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Chapter 22

What Lies Ahead?

Scientists Look into Their Crystal Balls

Chapter Outline

1. INTRODUCTION

Viral pathogenesis is a field in rapid evolution, reflecting the dynamic development of systems biology and the continuing introduction of new or improved methodologies. Therefore, this final chapter is dedicated to "futurism," a look at what lies ahead for this field. We have recruited a number of scientists to write short pieces where they are free to speculate on future developments in their respective areas of expertise.

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2. WHAT LIES AHEAD: INDIVIDUAL VISIONS

Chapter 22.1

Systems Analysis of Host–Virus Interactions

Ronald N. Germain

Ron Germain is Chief of the Laboratory of Systems Biology, NIAID, NIH, and Associate Director of the Trans-NIH Center for Human Immunology. Over the course of his career, he has studied the immune system at the molecular, cell, and more recently, organismal level. He is now using a combination of advanced imaging methods and systems approaches pioneered in his laboratory to better understand host antimicrobial defenses and their pathological manifestations.

Decades of detailed biological study have taught us that host defense against viral infection and spread relies on multiple components of the immune system, ranging from parenchymal cell production of interferons, to innate immune responses by both myeloid and lymphoid cells, to lymphocyte-dependent cellular and humoral adaptive immunity. In the evolutionary tug of war between host and pathogen, viruses have developed ways to circumvent or even to take advantage of the host response. It has also become clear that much of the damage and disruption of physiology caused by viral infections is not due to direct cytopathic effects of the virus itself, but to the side effects of misdirected or over-exuberant host immunity.

1. WHERE DO WE GO FROM HERE?

The multilayered nature of host defense to viruses, the complex interplay between the pathogen and host, and the immune system's impact on normal tissue function call for a more holistic approach to the study of viral pathogenesis going forward. Rather than drilling down to the function of specific viral proteins or looking for a singular putative mechanism of host protection as most studies have done for the past several decades, we need to think at a systems level, integrating information across biological scales (genes, proteins, cells, organs), across time, and across interaction directions (virus->host, host->virus, host->host).

How can this be accomplished? The exciting news is that we now have tools that permit us to collect quantitative data on each of these aspects of the host–pathogen interplay. In animal models, viruses that encode fluorescent proteins, advanced microscopes that allow deep, long-term, high-resolution imaging in many tissues and organs of living animals, and reporter systems that reveal the identity of immune cells and their molecular state, now permit investigators to observe viral spread and the concomitant host response in real time (Germain et al., 2012). Novel methods for tissue preparation allow even entire organs to be made accessible to high resolution confocal imaging in dozens of colors, enabling detailed phenotypic analysis of infiltrating immune cells, the location of virus, and the state of host cells (Gerner et al., 2012). Emerging tools for DNA and RNA in situ hybridization permits documentation of the transcriptional response to infection. The application of such tools can produce a detailed, four dimensional (volume and time) picture of how a virus spreads, and the nature of the host response. In animals given candidate vaccines or drugs, these tools can also be used to directly visualize the specific effects of the treatment on the infection and the host response, both immune and organ-related.

But as powerful as imaging has become, it is in combination with the emerging methods of systems biology that insight will be gained most rapidly. This is especially true for emerging or re-emerging infections that frequently have more severe effects on host homeostasis and for which new understanding of the infectious process and host response are most needed. Transcriptomic, proteomic, and metabolic analyses are becoming more and more accessible, powerful, and applicable even for the human host (Nakaya et al., 2012), allowing collection of data sets that can be compared with those from animal models to determine the extent to which the animal systems are suitable substrates for vaccine or drug development.

One sticking point going forward is the limited capacity of many investigators to integrate the enormous data sets these technologies can generate. It has become very challenging to interpret the data in the context of human genetic variation and microbiome differences that have critical impact on immune performance. Investigators just coming

into the field have a remarkable opportunity to address questions of viral pathogenesis in ways few established scientists are able to employ. However, this will only be the case if these rising scientists embrace the need for training in quantitative analysis. Also, the scientific enterprise must provide suitable incentives for the large multidisciplinary teams needed to collect and mine the many types of information required for a systems-level understanding of the infectious process (Germain et al., 2011).

Reductionist biology is not nearing its extinction point, but more and more the future will be about analyzing biology in an integrated and holistic manner. Imaging will help us understand cell dynamics, migration, positioning, the cell–cell interactions critical to immune effector responses, and the range over which cytokine and chemoattractants work. These are all facets of immunity that are central to determining what occurs in the face of viral infection and how various cells and factors contribute to maintaining host homeostasis or to disrupting it. Informatic analysis and computational modeling will provide new ways to integrate disparate data sets so that the yin and yang of immune function during an infection can be better understood. More precise understanding of the tipping points where augmentation or blockade could play a key role has the potential to improve health outcomes. Rising virologists will need to become "physiologists" with an appreciation for how the entire organism behaves in the face of infection. Such insight will determine the levers that are needed to reduce morbidity and mortality from infection in the absence of fully protective vaccines, and indeed, to determine what components of the immune response are best suited to protection. The future is both exciting and demanding—we will need to do our science in new ways to move forward in the most efficient manner. Although challenging, the systems biology approach has the potential to vastly accelerate our capacity to understand the origins of virus-induced pathology and to develop novel ways to prevent it.

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Chapter 22.2

New Cellular Assays

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Holden Maecker is an Associate Professor of Microbiology and Immunology, and Director of the Human Immune Monitoring Center, at Stanford University. His research focuses on cellular immune responses to chronic infections and cancer, and immune correlates of protection.

Because cellular immunity is so critical to the control of viral diseases, our understanding of viral pathogenesis has always been somewhat limited by our ability to measure the cellular immune response. One of the first assays of T-cell function, the enzyme-linked immunospot (ELISpot) technique, was revolutionary for its ability to quantitate antigen-specific cytokine production on a single-cell level. This was followed by intracellular cytokine staining (ICS) by flow cytometry, which was similarly applied to the detection of antigen-specific T cells a few years later. Since then, the growth of multicolor flow cytometry capabilities has led to ICS assays that provide information on multiple cytokines and phenotypic markers in combination. MHC-peptide multimer staining, as well as degranulation via CD107 export, can also be assessed together with ICS.

But the number of T-cell cytokines and differentiation markers of interest has grown tremendously over these years of T-cell assay development. Of interest to the protective capacity of virus-specific T cells is their expression of exhaustion markers such as PD-1, or costimulatory markers like CD28. Cytokines of relevance have expanded from the traditional IFN**γ**, to include IL-17, IL-22, and others. Intracellular levels of granzymes and perforin can be important to T-cell function. And chemokine receptors such as CCR6, CXCR3, and CCR5 can

give clues about T-cell cytokine profiles and functional patterns. There are also many markers to define regulatory T-cell subsets, which impact the function of effector T cells.

In addition to T-cell markers, recent evidence has shown that NK cells can have memory properties and contribute to control of infections. Dendritic cells are important for priming and sustaining T-cell responses, and may serve as vehicles of viral spread, in some virus infections such as dengue. In the B-cell lineage, the magnitude of plasmablasts has been correlated with dengue disease severity. Thus, there are reasons to quantitate and/or phenotype virtually every type of immune cell subset when looking for correlates of immunity and pathogenesis.

Fortunately, a technology has recently emerged that greatly increases the number of markers that can be simultaneously quantitated at the single-cell level compared to traditional flow cytometry. Mass cytometry, or CyTOF (for Cytometry by Time Of Flight), is based on the use of heavy metal ion labels, rather than fluorophores, for tagging antibodies and other probes (Tanner et al., 2008). The consequent readout by mass spectrometry yields two parallel benefits: many more channels can be simultaneously detected, with little to no spillover between them. The result is 40-plus parameter flow cytometry without the need to perform interchannel compensation.

Already, mass cytometry has been applied to the study of immune cell signaling capacities (Bendall et al., 2011; Bodenmiller et al., 2012), to phenotype the diversity of CD8 T cells (Newell et al., 2012), NK cells (Horowitz et al., 2013), and B cells (Bendall et al., 2014), to measure immune competence in cancer patients (Chang et al., 2014), and to find immune correlates of response to surgery (Gaudilliere et al.). A variation of the technique has even been adapted to reading cells in tissue sections, a kind of extension of immunohistochemistry with more than 40 parameters (Angelo et al., 2014).

With mass-tagged antibody conjugates now readily available for many markers, the ability to build high-dimensional panels has become easier, although expensive. Because of the relative lack of spillover, panel design is simpler, though not entirely fool-proof (Leipold et al., 2014). So what are the drawbacks of the technique? In addition to the cost and expertise required to set up and maintain the complex instrumentation, the two major drawbacks are acquisition speed and cell recovery. In other words, the CyTOF is slow in collecting cells, and the majority of cells are lost. A secondary drawback is sensitivity, since there is no channel in the mass cytometer that equals the sensitivity of phycoerythrin (PE) or similarly bright fluorochromes in fluorescence flow cytometry. Despite these drawbacks, the wealth of information collected by CyTOF is making it the technique of choice for both broad and deep profiling of immune cells. Many laboratories are using these methods to find cellular immune biomarkers of infection, pathogenesis, and immunity.

Of note, mass cytometry is not the only technique that can provide highly multiparametric data at the single-cell level. While it is technically possible to do whole-transcriptome RNAseq of single cells (Jaitin et al., 2014), a targeted version of this technology is also useful. Targeted RNAseq uses PCR to amplify a set of genes from cDNA of single cells. The amplified products from each cell are barcoded and pooled for deep sequencing. This provides an alternative to CyTOF in that it is not limited by the availability of good antibodies to target molecules. On the other hand, it generates transcript frequencies rather than protein abundance.

