MINI-SYMPOSIUM: EMERGING VIRAL INFECTIONS OF THE CENTRAL NERVOUS SYSTEM

Diagnostics and Discovery in Viral Central Nervous System Infections

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Abstract

The range of viruses implicated in central nervous system disease continues to grow with globalization of travel and trade, emergence and reemergence of zoonoses and investments in discovery science. Diagnosis of viral central nervous system infections is challenging in that brain tissue, where the pathogen concentration is likely to be highest, is not readily obtained and sensitive methods for molecular and serological detection of infection are not available in most clinical microbiology laboratories. Here we review these challenges and discuss how they may be addressed using advances in molecular, proteomic and immunological methods.

INTRODUCTION

The increasing emphasis on microbial diagnostics and discovery in the medical research community can be understood in terms of several observations that have implications for science and medicine. First, infectious agents are expanding beyond their usual geographic range and appearing in new contexts; thus, clinicians and public health officials must be prepared to detect and respond to the unexpected. Second, the advent of new antimicrobial drugs, therapeutic antibodies and vaccines holds promise that early and accurate diagnoses can have profound implications for medical management and public health. This is particularly true for viral infections where, until recently, opportunities for effective intervention were limited to HIV, hepatitis C and herpesvirus infections. Third, the spectrum of illness attributed to infection can extend beyond acute diseases such as pneumonia, diarrhea and meningitis/encephalitis to chronic disorders such as peptic ulcer, cancer and neuropsychiatric disorders like autism, attention-deficit/hyperactivity disorder (AD/HD) and schizophrenia. Fourth, insights into the role of the microbiome in nutrition, allergies, obesity, diabetes mellitus and autoimmunity will enable new strategies for modulating microflora through the use of antibiotics and probiotics. Finally, although there have been no recent examples of bioterrorism, the risk has only increased with growing political instability and increased accessibility of synthetic genomics that enable the creation or recreation of virulent pathogens.

Manifestations of disease are only rarely specific for individual infectious agents, particularly early in the course of illness when therapeutic intervention or containment of contagion by isolation is most likely to be effective. It is imperative therefore that clinicians and public health practitioners have actionable data that enable efficient triage.

Central nervous system (CNS) infections represent a diagnostic challenge not shared with enteric, pulmonary, blood-borne, musculocutaneous and genitourinary tract diseases because the tissue site of pathogen replication is not readily accessible. Infectious agents may enter the CNS hematogenously or via cranial or peripheral nerves. The incubation time with viruses that first replicate in the skin, respiratory or gastrointestinal tract typically ranges from a few days to a few weeks; however, incubation periods in excess of 1 year are reported for rabies. Herpesviruses, like herpes simplex viruses 1 and 2, and varicella zoster virus may be latent for several years before reactivating and infecting the CNS (1). An infectious cause of encephalitis is determined in less than 40% to 70% of cases worldwide (6, 10–13). In the last 10 years, the U.S. hospitalization rate for encephalitis cases was 7 per 100 000, totaling approximately 250 000 patients. The largest proportion of these hospitalizations resulted from viral infections and 14% resulted from herpes encephalitis alone (23). One retrospective cohort in Australia implicated an infectious agent in 38% of encephalitis cases, autoimmunity in 34% and no clear cause in 28% (18). A U.S. cohort attributed encephalitis to infections in 50% of cases, autoimmunity in 20% of cases and unknown causes in 30% of cases (23). Culture is of limited utility, particularly in viral infections. In recent studies, only 1.9% of cerebrospinal fluid (CSF) viral cultures were positive (12), and <0.1% of CSF cultures recovered viruses other than enteroviruses or herpesviruses (19). The differential diagnosis of viral encephalitis includes antineuronal autoimmune encephalitis (AIE), a group of immune-mediated disorders associated with multiple forms of encephalitis

and resulting from direct interaction between autoantibodies and neuronal surface or synaptic proteins. Disease often begins with a prodromal syndrome, followed by neurological symptoms including anxiety, psychosis, memory problems and agitation, and in severe cases progresses into abnormal movements, hypoventilation, autonomic instability and coma. In anti-NMDAR encephalitis cases, antibodies were always detected in CSF but were not found in serum in 14% of confirmed cases (9). Although some evidence exists in support of the hypothesis, the triggering of anti-NMDAR and other autoimmune encephalitides by viruses remains an unresolved issue (22, 25).

