the developing fetus in utero before birth. Measuring antibody isotypes binding to some 300 antigens, we found, as expected, a very high correlation between the IgG serum repertoires of each mother and the cord blood of her baby – this can be explained most easily by mother's transfer of her IgG.

The IgM and IgA repertoires, however, were surprising in three ways: First, there was a large amount of IgM antibodies and an appreciable amount of IgA; hence, healthy newborn humans were clearly producing antibodies in utero. Secondly, these IgM and IgA antibodies bound to self-antigens known to be recognized by IgG antibodies in persons afflicted later in life with major autoimmune diseases; it would thus appear that a disease my emerge from dysregulation of a healthy immune system. And thirdly, the IgM and IgA repertoires of the babies, unlike their IgG repertoires showed relatively less correlation to their mothers and greater correlation to the other babies; in other words, genetically diverse newborns had made IgM and IgA antibodies to very similar sets of self-molecules – different newborn humans come equipped with shared homuncular sets of autoreactive IgM and IgA antibodies [21].

The initial study of maternal-cord autoantibodies was expanded recently to a study of 71 mothers and their 104 newborns (including twins and triplets); we also studied antibody reactivities detectible in maternal colostrum [22]. The findings reported in the initial, small study were confirmed in this much larger set of subjects. In addition to serum and colostrum antibodies binding to self-antigens known to be associated later with autoimmune diseases, the autoantibody repertoires also contained antibodies associated with tumor states [22]. We do not know at present what might be the benefits of these autoantibody repertoires; but it is reasonable to hypothesize that autoreactivity to healthy body molecules might be associated with tissue maintenance and wound healing [13,14,23] and that autoimmunity to tumor-associated self-antigens could take part in tumor immunotherapy unleashed by checkpoint blockade [24].

Autoantibody repertoires can reflect the state of a transplanted tumor

To learn whether autoantibody profiles could disclose the states of tumors, we used the antigen microarray to study the sera of mice bearing transplantable, syngeneic tumors [25]. We found that profiles of IgG and IgM autoantibody repertoires distinguished between mice bearing metastatic and non-metastatic clones of the same tumor line, and that the curative resection or metastatic spread of a tumor was reflected by specific changes in homuncular profiles.

These laboratory observations indicated that multiplex profiling of autoantibody repertoires could contribute to individual medical care by revealing characteristic profiles shared with others in particular states of health or disease. The challenge was to translate the approach to reliable clinical use.

Part Two - Clinical Translation

Requirements for clinical medicine

In vivo and *ex vivo* experiments in an academic laboratory usually deploy experimental animals that are divided into statistically empowered test and control groups, and that are genetically uniform, fed with defined diets, raised in controlled environments free of pathogens and infections, and identical in age and gender. Laboratory studies involving humans or human materials are usually obtained from defined patient populations that are selected by experts at recognized clinical centers; discriminating test and control groups are determined by detailed inclusion and exclusion criteria; and group numbers are commensurate with the nature and the goals of the study. Moreover, statistically meaningful results are determined by optimally discriminating between test and control groups. The initial work devising and testing the antigen microarray on laboratory mice or on selected patient groups was done in our laboratory under such ideal conditions using single batches of microarray chips.

But individual patients do not come with a matched control group; they come to the doctor sporadically, without disease labels, and at different stages of their illness along with accompanying illnesses and variable and often inadequate medical histories. Patients in clinical practice are genetically diverse and differ markedly in occupation, socioeconomic status and styles of life. In addition to technological robustness, microarray antibody assays for human disease need to generate results that suit a standard nosological category across a wide range of patients suffering from a given disease; a useful analysis will have to lump together individuals who may differ in all aspects of life other than in their common disease. Moreover, informatics algorithms suitable to discriminate between defined test and control groups in academia are not necessarily suitable for the errant individual patient visiting a clinic.