Parallel measurements on thousands of immune cells create complex data to decipher for potential biomarkers. The development of visualization and statistical algorithms to help interpret CyTOF data continues to grow. Currently published clustering algorithms that have been applied to mass cytometry include SPADE (Simonds et al., 2011), PCA (Newell et al., 2012), viSNE (Amir et al., 2013), Citrus (Bruggner et al., 2014), and ACCENSE (Shekhar et al., 2014). The next few years will likely see a refinement and selection of these algorithms for those that perform best for particular questions.

In summary, it may be predicted that future studies of viral interaction with the immune system will be dominated by big-data approaches such as mass cytometry and single-cell targeted RNAseq. Computational methods to decipher these large data sets will be key to finding the biomarkers hidden within them.

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Chapter 22.3

The Virome: Our Viral Commensals

Martin J. Blaser

Martin J Blaser is Professor of Medicine and Microbiology, and Director of the Human Microbiome Program at the NYU School of Medicine. His work over the past 30 years has focused on human pathogens, including Campylobacter species and Helicobacter pylori*, which also are model systems for understanding interactions of residential bacteria with their human hosts. Most recently, he wrote "Missing Microbes," a book targeted to general audiences.*

Animals have had resident microbes ever since there have been animals, at least for 500 million years. These have mostly been prokaryotic bacteria and -archaea but wherever there are bacteria, there also are viruses that live with them. Whether in the ocean or in the human body (Reyes et al., 2012; Minot et al., 2011; Pride et al., 2012), bacteriophages are predators of their bacterial hosts.

These relationships are multifaceted and complex, marked by both competition and cooperation; the tension between these forces is ever-present and dynamic. Many bacterial species live within animal hosts, competing for niche, and viruses do the same within their bacterial hosts. In longstanding ecosystems, such as represented by the human body, viruses predictably affect the fitness of their immediate bacterial hosts (Reyes et al., 2013; Minot et al., 2013). Just as in the tundra, where the wolf keeps the caribou healthy, the inevitable force of viral predation affects the bacterial populations, and therefore the larger host in which they all reside (Reyes et al., 2010).

In addition to bacteriophages, which are the dominant viruses in humans, commensal viruses also live directly in our cells. New nucleotide-based approaches show that we are teeming with resident human viruses; between skin, mouth, nose, and vagina, and we each seem to be carrying 3–15 detectable DNA viruses at any time (Wylie et al., in press). Most of those with which we were familiar cause common, usually mild, but occasionally severe, infections. Cytomegalovirus and Epstein–Barr virus are two excellent examples. We focus on their ill effects, but most people are carrying these viruses silently for decades, essentially for life.

What are they doing? Are they just parasites, exploiting us for their own purposes, with relatively low biological cost to human fitness? Or are there benefits to our relationships, now mostly hidden? In the discipline of microbiology, most new organisms have been discovered as pathogens. "Pathogens" became our dominant mind-set for viruses, even for organisms that infect most of us, and persist for decades, 99% of the time without clinical consequence. Although some of the herpesviridae, JC virus, or certain papillomaviruses, for example, may cause illnesses and may be lethal, the net negative effects, integrated across the life span of the entire human population, are extremely low. When the cost side to human fitness is so low, it is possible that even a small benefit across most people may cumulatively be greater. Our drive toward "microbiological perfection," may be producing a

paradoxical worsening of health, because we are removing their benefits as well.

We are endowed by immunity, now divided into adaptive and innate, but there is increasing evidence for a third class, which may be called "microbial." It is our commensals, our long-time partners, which among their functions, help protect the motherland, that is, the human host. Many observations indicate that our commensal bacteria are part of the protection against invading organisms (Bohnoff et al., 1954), and already there are reports that some "commensal viruses" may help control serious infections (Barton et al., 2007; Barr et al., 2013).

I predict that the evidence for such relationships will grow in the coming years. Medical science has focused on the horizontal, because consequences often are severe, but numerically, the vertical may be at least as important. It is in the evolutionary interest of commensals, whether bacterial or viruses, to protect us most of the time. The tension comes when their necessity to be transmitted to the next host exceeds their protective properties.

A final, seemingly unpleasant thought: we all must die. To some scientists, this is just the consequence of aging. An alternative view is that clock-like markers and phenomena of aging have been selected; those species that have orderly senescence fare better than those in which the herds are subject to culling regardless of age. Pathogens can sweep through a population and kill young and old alike, in some cases leading to the extinction of the entire group. Clock-imposed senescence appears safer, as mathematical modeling indicates.

Commensals can contribute to aging. In humans, they may contribute to the causation of cancers (think *Helicobacter pylori,* EB-virus, Hepatitis B) and degenerative diseases (JK virus and prions)—most of which are log-linearly age-related. Our commensals may contribute to killing us safely (in an age-related manner), without epidemic risk to our community (Blaser and Webb, 2014). What is bad for the individual may be good for the species.

If the above biological premises are correct, what should we do? One implication is that our commensals (viruses included) may be beneficial for us early in life through reproductive age, but costly later. Rather than try to prevent infection early, we might welcome at least a part of it, while controlling the untoward consequences, and focus on better late-in-life control. Less-virulent commensals may protect against higher virulence organisms, even of the same species. As one example, perhaps children should be exposed to varicella-zoster virus early, as they had been for eons, but now controlling for the serious ill effects with antiviral drugs. Then with aging, we might boost VZV immunity by vaccination to prevent serious consequences. Such an approach would need to be tested.

In conclusion, there clearly are viral pathogens—they mostly are exogenous—that have crossed from other animals. Historically, these are the most dangerous. As SARS, Ebola, MERS, and variant influenza have shown us, their introduction will continue. However, we are also endowed with many viral commensals, with new types being discovered with regularity (Reyes et al., 2010, 2013; Minot et al., 2013; Wylie et al., in press). Our biological relationships with them are complex, and the same commensal virus can be symbiotic or pathogenic, depending on host context and timing; the biosphere is full of contingency. Deep understanding of the underlying biology of viral commensalism will allow us to harness our friendly microbes to improve human health.

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Chapter 22.4

Forward Genetics and Viral Pathogenesis

Bruce Beutler

Bruce Beutler directs the Center for the Genetics of Host Defense at UT Southwestern Medical Center in Dallas. He analyzes immune function in mammals by random germline mutagenesis. In 2011, he shared the Nobel Prize in Physiology or Medicine for discoveries concerning the activation of innate immunity.

How are we to understand precisely how viruses work, and how are we to defeat them? Genetics continues to lead the way to understanding the precise molecular interactions between virus and host. A number of remarkable tools have become available to probe precisely how viruses work.

Today it is possible to ask, "what proteins of the host are essential for virus X to complete its life cycle?" In mice, at the whole organism level, genetics can deliver answers to questions of this kind with greater speed than ever before. As soon as a new phenotype is observed (for example, instances of mice in which a normally virulent virus fails to proliferate), one can confidently state which mutation in the host genome is responsible for this phenotype. This new reality has stemmed from the development of massively parallel sequencing platforms, methods for high-speed genotyping, and new computational tools for this express purpose (Moresco and Beutler, 2013; Bull et al., 2013; Wang et al., 2015). The latter have been developed in our laboratory, and while a detailed description cannot be offered in this short essay, real-time forward genetic analysis in the mouse works approximately as follows.

Male mice of known sequence, homozygous at all loci (usually C57BL/6J background) are exposed to the mutagen ENU (N-ethyl-N-nitrosourea) which induces single base pair changes in the genomic DNA of spermatogonia. Several thousand mutations are transmitted to each sperm, causing coding changes in about 70 genes per haploid genome. These mutations are transmitted to G1 males by breeding the mutagenized sire to a C57BL/6J female. About 40 G1 males are produced each week, and each G1 male is subjected to deep, whole exome sequencing to detect all mutations that cause (or may cause) coding change. Sperms are preserved from this male, which is then used to produce G2 daughters. Ten G2 daughters are then backcrossed to the G1 sire to yield between 30 and 50 G3 progeny. Before the G3 progeny are released for screening, they are genotyped at all mutant sites to determine whether they are homozygous WT (REF), heterozygous (HET), or homozygous variant (VAR). Foreknowledge of genotype assures that when phenotype is measured, an immediate computational determination of linkage can be made, using recessive, semi-dominant, or dominant inheritance models. In general, linkage will be observed only if a phenotype has a genetic basis. And if linkage is observed, it often indicates unambiguous cause and effect (i.e., it shows that a particular mutation is responsible for the phenotype).

Because 40 G1 mice are processed each week, and each G1 mouse bears about 70 mutations, about 2800 mutations are investigated each week for their phenotypic consequences (and many phenotypes can actually be studied in parallel). Annually, more than 100,000 mutations can be examined. Over time, every gene is struck repeatedly by mutation, and many alleles can be tested for phenotypic effects. It does not take long until all genes have been examined in considerable depth, so that they may be considered "implicated" or "exonerated" in the phenomenon of interest. Where putative null alleles are concerned (those that cause premature truncation of a protein or aberrant splicing), three observations in the homozygous state are considered an ample test of the importance of a gene in a phenomenon of interest.

Not only simple (Mendelian) traits can be linked to mutations, but complex traits involving mutation at multiple loci can be solved as well, particularly if pedigrees of a large size are constructed for the purpose. As contrasted with quantitative trait locus mapping, ENU mutagenesis tends to produce monogenic phenotypes, but when complex phenotypes are observed, they are more easily solved owing to the limited number of mutations under

investigation. The very fact that complex traits do occur, even with so few as 70 mutations causing coding change in a given pedigree, suggests that interactions between naturally occurring mutations are abundant, and may represent a major source of a phenotype as it is observed in wild populations, or in humans. This, of course, would presumably apply to all phenotypes, including viral susceptibility phenotypes.