DIAGNOSTICS

Viral diagnosis was not a prominent service function in clinical microbiology laboratories until recently. This reflected resource intensive aspects of classical viral diagnostics, such as culture, electron microscopy or serology as well as the fact that results provided insights that had only minimal impact on clinical management. With development of antiviral drugs, molecular assays can now be employed to identify candidates for and monitor adequacy of responses to these drugs.

Despite the shift in diagnostics toward molecular assays, viral culture continues to be important because it is essential to test drugs, neutralization capacity of antibodies and vaccine responses as well as to develop stocks of virus for work in animal models. Some viruses can be propagated in immortalized cell lines whereas others can only be grown in primary or organotypical cultures. Still others require the use of antibodies or RNAi to suppress innate immune responses or must be inoculated into live animals such as suckling mice. Indeed, a virus better adapted to culture may outgrow a virus that is more abundant *in vivo*, obscuring detection of the latter. Accordingly, we prefer to use less biased molecular methods for discovery and reserve culture for follow-on studies.

Molecular assays

Molecular assays employed in clinical microbiology include polymerase chain reaction (PCR), isothermal amplification, DNA microarrays, *in situ* hybridization and sequencing. The most common are real-time PCR assays wherein the release of a fluorescent molecule during the course of DNA strand replication results in detection of a single viral target. These assays are exquisitely sensitive, specific, quantitative and inexpensive, and are used not only for differential diagnosis but also to follow response to antiviral therapy. Portable PCR systems have been developed for field applications; nonetheless, some investigators prefer isothermal amplification tests that do not require programmable thermal cyclers. PCR sensitivity is highest when primers and probe sequences perfectly match a selected, single genetic target. Indeed, these assays may fail to detect related viruses—a potentially daunting challenge in RNA virus infections where high mutation rates are characteristic. Consensus PCR assays wherein primers and/or probes contain wobble codes may succeed; however, they are typically less sensitive than specific PCR assays. Nested PCR tests that can employ consensus or specific primers in two sequential amplification reactions with either one (hemi-nested) or two (fully

nested) primers located 3′ with respect to the first primer set may accommodate sequence variation and be as sensitive as fluorescent real-time PCR assays. However, whereas in real-time assays reporter readings are taken indirectly without opening the reaction vessels, nested PCR systems are prone to contamination because of the transfer of amplified product from the first to the second nested reaction.

Multiplex PCR assays are increasing in popularity because they can be used to simultaneously address a wide range of candidate viral, bacterial, fungal and parasitic pathogens. This is particularly important early in the course of infection when signs and symptoms of disease are less specific. However, multiplex assays can be difficult to establish because different primers and probes require different reaction conditions for optimal performance. An additional challenge is the limited repertoire of fluorescent reporter molecules available for use. An alternative platform (Luminex xTag, Luminex Molecular Diagnostics, Austin, TX) employs flow cytometry to detect multiple PCR amplification products bound to matching oligonucleotides that are attached to fluorescent beads. By combining multiplex PCR amplification systems with various protocols for direct or indirect (tag-mediated) bead-based hybridization of the products, assay panels have been developed that detect more than 20 different genetic targets. Other PCR platforms employ mass spectroscopy to differentiate genetic targets based on product mass (IRIDICA, Abbott, Carlsbad, CA, USA) or the presence of tags conjugated to primers used in PCR that vary in mass (3). Such systems can detect up to 20 different genetic targets in a single reaction but are typically 10- to 100-fold less sensitive than real-time PCR.

Microarrays comprising millions of discrete oligonucleotide probes have the potential to detect all known viruses as well as viruses with limited homology to known viruses (17, 24); however, as they currently depend on a random (vs. specific) PCR amplification step followed by hybridization of the fluorescently labeled product, microarrays are even less sensitive than multiplex PCR systems. New platforms are in development that will detect viral sequence binding through changes in electrical conductance. These platforms will not require fluorescent scanners and may have improved sensitivity.

High-throughput sequencing, also known as next generation sequencing, has transformed medicine and virology by enabling viral discovery as well as diagnostics. Unlike PCR or array methods where the breadth of agents interrogated is limited by the capacity for multiplexing or known sequence data, high-throughput sequencing has the potential to simultaneously detect not only all viruses, but also bacteria, fungi and parasites. Furthermore, the time and resources previously required to clone and sequence entire viral genomes have been reduced from months to days.

