

Advances in Development of Countermeasures for Potential Biothreat Agents

Guest Editors: Phillip R. Pittman, Kelly T. McKee Jr., and Zygmunt F. Dembek





Advances in Development of Countermeasures for Potential Biothreat Agents

Advances in Preventive Medicine

Advances in Development of Countermeasures for Potential Biothreat Agents

Guest Editors: Phillip R. Pittman, Kelly T. McKee Jr.,
and Zygmunt F. Dembek



Copyright © 2012 Hindawi Publishing Corporation. All rights reserved.

This is a special issue published in "Advances in Preventive Medicine." All articles are open access articles distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Editorial Board

Francisco M. Averhoff, USA
Jim P. BATTERY, Australia
William C. S. Cho, Hong Kong
Myron Christodoulides, UK
Rhea Coler, USA
Guido Dietrich, Belgium
Isabelle Dimier-Poisson, France
Jean-Louis Excler, Switzerland
Germain J. Fernando, Australia
Elisabetta Franco, Italy
Tong-Ming Fu, USA

Ivaylo Gentshev, Germany
Magdalena Gherardi, Argentina
Mark Grabowsky, Switzerland
Gerardo E. Guillén Nieto, Cuba
Joel R. Haynes, USA
John Iskander, USA
Helena Käyhty, Finland
Gideon Kersten, The Netherlands
Katrin S. Kohl, USA
James P. McGettigan, USA
Sergio Costa Oliveira, Brazil

Ultan Power, UK
David Russell, Norway
Masaru Shimada, Japan
Jim Tartaglia, Canada
Michael Vajdy, USA
Ramesh Vemulapalli, USA
Michael Wallach, Australia
Shixia Wang, USA
Andrés Wigdorovitz, Argentina
Diane Williamson, UK
Lijuan Yuan, USA

Contents

Advances in Development of Countermeasures for Potential Biothreat Agents, Phillip R. Pittman, Kelly T. McKee Jr., and Zygmunt F. Dembek
Volume 2012, Article ID 570246, 2 pages

The Need for Continued Development of Ricin Countermeasures, Ronald B. Reisler and Leonard A. Smith
Volume 2012, Article ID 149737, 4 pages

Biosurveillance: A Review and Update, Nicholas E. Kman and Daniel J. Bachmann
Volume 2012, Article ID 301408, 9 pages

Advanced Development of the rF1V and rBV A/B Vaccines: Progress and Challenges, Mary Kate Hart, George A. Saviolakis, Susan L. Welkos, and Robert V. House
Volume 2012, Article ID 731604, 14 pages

The Role of Immune Correlates and Surrogate Markers in the Development of Vaccines and Immunotherapies for Plague, E. D. Williamson
Volume 2012, Article ID 365980, 7 pages

FDA Experience with Medical Countermeasures under the Animal Rule, Paul Aebersold
Volume 2012, Article ID 507571, 11 pages

Editorial

Advances in Development of Countermeasures for Potential Biothreat Agents

Phillip R. Pittman,¹ Kelly T. McKee Jr.,² and Zygmunt F. Dembek³

¹ *Department of Clinical Research, United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Frederick, MD 21704-5011, USA*

² *Public Health and Government Services, Quintiles Transnational Corporation, Durham, NC 27703, USA*

³ *Center for Disaster and Humanitarian Assistance Medicine, Uniformed Services University of The Health Sciences, Bethesda, MD 20814-4779, USA*

Correspondence should be addressed to Phillip R. Pittman, phillip.pittman@amedd.army.mil

Received 24 June 2012; Accepted 24 June 2012

Copyright © 2012 Phillip R. Pittman et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

September 11, 2001, was the day that the people of the United States of America came face to face with the specter of terrorism. Long considered secure by virtue of being bordered by friendly countries to the north and south and two great bodies of water to the east and west, the US landmass was suddenly no longer safe from the brutal and indiscriminate acts of extreme mass violence that had menaced other countries for decades. A few weeks later, and already shaken to its core, the anthrax spore letter attacks then awakened the American public to the reality of lethal biological terrorism. The autumn of 2001 was, indeed, a frightening and unsettling time.

Before this, the potential for use of live biological agents and toxins as weapons was viewed largely to be the purview of the US military; revelations of the large-scale Soviet program and discovery of weapons caches and warheads in Iraq after the first Gulf War had raised general awareness of the threat, but had done little to stimulate the political will necessary to invest in medical countermeasures before 2001. The anthrax letter attacks, however, served to crystallize the threat, and, for the first time, significant funds were allocated by the US Congress for biodefense.

The original goal of this special edition of *Advances in Preventive Medicine* was to provide a venue for highlighting some of the advances in biodefense research which have been made since the fall of 2001. Papers selected by the editors for inclusion include an overview of biosurveillance; a consideration of the regulatory hurdles needed to be overcome

for FDA licensure of potentially beneficial products; a discussion of correlates of immunity; and two papers highlighting progress in the development of new vaccines to counter biothreat agents.

A reliable surveillance program is a key component of the early detection algorithms necessary to institute countermeasures in a timely manner. The paper by Kman and Bachmann, "Biosurveillance: a review and update," offers a discussion of passive and active surveillance, and provides overviews of syndromic surveillance, laboratory surveillance, and environment surveillance, together with the systems used in these methodologies and their relative effectiveness.

The very nature of biologic agents used to incite terror proscribes traditional studies to assess efficacy of medical countermeasures against these agents in human volunteers. The paper by Aebersold, "FDA experience with medical countermeasures under the Animal Rule," offers a comprehensive review of the development of the regulatory framework established by the US Food and Drug Administration for licensing medical countermeasures against chemical, biological, and radiological threats, the so-called "Animal Rule," as well as experience in implementing this guidance in the approval of two drugs: pyridostigmine bromide, a drug licensed for the treatment of myasthenia gravis since 1955 and approved by FDA for prophylaxis against the lethal effects of Soman nerve agent poisoning, and Cyanokit, approved as an antidote for patients with known or suspected cyanide poisoning.

Critical to successful application of the Animal Rule is establishing correlates of protection that can be used to establish surrogate markers of efficacy in humans. The paper by Williamson, "The role of immune correlates and surrogate markers in the development of vaccines and immunotherapies for plague," provides insight into some of the challenges inherent in identifying such correlates, and approaches to evaluate and validate markers to achieve this objective.

In "Advanced development of rF1V and rBV A/B vaccines: progress and challenges," Hart et al. review their experience as Prime Systems contractor for the US Department of Defense in manufacturing, assay development, preclinical and clinical development and testing of vaccines for *Yersinia pestis*, plague, and *Clostridium botulinum* toxins A and B. The authors address obstacles inherent in navigating the requirements of the FDA Animal Rule as they seek to provide safe and effective products that meet the challenges of the modern warfighter. Ricin toxin has remained a menace as an agent of terror. In their paper, "The need for continued development of ricin countermeasures," the authors, Reisler and Smith, review the origin of the toxin, its pathophysiology, the history of the use of this category B biothreat agent, and the development of medical countermeasures including vaccines.

This brief collection of papers provides a taste of the challenges underlying development of countermeasures against biological weapons, as well as a sense for the progress that has been made since the early years of the 21st century. It is an unfortunate reality that biological agents are, and will continue to be, threats to public welfare and safety. Continued progress along the lines of the experiences shared here is vital to counter these threats, to remain a step ahead of those who would purposefully harm others through deployment of biological weapons, to prevent illness and death through stimulation of protective immunity, and to mitigate the consequences of their use among the vulnerable.

Disclaimer

The opinions expressed are those of the authors and should not be construed to represent those of the Department of Defense or Quintiles Transnational Corporation.

Conflict of Interests

The authors have no conflict of interests to report.