Will ENU mutagenesis offer an explanation of all the workings of a complex biological system and tell us precisely how we fight viruses, or how viruses take advantage of the host? It can only give us a good start. The future may call for even more sophisticated methods than forward genetics as we presently practice it. Already widespread is the use of CRISPR/Cas9 technology to modify the genome. It is quite possible to create many targeted mutations within a given model organism genome to probe interactions between pathways that might be thought redundant in function. Yet this innovation too will run its course, and in the future, we may rely upon synthetic genomics. Already it is possible to create designer microbes, with genomes that are modified as the investigator chooses. A complete chromosome of a eukaryotic organism (yeast) has been synthesized as well. Will the day come when we may synthesize the genome of a mouse, modified just as we choose? Very likely yes, and very likely such mice will answer questions that cannot be addressed using the current generation of genome-modifying technologies. Undoubtedly, a cascade of future technical advances will continue to elucidate the interactions between host and pathogen.

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Chapter 22.5

The Future of Synthetic Virology

Eckard Wimmer, Yutong Song, Oleks Gorbatsevych

Eckard Wimmer is Distinguished Professor of Molecular Genetics and Microbiology, Stony Brook University. For decades, he has studied the replication and pathogenesis of human RNA viruses, particularly poliovirus, and is known for the de novo chemical synthesis of poliovirus, the first organism generated on the basis of sequence information in the public domain.

Yutong Song is a Research Assistant Professor of Molecular Genetics and Microbiology, Stony Brook University School of Medicine, Stony Brook. His research focuses on translational and replication control of viral RNA, and pathogenesis of human RNA viruses such as poliovirus and Hepatitis C virus.

Oleks Gorbatsevych is a scientist at Stony Brook University. He developed software for designing synthetic genes, and his research is focused on the evolution of synthetic viruses.

The first test tube synthesis of a virus (Cello et al., 2002) caused a global uproar. One axiom in biology holds that proliferation of cellular organisms or viruses depends on the presence of a functional genome. The cell-free synthesis of poliovirus has violated this law: no natural template was required to recreate this organism. In 2002, few people were prepared to accept the new reality that viruses exist

TABLE 1 List of Virus Genomes in Chronological Order that Were Synthesized either Partially, or in toto in the Absence of Natural Templates

I.	2002	Poliovirus[a]
II.	2003	Phage PhiX174[b]
III.	2005	Recreation of the 1918 influenza Virus[c]
IV.	2005	Refactoring bacteriophage T7[d]
V.	2006	Codon deoptimized polioviruses[e,f]
VI.	2007	Reconstitution of an infect. Human endogenous retrovirus[g]
VII.	2007	Generation of infectious molecular clones of HIVcpz[h]
VIII.	2008	Codon pair deoptimized polioviruses[i]
IX.	2008	Bat SARS-like Coronavirus[j]
X.	2010	Codon pair deoptimized influenza virus[k]
XI.	2010	West Nile virus[l]
XII.	2012	Poliovirus: Discovery of novel regulatory elements by recoding[m]
XIII.	2013	Influenza virus HA and NA codon pair deoptimization[n]
XIV.	2014	Tobacco Mosaic virus[o]
XV.	2014	Respiratory syncytial virus: attenuation by recoding[p]

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as infectious particles in nature as well as entries in a database[1]. Not surprisingly the response of laymen and experts alike included praise, ethical concerns, ridicule, and fierce condemnation (Wimmer, 2006; Wimmer and Paul, 2011).

Total synthesis of viruses, meanwhile, has come of age. Yet the number of synthetic viruses (Table 1) is still modest because the technology needed to produce large segments of DNA that can be stitched together is far from efficient. Moreover, the price of error-free synthetic oligonucleotides (>100 nucleotides) is still significantly greater than predicted in 2004 (20,000 bp for $1). A revolution, however, is brewing (Notka et al., 2011; Kosuri and Church, 2014) even though the new technologies have not yet reached the commercial sector. It can be predicted that new strategies will drastically change research

1. http://www.ncbi.nlm.nih.gov/genomes/GenomesHome.cgi?taxid = 10239.

in molecular genetics and biological engineering: the tedious steps—construction of vectors, of site-specific mutants, etc.—will soon be replaced by fast and affordable DNA synthesis.

Template-free synthesis of RNA viruses, of course, relies on the chemical synthesis of genome-complementary (double stranded) DNA, referred to as "cDNA." cDNA, in turn, can be enzymatically transcribed into viral RNA and ultimately converted to infectious virus. The chemical synthesis of cDNA discussed here is reminiscent of the enzymatic synthesis of cDNA by retrovirus reverse transcriptase that was introduced by Charles Weissmann and his colleagues in 1978 (Taniguchi et al., 1978). Weissmann's strategy, termed "reverse genetics," has led to the revolutionary method allowing DNA-based genetic manipulations of RNA viruses. However, whereas the Weissmann strategy requires natural viral isolates to generate cDNA (Figure 1(A)), chemical synthesis only needs the genome sequence information readily available on the Internet (Figure 1(B)). Assuming the sequence of an RNA is known, we believe that the enzymatic synthesis of cDNAs will soon be replaced by chemical synthesis (Wimmer and Paul, 2011).

Owing to their properties as "quasi species" (very high spontaneous mutation frequency during each cycle of RNA replication), RNA virus genomes are generally much smaller (around 10,000 nucleotides) than DNA virus genomes (between 3000 and 1,500,000 bp). Moreover, among the terrestrial viruses, RNA viruses outnumber DNA viruses by a ratio of 3:1. Hence, it is not surprising that the chemical synthesis of viruses has so far targeted predominantly RNA viruses (Table 1). The expected revolution in DNA synthesis, and exciting novel strategies for manipulating and assembling large synthetic DNA molecules (Gibson et al., 2010), will likely result in the chemical syntheses of many more DNA viruses.

Table 1 summarizes examples of synthetic viruses in roughly chronological order. The question lingers: what was/is the purpose of synthesizing viruses independently of their natural isolates? Most importantly, chemical synthesis of viral genomes allows investigation of the structure and function of the organism's biology to an extent hitherto impossible. However, different virus synthesis projects have had different objectives that we will only briefly mention. Wimmer and Paul, 2011 have discussed most of these projects in some detail.

Syntheses I and II (Table 1) served as proof of principle. Regrettably, in synthesis I all discussion of societal implications of the work and possible applications were cut by the *Science* editors (Cello et al., 2002; Wimmer, 2006). The generation of phage PhiX174 described in Synthesis II "improved upon the methodology and dramatically shortened the time required for accurate assembly of 5–6 kb segments of DNA from synthetic oligonucleotides"—the entire synthesis of the phage consumed only 2 weeks (II, Table 1).

Syntheses III, VI, VII, and IX served to identify beyond doubt the history, identity and/or pathogenesis of important human pathogens (Wimmer and Paul, 2011). These include most notably the 1918 Spanish Flu Influenza virus, an organism that disappeared in the years after the devastating pandemic of 1918/1919 (III); the infectious Simian Immunodeficiency Virus SIVcpz (VII); and the infectious Bat SARS-Like CoronaVirus (IX). The latter studies "demonstrated the usefulness of genetics and whole-genome synthesis in the investigation of

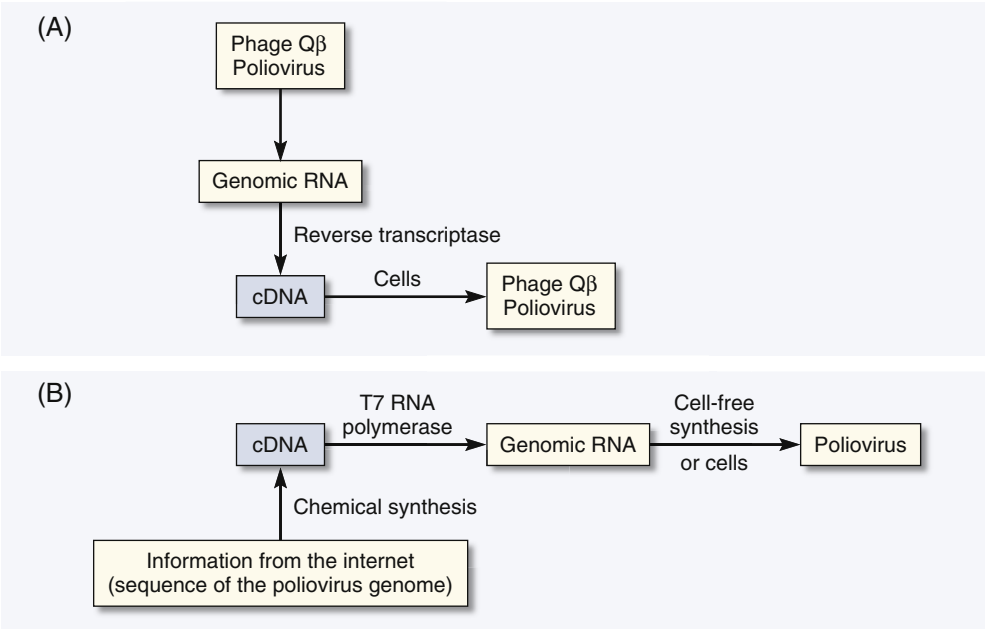

FIGURE 1 Two different strategies for generating RNA viruses via complementary DNA (cDNA) intermediates. (A) Synthesis of cDNA catalyzed with reverse transcriptase followed by transfection of the cDNA into host cells (Taniguchi et al., 1978). (B) Chemical synthesis of cDNA followed by transcription and incubation of the infectious viral RNA in a cell-free extract. *Modified from Ref. Cello et al. (2002).*

the trans species movement of zoonoses." The reconstitution of the Infectious Human Endogenous Retrovirus HERV-K (VI) from remnants in our human genome may yield valuable clues to the impact of endogenous retroviruses on human evolution.