Platforms in current use analyze libraries of amplified nucleic acids. However, some platforms in development will have the capacity to directly sequence nucleic acid. Irrespective of the platform, raw sequence reads are filtered for quality and redundancy before assembly into contiguous strings of sequence streams that are aligned to sequences in databases using algorithms that search for similarity at the nucleotide and deduced amino acid levels in all six potential reading frames. The alignments allow identification of known and novel agents, as well as detection of genetic features that may be associated with drug or vaccine resistance, or provide insight into provenance and evolution.

Host response

Molecular diagnostic methods are sensitive, but do not succeed where microbial nucleic acids are not present in an accessible sample or disease is triggered by an agent that is no longer present. Serology complements molecular diagnostics by providing insights into a subject's pathogen exposure history. It can test for the relevance of a discovery made using molecular methods by indicating whether an infection temporally correlates with the onset of disease. Serology can also help target molecular investigation. This advantage has become less important since the introduction of inexpensive unbiased high-throughput sequencing; however, immunohistochemistry and serology were critical in focusing PCR studies that resulted in the identification of Sin Nombre virus (15), Nipah virus (5) and West Nile virus in New York City in 1999 (2). Although a wide range of diagnostic platforms has been developed for detection of microbial nucleic acids, methods for detection of host responses to pathogens have lagged.

Serological assays provide evidence that a host has encountered and responded to a microbe through the activation of specific B-cells. An activated B-cell can respond to femtomolar antigen concentrations and generate up to 10^9 specific antibody molecules in a week; hence, antibody assays have potential for extraordinary sensitivity. Antibodies are also remarkably stable. To date, most serology has been performed using single antigenic target enzyme-linked immunosorbent assays (ELISA). More recently, bead-based systems have been employed that can address up to 100 antigenic targets simultaneously (ie, 100 individual pathogens, 100 individual antigenic targets for one pathogen, or some variation thereof). Additionally, arrays are established that comprise spotted recombinant proteins expressed *in vitro* in *Escherichia coli*, *Saccharomyces cerevesiae*, baculoviruses, or in cell-free, coupled transcription-translation systems. However, assay development is complex and time consuming; hence, rapid response to an emerging pathogen is impractical. Furthermore, none of these methods achieve the degree of multiplexing required to complement what has become standard in direct nucleic acid detection systems.

Burbelo *et al* have established a sensitive and quantitative liquid phase format assay for profiling antibody responses to antigen panels (4). Luciferase immunoprecipitation system (LIPS) assays express viral open reading frames fused to an enzyme reporter, *Renilla* luciferase, in eukaryotic cells. Fusion proteins harvested under native conditions are mixed with serum samples and immunoprecipitated using protein A/G magnetic beads. No secondary antibodies are required because the primary antibodies in serum bind directly to beads through interaction of their Fc region with staphylococcal protein A and streptococcal protein G. Protein A binds with high affinity to the Fc region of human (IgG1, IgG2, IgM, IgA, IgE), rabbit, guinea pig, dog and monkey immunoglobulins. Protein G binds with high affinity to the Fc region of human, rabbit, pig, horse, cow, mouse, goat and monkey immunoglobulins. Thus, a single LIPS assay can be used to screen many different species for evidence of exposure to infectious agents or for successful vaccination. Establishment of a new LIPS assay typically requires 10–15 days from the time that genomic sequence data for a particular virus become available. Over the past 2 years, we and our colleagues have designed and implemented LIPS assays for investigation of the pathogenesis and epidemiology of astroviruses, picornaviruses, parvoviruses, orbiviruses, hepaciviruses and pegiviruses and to profile exposure histories of individuals with HIV/AIDS.

Although LIPS technology enables rapid development of serological assays for virology, it is not a multiplex platform. One solution that can address this gap is phage display systems wherein libraries of peptides are reacted with sera and those that bind are characterized through DNA sequencing (26). Another potential solution is programmable peptide chip technology, an approach analogous to that used in building DNA microarrays but substituting oligopeptides for oligonucleotides. Commercial platforms are in development that comprise millions of oligopeptide features in a single assay. Such platforms have the potential to enable detection of humoral immune responses to all known vertebrate viruses, allowing surveys for exposure to all known human viral pathogens as well as those that emerge through zoonotic transmission. Through microfluidics, it may become feasible to probe an individual's immunological memory, providing insights that will facilitate differential diagnosis and research into the role of specific infectious agents in acute and chronic diseases.

