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The Definition and Measurement of Dangerous Research

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July 2004

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This paper was prepared as part of the Advanced Methods of Cooperative Security Program at the Center for International and Secuirty Studies at Maryland, with generous support from the John D. and Catherine T. MacArthur Foundation.

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I. Introduction to the Biological Research Security System Model

Both scientists and policy-makers are increasingly recognizing the potential and pitfalls of biotechnology in regards to biosecurity. The spread of biotechnology and biological research across the globe is revealing a great deal of information on the origins of human disease and microbial pathogenesis. There is great hope that the genetic, proteomic, and metabolomic information will yield new antimicrobial and immunological therapies and vaccines in upcoming years.

However, continued research into disease pathogenesis also has the potential to cause more harm than good without proper oversight. Although such a negative experimental outcome has not manifested itself yet, recent experiments into mouse host susceptibility to an engineered strain of mousepox and a smallpox complement inhibitor have pointed the way toward the need for greater debate, if not oversight, of scientific research into high-threat pathogens.[1]

Mindful of the possible threat of this "scientific inadvertence," security studies experts at the University of Maryland have proposed a legally binding, global oversight system to deal with the threat presented by advanced pathogens.[2] The so-called Biological Research Security System (BRSS) does not seek to ban any research. Rather, the BRSS wishes to develop legally enforceable "protective standards of prudence" by mandating independent peer review to assess not only the scientific merit and biosafety/physical security protocols for the research, but also its larger social consequences. Given the recent explosive growth in biodefense research funding and the access limitations to pathogen stocks based on nationalities, the lack of true research oversight is especially glaring.

One of the chief problems in devising such a system is coming up with a definition of dangerous research that captures relevant research without being unduly broad and sets clear, consistent standards for managing high-consequence research without being arbitrary or excessively rigid. As originally conceived in "Controlling Dangerous Pathogens: A Protective Oversight System," the BRSS would match the expected level of risk involved in proposed research with the level and extent of oversight that it would receive. The risk level would reflect three epidemiological parameters intrinsic to each pathogen -- transmissibility, infectivity, and lethality -- and would consider the probability that proposed research activities would significantly increase the level of danger on one or more of these dimensions. Figure 1 shows a three-dimensional conceptual scheme in which "potentially" dangerous research is reviewed only at the local level, "moderately" dangerous research is reviewed also at the national level, and "extremely" dangerous research is raised to the international level of oversight (qualitatively noted as the green, yellow, and red areas).

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[1] Cozzarelli, NR. "PNAS policy on publication of sensitive material in the life sciences," *Proceedings of the National Academy of Sciences* 100(4) (18 February 2003): 1463.
[2] Steinbruner, J. E.D. Harris, N. Gallagher, and S. Gunther. "Controlling Dangerous Pathogens: A Prototype Protective Oversight System," Center for International Security Studies at Maryland Working Paper (September 2003), esp. pp. 19-25. Available at http://ww.cissm.umd.edu/documents/pathogensmonograph.pdf.

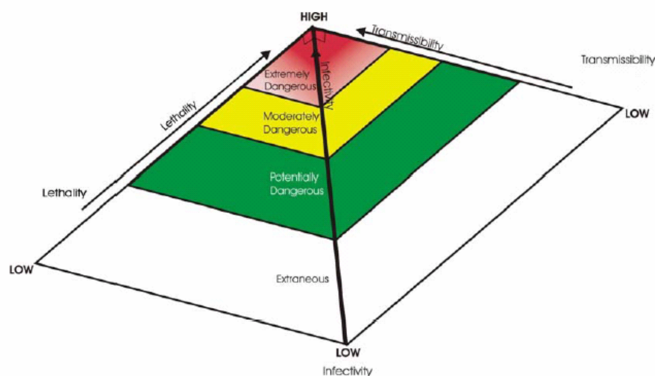

Figure 1 - 3D Danger Space

Figures 2-4 give illustrative lists of research activities that might fit into each oversight category.

Potentially Dangerous Activities (PDA):

- Work with listed agent— or exempt avirulent, attenuated, or vaccine strain of select agent — not covered by EDA/MDA
- Increasing virulence of non-listed agent
- Increasing transmissibility or environmental stability of non-listed agent
- Powder or aerosol production of non-listed agent
- Powder or aerosol dispersal of non-listed agent
- *De novo* synthesis of non-listed agent
- Genome transfer, genome replacement, or cellular reconstitution of non- listed agent

Figure 2 - Potentially Dangerous Activities Overseen by Local BRSS Committees

Moderately Dangerous Activities (MDA):

- Increasing virulence of listed agent or related agent
- Insertion of host genes into listed agent or related agent
- Increasing transmissibility or environmental stability of listed agent or related agent
- Powder or aerosol production of listed agent or related agent
- Powder or aerosol dispersal of listed agent or related agent
- *De novo* synthesis of listed agent or related agent
- Construction of antibiotic- or vaccine-resistant related agent
- Genome transfer, genome replacement, or cellular reconstitution of listed agent or related agent

Figure 3 - Moderately Dangerous Activities Overseen by National BRSS Committees

Extremely Dangerous Activities (EDA):

- Work with eradicated agent
- Work with revived extinct agent related to listed agent
- Work with agent requiring Biosafety Level-4
- *De novo* synthesis of above
- Expanding host range of disease agent to new host (in humans, other animals and plants) or changing the tissue range of a listed agent
- Construction of antibiotic- or vaccine-resistant listed agent

Figure 4 - Extremely Dangerous Activities Overseen by International BRSS Committees

A list-based approach to defining danger has the advantage of being more concrete and less ambiguous than the conceptual scheme. However, depending on how the list is structured, lists of candidate research activities can be excessively narrow in some regards, overly broad in others, and quickly outdated in a rapidly evolving field. Furthermore, while the list-based definition of dangerous research makes it easy to match proposals with the appropriate level of review, it provides the reviewers with little guidance as they try to assess the benefits and risks of a given proposal. Therefore, this paper examines the literature on two high-threat pathogens, influenza and pneumonic plague, to assess the operational difficulty of defining dangerous research in terms of standard quantitative measures for transmissibility, infectivity, and lethality.

A. The 3-D Definition of Danger

While no three parameters can capture the entire picture of what makes an infectious agent unique and dangerous, transmissibility, infectivity, and lethalty present a great deal of the basic biology of a pathogen.

1. Defining Transmissibility

Transmissibility is defined as the ease with which an organism spreads from person-to-person. It should not be confused with the ability of an organism to spread from the dispersion device to the index cases, or infectivity. Assuming an organism is reasonably pathogenic, transmissibility can be considered the most critical parameter in the pyramid definition of danger. It is what separates the ability of biological weapons to be a true weapon of mass destruction versus a special type of one-off bomb that has historically yielded mostly mass disruption.

Epidemiologists do not have standard ways of measuring the intrinsic transmissibility of pathogens. Transmissibility is also rarely quantified due to the difficulties in contact tracing and the relative novelty of epidemic modeling that takes into account contact tracing. Traditionally, transmissibility has been represented by R_0, or basic reproduction number, in epidemiological models. R_0 is defined as the number of cases that one case will directly infect when introduced into an entirely susceptible population. Although this definition works in a bioterror context – most populations would be entirely susceptible to bioterror agents – R_0 has the major drawback in research oversight of integrating the intrinsic biology of a pathogen with the circumstance of its release. The same organism may have different R_0's depending on whether it is released in a closed building or an open field. R_0's measured inside buildings, usually hospital wards, typically overestimate the true transmissibility of the organism because contacts in hospitals are necessarily very close and often; patients are often already sick or immunocompromised; and hospital personnel often aid in the spread of the disease around a ward.

R_0 can be determined in two different ways. The method used most often involves the use of seroprevalance data; $R_0 = 1/x^*$ where x^* is the fraction susceptible at equilibrium. This fraction can be estimated via seroprevalence data. The other method is far more laborious. $R_0 = \tau c \delta$, where

τ is the transmissibility of the pathogen; c is the average contact rate in the population; and δ is the expected time of removal from the infected population (from recovery). A measure for intrinsic transmissibility does exist (τ), but it is somewhat difficult to calculate. This subject will be covered in further detail in the concluding chapter.

For most epidemiologists, the combination of social and pathogenic factors in R_0 is of little to no consequence. Epidemiologists are chiefly concerned with the propagation and possible elimination of an epidemic. Environmental conditions of an outbreak may be relevant to the control of future outbreaks. R_0 captures the entirety of an epidemic and greatly simplifies foreseeing the future of an outbreak. Assuming little variance in R_0, an outbreak that has an $R_0 > 1$ will create an epidemic while one in which the basic reproduction rate is below stasis will eventually die out.

For the purposes of defining transmissibility for the BRSS, one would like to have a quantification of the intrinsic transmissibility of the organism. Arriving at such a number experimentally would require standard assays of transmissibility which are seldom available due to the possible lethality and/or virulence of most infectious agents. Hypothetical standard assays would have to be conducted in animal models which are rarely adequate for measuring the transmissibility in humans. The science of both understanding and measuring transmissibility has not progressed very far for these exact reasons. Possible changes to and investment in understanding the intrinsic transmissibility of an organism will be discussed in the Conclusion section.

For many of the bioterror agents, even R_0's are often not available. For instance, the last reported human-to-human transmission of pneumonic plague occurred in 1924. Ebola data is spotty due to the lack of knowledge of how it is spread and the fact that all human-to-human transmissions have occurred in African villages between persons who had multiple contacts. For the purposes of this paper, transmissibility will be quantified on a scale from 0 to 3, corresponding to none (0), low (1), medium(2), and high (3). A pathogen's place in the scale is not necessarily scientifically determined. Rather, it is based on both a review of anecdotal data from previous outbreaks and the current understanding of how each pathogen is spread. For example, the high annual incidence of influenza means that its transmissibility is necessarily very high, while no studies have shown the ability of anthrax to be contagious to any relevant degree.

2. Defining Infectivity

Infectivity is the most difficult parameter to measure and understand. Infectivity is formally defined as the ability of an organism to penetrate its host and establish an infection. The key to understanding infectivity revolves around the arbitrary definition of "infection." In the past, symptoms were a prerequisite before infections could be detected by doctors or epidemiologists. Infectious organisms that multiplied a great deal in the body but caused non-symptomatic infections could only be discovered after a histological examination of a corpse or biopsy. In the present era, pathogens can be detected in samples via polymerase chain reaction (PCR) and an infection can be defined as a threshold amount of DNA present in a tissue after a defined time period.

For the purposes of this paper, infectivity will be measured by dose of pathogen needed to kill 50% of infected hosts, or LD_{50} values. Ideally, the dose needed to simply establish infection in 50% of hosts, or ID_{50}, would be used for "infectivity." However, because 1) death is such a great discrete variable, 2) measuring an infection is fairly difficult and arbitrary, and 3) LD_{50} and ID_{50} values are generally correlated (especially in pathogens with such high lethality), LD_{50} values are more available and thus will be used as a measure of infectivity. Some may argue that measuring LD_{50} values conflates infectivity and lethality. This charge is somewhat true due to the lack of data on ID_{50} and the inability to get at any good ID_{50} data in the near future. Once again, the difficulties of defining infection per PCR, the ethics of testing in humans, and the inadequacies of animal models make ID_{50} a problematic measure. LD_{50} is the only conceivably relevant measure that exists in the literature at this time.

Another possible definition of infectivity – one that will not be used in this paper but that is nonetheless worth being considered – is that of pathogenicity or virulence. Here, infectivity is a direct measurement of the incapacitation caused by an infection. For instance, influenza has a much

greater economic consequence on a population than an equally transmissible commensal organism because it makes people sick for a week, even though both organisms generally would kill <1% of their infected hosts. Defining infectivity as such would necessarily force one to take into account other intrinsic economic consequences of an outbreak of a pathogen, including the environmental hardiness of the organism.[3] Although it would be desirable to measure the intrinsic economic consequences of a pathogen to avoid focusing solely on mortality, the difficulties surrounding this alternative definition of infectivity are necessarily quite great.

3. Defining Lethality

Lethality is perhaps the easiest parameter of the three to both measure and understand. Because death is a discrete variable and thus relatively easy to observe, some of the issues presented with measuring infectivity are mitigated in measuring lethality. Epidemiologists typically measure lethality by dividing the number of deaths caused by a pathogen by the number of infected cases. For example, in the 1997 Hong Kong H5N1 avian influenza outbreak, eighteen cases were noted with six deaths, leading epidemiologists to declare the Hong Kong H5N1 strain had a 33% lethality rate.

Measuring lethality is not uniformly simple. The greatest complicating variable comes in gaining an adequate representation of the denominator, the number of infected cases. That is, the organism must first produce a rather large, symptomatic infection if it is to be recorded in the lethality statistics (although occasionally pathogens are cultured from tissue or saliva samples from asymptomatic persons). Even today, patients that do not report symptoms would not be included in the infected cases measurement. Ideally, one would run PCRs on blood and/or tissue samples from anyone potentially exposed to a biological agent to get a true sense of the number of infected cases. Given that deaths due to an infectious agent are easier to monitor than overall infections, it is likely that most lethality statistics are biased upwards in those data derived from humans.

B. Defining the Project at Hand

The theory behind the 3-D and list-based definitions of danger works on paper. However, research regulation will necessarily be quite pathogen-specific and will require case studies. Given that, there is a great need to operationalize these definitions of danger for a small, test group of high-threat pathogens. This paper will profile past, present, and future work in the influenza A virus and plague bacteria that could be seen as dangerous. These pathogens were chosen for the unique challenges they present a research oversight system. At first pass, one is a virus while the other is a bacterium. Influenza is one of the most transmissible diseases known while pneumonic plague exhibits a rather low, albeit heterogeneous transmissibility. Plague is almost uniformly lethal when antibiotics are not administered. Influenza, on average, does not even kill 1% of those infected each year. Although susceptibility to countermeasures was not necessarily intended to be included in the 3-D definition of danger, it is important that this paper has elected a virus and a bacterium that vary in the efficacy and attainability of their candidate countermeasures, both vaccines and antimicrobials.

In attempting to come up with an initial definition of dangerous research, it is necessary to get a general sense of their place on the 3-D "danger terrain." Two pathogens are not enough to test the usefulness of this definition of danger. Thus, three other high-threat agents -- anthrax, smallpox, and ebola -- were added to the danger terrain for a total of five pathogens. By placing these agents according to their transmissibility, infectivity, and lethality, one can get a sense of the arbitrary cutoff values that will define whether research should be considered moderately or potentially dangerous (i.e., the green and yellow zones in Figure 1). It may be that such arbitrary cutoffs can never be operationalized. After all, it is difficult to say whether a certain point mutation in influenza's hemagglutinin gene will make the virus transmissible to a level that warrants placement into the red or yellow area. However, such arbitrary cutoffs are necessary to define the space covered by one

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[3] Here, compare anthrax with its spore life of up to 300 years and pneumonic plague with an environmental shelf life of <1 hour in sunlight.

region. In cases where there is no knowledge about previous research into an organism, a pathogen's place on the danger terrain will initially define the level of oversight required for proposed research into that organism.

One of the great complexities in defining dangerous research is figuring out a priori how to think about proposed research in a meaningful way. Will research proposed on R01 grants include enough detail to adequately place? Is it important to think before the fact about different types of experiments will be proposed? These questions are difficult to answer because of the distinct lack of "case law" in defining dangerous research. This paper will attempt to start the creation such case law through retrospective examination of research into influenza and plague.

Ideally, the BRSS would not have to develop an understanding of the danger of proposed research on an ad hoc basis. But to do otherwise would be almost impossible, for the body of possible research into a certain pathogen is almost infinite. Instead, a middle ground between planning for every contingency and overseeing actual research on a completely ad hoc basis must be reached.

At one level, this planning could be done entirely at the genomic level. Researchers often seek to disrupt or alter or monitor the function of certain genes to understand their role in pathogenesis. Based on past research into the role of certain genes or types of genes in disease pathogenesis, the BRSS could define dangerous research on a gene-by-gene basis as experiments are proposed. Although gene cross-regulation and expression are complex in influenza systems biology, a genomic-focused oversight system would not seem impossible since there are only 10 genes on 8 genetic segments and the entire genome is generally less than 14 kbp. However, such an oversight system might fail in bacterial agents such as *Yersinia pestis*, the causative agent of plague, which includes over 3800 plausible protein-coding genes in a genome over 4.6 Mb (with multiple plasmids) with many to-be-determined functions. A genomic oversight system would also only work for experiments that sought to alter genes previously defined through functional studies or sequence homologies.

A second way to think about research oversight would be a process-oriented system. As shown in Figures 2-4, the original paper proposing the BRSS predominantly used this strategy to define dangerous research.[4] Extreme danger is described as any "work with [an] eradicated agent" or "*de novo* synthesis" of an eradicated or extinct agent, while moderately dangerous activities include "insertion of host genes into listed agent" or "powder or aerosol production of listed agent." The process-oriented method is perhaps the best way to define danger when not speaking about any particular pathogen because it adequately balances ambiguity and specificity.

For example, in some cases, adding a host gene into a listed agent might be truly dangerous (as in the case of IL-4 mousepox). But the danger depends a great deal upon the host gene inserted into the pathogen. It is doubtful that a pathogen carrying a gene encoding for a defensin, for interferon-alpha, or for an antibody against the carrier agent would necessarily make the organism more pathogenic. To be sure, the experiment would have to be carried out, but the fact remains that all host genes are not created equal when it comes to increasing pathogenesis. The vagueness of certain laboratory processes can be a liability for process-oriented approach in arriving at a definition of danger.

Finally, dangerous research could be defined by objective. To a certain degree, the original paper proposing the BRSS used this strategy to sidestep the difficulty of defining dangerous research (see Figures 2-4, e.g., "increasing virulence of listed agent" or "increasing transmissibility or environmental stability of listed agent"). The October 2003 NAS Fink report, "Biotechnology Research in an Age of Terrorism: Confronting the Dual Use Dilemma," also used a similar strategy in defining the seven areas of research it was concerned about. It defined seven areas of high threat research, including countermeasure and detection evasion; weaponization information; increasing virulence and transmissibility; and altering the host range of pathogens.

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[4] Steinbruner, J and Harris, ED.

While few legitimate scientists would try to make a pathogen more virulent for its own sake, they might knowingly do so in order to study the molecular mechanisms of transmissibility, to design counter-measures for biodefense, or to achieve some other desirable purpose. Risk assessment could be complicated if the reviewers disagree among themselves or with the principle investigator about the probability and magnitude of an unexpectedly dangerous result. Deciding who defines what will qualify as "increasing transmissibility", for example, is perhaps an inescapable problem for any research oversight system. While this approach might work reasonably well for research that replicates past experiments or extends a line of research in an area that is either reasonably simple or relatively well understood, prediction will necessarily be difficult for much cutting-edge research with dangerous pathogens.

Clearly, each of these approaches has benefits and drawbacks that can be analyzed more concretely by exploring how they might be applied to specific cases. This paper will assess the state of knowledge about the genomic determinants of pathogenicity in influenza and pneumonic plague and will consider how a research oversight system might handle both past problematic research and potential new lines of dangerous research. Understanding of these two pathogens is not well enough developed for any one approach to provide a complete, unambiguous, and practical way to assess risk levels before the research is done, and there are good reasons to believe that a combination of strategies for defining danger will always be more satisfactory than exclusive reliance on a single approach. Nonetheless, a review of the literature about both influenza and pneumonic plague shows that it is possible to make judgments about lines of research that are clearly more problematic from a dual-use standpoint than others.

C. Where Do Candidate Pathogens Fit into the Danger Terrain?

A necessary first step in the definition of dangerous research is the definition of danger of the starting material. For the five selected pathogens, the lethality, infectivity, and transmissibility values are those generally cited in the literature with at least two sources being used to determine numerical values where available. It is worth noting that there is considerable strain diversity in the above values. As Aum Shinrikyo discovered, not all anthrax strains are lethal. Smallpox strains vary in lethality from below 30% lethality to as much as 90%+ lethality.[5] The 1918 strain of influenza killed over 2% of those infected and H5N1 avian influenza kills ~33% of those infected, while most other influenza strains are really only lethal in the very young and very old.

Transmissibility was somewhat arbitrarily determined through a review of the literature since there is no systematic, quantitative determinant for transmissibility. Infectivities noted in italics are aerosol "infective doses" noted from Franz et al. (1997) and should not be considered LD_{50}'s.

	Lethality	Infectivity (LD50)	Transmissibility (0-3)
Anthrax	90%[6]	2.4e3 – 5e4[7,8], 4.13e3[9]	0[10]
Smallpox	30%[11,12]	*10-100*[13] (1e9??)[14]	1.75[15,16] R_0=6-9[17]

[5] Fenner, F. et al. *Smallpox and its eradication.* Published by WHO, 1988. p. 5.

[6] Inglesby, T. V. et al. "Anthrax as a Biological Weapon." *JAMA.* 9 Jun 1999, 281(18):1735-45.
Morgan, Maria F. Anthrax: An Old Disease Returns as a Bioterrorism Weapon. *New Jersey Medicine.* September 2000, 97(9):1-3.

[7] Inglesby, T. V. et al. (1999)

[8] Ivins, BE. et al. "Comparative efficacy of experimental anthrax vaccine candidates against inhalational anthrax in rhesus macaques." Vaccine. 1998 Jul, 16(11-12):1141-8.

[9] Glassman, H.N. *Bacteriological Reviews.* 1966, 30:657-9.

[10] Inglesby, T. V. et al. (1999)
http://www.cdc.gov/ncidod/dbmd/diseaseinfo/anthrax_g.htm#Can%20anthrax%20be%20spread%20from%20person-to-person

[11] Henderson, DA. Et al. Smallpox as a Biological Weapon. *JAMA.* 9 June 1999, 281(22):2127-37.