Syntheses IV, V, VIII, X, XIII, XV, and XVI describe "designer viruses" in which large segments (20–40%) of the genome have been recoded with the purpose of either studying function (IV) or modifying expression of viral genetic elements. The latter has focused on expression of proteins by recoding open reading frames either through changing codon bias (V) or codon pair bias (VIII, X, XIII, XV, XVI). The authors of this commentary view codon pair deoptimization as a promising new strategy to design new vaccine candidates (VIII, Table 1). In synthesis XII, the entire ORF of the poliovirus genome was recoded, thereby mutating 1304 of 6249 nucleotides encoding the polyprotein. This has led to the discovery of two redundant RNA regulatory elements involved in RNA replication. In synthesis XI, seed virus for the development of vaccines was synthesized to avoid licensing problems by regulatory authorities. This proved to be an important bypass for creating a commercial product targeting human disease.

Finally, Cooper reported the synthesis of tobacco mosaic virus, TMV (synthesis XIV), which was the first plant virus originally discovered by Beijerinck in 1898. TMV is one of the most researched viruses of all time yet Cooper chose to title his publication: "Proof by synthesis of Tobacco Mosaic Virus." Curiously, he refers to the classical code of chemists who considered synthesis the ultimate proof for any deciphered chemical structure. Cello and his colleagues also made reference to this code (Cello et al., 2002), which will be significant in "proof reading" by synthesis the nucleotide sequences deposited in sequence databases (see also ref. 7).

We conclude by reminding the reader that synthetic biology represents a dual use dilemma in which the same technologies can be used legitimately for the benefit of humankind and misused for terrorism, a quandary referred to as "dual use research" (Wimmer and Paul, 2011; National Research Council, 2014). Nothing better matches this definition than the template-free synthesis of viruses. On the one hand, it advances our understanding of these organisms and leads to new methods to protect us from viral disease; yet, on the other hand it could be exploited with malicious intent. With optimism and enthusiasm, we predict that future applications of synthetic virology will be vastly more constructive than destructive.

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Chapter 22.6

Precision Medicine: Applications of Genetics to Pathogenesis and Treatment of Viral Diseases

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Genome-wide association studies (GWAS) and high-throughput DNA sequencing, often referred to as next generation sequencing (NGS), offer exciting new opportunities for the diagnosis and treatment of viral disease. These evolving technologies are leading to a new era of precision (also known as personalized) medicine. This approach is based on the fact that common variants in host genomes may have profound influence on the course of viral infections and the response to treatment. Two examples illustrate the potential of precision medicine: (1) host and viral genome variation in determining response to current standard of care for hepatitis C; and (2) use of NGS as a diagnostic test to identify microbial pathogens.

1. HOST GERMLINE VARIATION PREDICTS RESPONSE TO THERAPY IN HEPATITIS C

Until recently, the mainstay for standard of care for Hepatitis C had been antiviral drugs that required 48 weeks of therapy with pegylated interferon (PegIntron) and ribavirin, which were associated with significant side effects. Some patients achieved sustained viral response (SVR) with PegIntron/ribavirin, but many did not. Furthermore, patients of African-American and Hispanic descent did not respond as well, and it had not been possible to identify those individuals among all populations who were most likely to respond.

GWAS comparing responder and nonresponder populations identified a single nucleotide polymorphism near the interferon responsive gene IL28B that segregates with SVR and explains a significant proportion of the variation in response (Ge, D et al. Nature (2009) 461: 399). Patients homozygous for the C/C nucleotide showed a 78% successful outcome while those homozygous for the T/T nucleotide had a 26% successful outcome. The T/T genotype was more frequent in African-Americans, explaining their lower response rate.

These data are not new and therapy for hepatitis C is rapidly evolving toward potential cures. However, even with newer RNA polymerase and protease inhibitors, there will be variation in response based on host and viral genotype. Thus, use of GWAS is likely to have value in responder stratification for treatment of hepatitis C, and also for other virus diseases.

2. GENETIC VARIATION IN PATHOGENS TO PREDICT RESPONSE TO THERAPY: HEPATITIS C GENOTYPE AS AN EXAMPLE

There are six Hepatitis C sequence-specific genotypes, and these variants differ in their response to newly available antiviral therapies. These include viral RNA polymerase inhibitors such as sofosbuvir, and protease inhibitors such as simeprevir that impair viral entry into mammalian cells. As shown in Table 1 below, Hepatitis C genotype determines the best therapeutic strategy. Hepatitis C thus provides a valuable case study of how both host and pathogen genetic variation influence treatment choices and outcomes.

TABLE 1 Hepatitis C Viral Genotype Determines Therapeutic Approach to Treatment

Population	Recommended Regimens	FDA Approved?	Alternative Regimens	FDA Approved?
Genotype 1 interferon eligible	Sofosbuvir 400 mg daily + PEG/RBV × 12 weeks	Yes	Simeprevir 150 mg daily + PEG/RBV × 12 weeks then PEG/RBV × 12 weeks (genotype 1b or 1a without Q80K)	Yes
Genotype 1 interferon ineligible	Simeprevir 150 mg daily + sofosbuvir 400 mg daily + (with or without RBV) × 12 weeks	No	Sofosbuvir 400 mg daily + RBV × 24 weeks	Yes
Genotype 2	Sofosbuvir 400 mg daily + RBV × 12 weeks	Yes	None	
Genotype 3	Sofosbuvir 400 mg daily + RBV × 24 weeks	Yes	If interferon eligible: Sofosbuvir 400 mg daily + PEG/RBV × 12 weeks	No
Genotype 4 interferon eligible	Sofosbuvir 400 mg daily + PEG/RBV × 12 weeks	Yes	Simeprevir 150 mg daily + PEG/RBV × 12 weeks Then PEG/RBV × 12–36 weeks	No
Genotype 4 interferon ineligible	Sofosbuvir 400 mg daily + RBV × 24 weeks	No		
Genotype 5 or 6	Sofosbuvir 400 mg daily + PEG/RBV × 12 weeks	No	PEG/RBV × 48 weeks	Yes

3. USE OF NGS FOR IDENTIFICATION OF MICROBIAL PATHOGENS: IS IT POSSIBLE TO MOVE TO "ONE TEST" MICROBIAL DIAGNOSTICS WITH NGS?

Infectious disease has been a flagship for personalized diagnostics in medicine, with diverse strategies for identification of the microbial pathogen and sensitivities to antibiotics. In many cases, these diagnostic tests take days—for example at least 48 h of growth of blood cultures, followed by additional days required to culture the organism and determine sensitivities.

For GWAS, bodily tissues or fluids are obtained, and a DNA library is prepared representing all DNA in the sample—both host and microbial pathogen. Unbiased massively parallel sequencing is performed. Human DNA sequences are removed from consideration, and microbial sequences are aligned with a comprehensive database containing DNA sequences for all known human microbial pathogens. This enables rapid identification of the organism, and often predicts sensitivity to antibiotics.

The following example focuses on the use of NGS to identify a bacterial pathogen in an immune compromised host. However, viral sequences of nonpathogenic strains were also identified, and demonstrate that this approach should be equally applicable for rapid diagnosis of viral pathogens.

A 14-year-old boy with severe combined immunodeficiency disease developed fever and progressively worsening headache. A comprehensive diagnostic work-up including blood cultures, serology, and cerebrospinal fluid (CSF) was negative. Laboratory data were consistent with either a viral meningitis or potential autoimmune encephalitis, and he was discharged without antibiotic therapy.

The patient subsequently progressed, and was readmitted with progressive severe neurological disease. At this point, with informed consent, cerebrospinal fluid was obtained for unbiased massively parallel DNA sequencing. A total of 475 sequences among 3,063,784 total reads corresponded to the spirochete Leptospira, known to be sensitive to penicillin. NGS also identified several other microbes, including nonpathogenic viral species such as Anelloviridae, demonstrating the ability to detect viral sequences. After a total of 7 days of high-dose penicillin, the patient showed evidence of clinical improvement, and was ultimately discharged home with nearly full recovery to his premorbid state.

This single case report suggests the possibility of a future where a single test—unbiased massively parallel sequencing of relevant tissue or bodily fluid—could be used for rapid identification of microbial pathogens and sensitivities. It seems unlikely that this approach would supplant all conventional approaches to microbial pathogen detection. However, it may be a useful adjunct in cases such as this one. And to be provocative, it is conceivable that NGS could ultimately be the "single test" performed for microbial identification and sensitivity testing.

Chapter 22.7

Oncolytic Viruses

Stephanie L. Swift, David F. Stojdl

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David Stojdl is a senior scientist at the Children's Hospital of Eastern Ontario Research Institute and an Associate Professor at the University of Ottawa, Canada. He has a long-standing interest in oncolytic viruses, and has translated them into clinical immunotherapies as a co-founder of both Jennerex Biotherapeutics and Turnstone Biologics.

Viruses that selectively replicate in and lyse cancer cells, but leave normal cells intact, are known as "oncolytic." In fact,

oncolytic viruses (OVs) are multimodal therapeutics capable of not only lysing cancer cells, but also modulating the tumor microenvironment and collapsing tumor vasculature. This ability to engage multiple therapeutic pathways has clear potential benefits, since cancer treatments designed to apply therapeutic pressure against a single tumor target can trigger an antigenic shift and, ultimately, the development of tumor resistance.

Only relatively recently have we begun to understand that the immune responses triggered by OVs also have a critical role in their antitumor efficacy. Consequently, strategies designed to enhance OV immune engagement represent a key research focus.

Wild-type OVs are ideal agents to shift the tumor immune microenvironment from tolerogenic to antigenic, lysing tumor cells to create inflammatory conditions loaded with damage- and pathogen-associated molecular signals whose level increases as replication proceeds. Indeed, replication is required for efficacy, since nonreplicating viruses and virus-like particles typically fail to achieve tumor control. Ultimately, replicating OVs represent a self-limiting infection, since susceptible tumor cells are a finite population whose availability declines as oncolysis begins.

