Gain-of-Function Research: Background and Alternatives

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The field of virology, and to some extent the broader field of microbiology, widely relies on studies that involve gain or loss of function. In order to understand the role of such studies in virology, Dr. Kanta Subbarao from the Laboratory of Infectious Disease at the National Institute of Allergy and Infectious Diseases (NIAID) at the National Institutes of Health (NIH) gave an overview of the current scientific and technical approaches to the research on pandemic strains of influenza and Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) coronaviruses (CoV). As discussed in greater detail later in this chapter, many participants argued that the word choice of "gain-of-function" to describe the limited type of experiments covered by the U.S. deliberative process, particularly when coupled with a pause on even a smaller number of research projects, had generated concern that the policy would affect much broader areas of virology research.

TYPES OF GAIN-OF-FUNCTION (GOF) RESEARCH

Subbarao explained that routine virological methods involve experiments that aim to produce a gain of a desired function, such as higher yields for vaccine strains, but often also lead to loss of function, such as loss of the ability for a virus to replicate well, as a consequence. In other words, any selection process involving an alteration of genotypes and their resulting phenotypes is considered a type of Gain-of-Function (GoF) research, even if the U.S. policy is intended to apply to only a small subset of such work.

Subbarao emphasized that such experiments in virology are fundamental to understanding the biology, ecology, and pathogenesis of viruses and added that much basic knowledge is still lacking for SARS-CoV and MERS-CoV. Subbarao introduced the key questions that virologists ask at all stages of research on the emergence or re-emergence of a virus and specifically adapted these general questions to the three viruses of interest in the symposium (see Box 3-1). To answer these questions, virologists use gain- and loss-of-function experiments to understand the genetic makeup of viruses and the specifics of virus-host interaction. For instance, researchers now have advanced molecular technologies, such as reverse genetics, which allow them to produce de novo recombinant viruses from cloned cDNA, and deep sequencing that are critical for studying how viruses escape the host immune system and antiviral controls. Researchers also use targeted host or viral genome modification using small interfering RNA or the bacterial CRISPR-associated protein-9 nuclease as an editing tool.

BOX 3-1

General Virology Questions and Questions Specific to Influenza, SARS, and MERS Research. Why/how does the virus infect and kill mammals? What are the critical host range and virulence determinants of MERS-CoV?

During Session 3 of the symposium, Dr. Yoshihiro Kawaoka, from the University of Wisconsin-Madison, classified types of GoF research depending on the outcome of the experiments. The first category, which he called "gain of function research of concern," includes the generation of viruses with properties that do not exist in nature. The now famous example he gave is the production of H5N1 influenza A viruses that are airborne-transmissible among ferrets, compared to the non-airborne transmissible wild type. The second category deals with the generation of viruses that may be more pathogenic and/or transmissible than the wild type viruses but are still comparable to or less problematic than those existing in nature. Kawaoka argued that the majority of strains studied have low pathogenicity, but mutations found in natural isolates will improve their replication in mammalian cells. Finally, the third category, which is somewhere in between the two first categories, includes the generation of highly pathogenic and/or transmissible viruses in animal models that nevertheless do not appear to be a major public health concern. An example is the high-growth A/PR/8/34 influenza strain found to have increased pathogenicity in mice but not in humans. During the discussion, Dr. Thomas Briese, Columbia University, further described GoF research done in the laboratory as being a "proactive" approach to understand what will eventually happen in nature.

In Session 8 of the symposium, Dr. Ralph Baric, University of North Carolina and a member of the symposium planning committee, explained that GoF experiments for CoV research encompass a very diverse set of experiments that are critical to the development of broad-based vaccines and therapeutics. Like Subbarao and Kawaoka, Baric listed experiments important for the identification of determinants of pathogenesis and virulence, defined the virus-host interaction networks, and described the alleles responsible for susceptibility and the host response patterns that drive a pathogenic or protective responses. However, he specifically noted that transmissibility studies for SARS and MERS-CoV actually fall in a different category than influenza research because of fundamental biological differences between these viruses. He first explained that the SARS-CoV has evolved over the past ~800 years to efficiently infect human cells that expressed the ACE2 viral receptor. To illustrate this, he shared sequencing results obtained from the Chinese during the 2003 SARS-CoV pandemic that show the gradual changes in the amino acid sequence across the genome associated with the expending epidemic. Among the 16 mutations found at the end of the pandemic, two were associated with the increased efficiency of the civets' strains to use the ACE2 receptor to invade human cells. In vitro experiments on human airway epithelial (HAE) cells and in vivo experiments on transgenic mice showed that while the human strain can efficiently infect and replicate in cells expressing the human, bat, and civet ACE2 receptor, the civet strain cannot use the human ACE2 receptor. This demonstrates the human SARS-CoV strain evolved to maintain its capacity to replicate and cause expanding epidemics while keeping its capacity to cycle through civets and most likely retreat into the bat reservoir following the control of the epidemic. In most instances, GoF experiments looking at receptor interactions with SARS-CoV and MERS-CoV showed that in in vitro or in vivo models with a civet strain gain human ACE2 receptors but also lose the civet

