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Amending Koch's postulates for viral disease: When “growth in pure culture” leads to a loss of virulence

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ABSTRACT

It is a common laboratory practice to propagate viruses in cell culture. While convenient, these methodologies often result in unintentional genetic alterations, which have led to adaptation and even attenuation in animal models of disease. An example is the attenuation of hantaviruses (family: *Bunyaviridae*, genus: *Hantavirus*) when cultured *in vitro*. In this case, viruses propagated in the natural reservoir species cause disease in nonhuman primates that closely mimics the human disease, but passaging in cell culture attenuates these viruses to the extent that do not cause any measurable disease in nonhuman primates. As efforts to develop animal models progress, it will be important to take into account the influences that culture *in vitro* may have on the virulence of viruses. In this review we discuss this phenomenon in the context of past and recent examples in the published literature.

Contents

1. Introduction

Two manipulations commonly performed in virology laboratories may change the phenotype of a virus population. In the first, a virus is deliberately “adapted” to a new host, such as mice, through sequential passage from animal to animal. By recovering virus from diseased animals at each passage and inoculating it into

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a new cohort, researchers impose selective pressure and obtain a virus population more virulent for the new host. In the second setting, researchers "amplify" a virus by preparing a large stock in cell culture, such as Vero cells. Although this procedure is frequently considered only to increase the quantity of virus, some degree of selection will also take place, favoring members of the virus population that replicate best in the chosen cells.

Tissue culture passage may have unexpected results when the amplified stock is used in subsequent experiments, such as attempts to "model" a human disease in nonhuman primates (NHPs). Some viruses, such as Marburg or Ebola, cause a severe illness in NHPs, even when the inoculated agent has previously undergone multiple tissue culture passages. In contrast, when researchers have inoculated NHPs with cell culture preparations of the hantaviruses that cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), little or no illness has been observed. These outcomes have traditionally been attributed to an inherent resistance of NHPs to these viruses, but we have recently found that it was in fact the result of attenuation of the viruses in cell culture (Safronetz et al., 2014). In this article, we examine the possibility that other "failures" of viruses to cause disease in NHPs may have resulted from the inadvertent modification of the agent being studied.

2. Case study - hantavirus infection in nonhuman primates

The development of a NHP model for the study of hantaviral diseases has long been a goal in the field of emerging pathogens (Safronetz et al., 2014). The most prominent disease associated with hantavirus infection is hemorrhagic fever with renal syndrome (HFRS, caused by Old World hantaviruses), which is characterized by fever, renal insufficiencies and coagulation disorders. Several attempts to experimentally recreate the clinical features of HFRS in NHPs demonstrated that a variety of species were susceptible to infection, but did not develop overt signs of disease. After the characterization of hantavirus cardiopulmonary syndrome (HCPS, also referred to as hantavirus pulmonary syndrome (HPS)) and the discovery of highly pathogenic New World hantaviruses in 1993, efforts continued to model hantavirus diseases, but the outcomes were the same: inoculation of NHPs with New World hantaviruses amplified in cell culture resulted in asymptomatic, self-limiting infection (McElroy et al., 2002).

Hantaviruses are notoriously difficult to isolate from the reservoir hosts or diseased humans, and often require multiple blind passages in cell culture to obtain sufficiently high titers for further characterization and experimentation. Interestingly, propagation in cell culture may result in loss of the ability to reliably infect their natural reservoirs (Fulhorst et al., 1997). An example is provided by Puumala virus (PUUV), an etiological agent of a mild form of HFRS commonly referred to as nephropathia epidemica (NE), which is carried by the bank vole (*Clethrionomys glareolus*) (Lähdevirta et al., 1984). Genetic analysis revealed that point mutations in the nucleocapsid and polymerase genes accompanied adaptation to Vero cells in culture (Nemirov et al., 2003). Interestingly, when reintroduced into laboratory-reared bank voles, the Vero-propagated PUUV was unable to reliably establish infection. These findings led to the hypothesis that an accurate NHP model of nephropathia epidemica might require the inoculation of virus derived directly from bank voles, rather than virus propagated in cell culture. The pivotal article by Klingstrom and colleagues demonstrated just that: PUUV prepared from tissues of infected voles caused a mild disease in macaques, including low-grade fever, proteinuria and microhematuria as well as a transient viremia, resembling the human condition (Klingstrom et al., 2002).