Systemically delivered OVs can target both primary and disseminated metastatic disease (Breitbach et al., 2011; Russell et al., 2014), and can penetrate both peripheral and lymphoid compartments, unlocking the potential to activate different subtypes of memory CD8+ T-cell responses. While the duration of therapy from systemic OV infusion can be limited by pre-existing antiviral immunity, the de novo development of functional neutralizing antibodies against some OVs is surprisingly delayed, allowing virus to survive in the bloodstream for over a month during repeat intravenous administrations. Expanding the clinical development of such self-protective viruses has clear therapeutic value.

Immune responses are generated not only against the OV itself (ultimately leading to virus control, an important safety feature), but also against the virus-infected tumor cells. Indeed, OV-activated antitumor immunity can mediate objective clinical responses: in phase III trials with Amgen's T-VEC, a modified oncolytic herpes simplex virus encoding GM-CSF, tumor regression was observed both in directly injected tumors and distant noninjected tumors that harbored no detectable virus (Kaufman et al., 2010). Such immune activation can culminate in the establishment of memory populations that protect against tumor rechallenge. Finding new ways to enhance CD8+ T-cell memory formation will be critical for establishing long-term durable responses in patients.

One exciting approach that magnifies the differential immune response in favor of tumor over viral targets encodes a tumor-associated antigen (TAA) into the OV genome to create a bona fide OV vaccine (Bridle et al., 2010). Oncolytic vaccines can be designed to expand naturally established memory CD8+ T cells that recognize tumor-specific targets. For example, human cytomegalovirus (HCMV) antigens have been detected in several tumour types, including glioma and neuroblastoma, and approximately 70% of cancer patients have HCMV-specific memory CD8+ T cells established during a past infection that can be activated by an oncolytic vaccine encoding HCMV TAAs. Alternatively, tumor-reactive T cells can be artificially primed with another vaccine vector expressing a shared TAA, followed by OV infusion in a "prime-boost" regimen (Bridle et al., 2010).

Boosting immune system engagement will continue to be a central theme in the field of oncolytic virotherapy. While OVs can naturally condition the tumor microenvironment to attract T cells and maintain their local activity (Nishio et al., 2014), pairing OVs with other immunomodulatory therapies to promote antitumor immune responses is an exciting approach. Partner therapies may range from the more traditional chemo- and radiotherapies to cutting edge techniques, such as antibody-mediated immune checkpoint inhibition and adoptive cell therapy (ACT). Pairing an OV with anti-CTLA4 has already been shown to improve the control of metastatic melanoma beyond that observed with either treatment alone (Zamarin et al., 2014). Similarly, ACT has shown clinical benefit as a stand-alone treatment for multiple malignancies, and is now showing preclinical promise in combination with OVs (Rommelfanger et al., 2012). While either therapy alone may fail, coadministration of both can compensate for solo deficiencies and render heterogeneous tumors susceptible to therapy.

Oncolytic virotherapy represents an exciting approach for the treatment of malignant disease. Further efforts are needed to consolidate the ability of OVs to (1) activate immune cells against appropriate antigenic tumor targets, (2) improve recruitment and infiltration of immune cells into the tumor microenvironment, and (3) maintain their in situ activity. New therapeutic targets and strategies continue to be uncovered by host-virus screening programs (Mahoney et al., 2011), or informed by mathematical models (Bailey et al., 2013; Le Boeuf et al., 2013). Greater efforts to map the complex interactions between OVs and their host cells, in the context of an expanding immune response, will uncover new therapeutic opportunities. These initiatives will undoubtedly generate anticancer responses that not only substantially enhance tumor regression, but also extend tumor-free survival by protecting against regrowth and relapse.

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Chapter 22.8

Prions and Chronic Diseases

Stanley B. Prusiner

Stanley Prusiner is Director of the Institute for Neurodegenerative Diseases and professor of neurology at the University of California, San Francisco. Prusiner discovered prions—proteins that cause neurodegenerative diseases in animals and humans, for which he received the 1997 Nobel Prize in Physiology or Medicine.

Prions are proteins that adopt alternative conformations, which become self-propagating (Prusiner, 2013). Generally, one conformation is rich in β-sheet, a conformation that is prone to polymerization into amyloid fibrils.

Looking into the future of prion biology and diseases, a rich universe of previously unknown biology is beginning to emerge (Prusiner, 2014). The number of different physiological prions, particularly in mammals, is steadily increasing. Physiological prions play a role in various normal functions, ranging from long-term memory to innate immunity to metabolic adaption to fungal incompatibility (Xu et al., 2014). Similarly, the list of neurodegenerative diseases caused by prions is expanding (Jucker and Walker, 2013; Prusiner, 2013). Over the last 5 years, evidence has continued to accumulate, arguing that Alzheimer's and Parkinson's diseases as well as multiple system atrophy, the tauopathies, and Huntington's disease are caused by prions (Stöhr et al., 2014; Watts et al., 2013, 2014). It seems likely that in addition to Huntington's disease, some of the other polyQ (polyglutamine) disorders including the spinocerebellar ataxias will be prion diseases.

Several important new concepts have emerged from the study of prions. First, prions create a novel mechanism whereby physiological functions can be regulated almost instantaneously, by shifting the conformation of a protein from one structural state to another (Prusiner, 2014). Second, prions often feature in diseases where a particular protein accumulates inside cells, as found in neurofibrillary tangles, Lewy bodies, glial cytoplasmic inclusions, and nuclear inclusions. In other disorders, prions accumulate outside of cells, like the plaques in Alzheimer's and Creutzfeldt–Jakob diseases. Third, strains of prions with different phenotypes represent distinct conformations of these alternatively folded proteins. Fourth, the late onset of heritable neurodegenerative diseases seems likely to be explained by the conversion of the mutant causative protein into a prion as the precipitating event. Fifth, prion diseases are age dependent; this is likely due to the protein quality control machinery, which slowly becomes less efficient as organisms age.

While many explanations for late onset, heritable neurodegenerative diseases have been offered to explain their manifestations in the fifth, sixth, or even seventh decade of life, it seems more likely that the initial event is the formation of a sufficient number of prions to stimulate sustainable self-propagation (Prusiner, 2013). Although mutations in patients with familial neurodegenerative diseases have been demonstrated to cause these disorders by genetic linkage studies, explaining the late onset of these illnesses has remained problematic. One explanation is that a stochastic event results in a sufficient number of prions accumulating to initiate a sustainable infection. With aging, an increase in the frequency of random events that produce prions, in tandem with a decline in the protein quality control machinery, conspire to produce sustainable prion infections. This mechanism is applicable to both the inherited and sporadic prion diseases.

These new concepts may offer some novel approaches to developing both early diagnostics and effective therapeutics. Currently, there are no drugs that halt or even slow any neurodegenerative disease (Prusiner, 2014). Developing PET (positron emission tomography) reporters that can be used to establish the diagnosis early in the course of disease will be critical. Accurate and early diagnoses are likely to be critical in choosing appropriate therapeutics.

Although attempts to develop effective therapeutics for Alzheimer's and Parkinson's diseases have been both costly and unsuccessful, the discovery that these diseases are caused by prions offers new strategies for drug discovery. Notably, some point mutations have been found in the PrP protein that causes Creutzfeldt–Jakob disease, which have been shown to be dominant negatives (Prusiner, 2013). Deciphering the structural changes that such mutations initiate may give important insights that could inform novel therapeutic approaches. Drugs have been developed that extend the lives of wild-type and transgenic mice inoculated with mouse-passaged scrapie prions and chronic wasting disease prions, respectively (Berry et al., 2013), which gives promise of future therapeutics.

How many systemic diseases will be found to be caused by prions is unknown. Certainly, there is much interest in the possibility that adult-onset type II diabetes may be caused by prions. In such patients, the β-islet cells are often filled with amyloid fibrils composed of the protein amylin.

The area of prion biology and diseases is certainly "ripe" for increased investigation. Our knowledge of most physiological prions is in its infancy. Learning how such prions propagate is likely to offer novel approaches to therapeutics for such diseases like Alzheimer's and Parkinson's that are already prevalent and predicted to increase as human life expectancy continues to rise.

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Chapter 22.9

Pathogenesis Research and the HIV/AIDS Pandemic

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The global HIV/AIDS pandemic continues to exact an enormous toll, claiming 1.2 million lives in 2014 alone and 34 million since AIDS was recognized more than three decades ago. Worldwide, 37 million individuals live with HIV/AIDS. Despite these daunting statistics, the global deployment of proven treatment and prevention strategies has slowed the onslaught of HIV/AIDS, with both incident infections and deaths falling by more than one-third over the past decade. These successes did not come easily. They were the result of decades of innovation beginning with fundamental basic research that led to successful interventions. Indeed, basic research on HIV/AIDS is inexorably linked to the development of effective interventions for the disease. In this regard, the development of new tools for the

treatment and prevention of HIV has been facilitated by a detailed understanding of HIV pathogenesis.

Studies on viral pathogenesis underpin all HIV research, but perhaps most tangibly the field of therapeutics. Detailed knowledge of the replication cycle of HIV provided the first targets for antiretroviral drugs (ARVs). The first FDA-approved ARV, the nucleoside analog zidovudine, targeted the reverse transcriptase enzyme, a critical component of the HIV replication cycle that converts viral RNA to proviral DNA, thus allowing integration into the host cell genome. Zidovudine was first synthesized as an antineoplastic agent years prior to the discovery of HIV and was found to have activity against HIV during a screening process. As knowledge of the HIV replication cycle improved, new classes of therapeutic agents were developed. Notably, inhibitors of the HIV protease and integrase enzymes were synthesized and optimized based on detailed crystal structures of the target proteins. Similar investigations have yielded more than 30 licensed ARVs and ARV combinations, all dependent on an intimate knowledge of the viral replication cycle. By March of 2015, 15 million people were receiving ARVs, averting an estimated 7.8 million deaths between 2000 and 2014 (UN Joint Programme on HIV/AIDS (UNAIDS), 2015).