ACE2 receptor. Cell receptors for influenza viruses are relatively similar across different species, and this prompts a concern about possible increased transmission in humans from an influenza virus that is adapted for readier transmission in other mammals. By contrast, the ACE2 orthologue receptor interface for coronaviruses varies more markedly across different species.

APPLICATIONS OF GOF RESEARCH

Subbarao emphasized that current medical countermeasures are often insufficient largely because of resistance mechanisms that lead to "escape mutants," that is, drug-resistant strains. There is, therefore, a continual need to develop new antiviral drugs and additional options, such as immunotherapy, based on neutralizing monoclonal antibodies. Ultimately, GoF studies, which enhance viral yield and immunogenicity, are required for vaccine development. Molecular methods help with the characterization of antigenic variants, elucidate the biological basis for adverse outcomes associated with vaccine candidates, and determine the basis for attenuation and stability of vaccine candidates.

Subbarao also explained that one of the important applications of GoF research is the development of animal models, especially in the case of pathogens with pandemic potential, because to get approval to study a countermeasure compound in humans, the Food and Drug Administration's animal rule requires the presence of disease that mimics the human disease in an animal model. Influenza virus is unique in that its genome is fragmented; therefore, mouse models can be used to specifically identify viral determinants of virulence using single gene reassortment. Another type of GoF experiment, where the influenza virus is administered to ferrets and passaged a certain number of times, can lead to the characterization of molecular determinants of transmissibility. Subbarao reiterated that there are currently no small animal models to study MERS-CoV virulence factors or transmissibility and that lab strains of SARS-CoV need to be adapted to specific animal models to induce clinical signs of disease.

Baric, in Session 8 of the symposium, expanded on the complexity to use and optimize animal models for studying SARS- and MERS-CoV transmissibility and virulence. He referred to a study done in Subbarao's lab where a SARS-CoV strain was adapted by serial passages into a mouse model. As described earlier, the adaptation of the virus to the mouse ACE2 receptor decreases its interaction fitness with the human receptor but also does not induce a lethal phenotype in mice because supplemental mutations need to occur. Further experiments demonstrated that increased virulence and replication efficiency do not correlate with increased transmissibility in the mouse model, making the use of GoF research safe in these models.

GOF RESEARCH AS DEFINED BY THE U.S. GOVERNMENT

Many participants pointed out during the course of the meeting that the broad term "gain-of-function" needs some refinement that will differentiate the type of experiments typically performed for basic virological research from experiments that clearly raise concerns. When asked to define where virological research crosses the line into GoF research as defined by the U.S. government (White House, 2014a), Subbarao responded that "the term gain-of-function is

used by geneticists and is a vague and unsatisfactory term for microbiologists." This statement was echoed by Imperiale and many others during the discussion. Subbarao presented a list of experiments that encompass all influenza viruses, SARS-CoV, and MERS-CoV that can be reasonably anticipated to increase pathogenicity or transmissibility in mammalian species (see Box 3-2). Reflecting on this list, Dr. David Relman, Stanford University, and the panelists of Session 2 expressed the view that GoF experiments generating viruses with increased virulence, transmissibility, and pathogenicity would clearly define the line that would prompt the use of alternatives.

BOX 3-2

Where Does Virological Research Cross the Line into GoF Research as Defined by the U.S. Government? Adaptation of MERS-CoV to animal models Elucidating the molecular determinants of transmissibility by the airborne route (influenza)

Imperiale explained that, with respect to the GoF terminology, whenever researchers are working with RNA viruses, GoF mutations are naturally arising all the time and escape mutants isolated in the laboratory appear "every time someone is infected with influenza." He also commented that the term GoF was understood a certain way by attendees of this symposium, but when the public hears this term "they can't make that sort of nuanced distinction that we can make here" so the terminology should be revisited. Fineberg, the session moderator, after listening to this set of talks, asked whether proposed GoF experiments should be individually reviewed to make a better judgment. Subbarao proposed to first redefine the line because she is concerned that the pause in the current research "has swept far too many aspects of virologic research into the definition." Dr. Mark Denison, Vanderbilt University, suggested that a case-based approach should be considered for coronaviruses, for which a better understanding of the biology is needed. Along the same lines, Imperiale added that we should "take each individual case and call it what it is rather than try to come up with some acronym or two- or three-word term that can easily be misinterpreted." Baric reminded the audience during his talks that because there are currently no small animal models to study MERS-CoV, restrictions on this coronavirus should be lifted immediately.

