Immunoassay of infectious agents

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Immunoassays have evolved for a broad range of applications since the pioneering work of Yalow and Berson who developed the first competitive radioimmunoassay (RIA) for human insulin in 1959. Immunoassay detection of specific antigens and host-produced antibodies directed against such antigens constitutes one of the most widely used and successful methods for diagnosing infectious diseases (IDs). The number and variety of new assay systems that are continually being developed reflect the increasing demand for immunoassays possessing greater sensitivity, speed, and ease of use. This trend has been driven, in part, by the need for improved immunodiagnostic systems to perform rapid testing and counter emerging IDs and biothreat (BT) agents. Another factor driving this trend is the need to integrate immunoassays with more sensitive nucleic acid-based methods for a comprehensive approach. Here we examine the development of immunoassays, some of the key formats used for the detection and identification of BT/ID agents, and the application of these technologies under different scenarios.

OVERVIEW OF IMMUNOASSAY DEVELOPMENT

Immunoassays rely upon four components regardless of the application and underlying technology: (*i*) the antigen to be detected; (*ii*) the antibody or antiserum used for detection; (*iii*) the method to separate bound antigen and antibody complexes from unbound reactants (if a heterogeneous assay is used); and (*iv*) the detection method. At the most fundamental level, the efficacy of any given immunoassay is dependent on two major factors: the efficiency of antigen-antibody complex formation and the ability to detect these complexes.

A principle requirement for immunoassays is the availability of organic molecules that can bind to specific domains present on the target. Traditionally, antibodies have filled this role because they are relatively simple to produce and can be selected to possess the desired affinity characteristics. While polyclonal and monoclonal antibodies remain the most commonly used reagents for traditional immunoassays, other organic molecules show significant promise (1–3). These antibody "mimics" include random peptides and oligonucleotides (aptamers) whose tertiary structure produce binding sites that are capable of forming noncovalent linkages to proteins and other target molecules (1,3–5).

ANTIBODIES AND ALTERNATIVES

Polyclonal Antibodies

Producing high-quality antibodies by hyperimmunizing host animals has long been a standard method (6). This simple process can create antibodies of very high avidity directed against multiple epitopes present on an antigen (polyclonal response) that can subsequently be purified from serum or ascites fluid. Whole organisms, crude fractions, or individual proteins may be used for immunization, depending on the desired specificity of the antibody and the intended application. Adjuvants may enhance the response, depending, in part, on the immunogenicity of the selected antigen.

However, polyclonal antibodies have key disadvantages. First, laboratory animals are required and can vary considerably in their ability to respond to different antigens, resulting in significant variation among lots of antibodies. Second, the antibody response to a given antigen tends to be broad, covering both specific and cross-reactive epitopes. As a result, immunoassays based solely on polyclonal antibodies can be highly sensitive but may not be very specific.

Specificity of assays based on polyclonal antibodies can

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usually be increased by affinity purification (6). However, purification conditions must be balanced between selection of the most useful antibodies and loss of antibody activity due to the severity of the elution conditions and decreasing antibody yields resulting from an increasing number of manipulations.

Monoclonal Antibodies

With the advent of technologies to produce monoclonal antibodies in the mid 1970s (7), it became possible to develop diagnostic assays that were more specific and reproducible than those based on polyclonal reagents. Assay reproducibility was improved by the ability to generate large quantities of antibodies with little variation between lots. Assay specificity was improved by simplifying the selection of antibodies directed against chosen molecular targets.

A common disadvantage with the use of monoclonal antibodies or any mono-specific reagent used in diagnostics is that antigenic drift or shift may lead to changes in proteins such that the antibodies are no longer capable of binding to their respective biological agents (8). Similarly, antigens could be purposefully modified by genetic engineering or through active selection processes resulting in agents capable of evading detection systems (9–11). Another disadvantage with monoclonal antibodies is that these antibodies rarely possess affinities that match the avidities of good polyclonal antibodies. Therefore, diagnostic assays made with only one monoclonal antibody are often less sensitive than similar assays with polyclonal antibodies. To address such problems, cocktails of monoclonal antibodies directed against multiple epitopes are often necessary.

