Recent advances in diagnostic technology: applications in autoimmune and infectious diseases

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ABSTRACT

Biomarkers are used ubiquitously as indicators of biological health. The development of genomic and proteomic multiplex technologies have enormously amplified biomarker discovery and application to diagnostic and therapeutic decisions in clinical practice. New technologies are now available that simultaneously identify a wide spectrum of biomarkers and save time and costs. Multiplexed assays can be coupled to other disease specific indicators (i.e., cytokines, single nucleotide polymorphisms) in order to get more powerful information. However, there is an urgent need for validation/standardization of the new assays before they are adopted into clinical diagnostics.

It is worthy to note a new assay, T cell interferon gamma release (TIGRAs), which has recently been introduced in the diagnosis of latent tuberculosis infection. It seems to perform better than tuberculin skin test in patients with inflammatory rheumatic diseases.

In this review, we focus on advantages and limits of novel approaches to the detection of autoantibody profiles in autoimmune diseases or pathogen signatures in microbiology.

Introduction

Recent advances in genomics and proteomics applied to multiplexed technologies have opened up a new era in clinical laboratories, allowing the quick and efficient detection of a number of biomarker candidates worthy of being further characterized.

Autoantibody testing is an essential diagnostic step in autoimmunity. In several systemic and organ-specific autoimmune diseases the detection of serum autoantibodies represents one of the classification/diagnostic criteria but it is also useful in monitoring disease activity and organ damage in several conditions. Although there is still no evidence that all the autoantibodies are pathogenic, in recent years several studies have demonstrated that some autoantibodies can be detected in asymptomatic subjects even years before the development of full blown disease clinical manifestations (1). This finding raises the possibility of the predictive value of serum autoantibodies, further adding value to their detection. In addition, some autoantibodies may also be associated with an aggressive variant of the disease itself, thus offering a prognostic predictive value (2). The identification of patients suffering from an aggressive form of the disease and/or an early diagnosis is becoming more and more important since prompt and intense treatment has been reported to stop disease evolution or even to induce remission (3, 4). The diagnostic/prognostic importance of autoantibody testing stresses the need to have reliable and standardized techniques. In fact, inadequate use of laboratory tests is one of the most frequent problems in autoimmunity, leading to incorrect diagnoses, treatment and unnecessary costs.

Multiplexed microarray technology can also be used to quickly identify and genotype pathogens and drug resistance in microbiology, as well as to study the relationship between microorganism or host responses to other external stimuli such as drug, vaccine treatments or chronic diseases like rheumatic ones.

Another relevant aspect in rheumatology is the screening for latent tuberculosis infections before commencing anti-TNF-α therapy for chronic inflammatory diseases. T cell interferon gamma release assays (TIGRAs) offer a realistic alternative to the poor performance of tuberculin skin test for diagnosis of latent tuberculosis infection in this population but published data on TIGRA performance in this setting are scarce.

Recent advances in autoimmune diagnostics

Historically, the detection and analysis of autoantibodies has relied on a number of different technologies such as immunodiffusion, indirect immunofluorescence, particle aggregation, complement fixation, hemagglutination, counterimmunoelectrophoresis, radioimmunoassay, enzyme immunoassays (5). During the 1990s, the application of immunometric methods to increasingly advanced instruments in terms of analytical reliability and automation led to widespread use of antibody testing, with an increase in the volumes of tests performed in each clinical laboratory, and improvements in turn-around time (6).

Over the last few years, the advent of the "proteomic revolution" has opened up new horizons in the diagnosis of autoimmune diseases (5, 7-9). The goals of proteomic analyses include the elucidation of the molecular mechanisms that regulate cellular processes, the characterization of complex protein interacting networks and their perturbations, the discovery of biomarkers useful in the diagnosis and monitoring of disease, and for the identification of therapeutic targets (10-12). Therefore, the investigation of autoimmunity provides an interesting challenge in proteomic research, as autoimmune diseases are common disorders of unresolved aetiology that occur in a wide variety of manifestations (13). The possibility of simultaneously measuring a number of correlated analytes appears to be very interesting for analytical reasons (reduced volumes of biological samples, reagents and low costs), logistical/managerial reasons, and pathophysiological reasons (combination of markers in disease-oriented or organ-oriented profiling) (14). The final aim, for research and clinical purposes is therefore, to study the entire autoimmune process rather than its individual components. However, several problems are related to the possibility of simultaneously measuring a number of correlated analytes, involving both ethical and economical concerns.