*Phillip R. Pittman
Kelly T. McKee Jr.
Zygmunt F. Dembek*

Review Article

The Need for Continued Development of Ricin Countermeasures

Ronald B. Reisler¹ and Leonard A. Smith²

¹Division of Clinical Medicine, Clinical Research Management Inc., Team Ke'aki Tech, United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, MD 21702-5011, USA

²Division of Toxinology, United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, MD 21702-5011, USA

Correspondence should be addressed to Ronald B. Reisler, ronald.reisler@hhs.gov

Received 1 August 2011; Accepted 10 January 2012

Academic Editor: Phillip R. Pittman

Copyright © 2012 R. B. Reisler and L. A. Smith. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Ricin toxin, an extremely potent and heat-stable toxin produced from the bean of the ubiquitous *Ricinus communis* (castor bean plant), has been categorized by the US Centers for Disease Control and Prevention (CDC) as a category B biothreat agent that is moderately easy to disseminate. Ricin has the potential to be used as an agent of biological warfare and bioterrorism. Therefore, there is a critical need for continued development of ricin countermeasures. A safe and effective prophylactic vaccine against ricin that was FDA approved for “at risk” individuals would be an important first step in assuring the availability of medical countermeasures against ricin.

1. Introduction

In the aftermath of September 11, 2001, it has become increasingly clear that there is a need to enhance readiness against attack from both state sponsors and nonstate sponsors of bioterrorism. Ricin toxin, an extremely potent and heat-stable toxin produced from the bean of the *Ricinus communis* (castor bean plant) [1], has been categorized by the US Centers for Disease Control and Prevention (CDC) as a category B biothreat agent for biological warfare and bioterrorism [2]. In fact, according to Cookson and Nottingham, ricin was code named compound W and considered for weaponization during the US offensive Biological Warfare Program [3]. The US intelligence community believes that ricin was a component of the biowarfare program of the former Soviet Union, Iraq, and possibly other countries as well [4, 5].

Ricin toxin is relatively easy to produce and potentially lethal when delivered orally, intramuscularly, or through inhalation [4]. While the primary large-scale threat to US military personnel would be through powdered material that could be inhaled, ricin has been used successfully to assassinate individuals, to carry out suicide, and in 2003-2004, to terrorize US postal and Senate workers [4]. This paper reviews the rationale for development of ricin countermeasures

and the progress toward achieving effective ricin countermeasures.

2. Background

Ricin is a 65 kilodalton (kDa) polypeptide toxin comprised of two dissimilar polypeptide chains (an A-chain and a B-chain) held together by a disulfide bond [1, 4, 5]. The A-chain, ~32 kDa, targets the ribosome and is therefore a potent inhibitor of protein synthesis [4, 5]. Consequently, the A-chain has been classified as a ribosome-inactivating protein (RIP) [4, 5]. The B-chain, ~34 kDa, is a galactose or an N-acetylgalactosamine-binding lectin that attaches to cell-surface receptors [4, 5]. After binding and subsequent endocytosis, the holotoxin travels through the Golgi apparatus to the endoplasmic reticulum where the disulfide bond linking the A and B chains is reduced. Once the disulfide bond is broken, the A-chain molecule is transported to the cytosol where it inactivates the ribosome. In fact, just one ricin molecule per cell may be sufficient to permanently inhibit that cell from performing essential cellular protein synthesis [6].

Ricin holotoxin is lethal in mice, rabbits, and monkeys at parenteral doses of 5–25 $\mu\text{g}/\text{kg}$ [4]. By inhalation, ricin has an LD₅₀ in mice, rabbits, and monkeys of 3–17 $\mu\text{g}/\text{kg}$, and by

ingestion it has an LD₅₀ of 20 mg/kg [4]. When ricin toxin A (RTA) chain is separated from ricin toxin B (RTB) chain and is administered parenterally to mice, it has limited toxicity at lower doses. RTA is approximately 1000-fold less toxic than natural ricin at lower doses when administered parentally to mice [7].

Ricin toxin is a potential threat to humans by three distinct routes: aerosolized ricin via the pulmonary system, food and water via the gastrointestinal system, and bioweaponized munitions including improvised explosive devices, via skin wounds [8, 9]. For more than 120 years, researchers have been working on ways to both develop prophylaxis against ricin exposure and to effectively treat ricin postexposure [4].

3. Early Work on Ricin Vaccine and Pretreatment for Ricin Exposure

Initially, as early as the 1890s, Paul Ehrlich vaccinated mice with oral doses of ricin and then subsequently challenged the mice with subcutaneous lethal doses of ricin [10]. Later, in the 1940s, a formalin-inactivated holotoxin vaccine was developed by the US Army that enhanced survival in animals [11]. This vaccine candidate did not progress past preclinical testing. Pretreating animals with passive transfer of either IgG polyclonal antibody [12–14] or monoclonal directed against RTA, appeared to effectively protect them from lethal parenteral challenge to ricin [15–17]. Protection against a lethal dose of aerosolized ricin with passive transfer of either IgG polyclonal or monoclonal antibody directed against RTA has proved to be more difficult to achieve.

4. Progress toward a Prophylactic Ricin Vaccine

4.1. US Army Ricin Vaccine Development (1990s). Past attempts to produce a ricin vaccine with an Alhydrogel-adsorbed ricin toxoid [18–20] and a deglycosylated RTA (dgRTA) vaccine (Lot 01-0419964, PerImmune) [20–22] suggested that although both products can induce protective immunity against the toxin in animals, their use as vaccines was limited by safety concerns raised during preclinical development, the tendency to self-aggregate and precipitate from solution, and difficulties associated with process and product characterization during manufacturing. Thus, these vaccine candidates were limited to pre-clinical testing and never progressed to human clinical trials.

4.2. RiVax Recombinant Vaccine. RiVax, an investigational recombinant protein RTA vaccine, was developed based on studies with ricin and RTA [23, 24]. RiVax is essentially RTA with two simple amino-acid substitutions, one in the LDV amino acid sequence {amino acid residues 74–76}, hypothesized to play a key role in intact RTA-induced Vascular Leak Syndrome (amino acid 76: valine replaced by methionine), and the other in a ribotoxic site (amino acid 80: tyrosine replaced with alanine) [23]. RiVax was found to have sufficient preclinical safety data to proceed to a human phase I dose-escalating study [24, 25]. The human phase I study was de-

signed as follows: 15 healthy volunteers (three groups of five) were vaccinated three times with intramuscular (IM) RiVax (doses were either 10 μ g, 33 μ g, or 100 μ g) at monthly intervals [25]. Vitetta et al. demonstrated that RiVax was safe and elicits neutralizing antibody in a cell based assay. Vitetta et al. reported that in the low-dose group, one out of five had neutralizing antibody, in the intermediate dose group four out of five had neutralizing antibody, and in the high-dose group five out of five had neutralizing antibody. In the two higher dose groups, neutralizing antibody titers were similar but somewhat modest. Vitetta et al. estimated that the vaccine could protect against an injected dose of ricin of (0.3–3.0 mg) or approximately 1 to 10-fold the human LD₅₀. However, the duration of antibody titers after three vaccinations (range: 14–127 days) was suboptimal and not related to dosing group.

While initial RiVax phase I results were encouraging, vaccine formulation and stability remain problematic. Vitetta et al. required the use of four different vaccine lots during the course of the initial 15 subject phase I study [25]. Moreover, RiVax formulation required storage at -70°C in a buffer containing 50% glycerol. Therefore, Smallshaw and Vitetta subsequently developed a lyophilized formulation of the vaccine that retained immunogenicity when stored at 4°C [26, 27].