Our group took into account these findings in an effort to develop a NHP model of HCPS. We inoculated macaques with Sin Nombre virus (SNV), the primary agent of HCPS in North America, which was derived either from Vero cell culture or directly from tissue homogenates obtained from infected deer mice (*Peromyscus maniculatus*), the natural reservoir of SNV. Analogous to the previous PUUV study, the macaques that were inoculated with deer mouse-derived SNV developed HCPS, with 7 of 10 animals becoming severely ill and requiring euthanasia, with a disease that fully recapitulated the human condition (Safronetz et al., 2014). Similar to the 2002 study by McElroy et al., macaques which received the Vero-propagated SNV experienced only a self-limiting infection without visible signs of illness. Genetically, the SNV viruses utilized in these experiments differed by only a few mutations in the nucleocapsid and polymerase genes. Nevertheless, the loss of virulence associated with Vero cell culture highlights an important and potentially widespread problem in the field of virology.

3. Koch's postulates

Based in part on the earlier perceptions of Jakob Henle, and in consultation with Friedrich Loeffler, Robert Koch devised guidelines to demonstrate that certain human diseases were caused by specific micro-organisms (Table 1). As applied to viral agents, "Koch's Postulates" for establishing causation require virus isolation from a diseased organism, growth of the agent in pure culture, and the development of disease when the virus is re-introduced into a healthy organism (Koch, 1884; Rivers, 1937). This approach has been applied to microbes for over a century and is a current practice not only for identifying pathogenic viruses in diseased organisms, but for the isolation of viruses from their natural reservoirs and vectors that harbor them (see Table 2).

Although Koch was also instrumental in the birth of the field of virology, at the time he proposed his postulates, knowledge regarding viruses was in its infancy. As obligate intracellular organisms, the procedure of 'growth in pure culture' in virology differs substantially from the solid phase media cultures described by Koch for bacteriology. Multiple steps are required for a virus to replicate in cell culture, and each step may impose selective pressure on the population. Host cells are required for the propagation of viruses. This propagation inevitably results in a mixed population of viruses. For the purpose of this article we propose that 'pure culture' for virus isolation means propagating viruses using *in vitro* preparations, such as mammalian cell culture. Historically, viruses were isolated by inoculating susceptible laboratory animals or embryonated eggs with small quantities of homogenized tissues or fluids obtained from biological specimens. Utilizing modern *in vitro*

Table 1
Koch's postulates to identify the causative agent of an infectious disease.

- The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms[a]
- The microorganism must be isolated from a diseased organism and grown in pure culture
- The microorganism (from the pure culture) should cause disease when inoculated into a healthy organism
- The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent

[a] Koch dismissed the universal requirement of the first postulate following the discovery of asymptomatic carriers of diseases such as cholera.

Table 2
Examples of the alteration of viral virulence upon propagation in cell culture.

Virus	Outcome of cell culture passage
Sin Nombre hantavirus	Vero-passaged virus is completely attenuated in NHPs, whereas virus propagated in deer mice causes severe disease (Safronetz et al., 2014).
Puumala hantavirus	Virus passaged in the reservoir (bank vole) causes disease in NHPs, but virus passaged in Vero cells does not (Klingstrom et al., 2002).
Ebola virus	Accumulation of adenosine residues in the GP gene editing site upon passage in Vero cells leads to attenuation in guinea pigs (Volchkova et al., 2011).
Measles virus	Cell culture adapted viruses lose pathogenicity *in vivo* due to a loss in interferon antagonism (Bankamp et al., 2008). Passage in Vero cells results in a change in entry receptor usage and a decrease in pathogenicity *in vivo* (Dörig et al., 1993).
Foot and mouth disease virus	Passage in culture results in a receptor switch between $\alpha_v\beta_3$ integrin and heparan sulfate (Martínez et al., 1997).
Sindbis virus	Virus grown on mosquito cells demonstrated increased infectiousness for human dendritic cells when compared to virus grown on Chinese hamster cells (Klimstra et al., 2003).
Rift Valley fever virus	Virus passaged on mosquito cells retains virulence, whereas when the virus is passaged on Vero cells, *in vivo* virulence is lost (Weingartl et al., 2014b).