ARVs have similarly revolutionized HIV prevention, notably through prevention of mother-to-child transmission (PMTCT) programs, which averted 1.4 million infections since 2000 (UN Joint Programme on HIV/AIDS (UNAIDS), 2015). ARVs have also proved effective when used for pre-exposure prophylaxis (PrEP) for uninfected individuals, and as "treatment as prevention" or TasP when taken by HIV-infected people. Oral PrEP demonstrated more than 90% efficacy in preventing viral acquisition when taken as prescribed (Haberer et al., 2013). The landmark study HPTN 052 demonstrated the value of TasP, whereby antiretroviral therapy (ART) given to the infected partner in a serodiscordant couple lowers his or her viral load, thus reducing the risk of transmitting the virus to the uninfected sexual partner by 96% (Cohen et al., 2011). These ARV-based prevention modalities, combined with other interventions such as condoms and voluntary medical male circumcision, provide the building blocks of comprehensive prevention programs.

Understanding the pathogenic mechanisms of HIV infection and the details of the immune response to the virus are also critical to the development of a safe and effective vaccine to prevent HIV infection. In this regard, the development of an effective HIV vaccine is a formidable challenge due to the fact that the natural immune response to HIV is inadequate in controlling and certainly in eliminating the virus. Decades of disappointing clinical trials, first with vaccines aimed at the induction humoral immunity and then cell-based, "T-cell" vaccines, failed to produce an effective immune response. The RV-144 trial in Thailand offered the first signs of clinical efficacy, with a 31% reduction in viral acquisition (Rerks-Ngarm et al., 2009). The RV-144 regimen (canarypox vector prime, recombinant gp120 boost) appears to have elicited non- or weakly neutralizing antibodies against the V1V2 region of the envelope trimer. RV-144 represented the first moderately successful study of an HIV vaccine candidate in humans and reinvigorated the field of HIV vaccinology.

Simultaneously, researchers have deepened their understanding of the immune response to HIV, discovering broadly neutralizing antibodies (bNAbs) induced over the course of chronic infection. It is curious that these bNAbs are produced by only a minority of infected individuals (~20%), and usually after 2 or more years of infection (Liao et al., 2013). Serial blood samples taken from an acutely infected donor revealed the coevolution of viral mutations and a broadening humoral immune response to the virus. At the end of more than 2 years, a hypermutated, broadly reactive anti-HIV antibody evolved together with a highly mutated virus that was continually trying to escape the immune response. Therefore, in trying to evade the evolving antibodies, the virus ultimately stimulates bNAb production, a paradox of HIV immunology. Current research is aimed at determining if these bNAbs can prevent or treat HIV infection. If successful, the major challenge in HIV vaccinology will be to induce these antibodies via appropriate immunogens. Recently, the potential for bNAbs, whether infused directly or produced in vivo by gene inserts administered via viral vectors, to prevent or treat infection has been demonstrated in animal models (Balazs et al., 2014; Shingai et al., 2013; Barouch et al., 2013). In this regard, vaccinologists are attempting to recapitulate in a more rapid and expeditious manner the natural immune response seen in the minority of infected individuals through prime-boost vaccination regimens. Specifically, they are identifying viral envelope epitopes that induce bNAbs in natural infection, and expressing those as immunogens to induce B-cell maturation toward bNAb-producing cells. Thus, the understanding of the complexities of HIV pathogenesis has offered new hope for a moderately effective HIV vaccine.

Research toward a cure for HIV infection is also closely linked to an understanding of HIV pathogenesis. In pursuing a cure, it is important to first define the goal of the work. Simply put, a "cure" is an indefinite remission of disease following cessation of ART. In HIV, this could come in one of two forms—viral eradication or sustained virologic remission (SVR); for the latter the virus would remain at low levels in the absence of daily ART. The now famous case of the "Berlin Patient" offers some evidence that eradication is possible. Still, reliance on hematopoietic stem-cell transplant is unlikely to be feasible and certainly will be risky for the majority of infected individuals. Novel scientific approaches have employed gene-editing techniques to engineer ex vivo mutations into the CCR5 coreceptor for HIV on autologous T cells followed by re-infusion into the

donor host, thus rendering them "resistant" to HIV infection (Tebas et al., 2014). These efforts are at an early stage of discovery; however, they merit attention.

The case of the "Mississippi Child" offers evidence that an SVR is possible. The infant was infected with HIV in utero and started on ART within 30 h of birth. ART was continued through 18 months at which point the child was lost to follow up and stopped treatment. When the child represented to care 5 months later, there were no traces of replication competent virus, a status that persisted for 27 months before the virus ultimately rebounded (Persaud et al., 2013; National Institute of Allergy and Infectious Diseases (NIAID)). The case indicates that treatment soon after infection can minimize, but not eliminate HIV reservoirs resulting in long-term SVR. Translating this experience into adults could be possible, as evidenced by 14 patients in France treated during acute HIV infection who maintained an SVR after treatment interruption (Sáez-Cirión et al., 2013). However, these results need to be confirmed in additional studies. Establishing an SVR in the absence of continual ART for a broader population will likely require adjuvant therapies such as therapeutic vaccines or passive infusion of bNAbs following the initial suppression of viremia.

In summary, the development of effective interventions for the prevention and treatment of HIV infection, critical to any hope of controlling and ultimately ending the HIV/AIDS pandemic, is heavily dependent on an in-depth understanding of the viral and immune pathogenesis of HIV disease.

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Chapter 22.10

The Future of Viral Vaccines

Gary J. Nabel

Gary Nabel is Chief Scientific Officer, Sanofi, a global pharmaceutical company. Dr Nabel served as Director of the Vaccine Research Center of the National Institute of Allergy and Infectious Diseases, 1999–2012, where he guided research on the development of novel vaccine strategies against HIV and other emerging infectious diseases.

Progress in understanding the pathogenesis of viral infections has stimulated innovative approaches to the development of vaccines. This work builds upon insights from the basic sciences, including virology, microbiology, immunology, molecular biology, and genetics. In addition, advances in biotechnology are generating alternative platforms to elicit specific immune responses that facilitate

the development of next-generation vaccines. Finally, the tools of molecular medicine provide new insights into immune responses induced by vaccines, as well as associated adverse reactions. This understanding will accelerate clinical vaccine development and the identification of biomarkers that predict a successful protective response. Together, the underlying science lays the groundwork for the development of promising vaccines that can be both safe and efficacious in protecting against a variety of pathogens.

1. BUILDING ON BASIC SCIENCE

In the basic sciences, our understanding of T- and B-cell differentiation, as well as the details of antigen recognition and signaling, is leading to insights into the pathways of immune maturation. Recent research has also helped to identify structural features of antigens that are required to elicit a specific immune response. For example, it is now possible to identify the determinants of antigens that will engage germ-line B cells and promote their differentiation into memory B cells that produce broadly neutralizing antibodies to influenza (Lingwood et al., 2012). Similarly an understanding of T-cell biology, both maturation and signal transduction following interactions with antigen-presenting dendritic cells, have pointed to rational approaches to immunogen design. This has led to improved adjuvants, and ways to modulate the balance of Th1 or Th2 immunity. Progress in understanding innate immunity, including the TLR signaling, interferon activation and Rig-I stimulation provide tools to selectively activate or suppress these pathways as needed. In the future, the ability to develop small-molecule agonists or antagonists against these or immune-suppressive targets, such as checkpoint or TGF-beta inhibitors will further empower these efforts.

The ability to utilize biologics therapy, such as monoclonal antibodies, nanoparticles, or peptides, provides diverse and more effective ways to elicit durable T-cell responses that mediate protective cellular immunity. These approaches will increase the likelihood of success against many challenging infectious disease agents, such as cytomegalovirus, Ebola and Marburg viruses, or HIV-1. Similarly they can be harnessed to elicit protective antibody responses. Such tools could enable the development of a universal influenza vaccine that will better protect public health against strain drift and the emergence of new influenza virus pathogens from animal reservoirs.

Advances in DNA sequencing have already enabled more rapid and rational responses to evolving outbreaks and to the identification of the mutations that render vaccines ineffective. Such genetic analysis is now routinely performed for HIV-1, influenza, and Ebola viruses. As more is understood about the structural implications of these mutations, rational design of new vaccines to counter viral resistance will be enhanced. In addition, as the molecular epidemiology is further understood, it will become increasingly possible to develop preemptive vaccination strategies, as proposed in the past for avian influenza (Yang et al., 2007). This will be based on an understanding of viral evolution, human immunity, and predictive patterns of viral mutation. In addition to genetic sequencing of pathogens, much will be learned from the genetic polymorphisms of humans. When human genome sequencing becomes affordable and routine, it will undoubtedly provide insights into optimizing immunogenicity. It will also help to probe the causes of adverse responses that may limit the use of some vaccines or immunotherapies.