Recombinant Antibodies: Phage Display

In some instances, development of polyclonal and monoclonal antibodies by traditional techniques may be unsuccessful in producing reagents with the desired biological characteristics. Phage display is a technology with the potential to overcome this problem by exploiting the nearly limitless binding capability of the mammalian immune system (12). It is a recombinant DNA technique that couples the speed of bacterial growth with the ability to generate and display fusion proteins on filamentous phages. Genes encoding a phage protein are fused to the antigen-binding segment of antibodies. The antibody segment may encode either a fragment of an antibody molecule, consisting of the light chain disulfide bonded to the amino terminus of the heavy chain (Fab) or a synthetic peptide consisting of the amino terminus of the Fab light (V_L) chain joined to the carboxy terminus of the Fab heavy (V_H) chain single-chain antibody variable region fragment (scFv) (12,13). Phage display of Fab fragments or scFv provides a rapid and specific tool for identifying antibody sequences that can bind biological agents. An added advantage of recombinant fragments is that they can be produced in large quantities with little lot-to-lot variation through standard fermentation processes. Another key advantage of using phage display technology is that antibody variable domains can be more easily engineered with desired affinity characteristics (14). However, recombinant antibodies have three significant limitations: (*i*) they are less stable in vivo than natural antibodies; (*ii*) they are unable to cross-link antigens; and (*iii*) they can lack critical domains necessary for certain biological functions (15).

Random Peptides

Generation of short peptides with defined biological binding activity can be used to select reagents useful for diagnostic purposes. One system for producing peptides was originally designed for probing protein-protein interactions by displaying random peptide libraries on the surface of *Escherichia coli* flagella (4). A fusion protein made with the entire coding sequence of *E. coli* thioredoxin gene (*trxA*) and the dispensable region of the flagellin gene (*fliC*), the key structural protein of flagella, is a key component of this system (4). The fusion protein is efficiently transported and assembled into partially functional flagella on the bacterial cell surface. A library containing over 10^8 random dodecapeptides can thus be displayed on the exterior of *E. coli*. Because these peptides are inserted into the thioredoxin active-site loop, they are conformationally constrained. Bacteria displaying peptides with affinity to immobilized antibodies or antigens are then isolated by a panning technique. This technique has been used to map linear antibody epitopes and has some potential for rapidly selecting peptides that could serve as useful ligands in antigen-detection assays (4). One disadvantage of this in vivo procedure is that it often produces proteins that may be toxic to cells. A modification of this approach has been designed to produce large libraries of peptides fused to their encoding DNA (16). Peptides made using this procedure do not require living cells, thereby eliminating the effect toxic proteins can have on host cells.

Aptamers

Peptide ligands are not the only molecules that may serve as antibody mimics in immunoassays. Aptamers are single-stranded nucleic acids developed in vitro to bind proteins or other nucleic acids (3). They are most commonly produced by a technique called systematic evolution of ligands by exponential enrichment (SELEX) (17). Briefly, a large population of random single-stranded nucleic acid molecules is screened against an antigen of interest. Antigen-nucleic acid complexes are separated from free nucleic acid, and the selected oligonucleotides are amplified. Aptamers with the desired biological characteristics are acquired after several such rounds of sequential selection and amplification. These enriched oligonucleotides may have dissociation constants (K_d) of less than 1 nM—binding affinities equivalent to or better than many antibodies (18). Large-scale manufacturing of aptamers is also possible at costs that are similar to those for antibody production, making them viable alternatives as reagents.

A key concern with aptamers is stability. Free nucleic acids, particularly RNA, are subject to digestion by nucleases found in essentially all common biological and environmental samples. Aptamers can be stabilized by modifying sugars on the nucleoside triphosphates, use of appropriate production phosphoramidites, and by methylation of the 2′ OH groups on purine nucleosides (19–21).

ISSUES IN ASSAY DEVELOPMENT

Specificity

Regardless of the format, the specificity of an immunoassay is dependent on the reaction between antibody and antigen. Specificity of an antibody or antiserum is a function of the affinity and cross-reactivity of the antibody or antiserum and is a principal determinant for the quality of the assay. Factors such as the matrix in which the antigen is tested may also influence specificity and sensitivity by interfering with the antigen-antibody reaction. Other extrinsic factors may lead to false negative or false positive results depending on the immunoassay format. An example of this sort of problem is the false positive result that may be obtained with some fluorescent methods when testing biological samples that express high levels of endogenous fluorescent molecules. In addition to optimizing environmental parameters, it is also critical to optimize antibody quality to ensure maximum specificity and sensitivity. This is generally accomplished by testing potential detector antibodies against a panel of related "near neighbor" antigens or species to determine specificity and cross-reactivity of candidate detector antibodies. The results of these antibody optimization studies can be used to select antibodies with maximum specificity for the target antigen.

Cross-Reactivity

The cross-reactivity of an antibody plays a central role in the overall quality of an immunoassay. The obvious preference is to construct an assay around high-affinity antibodies that exhibit minimal cross-reactivity. In practice, it may be difficult if not impossible to achieve this specification, despite the advances in antibody production techniques. At the molecular level, there are two models for cross-reactivity to be considered when evaluating antibody specificity and immunoassay results. The first is cross-reactivity resulting from an antibody that binds to structurally distinct but similar epitopes present on different antigens. The second is cross-reactivity resulting from an antibody that binds to structurally identical epitopes on different antigens. While usually undesirable, a cross-reactive antibody may still provide useful diagnostic information.