Among the numerous systems developed over the past few years for proteomic analyses, mass spectrometry and, in particular, surface-enhanced laser desorption ionization-time of flight (SELDI-TOF), seem to be potentially interesting (14). However, many problems, particularly in the pre-analytical phase (sample collection and handling) have been described stressing the need for a careful evaluation of analytical performances of this technique (15). Several groups have employed the proteomic techniques of two-dimensional gel electrophoresis and mass spectrometry to identify proteins or protein ion mass peaks which are deregulated in tissues and fluids obtained from individuals suffering from multiple sclerosis, arthritis and other autoimmune diseases when compared with reference subjects or controls. These investigations have resulted in the identification of putative biomarker proteins and signature protein expression patterns characteristic for a specific autoimmune disease, and provide insights into putative mechanisms involved in the development and pathogenesis of these disorders.

There has been a recent proliferation of new technologies which are capable of identifying an increasing spectrum of autoantibodies and other biomarkers in autoimmune diseases. Of all the variations currently available, planar and non-planar autoantigen microarrays have attracted particular attention. Planar assays include systems constituted by microspots on slides, polystyrene microplates or nitrocellulose membranes, and linear immunoblot systems (16). Non-planar arrays include suspension arrays which use microparticles recognized by laser nephelometry (17), or laser fluorimetry in flow cytometers (18). These new advances are only the beginning of a rapid succession of newer technologies such as microfluidics, lab-on-a-chip platforms, and nanobarcode particle immunoassays (16). Of these, cell and tissue arrays, line immunoassays and addressable bead assays are already in use and their adoption by clinical diagnostic laboratories is steadily increasing (19).

An additional aspect of the technological evolution in autoantibody testing is the introduction of automation. The advantages offered by these automatized techniques are actually impressive: they can reduce in a significant manner the time and costs of the assays, and they also allow to carry out multiple detection in the case of the so-called multiplex systems (20). Moreover, most – if not all – of the new techniques appear to display a higher sensitivity in comparison to the previous ones. Multiple determination systems, therefore, could play a crucial role in allowing the identification of autoantibody signatures in the individual patient. This, in turn, may lead to a more accurate diagnosis and a targeted therapy.

There is, however, an urgent need to ensure that the rapid adoption of new technologies is attended by an appropriate balance of assay sensitivity and specificity. This is an essential requisite before formally accepting the new assays into the clinical diagnostic laboratory. For example, indirect immunofluorescence (IIF) for anti-nuclear antibody detection (ANA) is a time-consuming technique and may not be suitable because of the growing number of requests and the need to establish an early diagnosis and reduce time of hospitalization. To solve such a problem, automatized screening methods for ANA detection have been set up. These assays comprise the most relevant autoantigens coupled with a solid phase; antibody-antigen binding is then revealed by different techniques (immunoenzyme, chemiluminescence, etc.). The new methods have displayed good sensitivity and specificity for connective tissue diseases when tested on clinically defined groups of patients. However, a recent study showed that three of these new assays did not detect up to 30% of positive IIF samples in a series of 450 sera referred to the laboratory for ANA screening (21). This finding further points out the need for studies aimed to validate the sensitivity/specificity of the new assays versus the standard ones.

Challenges and opportunities for pathogen detection using multiplex microarrays

The main function of diagnostic microbiology laboratories is the detection and identification of microorganisms in a variety of samples of human, animal, food, industrial, or environmental

origin. Additionally, in clinical laboratories, drug susceptibility testing of the isolates to allow correct treatment decision is of major importance. A third and equally important activity is epidemiological typing of the isolated and identified microbial species. This requirement is basic for monitoring the routes of infection as well as for bacterial population studies, both essential in the setup of strategies to prevent or control infections in the community and in healthcare facilities.

During the past decade, the extensive research on microbial genomes and the development of new nucleic acid-based methodologies have resulted in the increasing use of molecular assays in microbiology laboratories (22). In particular, DNA microarrays are a powerful tool for the investigation of various aspects of prokaryotic biology because they allow simultaneous monitoring of the expression of all genes in any bacterium or virus. They offer a more holistic approach to studying cellular physiology and therefore complete the traditional "gene-by-gene" approach (23). Since the term DNA microarray was coined by the laboratory of DeRisi *et al*. (24) and Schena *et al*. (25), this technique has evolved from a very specialized method which was only available to a few people to become a common tool with many different and important applications in microbiology (26).