Ebola	30-90%[18]	*1-10*[19], <400[20]	1[21] R_0=1-2[22]
Pneumonic Plague	90-100%[23]	*100-500*[24], 1-9e4[25]	1[26]
Influenza	0.1 – 2.5%[27] (33%)[28]	1e4[29] *1-320* $TCID_{50}$[30]	2.75[31] R_0=9-15[32]
Danger Cutoffs	**0.1%, 5%, 30%**	**1e6, 1e4, 1e2**	**0.5, 1.5, 2.5**

Table 1 – Lethality, Infectivity, and Transmissibility Values for Select Pathogens

The setting of cutoff values shown in Table 1 for the three levels of dangerous research was, admittedly, a highly arbitrary, subjective process. For lethality, an organism that does not kill should be classed into the "extraneous" danger zone, and thus 0.1% is considered the first cutoff point. The second threshold could comprise any number between 5-10%, as agents that kill less than 10% of the time may not be so effective for bioterror use, depending on the transmissibility. The final threshold sits at the generally accepted smallpox lethality, since any agent that kills more than 30% of the time

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[12] Fenner, F. et al. *Smallpox and its Eradication*. Geneva: World Health Organization, 1988. p. 5.
[13] Infectious dose noted in: Franz, DR. et al. "Clinical Recognition and Management of Patients Exposed to Biological Warfare Agents." *Journal of the American Medical Association*. 6 August 1997, 278(5):399-411. Moran, Gregory J. "Biological Terrorism: Are We Prepared?" *Emergency Medicine*. November 15, 2001. Anecdotal evidence, see Wehrle, PF et al. An airborne outbreak of smallpox in a German hospital and its significance with respect to other recent outbreaks in Europe. *Bulletin of World Health Organization*. 1970; 43:669-679.
[14] Here, the non-human primate model for smallpox indicates 1e9 pfus are needed to form a smallpox-like infection although some anecdotal evidence indicates 10-100 virions is enough to cause disease. See, LeDuc JW et al. Smallpox research activities: US interagency collaboration, 2001. *Emerging Infectious Diseases*. 2002 July, 8(7):743-5.
[15] Henderson, D.A. et al.
[16] Eichner, M. and Dietz, K. "Transmission potential of smallpox: estimates based on detailed data from an outbreak." *American Journal of Epidemiology*. 2003, 158:110-7.
[17] Fraser, C. et al. "Factors that make an infectious disease outbreak controllable." *PNAS*. 20 April 2004. 101(16):6146-51.
[18] Feldman, H. and Klenk, H. "Filoviruses." Eds. Samuel Baron et al. *Medical Microbiology*. 4th ed. Accessed online at NCBI Bookshelf, (Clinical Manifestation section) http://www.ncbi.nlm.nih.gov/books/bv.fcgi?call=bv.View..ShowTOC&rid=mmed.TOC&depth=2
[19] (Infectious Dose) Franz, DR. et al.
[20] (Infectious Dose) Johnson, E. et al. Lethal experimental infections of rhesus macaques by aerosolized Ebola virus. *Int J. Exp. Pathol.* 1995 Aug. 76(4):227-36.
[21] Feldman, H. (Epidemiology section).
[22] Chowell, G. et al. "The Basic Reproductive Number of Ebola and the Effects of Public Health Measures: The Cases of Congo and Uganda." Forthcoming in the *Journal of Theoretical Biology*. Published online at http://people.cornell.edu/pages/gc82/ebola.pdf.
[23] Inglesby, T. V. et al. Plague as a Biological Weapon. *JAMA*. (May 3, 2000), 283(17):2281-2290.
[24] (Infectious Dose) Franz, DR. et al.
[25] Welkos, S. L., K. M. Davis, M. L. M. Pitt, P. L. Worsham, and A. M. Friedlander. 1995. Studies on the contribution of the F1 capsule-associated plasmid pFra to the virulence of *Yersinia pestis*. Contrib. Microbiol. Immunol. 13:299-305.
[26] Wu, Lien-the. *Treatise on Pneumonic Plague*. Berger-Levrault, 1926. p. 188, 298, 301.
[27] Kolata, Gina. *Flu*. New York: Farrar, Straus, and Giroux, 1999.
[28] Ibid.
[29] Chen, Dexiang. "Serum and Mucosal Immune Responses to an Inactivated Influenza Virus Vaccine Induced by Epidermal Powder Immunization." *J. Virology*. September 2001, 75(17): 7956-65.
[30] Snyder, M.H. et al. "Infectivity and Antigenicity of Live Avian-Human Influenza A Reassortant Virus: Comparison of Intranasal and Aerosol Routes in Squirrel Monkeys." *Journal of Infectious Diseases*. October 1986, 154(4):709-712.
[31] Fraser, C. et al.
[32] Ibid.

is subjectively considered to be of great danger. Infectivity danger cutoffs were determined based on the range of the five diseases defined here. All infectivity values are LD50s based on plaque forming units or the number of bacterial organisms in colony forming units delivered through an aerosol route. Transmissibility cutoff values were determined via anecdotal review and the placement of the five pathogens on the pyramid.

The difficulties associated with the 3-D method of defining danger will be further discussed in the final chapter. The next two chapters will include literature reviews of past and future potentially problematic research into influenza and pneumonic plague. Reviewing particular experiments allows one to start to ascertain the relevance of both the 3-D terrain and list-based definitions of danger. More importantly, examining specific experiments helps develop the case law that is currently lacking in defining dangerous research.

II. Influenza – "An Unvarying Disease Caused by a Varying Virus"

Despite the relative lack of bioterror media attention in comparison to anthrax or smallpox, influenza is a very important pathogen for BRSS research oversight. Its high transmissibility allows it to have unparalleled global reach. Even today, influenza annually infects 10-20% of the world's population, resulting in 250,000 to 500,000 deaths every year.[33] Due to strong immune selective pressure, the epidemiology of influenza is complex and viruses vary greatly from year to year. In 1918, 1957, and 1968, new influenza variants emerged that killed tens of millions of people.

Influenza viruses are typed by the two major antigens – hemagglutinin and neuraminidase – that protrude from the virion surface. Thus, the 1918 virus (Spanish flu) is known as H1N1; the 1957 virus (Asian flu) is H2N2 and the 1968 virus (Hong Kong flu) is H3N2. So far, fifteen different types of hemagglutinins and nine different types of neuraminidases have been identified in birds.[34] These "antigenic-shift" groups include many more slightly different types of hemagglutinins and neuraminidases that constitute "antigenic-drift" groups, or proteins that have different amino acid sequences but still can be typed by specific antibodies. With tools that can probe influenza's small but complex genome, scientists are just now beginning to understand what made those particular variants so lethal and generally why influenza is so dangerous.

This chapter will first discuss the BRSS's epidemiological parameters in regards to influenza, including different variants of influenza. It will then examine previous research that is illuminating the role of certain genes in influenza pathogenesis and try to place it in the context of the 3-D definition of danger. Finally, given the knowledge, this chapter will show the potential and problems of creating an influenza research oversight system for research not yet done. This chapter will specifically deal with the influenza A virus and not with the influenza B or C viruses.

A. Parameter Background

1. Transmissibility

Influenza is one of the most transmissible pathogens known to man. As Alfred W. Crosby, historian of the 1918 influenza, often remarks, "I know how not to get AIDS. I don't know how not to get the flu."[35] During the 1918 influenza pandemic, over 28% of the United States became infected, including 40% of the Navy and 36% of the Army. Overall, it is estimated that between 20-30% of the world's population became infected during the pandemic.[36,37] Between 10-20% of the world still becomes infected with influenza every year. Analysis of a 1978 outbreak in a boys boarding school yielded a R_0 of near 21.[38,39] Influenza's spread through sneezing and coughing and less intimate contact allows it to reach such a high prevalence. For these reasons, influenza was given almost the highest level of transmissibility on the 0-3 scale arbitrarily created for this paper.

Because the biological determinants of transmissibility are not well understood, influenza is a good candidate for engineering for nefarious purposes. Bioterrorists might prefer a pathogen as transmissible as influenza that killed a greater percentage of those infected. Although engineering any pathogen to such specifications would be quite difficult, so little is known about the science of transmissibility it would probably be more complicated to increase the transmissibility of an already-lethal pathogen. A better bet would be to take a highly transmissible platform pathogen such as influenza and increase the lethality of the pathogen. In other words, the natural evolution of influenza has already done the hard(er) parts.

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[33] Report by the Secretariat. *Influenza*. 56th World Health Assembly. 17 March 2003. Accessed online at http://www.who.int/gb/EB_WHA/PDF/WHA56/ea5623.pdf.

[34] Pringle, C.R. "Avian influenza viruses and human health." *Infectious Disease News*. February 2004. Accessed online at http://www.infectiousdiseasenews.com/200402/frameset.asp?article=guested.asp.

[35] Kolata, 6.

[36] Kolata, 7.

[37] Frost, W. (1920) *Public Health Rep.* 35:584-597.

[38] Communicable Diseases Surveillance Center. "Influenza in a boarding school." *British Medical Journal.* 4 March 1978: 587.

[39] Fraser, C. et al.

2. Lethality

Influenza's lethality has, to date, been the saving grace of the virus for humans. Influenza rarely kills more than 0.01% of those infected.[40] Major pandemics are noted for the increased lethality in the virus. For example, around 2.5% of those infected with the 1918 influenza virus died, including half a million Americans (Figure 5). Given the wide reach of influenza, a percentage point increase in lethality can represent many millions of people. Although data from China is sketchy, experts are certain that over 20 million people worldwide died during the 1918 influenza pandemic. Some high-ball estimates have even been put at 40-100 million. The high lethality of the 1918 pandemic alone was able to reduce the average American life expectancy from 51 to 39 for that year.

Influenza experts have been especially concerned with the increasing prevalence of the H5N1 avian influenza in Asia. H5N1 first emerged in Hong Kong in 1997 where it killed 6 of 18 infected cases for a whopping presumable lethality rate of 33%. As of 8 March 2004, this year's H5N1 outbreak has caused 22 deaths out of 32 laboratory-confirmed infected cases.[41] As noted in the previous chapter, lethality rates are often biased upwards due to an inability to detect all infected cases. Nonetheless, The lethality of the H5N1 virus is incredibly high given human influenza history. The avian virus has, thankfully, not demonstrated the ability to transmit between humans. Since a vaccine would not be ready for at least another 6-9 months, experts are actively intervening in the area to prevent the emergence of a human-to-human transmissible H5N1 virus.

Influenza's lethality can also vary depending on the target population. This variance occurs both between age groups and geographically separated people. Even though the 1918 influenza averaged a death rate of 2.5% globally, it killed an unbelievably high 20% of Western Samoans.[42] As far as age groups are concerned, influenza typically kills the very young and very old (Figure 6). The 1918 influenza virus demonstrated a unique ability to kill young adults, as indicated in Figure 6.[43] The molecular determinants, in either the virus or the immune system, of this heterogeneity in lethality are not well understood.

In sum, preventing the augmentation of the lethality of the virus should be the number one priority for any research oversight scheme for influenza.

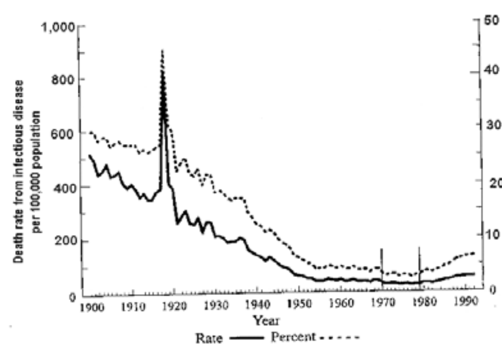

Figure 5 - 20th Century Mortality Rate from Infectious Disease[45]

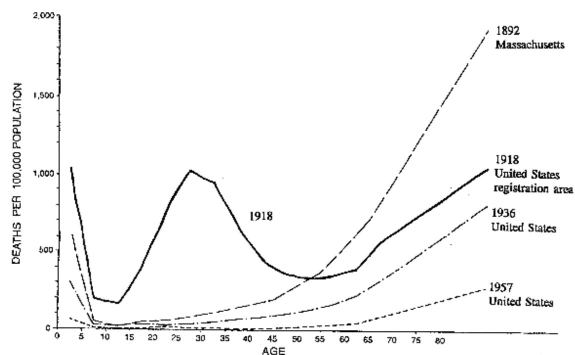

Figure 6 - Lethality Age Distribution[44]

3. Infectivity

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[40] Kilbourne, E. *Influenza*. 1987.
[41] World Health Organization. Communicable Disease Surveillance and Response. 8 March 2004. Accessed online at http://www.who.int/csr/disease/avian_influenza/country/cases_table_2004_03_08/en/.
[42] Kolata, 7.
[43] Lederberg, J. "Infectious Disease as an Evolutionary Paradigm." *Emerging Infectious Diseases*. October-December 1997. 3(4):417-423.
[44] Ibid.
[45] Ibid.

LD50s can only be obtained using human influenza viruses in some animal models. The BALB/c mouse has previously been used as an animal model for human H5N1 virus pathogenesis, including the determination of LD50s. H5N1 viruses do not require any adaptation to reproduce and cause illness in mice. However, many other influenza viruses do not kill readily and thus it is impossible to measure an LD_{50}.

Figure 7 shows the LD50s in BALB/c mice of 15 human H5N1 viruses isolated from the 1997 outbreak in Hong Kong.[46] The mouse LD50s only partially correlate with the case outcome in the human host. Upon further examination of the human data, the mouse model correlates better with the "average" human outcome. The age of the patient infected was a major determinant of the case outcome – three of the viruses that were highly pathogenic in mice were isolated from children younger than 4 years of age that had only mild symptoms. The 34-year old female that succumbed to a low pathogenicity virus had systemic lupus erythematosus. However, the article concludes that "at present it is not possible to distinguish between molecular determinants responsible for general virulence in mammals and those responsible for specific virulence in mice."[47]

The article's conclusion confirms the well-known fact in medical research that animal model data can only be taken with a grain of salt. Pathogens co-evolve with their specific host and will often exhibit different patterns of pathogenicity in different hosts. For this reason, Stanford influenza research Dr. Harry Greenberg remarked that the primary type of influenza research he'd be concerned about is research in human hosts.[48]

TABLE 1. Characteristics and mouse pathogenicity phenotypes of influenza A H5N1 viruses isolated from humans

H5N1 virus	Origin		Case outcome	Passage history[a]	Antigenic group[b]	Amino acids in HA1[c]	Mouse LD_{50}[d]	Mouse pathogenicity phenotype[e]
	Age (yr)	Sex						
HK/481/97	2	Male	Recovered	E2	A	NXA	$10^{1.7}$	High
HK/483/97	13	Female	MV[f]; died	C1E3	B	NXT	$10^{2.4}$	High
HK/485/97	24	Female	MV; recovered	C2E2	B	NXS	$10^{2.9}$	High
HK/491/97	4	Male	Recovered	C1E2	B	NXS	$10^{2.0}$	High
HK/503/97	1	Male	Recovered	C1E2	B	NXS	$10^{2.0}$	High
HK/514/97	25	Female	MV; died	CXE2	B	NXS	$<10^{1.5}$	High
HK/516/97	60	Female	MV; died	C2E2	A	NXA	$<10^{1.5}$	High
HK/532/97	14	Female	Recovered	CXE2	B	NXT	$<10^{1.5}$	High
HK/542/97	19	Female	MV; recovered	CXE3	B	NXS	$<10^{1.5}$	High
HK/156/97	3	Male	MV; died	E3	A	NXA	$10^{5.9}$	Intermediate
HK/486/97	5	Female	Recovered	C2E3	A	NXA	$>10^{6.5}$	Low
HK/488/97	2	Male	Recovered	C1E4	A	NXA	$>10^{6.5}$	Low
HK/507/97	3	Female	Recovered	C1E2	A	NXA	$>10^{6.5}$	Low
HK/538/97	3	Male	Recovered	CXE3	A	NXA	$>10^{6.5}$	Low
HK/97/98	34	Female	MV; died	CXE4	A	NXA	$>10^{6.5}$	Low

[a] Number of passages in a given host cell type: C, MDCK cells; E, embryonated eggs; X, unknown.
[b] As defined in Bender et al. (3).
[c] HA residues 154 to 156 encode a potential glycosylation site defined by NXS/T, where X is not proline. GenBank accession numbers for H5 HA sequences are AF036356 (30), AF046096-97 (27), and AF102671-82 (3).
[d] LD_{50}s were determined by inoculating groups of five lightly anesthetized mice intranasally with $10^{6.5}$ to $10^{1.5}$ EID_{50} of virus in a volume of 50 μl. Mice were checked daily for 14 days, and the LD_{50}s were calculated as previously described (17). LD_{50}s are expressed as the EID_{50} corresponding to 1 LD_{50}.
[e] Viruses with an LD_{50} of $>10^{6.5}$ were considered to be of low pathogenicity, and viruses with an LD_{50} of $<10^{3.0}$ were considered to be of high pathogenicity.
[f] MV, mechanical ventilation required.

Figure 7 - Heterogeneity of H5N1 LD50 in BALB/c Mouse Model

Infectious Dose Data

Because of the low lethality of influenza, true infectivity data, including ID_{50}s, are available. The first experiments with influenza in non-human primates did not even bother to measure the amount of influenza inoculated into the animals.[49, 50] These experiments only demonstrated the

[46] Katz, J.M et al. "Molecular Correlates of Influenza A H5N1 Virus Pathogenesis in Mice." *Journal of Virology*. 74(22):10807-10810.
[47] Ibid.
[48] Interview with Dr. Harry Greenberg. 23 February 2004.
[49] Burnet, F.M. "The influenza virus A infections of cynomolgus monkeys." *Australian Journal of Experimental Biology*. 1941, 19:281-290.
[50] Long, P.H. et al. "Etiology of influenza." *Journal of the American Medical Association*. 1931, 97:1122-27.

susceptibility, including possible lethality, of different monkey models to influenza.[51,52] Later studies quantitated the infectivity of influenza in respect to its 50% tissue culture infective dose or $TCID_{50}$. The $TCID_{50}$ is the amount of a virus that produces a cytopathic effect in 50% of the cultures inoculated. Other studies calculate the infectivity of influenza viruses with respect to its egg infectious dose or EID_{50}. Both $TCID_{50}$s and EID_{50}s can be converted into plaque forming units if a conversion formula is available.

One study found that three strains of human influenza virus could infect owl, cebus, and squirrel monkeys.[53] However, because only squirrel monkeys showed clinical symptoms similar to those of humans, the study concluded that squirrel monkeys would be a good primate model for human influenza. No ID_{50}s were determined in this study, although it is clear from the data that the ID_{50}s for these particular strains would be below 1e4 $TCID_{50}$.

Because squirrel monkeys showed clinical symptoms in response to infection with human influenza virus, they make an excellent model to study possible transmission of avian influenza viruses in primates. A follow-up study looked at the virulence of avian influenza viruses in squirrel monkeys and hamsters as well as ducks and ferrets.[54] The 10 avian influenza viruses exhibited a wide range of virulence in the squirrel monkeys. However, the study did not calculate the infectious doses and the disease-producing doses of either the avian or human influenza viruses because of the lack of monkeys.

A later study arrived at a possible ID_{50} dose in squirrel monkeys.[55] Groups of four squirrel monkeys were given doses of 1e2, 1e3, 1e4, 1e5, 1e6, or 1e7 $TCID_{50}$. The ID_{50} calculated for both intranasal and intratracheal administration of the avian-human influenza reassortant virus was **1e2.6 $TCID_{50}$**. Their results are interesting because infectivity was the same for both intranasal and intratracheal administration routes.

Another study demonstrated that Hong Kong H5N1 virus could infect ferrets.[56] The H5N1 virus was shown to produce a more severe respiratory disease than H3N2 virus, even though H3N2 virus replicate 100 to 1000 times more efficiently in the upper respiratory tract of ferrets. H5N1 virus was serially titrated in eggs to determine the EID_{50}. A 50% ferret infectious dose (FID_{50}) was determined by giving two ferrets each doses of 1e4, 1e3, and 1e2 EID_{50} and three ferrets a dose of 1e1 EID_{50}. The FID_{50} was determined to be approximately 1e2 EID_{50}. However, without a conversion from EID_{50} to PFU it is difficult to compare this to other data, as it is not known how H5N1 virus strains establish themselves in eggs.

Because of its low lethality, some infectivity data for influenza is available in humans. Couch et al. (1974) cites unpublished data that the 50% human infectious dose (HID_{50}) for intranasal administration of a influenza strain serologically identical to the A/Aichi/2/68 H3N2 strain is **320 $TCID_{50}$**.[57] Couch et al. (1971) had previously also cited unpublished data that the HID_{50} of (possibly

[51] Saslaw, S. and Carlisle, H.N. "Aerosol exposure of monkeys to influenza virus." *Proceedings of the Society of Experimental Biology and Medicine*. 1965, 119(3):838-843.
[52] Saslaw, S. et al. "Reactions of Monkeys to experimentally induced influenza virus A infection." *Journal of Experimental Medicine*. 1946, 84:113-125.
[53] Murphy, B.R. et al. "Evaluation of Three Strains of Influenza A Virus in Humans and in Owl, Cebus, and Squirrel Monkeys." *Infection and Immunity*. June 1980, 28(3):688-691.
[54] Murphy, B.R. et al. "Virulence of Avian Influenza A Viruses for Squirrel Monkeys." *Infection and Immunity*. September 1982. 37(3):1119-26.
[55] Snyder, M.H. et al. "Infectivity and Antigenicity of Live Avian-Human Influenza A Reassortant Virus: Comparison of Intranasal and Aerosol Routes in Squirrel Monkeys." *Journal of Infectious Diseases*. October 1986, 154(4):709-712.
[56] Zitzow, L.A. et al. "Pathogenesis of Avian Influenza A (H5N1) Viruses in Ferrets." *Journal of Virology*. May 2002, 76(9):4420-29.
[57] Couch, R.B. et al. "Correlated Studies of a Recombinant Influenza-Virus Vaccine. III. Protection Against Experimental Influenza in Man." *Journal of Infectious Diseases*. November 1971, 124(5):473-80.

the same strain of) influenza serologically identical to the A/Aichi/2/68 H3N2 strain was **127 $TCID_{50}$**.[58]

An experiment in which prisoners were administered A2/Bethesda/10/63 influenza virus via aerosol gave a wide range of infectivity results.[59] Of the 23 prisoners, only 4 developed clinically symptomatic influenza. However, one of those who became sick received a dose of only 1 $TCID_{50}$. The other three prisoners that became sick received doses of only 5 $TCID_{50}$. Meanwhile, those who received doses of 126, 78, and 59 $TCID_{50}$ did not become ill, perhaps because many of those men already had medium to high neutralizing antibody titers for influenza. The article concludes that half of the men with low antibody titers became infected with 0.6 to 3.0 $TCID_{50}$. These data correspond with infectivity data in mice where the "LD_{50} was 10 and the 50% infectious dose was 0.5 mouse infectious doses."[60] The article does note that 1) these minimal doses approached the limits of sensitivity of sampling and assay systems and that 2) it is likely that these estimated minimum infectious doses are falsely low because of loss of infectivity in the atomization and sampling process. If possible, new experiments are likely needed to understand the true infectivity of influenza in human and non-human primate populations. Access to proprietary data at firms that test influenza vaccines might also help establish influenza's infectivity in humans.

B. Highlighting Problematic Research That Has Been Done

Influenza research has advanced by leaps in bounds from the era when it took researchers tens of years to identify a virus, rather than a bacterium, as the etiological agent. This section will detail the basic pathogenesis of the influenza A virus before examining past research that is potentially problematic from a dual-use standpoint.

1. General Pathogenesis

Influenza is one of the few viruses that have a segmented genome. It carries its ten gene products on eight different gene segments. Each segment contains one gene, with two segments containing two related gene products. The gene segments and viral structure are depicted in Figure 8.

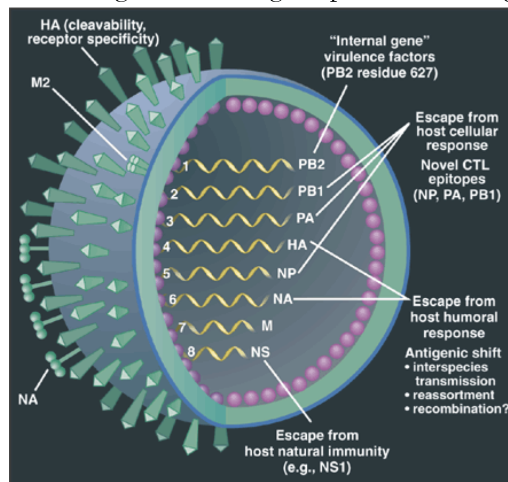

Figure 8 - Influenza Gene Function and Virion Structure[61]

Understanding of influenza genetics has progressed by leaps and bounds in the 1990s after the development of an influenza reverse genetics system. Reverse genetics allows the production of "live" viral RNA from cloned cDNA. With the development of reverse genetics, researchers can selectively alter or delete viral segments and incorporate those altered segments into new influenza virions.[62] This process puts rationally-designed live-attenuated virus vaccines within reach. Many influenza researchers want to replace the annual reassortment-based influenza vaccine production process with the reverse genetics

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[58] Couch, R.B. et al. "Induction of Partial Immunity to Influenza by a Neuraminidase-specific Vaccine." *Journal of Infectious Diseases*. April 1974, 129(4):411-20.
[59] Alford, R.H. et al. "Human Influenza Resulting From Aerosol Inhalation." *Proceedings of the Society for Experimental Biology and Medicine*. 1966, 122:800-4.
[60] Hood, A.M. "Infectivity of influenza virus aerosols." *Journal of Hygiene*. 1963, 61:331-335. Note: this article uses a method for approximating viral titers in eggs called the "egg-membrane piece technique" that I couldn't figure out. The technique is cited in Fazekas de St. Groth, S. et al. *J. Hyg. (Cambridge)*. 1958b, 56:535.
[61] Webster, R.G. "A Molecular Whodunit." *Science*. 7 September 2001, 293:1773-75.
[62] Neumann, G. and Kawaoka, Y. "Reverse Genetics of Influenza Virus." *Virology*. 2001, 287:243-250.

system. Progress in the reverse genetics model has also allowed the de novo synthesis of influenza virus. Researchers can selectively test which mutations in 1918 or H5N1 influenza make those particular strains so virulent.[63]

Hemagglutinin

The plurality of past research into influenza pathogenesis has focused on the role of hemagglutinin (HA). As the sole receptor binding protein on the surface of influenza, HA is responsible for the host and tissue tropism of individual influenza viruses. HA binds sialic acid (or N-acetylneuraminic acid), a carbohydrate found throughout the body. The protein is initially synthesized in an inactive form (HA_0) and must be cleaved by proteases in an infected cell to gain receptor-binding function (HA_1 and HA_2).[64] Tissue tropism is determined partially by the presence of the correct types of proteases.[65] HA is one of the three proteins used in the classification of influenza viruses (N and NP are the other proteins). As one of the primary targets for the humoral immune system, HA is very important for viral evasion of the immune system. HA is also the only protein against which neutralizing antibodies are made. Depending on the variant of HA, four to five antigenic sites have been identified for each HA. These sites are among the most variable sites in the HA protein in human strains.