Sensitivity

Sensitivity, along with specificity, is also a defining characteristic for an immunoassay and is a direct function of the antibody used. Greater sensitivity is generally achieved with higher affinity antibodies. Testing of detector antibodies against a panel of specific antigens that has been carefully titrated is commonly performed to determine the lower limit of sensitivity for each potential detector antibody. However, since sensitivity is dependent on the ability to discriminate signal from background measurement at low analyte concentration, sensitivity may also depend on the assay format and instrumentation used for detection. An assay using a format and/or instrumentation that has a high background signal in the absence of antigen is inherently less sensitive compared to an assay using a format and/or instrumentation with a lower background signal. Sensitivity is commonly determined using a statistically significant cut-off level above background (22–24). Thus, both sensitivity and accuracy may be affected by the precision of signal and background measurements, and it is possible to increase the accuracy of an assay by increasing the statistically significant cut-off level. However, this effectively decreases the sensitivity of the assay.

Signal Detection

Assay sensitivity is also dependent on the signal used for measurement. Depending on the nature of the signal, the reactants may be detected visually, electronically, chemically, or physically, and a wide range of instruments can detect the presence of these labels with a high degree of sensitivity. Enzymes are effective labels because they catalyze chemical reactions, which can produce a signal. Because a single enzyme molecule can catalyze many chemical reactions without being consumed in the reaction, these labels are effective at amplifying assay signals (25). Most enzyme-substrate reactions used for immunoassays utilize chromogenic, chemiluminescent, or fluorescent substrates that produce a signal detectable with the naked eye, a spectrophotometer, luminometer, or fluorometer (26–28). A disadvantage of enzyme-based assays is that both the enzymes and substrates may be unstable and require specialized storage to maintain activity. Fluorescent dyes and other organic and inorganic molecules capable of generating luminescent signals are also commonly used labels in immunoassays (29). Assays using these molecules are often more sensitive than enzyme-based immunoassays but require specialized instrumentation and often suffer from high background contamination due to the intrinsic fluorescent and luminescent qualities of some proteins and light-scattering effects. Signals for assays with these types of labels are amplified by integrating light signals over time and cyclic generation of photons. Other commonly used labels include gold, latex, and magnetic or paramagnetic particles. All are quite stable under a variety of environmental conditions and can be detected by visual inspection or instruments. However, these labels are essentially inert and therefore do not produce an amplified signal. Signal amplification is useful and desirable because it results in increased assay sensitivity. Increased signal strength can be attained by using amplifiable labels as described above or by using molecules capable of forming multiple bonds. These molecules can produce more complex lattices of signal-generating compounds or molecules. Biotin and avidin are examples of molecules exhibiting these characteristics. They have very high affinities for each other, developing almost irreversible bonds ($K_d = 10^{-15}$ M). In addition, avidin can bind as many as four biotin molecules, increasing the size of the complex. If biotin is bound to a signal-generating molecule or compound, the strength of the signal increases proportionally.

Optimization

Temperature, time, reagent concentration, kinetics, and

reagent quality are five key parameters affecting the performance of immunoassays. Successful assay development requires that each parameter be optimized. The most common optimization method is an empiric approach where one or two parameters are varied between experiments using a matrix format. Depending on the number of steps involved with an assay, this process can be time-consuming and complicated. However, for a given assay platform, many of the parameters such as time and temperature need only be optimized for a single assay. Subsequent assays will generally function well within the specification identified for the first assay.

For many immunodiagnostic assays, particularly heterogeneous assays, diffusion of molecules within an assay matrix is the rate-limiting step. Increasing the assay temperature generally increases the rate of diffusion. However, antibody affinity decreases as temperature increases, so assay speed and sensitivity must be balanced. Again, because diffusion is a rate-limiting step, increasing the incubation time for critical assay steps often results in greater assay sensitivity. The duration of key assay steps is more critical for nonequilibrium techniques, in which background signals may increase above acceptable levels if such steps are allowed to continue too long. With some assays, this effect can be minimized by adding compounds at the end of the reaction period that inhibit enzymatic reactions or inactivate the reactants. Another concern is reagent concentration, especially in development of homogeneous assays, where incorrect concentrations of antibody can lead to pro-zone effects, where signals are artificially low due to the stoichiometry of the reactions. In general, reagent concentration drives the sensitivity of the assay, but it can also affect the rates of false positive and false negative results and requires careful optimization. The efficiency of each step of the assay cascade is governed in part by the assay environment. This environment includes pH, ionic strength, and the presence or absence of additives, such as carrier proteins, detergents, enzyme inhibitors, and preservatives in the assay buffers. Each of these parameters must be examined and optimized for the desired application and requirements of the assay.