The essence of microarray technology is the parallel hybridization of a mixture of labelled nucleic acid called *target*, with thousands of individual nucleic acid species called *probes*, which can be identified by their spatial position in a single experiment. DNA microarrays can be used to interrogate a mixture of nucleic acids which is produced from a PCR reaction, or directly in the absence of amplification, when target nucleic acids are abundant (27). In the first case, three approaches are possible. One approach is to amplify one or more universal gene (*i.e*., 16S rRNA, 18S rRNA, 23S rRNA genes) and to screen for pathogen specific polymorphism. Given the simplicity of this strategy, it may be surprising that few examples exist in the literature, but some studies highlighted the fact that universal PCR can be challenging because of frequent and unexpected amplification of contaminating template DNA in negative control reactions. Furthermore, a false negative detection rate was observed and was attributed to secondary structure of the target DNA which prevents hybridization to the microarray probes. A second strategy for coupling PCR and microarray detection is to use multiplex PCR to amplify a number of discreet pathogen-specific genetic markers that are subsequently detected using a DNA microarray. This approach provides a greater degree of discrimination and can also provide useful subtyping information, but there is a practical limit to the number of primer sets that can be included in the PCR reaction. The third strategy for coupling PCR and microarray detectors is to use a random amplification. Wang *et al*. (28) provided the best example of this technique developing a microarray composed of 1,600 unique viral oligonucleotides derived from approximately 140 distinct viral genomes. They demonstrated the potential for combining relatively unbiased nucleic acid amplification with a relatively high-density microarray suitable for detecting and discriminating large classes of viruses as well as detecting previously uncharacterized members of these classes. However, while very powerful for many applications, it is unlikely that this method is suitable for amplifying pathogen DNA from tissue samples because the host DNA will be amplified concurrently with pathogen DNA and this will likely decrease assay sensitivity and may interfere with assay specificity.

Despite some methodological problems, DNA microarray technology is becoming one of the most popular tools in microbiology and many applications could be hypothesized in the future. This technology in fact enables microbiologists to perform global surveys of novel virulence factors, antimicrobial drug resistance genes and potential vaccine targets, by monitoring the transcription profile of pathogens in response to host environments (29). For example, recent studies used the microarray approach to monitor the gene expression of the malaria pathogen *Plasmodium falciparum* in host cells, and identify potential vaccine candidates or drug targets (29). Likewise, DNA microarrays can be used to study pathogen responses to various host systems such as different tissues and cell lines. Recently, Fradin *et al*. (30) demonstrated that *Candida albicans* uses several genes and transcriptional regulations to counteract the attack of neutrophils, enabling the pathogen to escape the innate immune system and cause infection. DNA microarrays can be applied also to study the host response to pathogen infections or to help evaluate the efficacy of anti-infectious drugs. For example, by using liver mRNA expression profiles Hayashida *et al*. (31) were able to successfully predict the efficacy of interferon treatment in chronic hepatitis C patients, before the therapy.

However, the most direct and most important application of this technology in daily practice is the identification of pathogens and genotyping based on the unique sequence signature detectable by the large number of probes on the array. A famous case in recent years was the identification of the new corona virus that caused severe acute respiratory syndrome. However, before DNA microarrays become a standard diagnostic tool in clinical laboratories, researchers, clinicians and microbiologists have to answer some questions such as what are the factors that influence probe design and performance, how a pathogen signature is measured and detected, what the specificity and sensitivity of an optimized detection platform is.

T cell-based diagnosis of tuberculosis infection in patients requiring anti-TNF therapy

Approximately a third of the world's population is infected with *Mycobacterium tuberculosis (M. tb)*. Persons infected with *M. tb* have a 10% life-time risk of developing active tuberculosis (TB), but immunosuppression greatly increases this risk. Tumor necrosis factor-alpha (TNF-α) is a key cytokine in protective host defence against *M. tb* playing an important role in the development and maintenance of the

granuloma which compartmentalises the bacilli during infection. The most serious adverse effect of TNF-α blockade is the development of life-threatening extra-pulmonary and disseminated TB. Therapeutic anti-TNF-α agents including etanercept (a TNF-α receptor antagonist), infliximab and adalimumab (humanised monoclonal antibodies) are increasingly being used as effective treatments in chronic inflammatory diseases such as rheumatoid arthritis, ankylosing spondylitis or Crohn's disease (32, 33). The higher risk of incident TB in patients on these drugs is being increasingly reported (34-38). Screening patients for latent TB infection (LTBI) before commencing anti-TNF-α agents is therefore essential. Treatment of LTBI with isoniazid is potentially hepatotoxic but because of the risk of severe forms of active TB in this group, it is recommended that patients with LTBI complete prophylactic treatment prior to commencing anti-TNF-α agents.