A second RiVax phase I trial in 30 subjects at three different dose levels, utilizing an alum adjuvant formulation, was supported by an FDA Orphan Products grant to University of Texas Southwestern (UTSW). As of March 29, 2011, enrollment for the second phase I trial [28] was complete [29]. In their SEC annual report filing, Soligenix reported that preliminary results from the second phase I trial indicated that RiVax appeared safe at all doses tested. To date, human immunogenicity data have not been reported. Soligenix also reported that they initiated a comprehensive program to evaluate the efficiency of RiVax in nonhuman primates at the Tulane University Health Sciences Center [29].

4.3. RVEc Recombinant Vaccine. USAMRIID has developed a recombinant RTA vaccine 1–33/44–198 (rRTA 1–33/44–198) (RVEc) produced in *Escherichia coli* [30–32]. Based on pre-clinical studies, including a pivotal repeated-dose toxicology study in New Zealand white rabbits conducted under GLP [33], this product was determined to have a reasonable safety profile for use in human studies. The pre-clinical testing demonstrated no detectable ribosome inactivating protein (RIP) activity [33] or evidence of vascular leak syndrome (VLS) [34]. A phase I ($N = 30$) first in human escalating, multiple-dose, and single-center study to evaluate the safety and immunogenicity of RVEc was launched at USAMRIID, Fort Detrick MD, in April 2011. The phase I study is expected to be completed by the first half of 2013 [35, 36].

5. Monoclonal Antibody Pre-Clinical Development and Proof of Concept for Postexposure Prophylaxis

Neal et al. reported that passive prophylactic administration (intraperitoneal {IP} injection) of GD12 (a murine IgG1

monoclonal antibody (Mab)—anti-RTA) when administered 24 h prior to challenge was sufficient to protect mice against intraperitoneal ricin challenge of 5 LD₅₀ [37]. Neal et al. further demonstrated that GD12 protected mice utilizing a backpack tumor delivery system after intragastric ricin challenge of 5 mg/kg. Neal et al. did not test GD12 in the setting of post-exposure prophylaxis. In a follow-up study, Neal et al. demonstrated similar protection in mice when two other monoclonal antibodies, R70 (anti-RTA) and 24B11 (anti-RTB), were passively administered using the so-called backpack tumor model [38]. The mice then survived challenge with intragastric ricin 5 mg/kg 12–24 h. In addition, R70 Mab protected mice after it was administered IP, 12–24 h before intragastric ricin challenge of 5 mg/kg.

Prigent et al. demonstrated that a combination of three Mabs (2 anti-RTB and 1 anti-RTA) to ricin protected mice when the three Mabs were administered intravenously (IV) within 7.5 h after ricin intranasal challenge of 5 LD₅₀ [39]. Thus, it would appear that Prigent et al. demonstrated a proof of concept for effective post-exposure prophylaxis to lethal-dose intranasal challenge to ricin [39].

6. Small Molecule Inhibitors: Preclinical Development and Pre-Exposure Prophylaxis

Stechmann et al. have recently reported on the successful identification of a selective small molecule inhibitor, Retro-2, that protected mice in a ricin nasal challenge model, when Retro-2 was administered IP one hour prior to challenge [40]. This small molecule inhibitor is attractive in that it does not act on the toxin itself, but rather it blocks retrograde transport of the toxin, a host-toxin interaction. Stechmann et al. argue that since Retro-2 blocks retrograde transport and does not act on the toxin or the host cell itself, there is a decreased likelihood that significant drug resistance will develop to Retro-2. Moreover, Retro-2 appears to be non-toxic to HeLa cells. Small molecule inhibitors offer another promising potential avenue for the development of effective prophylaxis against ricin toxin exposure [41].

7. Rationale for Continued Development of Ricin Countermeasures

Schep et al. have recently argued somewhat simplistically that although ricin is toxic, it does not deserve to be a priority in biological countermeasure development [9]. They maintain that bioterrorists do not possess the technical and logistical skills necessary to formulate and mill ricin powder. St. Georgiev similarly maintained that ricin is more compatible with a tool of assassination instead of a weapon of mass destruction [42]. However, Radosavljevic and Belojevic have recently formulated a much more compelling and comprehensive approach to biodefense prioritization and risk assessment [8]. Their approach incorporates all of the potential biothreat agents on the CDC biothreat agent list. Furthermore, their model considers quantitative and qualitative parameters in assessing risk and has four main components: perpetrators (government institutions/organizations, terrorist groups,

individuals); agent (CDC categories A, B, and C); means and media of delivery (air, food, water, fomites); target (direct and indirect) [8].

The US Armed Forces, Department of Homeland Security (DHS) personnel, first responders, FBI, local law enforcement personnel, CDC/HHS, the Environmental Protection Agency (EPA), and environmental clean-up crews all need adequate protection against potential biological warfare and bioterrorism. Therefore, there is a critical need for continued development of ricin countermeasures.

8. Conclusion

While small molecule inhibitors and Mabs for post-exposure treatment are still being evaluated in a pre-clinical setting, RiVax has been studied in two phase I clinical trials, and RVEc is currently in a phase I human trials. A safe and effective prophylactic vaccine against ricin that is FDA approved for “at risk” individuals should be an important first step in countering this 120-year-old threat.

Acknowledgments

The authors would like to acknowledge the Defense Threat Reduction Agency (DTRA), Joint Science and Technology Office for Chemical and Biological Defense (JSTO-CBD) for their continued support of the development of the RVEc vaccine. The views and opinions expressed in this paper are those of the authors and do not reflect official policy or position of the Department of the Army, Department of Defense, or the US Government. The opinions or assertions contained herein are those of the authors and are not to be construed as official policy or as reflecting the views of the Department of the Army or the Department of Defense.

References

- [1] G. A. Balint, “Ricin: the toxic protein of castor oil seeds,” *Toxicology*, vol. 2, no. 1, pp. 77–102, 1974.
- [2] Centers for Disease Control and Prevention, “Biological and chemical terrorism: strategic plan for preparedness and response,” *Morbidity and Mortality Weekly Report*, vol. 49, no. RR-4, pp. 1–14, 2000.
- [3] J. Cookson and J. Nottingham, *A Survey of Chemical and Biological Warfare*, vol. 6, Monthly Review Press, New York, NY, USA, 1969.
- [4] M. A. Poli, C. Roy, K. D. Huebner, D. R. Franz, and N. K. Jaax, “Ricin,” in *Medical Aspects of Biological Warfare*, Z. F. Dembek, Ed., pp. 323–335, Borden Institute, Washington, DC, USA, 2007.
- [5] J. Audi, M. Belson, M. Patel, J. Schier, and J. Osterloh, “Ricin poisoning a comprehensive review,” *Journal of the American Medical Association*, vol. 294, no. 18, pp. 2342–2351, 2005.
- [6] K. S. Eiklid, S. Olsnes, and A. Pihl, “Entry of lethal doses of abrin, ricin and modeccin into the cytosol of HeLa cells,” *Experimental Cell Research*, vol. 126, no. 2, pp. 321–326, 1980.
- [7] A. M. Soler-Rodriguez, J. W. Uhr, J. Richardson, and E. S. Vitetta, “The toxicity of chemically deglycosylated ricin A-chain in mice,” *International Journal of Immunopharmacology*, vol. 14, no. 2, pp. 281–291, 1992.