culture techniques, most viruses are now isolated by inoculating susceptible, generally immortalized, cells with biological material containing the desired agent. Accordingly, virus preparations are obtained by collecting supernatants or lysed cell homogenates. These methods facilitate obtaining high-titer virus stocks which can be concentrated and purified (e.g., using a sucrose gradient), and allow for the serial propagation and molecular characterization of viruses that can readily be grown in culture. While it is often assumed that the starting and final virus populations are the same, in fact there is always some degree of genetic change resulting from "adaptation" to the cultured cells. There are therefore several limitations for many viruses generated in this fashion, potentially reducing the biological relevance of *in vivo* studies performed with cell culture-derived viruses.

4. Laboratory-induced natural selection

The selective pressures experienced by a virus during replication in a mammalian host are not recapitulated during propagation in cell culture, permitting the appearance of viral variants that may not arise in the natural settings. These novel variants may outreplicate wild-type viruses encoding virulence factors, such as immune modulating factors, that are required for replication in a host species. As a consequence, passaging viruses in cell culture, chick embryos, or sometimes in animals has led to attenuation for humans and potential usefulness as prophylactic vaccines. One of the first vaccines to be produced by serial passaging was the oral polio vaccine (OPV), in which the accumulation of nucleotide changes during passaging at a sub-physiological temperature resulted in the loss of the ability of the virus to be neuroinvasive (Sabin et al., 1960). Following the success of the OPV, many other attenuated viral vaccines have been developed using this method, including vaccines for measles, mumps, rubella, rotavirus, yellow fever, rabies, varicella-zoster and Influenza viruses (Minor, 2015).

It should be noted that adaptation resulting in increased virulence for a laboratory animal can also be achieved when some viruses are serial passaged in those animals. Provided that an animal has suitable cellular receptors to allow the virus to attach and enter target cells, serial passage can result in increased virulence by selecting for variants which can replicate in the new *in vivo* environment and evade immune pressures (Novella et al., 2014). Increased virulence often reflects the acquired ability to suppress specific host immune responses to which the pathogen is otherwise susceptible. Both attenuation and adaptation to increase virulence are examples of natural selection ("survival of the fittest"), occurring at accelerated speeds due to the short replication cycle of viruses.

4.1. Adaptation and attenuation of Ebola virus

Studies of Ebola virus have demonstrated the process of natural selection leading to adaptation in laboratory animals. Low-passage isolates of Ebola Zaire-Mayinga virus obtained from human specimens and amplified in cell culture are uniformly lethal for NHPs, while common laboratory mice inoculated with the same viruses support only limited replication, and do not become ill. Passaging of Mayinga virus in suckling mice resulted in an adapted strain which was lethal in mature, immunocompetent mice, but somewhat attenuated for NHPs (Bray et al., 2001, 1998). Interestingly, the generation of large stocks of the mouse-adapted virus in Vero cells has resulted in a partial loss of its lethal phenotype in mice, presumably due to the lack of selective pressures in cell culture (M Bray, personal communication). Recent findings also demonstrate that genomic alterations of wild-type Ebola viruses occur during propagation in cell culture. Amplification in cell culture, particularly interferon-deficient cell lines including Vero cells, leads to an accumulation of a subpopulation of viruses containing eight adenosine residues at a crucial editing site within the viral glycoprotein gene (Volchkova et al., 2011). After a few passages, the "8A" mutated viruses essentially out-compete and replace wild-type "7A" viruses. This change been suggested to reduce pathogenicity of the virus for guinea pigs; however, these results appear to contradict observations in NHPs (Kugelman et al., 2012).

5. Mechanism of attenuation

A number of studies have examined changes that occur during culture and passage of viruses, to determine why a vaccine candidate is attenuated for humans, or why sequential passage of a virus in cell culture results in decreased virulence in animals. Several mechanisms, with potentially additive effects, have been identified.