RAPID IMMUNOASSAY FORMATS

Overview

Today, numerous immunoassay formats are available to measure almost any substance ranging from small molecules to complex cellular antigens. A partial list of standard methods includes the first such test, the radioimmunoassay (RIA), the enzyme immunoassay (EIA), which has largely replaced RIA, and derivatives of "sandwich" assays based on enzyme-linked immunosorbent assay (ELISA) methods, which were first developed in the 1970s using chromogenic substrates and which are now performed with chemiluminescent, electrochemiluminescent, and fluorescent substrates and labels, along with immunohistochemistry and immunoblotting techniques, direct and indirect immunofluorescence (commonly used in flow cytometry), and lateral flow diffusion, which is generally used for portable single determination applications (30–35). Of these, we will examine the major immunoassay formats used for rapid and specific detection of ID agents: ELISA and related formats, lateral flow techniques, fluorescent polarization, and flow cytometry.

Enzyme-Linked Immunosorbent Assay

Since the 1970s, the ELISA has been the standard against which immunoassay performance is measured (31). Perhaps the most widely used and best understood immunoassay, ELISAs have been developed in many formats and can be designed to detect either host antibodies produced in response to infection or antigens from the infecting agent.

ELISAs that detect biological agents are heterogeneous assays in which an agent or an agent-specific antigen is captured onto a plastic multiwell plate or tube by a "capture" antibody previously bound to the solid matrix. Bound antigen is then detected using a secondary "detector" antibody. The detector antibody can be directly labeled with a signal-generating molecule, or it can be detected with another antibody that is labeled with an enzyme. These enzymes catalyze a chemical reaction with a substrate that results in a colorimetric change. The intensity of this color can be measured by a spectrophotometer, which determines the optical density of the reaction, using a specific wavelength of light. Many ELISA formats require antibodies from two different species of animals so there is no direct interaction between sandwich layers. If the detector antibody were directly labeled with enzyme, antibodies from the same species could be used as both capture and detector reagents.

Many different enzyme-substrate combinations are effective at generating signals that can be detected using readily available readers or with the naked eye if readers are not available. Horseradish peroxidase (HRP) in the presence of 2,2′-azinobis(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS) absorbs light at 410 nm, producing a deep green color. This produces a particularly sensitive assay that can be interpreted without a spectrophotometer if necessary. Enzyme substrates, which produce a chemiluminescent signal, can be substituted for chromogenic substrates for chemiluminescent immunoassay (CLIA) detection, which may increase sensitivity with luminometer instrumentation (27,36). Similarly, detector antibodies can be directly labeled with chemiluminescent acridinium ester labels (37).

Major advantages of the ELISA are: (*i*) that they are commonly used and understood by clinical laboratories and physicians; (*ii*) they can be adapted for high-throughput laboratory use; (*iii*) the antibodies used in the assays do not require any special treatment or purification; and (*iv*) they are relatively inexpensive. Equipment requirements can range from simple manually operated devices to automated high-throughput systems. The major disadvantages of ELISA are that it is somewhat temperature-dependent, may be difficult to make quantitative, and is relatively time- and labor-intensive.

Electrochemiluminescence

Electrochemiluminescence (ECL) is a promising new technology for antigen and antibody detection, similar to ELISA except that the detector antibody is directly labeled with a

chemiluminescent label (33,34). One such system, ORIGEN®, (IGEN International, Gaithersburg, MD, USA), makes use of antigen-capture assays and a chemiluminescent label (ruthenium; Ru) and includes magnetic beads to concentrate target agents. These beads are coated with capture antibody, and in the presence of a biological agent, immune complexes are formed between the agent and the labeled detector antibody. Because of its small size, Ru can be easily conjugated to any protein ligand using standard chemistries without affecting immunoreactivity or solubility of the protein. In a typical agent-detection assay, a sample is added to a mixture of capture antibody-coated paramagnetic beads and a Ru-conjugated detector antibody. After a short incubation period, the analyzer draws the sample into the flow cell, captures and washes the magnetic beads, and measures the electrochemiluminescent signal.

The ECL analyzer consists of an electrochemical flow cell with a photon detector placed above the electrode. A magnet positioned below the electrode captures the magnetic bead-Ru-tagged immune complex. When an electric field is applied to the electrode, a rapid electron transfer reaction between the substrate (tripropylamine) and the Ru atom occurs, resulting in the production of photons. Excitation with as little as 1.5 V results in light emission, which in turn is sensed by the photon detector. The measurement of a single sample can be repeated numerous times in the analyzer, because the electron-transfer photon-release reaction regenerates the Ru. This results in an amplified signal and requires only a small volume of reagent per test. The magnetic beads provide a greater surface area than that of conventional ELISA, so the reaction does not suffer from the same surface steric and diffusion limitations. Instead, it occurs in a turbulent bead suspension, allowing for rapid reaction kinetics and a short incubation time. Detection limits of 200 fmol/L are feasible with a linear dynamic range spanning six orders of magnitude (38). Besides the ORIGEN system, ECL technology has been incorporated into the Elecsys® Immunoanalyzer (Roche Diagnostics, Indianapolis, IN, USA), NucliSens® amplification technology (Organon Teknika, Durham, NC), and the QPCR® System 5000 (PerkinElmer, Wellesley, MA, USA).