- [8] V. Radosavljevic and G. Belojevic, "A new model of bioterrorism risk assessment," *Biosecurity and Bioterrorism*, vol. 7, no. 4, pp. 443–451, 2009.
- [9] L. J. Schep, W. A. Temple, G. A. Butt, and M. D. Beasley, "Ricin as a weapon of mass terror—separating fact from fiction," *Environment International*, vol. 35, no. 8, pp. 1267–1271, 2009.
- [10] W. T. Porter, *An Introduction to Physiology*, The University Press, Cambridge, Mass, USA, 1906.
- [11] A. C. Cope, *Ricin in Summary Technical Report of Division 9 on Chemical Warfare and Related Problems*, chapter 12, Office of Scientific Research and Development, Washington, DC, USA, 1946, Parts I–II. National Defense Research Committee.
- [12] M. A. Poli, V. R. Rivera, M. L. M. Pitt, and P. Vogel, "Aerosolized specific antibody protects mice from lung injury associated with aerosolized ricin exposure," *Toxicon*, vol. 33, p. 289, 1996.
- [13] M. A. Poli, V. R. Rivera, M. L. M. Pitt, and P. Vogel, "Aerosolized specific antibody protects mice from lung injury associated with aerosolized ricin exposure," *Toxicon*, vol. 34, no. 9, pp. 1037–1044, 1996.
- [14] P. V. Lemley, P. Amanatides, D. Creasia, J. Ruvo, A. O'Neill, and D. C. Stafford, "Chicken antitoxin neutralizes ricin *in vitro* and *in vivo*," *Toxicon*, vol. 31, p. 527, 1993.
- [15] P. V. Lemley and D. C. Wright, "Active immunization after passive monoclonal antibody and ricin toxin challenge," *Toxicon*, vol. 30, p. 531, 1992.
- [16] P. V. Lemley, P. Amanatides, and D. C. Wright, "Identification and characterization of a monoclonal antibody that neutralizes ricin toxicity *in vitro* and *in vivo*," *Hybridoma*, vol. 13, no. 5, pp. 417–421, 1994.
- [17] P. V. Lemley and D. C. Wright, "Mice are actively immunized after passive monoclonal antibody prophylaxis and ricin toxin challenge," *Immunology*, vol. 76, no. 3, pp. 511–513, 1992.
- [18] A. Assaad, J. Hewetson, O. Parker et al., "A ricin toxoid attenuates acute pulmonary toxicity and improves survival in rats exposed to a lethal dose of ricin aerosol," *Toxicon*, vol. 33, p. 277, 1995.
- [19] J. F. Hewetson, V. R. Rivera, P. V. Lemley, M. L. Pitt, D. A. Creasia, and W. L. Thompson, "A formalinized toxoid for protection of mice from inhaled ricin," *Vaccine Research*, vol. 4, no. 4, pp. 179–187, 1995.
- [20] R. B. Reisler, "Development and pre-clinical evaluation of a recombinant ricin vaccine (RVEc)," in *Proceedings of the 9th ASM Biodefense and Emerging Diseases Research Meeting*, Poster 210 (H), Washington, DC, USA, February 2011.
- [21] J. F. Hewetson et al., "Immune response and protection of rats and mice from ricin aerosol exposure after vaccination with deglycosylated ricin A chain," *Fundamental and Applied Toxicology*, vol. 30, supplement, article 59, 1996.
- [22] P. V. Lemley, D. A. Creasia, M. Plesha, D. Walters, and D. O'Neill, "Ricin sub-unit vaccination in mice and protection from challenge," *Toxicon*, vol. 32, pp. 545–546, 1994.
- [23] J. E. Smallshaw, A. Firan, J. R. Fulmer, S. L. Ruback, V. Ghetie, and E. S. Vitetta, "A novel recombinant vaccine which protects mice against ricin intoxication," *Vaccine*, vol. 20, no. 27–28, pp. 3422–3427, 2002.
- [24] J. E. Smallshaw, J. A. Richardson, S. Pincus, J. Schindler, and E. S. Vitetta, "Preclinical toxicity and efficacy testing of RiVax, a recombinant protein vaccine against ricin," *Vaccine*, vol. 23, no. 39, pp. 4775–4784, 2005.
- [25] E. S. Vitetta, J. E. Smallshaw, E. Coleman et al., "A pilot clinical trial of a recombinant ricin vaccine in normal humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 7, pp. 2268–2273, 2006.
- [26] J. E. Smallshaw and E. S. Vitetta, "A lyophilized formulation of RiVax, a recombinant ricin subunit vaccine, retains immunogenicity," *Vaccine*, vol. 28, no. 12, pp. 2428–2435, 2010.
- [27] P. S. Marconescu, J. E. Smallshaw, L. M. Pop, S. L. Ruback, and E. S. Vitetta, "Intradermal administration of RiVax protects mice from mucosal and systemic ricin intoxication," *Vaccine*, vol. 28, no. 32, pp. 5315–5322, 2010.
- [28] Phase 1B Study of RiVax. ClinicalTrials.gov, 2011 <http://www.clinicaltrials.gov/ct2/show/NCT00812071?term=rivax&rank=1>.
- [29] Soligenix Annual Report 2010, 2011, http://www.sec.gov/Archives/edgar/data/812796/000121390011001567/f10k2010_soligenix.htm.
- [30] M. A. Olson, J. H. Carra, V. Roxas-Duncan, R. W. Wannemacher, L. A. Smith, and C. B. Millard, "Finding a new vaccine in the ricin protein fold," *Protein Engineering, Design and Selection*, vol. 17, no. 4, pp. 391–397, 2004.
- [31] J. H. Carra, R. W. Wannemacher, R. F. Tammariello et al., "Improved formulation of a recombinant ricin A-chain vaccine increases its stability and effective antigenicity," *Vaccine*, vol. 25, no. 21, pp. 4149–4158, 2007.
- [32] M. M. Meagher, J. G. Seravalli, S. T. Swanson et al., "Process development and cGMP manufacturing of a recombinant ricin vaccine: an effective and stable recombinant ricin a-chain vaccine-RVEc," *Biotechnology Progress*, vol. 27, no. 4, p. 1036, 2011.
- [33] D. E. McLain, T. L. Horn, C. J. Detrisac, C. Y. Lindsey, and L. A. Smith, "Progress in biological threat agent vaccine development: a repeat-dose toxicity study of a recombinant ricin toxin A-chain (rRTA) 1-33/44-198 vaccine (RVEc) in male and female New Zealand white rabbits," *International Journal of Toxicology*, vol. 30, no. 2, pp. 143–152, 2011.
- [34] A. Porter, G. Phillips, L. Smith et al., "Evaluation of a ricin vaccine candidate (RVEc) for human toxicity using an *in vitro* vascular leak assay," *Toxicon*, vol. 58, no. 1, pp. 68–75, 2011.
- [35] RVEc Phase I Human Clinical Trial, 2011, <http://www.usamriid.army.mil/newsroom.cfm>.
- [36] Safety and Immunogenicity Study of Recombinant Ricin Toxin A-Chain Vaccine (RVEc), 2011, <http://www.clinicaltrials.gov/ct2/show/NCT01317667?term=rvec&rank=1>.
- [37] L. M. Neal, J. O'Hara, R. N. Brey, and N. J. Mantis, "A monoclonal immunoglobulin G antibody directed against an immunodominant linear epitope on the ricin A chain confers systemic and mucosal immunity to ricin," *Infection and Immunity*, vol. 78, no. 1, pp. 552–561, 2010.
- [38] L. M. Neal, E. A. McCarthy, C. R. Morris, and N. J. Mantis, "Vaccine-induced intestinal immunity to ricin toxin in the absence of secretory IgA," *Vaccine*, vol. 29, no. 4, pp. 681–689, 2011.
- [39] J. Prigent, L. Panigai, P. Lamourette et al., "Neutralising antibodies against Ricin Toxin," *Plos ONE*, vol. 6, no. 5, Article ID e20166, 2011.
- [40] B. Stechmann, S. K. Bai, E. Gobbo et al., "Inhibition of retrograde transport protects mice from lethal ricin challenge," *Cell*, vol. 141, no. 2, pp. 231–242, 2010.
- [41] P. G. Wahome, J. D. Robertus, and N. J. Mantis, "Small-molecule inhibitors of ricin and shiga toxins," *Current Topics in Microbiology and Immunology*, vol. 357, pp. 179–207, 2012.
- [42] V. St. Georgiev, "National institute of allergy and infectious diseases," in *Impact on Global Health*, vol. 2, pp. 274–277, Humana Press, New York, NY, USA, 2009.