There are two principle ways in which changes in HA have been shown to make influenza more dangerous. The first is by adding basic residues in the HA connecting peptide. This change makes the cleavage site more accessible to more proteases and increases the tissue tropism of the virus.[66] The addition of basic residues is thought to account for the increased pathogenicity of H5 and H7 avian influenza viruses. These additional residues were not discovered in the 1918 pandemic influenza virus. This subject will be covered more in a later section on H5N1 research.

The second way HA increased influenza virulence is through evasion of the humoral immune response, especially preexisting immunity. This role of adaptive immune evasion presents less of a problem for research oversight, since it would be quite difficult for a human to create an entirely novel HA that retained its receptor binding function and evaded preexisting humoral immunity. It would probably be easier to use a HA sequence from a preexisting influenza virus, such as an H2, that has not re-emerged in the human population for quite some time but has previously shown the ability to produce an infection in humans. In sum, it would be difficult for any lab to produce any problematic knowledge on evasion of the humoral immune system because residual immunity in the population is not known at such a level. Furthermore, the information on the strains that the human population retains little preexisting immunity is already out in the open.

Neuraminidase

Neuraminidase is the other major protein on the outside of the influenza virus. Its role is to cleave N-acetylneuraminic acid residues from the surface of an infected cell to allow the virus to successfully bud from the cell. Neuraminidase is also thought to allow the influenza virus to penetrate mucosal surfaces. Antibodies created against neuraminidase do not neutralize the virus, but do prevent the virus from budding. The selection pressure encourages genetic drift in the neuraminidase gene and makes it one of the more variable influenza proteins. Two influenza antivirals – zanamivir and oseltamivir – use neuraminidase inhibition to prevent viral budding.

Unlike work with the addition of basic residues to the HA cleavage site, research in neuraminidase has demonstrated few single changes that markedly alter pathogenesis. One exception to this observation is the finding that the loss of a glycosylation site at residue 146 in the WSN/33

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[63] Hatta, M. et al. "Reverse genetics approach towards understanding pathogenesis of H5N1 Hong Kong influenza A virus infection." *Philos. Trans. R. Soc. Lond.* B. 2001, 356:1841-43.
[64] Taubenberger, J. "Influenza virus hemagglutinin cleavage into HA1, HA2: no laughing matter." *PNAS*. 1998 Aug 18, 95(17):9713-5.
[65] Rott, R. et al. *American Journal of Respiratory and Critical Care Medicine*. 1995, 152:S16-S19.
[66] Steinhauer, D.A. and Skehel, J.J. "Genetics of Influenza Viruses." *Annual Review of Genetics*. 2002, 36:305-32.

influenza strain makes the virus especially virulent and neurotropic in mice.[67] This finding, although not directly relevant to influenza's pathogenicity in humans, points to the cooperativity between HA and NA in determining tissue tropism.

HA and NA cooperativity has also been found in experiments attempting to create NA mutants that were resistant to antivirals that targeted NA activity. Scientists found that, although some NA mutants were isolated, most antiviral-resistant viruses had mutations that mapped to the HA gene, in residues next to those involved in receptor binding.[68] It is thought that, to compensate for the inhibition of activity of NA, influenza viruses modulated the binding affinity of their HA so that the virus did not require NA activity to bud successfully from an infected cell.

Another paper points to a possible role for NA cooperativity with HA in determining influenza virus tissue tropism. Goto and Kawaoka (1998) demonstrated how the NA protein of influenza strain A/WSN/33 (H1N1) helps in the cleavage of the viral HA protein. The WSN/33 variant was created after one of the first influenza viruses ever isolated was forced to replicate in mouse brain, a tissue influenza rarely infects.[69] In the A/WSN/33 strain, NA sequesters plasminogen on the cell surface, thus activating the serine protease plasmin, which cleaves and activates viral HA.[70] This study demonstrates the inherently complex way in which influenza viruses can find ways to replicate in systems to which they are not accustomed. It also hints at the overwhelming difficulties in trying to type certain research, especially on a gene-by-gene basis, into different danger categories. Thankfully, it is doubtful that this particular research would be especially dangerous as all experiments were done in mouse models and would not necessarily lead to any conclusions about influenza pathogenesis in humans.

Antigenic Changes in HA and NA

Before discussing other genes, it is worth commenting on the role of the immune system in shaping hemagglutinin and neuraminidase. Influenza A viruses are typed by their HA and NA repertoire because of the critical role these proteins play in the elimination of the virus from the body. Changes in the repertoire of HA and NA are differentiated into antigenic shifts and antigenic drifts. Antigenic shifts occur when the HA or NA of an influenza virus are not recognized by panels of antibodies for other types of influenza. Antigenic drifts account for any changes in the exact sequence of either HA or NA, but occur only when no changes in the antigenic repertoire are denoted from the antibody tests.

Antigenic shifts are thought to result from genetic reassortments of two different influenza viruses, usually one human and one animal strain. Influenza viruses are the only segmented virus known to undergo genetic reassortments. In the reassortment, animal and human influenza viruses coinfect the same cell and virions with combinations of the different gene segments emerge. Controlled genetic reassortment is currently the FDA approved method for the creation of influenza vaccine. Pandemic influenza has resulted in three cases in which an influenza virus has undergone an antigenic shift – H1N1 in 1918, H2N2 in 1957, and H3N2 in 1968. Interestingly, pandemic influenza did not result in the swine flu scare of 1976, when a H1N1 virus emerged in Fort Dix, New Jersey. Influenza experts are currently very concerned about the possibility of another influenza pandemic should the H5N1 avian virus gain the ability to spread from person-to-person.

Although less alarming, antigenic drifts are also troublesome. Antigenic drifts occur when there are repeated minor antigen changes but the virus retains its serological relationship with the dominant virus in the population. Antigenic drift is largely responsible for the influenza epidemics

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[67] Taubenberger, J. "Influenza virus hemagglutinin cleavage into HA1, HA2: no laughing matter." *PNAS*. 1998 Aug 18, 95(17):9713-5.
[68] McKimm-Breschkin, J.L. "Resistance of influenza viruses to neuraminidase inhibitors – a review." *Antiviral Research*. 2000, 47:1-17.
[69] Taubenberger, J. (1998)
[70] Goto, H. and Kawaoka, Y. "A novel mechanism for the acquisition of virulence by a human influenza A virus." *PNAS*. 18 Aug 1998, 95(17): 10224-28.

that occur every year. Since enough people have preexisting immunity, antigenic drift influenza viruses do not cause the pandemics that antigenic shift viruses do. However, antigenic drifts have caused influenza viruses to substantially lower the protection conferred by vaccination. In 1947 a worldwide influenza epidemic occurred in which no antigenic shift occurred, even though the vaccine was protective in 1943-44 and 1944-45 epidemics. A recent study showed that, even though both the 1947 virus and the 1943 vaccine strain virus were both H1N1 viruses, the intrasubtypic antigenic variation (especially in the HA protein) was enough that the vaccine gave no cross-protection to the new strain.[71]

Our knowledge of antigenic shifts and drifts in influenza points to a way to oversee some types of influenza experiments. Primarily, genetic reassortment of animal and human influenza virus should be placed into a high category of danger. This recommendation fits into the oversight scheme initially proposed for the BRSS, as genetic reassortment between animal and human viruses would expand the host range or tissue range of influenza virus. Of course, sans human host selective pressures and large numbers of recombinant virus particles, there is a reasonable chance that reassorted virus would not immediately have the ability to attach, fuse, or replicate in human cells. Furthermore, the technology and knowledge necessary to do this experiment has existed for quite some time. As noted above, the natural genetic reassortment process is the exact method used for the production of 6+2 vaccine (in which the candidate virus is coincubated in eggs with a stock strain of A/Puerto Rico/8/34 or H1N1 virus in hopes that the candidate HA and NA genes reassort with stock versions of the other six genes). A researcher that might plausibly create a new pandemic influenza strain would just need 1) to replace one of the human strains with the H5N1 or other animal influenza virus, 2) some eggs, and 3) a reasonable amount of luck (a little more than is needed to make the vaccine each year). Of course, testing and isolating virulent strains would require work in relevant host organisms. Given this procedure carries with it the greatest possibility of creating of a pandemic influenza strain that could kill millions of people, it is a strong candidate for the moderately or extremely dangerous activities area of the BRSS.

Even though intrasubtypic changes in HA or NA have been shown to produce influenza virus that could evade the humoral immune system, it is difficult to operationalize this fact into research oversight. A priori, there is no way to tell whether a point mutation will confer enough of a change in HA or NA to evade vaccine or preexisting immunity. Immune responses can vary greatly from individual to individual. Any research that sought to alter human HA or NA proteins with targeted or random mutagenesis would not technically become definitely dangerous until introduced into a human host. Nonetheless, it is likely that experiments that altered HA or NA would need to test the recombinant virus in animal models or in human cell cultures. And, although the probability of creating a truly dangerous strain is very low, some oversight – local or national, depending on the parent strain of virus and the degree of changes planned – would be needed to ensure that research was being conducted in a responsible manner and to deal with any potentially dangerous results before they were submitted for publication.

Other Influenza Genes

Research studies have shown that the removal of any one gene segment from wild type influenza virus severely attenuates the activity of the virus. Thus, it is important to review research that characterizes the role of other genes in influenza pathogenesis.

NS1

The non-structural gene of influenza has been shown to be an important contributor to influenza pathogenesis. Viral genes that do not directly contribute to virion structure or polymerase/replication are often used for immunoevasion. NS1 is no exception. After years of

[71] Kilbourne, E.D. et al. "The total influenza vaccine failure of 1947 revisited: major intrasubtypic antigenic change can explain failure of vaccine in a post-World War II epidemic." *PNAS*. 6 August 2002, 99(16):10748-52.

debate about the role of the non-structural genes, Peter Palese's group showed that influenza viruses without the NS1 gene could replicate in interferon-deficient systems, suggesting that NS1 was involved in inhibiting the cellular interferon response.[72] The group has since gone on to create rationally-designed attenuated vaccine strains that lack the NS1 gene.[73] Further studies showed that NS1 binds double-stranded RNA and prevents the activation of interferon-regulatory factor 3 and NF-kB and subsequent induction of IFN-alpha and IFN-beta.[74,75,76] Structural studies into NS1 have shown its interferon regulating activity is localized to its N-terminus and that the remaining two-thirds of the C-terminus helps stabilize dimeric interactions.[77,78] The NS1 gene has also been shown to both induce and down-regulate apoptosis in different systems, depending on the expression of other viral genes.[79,80] Substitution of a functional NS1 gene for a non-functional one has been shown to improve the growth kinetics and yield of various influenza viruses in African green monkey kidney cell lines.[81] Influenza virus without NS1 is attenuated in mice, but is able to kill mice with deficient interferon signalling.[82] NS1 (along with E3L of vaccinia) has shown to be a natural strategy of influenza viruses to inhibit the newly discovered innate immunity defense of RNA silencing.[83] NS1 has also been implicated as one of the chief molecular determinants of the increased virulence of both 1918 and H5N1 influenza. Alterations of the NS1 gene, if cleared in human hosts or cell culture, should be reviewed as potentially or moderately dangerous activities for potentially increasing the virulence of a listed agent.

Polymerase Associate Genes (PA, PB1, PB2)

Three gene segments of the influenza genome hold the entire polymerase complex. These genes are named based upon their acidic (PA) or basic properties (PB). The PB1 protein is responsible for endonuclease and polymerase activity. The PB2 subunit appears to be a cap-binding protein involved in the initiation of transcription. The PA subunit is responsible for the replication activity of the polymerase and exhibits protease activity. The lack of a proofreading capability in the influenza polymerase complex allows influenza to create its incredible genetic diversity. One in every

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[72] Garcia-Sastre, A. et al. "Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems." *Virology*. 1998 December 20, 252(2):324-30.
[73] Talon, J. et al. "Influenza A and B viruses expressing altered NS1 proteins: a vaccine approach." *PNAS*. 11 Apr 2000, 97(8):4309-14.
[74] Talon, J. et al. "Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein." *J. Virol.* 2000 Sep, 74(17):7989-96.
[75] Wang, X et al. "Influenza A virus NS1 protein prevents activation of NF-kB and induction of alpha/beta interferon." *J. Virol.* 2000 Dec, 74(24):11566-73.
[76] Using the assay derived for NS1 inhibition of IFN pathways, ebola protein VP35 has also been shown to execute a similar function in ebola pathogenesis.
[77] Salvatore, M. et al. "Effects of influenza A virus NS1 protein on protein expression: the NS1 protein enhances translation and is not required for shutoff of host protein synthesis." *J. Virol.* 2002 Feb, 76(3):1206-12.
[78] Wang, X. et al. "Functional replacement of the carboxy-terminal two-thirds of the influenza A virus NS1 protein with short heterologous dimerization domains." *J. Virol.* 2002 Dec, 76(24):12951-62.
[79] Schultz-Cherry, S. et al. "Influenza virus NS1 protein induces apoptosis in cultured cells." *J. Virol.* 2001 September, 75(17):7875-81.
[80] Zhirnov, O.P. et al. "NS1 protein of influenza A virus down-regulates apoptosis." *J. Virol.* 2002 Feb, 76(4):1617-25.
[81] Ozaki, H. et al. "Generation of High-Yielding Influenza A Viruses in African Green Monkey Kidney (Vero) Cells by Reverse Genetics." *Journal of Virology*. February 2004, 78(4):1851-57.
[82] Garcia-Sastre, A. et al. (1998)
[83] Li, W.X. et al. "Inteferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing." *PNAS*. 2004 Feb 3, 101(5):1350-5.

five virions created is likely to contain a single mutation in its 13,500 nt genome.[84] It should be noted that much of this model for polymerase function is under debate in the literature.

Variations in each protein have been shown to affect influenza replication and pathogenesis. A F130Y mutant in PB2 protein exhibited higher replication rates than wild-type PB2, although this does not mean there would be any change in vivo.[85] A reading frame shift in PB1 can create a novel protein, PB1-F2, which localizes in mitochondria and causes apoptosis in infected cells.[86] However, given the lack of conservation of this open reading frame across influenza strains, the in vivo relevance of such an apoptotic protein is not currently known. Multiple PB1 and PB2 (as well as NP) mutations are thought to be the cause of the temperature sensitive phenotype of the cold-adapted influenza virus used in MedImmune's FluMist vaccine.[87]

Most importantly, a mutation at position 627 in PB2 has been shown to play a role in determining host range of influenza viruses and may account for the high pathogenicity of H5N1 avian viruses in humans. Subbarao et al. (1993) examined a strain of reassorted influenza that could replicate in avian tissue but not in canine kidney cells.[88] They found that the host restriction phenotype was due to a single amino acid substitution from Glu to Lys at amino acid residue 627. Furthermore, they found that all avian influenza viruses known at the time had a Glu at position 627, while all human influenza viruses had a lysine. These facts suggested that PB2 residue 627, among other amino acids, may play a determining role in virus host range. This mutation will be covered a bit further in the section on research into H5N1 pathogenesis.

Recently, the 3D structure of the influenza polymerase complex was determined by electron microscopy and image process of recombinant ribonucleoproteins at a resolution of 23 angstroms.[89] However, it is unlikely that any information revealed in this structural analysis would be of any use to bioterrorists.

Matrix Genes

Gene segment 7 generates the influenza matrix protein (M1) and M2 protein. The M1 protein plays an important role in virus assembly, while the M2 protein functions as a small transmembrane ion channel that tells the virion to disassemble.[90] The M2 protein is sensitive to pH changes and only activates when the influenza virus enters acidic endosomes after viral entry. The acidification of the viral interior by the M2 protein causes the virus to disassemble and allows the polymerase proteins to traffic to the nucleus. The antivirals amantadine and rimantadine inhibit the activity of the M2 protein and prevent the acidification of the virus and subsequent disassembly. The specific amino acids that cause antiviral resistance in influenza have been identified.[91] Antiviral resistance is, to date, the major form of dangerous research in either the M1 or M2 protein.

2. Determinants of 1918 Influenza Pathogenesis

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[84] Parvin, J.D. et al. "Rapid RNA sequencing using double-stranded template DNA, SP6 polymerase, and 3'-deoxynucleotide triphosphates." *DNA*. 1986, 5:167-71.
[85] Gastaminza, P. et al. "Mutations in the N-terminal Region of Influenza Virus PB2 Protein Affect Virus RNA Replication but Not Transcription." *J. of Virol.* May 2003, 77(9):5098-5108.
[86] Chen, W. et al. "A novel influenza A virus mitochondrial protein that induces cell death." *Nature Medicine*. December 2001, 7(12):1306-1312.
[87] Jin, H. et al. "Imparting temperature sensitivity and attenuation in ferrets to A/Puerto Rico/8/34 influenza virus by transferring the genetic signature for temperature sensitivity from cold-adapted A/Ann Arbor/6/60." *Journal of Virology*. January 2004, 78(2):995-8.
[88] Subbarao, E.K. et al. "A single amino acid in the PB2 gene of influenza A virus is a determinant of host range." *Journal of Virology.* 1993 April, 67(4):1761-4.
[89] Area, E. et al. "3D Structure of the influenza virus polymerase complex: localization of subunit domains." *PNAS*. 2004 Jan 6, 101(1):308-13.
[90] Steinhauer, D.A. "Genetics of Influenza Viruses." *Annual Reviews of Genetics*. 2002, 36:305-32.
[91] Hay, A.J. et al. "The molecular basis of the specific anti-influenza action of amantadine." *EMBO J.* 4:3021-24.

New research into the 1918 influenza pandemic opens the possibility of engineering the influenza virus or at least recreating the 1918 virus itself. The lab of molecular virologist Jeffrey Taubenberger has spent the last few years carefully pulling out segments of the 1918 influenza genome from samples either frozen in tundra or embedded in paraffin during the pandemic. Five of the eight RNA gene segments of the 1918 influenza virus have been sequenced to date. Almost all of the analysis into the molecular determinants of the pathogenesis of the 1918 influenza virus has been done on the genomic level. The 1918 influenza gene segments have been lifted directly from human tissues and have not been passaged through chicken eggs, unlike many of the 1957 and 1968 pandemic influenza strains. Passaging results in alterations in the genome that would be due to random drift and not due authentic selection pressures. Thus, the genetic profile of 1918 influenza contains fewer adulterations than many later egg-passaged strains, including other pandemic influenza strains.

Hemagglutinin

The initial sequence determination of 1918 influenza hemagglutinins showed that their H1s were the closest mammalian H1s to their avian counterparts.[92] However, the 1918 HA1 sequence showed many more amino acid differences to its closest avian relative than the 1957 H2, 1968 H3, or 1997 H5 genes. Not surprisingly, the 1918 influenza virus is thought to be a very close relative to the common mammalian influenza virus ancestor.

Sequences of the HA1 part of the hemagglutinin gene from five different 1918 influenza cases exhibited 98.9 to 99.8% sequence homology.[93] Despite the passage through chicken eggs, the 1957 and 1968 pandemic viruses also demonstrated similar genetic homogeneity. One of the few differences between the 1918 strains mapped to the receptor-binding site of HA, suggesting that two different strains of influenza were circulating at the time. Critically, this difference occurred at the same amino acid that separates avian and swine influenza viruses, E190D.[94] Avian influenza HA bind alpha 2-3 sialic acid receptors while human influenza HA bind alpha 2-6 sialic acid receptors. Swine influenza viruses have the ability to bind both types of sialic acid receptors. Many influenza experts believe pigs are an intermediary in the spread of avian influenza viruses into the human population due to its receptor binding range.

The E190D polymorphism suggests that one of the variants of the 1918 influenza virus had the capability of binding both avian and human sialic acid receptors and, more importantly, that the E190D is the critical change needed to allow viral replication in the human respiratory tract. The co-circulating strains also differed in sequence at a key antigen-determining region of the HA1 gene (G225D). It is not known whether this polymorphism would change the immunological response to the epitope. Furthermore, it is not clear how this antigenic difference would help would-be bioterrorists today, unless one variant took advantage of an antigenic signature that the immune system could not respond to (through self-deletion, etc).

Recently, the crystal structure of the uncleaved 1918 HA gene was determined at 3.0 angstrom resolution.[95] When compared to three other crystal structures of influenza HA, the uncleaved 1918 HA was most closely related to the uncleaved avian H5 subtype. The 1918 HA0 binding site for sialic acid receptors was also more related to the avian H5 subtype binding site. Without going into much more detail, needless to say, the crystal structure of the 1918 HA0 has lead to a number of hypotheses about the contribution of certain amino acids to its role in 1918 influenza

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[92] Reid A.H. et al. "Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene." *PNAS*. February 1999, 96: 1651-56.

[93] Reid, A.H. et al. "1918 Influenza Pandemic Caused by Highly Conserved Viruses with Two Receptor-Binding Variants." *Emerging Infectious Diseases*. October 2003, 9(10):1249-1253.

[94] Reid, A.H. et al. (1999)

[95] Stevens, J. et al. "Structure of the Uncleaved Human H1 Hemagglutinin from the Extinct 1918 Influenza Virus." *Science*. Epub 5 February 2004. Accessed online at http://www.sciencemag.org/cgi/rapidpdf/1093373v1.pdf.

virulence. However, it is unlikely that structural studies that do not include mutational analysis need to be included in the BRSS.

Neuraminidase

Similar genetic sequence homologies and ancestries were found for the neuraminidase gene segment sequence of the 1918 influenza genome.[96] The 1918 influenza NA appears to be an intermediate between mammal and avian influenza, suggesting that the 1918 virus was introduced into mammals just before the pandemic. Again, although the 1918 influenza NA did not vary much from its closest avian relative, it varied more than the 1957 pandemic N2 and 1997 Hong Kong N1 did to their closest avian relatives. Sequencing also showed that the 1918 neuraminidase did not have the stalk deletions that are present in chicken influenza viruses and that previous studies had predicted. Nor did sequencing give many clues about the minimal changes necessary to allow avian N1 to function in a mammalian host. Ultimately, it seems that the greatest contribution NA made to the high pathogenicity of the 1918 influenza genome is through its antigenic novelty.

Sequencing did show a possibly interesting change at residue 354. The 1918 influenza NA had an Asp at this position, while all other human influenza viruses have a Gly at position 354. Only three other influenza strains, regardless of subtype, have an amino acid other than Gly at that position. It is not know whether this change confers a functional alteration in neuraminidase activity, although studies are currently being conducted in recombinant virus with 1918 influenza genes.

