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Microarray profiling of antiviral antibodies for the development of diagnostics, vaccines, and therapeutics

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Abstract

Multiplex analysis of antiviral antibody (Ab) responses provides a potentially powerful strategy for viral diagnosis, prognostication, and development of vaccines and prophylactic Abs. In the coming years, advancements in proteomic technologies will provide even more robust methods to characterize antiviral Ab responses. Biomedical researchers will be faced with the exciting challenge of identifying antiviral Ab specificities that correlate with improved outcomes and efficacious interventions, and translating the findings into more effective diagnostics, prophylactics, and therapeutics.

Keywords: Microarray profiling; Antiviral antibodies; SPOT-synthesis technique

Introduction

Viral infections cause significant morbidity and mortality. Better understanding of antiviral immune responses would facilitate development of more effective diagnostics and therapeutics. Antibody (Ab) responses are an important component of antiviral host defenses. Proteomic technologies represent a powerful strategy to profile antiviral Ab responses and have the potential to guide development of diagnostic and prognostic tests, preventive and therapeutic vaccines, and prophylactic Abs.

Roles of antiviral antibodies in control of infection

Antiviral B cell responses contribute to antiviral defenses through a variety of mechanisms. Many functions are associated with certain Ab isotypes. Some antiviral Abs provide protection by neutralizing virions; that is, by blocking the binding of viral particles to cell surface receptors or by preventing their fusion to host cells (reviewed in Ref. [1]). Other Abs are effective by agglutinating virions. Abs also direct the lysis of virions or infected cells through the complement cascade. By binding to Fc receptors on the surface of dendritic cells and macrophages, Abs are involved in opsonization of viruses and infected cells, thereby enhancing the presentation of viral antigens (Ags) to T cells. Antibody-mediated cytotoxicity can occur when Abs bind simultaneously to viral Ags on the surface of infected cells and to the Fc receptors on macrophages, neutrophils, eosinophils, or natural killer cells, thereby directing these cells to kill infected targets. While many Abs made in response to viral Ags contribute to antiviral defenses, certain antiviral Abs can be detrimental to host defenses [2,3].

Since Abs made in response to viral infections can vary in titer and isotype for any given epitope, it has become necessary to develop technologies to quantify and characterize Abs directed against individual epitopes. Since different Abs binding to the same epitope may have different structural requirements for binding, methods are being developed to define these properties.

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Solid phase arrays synthesized in situ

The PEPSCAN technique developed by Geysen [4,5] is arguably the most successful approach to date for epitope mapping. Distinct peptides are synthesized by traditional Boc or Fmoc chemistry on each of 96 plastic rods arranged to fit the wells of a microtiter dish. To "scan" the sequence of a protein, a series of overlapping 6–47 residue peptides can be synthesized. Substitutions can be introduced at one or more residues, and more complex combinatorial libraries with mixed substitutions can be generated. For each experiment, sets of 96 pins are blocked against nonspecific binding and then incubated in a tray of diluted serum. As in an ELISA, binding of Abs to pins is detected using peroxidase-conjugated secondary Abs and a colorimetric substrate. This technology was originally applied to define the epitopes of the coat protein from foot-and-mouth disease virus [4]. Since then, almost 300 papers describing mapping of viral epitopes have utilized this technique.

Despite its success, this method has significant drawbacks. Tests must be performed sequentially. Each set of pins can be used only once per day and then regenerated for the next sample. Incomplete removal of Abs and peptide degradation lead to incremental loss of reactivity with each use and generally limit the use of each set of pins to 50 tests [5,6].

With an average purity for each peptide of >70% [6], the possibility exists for immune reactivity to contaminating peptides, as has been observed in other assays [7]. Further purification of the peptides would require their release from the pins.

SPOT synthesis, developed by Frank [8,9], is another popular approach for generation of peptide arrays. It allows similar manual or semiautomated stepwise synthesis on derivatized cellulose membranes. Nontranslated amino acids (e.g., citrulline, D-alanine, or phosphoserine) have been incorporated into such peptides. Larger sheets allow over 5000 spots [10] with peptides as long as 38 residues [11], but miniaturization is limited because of spreading of reagents in the porous membrane [8]. After exposure to diluted Abs and washes, detection is performed using chemiluminescence, radioactivity, or fluorescence. If the bound Abs can be completely removed, these arrays can be reused [8].

The SPOT-synthesis technique has expanded beyond simply the mapping of linear epitopes. The dissociation constants of Abs have been measured utilizing anthranilic acid-labeled peptides and fluorescence energy transfer [12]. Low affinity interactions can be detected with dissociation constants around 10^{-4} [10]. This property has allowed detection of binding to discontinuous peptides [13,14].