5.1. Rise of the mutants

The most obvious mechanism of attenuation is the accumulation of mutations brought about by differences in selective pressures between the normal biological context of the virus in a host and replication in cell culture. Viruses in nature are subjected to pressures exerted by the immune response and by infection of specific cell types, and although mutations accumulate and quasispecies may form, biological pressure limits the fitness of viruses that might be able to replicate in a less stringent system. Removing this pressure through propagation in cell culture, particularly in deficient cell lines like Vero cells, results in 'freedom' for mutant viruses to accumulate to a greater extent than would be possible *in vivo*. These mutant viruses may be more effectively targeted by

Table 3
Human viral diseases which researchers have failed to recapitulate through experimental inoculation of laboratory primates, suggesting that the virus may need to be propagated in its natural reservoir or in a vector species to remain pathogenic.

Disease	Etiological agent	Reservoir or host
Hemorrhagic fever with renal syndrome	Hantaan and Dobrava viruses	Rodents (e.g. *Apodemus* species)
Crimean-Congo hemorrhagic fever (CCHF)	CCHF virus	Ruminants, *Hyalomma* species ticks
Lujo hemorrhagic fever	Lujo virus	Unknown
Severe-acute respiratory syndrome (SARS)	SARS coronavirus	Bats
Dengue hemorrhagic fever	Dengue virus	*Aedes* mosquitos
Severe fever with thrombocytopenia (SFTS)	SFTS virus	*Haemaphysalis* ticks

the immune system, and are therefore attenuated when reintroduced into a host animal. A specific example is provided by the adaptation of vaccine strains of measles virus. While gaining fitness for replication in cell culture, these viruses no longer need to antagonize and evade the innate immune defenses, as this immune pressure is not present in many cell culture systems. These tissue culture-passaged viruses therefore undergo regressive evolution and mutations arise in both the P and V proteins, which allow the wild-type virus to inhibit type I and type II interferon signaling, and the adapted virus loses its pathogenicity in rhesus macaques (Bankamp et al., 2008).

5.2. Receptor switching

The receptor(s) to which viruses bind influence disease by being a primary determinant of cell-type tropism, and viruses often alter the function of their receptor. Culturing viruses can therefore lead to mutations that alter receptor usage, tropism and pathogenesis. Measles virus also provides an example. Wild-type strains of measles virus, propagated in marmoset B-cell cultures, are pathogenic for NHPs, while some laboratory-passaged viruses are not, and this phenotypic difference is attributable to differences in receptor usage (Kobune et al., 1990). Repeated passaging of measles virus in Vero cells induces several mutations that allow the virus to utilize CD46, which is expressed on many cell types, whereas clinical isolates utilize CD150, found on lymphocytes (Dörig et al., 1993; Shibahara et al., 1994; Tatsuo et al., 2000).

Another example is provided by foot and mouth disease virus (FMDV). Naturally isolated viruses bind $\alpha_v\beta_3$ integrins for entry (coincidentally, the same receptor used by pathogenic hantaviruses) (Berinstein et al., 1995). In contrast, FMDV serially passaged in baby hamster kidney-21 (BHK-21) cells loses the restriction for integrin binding by acquiring mutations in the RGD sequence, while gaining the ability to use heparan sulfate as a receptor (Martínez et al., 1997). Wild-type viruses enters cells via a clathrin-mediated event, whereas heparan sulfate-binding viruses enter via a caveolae-mediated mechanism, sequestering them to different areas of the cell. This change is associated with attenuation of the virus for cattle (O'Donnell et al., 2008; Sa-Carvalho et al., 1997).

5.3. Temperature sensitivity

Attenuation may also be induced by changing the temperature at which the virus is propagated; perhaps the most notable example is influenza virus. Mammals have a range of normal body temperatures, both between species, and within an individual host. The temperature at which a virus replicates in its host may therefore be different than the typical 37 °C temperature used in tissue culture. Also, a virus replicating in the respiratory tract would be subjected to temperatures lower than that of a virus replicating systemically. Similarly, viruses that replicate in arthropods such as mosquitoes and ticks would be accustomed to much different temperatures in a mammalian host or in cell culture. Temperature differences impose selective pressure favoring certain novel variants resulting from mutation, that may produce conformational changes in proteins, increased or decreased protein dynamics, including protease cleavage and other enzymatic activities, or interactions between macromolecules.