Current screening recommendations are that patients should have a clinical examination, their history of any prior TB treatment or TB exposure checked, a chest radiograph and, if appropriate, a tuberculin skin test (TST) (39, 40). However, accurate diagnosis of LTBI with the TST is very difficult since diagnostic sensitivity is greatly reduced in those with weakened cellular immunity (41) which includes most of the patients who are eligible for anti-TNF-α therapy as they are already taking immunosuppressive therapies such as methotrexate (42). The TST is a measure of delayed-type hypersensitivity reaction to intradermal inoculation of purified protein derivative (PPD), a crude mixture of more than 200 *M. tb* proteins. Because antigens within PPD are also found in other mycobacteria, the TST also suffers from poor specificity in bacille Calmette-Guérin (BCG)-vaccinated persons and in those infected with environmental mycobacteria.

The advent of T cell interferon gamma release assays (TIGRAs) offers a realistic alternative to the TST for diagnosis of *M. tb* infection. There are currently two forms of TIGRAs which measure *M. tb*-specific interferon gamma (IFN-γ) response *in vitro*: IFN-γ secretion measured in whole blood by the enzyme-linked immunosorbent (ELISA) assay, and the enumeration of T cells secreting IFN-γ measured by the enzyme-linked immunospot (ELISpot) assay. These are both commercially available as QuantiFERON®-TB Gold (Cellestis, Carnegie, Australia) and T-SPOT®-TB (Oxford Immunotec, Oxford, United Kingdom) based on the Lalvani ELISpot assay, respectively. There is a large body of published literature in non-immunosuppressed populations which confirms that, compared with the TST, TIGRAs are more specific for diagnosis of *M. tb* infection (43). From studies in active TB and from studies correlating ELISpot results with TST and TB exposure in recently exposed contacts, ELISpot is likely to be more sensitive than the TST (39). Whilst the literature in immunocompromised populations is limited, TIGRAs in HIV co-infected persons do not seem affected by HIV infection status (43).

Published data on TIGRA performance in the diagnosis of LTBI in chronic inflammatory diseases is scarce. A recently reported prospective study of 142 patients treated for inflammatory rheumatic conditions found that QuantiFERON®-TB Gold in-tube performed better in the diagnosis of LTBI than TST (44). The IFN-γ ELISA was more closely associated with the presence of risk factors for LTBI than the TST (p = 0.04). However, whilst neither corticosteroids or disease modifying antirheumatic drugs (DMARDS) significantly affected IFN-γ response, the odds for a positive IFN-γ result was decreased in patients treated with TNF-α inhibitors (adjusted odds ratio = 0.19 (95% CI 0.05 to 0.76) p = 0.019). A second study in 68 patients with chronic inflammatory diseases reported that agreement between TST and an IFN-γ assay was poor (κ = 0.18) (45).

The performance of each TIGRA is assessed through the use of an internal control which measures the IFN-γ response against phytohaemagglutinin (PHA). A negative response against this mitogen in the context of negative responses against the *M.tb*-specific antigens renders the TIGRA indeterminate. This positive control becomes especially important in testing persons with weakened cellular immunity. To date, the indeterminate rate in patients on immunosuppressive therapy has been below 10% (44-46). However, we cannot yet draw conclusions on the indeterminate rate of TIGRAs in this population based on less than 130 people.

The evidence-base on the performance of TIGRAs in diagnosing LTBI in persons with chronic inflammatory diseases, especially in those already on immunosuppressive therapies, must be expanded to assess whether these new tests can replace the unreliable TST. Prospective trials are urgently needed to determine the prognostic value of positive TIGRA results in immunosuppressed populations with LTBI, and especially in patients who are candidates for anti-TNF-α agents.

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