The ECL system has been demonstrated to be effective for detecting both toxins and infectious disease (ID) agents (32,39) and could potentially be used with any biological agent, as long as high-quality, high-affinity antibodies or other ligands to those agents are available. While in general, ECL assays are simple, rapid, and sensitive, assay sensitivities may vary significantly depending on the sample matrices encountered. Because of this, matrix-specific positive and negative control samples are used to establish standard curves and cutoff values. The major limitations of ECL assays are associated with the instrumentation itself and the time required to analyze each assay tube.

Time-Resolved Fluorescence

Another promising assay technology is time-resolved fluorescence (TRF), a Food and Drug Administration (FDA)-approved immunodiagnostic technology used to detect agent-specific antibodies, microorganisms, drugs, and therapeutic agents (40–42). TRF assays are sandwich-type assays similar to those used for ELISA and ECL, except that the detector antibodies are directly labeled with lanthanide chelates such as europium, samarium, terbium, and dysprosium. Its strengths derive from its sensitivity, a similarity shared with the commonly used ELISA techniques, and the potential for multiplexing. TRF exploits the differential fluorescence life span of lanthanide chelate labels compared to background fluorescence. The labels have an intense long-lived fluorescence signal and a large Stokes shift, resulting in assays with a very high signal-to-noise ratio and excellent sensitivity (43). TRF produces its signal through the excitation of the lanthanide chelate by a specific wavelength of light. Fluorescence is initiated in TRF with a pulse of excitation energy, repeatedly and reproducibly. In 1 s, the fluorescent material can be pulse-excited over 1000 times with an accumulation of the generated signal.

One commercially available TRF format is the dissociation-enhanced lanthanide fluorescence immunoassay (Delfia®; PerkinElmer) in which long-lasting fluorescent micelles are formed by the dissociation of the complex-bound chelate after adding a low pH enhancement solution. The manufacturer claims detection limits as low as 10^{-17} moles of europium with a dynamic range of at 4 logs. A typical agent-detection assay is similar to an ELISA except that the detector antibody is labeled directly with europium. Because the detector antibody is directly labeled and the signal generated is so strong, the assay can be completed in less time than a colorimetric assay (2.2 vs. 3.5 h).

Lateral Flow Assays

Lateral flow assays have been available on the commercial market for many years, first developed to detect abused drugs and for pregnancy testing (44). Also known as “hand-held” assays (HHA), they are simple to use and require minimal training. In most cases, the manufacturer provides simple instructions that include pictures of positive and negative results. With shelf lives generally over 2 years, HHAs do not require special storage conditions, however, high humidity and heat will degrade performance.

The assays are typically designed on nitrocellulose or nylon membranes contained within a plastic or cardboard housing. The method used for determining if an assay is positive depends on if it is a competitive or an antigen-capture assay. In the antigen-capture format, a capture antibody is bound to the membrane, and a second labeled antibody is placed on a sample application pad. As the sample migrates down the membrane by capillary action, antigen present in the sample binds to the labeled antibody and is captured as the complex passes the bound antibody. Colloidal gold, carbon, paramagnetic, or colored latex beads are commonly used particles that create a visible line in the capture zone of the assay membrane for a positive result. In the competitive format, antigen in the sample competes with a labeled antigen for binding sites on the capture antibody. If a line fails to appear, the test is positive. The competitive format is frequently used to analyze small molecules with a limited number of epitopes.

A key limitation of HHAs is that assessment of a result is strictly qualitative and subject to the interpretation of the

user. Another limiting factor is sensitivity, which is at least 1 log worse than a similar ELISA. Several approaches are being explored to retain the simplicity of the HHA format while incorporating quantitative detection and improved sensitivity. Incorporation of fluorescent microspheres into modified versions of existing lateral flow assays permits the assessment of the result by a compatible reader. One such reader, the Rapid Analyte Measurement Platform Reader or RAMP™ Reader, produced by Response Biomedical Corporation (Burnaby, BC, Canada), allows for quantitative interpretation of the lateral flow assay and has been demonstrated for clinical and biodefense applications. Up-converting phosphors have also been used to make quantitative assays and also increase sensitivity (45,46). Another promising approach uses paramagnetic particles as the label, with the magnetic flux sensed within the capture zone [Magnetic Assay Reader (MAR); Quantum Design, San Diego, CA, USA]. This approach has improved sensitivity by as much as several orders of magnitude over more traditional lateral flow assays, while also permitting a quantitative measurement of antigen.