Academic experimentalists may come up with novel observations, creative ideas and exciting data, but they are not usually suited in mindset or technology to solve the problems inherent in translating innovation from the controlled laboratory to the demands of the real-

life clinic. Moreover, academia almost always lacks the funds to do the job. To effect the translation, it was clear that the microarray device and the informatics analysis had to be transferred out of academia and placed in the hands of experienced diagnostic innovators. Yeda, the development arm of the Weizmann Institute of Science licensed a startup company, now called ImmunArray [26], to develop our rudimentary, homegrown antigen microarray into a clinically reliable assay.

Management and problem solving

Translational medicine, to succeed, has to bridge a gap between different cultures of thought and action; the translational enterprise depends on productive interactions between people of different mindsets and training; Persons in manufacturing, commercial development and entrepreneurship and academic scientists, informatics experts and physicians must understand one another and work in concert with the requirements of regulators and the needs of patients. Successful translation requires carefully executed financing, teamwork, mutual understanding and open communication among the various groups. Unfortunately, these requirements are not easy to satisfy. Discoveries and innovations that should benefit patients and society may fail to materialize because of mismanagement of the translational process; scientists and developers may misinterpret their common interests. Obviously, a potentially promising project may turn out to be flawed intrinsically and fail despite good management. Fortunately for the iChip®, ImmunArray Ltd is both experienced and well-managed and has been financed along the way by individuals with dedication and foresight. Briefly, ImmunArray was able to create a team that could identify and solve the technical problems in making a robust and reliable microarray device: devising a special slide-coating process; switching to non-contact printing; optimizing antigens and samples and their interactions; and developing the informatics needed to analyze, validate and communicate the clinical results. Details of iChip® development and the operation of the iChip® platform can be seen in a published paper [27] and on the ImmunArray website [26].

Part Three - The Rationale for Autoantibody Profiling

The Immune system surveys body state and manages inflammation

What is the function of the immune system, anyway? Why does the immune system need a homunculus? What can one expect to gain from profiling autoimmune repertoires?

Let's start with the first question – the function of the immune system: The Clonal Selection Theory enunciated by Burnett saw the immune system as a Department of Defense, only – its task was simply to rid the body of foreign invaders like bacteria, viruses and other parasites [28]. According to Burnett's view, the true agents of the immune system were the lymphocytes; the unique and defining characteristic of the immune system was the ability to detect antigens, and lymphocyte antigen receptors and antibodies were the only way to do it. Antigen receptors and antibodies were proposed to be generated somatically and randomly during lymphocyte development, and those clones of lymphocytes that happened to interact with self-antigens were thought to be purged from the repertoire. As a result of this negative selection, surviving lymphocytes could only be those that recognize and respond to foreign antigens; accidental recognition of self-antigens was punished by autoimmune disease [28]. The function of the immune system was assumed to discriminate self (ignore) from not-self or foreign (destroy) – a binary discrimination [29]. In contrast to expectations, however, healthy immune systems were found to contain lymphocytes able to recognize and respond to self-antigens [7]. As a consequence, Polly Matzinger proposed that the function of the immune system was to distinguish between danger and not-danger [30] – self-reactivity was acceptable and necessary to assess danger. Quite simply, the immune system was proposed to be mobilized into action by sensing danger signals [31].

Foreign and danger, however, are words, concepts of mind, not functional expressions of biological activity; an antigen receptor that binds an antigen has no way of knowing whether the antigen is self or foreign; or whether the antigen marks danger – what binds, binds; only a human observer can append conceptual categories like self-not-self or danger-not-danger. Moreover, most of the cells included in the immune system – macrophages, dendritic cells, neutrophils, eosinophils, and the like – don't bear antigen receptors and don't respond to antigens, either self or foreign. So what does the immune system actually do?