6. A laboratory-based natural setting

The observation that propagation in tissue culture may result in a change in virulence highlights the disadvantages of isolating and passaging infectious agents outside of their natural systems. Recognizing that natural selection invariably accompanies any mode of virus propagation, researchers should examine procedures and choose those that are least likely to introduce unwanted changes in phenotype, to ensure accurate modeling of virus-host interactions. Some laboratories have established colonies of the natural reservoir species of certain viruses, such as the rodents that carry hantaviruses, and viruses may also be propagated in mosquitoes or ticks. Although propagation of viruses in this manner can be difficult, it may circumvent adaptations that can result in decreased pathogenicity. For example, the SNV preparation we used to develop the NHP model for HCPS was initially characterized by Dr. Brian Hjelle and colleagues in the late 90's (Botten et al., 2000). Continuous passage of this virus in deer mice at the University of New Mexico and more recently at Rocky Mountain Laboratories for almost two decades has not altered the viral phenotype in these mice.

7. Unexpected effects of virus propagation in cell culture

In cases when it is not possible to propagate a virus in its natural host or vector species, undesired selective pressure may be avoided by infecting cells derived from the host or vector. For example, flaviviruses and alphaviruses grown on mosquito cells interact differently with the human innate immune response than viruses grown on mammalian cells (Shabman et al., 2007; Silva et al., 2007). Similarly, Sindbis virus grown on the C6/36 insect cell line is much more infectious in human dendritic cells than the same virus propagated in Chinese hamster ovary (CHO) cells, even though both viruses use the same receptors (DC-SIGN/L-SIGN) (Klimstra et al., 2003). In some cases, phenotypic changes have been linked to differences in the makeup of the virus particles as they bud through membranes of cells derived from a natural host, instead of the typical cells used for virus propagation. For example, when Rift Valley fever virus (RVFV) is propagated on mosquito cells, it incorporates an additional viral glycoprotein into its virion, but not when the virus is grown in mammalian cells. The incorporation of this protein appears to be necessary for productive infections in ruminants, as viremia occurs when goats and sheep are inoculated with virus propagated in C6/36 cells, but not when they are inoculated with a Vero-derived RVFV isolate (Weingartl et al., 2014b) (Weingartl et al., 2014a).

These examples highlight that the adaptation of viruses to cell culture potentially influences the outcome of subsequent *in vivo* studies, and raise the question whether propagation strategies used for other viruses, such as Crimean-Congo hemorrhagic fever virus (CCHFV), dengue, severe acute respiratory syndrome (SARS) coronavirus, have been responsible for the inability of researchers to recapitulate these diseases in NHPs (Table 3). For example, failed attempts to model CCHF in NHPs may largely be due to the lengthy passage history of the commonly used laboratory strain, which includes suckling mice as well as cell culture (Fagbami et al., 1975; Gonzalez et al., 1995.). Similar conclusions may be drawn from NHP experiments with many seasonal strains of influenza virus or the etiological agents of hemorrhagic fever with renal syndrome (Old World hantaviruses), which similarly have a long and in many cases undefined passage history (Bouvier and Lowen, 2010; Groen et al., 1995).

Low-passage viruses may also fail to cause disease in NHPs, and in these situations the inherent resistance of the animal species utilized must also be considered. For example, recent attempts at modelling Lujo virus hemorrhagic fever utilized a low-passage isolate, but failed to recreate any clinical indicator of disease in macaques following inoculation by various routes (Safronetz, Feldmann unpublished data). However, similar experiments with low-passage Lassa virus, Lujo's closest relative, have successfully recreated the principal features of Lassa fever in NHPs (Callis et al., 1982).

8. Concluding remarks

Although modern laboratories possess advanced tools and techniques for virus isolation and propagation, those procedures may impose unrecognized selective pressures on virus populations, leading to the loss of the viral phenotypes needed for the development of animal models that accurately recapitulate human disease. Researchers should therefore strive to limit viral adaptation or attenuation during the propagation process and to preserve as much as possible the integrity of the original virus, so that studies will more accurately reflect natural host-pathogen interactions. The result may be the generation of animal models of disease that will yield important pathophysiological and immunological data on disease mechanisms, providing improved predictive values for medical countermeasures.

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