Other Assay Formats

Flow cytometry. Flow cytometry (FC) has many applications in biomedical research and is commonplace in most large clinical laboratories. Several companies including Luminex (Austin, TX, USA) and BD Biosciences (San Jose, CA, USA) now market systems and reagents for ID agents.

The technique works by placing biological samples (cells or other particles) into a liquid suspension. A fluorescent probe, the choice of which is based on its ability to bind to the particles of interest, is added to the solution, which is then streamed past a laser beam where the probe is excited. A detector analyzes the fluorescent properties of the sample as it passes through the laser beam.

A major strength of this technology is its ability to be multiplexed with little or no loss of sensitivity (47). Using the same laser excitation source, the fluorescence may be split into different color components so that several different fluorophores can be measured simultaneously and analyzed by specialized software. However, FC has several disadvantages. Assays typically lack the sensitivity of those based on ECL or TRF. The system itself is complicated, requiring extensive training and expertise to operate. Optimization of the assays can be tedious, and many user-defined parameters must be adjusted individually. It is also subject to sample matrix problems, with serum and blood being particularly problematic.

Fluorescence polarization. Fluorescence polarization (FP) is a phenomenon seen when polarized light excites a fluorescent dye causing photons to be emitted in the same plane as the exciting light. First described in 1926 by Perrin (48), FP occurs if a fluorescent molecule does not change its relative position between excitation and photon emission. By exploiting this property, it is possible to measure the binding of a fluorescent probe to an antigen in solution. When an individual probe binds to an antigen molecule, there is a decrease in the rotation of the probe in solution, increasing the FP value of the sample. Large molecules labeled with fluorescent tags are problematic, because they are more likely to emit polarized light in solution than small molecules and are more likely to move in solution. The size of the probe is one of the key variables for assay development due to the propensity of large probes to emit polarized light in solution (49). Smaller probes produce greater FP values than larger probes due to the loss of orbital rotation that occurs upon binding (49).

FP assays are typically very simple to perform. A fluorescent tag-labeled probe (antibody or other ligand) is mixed with an unknown sample. If the fluorescent probe binds to material in the sample, there will be a detectable change in polarized light emitted from the sample. Positive binding events result in an increase in the FP value because of a decrease in orbital rotation. FP assays based on negative binding events can also be developed. Typically, such assays measure enzymatic activity of a molecule. In this instance, cleavage of a fluorescent-labeled probe causes a decrease in FP value because of an increase in orbital rotation.

Over 50 FP assays are commercially available for detecting various molecules including proteins (49,50), nucleic acids (51), serum antibodies (52), and enzyme activity (53–55). However, as with any technology, there are advantages and disadvantages to FP. A key advantage is that minimal sample preparation is required. An FP assay can be carried out in any matrix in which the probe and antigen can interact. Analyses have been conducted in phosphate-buffered saline (PBS), sera, milk, and other solvents without any sample processing (52,56). Because of the limited need for sample processing, FP antibody detection assays are particularly useful for high-throughput screening. A key disadvantage is the limited number of small molecules suitable as probes. However, suitable probes may ultimately be developed from aptamers, random peptides, and recombinant antibodies, thereby expanding the array of potential assays.

CASE STUDIES: IMMUNOASSAYS AND INFECTIOUS DISEASES

For emerging infections, there are roles for immunoassay methods both in identification and in seroepidemiology. While nucleic acid methods are now often preferred for identification of novel pathogens, immunologic methods still have considerable utility. Historically, many unknown pathogens were originally identified by antibody cross-reactivity, and some programs, such as the Rockefeller Foundation virus program in the mid-twentieth century, used this as a pathogen discovery tool. In the Rockefeller Foundation program, putative viruses were isolated from natural sources (such as infected animals or people) by growth in animals or tissue culture; adult animals were then inoculated with the virus in order to provide antibodies for virus identification and research purposes, or alternatively, patients' sera might be used (57). Immunoassay for this purpose is still invaluable today, building on a large base of reagents and experience.

Emerging infections are those that have recently appeared in the population or that are rapidly increasing their incidence or geographic range, often manifesting as unexpected outbreaks of disease (58). In many cases, the first outbreaks are surprises, often a dramatic disease new to medical knowl-

edge, such as Ebola in 1976, hantavirus pulmonary syndrome in the Four Corners region of the southwestern U.S. in 1993, hemolytic uremic syndrome caused by certain strains of *Escherichia coli*, or severe acute respiratory syndrome (SARS) in 2003. One key force driving the development of immunoassays has been the need to rapidly detect and identify clinically important pathogens, as demonstrated by the following vignettes.