In physiological terms, we can say that the immune system deals with inflammation; inflammation is a network of processes triggered by injury, infection or other malfunction. The outcome of inflammation, suitably organized and controlled, is healing and restoration of health [32]. Healthy inflammation, in other words, is the process by which the body deals with entropy - the inevitable wear and tear and the accidents of existence in the world. I have proposed that the immune system mediates and controls inflammation, its onset, evolution and resolution [14,32]. Destructive components of the inflammatory network can destroy foreign pathogens invading from without and tumor cells arising from within the body. Regenerative components of the inflammatory network, in contrast, can heal the body by stimulating scar tissue and angiogenesis, and by activating the cell movement, proliferation and differentiation needed for recovery from injury or infection. The immune system also regulates our essential symbiosis with the microbiome – our health-giving resident bacteria and viruses [33]. Components of the immune system also influence metabolism and the metabolome [34,35]. From this point of view, the immune system is not only the body's department of defense, but also its department of maintenance and welfare [14]. Inflammation is involved in all aspects of body maintenance as well as in defense; since the immune system manages inflammation, we can see why the immune system is turning out to be involved in most conditions of medical interest. Obviously, inappropriate or poorly managed inflammation can itself cause disease – autoimmune diseases or other chronic or recurrent inflammations.

The realization that the immune system manages inflammation provides the framework for answering the other two questions:

Why does the immune system need a homunculus? Quite simply, immune repertoires in different individuals are biased to react strongly to certain common self-antigens because the expression of these particular self-molecules (and not others) can inform the immune system about states of cells and tissues in need of immune attention [13,14]. A telling example of molecules that help disclose tissue state are heat shock proteins (HSP). HSP molecules, because of their essential chaperone functions, are reliable biomarker signals for immune-mediated inflammation and resolution. For example, HSP60 and its peptides interact with a variety of lymphocyte antigen receptors and innate cell receptors [36] and can be used to modulate inflammation and inflammatory diseases [37-39]. This brings us to the

third question: What we can gain from multiplex profiling of antibody repertoires by the iChip® can now be put into perspective.

The iChip® discloses repertoire information about body state

Physicians need to know about the state of particular cells and tissues of the body to be able to make accurate diagnoses, monitor disease and healing, predict responses to treatments, and treat and advise the patient correctly [40]. Profiling the autoantibody repertoire by the iChip® can disclose and monitor these states and thus help the physician help the patient. The autoantibody repertoires of the immune system are dynamic and are modified by the changing states of the body in health and illness; the iChip®, as we have seen in our pre-clinical studies, can reflect these states [25].

It is true that autoantibody repertoires constitute only a part of the information about the state of an individual encoded in the immune system. Nevertheless, autoantibody repertoires are relatively accessible to study, especially using the iChip® [27]. A drop of serum or body fluid can profile meaningful information about individuals and collectives of individuals in health and in states requiring protective or restorative inflammation. The immune system is privy to body's deepest secretes as well as to its overt ills. Quite simply, inflammation is a factor in many, if not in most conditions of medical interest; iChip® analysis of autoantibody repertoires can thus serve as a guide to a wide range of medical interests. We just have to discover which autoantibody profiles are indicative of which body states – we have to prepare accurate profile maps.

Applying the iChip® to SLE

The first clinical application of the iChip® has been to rule-out a diagnosis of SLE [27]: why SLE and why begin with ruling it out?

SLE exemplifies the difficulty of defining a complex disease and diagnosing it: SLE is an autoimmune disease, yet no single autoantibody or autoimmune reaction is shared by all patients who appear to suffer from the disease [41]. SLE is an accepted nosological category, yet not all SLE patients suffer from a uniform set of clinical manifestations – skin, blood vessels, plasma proteins, joints, kidneys, heart and brain may be affected to different degrees in different patients. Indeed, a diagnosis of SLE rests, at present, on four or more manifestations out of a somewhat subjective and often debated list of eleven criteria [42]. Certain items on the list can also appear in healthy persons or accompany diseases other than SLE [43]; SLE can overlap with rheumatoid arthritis, scleroderma, phospholipid syndrome, and other inflammatory conditions of autoimmune or unknown etiology. The diagnosis of SLE ultimately rests on expert clinical judgment; but judgment can be less than prefect and even controversial. An objective criterion for aid in diagnosing SLE would clearly be useful. Variability in diagnosis certainly complicates therapy and hinders the development of new therapeutic modalities. An iChip® test to help diagnose SLE is clearly in order.