Review Article

Biosurveillance: A Review and Update

Nicholas E. Kman¹ and Daniel J. Bachmann²

¹ Ohio Task Force 1, FEMA Urban Search and Rescue and Emergency Medicine, The Ohio State University Medical Center, 4813 Cramblett Hall, 456 West Tenth Avenue, Columbus, OH 43210, USA

² Emergency Medicine, The Ohio State University Medical Center, 4734 Cramblett Hall, 456 West Tenth Avenue, Columbus, OH 43210, USA

Correspondence should be addressed to Nicholas E. Kman, nicholas.kman@osumc.edu

Received 30 April 2011; Revised 18 September 2011; Accepted 10 November 2011

Academic Editor: Zygmunt F. Dembek

Copyright © 2012 N. E. Kman and D. J. Bachmann. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Since the terrorist attacks and anthrax release in 2001, almost \$32 billion has been allocated to biodefense and biosurveillance in the USA alone. Surveillance in health care refers to the continual systematic collection, analysis, interpretation, and dissemination of data. When attempting to detect agents of bioterrorism, surveillance can occur in several ways. Syndromic surveillance occurs by monitoring clinical manifestations of certain illnesses. Laboratory surveillance occurs by looking for certain markers or laboratory data, and environmental surveillance is the process by which the ambient air or environment is continually sampled for the presence of biological agents. This paper focuses on the ways by which we detect bioterrorism agents and the effectiveness of these systems.

1. Introduction

Since the terrorist attacks of September 11, 2001, and the anthrax release in the following month, there has been a heightened interest in bioterrorism surveillance. The years immediately following these attacks were met with increased awareness and funding from the federal government. This paper will focus on the methods that we can use to prepare ourselves and detect these bioagent attacks.

The anthrax attacks of 2001, the SARS outbreak in 2004, and the recent H1N1 Influenza outbreak remind us that an essential component of preparedness for bioterrorism includes surveillance methods that can detect and monitor the course of an outbreak and thus minimize associated morbidity and mortality [1]. Surveillance of a population can be achieved in several ways. Syndromic surveillance occurs by monitoring clinical manifestations of certain illnesses. This type of surveillance occurs when health-related data, like *International Classification of Diseases Ninth Revision* (ICD-9) codes, are analyzed to signal possibility of an outbreak. Laboratory surveillance occurs by looking for certain markers or laboratory data. The Laboratory Response Network (LRN) is the United States' laboratory

system for detecting, confirming, and reporting bioterrorism agents. Within the LRN, sentinel laboratories are tasked with singling out suspicious specimens for further testing in higher-tier labs. Environmental surveillance is the process by which the ambient air or the environment is continually sampled for the presence of biological agents [2].

Unfortunately, the practice of bioterrorism surveillance remains poorly studied. A recent systematic review of 29 biosurveillance systems concluded that there is insufficient evidence to determine which of these systems is best [1]. One thing is known. Whether it is an astute clinician like the one who made the first diagnosis of anthrax in 2001 or the complex chemical lab techniques that are used to detect plague, we must maintain our ability to identify and respond to a biologic terrorist attack.

2. Background

Surveillance is recognized as the single most important public health instrument for identifying public health events of global concern, particularly infectious diseases that are emerging [3]. Not only is the use of surveillance helpful

for bioterror attacks, but also the information generated by surveillance systems is also useful in the recognition and response to emerging infectious diseases. These epidemics are not related to traditional bioterror agents but their public health significance can be equally alarming. The recent H1N1 Influenza outbreak is a prime example of this.

The four functions of basic surveillance include (1) detecting cases of disease in specific populations and reporting the information, (2) analyzing and confirming reported case information to detect outbreaks, (3) providing timely and appropriate responses at the local/regional level to allow appropriate national level prevention and control of disease outbreaks, and (4) providing epidemiologic intelligence information to assist in long-term management of public health and health-care policies and programs [3].

Surveillance in health care refers to the continual systematic collection, analysis, interpretation, and dissemination of data [4]. Early methods of public health surveillance have been passive and voluntary. This process occurred when patients were diagnosed with a reportable communicable disease and local health departments were notified by clinicians, hospitals, or laboratories. Time would pass as information meandered through local and state health departments. Although many of the key components of surveillance occur at the local level, it takes many working parts for this to occur in a timely fashion.

Passive surveillance is an important component to global biosurveillance. It has the advantages of being inexpensive, easy to implement, and free of technologic barriers. However, it likely is not rapid and accurate enough to be used alone to respond to a bioterrorist attack. Passive surveillance is used best with other methods to quickly identify the treat and institute public health protection measures such as immunization, prophylaxis, and quarantine.

Active surveillance is the method of tracking emerging infectious disease threats. Active surveillance involves outreach to actively collect disease information from specific groups, such as sentinel medical providers or hospitals. Typically, active surveillance is undertaken to look for a specific disease. Active surveillance is more labor intensive and requires more public health resources than passive surveillance [5].

Systems for bioterrorism surveillance for public health require 3 key features: timeliness, high sensitivity and specificity, and routine analysis of data [1]. Timeliness of diagnosis is vital as the effectiveness of most treatments hinges on early detection. To this end, the electronic collection and reporting of surveillance data has improved detection as compared with manual methods [1]. High sensitivity is necessary as, without this, systems may fail to detect cases of bioterrorism-related illness which could result in delays in detection. On the opposite end, systems with inadequate specificity may have frequent false alarms, which will result in costly public health responses. Using the example of a food-borne illness outbreak, a system with low sensitivity may miss the sentinel cases and not identify the trend until the outbreak is already widespread. This compromises the ability of the surveillance system to adequately mount an effective public health response to the outbreak. Using the

same example, a system with low specificity may identify cases which are not truly related to an outbreak resulting in an unnecessary public health response with diversion of resources from other true outbreaks. Sensitivity and specificity are typically inversely related such that optimization of one characteristic is at some expense to the other. Striking the optimal balance between these two characteristics for any given surveillance system is difficult [1].

3. Syndromic Surveillance

The first key to identifying a potential bioterrorism event is to maintain a strong index of suspicion. The initial cases of West Nile Fever Virus in 1999 and the deliberate release of anthrax in 2001 were ultimately diagnosed by astute clinicians working hand-in-hand with lab technicians, not by public health surveillance systems. Syndromic data are gathered before laboratory results are reported; therefore, health departments may be able to recognize increases in disease incidence before formal diagnoses are made and to respond to outbreaks early in their course. For this reason, the CDC, state and local public health agencies, and the US Government and military have invested heavily in syndromic surveillance.

Methods of syndromic surveillance include many clues and data points which public health personnel can use to identify patterns. Data sources such as nurse hotline calls, over-the-counter medication purchases, and chief complaints from emergency-department visits can monitor illness clusters [6]. Some other clues to suspicious events include sharp rises in the frequency or severity of communicable diseases, including those in animals. Additional red flags include an unusual cluster or age distribution, occurrence of rare diseases, presence or lack of exposure history, travel to an endemic location, unexplained deaths, or pathogens with unusual antimicrobial resistance [7].

In response to the events of 2001, new types of surveillance systems were developed to detect epidemics through population-based reporting of symptoms tracked by time and region [8]. Many cities and states in the United States use syndromic surveillance, which monitors nonspecific, prediagnostic indicators for disease outbreaks in near real-time to provide an early warning of infectious disease outbreaks in their communities. Syndromic surveillance systems (SSS) monitor descriptive data from clinical diagnoses, chief complaints, and behaviors (e.g., school and work absenteeism, illness-related 911 calls, emergency room admissions for symptoms indicative of infectious disease) to infer patterns suggestive of an outbreak [9]. A comparison of syndromic surveillance with traditional clinical recognition is presented in Table 1 [10].