Emerging Infections: Hantavirus Pulmonary Syndrome and West Nile Fever

The original outbreak of hantavirus pulmonary syndrome in the southwestern U.S. in 1993 is an example of the utility of immunoassays. In the summer of 1993, patients, mostly young and previously healthy adults, were admitted to area hospitals with fever and acute respiratory distress. More than half the patients subsequently died of respiratory failure. Known causes, such as influenza or plague, were rapidly ruled out, but initially, no etiologic agent could be identified. Serologic reactivity provided the initial clues. At the Centers for Disease Control (CDC), Ksiazek and colleagues tested patient sera against a broad range of antigens (59). All were negative except for cross-reactions with hantaviral antigens. This information, which suggested that the likely agent was a previously unrecognized hantavirus, was used to design more specific probes, including additional immunoassays (60,61) and nucleic acid probes based initially on conserved hantavirus sequences (61,62). While the availability of more nucleic acid sequence data and rapid DNA sequencing methods since then have greatly enhanced the use of nucleic acid analysis, especially using conserved or family-specific primers, for identification of unknown pathogens (63–65), immunoassay still remains a useful complementary approach. Biotechnology now makes it possible to produce unlimited quantities of test antigens without having to grow the pathogen, and a large number of potential antigens can be screened rapidly for reactivity with sera or, in other formats (such as antigen immunoassays), for possible presence of cross-reactive antigens (putative agent) in clinical samples.

In addition, many of these agents cause acute infections, meaning that clinical samples may no longer contain detectable agent if collected too late in the course of infection. Serology may provide useful information that the patient had been infected even though methods to detect the agent itself, such as culture or PCR, may prove negative in this situation (this may also be true of clinical samples that have not been appropriately collected or handled). Comparison of serology and nucleic acid testing (or culture) can also be used to identify chronic shedding and possibly help to identify the most likely sources of the infection. After a hantavirus was identified as the cause of the pulmonary syndrome in 1993, investigators (knowing that many hantaviruses are natural infections of rodents) trapped and tested a variety of wild rodents near patients' homes. The initial testing was done by serology, and several rodent species tested positive, with the highest frequency in *Peromyscus maniculatus* (deer mouse). RT-PCR also gave positive results in several species, but *P. maniculatus* had a high frequency of concurrent seropositivity and RT-PCR positivity, indicating that these animals were chronically shedding the virus (61).

Once an agent is identified and reliable test antigens become available, serosurveys can be used to determine the prevalence of infection in the population. This classic application is still of prime importance in understanding the natural history and epidemiology of infections, and a tremendous amount of epidemiologically useful information can be obtained from well-designed serosurveys (66). For many IDs, such studies have proven invaluable for identifying potential risk factors and age at infection, as well as overall prevalence. If certain conditions are satisfied, average age at infection can be used to estimate the transmissibility of the infection. Repeated sampling or cohort studies can be used to determine the rate of new infections.

Seroepidemiology is often useful to identify the relative proportions of symptomatic and asymptomatic infections. A recent example of this technique with an emerging infection is from the 1999 outbreak of West Nile virus in New York City. This outbreak is believed to represent the initial introduction of the virus into the U.S. Surveillance identified 59 patients, with 7 deaths, in the initial outbreak (67). A serosurvey of 677 individuals near the epicenter identified 19 seropositives, only 6 of whom had reported symptoms (68). From these data, the authors calculated that there were probably thousands of West Nile infections during the outbreak, most of them asymptomatic (68).

Pandemic Infections: AIDS and Human Immunodeficiency Virus

Perhaps no pathogen has resulted in the development of more diagnostic immunoassays than human immunodeficiency virus 1 (HIV-1). As a result of the spread of this virus through transfusions during the 1980s, coupled with the medical significance and potential consequence of a positive diagnosis and the wide genetic variability inherent in HIV-1, these immunoassays have been subjected to unusually intense scrutiny by regulatory groups and public health agencies (69–71). This is clearly evidenced by the establishment of exacting sensitivity and specificity standards and ongoing validation testing, to ensure that every positive case, regardless of genotype or variant, is diagnosed, while at the same time minimizing false positive results.

The present range of available immunoassays for HIV is extensive (72). Immunoassays have been developed to detect anti-HIV antibodies or viral antigens present in serum, plasma, dried blood spots, urine, and saliva. Assay formats range from EIAs, ELISA-based Western blot assays, and immunofluorescence assays. The history of HIV-1 immunoassays illustrates the advances in technologies to detect and identify infectious agents. The first HIV-1 EIA antibody-screening test was licensed in 1985 and was developed initially to protect the blood supply (73). Although many other immunoassays have been created since that time (74), the EIA remains the most widely used serologic test for detecting antibodies to HIV-1.