In clinical microbiology, serology is typically interpreted to mean the study of B-cell responses to infectious agents. However, it can be taken in a larger sense to represent any biomarker for infection that is present in serum. The most common approaches are bead-based assays for detection and quantitation of cytokines and chemokines that indicate immune activation consistent with infection. The initial hope was that patterns of expression would be sufficiently distinctive as to allow differential diagnosis by serving as surrogate assays for infection with specific infectious agents. This has not yet proven true; nonetheless, cytokine profiles have been used for insights into pathogenesis and prognosis (eg, cytokine storm), indications of genetic differences underlying difference in innate and adaptive immunity and as biomarkers for viral reactivation.

Even more robust than the cytokine platforms currently limited to analysis of 50–70 molecules are the biomarker discovery platforms that quantitate levels of specific host RNA populations in tissues and body fluids through use of microarrays or RNA sequencing (transcriptomics), host proteins (proteomics) or products of metabolic processes (metabolomics) through mass spectroscopy. Together, these assays comprise the components of the field of systems biology wherein investigators integrate large datasets for insights into the impact of environmental perturbations on function at the level of individual cells, organs or the entire organism. These insights have the potential to lead to treatment strategies that modify host response rather than targeting the infectious agent. They can also be used to identify genetic and epigenetic determinants of host response that influence the outcome of infection.

Host factors can have a profound impact on susceptibility to infection and the consequences of infection. Agents that normally cause no or only mild disease can have severe consequences in individuals with a range of immunodeficiencies, due to genetic mutations, age, malnutrition, HIV/AIDS or complications of cancer treatment or transplantation. Thus, the principle whereby investigators look for clusters of diseases associated with candidate pathogens must be expanded from simple models wherein cases are closely linked in time and space to include clusters based on other demographic factors. A classic example is the association between progressive multifocal leuokoencephalopathy due to JC virus and immunosuppression in the context of HIV/AIDS or

therapy for multiple sclerosis. Pathogenic mechanisms may be direct or indirect, readily manifest or subtle. Viruses can cause tissue damage as a direct result of replication or via innate or adaptive immune responses to microbial gene products. Microorganisms can also induce neoplasia through interference with cell cycle controls. Although not yet confirmed in human disease, work in animal models indicates that viruses can reduce the production of hormones or neurotransmitters that are vital to normal host physiology, and that they can do so without causing any apparent cell or organ damage.

Some of the most intriguing concepts in viral pathogenesis involve complex mechanisms that include infection with other microbes (other viruses, bacteria or parasites), immunogenetic variation or a timing component. The most straightforward instances are those where viral infection results in immunosuppression that enables opportunistic infections. The most dramatic example is HIV/AIDS where disease may present with *Pneumocystis carinii* pneumonia, CNS infection with *Toxoplasma gondii* or *Cryptococcus neoformans* or Kaposi sarcoma. A related phenomenon is expansion of the enteric virome in Rhesus macaques infected with simian immunodeficiency virus (SIV) (8). Bacterial superinfection has also been implicated in the increased morbidity and mortality associated with influenza infection (16, 24). Helminth infection in mice can result in reactivation of gamma herpesviruses through induction of interleukin (IL)-4 (20).

We and others have built models of neurodevelopmental disorders based on gestational viral infections wherein the timing of maternal inoculation with influenza virus or the viral mimic polyinosinic:polycytidylic acid (poly I:C or double-stranded RNA) results in abnormalities in social and locomotor behaviors in the offspring (14). These effects are associated with a reduction in neuronal stem cells that is found in offspring of wild-type mice but not in offspring of knockout mice missing the receptor for poly I:C, toll-like receptor (TLR)3; the effects of poly I:C can also be abrogated through the use of nonsteroidal anti-inflammatory drugs (7). The relevance of this model to human disease is underscored by the observations that mothers who report fever during pregnancy and the presence of elevated levels of pro-inflammatory cytokines in cord blood are both associated with an increased risk for autism spectrum disorder in offspring (Hornig and Lipkin, unpublished data). Our ability to detect associations between environmental exposures and disorders that may only manifest in later life depends on access to prospectively collected questionnaire data and clinical samples in large cohorts. The Center for Infection and Immunity works with the Norwegian Institute of Public Health in a program that includes such questionnaire data and samples obtained beginning at the first prenatal visit (approximately 17 weeks gestation). The cohort represents 114 500 children as well as their mothers and fathers and is linked to a national patient registry that records all clinic and hospital visits (21). In reviewing such registries, investigators have an unprecedented resource with which to examine how infection, intoxication, and genetic and epigenetic factors influence risk and resilience for diseases in any organ system.

ACKNOWLEDGMENTS

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