Non-structural Genes

Research into the role of the 1918 pandemic virus NS1 and NEP genes has been creating more questions than it answers. The NS gene segment was the third gene segment to be fully sequenced by Dr. Taubenberger's team. Phylogenetic analysis of the sequence tentatively put the NS gene segment at the root of the swine clade, rather than on the fringe of the mammalian clade as the HA and NA phylogenetic analysis indicated.[97] This tentative placement would mean that all swine and human NS genes were originally derived from the 1918 NS gene, giving it a possible dominant role in 1918 virulence. However, there is considerable uncertainty in the phylogenetic tree so this finding should not be taken too strongly. Because of the role of the NS1 gene in influenza lung pathology, Basler et al. (2001) added the 1918 NS1 gene or entire 1918 NS gene segment into stock influenza viruses. Although the recombinant viruses grew fine in culture, the resulting viruses were attenuated in virulence in mice. It is not known whether the attenuation is due to inability to interact with mouse host factors or poor protein stability. However, a earlier experiment in which a recombinant human influenza virus with an avian NS1 gene was attenuated indicates that the NS1 gene may be highly evolved for specific hosts.[98]

Because of the possible host specificity of the 1918 NS1 gene for evading the human immune system, Geiss et al. (2002) examined the role of the NS1 gene in altering gene expression in human lung epithelial cells.[99] The study compared both gene expression in cells infected with stock human influenza virus to those strains without NS1 genes as well as a recombinant influenza strain with the 1918 NS1 gene. The researchers found that NS1 plays an important role in inhibiting interferon, cytokine, and NF-κB pathways. More importantly, recombinant influenza virus with 1918 NS1 was more effective at inhibiting interferon-stimulated genes than its parental influenza virus, although the overall cellular response was similar. Equivocal gene expression and mouse model data

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[96] Reid, A.H. et al. "Characterization of the 1918 'Spanish' influenza virus neuraminidase gene." *PNAS*. 6 June 2000, 97(12):6785-90.
[97] Basler, C.F. et al. "Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes." *PNAS*. 27 February 2001, 98(5):2746-51.
[98] Treanor, J.J. et al. (1989) *Virology*. 171:1-9.
[99] Geiss, G.K. et al. "Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: The role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza." *PNAS*. 6 August 2002, 99(16):10736-41.

indicate that the role of the NS1 gene in determining the high lethality of the 1918 influenza pandemic is by no means certain but looks promising.

Research Into Countermeasures Against 1918 Influenza

Despite the dual-use dilemma surrounding the sequencing of the highly pathogenic 1918 virus, recombinant studies have given some fairly encouraging results. Under BSL-3 level conditions, Tumpey et al. (2002) created a recombinant influenza virus containing different combinations of the hemagglutinin, neuraminidase, and matrix genes from the 1918 influenza virus. The recombinant viruses were able to kill mice successfully, a phenomenon that is normally reserved for H5 influenza viruses. Tellingly, recombinant viruses that bore only 1918 HA or 1918 NA were attenuated in the mice, further suggesting a role of HA and NA cooperativity in viral pathogenesis. More importantly, the recombinant influenza virus growth was successfully inhibited both in cell culture and in vivo by NA antivirals/inhibitors, zanamivir and oseltamivir, and by M2 ion-channel antivirals/inhibitors, amantadine and rimantadine. Even if it takes 6-9 months to create a new vaccine, in the event of a bioterrorist release of 1918 pandemic influenza virus, antivirals will be able to confer some protection.

A very recent study indicates a possible vaccine that might control an outbreak of 1918 influenza. Frustrated by the lack of information given by sequence analysis, Tumpey et al. (2004) inserted between two to five gene segments from the 1918 pandemic virus into the A/WSN/33 influenza virus.[100] Once again, recombinant viruses containing the HA, NA, M, NS, and NP genes (or just the HA and NA genes) from 1918 pandemic influenza were very lethal in mice. However, introducing the 1918 M, NS, or NP genes did not significantly increase the virulence of the 1918 HA/NA:WSN virus. Furthermore, the introduction of the HA, NA, and M genes from the 1918 virus was able to overcome the attenuation noted in the 1918 NS:WSN virus. The 1918 HA/NA:WSN virus was also recovered in mouse brain, despite the fact that the 1918 HA and NA genes have not been adapted for mouse neurotropism. Neither did the 1918 HA/NA:WSN require exogenous trypsin to grow in MDCK cells, suggesting that HA is being cleaved through some other protease. The study also identified two candidate vaccines that could protect against 1918 influenza virus should it ever reemerge or be used in a bioterrorist attack. A homologous inactivated 1918 HA/NA:WSN strain protected against recombinant viral challenge as did the non-pathogenic Sw/Iowa/30 strain.

3. Determinants of H5N1 Virus Pathogenesis

With the recent emergence of a highly pathogenic avian influenza virus and growing fears about a new influenza pandemic, virologists have been spending a great deal of effort in trying to understand the molecular pathogenesis of the H5N1 avian influenza viruses. Because of the concomitant emergence of the virus with new genomic and proteomic technologies, a great deal is coming to light about how H5N1 viruses are different than annual epidemic influenza. The dual-use results from that work will be described in this section.

One of the major reasons for H5N1's increased pathogenesis is the presence of additional basic residues in the cleavage domain of the HA gene. Hatta et al. (2001) demonstrated the role of the additional residues in creating reassortants of avirulent and virulent H5N1 viruses.[101] When compared with a consensus avian HA, one of the H5N1 HA's had four extra basic amino acid residues and produced systemic lethal infection in mice (while the virus bearing the consensus avian HA did not). A single amino acid change in another H5N1 virus HA also gave the recombinant H5N1 virus the ability to produce a lethal infection in mice. Pathogenicity also depended upon a single amino acid change in the PB2 gene at position 627 that presumably altered the avian influenza

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[100] Tumpey, T.M. et al. "Pathogenicity and immunogenicity of influenza viruses with genes from the 1918 pandemic virus." *PNAS*. 2 March 2004, 101(9):3166-3171.

[101] Hatta, M. et al. "Molecular Basis for High Virulence of Hong Kong H5N1 Influenza A Viruses." *Science*. 7 September 2001, 293:1840-42.

virus host range. The Glu to Lys change in PB2 had previously been implicated in determining the host range of avian and human influenza viruses.

Harvey et al. (2004) used the recently determined crystal structure of the H5 HA protein to better understand the binding properties of H5 viruses.[102] The research team cloned the H5 gene from the index H5N1 influenza case and altered nucleotides in its receptor binding site. They demonstrated that changes in residues 226 and 228 increased the binding efficiency of H5 HA. They also showed that there was no genetic barrier via internal influenza genes (notably, the M2 ion channel) to the reassortment of H5 HA to human influenza viruses.

An experiment into the H5N1 NS1 gene has also indicated a potential molecular mechanism of its increased lethality and pathogenesis. Seo et al. (2002) showed that the NS1 in H5N1 influenza viruses more effectively counteracts host antiviral cytokine response by suppressing interferon and tumor necrosis factor.[103] Furthermore, the introduction of the H5N1 NS1 into an avirulent influenza strain enhanced the pathogenicity of the recombinant virus in swine hosts. The H5N1 NS1 is characterized by a single amino acid change at position 92. By changing the amino acid from glutamic acid to aspartic acid, the researchers were able to abrogate the effect of the H5N1 NS1. Thus, although tests were conducted in swine rather than human hosts, it appears as though a single amino acid change in the NS1 gene greatly increased the pathogenicity of the H5N1 influenza virus.[104]

D. Highlighting Problematic Research That Could Be Done

One of the first considerations about future influenza research is the ability of the virus to express another gene. Here, influenza is a unique case since it contains a segmented genome. Although we lack a great deal of knowledge on influenza viral assembly, it appears that it is not a random process.[105] Influenza is able to consistently package eight viral genome segments into its capsid. Dr. Palese's group previously demonstrated that space cannot be the reason for influenza's eight gene segments. Using the reverse genetics system and experimental growth conditions, they engineered an influenza virus that packaged nine different RNA segments rather than the usual eight.[106] Thus, it is conceivably possible to add a ninth gene segment that might carry an additional foreign gene.

In attempting to use the influenza virus as a gene delivery system for foreign genes, labs have use a variety of strategies. One of the first such efforts used the reverse genetics system to express the V3 loop of gp120 in the loop of antigenic site B of HA.[107] Another study truncated the NS1 gene to create room for a Her2/neu cytotoxic T-cell epitope, a very small protein.[108] Epitopes from lymphocytic choriomeningitis virus and HIV's gp41, have also been expressed in influenza viral vectors.

One of the first studies to express a foreign protein in influenza placed a self-cleaving protein between the NA gene and a foreign chloramphenicol acetyltransferase gene to produce two

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[102] Harvey, R. et al. "Restrictions to the adaptation of influenza A virus H5 hemagglutinin to the human host." *Journal of Virology*. Jan 2004, 78(1):502-7.

[103] Seo S.H. et al. "Lethal H5N1 influenza viruses escape host anti-viral cytokine responses." *Nature Medicine*. 2002 September, 8(9):950-54.

[104] Many scientists consider swine to be more representative host for humans than mice. See, Palese, P. et al. "The makings of a killer." *Nature Medicine*. 2002 September, 8(9):927-28.

[105] Fujii, Y. et al. "Selective incorporation of influenza virus RNA segments into virions." *PNAS*. 18 February 2003, 100(4):2002-07.

[106] Enami, M. et al. "An influenza virus containing nine different RNA segments." *Virology*. November 1991, 185(1):291-8.

[107] Li, S. et al. "Chimeric influenza virus induces neutralizing antibodies and cytotoxic T cells against human immunodeficiency virus type 1." *Journal of Virology*. November 1993, 67(11):6659-66.

[108] Efferson, C.L. et al. "Activation of Tumor Antigen-Specific Cytotoxic T-Lymphocytes (CTLs) by Human Dendritic Cells Infected with an Attenuated Influenza A Virus Expressing a CTL epitope Derived from the Her-2/neu Proto-Oncogene." *Journal of Virology*. July 2003, 77(13):7411-7424.

proteins on one gene segment.[109] Another study replaced the NA gene with green fluorescent protein (GFP).[110] The resultant recombinant virus was highly attenuated but exhibited GFP expression in vitro and in vivo. Another study hollowed out the coding regions from HA and NA and replaced them with vesicular stomatitis virus glycoprotein and GFP.[111] Even without HA and NA, the recombinant virus reproduced in cell culture, largely due to the new viral envelope protein, and expressed GFP in vitro.

These studies indicate that the insertion of foreign genes into influenza, although possible, is an unlikely method of engineering in the near future. As Dr. Harry Greenberg noted, influenza is "not ready for prime time as an expression vector and may never be."[112] Little pieces of protein can be inserted into the genome, but, to date, no whole proteins on the scale of interleukins have been inserted into influenza without first deleting the coding region from a native influenza protein. Although this is a comforting fact from the perspective of engineered bioterrorism, it does not mean that scientists will not find a way to express additional foreign genes or participate in research that attempts to do so. From a research oversight perspective, local or national oversight – depending on the gene proposed to be inserted – will be needed on any research attempting to insert entire foreign genes into influenza without deleting a native influenza protein first.

Protecting against dissemination of information that added to influenza's ability to evade the immune system would appear to be a high priority for any research oversight system. Here, evasion of innate immunity would be especially critical. Ecological modeling work has shown the importance of non-specific innate immunity, above and beyond adaptive immunity, in shaping the antigenic signature of influenza.[113] Other research showed that the decline of influenza viral replication corresponded closely to the level of circulating interferon.[114] It is currently believed that influenza has only one protein that functions in evading the innate immune system, NS1. Much research has shown the importance of this gene in determining influenza virulence and pathogenesis. It is doubtful though that NS1 could be replaced with another non-specific innate immunity evasion protein. NS1 is highly evolved to prevent the antiviral response of host cells to double-stranded RNA and any interferon inhibitor will likely need to retain this function. However, when foreign genes can successfully be added to influenza virus, those genes that inhibit innate immunity will be a high priority for any research oversight system for their potential in increasing influenza's lethality. Research that alters NS1 must also be closely monitored.

Although probably not as important as innate immunity, adaptive immunity certainly plays a role in the control of influenza. Indeed, much of the defense against most influenza strains comes from preexisting immunity in the form of neutralizing antibodies against HA. The role of cellular immunity in guarding against influenza is less clear than that of humoral immunity. Cytotoxic T-cells lyse targets presenting epitopes from HA, NP, M, and PB2 influenza proteins. However, a 2002 review on influenza stated that "the relative importance of each of the two effector arms of the immune system is not well defined for influenza."[115]

Aside from point mutations in HA or NA or in cellular immunity epitopes that fundamentally alter the nature of the immune response, it is doubtful that influenza can be

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[109] Percy, N. et al. "Expression of a foreign protein by influenza A virus." *Journal of Virology*. July 1994, 68(7):4486-92.

[110] Shinya, K. et al. "Characterization of a Neuraminidase-Deficient Influenza A Virus as a Potential Gene Delivery Vector and a Live Vaccine." *Journal of Virology*. March 2004, 78(6):3083-88.

[111] Watanabe, T. et al. "Exploitation of Nucleic Acid Packaging Signals to Generate a Novel Influenza Virus-Based Vector Stably Expressing Two Foreign Genes." *Journal of Virology.* October 2003, 77(19):10575-83.

[112] Greenberg, Harry. Email communication. 11 March 2004.

[113] Ferguson, N.M. et al. "Ecological and immunological determinants of influenza evolution." *Nature*. 27 March 2003, 422:428-433.

[114] Richman, D.D. et al "Three strains of influenza A virus (H3N2): Interferon sensitivity *in vitro* and interferon production in volunteers." *J. Clin. Microbiol.* 1976, 3:223-226.

[115] Hilleman, M.R. "Realities and enigmas of human viral influenza: pathogenesis, epidemiology, and control." *Vaccine*. 19 August 2002, 20(25-26):3068-87.

engineered to evade adaptive immunity (in absence of the ability to add interleukins or immunoregulators into the influenza genome). Nor would any research with altered HA or NA in animal hosts indicate an ability of that virus to evade the adaptive human immune system. However, experiments that generated alterations in peptide epitopes that then showed the recombinant viruses were not attenuated in cell culture or in animal models might warrant local oversight. The length from experiment to dangerous pathogen in that case is necessarily quite long though, especially given that no research can adequately comprehend the entire preexisting immunity that exists in the general human population. Generally speaking, oversight for adaptive immune evasion is likely very difficult and may be left to theoretical epitope generators and MHC-binding algorithms.

E. Difficulties in Creating a Oversight System for Influenza Research

That is the good news. Unfortunately, there are many problems that remain to be solved in overseeing influenza research. Drs. Harry Greenberg and Ann Arvin commented that the proposed research oversight system was, "from a scientific perspective, not feasible" and that all base pairs in influenza were essentially equal.[116] Indeed, the first problem an influenza oversight system would face is that influenza genome regulation is quite complex. As demonstrated in research on almost every influenza gene, single amino acid changes can alter the host range, tissue tropism, and overall pathogenicity of influenza viruses. This capacity for change at the single base pair level makes influenza research both potentially dangerous and unpredictable. When combined with the complex coding strategies of influenza viruses (Figure 9; here, influenza A, B, and C viruses are included) and the general lack of structural information, the capacity for change at the single amino acid level makes influenza research almost entirely unpredictable. As Chen et al. (2001) found in their discovery of the pro-apoptotic PB1-F2 protein in a second reading frame, influenza regulates expression of its genome in a myriad of ways (Figure 9). This complexity means that editing a single base pair may not just have ramifications for protein function, but may also influence the expression of novel proteins not yet discovered.

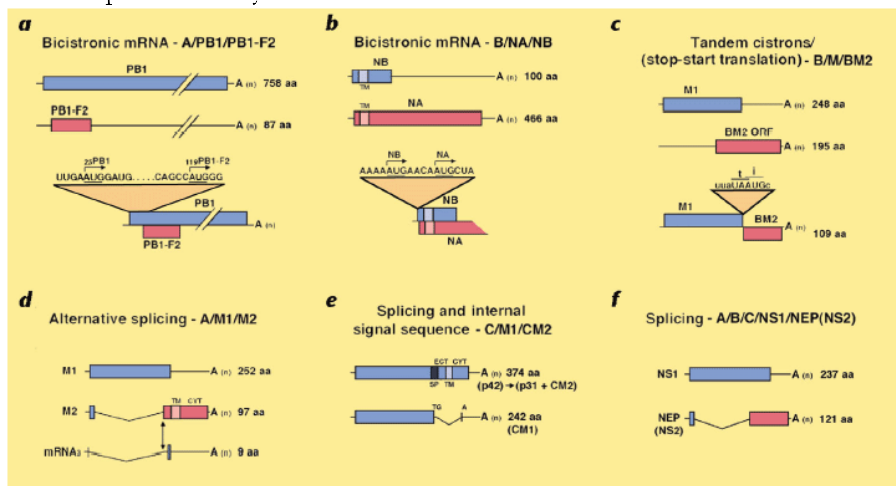

Figure 9 - Complex Coding Strategies of Influenza A, B, and C Viruses[117]

The problem of single amino acid changes is magnified by the lack of knowledge about the interactions of influenza proteins in modulating virulence. Of course, we are only aware of some protein interactions and there is no measure of our ignorance. Here, the interactions and coevolution of HA and NA proteins in determining viral entry and exit is worth noting. Some labs have essentially shown that NA may not be necessary for influenza to locally reproduce if the virus

[116] Interview with the author. 23 Feb 2004.
[117] Chen, W. et al. "A novel influenza A virus mitochondrial protein that induces cell death." *Nature Medicine*. December 2001, 7(12):1306-1312.

possesses the correct HA.[118,119] Also, given the interactions between PA, PB1, and PB2, and that base pair changes in these proteins have significantly altered viral properties, it is likely that predicting the impact on host and tissue tropism from proposed research would be very difficult.

Influenza virulence has consistently been shown to be a polygenic trait. For example, the high virulence of the mouse-adapted A/FM/1/47-MA strain was found to be due to mutations on genome segments 4, 5, 7, and 8.[120] Another study showed that a strain's virulence was due to host-independent mutations in HA, NA, and M1 genes, as well as host-dependent mutations in PB1 and PB2 genes.[121] The loss of virulence in the H2N2 vaccine strain A/AA/6/60 was traced to mutations in the PB1, PB2, M, and NS genes.[122] The polygenic aspect of influenza virulence may indicate a synergistic need for protein interaction or may mean that influenza virulence is simply the sum of its individual parts doing their individual jobs. As of now we simply do not know.

Another indication of the lack of knowledge about influenza and resultant difficulties in defining dangerous research comes in the form of environmental influences on influenza infection. The seasonality of epidemics is especially poorly understood. It is well established that almost all influenza epidemics and outbreaks occur in the winter. Although some research indicate that this is due to the creation of critical populations in school and work – typically secondary influenza outbreaks do not begin until school children return from winter break and then are seen ~2 weeks later in the adult population – it is not well understood why influenza chooses winter over other seasons. Is influenza transmission dependent on cold, dry weather? Or does the winter increase host susceptibility? In the effort to determine dangerous research that may increase influenza's transmissibility, infectivity, and lethality, the influence of environmental effects must be taken into account. The same goes for determining standard measures of transmissibility, infectivity, and lethality. Ultimately, should the deliberate use of influenza occur, it would happen in *some* environmental context. And, the effect of environmental influences on one mutation versus another may differ. Some effort must be made to understand the environmental influences in modulating these parameters, although it is quite difficult to imagine how to begin to implement such measurements.

A further problem remaining to be solved in influenza research (and research into other pathogens) is that of the relevancy of animal models. Influenza research has both advanced and suffered because of the use of many different animal models.[123] Seo et al. (2002) noted this problem in studying the role of interferon in influenza infections:

> "An appropriate small animal model would greatly facilitate studies of influenza virus pathogenesis. However, our experiments indicate that studies in pigs and ferrets are more informative than those in mice, because most inbred mice are deficient in one or more interferon pathways. The ferret is the currently accepted model for determining influenza pathogenesis. However, the miniature pig is recognized as an intermediate influenza virus host and is susceptible to all influenza subtypes. Reagents are increasingly available for immunological analysis of these animals and we anticipate their increased use in studies of pathogenesis."[124]

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[118] Wagner, R. et al. "Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenza virus growth: a study by reverse genetics." *J. Virol.* 2000 July, 74(14):6316-23.
[119] Gubareva, L.V. et al. "A release-competent influenza A virus mutant lacking the coding capacity for the neuraminidase active site." *J.Gen. Virol.* 2002 November, 83(Pt 11):2683-92.
[120] Brown, E.G. "Increased virulence of a mouse-adapted variant of influenza A/FM/1/47 virus is controlled by mutations in genome segments 4, 5, 7, 8." *J. Virology.* 1990 September, 64(9):4523-33.
[121] Brown, E.G. et al. "Genetic analysis of mouse-adapated influenza A virus identifies roles for the NA, PB1, PB2 genes in virulence." *Virus Research.* 1999 May, 61(1):63-76.
[122] Herlocher, M.L. et al. "Sequence comparisons of A/AA/6/60 influenza viruses: mutations which may contribute to attenuation." *Virus Research.* 1996 Jun, 42(1-2):11-25.
[123] McIntosh, J. and Selbie, F.R. "The pathogenicity to animals of viruses isolated from cases of human influenza." *British Journal of Experimental Pathology.* 1937, 18:334-44.
[124] Seo S.H. et al.

The problem of balancing results from pigs, ferrets, and mice has in some part been reduced by the recent growth in work in the mouse model, partly due to the pathogenicity of human H5N1 and 1918 influenza viruses in mice. However, one is never certain of the relevancy of results of human pathogens in animal hosts. The only restrictions Dr. Harry Greenberg said he might put on influenza research involves the publication of the complete 1918 influenza genome or work in human or non-human primate hosts. Virulence is a hallmark of specific host-pathogen coevolution. Thus, the LD50s of influenza virus in mice may or may not correspond to those found in man. Furthermore, the physiological characteristics of mice preclude the measure of transmission rates between mice. Thus, it would seem as though we are far away from some relevant, standard measure of some of these parameters.

The two main problems faced by a influenza research oversight system are that, 1) given the complex regulation of the influenza genome and interactions of the influenza proteome, it is very difficult to predict the effect of a single base pair change; and 2) extrinsic effects on virulence, whether from environmental influences or host response, will complicate any effort at deriving standard measures of lethality, infectivity, and transmissibility. On the genome complexity issue, past research does point to certain genes, such as HA and NS1, as being more potentially "dangerous" than others. However, that may simply be due to not enough work poking around in other genes.

E. Specific Recommendations for Influenza Research

The BRSS must seek a balance between not overseeing research because of perceived futility and reviewing too much research into too high of a threat level. The distance of the link between research done in vitro and its effects in vivo is also quite arbitrary and requires some cutoff. Past research into influenza gives us clues about experiments that should register quite high on any influenza research regulating scheme. This list should include experiments that reassort gene segments between animal and human influenza viruses and then screen those viruses in human tissue cultures (esp. with microarray expression data), swine, or non-human primates. Research seeking to add basic residues into the cleavage site of HA in an influenza virus and then testing the tissue tropism of such a virus in human cell cultures, mice, swine, or non-human primates deserve the designation of moderately or potentially dangerous research. Experiments that propose to make random or directed alterations to the NS1 gene and measure pathogenesis in vivo or gene regulation in vivo or in vitro could potentially be reviewed as moderately or potentially dangerous. Infecting non-human primate hosts with constructs from the 1918 pandemic influenza virus (esp. testing the role of its NS1 and HA genes in those hosts) might merit moderately or even extremely dangerous categorization. Further examinations of the role of amino acid residue 627 and/or other residues in the polymerase complex should fall under the lower level of potentially-dangerous research. Any examination of the molecular mechanisms of a new highly pathogenic influenza virus in any host would initially merit potentially dangerous work because of the complete uncertainty of what one might find. Finally, research into the innate immunity mechanisms of influenza clearance would also fall into the potentially dangerous category because of the possibility of using that host information to engineer influenza, should influenza be able to carry more innate immunity regulating genes.

This list is by no means exhaustive and encompasses only a preliminary effort at typing different forms of research into influenza that have shown danger potential in the past. This list also categorizes much research on a gene-by-gene basis. A different paradigm might include technical achievements or a process-oriented approach, such as 1) any effort to add foreign genes into influenza without first attenuating the virus by creating space or 2) any experiments in non-human primates or human hosts as being moderately dangerous. Work to develop standard methods of measuring transmissibility and infectivity must also be undertaken. The top priority in overseeing influenza research must be the oversight of research that directly shows how to increase influenza's lethality, most likely through innate immunoevasion or brain tissue tropism.