Our first clinical application for an iChip® has been to rule out SLE, rather than to diagnose it positively. There are two reasons for this decision: First, an earlier laboratory study indicated that SLE might be characterized by a signature of antibody reactivities that is not influenced by duration of disease, state of activity or remission, or range of clinical manifestations [44]. This finding suggested that a patient without this SLE signature would not be likely to be suffering from SLE. Thus we set out to develop an iChip® SLE RULE-OUT profile to identify a set of autoantibody reactivities without which a diagnosis of SLE is very unlikely, irrespective of its particular manifestations; unless the patient manifested this essential serologic signature, the patient was less likely to be suffering from SLE. In contrast, a positive diagnosis of SLE requires that SLE be distinguished from other diseases like rheumatoid arthritis or scleroderma that might overlap with SLE in certain serologic manifestations. Ruling-out SLE should be more clear-cut than ruling it in.

Secondly, many patients and physicians worry about possible SLE because the disease has so many different clinical manifestations – an SLE test result that effectively rules-out SLE can alleviate worry, reduce costly and inappropriate testing and prevent unnecessary treatments that can entail undesirable side effects; moreover, ruling out SLE can direct diagnostic efforts to other possibilities. So a rule-out test was deemed a good place to start with the iChip®. The actual development of the SLE Key Rule-Out iChip® assay has been published and there is no need to repeat the details of how the microarray and the informatics enabled us to scan many hundreds of IgM and IgG autoantibody specificities in SLE patients and controls and to finally arrive at a multiplex autoantibody profile of a manageable number of antigens; this profile represents a robust SLE signature and is able to rule-out SLE with a high degree of sensitivity [27]. At present, the test aids practicing physicians who treat individual patients and is being increasingly used to help with clinical decisions. We are now engaged in developing an expanded iChip® profile that maps the state of activity of SLE in the individual patient and that can alert the physician to an impending exacerbation or relapse. We are also planning to develop autoantibody profiles that can be used to diagnose SLE and possibly to distinguish it from other, clinically overlapping diseases. Antigen-microarray profiling of autoimmune diseases including SLE has been reported by others [45-47], but only ImmunArray Ltd, until now, has been able to develop an antigen microarray that is in clinical use [48].

Returning to Basics

Beyond diagnosis, an iChip® profile study comparing SLE with other inflammatory diseases could lead to new ideas about fundamental immunological relationships between diseases that may share related autoantibody profiles. Is there an underlying immune system signature shared by clinically different inflammatory conditions? Do variable or different clinical manifestations result from add-on autoreactivities, from genetic predispositions or from microbiome or environmental factors? Development of iChip® profiling and its successful translation from the laboratory to the clinic thus can feedback to provide basic science with new experimental questions. Clearly, laboratory research and clinical development are partners in the scientific enterprise.

Acknowledgements

I thank Donna Edmonds, a veteran diagnostic executive who knows the immune response to be a key player in most all diseases and who appreciates the need for a multiplexed approach to patient management. She put ImmunArray Ltd together and assured that it was ideally led, financed and directed to translate iChip® profiling into a clinical reality. Pennina Safer, Scott Batty, Keren Jakobi and the present team of ImmunArray Ltd, and previous work by Eli Sahar, Idan Tamir and Vered Daniel-Carmel, made it happen. Francisco Quintana and Yifat Merbl, as students, developed the laboratory prototype; Eytan Domany, a close collaborator, initiated the informatics. I am a consultant to ImmunArray Ltd.

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