Throughout the symposium, particularly in the final discussion session, there were calls for a clearer definition of precisely what types of experiments are really of concern. Dr. Tom Inglesby of the UPMC Center for Health Security noted that he thought that the origin of the term "gain-of-function" goes back to a 2012 meeting that he convened for the NIH on this topic. The term was used to replace more descriptive terms that indicated concerns about research that generates strains of respiratory viruses that are highly transmissible and highly pathogenic. According to Inglesby, this was the provenance of the term, and he suggested that it could be retired with something more descriptive. Dr. Gerald Epstein of the Department of Homeland Security also called for clarifying which experiments are of most concern. GoF is clearly not the right descriptor, and he stated that it would be a tremendous service to have terminology that accurately describes those things about which we are most concerned. The same point was made by others at various times during the workshop (see in particular the summary of Relman's talk in Chapter 5).

ALTERNATIVES TO GOF RESEARCH

The essence of the debate around the risks and benefits of GoF research and the concerns it raises have naturally encouraged virologists on both sides of the debate to consider alternative methodological approaches. During his talk, Kawaoka discussed alternatives to GoF research mostly applicable to influenza research, such as loss-of-function research, use of low pathogenicity viruses, and phenotypic analyses. He further cited a review paper in which Lipsitch and Galvani (2014) stated that "alternative scientific approaches are not only less risky, but also more likely to generate results that can be readily translated into public health benefits." However, Kawaoka argued through specific examples that alternatives do not always provide the full answer to key questions. For instance, he cited work by Tumpey et al. (2007) and Imai et al. (2012) on mutations responsible for the loss of transmission capabilities of the 1918 influenza strain between ferrets and noted that this work required GoF research because a loss-of-function approach did not provide the complete picture. In addition, although working with low pathogenic avian influenza viruses provides a safer approach, Kawaoka explained that "highly pathogenic avian influenza differ from low pathogenic viruses in their kinetics of virus replication and tropism" and therefore the data can be misleading. Other alternatives discussed by Kawaoka and Dr. Robert Lamb, Northwestern University, in Session 8 of the symposium were cited from the recent review paper by Lipsitch and Galvani (Box 3.3). Kawaoka concluded that even if these approaches offer safer alternatives to GoF research of concern, for some questions researchers cannot rely solely on them because the phenotype of and the molecular basis for these new traits have been identified by GoF research but not by alternative approaches.

BOX 3-3

Alternative Research Methods with Potentially Less Risk. Molecular dynamical modeling of influenza proteins and interactions with inhibitors and receptor In vitro studies of specific properties required for human adaptation, using single proteins

Alternatives to in vivo models have also been attempted to study SARS-CoV. Baric presented the work by Deng et al. (2014), who proposed to optimize a safer mouse model for in vivo drug screening using the non-pathogenic recombinant Sindbis virus (alphavirus) expressing a SARS proteinase. Although the investigators succeeded in enhancing mouse survival when the virus was mutated in the protease site, targeting the engineered virus with protease inhibitor failed to protect the mice. A few reasons might explain the results and constitute challenges of using alternative viral strains such as virus tropism, bioavailability of the drug, and virus titer in the targeted organ. Baric concluded that this type of indirect model can lead to misinformation that can complicate downstream development of treatment.

When discussing risk mitigation, Imperiale said he believed that "you can develop safer approaches to do these types of experiments; it just needs a little bit of imagination on the part of researchers." An example that was cited several times during the symposium is the work by Garcia-Sastre and others (Langlois et al., 2013). The group exploited species-specific endogenous small RNAs, which can shut down some basic functions, such as replication, found in the human and mouse respiratory tract but not in the ferret. Its engineered influenza A strain, which contained this specific microRNA target site, did not prevent influenza replication and

transmissibility in ferrets, but it did attenuate influenza pathogenicity in mice and presumably in humans. Imperiale and later Kawaoka agreed that it constitutes a promising approach. During his talk in Session 8, Lamb also listed some mitigation and reversibility approaches, such as the use of:

- Viruses with drug sensitivity (if not studying drug resistance)
- Vaccinations for strains used as genetic backbone, if possible
- Existing virus where immunity is widespread
- Mutation that confers acid stability (Zaraket et al., 2013)
- Mutation in HA multi-basic cleavage site (depends on GOF sought)