2. IMPROVEMENTS IN VACCINE DELIVERY

Viral pathogenesis, particularly the study of viral assembly, has contributed to a better understanding of synthetic biology, which will facilitate new approaches to the production of safe and more effective vaccines. The increased success of virus-like particles as vaccines is encouraging, because the immunogen closely resembles native virus and elicits effective immunity while the particle is unable to replicate and cause adverse responses. Next-generation improvements, involving the use of synthetic biology and nanotechnology (Kanekiyo et al., 2013), are expected to create scaffolds and antigen presentation surfaces that will better expose specific epitopes on viral protein. This will make it possible to target responses to subdominant epitopes not normally recognized in the immune response. These advances may permit targeting of highly conserved and vulnerable structures—normally protected by the virus—that can induce broad protective responses immune responses (reviewed in refs (Nabel and Fauci, 2010; Nabel, 2013)). Finally, as the methodology for expressing engineered molecules improves, it is increasingly possible to modulate immune function through the expression of novel molecules, or through genetic delivery of antibodies (Johnson et al., 2009; Balazs et al., 2011). Bi-specific antibodies allow for dual targeting against different epitopes on the surface of pathogens (Byrne et al., 2013). They also provide a mechanism to redirect immune cells that normally do not respond to a specific antigen. The tool of gene delivery can be used to produce selected antibodies in subjects who cannot be induced to make them. For example, this will enhance the induction of neutralizing influenza antibodies in the elderly (Limberis et al., 2013; Balazs et al., 2013), or by modulating the specificity of T cells to recognize antigenic determinants using chimeric antigen receptors for cancer vaccines (Jensen and Riddell, 2014). While the T-cell chimeric antigen receptor approach has currently been directed to cancer immunotherapy, it may also prove efficacious against infectious disease targets.

3. ADVANCED ANALYTICS AND THE HUMAN IMMUNE RESPONSE

The ability to interrogate human immune responses has expanded greatly in recent years. Powerful technologies—such as flow cytometry and nanofluidics—now enable detailed qualitative and quantitative interrogation of human immune responses. Similarly, the ability to perform deep sequencing of selected tissues or immune cells provides insight into the ontogeny of the adaptive immune response. At the same time, modern imaging techniques have been applied in vivo and are facilitating an understanding of the trafficking of these cells in response to specific stimuli. This information will lead both to a better understanding of the mechanisms of immune protection as well as to the definition of correlates of immunity against specific pathogens. It will also facilitate the development of new vaccines, or improvements of existing vaccines, which will confer broader reactivity and fewer adverse effects in the large numbers of people who can benefit from them.

Finally, it is important to recognize that global surveillance of viral infections, as well as rapid international vaccine distribution, will be greatly assisted by advances in pathogen detection, molecular definition of microbial resistance, and infectious disease surveillance. The ability of electronic and Web-based monitoring will facilitate efforts to distribute vaccines to populations at risk, particularly when unexpected outbreaks occur. The recent epidemic spread of Ebola virus in western Africa highlights the rapidity with which emerging pathogens can spread across international borders. Vaccines represent an essential tool to counter the pandemic spread of infectious diseases and preserve the public health. The welfare of people throughout the world has become increasingly dependent on our ability to provide effective countermeasures against emerging infectious disease threats. In the future, the more effective and efficient development of vaccines, as well as more timely distribution, will increasingly protect the public against these pathogens.

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Chapter 22.11

Emerging Viruses

W. Ian Lipkin

W. Ian Lipkin is the John Snow Professor of Epidemiology, Professor of Neurology and Pathology, and Director of the Center for Infection and Immunity at Columbia University. He pioneered the use of molecular methods in identifying viruses and other infectious agents in acute and chronic diseases, and in responding to infectious disease outbreaks including West Nile encephalitis, SARS, MERS, and Ebola.

In the late 1990s, I attended a retirement symposium for an eminent virologist at which a speaker of the same vintage bemoaned the end of the golden age of virology. In a

more recent symposium honoring the life of the late Hilary Koprowski, a nonvirologist speaker asked whether viruses are alive. As you are reading the epilogue of this book, you would have firm evidence to dispute the waning status of virology and to respond "no" to the second. You would likely add that although viruses are not living things, they are critical to life. Viral sequences comprise 8% of our genomes and are far from inert. The endogenous retroviral element syncytin, for example, is essential for placental development and embryo survival (Mi et al., 2000). The field of medical virology is alive and vibrant. New viruses continue to emerge, posing threats to public health, food security, and commerce. Viral databases are rapidly expanding as investigators survey animals and environments using ever more efficient and inexpensive sequencing platforms. With the introduction of new antiviral drugs, therapeutic antibodies and vaccines, viral diagnosis has become more than an arcane academic exercise. Evidence is mounting that viral infections contribute to chronic diseases including neurodevelopmental disorders and some forms of cancer. Viruses have been harnessed in oncology (Miest and Cattaneo, 2014) and gene replacement therapy. On a global scale, there is growing appreciation that viruses contribute to elemental cycling and oceanic carbon sequestration through effects on phytoplankton (Suttle, 2007). Given the allotted space and the focus of this book on pathogenesis, I can only touch on a few predictions with medical applications but encourage the reader to think more broadly about the implication of viruses and virology.

Globalization of travel and trade, loss of wildlife habitats, growth of megacities, mass migrations due to economic privation and political instability and changes in the distribution of mosquitoes due to climate change will continue to enable the emergence of viruses that might otherwise remain sequestered (Lipkin, 2013). We recently estimated that mammals alone harbor more than 300,000 new viruses (Anthony et al., 2013). At present, we have no way of ascertaining from viral sequence data alone which of them poses substantive threats to humans, wildlife, or domestic animals. However, investments in sequencing, bioinformatics, and systems biology may translate into algorithms that allow us to assess potential for host switching and pathogenicity. At minimum, knowledge of which viruses are circulating and where will enable targeted surveillance in populations at risk for exposure. Surveillance will also become increasingly efficient as diagnostic capacity improves in the developing world. It will be critical to address potential concerns regarding sovereignty and intellectual property if we are to ensure that investigators will be amenable to sharing data and isolates. Public enthusiasm for social media may lead to a global viral equivalent of the American Gut Project wherein citizen scientists share fecal samples and data to develop a human bacteriome database linked to information concerning diet, geography, season, health, and disease (http://humanfoodproject.com/americangut/). A key question that remains to be addressed is whether there are commensal or symbiotic viral flora. Do viruses, like bacteria, have a role in priming or regulating the immune system? Do viruses (primarily bacteriophage) regulate the composition or abundance of bacterial flora? If so, is there an optimum viral microflora and how is it established? Can it be modified?

Improvements in diagnostics and insights into the role of viruses in health and disease will provide incentives for the development of antiviral drugs and vaccines. Our armamentarium for herpesviruses, HIV and HCV will expand to include drugs that address not only chronic infections like HPV and HBV that have life threatening sequelae but also acute, self-limited infections (e.g., rhinoviruses) that interfere with activities of daily living and productivity. At present, antiviral discovery typically begins with screening of massive compound libraries. Compounds with activity are then modified and optimized through medicinal chemistry. This brute force approach will ultimately give way to more elegant strategies for rational drug design based in genomics, proteomics, structural biology, and cellular biology. These drugs will target not only the viruses themselves, but also host responses that contribute to viral replication, morbidity, and mortality. Insights into viral biology and evolution and host response will profoundly impact vaccine research. Vaccines will be optimized to expedite the development of protective immune responses, enhance immunity in the very young and the very old, and to increase the duration of protection. Vaccines will target conserved conformational domains to enable immunity to representatives of higher order viral taxa rather than only specific strains. New platforms will be established that facilitate inexpensive, rapid production and atraumatic immunization.

Finally, one wonders what Peter Medawar, reported to have described viruses as bad news wrapped up in protein, would make of the deliberate use of viruses in medicine. Viruses are ideal vectors for intracellular delivery of genetic information. As extracellular and intracellular determinants of tropism are defined, I anticipate that viruses will become increasingly important tools for targeted destruction of neoplastic cells and expression of RNA and proteins that enhance cell function.

In summary, virology is alive and well. As Timbuk3 sang in 1986, "The future's so bright, I gotta wear shades."

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Chapter 22.12

Pandemics: What Everyone Needs to Know

Peter Doherty

Peter Doherty works at the University of Melbourne and St Jude Children's Research Hospital. He has broad interests in virus pathogenesis, latterly influenza, and shared the 1996 Nobel Prize in Physiology or Medicine for discoveries about T-cellmediated immunity. In his spare time, he writes science books for lay audiences, including "Pandemics: What Everyone Needs to Know" and "Their Fate is Our Fate: How Birds Foretell Threats to Our Health and Our World."

Back in 2012 I was invited to write for the *What everyone needs to know* series published by Oxford University Press (Doherty, 2013). The subjects range from: *China in the twenty-first Century*, to *The Catholic Church*, to *Food Politics*, and so on. My charge was: *Pandemics*. I thought about this for a while as, being a laboratory scientist rather than a public health medico or an epidemiologist, I wondered whether I was the right person for the job. But then, perhaps in a triumph of optimism over rationality, I decided that, after working for 50 years on viruses and immunity, I might just have something useful to say. Also, since back in 1996, the Swedes made me more famous than I deserve to be, I have been covering a much broader remit in public lectures, talking to legislators and so forth than was the case when I was just known in my academic field.

It also occurred to me that any informed individual is likely to take a different angle. Moreover, I do know something about the influenza A viruses that are, after all, the most likely cause of future pandemics. Another motivation was that this gave me a chance to talk about how great the science of infectious disease has become over the past decades. I am less than enthusiastic about the fear mongering, "shock horror be afraid" scenarios that drive many books and movies on emerging pathogens. On the other hand, I also wanted to make the case that, in these days of cost-cutting governments, it is essential that we keep our public health services strong and that we continue to fund research on dangerous pathogens at a good level. Perhaps it is our evolutionary history, but we seem more enthusiastic about military spending to keep "bad guys" in their place than we are about preparing for an attack by "bad bugs." It is also important that, with all the media hype about "gain-of-function experiments" we do not impose excessive regulation that discourages talented people from working with exotic viruses.

Writing a "lay" book on such a subject has its challenges. It helped that my medical infectious disease colleagues were happy to talk and review whatever I wrote. Then this particular series is done in a Question and Answer format: it is odd to sit in front of a computer alone, invent questions, and then provide answers. This is probably how paranoids and conspiracy theorists operate! And you are subject to editors trained in a literature rather than science. That led, for example, to a challenging chapter summarizing infection and immunity for a general reader.