Fodor et al. [15] described miniaturized peptide arrays generated using light-directed chemical synthesis on nonporous surfaces, such as glass slides. The recent addition of spatially addressable lighting systems to this process [16,17] provides an alternative to the use of photolithographic masks. The resulting microarrays have 1300–2700 features/cm^2. Such arrays have the potential to be manufactured in large quantities. Arrays generated by in situ chemical synthesis still have several limitations. The length of the peptides synthesized is limited, and their purity is difficult to control.

Recently, it has become possible to produce arrays of polypeptides synthesized in situ using biomolecular methods including (i) PCR and a cell-free transcription and translation system [18], (ii) arrays of living yeast transformants [19], and (iii) printing of cDNAs in expression vectors followed by incubation with adherent cells [20]. These methods offer the power to screen libraries of Ags. However, control over the quantities of Ag generated in each feature may be limited.

Solid phase arrays with independently synthesized peptides and proteins

With spotted arrays, the variety of features on one array can be expanded beyond peptides to include whole proteins, DNA, RNA, complexes of molecules, column fractions, and crude extracts. Spotting techniques include use of ink jets and printing pins (reviewed in Refs. [21–23]). The dispensed Ags may be attached to derivatized glass, nitrocellulose, or other supports via adsorption, covalent bonds, electrostatic charge, or affinity reagents. With a current capacity of 14,000 features, chips can be made that encompass the entire viral "proteome," including overlapping peptides of even the largest viruses that encode 300 polypeptides.

Initial development of Ag microarrays on derivatized glass slides was described using nonviral Ags [24,25]. Mezzasoma et al. [26] recently demonstrated the use of similar microarrays for quantitation of Abs against viral proteins. Incorporating internal calibration into their studies of Abs against cytomegalovirus and herpes simplex virus types 1 and 2, they reported coefficients of variation of less than 10% within and between slides of identical batches, with slightly higher variation between slides of different batches. They also observed linear dose responses and strong correlation ($r^2 > 0.97$) with ELISA data.

We obtained similar results with monoclonal antibodies (mAbs) and antisera using our Ag microarrays containing HIV and SIV proteins and peptides [27]. These Ag microarrays detect nanogram per milliliter quantities of Abs against simian–human immunodeficiency virus (SHIV) peptides and proteins. Reactivity was observed in diluted sera from rhesus monkeys after they were immunized with a recombinant vaccinia virus that expresses SHIV Gag, Pol, and Env (Fig. 1B) and again after challenge with a pathogenic strain of SHIV (Fig. 1C). The Abs reacted with whole proteins as well as peptides from immunodominant and novel epitopes. For one set of monkeys that received a vaccine with gag-pol only, we employed Statistical Analysis of Microarrays [28] to find patterns of reactivity 22 weeks after challenge that distin-

guished surviving animals from those that succumbed at week 55.

The technology of printed microarrays is in its infancy. Several hurdles remain to be overcome. Denaturation of certain Ags from drying on or binding to solid supports might be avoided through improvements in solvents, surface chemistries, and the use of tag-based technologies. Internal standards would further reduce variability. Sensitivity might be improved with new detection methods or rolling circle amplification [29].

Although Ag microarrays are currently configured to assay levels of Ab to specific Ags, they have potential use for additional measurements. To link Ab reactivity to effector functions, we are performing multiplex isotype analysis using isotype-specific secondary reagents conjugated to spectrally resolvable fluorophores [25]. It may also be possible to measure the association or dissociation constants for Abs bound to specific features. Epitopes exposed on native proteins or virions might be revealed in competition experiments in which preincubation of Ab with native Ag would result in loss of reactivity to specific features [30].