F. **General Recommendations from Review of Influenza Research**

As noted in this chapter, scientists have discovered some of the individual genetic polymorphisms that can cause one strain of influenza to be more pathogenic than another. The knowledge gleaned from past influenza research will form the basis of the case law for the definition of danger of future proposed influenza research. However, it comes clear from a review of the influenza literature that a definition of danger based at the level of genetic determinism is simply unattainable. The level and detail of knowledge required for such a system is simply too great. Rather, a broad definition of danger must be made at a lower resolution by laboratory processes or grouping of genes be function. In influenza, the higher priority genes for research oversight (or most dangerous genes) are those involved in immunoevasion, specifically innate and humoral immunity.[125] Beyond that, the jump from polymorphic to enhanced activity in vitro and in animal models to human relevance is perhaps too great. The theory of how alterations in polymerase genes could affect pathogenesis is not as well established as how polymorphisms that target antimicrobial or vaccine resistance or immunoevasion will affect pathogenesis. This review of the influenza literature argues for a definition of danger that relies upon a combination of process and, where possible, objective-based definitions of danger with genomic knowledge forming an imperfect basis for individual case law and decisions.

Candidate Influenza Research Activities	Review Level
Genetic Reassortment of Highly Lethal Animal Influenza Virus with Human Influenza	National - International
Alteration of HA & NA in undetermined fashion with challenge in animal model	Local
Addition of basic residues into HA cleavage loop with challenge in animal model	National
Undetermined NS1 alteration with challenge in animal model	National
Challenge in animal model with unscreened or potentially dangerous changes in both HA & NS	National - International
Characterization of any gene from highly pathogenic avian influenza	Local
Innate Immunity protection against Influenza	Local

Table 1 – Summary of Candidate Influenza Research Activities and Expected Level of Review

[125] Although theoretically posible, altering T-cell epitopes would prove to be an enormous task.

III. Pneumonic Plague

Like influenza, pneumonic plague is a complex disease for which transmissibility, lethality, and infectivity are difficult to understand and measure. However, unlike influenza, pneumonic plague is difficult to understand because of the scarcity of data and relative infrequency of occurrence. Pneumonic plague is a rare disease while influenza is relatively commonplace and owes the incomprehension about its viral pathogenesis to its impressive heterogeneity. Historically speaking, pneumonic plague has required the environmental conditions to allow human-to-human propagation through coughing. Modern public health measures have greatly reduced the threat presented by both bubonic and pneumonic plague such that any outbreak warrants impressive news coverage, as indicated by the 1994 bubonic plague outbreak in Surat, India. The last 75 years have witnessed very few pneumonic plague cases, and thus it has been difficult to dissect its pathogenesis in humans. Indeed, the last reported human-to-human transmission of pneumonic plague in the United States occurred in 1924.[126]

To understand pneumonic plague, one must first differentiate it from bubonic and septicemic plague. Bubonic plague is by far the most common cause of plague cases, accounting for 84% of the 390 plague cases in the United States from 1947 to 1996 and about 2000 cases worldwide each year. Bubonic plague has also accounted for the dominant share of the 200 million deaths attributed to plague throughout history.[127,128,129] Bubonic plague occurs when a flea infected with *Yersinia pestis*, the causative agent of plague, bites and regurgitates bacteria into a human host. The name 'bubonic plague' comes from the large bubo, or swollen lymph node, that is hallmark sign of the disease. Septicemic plague occurs when the bacteria find their way to the bloodstream and multiply en masse, causing toxic shock or sepsis. Septicemic plague cases accounted for 13% of the plague cases in the United States between 1947 and 1996. Pneumonic plague occurs when plague bacteria are inhaled into the lungs and account for the remaining 2-3% of cases in the United States. It is generally accepted that any one strain of *Y. pestis* can cause all three types of plague. The differences between bubonic, septicemic, and pneumonic plague are not due to differences in the bacteria but rather the location of the bacteria in the human body.

Biosecurity analysts are most concerned about outbreaks of pneumonic plague for two reasons. First, neither bubonic nor septicemic plague involves human-to-human transmission. Bubonic plague has historically been associated with surges in rat and flea populations that force hungry fleas to feed on humans. Some authors believe that the spread of plague during the Black Death is best accounted for by transmission between humans by human fleas.[130] Secondly, pneumonic plague is associated with fatality rates in excess of 90% without antibiotics – 57% in the few cases in antibiotic era in the United States – while bubonic and septicemic plague have killed only 14% and 22%, respectively, of those infected in the antibiotic era.[131]

Understanding bubonic and septicemic plague is important because all three types of plague have been associated with one another. It is generally thought that pneumonic plague cases first occur when flea-transmitted plague bacteria find their way to the lungs. These cases are called secondary pneumonic plague. When the bacteria divide in the lungs and the human host coughs, primary pneumonic plague, or plague that results from direct human-to-human transmission, can occur. Thus, pneumonic plague outbreaks are generally thought to require first the presence of both bubonic and septicemic plague.

[126] Inglesby, T.V. et al. "Plague as a biological weapon." *JAMA*. 3 May 2000, 283(17):2281-2290.
[127] Ibid.
[128] Ratsitorahina, M. et al. "Epidemiological and diagnostic aspects of the outbreak of pneumonic plague in Madagascar." *Lancet*. 8 Jan 2000, 355:111-113.
[129] Perry, R.D. et al. "Yersinia pestis – etiologic agent of plague." *Clinical Microbiological Reviews*. 1997, 10:35-66.
[130] Scott, S. et al. *Biology of Plagues: evidence from historical populations*. Cambridge: Cambridge University Press, 2001.
[131] Inglesby, T.V. et al. (2000)

Understanding the threat presented by pneumonic plague is complicated by the presence of "pulmonary plague," or septicemic plague of the respiratory tract. Pulmonary plague occurs when *Y. pestis* enters the respiratory tract but does not enter the lungs. It is not counted as an official pneumonic plague case, although it is difficult to differentiate the two clinically. Pulmonary plague is also associated with a high lethality but is much less transmissible than pneumonic plague. For this reason, it is thought pulmonary plague cases may account for the slackening or spontaneous decline associated with pneumonic plague outbreaks.

Genetic Pathogenesis of Plague

The genetic basis of the pathogenicity of plague is a tale of rapid microbial evolution. The organism *Y. pestis* has only recently evolved from *Y. pseudotuberculosis*.[132] *Y. pestis, Y. pseudotuberculosis*, and *Y. enterocolitica* can all cause disease but differ in their entry mechanisms and niches. *Y. pseudotuberculosis* and *Y. enterocolitica* are only pathogenic in the gut. *Y. pestis* has evolved the ability to establish itself parenterally. The evolution of this capability has required *Y. pestis* to both inactivate and gain new genes. The genome of *Y. pestis* consists of a 4.38 Mb chromosome and three plasmids of 9.5, 70, and 100 kb. The main pathogenic factors are encoded on the bacterial plasmids and will be the focus of this chapter.

The 9.5 kb pPCP1 plasmid encodes for the plasminogen activator protease (*Pla*), the bacteriocin pesticin (*pst*), and the pesticin immunity protein (*pim*). The plasminogen activator allows *Y. pestis* to establish itself subcutaneously and is believed to be the cause of the telltale clotting symptoms associated with plague. The 70kb pCD1 plasmid carries genes associated with the low-calcium response stimulon (LCRS). These genes are the main focus of study on plague pathogenesis and include the LcrV antigen, Yersinia outer membrane proteins (Yops), the Yop secretion apparatus (Ysc), and the specific Yop chaperons (Syc), as well as other proteins that help with protein regulation and secretion. The 100 kb pMT1 plasmid (sometimes called pFra) encodes for the fraction 1 (F1) protein capsule and murine exotoxin. The F1 capsule is an antiphagocytic envelope that protects plague from macrophages and other immune cells. It is also one of the main plague immunogens.

A. Parameter Background

1. Transmissibility

A great amount of debate surrounds the level of transmissibility of pneumonic plague. Those who argue that it is quite high point to the 1910-11 Manchurian outbreak, in which tens of thousands of people died. The potential for rapid transmission led to plague's placement as one of only three diseases that must be reported to the World Health Organization (WHO) under the 1969 International Health Regulations.[133] Because of its 1-6 day incubation period, pneumonic plague has the capability of spreading far and wide should an asymptomatic infected person board an airplane.[134]

Others believe that the Manchurian outbreak was an anomaly in plague history and that pneumonic plague is not so transmissible. In an *Annals of Internal Medicine* editorial published shortly after the 1994 Surat outbreak, the editor remarked, "The extreme measures taken by some persons and governments in response to the initial recent reports from India can largely be attributed to the widespread impression that pneumonic plague is not only deadly but also highly contagious in all circumstances. The latter impression, however, is not supported by the evidence."[135]

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[132] All of this information is taken from Perry, RD. and Fetherstone, JD. "*Yersinia pestis* – etiologic agent of plague." *Clinical Microbiological Reviews*. Jan 1997, 10(1):35-66.

[133] Enserink, M. "A Global Fire Brigade Responds to Disease Outbreaks." *Science*. 12 March 2004, 303:1605-6.

[134] Fritz, C.L. et al. "Surveillance for pneumonic plague in the United States during an international emergency: a model for the control of imported emerging disease." *Emerging Infectious Diseases*. 1996, 2:30-6.

[135] "Plague in India: A New Warning from an Old Nemesis." *Annals of Internal Medicine*. 15 Jan 1995, 122(2):151-153.

As noted in the *Annals* editorial, "no secondary plague cases resulting from person-to-person spread have been reported since 1925, despite the occurrence of at least 37 pneumonic plague cases in the interim (including at least six primary pneumonic cases)." It is also worth noting that there is a low incidence of the disease among laboratory workers, even though plague does not require BSL-4 protection (and chances are that the requisite BSL-3 level of protection was not to be found in earlier decades when plague was studied more extensively).[136] Both Drs. Stanley Falkow and Harry Greenberg went so far as to say that they don't consider pneumonic plague to be very transmissible in an age of antibiotics.[137]

Indeed, other anecdotal evidence, such as that of Dr. Lien the-Wu, suggests the transmissibility of plague is low. Dr. Wu's *Treatise on Pneumonic Plague* is perhaps the best volume of evidence on the general transmissibility of pneumonic plague. Because the treatise was published in 1926, no R_o's are calculated for different outbreaks. However, due to Wu's prominent role in the International Plague Conference at Mukden in 1911 and his chronological and physical proximity to the number of large pneumonic plague outbreaks in the 1910s and 1920s, Wu's work is most likely the best to derive a general understanding of both the transmissibility and human pathogenesis of pneumonic plague. With regard to transmissibility, Wu believes that transmission of pneumonic plague requires fairly close contact. Although Wu notes that "the most important method of pneumonic plague infection is the cough of patients," in one South African epidemic (1914-23), Wu comments that "it seems probable that kissing was the mode of infection of the majority."[138] Most of the affected persons in pneumonic plague outbreaks are family members and/or health care workers. Wu writes:

> the number of victims among the persons isolated after having been found in contact with plague patients is, as a rule, very low. Thus, in the 1920-21 epidemic, only 8 per cent of the contacts isolate at Harbin developed plague….at Dalainor, where the same discipline among the rough miners could not be maintained, the incidence of cases did not reach higher than 21.9 percent."[139,140]

Wu also notes that overcrowding, bad ventilation, the proximity of a case to a patient, and the duration of exposure are the main risk factors for pneumonic plague.[141] Another source notes that Chinese coolies were "packed like sardines…into overcrowded inns" during the 1910-11 Manchurian Plague.[142] The disease is certainly not as transmissible as influenza and is most likely less transmissible that smallpox. Wu agrees with Dr. Greenberg's assertion about the threat of pneumonic plague by writing, "there is probably no infectious disease which theoretically is so easy to suppress as lung plague" – even in a pre-antibiotic era.[143]

Wu notes the strong influence of environmental factors on the spread of pneumonic plague. The Manchurian plague epidemic occurred during such extreme cold that blood from the autopsies "formed icicles as it flowed…over the edges of the table."[144] Although pneumonic plague outbreaks have occurred in all seasons, the majority of evidence suggests that pneumonic plague propagates better in colder, drier areas where people live close together. Teague and Barber note that the Manchurian outbreak occurred when temperatures were 30 degrees Celsius below zero, while in a

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[136] Burmeister, R.W. et al. "Laboratory-acquired pneumonic plague: report of a case and review of previous cases." May 1962, 56(5):789-800.
[137] Interviews with the author. 23 February 2004.
[138] Wu, Lien-the. *Treatise on Pneumonic Plague.* Geneva: World Health Organization, 1926. p. 175.
[139] Wu, 301.
[140] Such cohort studies depend greatly upon who is classified as a contact and thus these data are merely suggestive.
[141] Admittedly, these conditions could be found in public transportation systems or sporting arenas.
[142] Chernin, 299.
[143] Wu, 181.
[144] Chermin, 308.

contemporaneous plague outbreak in which most cases were bubonic – only 2-5% of cases were pneumonic – the temperature was 30 degrees Celsius. They believe that plague droplets remain suspended in the air longer in very cold temperatures, allowing the plague to stay alive and infective. They note the largest epidemic of pneumonic plague in India (with 1,400 deaths) occurred in Kashmir "at an elevation of 1,524 meters above the sea level during very cold weather."[145]

No one knows exactly why the environment alters the case type but a number of hypotheses exist in the literature. Certainly, cold weather combined with poverty causes greater overcrowding of housing units and thus increases the probability of transmission. Other theories speculate that, with the decline of flea populations in very cold weather, person-to-person transmission is the only way to keep up an epidemic and thus makes a greater contribution to plague cases. This theory is supported by evidence that bubonic cases sometimes drop off in pneumonic outbreaks, even though bubonic cases are needed as a base of infection at other times.

Also, Wu does not consider pneumonic plague to be very transmissible because of his observation of the phenomenon of spontaneous decline of pneumonic plague outbreaks. Wu cites studies that argue that the roles played by doctors and health authorities in stopping an epidemic is very small compared with the quick natural course of pneumonic plague outbreaks.[146] Wu himself writes that it is not clear whether pneumonic plague can maintain an epidemic without a base of bubonic plague cases to support it.[147] It is thought that pneumonic plague killed and incapacitated too quickly for it to propagate itself for more than a few months in the pre-antibiotic era.

Biological evidence also suggests that the transmissibility of plague should not be considered high. Wu notes a non-infective period of 4-24 hours after the onset of symptoms, which includes blood in the sputum. He writes, "Very rarely, the sufferers are infective before they cough and expectorate."[148] In transmissibility, pneumonic plague is very similar to SARS, which was defeated in a few months simply through contact tracing and isolation (and no antimicrobials). Thus, public health authorities have a greater chance to stop transmission if a pneumonic plague outbreak is identified. One could also argue that patients in the 1910s and 1920s were less likely to seek treatment for blood in the sputum, as infectious diseases were more common and there was less that could be done about it. However, several reports have described "symptomless carriers" that may have been able to pass the bacteria on to others.[149] The relevance of symptomless carriers in the propagation of pneumonic plague is uncertain.

Experiments by Toyoda and Yasuda in which agar bacterial culture plates were held at different distances from patients found that 1) coughing was very necessary to transmit plague bacteria and 2) plague bacteria could only be expelled a distance of 3.67 feet by coughing.[150] These facts suggest that, like SARS, pneumonic plague is spread through respiratory droplets and not aerosols. Strong and Teague also found that only coughing could spread plague bacteria and that normal breathing was insufficient. The CDC bioterrorism website notes that "becoming infected [with plague] usually requires direct and close (within 6 feet) contact with the ill person or animal."[151] Other researchers also found that *prolonged* close contact was the critical factor in transmission of pneumonic plague.[152] However, Wu cites some anomalous cases in which plague was transmitted after a very brief contact.

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[145] Kellogg, W.H. et al. "An epidemic of pneumonic plague." *Journal of Public Health*. 1919, pp. 599-606.
[146] Wu, 188.
[147] A noted exception is the 1910-11 Manchurian outbreak, in which there were no noted bubonic cases in rats or humans. (Chermin, 307)
[148] Wu, 297.
[149] Wu (1926) described this, as does Tieh, TH (1948).
[150] Chermin, 307.
[151] FAQ About Plague. CDC. http://www.bt.cdc.gov/agent/plague/faq.asp.
[152] Wu.

Wu's account of the spread of pneumonic plague is also backed up by data from a more recent outbreak in Madagascar.[153] In the 1997 outbreak, a village healer became sick after intimately treating a patient with secondary pneumonic plague.[154] The healer's family then spread the bacteria to a number of other people in the village. Overall, 18 infected cases were noted with 8 deaths. After the outbreak, the WHO tested 154 contacts of those infected and found that only 13 were seropositive for anti-F1 IgG, yielding a infection rate in the contact population of 8.4%. The researchers concluded that the "risk of spreading pneumonic plague is actually not as high as may be thought."

Another excellent account of the transmissibility of plague – and the debate surrounding it -- can be found in the account of laboratory-acquired pneumonic plague by Burmeister et al. (1962):

> Patients with pneumonic plague are considered to be extremely infectious. The origin of this concept is buried in antiquity and was reconfirmed during the plague pandemic which began in the Yunnan Province of China in 1894. During the first and most devastating epidemic in Manchuria in 1910-11 at least 60,000 people died. Epidemics occurred in central China in 1917-18 and again in Manchuria in 1920-21, resulting in 10,000 fatalities; yet the next pneumonic plague outbreak in Manchuria was a limited one of 39 cases in Mukden in 1946. In India where numerous cases of bubonic plague have occurred pneumonic outbreaks have been rare and limited. Probably the largest was in Kashmir during the winter of 1903-04 when 1,443 cases occurred. An analysis of the Rangoon epidemic of 1947 led Wynne-Griffith to state "the disease is not particularly infectious." Some 100 intimate contacts of his 16 cases suffered no disease. Similar data can be found in a report of the outbreak in Ecuador in 1939, and Wu Lien-the mentions a number of such occurrences. These contrasting situations suggest that statements regarding the infectiousness of pneumonic plague require qualification and that certain factors limiting natural outbreaks may also be responsible for the rarity of infection under laboratory conditions.[155]

Burmeister et al. (1962) also note that the administration of antibiotics can "curtail further transmission to contacts…even when such treatment does not save the life of the patient."

Transmissibility is, of course, difficult to study because of physiological differences between animal models and humans and the ethical restrictions on experimenting in humans. It should, however, be noted that some experiments in animal models – including guinea pigs, lemurs, and non-human primates – have shown the "potential for cross-infection of control animals from infected animals showing the symptoms of pneumonic disease."[156] It goes without saying that the molecular determinants of transmissibility in plague are not understood. More research should focus on the transmissibility in animal models to elucidate possible molecular determinants of infectiousness.

Given the anecdotal evidence listed above, the transmissibility of pneumonic plague is considered low in the modern era and thus it received a transmissibility index score of 1 on the arbitrary scale of 0-3 created for this paper. Although no R_0s are available for pneumonic plague, given its similarities to SARS in regards to transmissibility, it should be noted that SARS had an fairly low R_0 of ~3 during the 2003 outbreak.

2. Infectivity

The numbers for pneumonic plague's infectivity are subject to questions about strains and animal models and thus could conceivably create wide ranges. The plague strain most often studied in animal models is the CO92 strain. CO92 was isolated from a pneumonic plague patient in 1992 in Colorado. Pneumonic plague is most often studied in mouse models, since they are much cheaper and easier to study than non-human primates. However, as with influenza, due to physiological differences, mice cannot transmit pneumonic plague to each other.

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[153] Ratsitorahina, M. et al. "Epidemiological and diagnostic aspects of the outbreak of pneumonic plague in Madagascar." *Lancet*. 8 Jan 2000, 355: 111-13.

[154] The traditional treatment included "incising the patient's epigastric region and sucking out some blood."

[155] Burmeister, R.W. et al. "Laboratory-acquired pneumonic plague: report of a case and review of previous cases." May 1962, 56(5), 796.

[156] Titball, 1000.

The LD_{50} of the CO92 strain delivered via aerosol in Swiss Webster mice is **2.3e4** colony forming units (CFU) – the corresponding subcutaneous injection LD_{50} is 1.9 CFU.[157] In Hartley guinea pigs, the CO92 strain aerosol LD_{50} is **4e4** CFU. In BALB/c mice, the CO92 strain has an aerosol LD_{50} of **6e4** CFU.[158] Strains of plague lacking the protective F1 capsule demonstrated similar aerosol LD_{50} numbers in mice (3.7e4 CFU) but deviated more in guinea pigs (>7e5 CFU).

Studies in the United Kingdom using Porton outbred mice and the GB plague strain report a different aerosol LD_{50} (~**1e2** CFU), although it is not known why.[159] Porton Down plague scientist Dr. Diane Williamson does not believe the difference is attributable to a significant difference in virulence between GB and other plague strains. But she does not have an alternative explanation for the low aerosol LD_{50} of GB.[160]

The 100-kb plasmid that contains the F1-capsule also carries a murine exotoxin, or phospholipase D, that is lethal in mice and rats but shows no effect in other animal hosts.[161] The murine exotoxin has since been shown to be necessary for *Y. pestis* survival in its chief flea vector, *Xenopsylla cheopis*.[162] Thus, there is some concern about the relevance of murine models in plague research, even though they remain the most commonly used model.[163]

Other results indicate that the LD_{50} for intranasal administration of the GB strain of *Y. pestis* is 1330 CFU in Balb/C mice; 922 CFU in NIH/S mice; and 6391 CFU in Porton mice.[164] An earlier paper put the aerosol LD_{50} for guinea pigs at ~**7e3** CFU of plague cultured in vitro and ~**1e4** CFU of plague cultured in a guinea pig.[165] Burmeister et al. (1962) recorded a LD_{50} of 3.2 CFU for intra-tracheal inoculation in guinea pigs (presumably inducing pulmonary plague). Finally, a variant of the vaccine strain EV76 (EV51f) can kill African green vervet monkeys (*C. aethiops*) with no dosage-lethality relationship but does not kill macaques (*Macaca mulatta*) or guinea pigs when administered subcutaneously.[166]

Data on the infectivity of pneumonic plague in non-human primates are difficult to find. Friedlander's group demonstrated that both F1-positive and F1-negative CO92 plague strains could kill African green monkeys. Unfortunately, the doses they administered – ranging from 1.4e2 to 9.2e4 CFUs – killed every monkey in the study and thus no LD_{50} was determined. Ehrenhranz and Meyer (1955) determined an LD_{50} of 100 organisms in *Macacus rhesus* via intratracheal instillation.[167] Intratracheal instillation requires delivery of 1 mL of infective inoculum through a catheter in the trachea and might induce pulmonary plague. Intratracheal instillation is also perhaps not very applicable to aerosol release bioterrorism scenarios. Speck and Wolochow (1957) determined a LD_{50} of **2e4** in *Macacus rhesus* (the same value was used for *C. aethiops*, but only because of the value

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[157] Friedlander, A.M. et al. "Relationship between virulence and immunity as revealed in recent studies of the F1 capsule of *Yersinia pestis*." *Clinical Infectious Diseases*. 1995, 21(Suppl 2):S178-81.

[158] Worsham, P.L. et al. "Pestoides F, a Yersinia pestis strain lacking plasminogen activator, is virulent by the aerosol route." *Adv. Exp. Med. Biol.* 2003, 529:129-131.

[159] Williamson, E.D. et al. "A subunit vaccine elicits IgG in serum, spleen cell cultures and bronchial washings and protects immunized animals against pneumonic plague." *Vaccine*. 1997, 15(10):1079-84.

[160] Email correspondence with Dr. Diane Williamson. 28 April 2004.

[161] Brubaker, R.R. "Factors promoting acute and chronic diseases caused by Yersiniae." *Clinical Microbiology Reviews*. 1991, 4:309-324.

[162] Hinnebusch, B.J. et al. "Role of Yersinia Murine Toxin in Survival of *Yersinia pestis* in the Midgut of the Flea Vector." *Science*. 296(5568):733-735.

[163] Titball, 1003.

[164] Russell, P. et al. "A comparison of Plague vaccine, USP and EV76 vaccine induced protection against *Yersinia pestis* in a murine model." *Vaccine*. 1995, 13(16):1551-6.

[165] Burmeister, R.W. et al. "Laboratory-acquired pneumonic plague: report of a case and review of previous cases." May 1962, 56(5):789-800.

[166] Hallett, A.F. et al. "Pathogenicity and Immunogenic Efficacy of a Live Attenuated Plague Vaccine in Vervet Monkeys." *Infection and Immunity*. Dec 1973, 8(6):876-881.