One surprise was to find that there is no universal definition of what constitutes a pandemic versus an epidemic. A pandemic alert is sounded for a novel influenza A virus when it spreads between two WHO regions. Look at a WHO map and you will realize this is a pretty arbitrary definition. When an escape mutant of a currently circulating flu virus goes global that is described as a "seasonal" epidemic though, for the same situation with the noroviruses, it is called a pandemic.

The disease I got half wrong was Ebola. Going on past history, my view was that any Ebola outbreak would be jumped on fast and quickly contained. We now know that this is not necessarily so. The lesson: we cannot expect already overstretched missionary and volunteer organizations, like Doctors Without Borders, to handle something like this. Was the dilatory Western response a direct consequence of financial cutbacks? I do not know, but we just did not get enough well qualified "boots on the ground" soon enough. I have also realized that the world needs a new economic model to bring antiviral drugs and vaccines to the post development/human trial phase for potential pandemic risk pathogens, so we can go into immediate, large-scale production in the face of a dangerous outbreak. I am in no doubt that could have been "ready to go" for Ebola, but there just was not the money to do it. This is

a global responsibility, and we cannot expect that "big pharma" will, without financial compensation, take up the challenge.

Pandemics: what everyone needs to know was published in October 2013. Even with all the publicity around the Ebola outbreak, sales "grumble slowly." *China in the twenty-first century* is doing a lot better! It is hard to get people to engage with science and, as anyone who frequents bookstores knows, the whole science section is usually smaller than that for "alternative medicine." I think *Pandemics* is readable, honest, and informative, but it will never hit the sales heights of the "be terrified" genre!

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Chapter 22.13

One Health

Thomas P. Monath

Tom Monath is Chief Science Officer of BioProtection Systems/NewLink Genetics Corporation, where he is developing a vaccine against Ebola virus. His career spans 20 years in vaccine development in the biotechnology industry, preceded by 25 years at CDC and USAMRIID leading research on arbovirus epidemiology and pathogenesis.

One Health is a conceptual framework that seeks to establish "collaborative efforts of multiple disciplines…" including physicians, veterinarians, environmental and climate scientists and others "…working locally, nationally and globally to attain optimal health for people, animals and our environment." While not a new concept, One Health has gained considerable traction in the past 5 years, and has been embraced by academic institutions, professional societies, and governments (AVMA, 2008).

Underlying this momentum is the fact that zoonoses (diseases transmissible from animals to humans) represent ~60% of all infectious pathogens of human beings and 70% of all emerging infectious diseases. At this writing, a horrific epidemic of Ebola virus disease, a virus carried by fruit bats but capable of interhuman contact spread, has riveted the world's attention. Among emerging infections, viral pathogens are over-represented, since these agents are associated with host species having high population turnover and density surges; evolve genetic changes rapidly, permitting adaptation to new hosts and vectors (species jumping); may be infectious at very low doses due to their high replicative capacity; and are often shed in secretions. The importance of zoonotic viral infections to human and animal health, the complexity of virus life cycles, and the multifactorial causes of disease emergence, underlie the need to integrate a variety of scientific disciplines in their study.

In addition to the viruses that are known to infect both animals and humans, there are many viruses that cause disease only in animals or that circulate silently in animals. These include some that are related genetically to human pathogens, such as the hepatitis C-related flavivirus in dogs. Understanding these agents and their natural history can prepare us for new disease emergences and requires collaborative efforts across scientific disciplines.

Viral pathogenesis is a key field of investigation in a One Health approach to zoonotic and emerging diseases. This field encompasses the evolution of viruses, including genetic changes through immune pressure, mutation, recombination, and reassortment that may change transmission, receptor usage and host range, vector competence, and virulence. Examples are too numerous to cite in this brief account, but influenza viruses, SARS coronavirus, New World arenaviruses, and chikungunya virus provide examples. Minor changes in viral genes encoding ligands for cell receptors may result in a shift in cell tropism and host range from an animal reservoir to humans, or a shift in vector competence, causing increased virus transmission to humans. Elucidating the factors underlying such changes requires collaborative efforts of molecular virologists and cell biologists, as well as experts on the responses to infection of individual

organisms and species of vertebrates, insects, and ticks. The same conclusions concerning a multidisciplinary approach apply to understanding disease expression through systems biology. Signal transduction pathways cause pro-inflammatory changes following viral infections, as well as the innate and adaptive immune responses to viral proteins, topics covered extensively in this book.

Animal models are widely used in the study of viral pathogenesis. This is the realm of Comparative Medicine, a distinct discipline of experimental medicine designed to translate information from animal models to human disease, and arguably the clearest example of One Health principles. There are also numerous examples of important diseases only affecting animals that provide model systems for understanding disease emergence and pathogenesis. For example, bluetongue and related orbiviruses (Epizootic Hemorrhagic Disease of deer, and African horse sickness) provide interesting model systems for study of virus movement, chronic infection, hemorrhagic fever pathogenesis, and virus evolution and antigenic variation. Porcine respiratory and reproductive syndrome, an important pathogen of swine, is a model for studies of the molecular basis for viral virulence, which can explain the emergence of epizootics.

A few important animal diseases are mentioned in this book, especially as they relate to analogous diseases in humans (e.g., prion diseases), but the omission of many important examples perhaps reflects the need for a broader, One Health approach to the study of pathogenesis. Similarly, arthropod vectors of viral infections remain a relatively understudied area of viral pathogenesis. Vectors are critical to an understanding of virus transmission, persistence in nature, and evolution. Once again, this illustrates the need to integrate disciplines of entomology and insect taxonomy, physiology, pathology, and ecology.

Prevention and control through vaccines, antiviral therapy, vector control, and other strategies also rely on effective One Health interactions. A recent review emphasizes how vaccine development and utilization can benefit from such interactions (Monath, 2013). Hopefully, in the future, the One Health vision will increasingly inform both basic and applied research in viral pathogenesis.

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Chapter 22.14

Controversial Policy Issues

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Public policy attempts to match the concerns of the public with the work that scientists do. The goal of policy analysis is to inform and—in some cases—influence decision makers, including politicians, research administrators, and companies that develop biomedical products. How does policy intersect with viral pathogenesis, particularly research in this field? In this brief commentary I will focus on some examples where controversy has developed at the nexus of research and public health. Salient issues discussed are: dual use research; biosecurity; gain-of-function experiments; and public policies including those regarding "Select Agents."

As defined by the U.S. National Institutes of Health, "Dual Use Research of Concern (DURC) is life sciences research that, based on current understanding, can be reasonably anticipated to provide knowledge, information, products, or technologies that could be directly misapplied to pose a significant threat with broad potential consequences to public health" (NIH OSP, ND). Dual use research poses a difficult problem for scientists, especially virologists, because it is poorly defined. One approach has

1. The opinions expressed in this commentary are those of the author and not necessarily those of EMBO.

been provided by the Fink Committee report of the US National Academy of Sciences, which delimited dual use research to a fairly specific set of experiments of concern, including experiments that involve microbes (Committee on Research Standards and Practices, 2004).

Others have approached the problem of biosecurity by focusing on the trade-offs between potential benefits and potential risks, including ways to mitigate some of those risks (Garfinkel et al., 2007; AAAS, 2013; Baltimore et al, 2015; Dupres et al, 2015). The idea that benefits should be shared has been accepted, though it remains difficult to implement. But the idea that risks should be equally shared is more difficult to analyze; some work has been done on this problem by ethicists, but policy options for sharing risk remain scarce. Stated broadly, there are clearly risks of doing nothing against the risks of doing something. For example, there is almost no question that we must be doing more in the area of influenza virus research. But what are the trade-offs in researching smallpox, poliovirus, or Ebola?

Going further than naturally occurring microbes, gain-of-function experiments provide a current example of these issues. Recent experiments with influenza virus have sought to identify the genetic determinants of influenza virus that are required to produce a global pandemic. But this work led to an explosion of controversy, whether they should have been permitted in the first place, and in the second place, whether they should be published (Fouchier et al., 2013; Lipsitch and Galvani, 2014). At what level should such experiments be subject to oversight and approval? Is the local institutional biosafety committee sufficient? Are there societal concerns that would be mitigated by having such approvals at a national level?

The National Science Advisory Board for Biosecurity (NSABB) was established by the US government in 2004 to deal with general biosecurity issues. Recently, there has been a shake-up in the composition of the board, and an apparent move away from oversight of experiments, particularly gain of function (Begley, 2014). At the same time, it is clear that the overall safety of laboratories with respect to microbiological work is excellent. How this may change as experiments become more complex and more new researchers enter the field remains to be seen.

One approach to risk mitigation is the control of potential dangerous microbes. In the United States, the Select Agent Program lists viral, bacterial, and toxic agents that require special efforts and oversight (National Select Agent Registry, ND). The lists and accompanying rules were drafted during a time of understandable concern about new terrorist attacks following the events of September 11, 2001. Naturally, this list and accompanying rules is highly controversial. For instance, which agents should be on those lists? Certainly most researchers would agree some kind of additional oversight is important. But should the list of agents be expanded? Using what kind of analysis?

These broad policy issues do not respect borders, and international treaties present interesting and complex policy issues. For example, under the Convention on Biological Diversity (CBD, ND), treaties relating to biosafety broadly defined (Cartagena Protocol, ND) and to access and benefit sharing (Nagoya Protocol, ND), plus technical assessment processes to inform the main Convention (SBSTTA, ND), may have direct impacts on some scientific research. Although, the United States is not a party to the Convention, it is important for scientists to understand the basics of such Conventions if they are doing any work outside of national borders.

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