The general methodology of an EIA has remained unchanged, despite advances in technology and considerable

improvements in sensitivity and specificity over the years. Briefly, HIV antigens (often a purified viral lysate) attached to a microtiter plate or bead serves as the test platform. The sample to be tested is added along with a labeled conjugate that binds to anti-HIV antibodies. Detection is by colorimetric methods. While the viral lysate provides a cocktail of epitopes able to cross-react with various virus subtypes, it also contains antigens from cells in which the virus was propagated, which may result in some false reactivity. Use of recombinant and synthetic peptides (*env*, *gag,* and in some cases *pol* proteins) minimizes the nonspecific reactivity but increases the risk of not detecting antibodies to HIV-2 and certain strains of HIV-1.

Samples that are repeatedly reactive by EIA are then retested using a more specific assay, the most common form of which is the Western blot assay. Often referred to as the "gold standard" in HIV diagnostic testing, it is the key supplemental assay for detecting anti-HIV antibodies (75,76). A typical assay is manufactured from HIV-1 propagated in a suitable cell line. The separated HIV-1 proteins are electrotransferred to a nitrocellulose membrane. If HIV-1 antibodies are present in the specimen, they will bind to the viral antigens present on the nitrocellulose strips. Visualization of the human immunoglobulins (Ig) specifically bound to HIV-1 proteins is accomplished using a series of reactions with goat anti-human IgG conjugated with biotin, avidin conjugated with HRP, and the HRP substrate, 4-chloro-1-naphthol. If antibodies to any of the major HIV-1 antigens are present in the specimen in sufficient concentration, bands corresponding one or more of the following HIV-1 proteins (p) or glycoproteins (gp) will be seen on the nitrocellulose strip: p17, p24, p31, gp41, p51, p55, p66, gp120, and gp160. Individuals with repeated indeterminate results may undergo further testing using molecular assays, such as PCR, to help resolve infection status.

A number of other HIV-1 immunoassays have been developed for specific applications and supplemental testing. One such class of tests is the immunofluorescent antibody (IFA) method, which is designed to detect HIV-1 virus-specific antibodies. While simple to perform and requiring minimal equipment and reagents, significant expertise is necessary to interpret the results (77). Briefly, the serum or plasma specimen to be tested is added to a slide well containing fixed human T cells that express HIV-1 antigens. If HIV-1 antibodies are present in the specimen, they will bind to the viral antigens on the slide. Anti-human Ig conjugated to fluorescein isothiocyanate (FITC) is added, and the slide is then viewed under a microscope with UV light. If antibodies to HIV-1 are present, a characteristic pattern of fluorescence will be visible. An example of such a test is the Fluorognost™ HIV-1 IFA (Sanochemia, Vienna, Austria).

Another class of supplemental tests with broad implications is rapid immunoassays. The first such assay, the Murex Single-Use Diagnostic System (SUDS) HIV-1 test (Abbott Laboratories, Abbott Park, IL, USA), was approved by the FDA in 1992. It is a microfiltration EIA incorporating HIV-1 *gag*-encoded antigens and a synthetic peptide representing a conserved and immunodominant sequence from the HIV-1 transmembrane protein, which is fixed on latex particles (78). Bound anti-HIV-1 antibodies are detected with alkaline phosphatase-labeled anti-human Ig conjugate and color development, using 5-bromo-4-chloro-3-indolylphosphate. Within the last year, two more rapid assays, one a lateral flow immunoassay device and the other a membrane immunoreactive test device, have been approved for non-blood donor diagnostic screening (http://www.fda.gov/cber/products/testkits.htm). A number of other blood, urine, and oral fluid rapid assays currently are being evaluated (79,80).

A direct detection assay is based on the p24 antigen. One of the core proteins of HIV-1, p24 antigen can be detected in the serum of individuals as early as 16 days postinfection, prior to the detection of antibodies (81,82). The amount of antigen detected by this assay is highly variable, and therefore, it is not routinely used for diagnostic purposes. However, it can readily be automated and has been used to improve the screening of blood products obtained from donors, because it narrows the EIA detection window period following the onset of infection.

CONCLUSIONS

Immunoassays have progressed dramatically in scope and utility since their introduction in 1959. In recent years, development has been driven by the need to detect and identify dangerous pathogens and other select agents for both civilian and military applications. Conventional ELISA has been the predominant technology used for such assays, with CLIA, ECL, and TRF formats as the more recent and promising approaches. Multiplexed flow cytometry methods are also emerging as laboratory-based assays. However, the need for more rapid, portable, and sensitive assays remains as urgent as ever, providing the impetus for the development of next generation technologies. A diverse array of prototypes, ranging from microarrays (83) to silicon chip microcantilevers (84) and genetically engineered chemiluminescent biological sensors (85), are currently being developed, indicating that further improvements are on the horizon.

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