The most important determinants of detection for any given SSS were analyzed in a methodological review of 35 evaluations of outbreak detection in automated SSSs [11]. These determinants are key to taking one or more high-volume data feeds and differentiating the outbreak cases or "signal" from the baseline cases or "noise." The determinants were subdivided into characteristics of the system and

TABLE 1: Characteristics of bioterrorism-related epidemics that affect detection through clinical recognition versus syndromic surveillance.

Characteristics ^a	Clinical recognition ^b	Syndromic surveillance ^c
Duration and variability of incubation period	Broader distribution of incubation period increases likelihood that patients with short incubation-period disease would be diagnosed before a statistical threshold of syndromic cases is exceeded.	More narrow distribution of incubation period which leads to a steeper epidemic curve in the initial phases increase likelihood that statistical threshold would be exceeded sooner.
Duration of nonspecific prodromal phase	Shorter prodrome increases likelihood of recognition or diagnosis at more severe or fulminant stage.	Longer prodrome increases likelihood that increase in syndromic manifestations would be detectable and that recognition of more severe stage (at which a diagnosis is more apt to be made) would be delayed.
Presence or absence of clinical sign that would heighten suspicion of diagnosis	Presence increases likelihood of earlier clinical recognition and diagnosis (e.g., mediastinal widening on chest X-ray in inhalational anthrax or multiple cases of rare disease presenting at similar time).	Absence decreases likelihood that diagnosis would be considered clinically, increasing opportunity for earlier detection by means of syndromic surveillance.
Likelihood of making diagnosis in the course of routine clinical evaluation	If diagnosis is apt to be made in the course of a routine diagnostic evaluation (not dependent on clinical suspicion of specific bioterrorism infection), early diagnosis through clinical care is likely.	If diagnosis is dependent on the use of a special test that is unlikely to be ordered in the absence of clinical suspicion of diagnosis, then diagnosis in clinical care may be delayed, increasing the opportunity for early detection through syndromic surveillance.

^aInfection or disease attributes that may affect detection of an epidemic.

^bIncreases likelihood of initial detection through routine clinical care and reporting.

^cIncreases likelihood of initial detection through syndromic surveillance.

characteristics of the outbreak being monitored. While evaluations using natural outbreaks were best suited to answer qualitative questions, simulated outbreaks were also useful to allow greater flexibility and increased quantitative results [11].

The influential system characteristics identified included representativeness or sampling approach of the system, the outbreak detection algorithm, and the specificity of the algorithm. For example, systems that monitor a larger proportion of the population have a higher sensitivity for detecting an outbreak. Similarly, systems that only monitor one type of clinical setting—such as ED visits only—were less sensitive. Furthermore, the studies that relied on simulated outbreaks suggested that temporal surveillance was more sensitive when the algorithm considered multiple days of data at each decision point versus data from each day individually. Important determinants related to the outbreak included magnitude and shape of the signal and timing of the outbreak. Intuitively, signals with a rapid rise over a short period of time improved outbreak detection as compared with those that rose more slowly over time. The ideal magnitude of the signal for consistent detection is not clear. The studies indicated magnitudes ranging from 10% up to as much as 60%. Similarly, the influence of the timing of the signal was not consistent, though there was a better detection when the outbreak occurred in context of a lower baseline of activity [11]. Based on these characteristics, one could envision an ideal SSS that monitored a large population at

multiple clinical venues over multiple days at a time and flagged signals with rapid rise over a low baseline to at least a magnitude of 10%.

Almost immediately after the terrorist attacks of September 11, 2001, The New York City Department of Health and Mental Hygiene (NYCDOHMH) collaborated with the CDC to initiate an emergency-department-based syndromic surveillance for agents [12]. The system looked for symptoms that could be associated with a bioagent release such as respiratory distress, rash, gastrointestinal symptoms, neurologic impairment, and sepsis. Providers filled out forms with patient data that were analyzed by epidemiologists. This system was up and running in 15 New York City ED's within 2 days of its conception.

Syndromic surveillance systems monitor health care utilization patterns using data collected in real time, usually electronically. One example of a SSS is the Electronic Surveillance System for the Early Notification of Community-Based Epidemics (ESSENCE), which automatically downloads ICD-9 codes from U.S. Department of Defense health care facilities [1]. This novel use of ICD-9 codes is one way to group patient visits into syndromes. There are more than 10,000 ICD-9 codes available [13]. Patient visits are grouped by ESSENCE algorithms into one of eight syndromes based on lists of selected ICD-9 codes. If an increase in number of visits for a syndrome is noted, the clinic can be contacted for more information and an investigation can be launched.

Started in November of 2003, BioSense is a CDC Internet-based syndromic surveillance application designed for the early detection of intentional and natural infectious disease outbreaks [12]. BioSense receives data electronically from several sources. The Department of Veterans Affairs and Department of Defense provide ICD-9 codes for visits to their facilities. Retail pharmacies provide sales information on over-the-counter medications, and Laboratory Corporation of America provides information on laboratory tests ordered. After examination by CDC analysts, public health officials can access their summary reports.

Current SSSs monitor the average pattern of patients reporting to primary care physicians or emergency-departments and signal an alarm whenever the pattern changes. Reporting sources include emergency-departments, intensive care units, hospital admission and discharge systems, and laboratories [8]. The Rapid Syndrome Validation Project (RSVP) relies on physicians to enter data on patients presenting with a syndrome of interest into a computer that has a touch-screen interface with RSVP [14].

The Emergency Department is the most common clinical source for surveillance data, though other sources of data have been proven to be useful. The Real-time Outbreak and Disease Surveillance Laboratory (RODS) Pennsylvania is the biosurveillance system for the Commonwealth of Pennsylvania. In production since 1999, it monitors 3 million visits to emergency rooms from 137 emergency-departments a year and simultaneously monitors 1262 retail stores in Pennsylvania for disease outbreaks. By utilizing the National Retail Data Monitor (NRDM), they have found a strong correlation that exists between the purchase of over-the-counter (OTC) medications and emergency room visits for constitutional illnesses. This information is useful for predicting coming epidemics as the tracking patterns of influenza and seasonal gastrointestinal illnesses often precede trends in hospital data [15]. One study demonstrated that OTC electrolyte sales preceded hospital visits for gastrointestinal and respiratory illnesses by 2.4 weeks [16].

The Connecticut Department of Public Health has been effectively using an SSS based on unscheduled hospital admissions since 2001. The Hospital Admission Syndromic Surveillance (HASS) system monitors 32 Connecticut-based acute-care hospitals with required reporting for eleven syndromic categories. Daily monitoring of data with weekly comprehensive analysis allows identification of disease clusters and routine public health followup for further action or response [17].

Syndromic surveillance efforts have been expanded to include outpatient monitoring also. This type of system takes advantage of the experience of ambulatory care physicians, who are also likely to be among the first to encounter patients during the prodrome of any potential bioterrorism-related illness. One such system developed with a private large ambulatory multispecialty group practice in Eastern Massachusetts demonstrated that surveillance coverage of 5–10% of a region's population may be adequate to detect significant clusters of interest. Several ideal components of this particular system included the automated collection of information, the use of preexisting data from a standard

healthcare database, and the minimal cost for its implementation and continuous administration [18].

Although most systems for syndromic surveillance are continuously collecting, analyzing, and reporting data, some systems are designed for short-term use at mass-gatherings thought to be terrorist targets. These SSSs are referred to as event-based or “drop-in” surveillance [1]. One such “drop-in” surveillance system studied by the Bioterrorism Preparedness and Response Program demonstrated fair-to-good agreement of patient classification into an appropriate syndrome category when comparing use of Emergency Department chief complaints to discharge diagnoses. The findings were suggestive that use of discharge diagnoses may increase surveillance validity for “drop-in” and even possibly automated surveillance systems [19]. It is thought that syndromic surveillance systems are best used synergistically with laboratory surveillance.