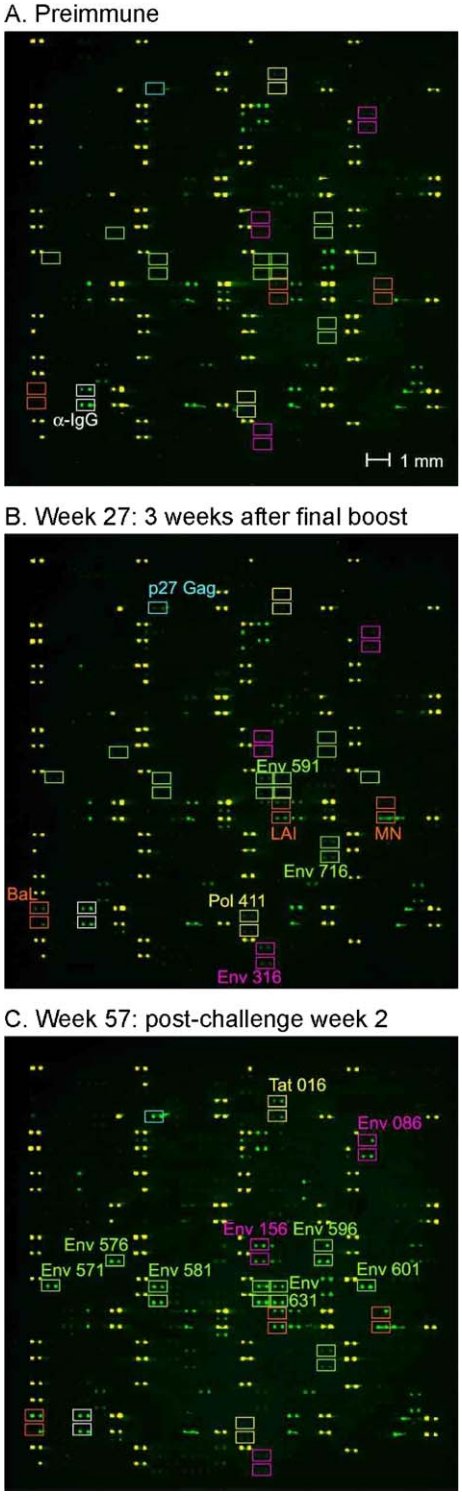

Fig. 1. Representative microarrays spotted with SHIV antigens and probed with sera from a rhesus macaque in a HIV vaccine trial. Sera were obtained before immunization (A); 3 weeks after the third and final immunization with a recombinant vaccinia Ankara virus expressing Gag, Pol, and Env (B); and 2 weeks after challenge with a pathogenic strain of simian–human immunodeficiency virus, SHIV-89.6P (C). Bound Abs were revealed with Cy3-conjugated Ab specific for macaque IgG (green features). Directly conjugated features (yellow) serve as reference markers to orient arrays. Selected features are labeled at the first time point when the signal is significantly above preimmune levels. Anti-monkey IgG features (α-IgG, inside white boxes) were reactive in all slides. Orange boxes and labels designate gp120 Env proteins from various clade B strains: BaL, LAI/IIIB, and MN. Purple and green boxes and labels demarcate peptides derived from gp120 Env and gp41 Env, respectively. SIV capsid protein p27 Gag is indicated in blue. Peptides from Pol and Tat are marked with yellow boxes.

Emerging technologies

A variety of techniques centered on individually addressable markers have been developed. Such technologies enable coupling of Ags to addressable tags while avoiding drying and binding to solid supports, which can denature and/or interfere with certain epitopes. These technologies include LabMAP™ bead arrays from Luminex (Austin, TX, USA) [31,32], Nanobarcode™ microrods from SurroMed (Mountain View, CA, USA) [33], and Qdot™ conjugated nanoparticles from Quantum Dot Corp. (Hayward, CA, USA) [34]. One disadvantage of these technologies is that each Ag must be coupled to an addressable tag in a separate reaction. Carbon nanotubes, which have recently been demonstrated in sensors for Ab–Ag interactions [35], have promise for use in multiplex devices that avoid those issues [36,37]. Ultimately, fluid-phase assays using addressable tags will likely supercede solid surface arrays.

General applications

The range of Abs produced in antiviral immune responses varies depending on the individual infected, the route of infection, and the microbe itself. A better understanding of the specificities and isotypes of Abs made in successful and unsuccessful immune responses against a viral pathogen

would likely provide useful information for the development of effective diagnostics, vaccines, and therapeutics.

The response to hepatitis B virus serves as an example of how Ab specificity can reflect disease status [38]. Abs against the viral envelope are present in patients with acute disease who clear the virus and recover, while patients with chronic infections lack such Abs. Anti-envelope Abs block infection and/or aid in clearance. Unlike patients with chronic hepatitis B virus infection, patients with acute disease also mount strong virus-specific T cell responses that include cytotoxic cells. Both groups of patients exhibit Abs against viral nucleocapsid Ags.

Diagnostics for detection of viral infection

There are four general methods for detecting viral infection: viral culture, serology, tests for viral Ag, and assays for viral nucleic acid (reviewed in Ref. [39]). Culture is frequently a slow process that requires conditions specific to each virus. Ag detection is based on analysis of blood or other biologic specimens with antiviral Abs. For detection of viral nucleic acids, polymerase chain reaction (PCR)-based assays require small samples and can rapidly provide sensitive and specific results [40]. Multiplex PCR and gene chip methods have the added advantage of assaying for a broad spectrum of viruses simultaneously [41,42]. For many viruses, nucleic acids are often detectable before antiviral Abs, making PCR an attractive method for diagnostics. However, for some infections that are transient and generate low levels of virus, including arboviruses such as West Nile virus, assays for antiviral Abs have still proven to be more sensitive [39,43].