[167] Ehrenkranz, N.J. and Meyer, K.F. "Studies on Immunization Against Plague VIII. Study of Three Immunizing Preparations in Protecting Primates Against Pneumonic Plague." *Journal of Infectious Diseases*. 1955, 96:138-144.

determined in rhesus monkeys).[168] Speck and Wolochow used a Wellstype atomizer to create an aerosol, so their number is perhaps the more similar to a pneumonic plague outbreak in humans. The discussion of their results includes an excellent commentary on the technical aspects of experimental pneumonic plague infections:

> The method of infecting the monkeys, differing from the others cited, warrants some comment… The further factor of retention, though not taken into account in these figures bears directly on the actual number of organisms required to cause infection. Goldberg and Leif (1950) determined that 30% of the radioactivity of P^{32} tagged avirulent *Past. Pestis* was retained in the mouse lung, while Harper and Morton (1953) found that between 17 and 79% of the radioactivity of P^{32} tagged *B. globigii* spores (1 to 6.1 microns) was retained in monkey lungs. This indicates that a significant proportion of the inhaled organisms is available in the lung to initiate infection. Variability in this method may occur in the *assay of the aerosol, the mechanics of exposure, and susceptibility and respiratory peculiarities of the animal.* Calculation of individual doses on the basis of respiratory volume adds another difficulty in treating the results quantitatively, and often requires somewhat arbitrary grouping.
>
> Other methods of inducing pneumonic infections, on the other hand, involve the artifacts of anesthesia (and its effects on pulmonary physiology) and intratracheal intubation where trauma may actually lead to submucosal injection, and installation of fluid which may for some time supply the organisms with culture medium outside the normal defense mechanisms of the host. This seems especially significant with [*Y. pestis*] which is much more virulent when injected into tissue, and which multiplies enormously in the alveoli in the early stages of pneumonic infection. The pertinence of some of the characteristics of these modes of infection becomes apparent when one compares the report of Ehrenkranz and Meyer (1955) [to this report] that "The intratracheal instillation of approximately 100 virulent [*Y. pestis*] was lethal to more than 50%" of their monkeys and this report which indicates an LD_{50} of about 20,000 inhaled cells. While strains of the organism used in the two studies differed, both were highly virulent for the albino mouse and the guinea pig by parenteral inoculation. Considering even a minimum retention of 5% of the inhaled 20,000 organisms in the LD_{50} cited above, one arrives at a retained dose of 1000 organisms so that the retained inhaled LD_{50} is at least 10 times the instilled "50% lethal" dose of Ehrenkanz and Meyer. This difference clearly indicates a difference in the infectious process, at least during the early hours of the infection, which may be due to any or several of the factors referred to above. It is remarkable that an occasional animal was resistant to enormous doses administered by either method; these must be considered when analyzing the results of any immunizing procedure. (Emphasis added)

Thus, many factors – including strain, animal model, and infection procedure (anesthetization, intubation, aerosolization, etc.) – have to be taken into consideration when creating experimental pneumonic plague infection in animal models. Here, the heterogeneity of response in different monkey species should be noted. Nineteenth century and early twentieth century reports noted that high susceptibility to plague in langur monkeys (*Semnopithecus entellus*) and irregular susceptibility of *Cynomolgus philippinensis*.[169] Other data indicate that subcutaneous infection with 1,000 to 100,000 plague bacteria produce fatal infection only 10% of the time in *Macacus mulatta* and *Cynomolgus philippinensis*. A dose less than 1000 organisms of strain 195/P never produced disease in these two monkey species, while as few as 100 to 1000 plague organisms can kill some langur monkeys.[170] The genetic basis of this difference in host susceptibility is not currently known.

It is also worth commenting on the efficacy of aerosolization as a major known determinant of infectivity. The prominent plague scientist of the 1950s, KF Meyer estimated that only 10% of the bacteria administered intranasally in his pneumonic plague experiments reached deeper respiratory passages.[171] This estimate differs from those listed in the passage above (30% and 17-79%). Any

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[168] Speck, R.S. and Wolochow, H. "Studies on the experimental epidemiology of respiratory infections VIII. Experimental pneumonic plague in *Macacus rhesus*." *Journal of Infectious Diseases*. 1957, 100:58-69.
[169] Chen, T.H. and Meyer, K.F. "Susceptibility of the langur monkey (*Semnopithecus entellus*) to experimental plague: pathology and immunity." *Journal of Infectious Diseases*. 1965, 115:456-464.
[170] Ibid.
[171] Meyer, K.F. et al. "Prophylactic immunization and specific therapy of experimental pneumonic plague." *American review of tuberculosis and respiratory diseases*. 1948, 57:312-321.

technology or research (and its open publication) that showed how to better aerosolize plague would increase the infectivity of plague from a practical standpoint. It should also be noted that a Pubmed search for "experimental plague primate" yields a number of hits from Russian journals. Any research oversight system should try to capitalize on work previously done by the Russian bioweapons program.

Keeping in mind the complexities of deriving an aerosol LD_{50} of plague in relevant animal models, the best (and most) work done in this area settles on a value around **2e4** plague organisms. This value is therefore taken as the relevant LD_{50} of plague.

3. Lethality

As with influenza, pneumonic plague's lethality is by far the easiest measure to understand and to measure. Like inhalational anthrax or other toxic bacteria that find themselves settled in the lungs, all evidence points to *at least* an 90% lethality rate for primary pneumonic plague. An epidemic in Manchuria in 1910-11 (the pre-antibiotic era) indicated a lethality rate of "nearly 100%".[172] Other sources put the Manchurian plague epidemic's lethality at exactly 100% – 43,942 cases and 43,942 deaths.[173,174] Between 1925 and 1948, there were reports of only 9 recoveries worldwide from pneumonic plague, and all recoveries had received a vaccine or antibiotic.[175] Lien-the Wu wrote in 1926 that "for all practical purposes, the prognosis of primary pneumonic plague may be considered as well-nigh hopeless."[176]

The most important determinant of lethality of pneumonic plague in the modern era is the speed with which antibiotics are delivered to a patient. General consensus says that antibiotics must be delivered within 24 hours of symptom onset to be effective against pneumonic plague.[177] Another study indicates that as few as 20 hours may pass before antibiotics lose their efficacy.[178] Of thirteen pneumonic plague cases in one Madagascar hospital, the two that died (15.4% lethality) received antibiotics at 24 and 40 hours after onset of illness – all survivors received antibiotics before the 24-hour mark.[179] In 1970, the WHO estimated that the airborne release of 50kg of *Y. pestis* over a city of 5 million would result in 150,000 cases of plague and 36,000 deaths, yielding a 24% estimated mortality rate in the antibiotic era.[180]

Generally speaking, plague is uniformly lethal if it reaches the lungs in most animal hosts. The only variance in pneumonic plague's lethality comes from the rapidity of antibiotic delivery. Aside from antibiotic resistance or increasing the efficiency of delivery into the lungs, there is little to gain in engineering plague to be more lethal.

4. Conclusions

Aside from antibiotic resistance, the greatest potential for dangerous research in plague comes in increasing transmissibility. However, the science of transmissibility and the animal models for transmissibility are not well understood. In the area of infectivity and (possibly) transmissibility, the greatest threat comes from information on the initial aerosolization of plague. The Japanese Unit 731 decided not to bother with aerosolizing plague bacteria and used the flea vector to disperse plague in their World War II tests in Manchuria. On the other hand, according to Ken Alibek's

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[172] Inglesby, T.V. et al. (2000)
[173] Chernin, E. "Richard Pearson Strong and the Manchurian Epidemic of Pneumonic Plague, 1910-1911." *Journal of the History of Medicine and Allied Sciences*. 1989, 44: 296-319.
[174] Tieh, T.H. Et al. "Primary pneumonic plague in Mukden, 1946, and report of 39 with 3 recoveries." *Journal of Infectious Diseases*. 1948, 82(1):52-58.
[175] Ibid.
[176] Ibid.
[177] Meyer, K. "Pneumonic plague." *Bacteriological Reviews*. 1961, 25:249-261.
[178] McCrumb, F.R. et al. "Chloramphenicol and Terramycin in the Treatment of Pneumonic Plague." *American Journal of Medicine*. 1953, 14:284-293.
[179] Ibid.
[180] *Health Aspects of Chemical and Biological Weapons*. Geneva: World Health Organization, 1970. pp. 98-109.

Biohazard and multiple defectors' accounts, the Soviets succeeded in weaponizing plague and made it one of their highest priority weapons. Keeping aerosolization information from terrorists should be a top priority for those concerned with the dual-use dilemma in biodefense.

Although not explicitly included in the three-parameter model of lethality, infectivity, and transmissibility, another area of potentially dangerous research in plague might be its environmental hardiness. Here, it is difficult to come up with any definite recommendations. On one hand, plague bacteria decay rapidly in the environment. Most estimates give plague a viability of only one hour in the presence of sunlight or less than 5 days when dried on different surfaces (although plague DNA evidently can persist for 400 years in dental pulp).[181,182,183] Anthrax, on the other hand, can survive in the environment for tens, if not hundreds, of years and presents a major problem for decontamination after an attack. Some of the buildings affected by the October 2001 attacks are still closed. Furthermore, a hardier plague may be more transmissible if fewer of the organisms were damaged by sunlight as they were transmitted from one person to the next.

On the other hand, it is not clear if a hardier plague would present much of a danger. After all, plague does not infect cutaneously as anthrax does. Plague requires direct delivery into the blood or lungs.

Plague could still infect via an open cut in a hand, and, as noted in many animal studies, the LD_{50} of plague injected subcutaneously is extremely low (1.9 CFU in most mice). In an age of liability concerns, it is doubtful that occupants would return to a building that was still contaminated with plague bacteria, even if the chance of infection was quite low. Thus, it is likely that environmental hardiness should be considered as a potentially dangerous area of plague research for the economic consequences alone. However, as with transmissibility, the science of making non-spore-forming bacteria equally hardy as spore-forming bacteria is not well understood, and thus it is difficult to make any definite recommendations beyond the theoretical.

B. Highlighting Problematic Research That Has Been Done

Increasing presentation or activity of virulence factors

F1 capsule presentation

Generally speaking, most alterations to plague do not create a strain that is more virulent than the original strain. This phenomenon may be due to the extremely low subcutaneous LD_{50} and because aerosols are difficult to create so pneumonic plague has been studied less. However, Welkos et al. (1995) were able to create two strains of plague via random mutagenesis of the CO92 parental strain that had, albeit marginally, lower subcutaneous LD_{50}s in Swiss Webster mice (0.5 and 1.7 CFU v. CO92's 1.9 CFU).[184] The strain with an LD_{50} of 0.5 CFU showed increased presentation of the F1 capsule as did two other strains that demonstrated lower LD_{50}s than similar mutants. No aerosol tests were done on these particular bacteria, so it is not known whether increased presentation of the F1 capsule would have any effect on the infectivity of pneumonic plague. To be a net positive for the bacteria, the antiphagocytic effect of the F1 capsule would have to outweigh its immunogenic effect, for F1 is the principal target of plague vaccines. Given the data in this experiment, it would seem as though such was the case. Of course, a plague bacterium that expressed extra F1 would seem to be more susceptible to plague vaccine, so the utility of this research for bioterrorists would depend upon the use of vaccine.

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[181] Inglesby, T.V. et al. (2000)
[182] Rose, L.J. et al. "Survival of *Yersinia pestis* on Environmental Surfaces." *Applied and Environmental Microbiology.* April 2003, 69(4):2166-2171.
[183]Drancourt, M. et al. "Detection of 400-year-old Yersinia pestis DNA in human dental pulp: an approach to the diagnosis of ancient septicemia." *PNAS*. 1998 Oct 13, 95(121):12637-40.
[184] Welkos, S.L. et al. "Studies on the Contribution of the F1 Capsule-Associated Plasmid pFra to the Virulence of *Yersinia pestis*." *Contrib Microbiol Immunol.* 1995, 13:299-305.

Pla protease activity

Another important plasmid-carried virulence factor of *Y. pestis* that separates it from its evolutionary cousin *Y. pseudotuberculosis* is the Pla protease. The Pla protease is thought to be critical for invasion after subcutaneous infection. Pla is also a potent activator of plasminogen (from whence it gets its name), which causes the telltale blood clots of a plague infection. Plague bacteria without the Pla protease plasmid have a million-fold higher LD_{50} than wild-type bacteria superficially injected into mice.[185] Intravenuous infections/injections show little requirement for the Pla protease. Pla mutants are able to establish local infections when subcutaneously injected but cannot spread to other tissues and are thus avirulent. Experiments on the biochemical and catalytic properties of the Pla proteases demonstrated that the protease has a strong affinity (K_m of 145nM) but low catalytic activity (k_{cat} of 0.21 min^{-1}) for plasminogen versus the human plasminogen activator urokinase (k_{cat} of 89 min^{-1}).[186] Thus, there exists, at least theoretically, plasticity in Pla's design that could allow it to be engineered to become a more effective pathogenic factor. Other experiments suggest that the major role of the Pla protease is systemic dissemination of the bacteria from local infection sites through the destruction of plasmin.

Pla is not thought to play as critical a role in pneumonic plague as in subcutaneous infections, but experimental data are suggesting it is still an important virulence determinant in pneumonic plague. While plague bacteria without the Pla-carrying plasmid had a million fold higher LD_{50} for subcutaneous infections in mice, they had only a 5-fold higher LD_{50} for aerosol infections in mice (2.3e4 vs. 1e5).[187] In another experiment, Pla-lacking plague bacteria (Pestoides F) showed aerosol pathogenesis with an LD_{50} not radically different from the wild-type CO92 strain in BALB/c mice (7.3e4 vs. 6.0e4).[188] Surprisingly, Pla-lacking plague bacteria also did not have a higher LD_{50} for either aerosolized or subcutaneously inoculation in guinea pigs.[189]

It appears as though no aerosol studies have been conducted in monkeys with plague bacteria lacking only the Pla protease. However, one study found that no *C. aethiops* monkeys died when exposed to doses ranging from 3.5e7 – 1.8e8 of aerosolized *Y. pestis* lacking both the Pla protease and the iron transport and binding locus *Pgm*.[190] When compared to the data for bacteria lacking only *Pgm*, these data suggest that Pla-mutants are attenuated compared to wild-type plague bacteria in aerosol infections in monkeys. Pla may play a role in establishing infection in monkey lungs, but the data are inconclusive.

Because of the demonstrated importance of active Pla in both experimental bubonic and pneumonic plague infections, it is not unreasonable to conclude that a more active Pla could create a more virulent bacteria. A more active Pla could allow bubonic plague infections to become more lethal or could allow pneumonic plague infections to proceed more rapidly. Research into the structural determinants of Pla protease activity could unwittingly create a more active Pla. The low kinetic activity of Pla relative to human urokinase suggests that there is room for improvement in Pla activity. Because of the low potential for danger in this research, research into the molecular and structural determinants of Pla activity would only require a low, local level of oversight.

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[185] Brubaker, R.R. et al. "Pasteurella pestis – role of pesticin I and iron in experimental plague." *Science*. 1965, 149(3682):422.
[186] Sodeinde, O.A. et al. "A surface protease and the invasive character of plague." *Science*. 6 November 1992, 258: 1004-7.
[187] Welkos, S.L. et al. "Studies on the role of plasminogen activator in systemic infection by virulent *Yersinia pestis* strain CO92." *Microbial Pathogenesis*. 1997, 23:211-23.
[188] Worsham, P.L. et al. "Pestoides F, a Yersinia pestis strain lacking plasminogen activator, is virulent by the aerosol route." *Adv. Exp. Med. Biol.* 2003, 529:129-131.
[189] Samoilova, S.V. Et al. "Virulence of pPst+ and pPst- strains of *Yersinia pestis* for guinea pigs." *Journal of Medical Microbiology*. 1996 December, 45(6):440-4.
[190] Welkos, S.L. et al. "Determination of the virulence of the pigmentation-deficient and pigmentation-/plasminogen activator-deficient strains of *Yersinia pestis* in non-human primate and mouse models of pneumonic plague." *Vaccine*. 2002, 20:2206-2214.

Therapy and Prophylaxis Resistance
Antibiotic Resistance

Given that antibiotics must be administered during the first 24 hours of symptoms, resistance to antibiotics would be one of the priority areas for a research oversight system for plague. An antibiotic-resistance strain of plague would most likely achieve higher lethality than the ~50% seen in the antibiotic era. Unfortunately, the methods and techniques for the conferral of antibiotic resistance to Gram-negative bacteria are already commonplace in any microbiology laboratory. The BRSS would have a difficult time fixing this problem since the information and techniques are already widespread. However, by including all legitimate scientists under the umbrella of research oversight, the BRSS could instantly delegitimize dangerous work done outside its aegis.

Thankfully, there are a number of antibiotic options when it comes to pneumonic plague therapy. Although gentamicin, doxycycline, and tetracycline have shown similar efficacy in humans and animal models, streptomycin is generally considered to be the top antibiotic to treat plague.[191] The fluoroquinolones have shown strong efficacy in animal models, but they are not FDA-approved for human plague since there have been no trials (and no cases to conduct them on). The beta-lactam class of antibiotics demonstrated poor efficacy when administered late in a mouse model of pneumonic plague and have shown rapid clinical deterioration in pneumonic plague patients.[192]

Resistance is, of course, another question. McCrumb et al. (1953) note the emergence of streptomycin-resistance in other gram-negatives and in experimental pneumonic plague infections as far back as 1953 in their article on antibiotic treatment of pneumonic plague.[193] Plague strains naturally resistant to tetracycline have occasionally been noted, including a strain carrying a multi-drug resistance plasmid with activity against tetracycline, streptomycin, chloramphenicol, and sulfonamides.[194]

The most alarming precedent for dangerous research into antibiotic-resistant plague comes from the former Soviet bioweapons program. Dr. Sergei Popov asserts that the Soviet Union successfully engineered anthrax that was resistant against ten antibiotics and a strain of plague that was resistant to "almost ten antibiotics."[195] Meanwhile, the first Russian bioweapons defector, Dr. Vladimir Pasechnik, has stated that he engineered "a 'hot' plague that could resist huge combined doses of fifteen different antibiotics" in less than five years.[196] The Working Group on Civilian Biodefense *JAMA* review also noted an article published by Russian scientists describing a quinolone-resistant plague bacterium.[197]

The same Russian group published a similar article recently on the development of antibiotic resistance in plague.[198] The scientists demonstrated the spontaneous development of rifampicin and nalidixic acid resistance in two strains of plague, one from a marmot and another from a human. While rifampicin resistance slightly altered the virulence of the strains, nalidixic acid resistance did not alter strain virulence. However, neither resistant strain showed any difference in LD_{50}s from the parental strains, even when the mice were treated with the appropriate antibiotic. Furthermore, nalidixic acid resistant strains were cross-resistant to fluoroquinolones, including ciprofloxacin, ofloxacin, pefloxacin, and lomefloxacin. And, F1-negative mutants of both the parental and

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[191] Inglesby, T.V. et al., 2286. (2000)
[192] Byrne, W.R. et al. "Antibiotic Treatment of Experimental Pneumonic Plague in Mice." *Antimicrobial Agents and Chemotherapy*. Mar 1998, 42(3):675-81.
[193] McCrumb, F.R. 1953.
[194] Inglesby, T.V. et al. (2000)
[195] *NOVA Online Bioterror Sergei Popov*, Accessed online (15 January 2003) at http://www.pbs.org/wgbh/nova/bioterror/biow_popov.html.
[196] Cooper, S. "Life in the pursuit of death," *Seed* (Jan/Feb 2003): 72.
[197] Ryzhko, I.V. et al. "Virulence of rifampicin and quinolone resistant mutants of strains of plague microbe with Fra+ and Fra- phenotypes." *Antibiot Khimioter*. 1994, 39:32-36.
[198] Ryzhko, I.V. et al. "[Formation of virulent antigen-modified mutants (Fra-, Fra-Tox-) of plague bacteria to rifampicin and quinolones]." *Antibiot Khimioter*. 2003, 48(4):19-23.

antibiotic-resistance strains "were able to overcome specific immune reaction" or to circumvent an F1-directed vaccine.

Vaccine Resistance

There is already a great deal of precedent for the Russian scientists' ability to evade a "specific immune reaction" by creating F1-negative "antigen modified strains." Indeed, many past vaccines (as well as diagnostic tests) have been based largely around the F1 antigen. Early studies indicated that serum from humans immunized with purified F1 protein could passively protect mice infected with 100 LD_{50}s of plague.[199]

Most plague vaccines have been based off killed whole-cell technology. A killed whole-cell vaccine was first experimentally used in 1897 and later licensed for human use in 1946 in the United States using the 195/P strain. Production of the killed whole-cell vaccine in the United States was discontinued in 1999, and there is no licensed vaccine available in the United States; however, Australian manufacturers produce a similar vaccine.[200] The killed vaccine is not appropriate for post-exposure use because several months are needed to complete the primary vaccination schedule. Neither has the vaccine been tested in children and pregnant women. Booster immunizations may be required every 6 months.[201] Although the vaccine is also no longer licensed for humans, a live-attenuated vaccine for plague (strain EV76) has also been used in the former Soviet Union and French colonies.

The low incidence of plague means that none of these vaccines have been subjected to randomized, clinically controlled field studies in humans.[202] Surrogate markers and animal models will have to be used to prove vaccine efficacy and safety. More importantly, the effectiveness of killed vaccines against pneumonic plague is uncertain as cases of pneumonic plague have been noted in vaccinated persons, and 100% of twice-vaccinated mice died when challenged with 100 aerosol LD_{50}s of plague.[203]

The live EV76 vaccine strain has shown the ability to protect mice from aerosol challenge. Although the reason for this protective difference is not known, it has been suggested that it may be due to 1) differences in V antigen presentation, 2) structural changes in the F1 capsule antigen, and/or 3) a sustained exposure from a possible local infection with the live EV76 strain. Of course, the extra protection comes at the price of safety; the EV76 strain sometimes causes diseases in animals, including ~1% fatality rate in mice, and has resulted in hospitalizations in the former Soviet Union.[204]

The whole-cell vaccine is generally thought to protect via the F1 capsule antigen because only scarce amounts of the V antigen are present in the vaccine.[205] Furthermore, the titer of F1 antibody correlates with protection against plague in animal models.[206] The principal antibody response against plague is directed against the F1 antigen.[207]

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[199] Meyer, K.F. et al. "Measurement of protective serum antibodies in human volunteers inoculated with plague prophylactics." *Stanford Medical Bulletin*. 1948, 6:75-79.
[200] Titball, R.W. p. 1004.
[201] Ibid.
[202] Ibid.
[203] Lawton, D. et al. "Biosynthesis and purification of V and W antigens in *Pasteurella pestis*." *Journal of Immunology*. 1963, 91:179-184.
[204] Meyer, K.F. et al. "Plague immunization I. Past and present trends." *Journal of Infectious Diseases*. 1974, 129:S13-18.
[205] Williamson, E.D. et al. "A new improved sub-unit vaccine for plague: the basis of protection." *FEMS Immunol Med Microbiol*. 1995, 12:223-230.
[206] Williams, J.E. et al. "Measuring the efficacy of vaccination in affording protection against plague." *Bulletin of the World Health Organization*. 1979, 57:309-313.
[207] Williams, J.E. et al. "Application of enzyme immunoassays for the confirmation of clinically suspect plague in Namibia." *Bulletin of the World Health Organization*. 1982, 64:745-752.