4. Alternative Surveillance Systems

The Centers for Disease Control and Prevention (CDC) has pioneered surveillance systems for monitoring other indicators of disease beside the traditional symptom- and diagnosis-based data used for clinical and syndromic surveillance. One such system is the Early Aberration Reporting System (EARS). This is a free tool which has been utilized and modified in both cities (Boston, NYC, Los Angeles) and in state public health agencies (Georgia, Florida, Tennessee, North Carolina, and Mississippi). It uses nontraditional public health data sources including school absenteeism rates, over-the-counter medication sales, 911 calls, veterinary data, and ambulance run data [20].

One novel epidemiologic surveillance approach has been developed by Google Inc and the CDC during the influenza season of 2007-08. This system monitored the health-seeking behavior of millions of users per day in the form of queries to online search engines. Ginsberg et al. demonstrated use of their model to estimate influenza-like illness within 85–96% of CDC-reported actual illness prevalence for the mid-Atlantic region of the USA. The advantages of this internet-based system were that illness statistics were available with a reporting lag of only one day, compared to the 7–14 day lag of CDC surveillance reports [21].

Though the Google surveillance system was specifically designed to monitor for influenza-like illness, the concept is more broadly applicable to other infectious pathogens such as bioterror agents. In addition to earlier detection of outbreaks, other advantages include freely available information to both the public and the government officials, automated processing with near real-time dissemination, and relative inexpensiveness for operation. Unfortunately, the specificity of internet-based surveillance remains unclear and could create more issues related to a high false-positive rate. These systems also require large populations with adequate internet access across regions and socioeconomic classes [22].

Another emerging example of a web-based surveillance system is the HealthMap Project. This collaborative undertaking performs extraction, categorization, filtration, and

integration of aggregated reports from multistream real-time internet surveillance data [23]. The round-the-clock process involves automated data mining assisted by analyst review and reclassification. This system specifically focuses on identifying the “breaking news” trends to avoid overwhelming public health officials with low-impact problems [23]. The HealthMap system was applied to the H1N1 outbreak of 2009 with impressive results. The time difference between report of suspected cases and confirmed cases of H1N1 influenza was tracked by country with an overall median lag time of 12 days [24]. This time period can and will significantly alter the impact of the subsequent public health response. Further integration of these types of innovative systems with more traditional surveillance offers the greatest promise for future surveillance of emerging diseases [24].

The true utility of the SSSs is the dissemination and integration of its main output: surveillance data. In 2007, the CDC's Office of Critical Information Integration and Exchange created the CDC created the BioPHusion Center with a mission to provide a CDC-wide resource that facilitates the exchange, integration, and visualization of relevant information from a variety of sources to enhance agency and programmatic situational awareness for decision-making and early event detection. Its goal is to share timely and actionable information to public health programs and leaders at the national, state, local, tribal, and global levels. They use data from a wide variety of governmental, private, and other sources to create an integrated daily report of potential events available through their Public Health Information Integration Portal [25]. Other publicly accessed CDC resources for information exchange include the Epidemic Information Exchange (Epi-X) and the Public Health Information Network (PHIN).

5. Laboratory Surveillance

Clinical laboratories have been the cornerstone of diagnosis in infectious diseases of public health importance. In 1999, the CDC, the Federal Bureau of Investigation (FBI), and the Association of Public Health Laboratories (APHL) established the Laboratory Response Network (LRN) of about 120 laboratories [26]. The RODS laboratory is an example of one such system used for active surveillance as well as research efforts in the field of biosurveillance [15]. The mission of the LRN is to maintain an integrated network of laboratories that are fully equipped to respond to acts of chemical or biological terrorism, emerging infectious diseases, and other public health emergencies [26, 27]. In addition to identifying agents, the LRN is responsible for developing protocols for the handling, identifying, and reporting of potential biological agents to other national security agencies [2].

The LRN includes federal laboratories (CDC), state and local public health labs, military labs (the United States Army Medical Research Institute for Infectious Diseases (USAMRIID)), food testing (FDA), environmental laboratories, veterinary laboratories (United States Department of Agriculture), and international laboratories (Canada, the United Kingdom, and Australia) [2]. The laboratories

involved in the LRN are divided into levels A through D, based on capabilities and function. Table 2 describes the levels of labs and their function [2, 26, 27].

As described in Table 2 above, sentinel laboratories are the first tier of the LRN and are responsible for sorting through their daily routine clinical tests to find suspicious biothreat specimens. The response to a local outbreak is the first and, perhaps, most important level. Sentinel laboratories must operate using Biosafety Level 2 procedures and possess a class II certified biological safety cabinet [27]. These labs are staffed by workers with only basic sentinel lab training. When a suspected biothreat agent is identified by one of these workers, it is sent to the local and state public health labs that comprise the second tier of the LRN. These reference laboratories then perform rapid confirmatory testing while maintaining biosafety level (BSL-3) facilities [7]. Once this threat is confirmed, it is passed on to the third tier in the LRN, the national laboratories. The national laboratories are equipped with the most secure containment labs (BSL-4) that they can use if necessitated by the agent.

Effective communication between the laboratory divisions is essential to preparedness. Ongoing dialogue between clinicians, sentinel laboratories, and LRN reference laboratories is essential to confirm the diagnosis quickly. In the USA, if a sentinel laboratory cannot rule out a bioterrorism agent, then it must be referred to an LRN reference laboratory [7]. For cases garnering high suspicion, state public officials are typically contacted and specimens are transported under the jurisdiction of law enforcement.

The following principles described by Pien et al. guide clinicians with respect to sentinel laboratory evaluation of potential bioterrorism agents [7]. (1) The initial evaluating physician should obtain optimum specimen collection instructions from the sentinel laboratory and alert them to the possibility of dangerous pathogen. (2) To maximize speed, accuracy, and safety, sentinel laboratories should limit culture manipulation to what is required by LRN reference labs. (3) Labs are to not inoculate highly suspected smallpox, hemorrhagic fever viruses, alphaviruses, or any unknown viral agents of potential bioterrorism into cell culture. Local public health authorities or the CDC should be contacted prior to collection. (4) Labs are directed to not send environmental (e.g., packages, powders, letters, soil, or water), food, animal, or plant specimens to sentinel laboratories for analysis. Instead, these should be referred directly to a LRN reference laboratory. (5) Finally, to reduce the risk of laboratory-acquired infection, restrict manipulation of certain potential agents (e.g., *Francisella tularensis*, *Brucella* species, *Coxiella burnetii*, *Burkholderia mallei*, and *Burkholderia pseudomallei*) to environments under certified class II biological safety cabinet or BSL-3 conditions [7].

The CDC is responsible for monitoring the reference labs via regular proficiency training [30]. It cannot do the entire job alone and delegates some of the work to states local municipalities. This introduces some pitfalls and inconsistencies to the LRN. Many states and locals have different laws regulating this reporting. Additionally, as some of the sentinel labs are privately owned, only moderate oversight can occur at this level. To this end, a recent survey

TABLE 2: Laboratory divisions within the laboratory response network.

