Antiviral Research 137 (2017) 1–5

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

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

Joseph Prescott [a, **], Heinz Feldmann [a, c], David Safronetz [b, c, *]

[a] Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 South 4th Street, Hamilton, MT 59840, USA
[b] Zoonotic Diseases and Special Pathogens, National Microbiology Laboratory, Public Health Agency of Canada, 1015 Arlington Street, Winnipeg, MB R3E 3R2, Canada
[c] Department of Medical Microbiology, University of Manitoba, 745 Bannatyne Avenue, Winnipeg, MB R3E 0J9, Canada

ARTICLE INFO

Article history:
Received 3 May 2016
Accepted 5 November 2016
Available online 8 November 2016

Keywords:
Virus
Viral pathogens
Cell culture
Attenuation
Adaptation
Disease modeling

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 1
2. Case study - hantavirus infection in nonhuman primates 2
3. Koch's postulates 2
4. Laboratory-induced natural selection 3
   4.1. Adaptation and attenuation of Ebola virus 3
5. Mechanism of attenuation 3
   5.1. Rise of the mutants 3
   5.2. Receptor switching 4
   5.3. Temperature sensitivity 4
6. A laboratory-based natural setting 4
7. Unexpected effects of virus propagation in cell culture 4
8. Concluding remarks 5
References 5

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

* Corresponding author. Zoonotic Diseases and Special Pathogens, National Microbiology Laboratory, Public Health Agency of Canada, 1015 Arlington Street, Winnipeg, MB R3E 3R2, Canada.
** Corresponding author. Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 South 4th Street, Hamilton, MT 59840, USA.
E-mail addresses: prescottjb@niaid.nih.gov (J. Prescott), david.safronetz@phac-aspc.gc.ca (D. Safronetz).

http://dx.doi.org/10.1016/j.antiviral.2016.11.002
0166-3542/

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.