Arthur M. Friedlander's group has shown that the F1 capsule protein of *Y. pestis* is not essential for virulence and lethality in both mice and non-human primates.[208,209] The F1 capsule presumably contributes to virulence by allowing the plague bacteria to escape phagocytosis. Along with the V antigen, the F1 protein is one of the main plague immunogens and is an important component of the previously lisensed plague vaccine.[210] A previous study found that F1-negative plague was virulent in mice but attenuated in guinea pigs.[211] Only one F1-negative plague infection has ever been reported in humans.[212]

Friedlander's group demonstrated that the aerosol LD_{50} of plague lacking the F1 capsule was not significantly different from that of comparable F1+ plague strains in mice and non-human primates and only slightly changed in guinea pigs. Although the experiment did not necessarily show the role of the F1 capsule in the human host, it gives preliminary data that one could create F1-negative plague that might evade currently available vaccines. Data from the Russian group showing F1-negative strains evade the second-generation Russian vaccine also strongly support this conclusion.[213]

Interest in creating a new plague vaccine has increased due to the lack of protection for pneumonic plague by the whole-cell killed vaccine and the rising possibility of naturally-occurring multi-drug resistant bubonic plague.[214] Next-generation plague vaccines use recombinant subunits of F1 and V antigens to elicit a systemic immune response. The V antigen is a secreted protein that is thought to play an important role in plague pathogenesis and immunomodulation. Immunological response against the V antigen has shown the ability to protect against F1-negative strains such as the Java9 strain. One experiment demonstrated that recombinant V antigen could protect mice from 1000 or greater aerosol LD_{50}s of plague.[215] A recombinant F1+V vaccine has also shown the ability to protect guinea pigs from 10 LD_{50}s using similar epitopes as those used to protect mice.[216]

In addition to creating protective antibodies, immunization with the V antigen also elicits the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha and interferon-gamma.[217] Although T-cell memory is involved, the F1+V subunit vaccine has shown a Th2 bias toward IgG1 antibodies.[218] The majority of protection against plague seems to be antibody-mediated. Other research shows that a Th1 response (IgG2b antibodies) can protect against plague

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[208] Friedlander, A.M. et al. "Relationship between virulence and immunity as revealed in recent studies of the F1 capsule of *Yersinia pestis*." *Clinical Infectious Diseases.* 1995, 21(Suppl 2):S178-81.
[209] Davis, K.J et al. "Pathology of Experimental Pneumonic Plague Produced by Fraction 1-Positive and Fraction 1-Negative *Yersinia pestis* in African Green Monkeys (*Cercopithecus aethiops*)." *Arch Pathol Lab Med.* February 1996, 120:156-163.
[210] Ibid.
[211] Kutyrev, V.V. et al. "Genetic analysis and modeling of the virulence of *Yersinia pestis*." *Mol Gen Mikrobiol Virusol.* 1989 Aug, 8:42-7.
[212] Winter, C.C. et al. "An unusual strain of *Pasteurella pestis* isolated from a fatal human case of plague." *Bull World Health Organ.* 1960, 23:408-9.
[213] Ryzhko, I.V. et al. "[Formation of virulent antigen-modified mutants (Fra-, Fra-Tox-) of plague bacteria to rifampicin and quinolones]." *Antibiot Khimioter.* 2003, 48(4):19-23.
[214] "Mouse Model Mimics Real-World Plague Infection." NIAID Website. http://www2.niaid.nih.gov/newsroom/releases/plaguemouse.htm (24 March 2004).
[215] Anderson, G.W. et al. "Recombinant V antigen protects mice against pneumonic and bubonic plague caused by F1-capsule-positive and –negative strains of *Yersinia pestis*. *Infection and Immunity.* 1996, 64:4580-4585.
[216] Jones, SM. et al. "Protective efficacy of a fully recombinant plague vaccine in the guinea pig." *Vaccine.* 8 September 2003, 21(25-26):3912-3918.
[217] Nakajima, R. et al. "Suppression of cytokines in mice by protein A-V antigen fusion peptide and restoration of synthesis by active immunization. *Infection and Immunity.* 1995, 63:3021-3029.
[218] Williamson, R.A. et al. "An IgG1 titre to the F1 and V antigens correlates with protection against plague in the mouse model." *Clin Exp Immunol.* 1999, 116:107-14.

in mice when passively transferred (although it should be noted that this study did not examine pneumonic plague).[219]

Given the low natural incidence of plague, passive protection by human antibodies in mice is likely to be a correlate of protection for approval of any future plague vaccines. The role of Th1/Th2 is further clouded by a study that shows coencapsulation of F1+V antigens with either IL-4 (the predominant Th2 cytokine) or IFN-gamma (the quintessential Th1 cytokine) reduces the efficacy of the intranasal vaccine. However, coencapsulation with IL-6 (a mucosal protection activator) elicited complete protection in the pneumonic plague mouse model.[220] Thus, it is not certain how Th1-Th2 immunomodulation could be used to boost or circumvent the immune response, although it seems that slating the immune response toward the Th1 would reduce its ability to respond to plague, given the importance of IgG1 antibody in protecting mice.

Recently, a single dose F1+V subunit vaccine demonstrated the ability to protect against pneumonic plague.[221,222] It is thought that the strong protection conferred by the F1+V subunit vaccine might be due to different kinetics in the development of antibody to each protein (anti-F1 antibody shows early, anti-V antibody titer rises weeks later in the response). Next-next generation plague vaccines may see the subunits microencapsulated and preferentially delivered to mucosal sites to elicit protective antibodies.[223] Although initial microencapsulated subunit antigens only elicited a 66% survival rate in mice, the addition of cholera toxin B as an adjuvant achieved 100% protection.[224]

Although the F1+V vaccine is yet to be approved, circumventing it is likely to be more difficult given that 1) there are two antigens instead of just the F1 antigen, and 2) the V-antigen is considered to be an "absolutely essential" virulence factor of *Y. pestis*.[225,226,227] Still, there is some worry that even the F1+V subunit vaccine may not be a final answer. As noted in Heath et al. (1998):

> Indeed, isolates deficient in V, determined immunologically, have been cultured from immunized animals infected with V-containing, wild-type, *Y. pestis*. Furthermore, variability in the structural gene for the V-antigen has described for *Y. pseudotuberculosis* and *Y. enterocolitica*, although to date, this has not been reported for *Y. pestis*. Most significantly, serum raised to one V type of *Y. enterocolitica*, while conferring passive protection to the homologous V type strain did not protect against either other strains of *Y. enterocolitica* having a different V antigen type or against *Y. pseudotuberculosis,* which also possessed the different V type.[228]

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[219] Elvin, SJ. et al. "The F1 and V subunit vaccine protects against plague in the absence of IL-4 driven immune responses." *Microb Pathog.* 2000, 29:223-230.
[220] Griffin, K.F. et al. "Protection against plague following immunization with microencapsulated V antigen is reduced by co-encapsulation with IFN-gamma or IL-4, but not IL-6." *Vaccine.* 1 November 2002, 20(31-32):3650-57.
[221] Anderson, G.W. et al. "Short- and long-term efficacy of single-dose subunit vaccines against *Yersinia pestis* in mice." *American Journal of Tropical Medicine and Hygiene.* 1998, 58:793-9.
[222] Williamson, E.D. et al. "A single dose sub-unit vaccine protects against pneumonic plague." *Vaccine.* 2001, 19:566-571.
[223] Titball, R.W. et al. "Vaccination against bubonic and pneumonic plague." *Vaccine.* 2001, 19:4175-4184.
[224] Eyles, JE. et al. "Intranasal administration of poly (lactic acid) microsphere co-encapsulated *Yersinia pestis* sub-units confers protection from pneumonic plague in the mouse." *Vaccine.* 1998, 16:698-707.
[225] Fields, K.A. et al. "Virulence role of V antigen of *Yersinia pestis* at the Bacterial Surface." *Infection and Immunity.* October 1999, 67(10):5395-5408.
[226] Fields, K.A. et al. "LcrV of *Yersinia pestis* enters infected eukaryotic cells by a virulence plasmid-independent mechanism." *Infection and Immunity.* September 1999, 67(9):4801-13.
[227] Heath, D.G. et al. "Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine." *Vaccine.* 1998, 16(11-12):1131-1137.
[228] Ibid.

Thus, it seems possible that a F1-negative *Y. pestis* with a polymorphic V-antigen could circumvent the next-generation F1+V vaccine through physical antigen evasion. Thankfully, the exact steps necessary to create such a plague strain are not known although some experiments indicate that repeated passage of plague in vaccinated animals might yield polymorphic strains. Right now, F1-negative strains have only been acquired through this selection pressure.[229,230] However, the potential for the creation of a polymorphic V-antigen-bearing plague resistant to the next generation vaccine means research into V-antigen (especially research creating mutational variants) deserves a medium to high priority status under any research oversight system for plague.

C. Highlighting Problematic Research That Could Be Done

V antigen and IL-10

The V antigen, or LcrV (for low calcium response V), is a secreted protein that is thought to help in multiple functions of the Type III secretion system of plague, including regulation of the Yop virulence factors. Importantly, it is thought to be essential for plague pathogenesis and the onset of the low calcium response.[231,232] It is also thought to function as an immunomodulator by inhibiting production of pro-inflammatory cytokines such as interferon-gamma and tumor necrosis factor-alpha.[233]

One theory points to a role for V antigen in eliciting anti-inflammatory IL-10 production by host immune cells, thereby inhibiting production of pro-inflammatory cytokines and preventing a systemic immune response. [234,235] Among other data, mice infected with avirulent plague that lacked the plasmid that bears the V antigen showed a robust TNF-alpha and IFN-gamma response.[236] Co-administration of pure V-antigen exacerbated infections of *Listeria monocytogenes* and *Salmonella typhimurium* in mice.[237] Co-administration of IFN-gamma and TNF-alpha helped protect against 10 minimal lethal doses of *lcrV*+ plague bacteria.[238] As a recent review stated, "the generalized Yersinia-induced paralysis of professional phagocytes that occurs in vivo is caused by the systemic down-regulation of inflammation," putatively by IL-10.[239] The same review argues that the role of the V-

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[229] Andrews, G.P. et al. "Fraction 1 capsular antigen (F1) purification from *Yersinia pestis* CO92 and an *Escherichia coli* recombinant strain and efficacy against lethal plague challenge." *Infection and Immunity*. 1996, 64:2180-2187.

[230] Anderson, G.W. et al. "Protection of mice from fatal bubonic plague and pneumonic plague by passive immunization with monoclonal antibodies against the F1 protein of Yersinia pestis." *American Journal of Tropical Medicine and Hygiene*. April 1997, 56(4):471-3.

[231] Burrows, T.W. et al. "The basis of virulence in *Pasteurella pestis*: an antigen determining virulence." *British Journal of Experimental Pathology*. 1956, 37:481-493.

[232] Price, S.B. Et al. "The *Yersinia pestis* V antigen is a regulatory protein necessary for Ca2+-dependent growth and maximal expression of low-Ca2+ response virulence genes." *Journal of Bacteriology*. April 1991, 173(8):2649-2657.

[233] Elvin, S.J. et al. "The F1 and V subunit vaccine protects against plague in the absence of IL-4 driven immune responses." *Microb Pathog*. 2000, 29:223-230.

[234] Nedialkov, Y.A. et al. "Resistance to lipopolysaccharide mediated by the *Yersinia pestis* V-antigen-polyhistidine fusion peptide: amplification of interleukin-10." *Infect Immun*. 1997 April, 65(4):1196-203.

[235] Sing, A. et al. "Yersinia enterocolitica evasion of the host innate immune response by V antigen-induced IL-10 production of macrophages is abrogated in IL-10 deficient mice." *Journal of Immunology*. 2002 February 1. 168(3):1315-21.

[236] Nakajima, R. et al. "Association between virulence of *Yersinia pestis* and suppression of amma interferon and tumor necrosis factor alpha." *Infection and Immunity*. 61:23-31.

[237] Nakajima, R. et al. "Suppression of cytokines in mice by protein A-V antigen fusion and restoration of synthesis by active immunization." *Infection and Immunity*. 1995, 63:3021-3029.

[238]Nakajima, R. et al. "Association between virulence of *Yersinia pestis* and suppression of amma interferon and tumor necrosis factor alpha." *Infection and Immunity*. 61:23-31.

[239] Brubaker, R.R. "Interleukin-10 and Inhibtion of Innate Immunity to Yersiniae: Roles of Yops and LcrV (V Antigen)." *Infection and Immunity*. July 2003, 71(7):3673-3681.

antigen as a "long-range missile capable of systemic immunosuppression" is what separates *Yersinia pestis* from its enteropathogenic and chronic disease inducing cousins *Y. enterocolitica* and *Y. pseudomonas* as well as from the more innocuous *Pseudomonas aeruginosa*, which bears a homologous protein (PcrV). Of course, the role for the V-antigen as one of the (if not the) chief immunosuppressants in plague is under considerable debate.[240]

Despite the debate over the complete role of V-antigen in plague pathogenesis, structural studies give information about what parts of V-antigen carry out its already-defined roles in pathogenesis. Price et al. (1991) removed bases 51 through 645 in *lcrV* and replaced them with 61 new nucleotides. The resultant plague organism was avirulent in mice.[241] A fusion protein of staphylococcal protein A and plague's V-antigen (PAV) that lacked the first 67 residues (N-terminus) of the V-antigen was able to suppress TNF-alpha and IFN-gamma production such that avirulent plague, salmonella, and *Listeria monocytogenes* were able to survive in mice.[242] Earlier studies with the PAV fusion peptide demonstrated that rabbit-derived protective antibodies are not directed against the 67 N-terminus residues but that one protective epitope exists between residues 168 to 275.[243] In creating the first V-antigen directed monoclonal antibody that protects against fully virulent GB plague bacteria, Hill et al. (1997) also found evidence for a protective epitope between residues 135 and 275 (or possibly 245) and a minor protective region between residues 2 and 135.[244] Sequencing analyses suggest that "antibodies recognizing the domain [in the variable region of the antigen between residues 225 and 232 (in *Y. pseudotuberculosis* V antigen numbering) are probably able to neutralize the biological function of the V antigen."[245] Other research shows that the first 125 residues of the V-antigen are likely required for its secretion.[246] Analysis of overlapping peptide sequences derived from V antigen suggests that the protective epitope is conformational and not a linear peptide.[247] Recently, the structure of the V-antigen was determined at a 2.2 angstrom resolution.[248] Thus, researchers are closing in on the important epitopes in the V-antigen that both allow it to confer protection and evade animal model immune systems.

Like influenza's NS1 gene, future research into plague's V-antigen most likely contains the most dangerous research into plague. Now that the V-antigen is shown to play an important role in inducing both a local and systemic anti-inflammatory response, research will likely follow in areas related to the mechanism of this induction, such as looking for the receptor and/or molecular targets for V-antigen. It is also likely that the V-antigen has co-evolved more with the mouse's immune system or even the flea's primitive immune system rather than the human immune system. For the purposes of killing as many humans as possible, there is likely a bit of evolutionary plasticity in this protein. In looking for the mechanism of V-antigen's action, it is possible that scientists may create mutants of V-antigen that show an increased ability to elicit IL-10 production or perhaps that evade the F1+V subunit vaccine. The potential for these results require that certain research into the V-

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[240] Cornelis, G.R. et al. "The Virulence Plasmid of *Yersinia*, an Antihost Genome." *Microbiology and Molecular Biology Reviews*. December 1998, 62(4):1315-52.
[241] Price, S.B. et al.
[242] Nakajima, R. et al. "Suppression of cytokines in mice by protein A-V antigen fusion and restoration of synthesis by active immunization." *Infection and Immunity*. 1995, 63:3021-3029.
[243] Motin, V.L. et al. "Passive immunity to Yersiniae Mediated by Anti-Recombinant V Antigen and Protein A-V Antigen Fusion Peptide." *Infection and Immunity*. October 1994, 62(10): 4192-4201.
[244] Hill, J. et al. "Regions of *Yersinia pestis* V antigen that contribute to protection against plague identified by passive and active immunization." *Infection and Immunity*. November 1997, 65(11):4476-4482.
[245] Roggenkamp, A. et al. "Passive immunity to infection with *Yersinia* spp. Mediated By Anti-Recombinant V Antigen is Dependent on Polymorphism of Vantigen." *Infection and Immunity*. Feb 1997, 65(2):446-451.
[246] Skrzypek, F. et al. "Differential effects of deletions in *lcrV* on the secretion of V antigen, regulation of the low-Ca2+ response, and virulence of *Yersinia pestis*." *Journal of Bacteriology*. 1995. 177:2530-2542.
[247] Pullen, J.K. et al. "Analysis of *Yersinia pestis* V protein for the Presence of Linear Antibody Epitopes." *Infection and Immunity*. Feb 1998, 66(2):521-527.
[248] Derewenda, U. et al. "The structure of *Yersinia pestis* V-antigen, an essential virulence factor and mediator of immunity against plague." *Structure*. 2004 Feb, 12(2):301-6.

antigen be reviewed at the national or international level of oversight. Per the original table in the BRSS paper, this research would deserve international level of oversight. However, since creating a vaccine-resistant strain might not be their original objective, scientists proposing experiments that alter V-antigen might not readily acquiesce to such a high standard of oversight. This research also indicates that the addition of any immunomodulators (esp. IL-10 or TGF-beta) into plague bacteria to show their role in immunoevasion would also warrant national or international level of oversight, as these strains might evade vaccines.

D. Conclusions

It is quite difficult to make many solid recommendations on plague. On one hand, plague is a highly evolved organism. When plague mutants are assayed for their LD_{50}, they are very rarely more virulent than the parental strain. Of course, the pneumonic form of the disease is tested much less, due to the natural burden of bubonic plague and the difficulties associated with aerosolization versus subcutaneous injection. With increased attention for biodefense concerns, the relative lack of pneumonic plague data is likely to change, presenting a greater challenge to research oversight. On the other hand, the genome of plague is infinitely more complex than that of influenza. It has 300-fold more genes than influenza and many of them have not been studied for virulence determinance or interactions with each other. Indeed, this chapter has only dealt with a handful of these genes. In addition, plague's genome was only recently sequenced.[249] Thus, our ignorance in the ways of making plague more transmissible, lethal, or infectious is substantial. There are likely many ways to make plague more pathogenic in humans, especially given the evolutionary constraints flea transmission has put on the organism. Furthermore, research into *Y. pestis'* cousins, *Y. pseudotuberculosis* and *Y. enterocolitica*, may also provide information that will help make a more virulent plague bacteria. There will be a need to guard multiple fronts when it comes to plague research oversight.

A necessary first step for the BRSS is gaining more access to information and experiments performed by the old Soviet bioweapons program as well as an inventory of their old strains. It is important for biodefense scientists to understand why the Soviets saw it as such a high priority agent and to build defenses that will protect against Soviet strains that may have escaped into other laboratories. Increasing transparency and access to their laboratory experiments would be a crucial diplomatic first step for any research oversight system.

Antibiotic resistance comprises the most dangerous research into the plague organism. The technology and knowledge needed to confer antibiotic resistance are, of course, commonplace in microbiological laboratories. Since research oversight cannot turn back the hands of time, research oversight's greatest contribution could come through 1) overseeing new work into antibiotic resistance, especially multi-drug resistance mechanisms, and 2) delegitimizing any work involving antibiotic resistance done outside its purview.

On the vaccine front, it is also difficult to make any strong recommendations, since the current state of plague vaccine science is in flux. It does appear as though the F1+V subunit vaccine shows good protection and will likely be approved for use in humans. However, the potential for F1-negative plague bacteria with polymorphic V-antigen mean that the F1+V vaccine may not constitute a true vaccine. More research is needed into the structural and molecular determinants of both V-antigen activity and role as a protective antigen. This research will need to be reviewed at a national or international level of oversight depending on the exact experiments proposed.

There is a potential for harmful research to be undertaken in increasing the transmissibility of plague, but the science of transmissibility is so poorly understood that it is impossible to make any recommendations. On infectivity, it is not known why **2e4** aerosolized organisms are needed to infect mice or guinea pigs. It could be that some of the organisms are phagocytosed and killed

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[249] Parkhill, J. et al. "Genome sequence of *Yersinia pestis*, the causative agent of plague." *Nature*. 4 October 2001, 413:523-527.

before they activate their immunoevasion genes. Or, some of the organisms might not have found a niche that has adequate resources due to the aerosolization protocol before they degrade.

Aside from lethality rate-increasing work on antibiotic and vaccine resistance, the next greatest potential for dangerous research in plague comes in advances in and openness about aerosolization technology and, possibly, in environmental hardiness. Aerosolization technology was not reviewed to a great extent for this paper and the important details of plague aerosolization do not appear to be in the open literature. However, the materials and methods of many of the papers from the 1950s on creating experimental pneumonic plague infections contains enough information to create a localized aerosol, provided one can obtain the different nebulizers and atomizers. Advances in aerosolization technology have increased the stability of plague in aerosol and increased the infectivity of the organism.[250]

Druett et al. (1956) demonstrated that conclusions reached from experiments on the aerosolization of anthrax spores hold true for plague bacteria as well (although the quantitative aspects of the two studies were quite different overall).[251] They found that particles 1 micron in size kill faster than particles 12 microns in size and had a ~2.5 fold lower LD_{50} and ~9.5 fold lower LD_{75} (note the difference in LD_{50} was 17-fold in anthrax). They also demonstrated that, while there was no positive correlation between deaths in control contacts of guinea pigs infected and particle dosage, deaths in control contact animals (i.e., uninfected animals placed in the cage with infected animals) were about four times greater if the animals had been in contact with 1 micron infected neighbor animals vs. those infected with 12 micron sized particles. However, their experiment was unable to produce any tertiary infections in the guinea pigs, irrespective of particle size. Although their experiment demonstrated no data in monkey models, they note that "the disease takes longer to develop after exposure to large particles" in monkeys.

Environmental hardiness does not make the lethality, infectivity, or transmissibility definition of danger, but the potential for economic damage after a plague infection would be substantial, should a hardier plague bacterium be engineered. As with the science of transmissibility, it is very difficult to conjure up an experiment that would increase the environmental hardiness of plague a priori. The BRSS should recognize that this is one of the areas of potential harm in plague research while reviewing any proposed research at the local level. Since a Pubmed search for "sunlight and pestis" yielded mostly Russian articles, further understanding and access to current and past Russian research is highly recommended.

Many questions remain unanswered in pneumonic plague history and pathogenesis. For instance, it is not known why the Manchurian pneumonic plague outbreak was so anomalous and, anecdotally, did not require a base of bubonic plague cases. Questions still remain about the environmental versus genetic aspects of pneumonic plague outbreaks. To date, it would seem most large pneumonic outbreaks have occurred in areas of great poverty during the winter in the pre-antibiotic era. It is not known whether past outbreaks of pneumonic plague are comparable to a potential bioterrorist attack in the modern era. It is likely that only further research in animal models will create a new understanding of plague outbreaks. But, as with influenza, the usual caveats for research in pathogenesis in animal models apply in the case of plague.

The low natural case load of pneumonic plague also provides an important caveat for plague research oversight. Almost all of the advances made in plague have been made by research in animal models or human cell lines. Only the safety of vaccines can be shown in the complete human host. Thankfully, we are not afforded the human experiments of H5N1 influenza in Hong Kong that allow study on pathogenesis, propagation, and defense. But the relevance of plague research in animal models to a true pneumonic plague outbreak in humans might never be known to a level comparable with that of influenza.

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[250] Davis, Christopher. Personal communication. 18 May 2004.

[251] Druett, H.A. et al. "Studies on respiratory infection II. The influence of aerosol particle on infection of the guinea-pig with *Pasteurella pestis*." *Journal of Hygiene*. March 1956, 54(1):37-48.

In sum, it is more difficult to make strong recommendations on priority areas of oversight for plague research than for influenza. The bacterium kills so well that there is little point in increasing its lethality potential, beyond circumvention of antibiotics and vaccines. Infectivity determinants are not well-understood and thus aerosolization information is the only priority area of oversight. And, the difficulty of studying transmissibility in animal models makes it impossible to convey any recommendations on plague's infectiousness.

E. General Recommendations from Review of Plague Research

The review of literature on pneumonic plague does contain an important lesson for general research oversight of dangerous research into pathogens. This chapter argued that there was little point in overseeing research that increased the lethality of plague because the lethality of plague sans antibiotics was already so high. It is only with the introduction of antibiotics that research into increasing the lethality of plague, namely through antibiotic resistance, becomes truly dangerous. One could call this fact the "potential definition of danger" because danger increases as the potential for increases in a parameter becomes greater. The potential definition of danger thus becomes a compelling dilemma for any quantitative definition of danger based on parameters.