Diagnostics for assessment of immune status

Assays for antiviral Abs will likely prove useful for assessment of markers for antiviral immune status. Ab isotype is often informative (reviewed in Ref. [39]). High levels of virus-specific IgM are usually indicative of acute infection, whereas virus-specific IgG may be present during or long after infection. Such is the case for Epstein–Barr virus (EBV), where infectious mononucleosis, but not convalescent mononucleosis, is marked by IgM against viral capsid Ag. However, IgG against the same Ag is often detected during and after infection. Abs specific for Epstein–Barr nuclear antigen appear during the convalescence stage and remain for life [44]. Therefore, as for hepatitis B virus, EBV Ab specificity can reflect immune status long after viral nucleic acid is no longer detectable.

In chronic infections in which viral Ags persist, markers for prognosis may be discovered by analysis of correlations between Ab reactivity and long-term health. For example, IgM against core proteins of hepatitis B and C viruses (HBV and HCV) correlate with persistent infection and increased likelihood for liver damage [45–49]. The Ab assays used in most of these studies have been limited to whole protein Ags. In developing assays for diagnostic Ab, Ag microarrays offer the ability to monitor Ab responses in greater detail: including detection of reactivities against large panels of viral epitopes and multiplex isotype analysis. Long-term studies will be necessary to obtain correlations with disease parameters including viral genotype, viral titers, and clinical course.

Development of vaccines

Although vaccines have been developed against several viruses, tremendous need exists for effective vaccines against many viruses including HIV and HCV. The purpose of vaccines is to induce immunological memory to accelerate both humoral and cell-mediated responses upon challenge by a pathogen. The optimal Ab responses elicited by vaccines are usually against neutralizing epitopes. Ag microarrays can be employed throughout the vaccine development process. Applications include mapping epitopes associated with effective immune responses and testing the ability of experimental vaccines to generate specific Ab responses against those epitopes both post-immunization and post-challenge.

Antigenic diversity exists in many viruses, such as HIV, and presents additional challenges in the development of vaccines. An ideal vaccine would elicit a response capable of neutralizing a broad spectrum of variants. One approach is to develop vaccines that target invariant epitopes. Another approach is to generate a cocktail vaccine that includes epitopes from a variety of strains. To test a candidate vaccine for the breadth of its specificity, Ags from numerous strains can be included on a microarray.

Studies of immune responses that are associated with different clinical outcomes can provide direction for the development of vaccines. Patients who effectively control viral infections may have reactivity against epitopes that are critical targets of neutralizing Abs and/or T-cell responses. This is the approach of several studies that compared the epitope specificity and neutralizing capability of anti-HIV Abs from HIV-positive patients who rapidly progress to AIDS with infected long-term survivors [50–57].

Microarray results can be combined with Ab neutralization titers, quantitation of viral load, and other clinical parameters to identify correlations with Ab profiles. In our SHIV study (Fig. 1), for monkeys vaccine composed only of gag and pol, we identified antiviral Ab profiles 22 weeks after challenge that distinguished surviving animals from those that succumbed at week 55 [27]. We found that survivors had higher concentrations of Abs directed against a broad spectrum of viral epitopes. This represents an example of how profiles encompassing Ab specificities to several epitopes rather than a single epitope can provide prognostic value.

Prophylactic and therapeutic antibodies

Passive transfer of normal or hyperimmune Ig is another approach for the prevention and treatment of viral infections (reviewed in Refs. [58–60]). For immunocompromised patients, polyclonal intravenous Ig derived from the sera of human donors, with and without antiviral specificity, has been used to prevent infections by HBV, measles, rabies, cytomegalovirus, respiratory syncytial virus (RSV), and varicella zoster virus [58–60]. With lower risks and potential for greater efficacy, human or "humanized" mAbs have become a more attractive method in individuals with high risk for exposure to life-threatening viral pathogens. Recently, the U.S. Food and Drug Administration approved the mAb palivizumab (Synagis™) for the prevention of RSV infections in high-risk infants [61]. This humanized IgG1 binds to the F-protein from both the type A and B isolates of RSV [62]. Palivizumab has also shown promise as a treatment during RSV infection [63].

Development of such Abs requires methods for screening candidates. Ag microarrays and other proteomic technologies can assist in this process by helping define the specificities of protective Abs. Ag microarrays may also be used to identify small molecules that bind viral Ags [64].

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