Imagine a highly transmissible pathogen that killed 100% of the time (with or without countermeasures) and infected at very low doses. Although this pathogen would indeed be very dangerous, one might consider research into this pathogen considerably less dangerous from a dual-use information standpoint. Unfortunately, the potential definition of danger does not function from an operational standpoint for extremely benign 'pathogens' or commensal bacteria. Research oversight cannot expand to contain research into non-communicable bacteria or viruses that only infect at high doses and rarely kill, even though these pathogens contain the greatest potential increase in a parameter. When defining danger for research oversight, one begins to wonder whether the initial parameters of a pathogen or the potential increases in those parameters define dangerous research.

Two potential solutions might help manage this dilemma. The first, more complicated, solution would be to define danger based on a nexus of three parameters. If at least one parameter for the pathogen falls in a yellow or red zone in the 3-D danger terrain (Figure 1), then research into either of the other parameters could be defined as dangerous or moved up to higher levels of review. Influenza provides a reasonable model for this solution. Influenza's transmissibility and infectivity are rather high, while its lethality is rather low. As discussed in Chapter 2, research into the determinants of influenza's lethality is therefore potentially quite dangerous and merit extra oversight. Research into the infectivity or transmissibility of anthrax provides another example of dangerous research that would merit a higher level of review. Since anthrax's lethality is so high, research into the other parameters (in addition to antibiotic resistance) could be considered quite dangerous. Admittedly, such a definition of danger would be quite complicated, but it allows a semi-quantitative definition of danger to exist and should not overly burden research oversight with experiments that examined some commensal bacteria where all three parameters might be quite low.

A second option would be to demote the quantitative definition of danger, along with the 3-D danger terrain, to a model of thinking about dangerous research rather than a legal definition of danger. In its place would be the process-based and, where possible, objective-based definition of danger outlined by lists of certain research activities (Figures 2-4). Even though a quantitative definition of danger based on parameters is preferable to a qualitative definition, this approach retains a number of advantages over the first option. First, it is very difficult to know at a quantitative level how proposed research will change a parameter of a pathogen. If a pathogen's lethality is currently in the white or green area defined by the 3-D danger terrain and an experiment proposes to make novel mutations in pathogenicity-determining gene, will those mutations increase the lethality of the pathogen to a yellow or red level? Should the probability of different magnitudes of changes be factored into the definition of danger or does a small chance of a worst-case scenario mean that research is reviewed at the highest level? Will reviewers even be able to know whether lethality, infectivity, or transmissibility will be affected by the proposed alterations? Asking reviewers

to answer these questions might be too great a task and might argue for backing away from a quantitative definition of danger. The lists of research activities, if appropriately defined, are more easily applied to proposed research and can adequately capture the different levels of danger without requiring an exact quantification of a parameter or a change in the parameter.

Candidate Plague Research Activities	Review Level
Easier or More Efficient Aerosolization	National - International
Mechanisms of Multi-drug Resistance	International
Antibiotic Resistance Mechanism	National - International
Working with F1-negative Variants (before approval of F1 – V antigen vaccine), including non-human primate aerosol challenge	Local - National
Increase Pla protease efficiency (molecular evolution, etc.) with non-human primate aerosol challenge	Local
V – antigen Plasticity Experiments (chiefly, binding/active site, neutralization sites)	National - International
IL-10 plague challenge in non-human primates	National - International
Environmental Hardiness	Local - National
Increased transmissibility	National - International

Table 2 – Summary of Candidate Plague Research Activities and Expected Level of Review

IV. Conclusions

Recommendations

1. **Include Countermeasures**

In this first effort to define danger in the BRSS, it seemed worthwhile to try to avoid defining danger in terms of circumventing countermeasures. The BRSS was to deal with the inherent transmissibility, lethality, and infectivity of a pathogen. However, epidemics do not happen in a vacuum. Medical countermeasures, when available, critically impact the course of an epidemic and thus deserve to be included in any definition of dangerous research.

The dangerous research activities lists included countermeasure evasion in their definition of dangerous research. Support for the inclusion of countermeasure resistance in any definition of danger can also be found in the more quantitative 3-D danger terrain. As detailed in Chapter 3, potential increases in parameters often comprise a better definition of danger for research oversight than a pathogen's initial starting conditions. For many category A agents lethality reaches a low enough level where potential increases become a problem only when antibiotics or vaccines are taken into account. For instance, pneumonic plague kills almost 100% of the time when antibiotics are not used, but it may not kill at all if antibiotics are administered rapidly. There is no point in altering molecular determinants of pathogenicity to increase the virulence of pneumonic plague if 1) it already kills at 100% rate if antibiotics are not available, and 2) it won't kill at all provided that antibiotics are administered rapidly enough to eliminate the hypervirulent strain of plague. Countermeasure resistance therefore comprises the most dangerous form of research into these pathogens.

The experiment that, arguably, began the endeavor to create a BRSS also illustrates the importance of taking into account medical countermeasures. Although the Australian IL-4 mousepox experiment did show an increased lethality of IL-4 mousepox relative to normal mousepox, the experiment is considered important insofar as it showed how to evade the smallpox vaccine.[252] The generally accepted lethality rate for smallpox is 30%.[253] The marginal impact of increasing lethality or virulence in smallpox is minute compared to the marginal impact of constructing a vaccine- or cidofovir-resistant strain. The considerable impact of the IL-4 mousepox article followed directly from the proof-of-principle that exogenous IL-4 could make smallpox vaccine-resistant.

2. **Build the Science of Transmissibility**

A lynchpin of any future BRSS is the development of standard measurements of transmissibility and the understanding of at least some molecular determinants of transmissibility. Transmissibility is perhaps the most important parameter in epidemiology. It separates a mass contagion from a one-off bomb dispersal. Currently, there is only one quantitative account of intrinsic transmissibility (the tau or beta component of R_o), and it is seldom measured. There is little understanding about what makes one organism transmissible while another not transmissible or of what factors increase transmissibility.

A necessary first step in gaining an understanding of transmissibility lies in the development of a standard measure. Epidemiological analysis classically relies upon Ro as a measure of transmissibility. Unfortunately, R_o conflates the intrinsic communicability of an agent with the social and environmental conditions of its outbreak. The calculated R_o transmissibility of, say, influenza

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[252] Jackson, R.J. Et al. "Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox." *Journal of Virology*. 2001 February, 75(3):1205-10.

[253] Henderson, D.A. Et al. "Smallpox as a biological weapon: medical and public health management." *JAMA*. 9 June 1999, 281(22):2127-37.

will be different in New York or Hong Kong than it will be in North Dakota. Ideally, epidemiologists would be able to create a standard measure of the intrinsic communicability of an organism.

The best approximation of intrinsic transmissibility can be calculated by deconstructing the R_o equation, $R_o = \tau c \delta$. As noted in the introductory chapter, deconstructing this equation is a laborious process. The main issue in determining the intrinsic transmissibility of the organism is understanding the contact rate. For sexually-transmitted diseases such as HIV, contact rates are easier to determine because of the discrete nature of the contact. For airborne diseases such as influenza or droplet-transmitted diseases such as pneumonic plague, it is far harder to calculate the contact rate because it is not obvious what counts as a contact and what does not. Nonetheless, epidemiologists have long dealt with this problem in outbreaks and have kept historical records of most contacts of infected persons during these outbreaks. This area of research is ripe for further exploration. For example, Meyers et al. (2003) was able to explain the difference in the Toronto and Vancouver outbreaks of SARS based on theoretical contact networks and intrinsic transmissibility indices that were approximated from other SARS outbreaks. Epidemiologists should revisit their historical records of contacts and Ro's to deconstruct the intrinsic transmissibility of organisms to get the science of transmissibility off the ground.

Outside of analyzing historical data, developing standard measures of transmissibility will be very difficult because of the reliance on animal models. The communicability of a disease is intimately related to the physiology of humans. Unfortunately, mice simply do not have the same physiology as humans and seldom cough.[254] The future study of transmissibility will most likely rely upon expensive non-human primate models.

The relevance of animal models will be further tested by the tiny natural incidence of bioterror agents in humans. If a certain strain of pneumonic plague is highly transmissible in an animal model, does that mean anything in human hosts? There is perhaps no better example of the questionable utility of animal models in transmissibility than the current H5N1 avian influenza in Asia. These strains of influenza have shown a remarkable ability to spread in chickens or other fowl, but have consistently failed to achieve human-to-human transmission. The influenza virus has been studied enough to understand parts of the molecular basis of this transmission phenomenon. It is likely that a similar degree of knowledge of molecular pathogenesis of other pathogens will be necessary for animal models to be considered relevant in studies of transmission. However, unlike avian influenza, we are unlikely to be afforded natural human experimentation in establishing the relevant transmissibility of bioterror agents in humans.

One possibility to further the study of transmissibility and infectivity is the testing of avirulent strains among humans. Genes encoding for virulence in animal models could be selectively knocked out of a microbe to create an avirulent strain. Of course, virulence is deeply related to communicability. Most evolutionary theory in infectious diseases holds that virulence is kept as a trait provided that it increases the transmissibility of the microbe.[255] The relevance of experimental results with avirulent organisms would therefore be called into question. Furthermore, concerns over liability would plague any such program as there is no way to be certain that a strain was apathogenic. Novel pathogenicities may be uncovered. And, the combined need and danger of establishing infections in the lungs would be highly problematic. Commensal bacteria such as *Mycobacterium avium* can become pathogenic if they establish in the lungs. Nonetheless, testing of avirulent organisms in humans remains one of the few options for the establishment of some standard for measuring transmissibility in humans.

It should be also noted that if a science of transmissibility is created, the BRSS will undoubtedly need to oversee the research. Transmissibility will be uncharted territory. It will be of

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[254] For example, nasal hairs are sometimes cited as a reason for the inapplicability of mouse models for the transmission of human disease.

[255] Hooper, J. "A New Germ Theory." *The Atlantic Monthly*. February 1999.
Ewald, P. *Evolution of Infectious Disease.* Oxford University Press, 1996.

the utmost importance to oversee this research to mitigate the chance of openly publishing dangerous research. As far as the dual-use research question is concerned, ignorance is bliss in transmissibility.

3. **Recognize the Threat Presented by Host Susceptibility and Immunology**

Although presumably falling under both the lethality index and the point above on including countermeasures, the dual use nature of host susceptibility and immunology is only partially captured by the BRSS, as currently proposed. As Stanley Falkow noted, adding IL-4 into mousepox does not necessarily make the virus any more pathogenic, rather it increased host susceptibility by suppressing the relevant arm of the immune system. The same would be true of a plague bacteria that carried a plasmid bearing an IL-10 gene. While the lists of dangerous research activities may include such research in catch-all phrases such as "increasing virulence in a listed agent," the distinction between a pathogen's virulence and host susceptibility, as well as the type of research being regulated, merits discussion.

Research into immunomodulation and immunoevasion are revealing strategies of organisms that allow them to sculpt the immune response to their evolutionary benefit. Vaccinologists have long been interested in the Th1 v. Th2 (as well as the Th0 v. Th3 v. Tr1) responses of the adaptive immune system in response to candidate vaccines. Research into the immune response to candidate pathogens will undoubtedly lead to dual-use information that will be difficult to regulate because no super-pathogen has been created.

The dual-use dilemma presented by immunology research comes not only from research into dangerous pathogens. Tumor immunology research has unveiled a number of strategies used by cancer to evade the immune system. Immunoevasion and immunomodulation are the basis of the discovery of therapies for autoimmune disorders. Any research oversight system faces a difficult dilemma in determining the exact relevance of immunotherapy research in creating dangerous pathogens.

Innate immunity also presents itself as both a potential problem and solution. Biodefense experts such as Ken Alibek have been pushing for more strategies that boost innate immunity in biodefense.[256] An expert panel convened by the National Institute of Allergy and Infectious Diseases on immunity and biodefense put innate immunology research as a high priority in future biodefense research.[257] Learning how to boost innate immunity is critical for biodefense and immunology research. But since all aspects of the immune system are a mix of positive and negative signals, such research will likely show how to both reduce and enhance the actions of innate immunity. The trouble with innate immunity is that it does not appear to be as highly adapted to individual pathogens as the adaptive immune system. There is no Th1 v. Th2 balance to worry about. The methods used by one organism to evade the innate immune system will also generally work just as well for a listed bioterror agent. How to deal with the spread of this generalized dual-use information will remain a problem for research oversight.

4. **Keep Weaponization Information Under Control**

Even if terrorists can make an especially lethal, infectious, and/or transmissible bioterror agent, delivery will always be the rate-limiting step of bioterrorism. Aum Shinrikyo tried to develop anthrax as a biological weapon but could not adequately weaponize or deliver (or pick the right strain of) the bacterium. Terrorists also have to worry about blowback of the weapon once it is adequately weaponized. These reasons may account for the relative lack of use of biological weapons throughout history.

Because the establishment of a productive infection with most bioterror agents is all about location, weaponization information impacts the BRSS through the infectivity index. As noted in the

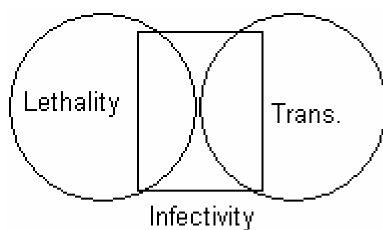
[256] Alibek, K. *Biohazard.* New York: Dell Publishing, 1999.
[257] Summary of the NIAID Expert Panel on Immunity and Biodefense. June 17, 2002. Accessed online at http://www.niaid.nih.gov/publications/pdf/biodimmunpan.pdf.

pneumonic plague chapter, the amount of radioactivity recovered from labeled particles in monkey lungs varied greatly (17-79%). Research that indicated how to consistently achieve the upper end of that range should be reviewed as very dangerous, as it could lower the de facto LD_{50} experimentally. New work by Dean Wilkening on modeling the uncertainties of a biological attack suggests that the "success" of an aerosolized anthrax attack on Washington DC may differ by as much as 10^4-fold in persons affected due to differences in biological agent preparation and dispersal efficiency. Research that lowered the LD_{50} and other variations in strains only accounted for a 10-fold level of uncertainty.[258] Although preventing dissemination of this information will prove increasingly difficult with the new expansion of biodefense research and creation of new research centers, weaponization information must stay outside of the public view and should be considered a priority for research oversight.

5. **Reexamine How Infectivity Impacts Danger**

Questions exist on the usefulness of infectivity as one of the main parameters in the definition of danger. As noted in the first chapter, infectivity is hard to measure and depends greatly on the animal model. Because of the need for a discrete measurement of "infection" (i.e., death), LD_{50}'s were used as the operational definition of infectivity in this exercise. Admittedly, this measure of infectivity combines infectivity with lethality. However, because many listed pathogens kill so efficiently (influenza aside), the benefits of investing in measuring ID_{50}s instead of LD_{50}s have not been represented as obviously in this paper. It would be more useful to use ID_{50}s in non-listed agents that do not kill so readily but nonetheless present problems for research oversight given the potential definition of danger.

Figure 100 - Infectivity Overlaps with Lethality and Transmissibility



For transmissible agents, infectivity will also, inevitably, be included in the transmissibility of the organism. By nature, diseases that require fewer organisms to infect should prove more transmissible. The only way to separate infectivity from transmissibility (for communicable agents) would be to separate the so-called "input" and "output" functions of transmissibility. In this sense, infectivity could be considered the input of an infection into a host while transmissibility would be a measurement of the organisms ability to shed itself productively from the host. For example, take two different strains of influenza. One strain may have a genetic property that allows it to better establish infection or have a higher infectivity than the other strain. The second strain may have a tissue tropism or attachment receptors that allows it to be expelled from the lungs at a faster rate and would be considered more transmissible. Of course, the science of transmissibility has not progressed to such a level; nor is it known if such a level of understanding is even attainable. Furthermore, it is not clear what benefits would be derived from such an exercise, as the two are so often intimately related.

The development of standards for input and output of infection might seem laughable given the chaotic and random nature by which infections are communicated between people. How could one determine what was a standard method of communicability? Although one should not be too dismissive of a new kind of science that may yet create such standards, it is difficult to imagine much gain at the interface of transmissibility and infectivity. However, measuring the true infectivity (via either ID_{50}s or LD_{50}s) of different communicable agents could be one of the starting points for the new science of transmissibility.

[258] Wilkening, D. "Uncertainties in Biological Weapons Attack." Talk at CISAC Science Seminar and forthcoming paper. 27 April 2004.

Infectivity might be considered a useful parameter for separating non-communicable pathogens or the initial dispersion of a communicable disease, where the output function, or transmissibility, is zero. In other words, the ability of these pathogens to establish infection in others is entirely dependent on their input function, or infectivity. Infectivity would be a useful parameter for separating pathogens such as tularemia from inhalational anthrax. Both pathogens are non-communicable bacteria. However, the infectivity of tularemia is considered to be far greater than anthrax's, as only ten organisms of tularemia are required to establish infection, while the LD_{50} of anthrax is between 1e4-1e5 bacteria (note: lethality could also separate inhalational anthrax (80-90%) from tularemia (10%)). Differences of 3-4 orders of magnitude should indeed by captured by the BRSS, for a strain of anthrax that produced infections as readily as tularemia would be very dangerous. However, the chaotic and random nature of the spread of an aerosolized biological agent suggests that another infectivity measure may be worth considering.

Dean Wilkening's work in modeling the uncertainties associated biological attacks argues for measurement of the lower tails of infectivity or the ID_{10}s and ID_5s. These tails of the so-called infectivity probit slopes critically impact the number of people infected in an aerosol dispersion of a biological agent since the number of people that come in contact with only a few organisms is far larger than the number of people that see doses higher than the ID_{50}. The ID_5 dose can also drastically impact the number of infected if there is an age-dependency of infection. For instance, during the 2001 anthrax attacks, scientists re-learned the meaning of LD_{50} curves when 94-year-old Ottilie Lundgren died from inhalational anthrax after receiving mail with undetectable levels of anthrax. Wilkening estimates that the probit slope of an infectivity curve can affect the number infected by an aerosol release of anthrax by 3 to 30-fold. Unfortunately, LD_{20} or ID_{20} are seldom measured because so many non-human primates are required and thus the cost of such experiments is very high. For example, one of the few experiments that attempted to measure the full lethal dose curve of inhalational anthrax in a monkey consumed 1200 monkeys. A similar experiment conducted today would cost near ten million dollars for the monkeys alone.

Before infectivity can be fully accepted or discarded from the BRSS, a new conversation is needed on the aspect of danger that infectivity is to capture. As noted in the introductory chapter, there are two immediate aspects that infectivity could capture: 1) the ability of an organism to establish infection or 2) the incapacitation caused by a non-lethal infection (perhaps an economic measure). This conversation should also include a discussion on the usefulness of infectivity in 1) high lethality v. low lethality agents and 2) communicable vs. non-communicable agents. This author would argue that infectivity is more useful as a parameter for non-communicable agents, but may be necessary to measure at lower levels, such as ID_{10}, for all pathogens.

Long-Term Dilemmas

As noted in the introductory chapter and the issues detailed above, the BRSS is not a silver bullet. After profiling prospective oversight over influenza and pneumonic plague research, even more "seemingly intractable problems" of the BRSS present themselves for further discussion.

1. **The Host-Pathogen Relationship**

Beyond the threat presented by host susceptibility is the difficult dilemma of the host-pathogen relationship. Pathogens are highly tuned and evolved to their respective host(s). Indeed, pathogenicity does not exist in the absence of a specific host. Thus, simply overseeing research into pathogens could be considered insufficient. As Stanley Falkow remarked, "if you want to restrict access to the information about pathogens, then you have to restrict access to information about the host." Following this dictum would entail not publishing the human genome, provided one wished to prevent the open publication of the 1918 influenza genome.

Of course, the problem cannot be seen as so stark. After all, the design of the proposed research oversight system is to prevent open dissemination of information that necessarily draws a straight line to a more dangerous pathogen. It is theoretically possible to allow open publication of human genomes and proteomes if one is able to restrict the most relevant, dangerous information on

pathogens. But, the problem of using open information about the human organism to 1) create a more virulent pathogen or 2) turn the body against itself will be with us for awhile.

Ultimately, the dilemma centers around the degree of proof required by bioterrorists to produce a dangerous pathogen. Legitimate research that seeks to combine transmissible pathogens or vectors with potentially dangerous cytokines or human proteins should be overseen. This research could potentially provide direct evidence for the creation of an extremely dangerous pathogen. What is more problematic is the connection of potentially dangerous work in disparate fields. Bioterrorists may not need a laboratory to give them direct evidence or proof that the combination of a highly transmissible organism with gene X (researched perhaps exclusively in immunology or neurobiology) will prove destructive. To be sure, problems with gene expression abound. However, the inexorable march of biotechnology and know-how may one day mitigate such speed bumps in the creation of dangerous pathogens.

2. **Signaling Danger?**

By categorizing what proposed research would be dangerous, research oversight might also signal or prioritize for bioterrorists steps to make a more dangerous pathogen. This project in itself is inherently dual-use. Defenders of the current project may say that all of the information was gathered from the open literature, and thus it creates no more danger than was already out there. Some dangerous procedures such as conferring antibiotic resistance to high threat agents are quite obvious. Other steps may not be so obvious. After all, this project took many months to complete and a reasonable amount of literacy in microbiology, virology, and immunology. It collated many of the most relevant, potentially dangerous articles in influenza and pneumonic plague to point out high priority areas for research oversight. Depending on how openly published the rubric of rules of danger for proposed research oversight is, research oversight may create more danger than it prevents. On one hand, research oversight may be judiciously limited to a panel of international trusted experts that participate in closed meetings and pass down decisions about potential danger of proposed research. Such an arrangement would contain the signaling danger presented by research oversight. On the other hand, as the initial BRSS paper argued, transparency is critical to the endeavor and one would prefer to have all labs know how to distinguish acceptable proposals from illegitimate/dangerous ones before research proposals are submitted. A dilemma of security and secrecy v. widespread distribution of danger signals is seemingly inescapable.

Of equal importance, questions exist about the use of a system if one can a priori tell the danger level of proposed research. Would not a sufficiently competent bioterrorist also be able to tell? Certainly, one does not want to churn out cookbooks in research journals. But if a priori one can discern a particular danger level of an experiment *and* one is not a top expert in the field, is not research oversight somewhat diminished? If an M.S. in biology can make an educated guess that it might be deleterious to insert a Th2-skewing immunodulator gene into a pathogenic intracellular bacteria, then it would seem that somewhat competent bioterrorists might also be able to figure it out. It could be that resolving that uncertainty is the value-added the basic researchers are giving to the terrorist. But the greater the uncertainty, the more problematic the definition of danger and the greater contention about the use of the research oversight system (not to mention increasing costs). As uncertainty increases it becomes even more difficult to put research into a certain danger category. Once again, tensions exist as a result of the necessary expertise required for research oversight and the amount of uncertainty in experimental results.

3. **Dealing with Agents under the Radar**

It was argued at the end of Chapter 3 that – at least in terms of research oversight – the potential to increase danger is almost as important as the current level of danger of a pathogen. One can think of this as the "diminishing returns" argument. Research that shows how to make a more virulent pneumonic plague almost does not warrant oversight, provided the work does not show how to evade a vaccine or antibiotics. The existence of antibiotics and vaccines for pathogens reduces their de facto lethality (as well as their de facto infectivity and transmissibility), opening up the

potential for danger. Research that creates antibiotic or vaccine resistant strains thus becomes the highest priority for oversight. Similarly, oversight of research that might potentially show how to make a more lethal influenza would be of the highest priority because 1) transmissibility is so poorly understood and 2) the lethality of influenza is so low.

Undoubtedly, to have research be truly dangerous one or two of the parameters must be elevated such that the increase of the third parameter creates a highly pathogenic, infectious organism. The true level of danger also depends on the type of research being done on the pathogen. Random mutagenesis of apathogenic *E. coli* is much less likely to create a monstrous strain than similar research done on influenza or smallpox. But, including within the definition of danger the potential for increases means that the whole array of microbial life is in play for research oversight, including grey areas such as research into tuberculosis or Category B/C agents. The list of candidate research activities does take this problem into account by including and giving a low priority to "Increasing virulence of non-listed agent" and other activities with non-listed agents. Nonetheless, the operational requirements of overseeing so much research and developing *specific* guidelines for their oversight will likely present a problem for the BRSS. Defining danger broadly or specifically is a continuing dilemma for the BRSS.