Level A Laboratory (Sentinel Labs)	Tier 1	Approximately 2300 hospital and clinic labs were likely first to receive specimens. Role is to rule out and refer to a lab within LRN to confirm a diagnosis.
Level B Laboratory (Reference Labs)	Tier 2	Increased capabilities to confirm diagnoses of biological agent. County public health labs where role is confirmatory testing, initial susceptibility testing, and referral.
Level C Laboratory (Reference Labs)	Tier 2	Much like level B, State public health labs that confirm diagnosis and refer to national laboratory. There are approximately 160 reference labs (B and C).
Level D Laboratory (National Labs)	Tier 3	National laboratories whose primary responsibility is to further characterize the agent (CDC, USAMRIID have biosafety level IV (BSL-4) capabilities).

showed that only 73.8% of reporting labs indicated that they had sufficient personnel, equipment, and training to respond to a bioterrorism event [27]. Another study performed exercises with three category A organisms of bioterrorism (Anthrax, Plague, and Tularemia). In this study, sentinel laboratories only correctly identified 84% of bioterrorism agents [26]. This study showed that sentinel lab performance is improving, but still not likely at this optimal goal.

6. Environmental Surveillance

There are two categories of environmental detection systems currently in existence, the remote or standoff detection of aerosol clouds and the point detection systems of the environment [2].

6.1. Remote Detection Systems. One way that remote detection systems monitor for potential biothreats from a distance is by the observation of aerosolized masses or clouds. Finding and evaluating the contents of a cloud is referred to as “standoff” detection [28]. On its most basic level, these detectors aim to alert military or civilian public health personnel to the presence of an approaching cloud. After the initial identification of the cloud, a more detailed assessment of the contents, such as water droplets, inert inorganic material, dead biotic particulates, or nonpathogenic microbes, is pursued [28]. Remote or standoff detection surveillance systems include cloud recognition by Doppler radio and radar, the Army’s long- and short-range biological standoff detection systems. The Army’s standoff detection systems are capable of detecting aerosol clouds from long distances, as well as determining their composition using ultraviolet light reflectance [2].

6.2. Point Detection Systems. Point detection systems are those that sample an environmental source, attempting to detect and identify the agent. Specific identification of a biologic agent by rapid diagnostics at the site of the attack can be done using immunologic assays, genetic assays, and mass spectrometry [2]. These systems can further be differentiated by the type and location of sample collected. For example, The Interim Biological Agent Detector is used

on US naval ships to monitor the air for an increase in particulate concentrations [1]. Biowatch is an example of an environmental detection system that takes aerosol samples from locations in fixed sites, such as airports or public buildings.

6.3. Biowatch. In July of 2003, the Department of Homeland Security (DHS), the Environmental Protection Agency (EPA), and the CDC introduced the Biowatch program—a federal monitoring system intended to speed detection of specific biological agents that could be released in aerosolized form during a biological attack. Biowatch air sampling devices are deployed in 31 major U.S. cities. The air samples typically are tested daily for signs of the particular biological agents being monitored [2, 29].

The core purpose and intent of Biowatch is to hasten the public health response to a covert bioattack. This would allow rapid distribution of medical countermeasures, like antibiotics or vaccinations, thereby saving lives [30]. To this end, there are 500 air filters in these 31 urban areas that work as Biowatch sensors. These sensors have also been deployed to select indoor venues and are used to monitor mass-gathering events, such as the Super Bowl. This nationwide surveillance system uses distributed aerosol collectors to capture airborne particles onto removable dry filters that are transported daily to LRN laboratories for analysis [30]. An expanded deployment of the same technology in 2005 was referred to as Generation 2 Biowatch. Generation 2 Biowatch reportedly can sample and report detection from 10 to 36 hours [31]. Biowatch sensors are intended to be integrated into a complex network of environmental monitoring, medical surveillance activities, and public health response. It is thought that this integration of public awareness information, as well as syndromic, laboratory, and environmental surveillance technologies and systems, would be the best defense against a bioterrorist attack [2, 31].

Biowatch is not perfect. As currently operated, Biowatch filters are collected every 24 hours and delivered to local laboratories, where they are analyzed according to prescribed protocols. If this analysis recognizes one of the five biothreat agents that the system is designed to detect, it is termed a Biowatch Actionable Result (BAR). Laboratories report BARs to local public health officials, who must then decide how

to respond. This decision is not taken lightly. The decision to treat a BAR as evidence of a bioattack could have huge consequences if it were a false alarm, including destructive impacts on the community's confidence in the public health system. Since 2003, there have been a number of BARs, though none have been the result of a biological attack. In some BAR cases, Biowatch samples contained material that was genetically similar to that found in Biowatch target organisms. These cases turned out to be from microbes that are present in the ambient environment but do not represent a threat to humans. Progress has been made in developing lab tests that distinguish these close relatives of bioweapons and work on more specific lab assays is ongoing [31].

Warnings from Biowatch would only be timelier than current health care systems under specific circumstances. Those are if a large-scale aerosol attack were to use certain biological agents and occur where Biowatch is deployed and if Biowatch successfully detects the biological agent [29].

Generation 3 Biowatch is currently in development. The next evolution of environmental sensor technology has been referred to as a "lab in a box" [31]. Gen 3 Biowatch would be more sophisticated than the current Biowatch sensors, with the ability to automatically collect outdoor air samples, perform molecular analysis of the samples, and report the results electronically to provide near-real time reporting. The target requirements for Generation 3 are reduction of time to diagnosis to 4 hours, increasing targeted bioterror agents monitored, reducing unit procurement costs down to \$80,000 per detector unit, and detection sensitivity and false-positive rates remaining consistent with the current system's performance [31].

7. Cost/Benefit

A bioterrorist incident is considered a low probability but high-cost event. The costs are high, because many agents go undetected until the onset of symptoms when treatment is less effective and more expensive [32]. That said, it is not economically feasible to the government to undertake a blanket deployment of biosensors [2]. Despite the growing cost, Congress continues to pass legislation intended to strengthen the nation's biological surveillance by increasing funding of federal and state biological surveillance. A 2007 DHS report documented that since the events of 2001, almost \$32 billion has been allocated to biodefense and biosurveillance in the USA alone [3].

As of 2005, Biowatch costs per year were approximately \$13,672,096. This figure includes labor costs, site upgrades, supplies, travel, training, and other operation and maintenance costs [32]. Most agree that this cost is justified if the probability of a bioterrorism incident remains high as the benefits of Biowatch improve.

As the Biowatch network is presently planned to expand with greater capability, this will increase the costs of the Biowatch Generation 3 system as compared to the currently deployed Generations 1 and 2 systems. Considering the operational complexity of current US biosurveillance systems, it is imperative that the operational advantages and feasibility

of the proposed system be carefully evaluated and that actual performance of Generation 3 be tested in field conditions before large technology acquisition investments are made. The Department of Homeland Security will continue to work collaboratively to conduct and oversee developmental and operational tests of Biowatch 3 [31].

8. Limitations/Current Challenges

Despite the massive increase in funding and resources that has catapulted our capability for increased biosurveillance over the past decade, both in the US and abroad, there remain several challenges that must be addressed. The value of disease surveillance systems to public health officials is greatest when several systems are used together. The primary limitation of disease surveillance at this point is the limited coordination and lack of interoperability among the various private and federal surveillance systems.

As part of the 9/11 Commission Act, the National Biosurveillance Integration Center (NBIC) was created within the Department of Homeland Security to integrate information and support an interagency biosurveillance community. A 2009 report from the US Government Accountability Office on the state of biosurveillance and resource use concluded that there exists confusion, uncertainty, and skepticism around the value of the interagency community, as well as the mission and purpose of the NBIC within that community. Furthermore, there was a lack of clarity about roles, responsibilities, joint strategies, policies, and procedures for operating across agency borders [33].

Each individual system provides useful information, though no single system is complete [5]. Since it is possible to travel to most places in the world in less time than the incubation period for many infectious diseases, our networks must be expanded to allow for global surveillance [9]. The World Health Organization produced a major overhaul of their International Health Regulations in 2005 with a specific focus on the coordination of the global public health response to natural disasters, accidental release, or deliberate use of biological and chemical agents that can affect global public health [3]. But this cooperation must exist at every level—local, state, federal, and international—to maximize the effects of surveillance.

Another challenge of our current surveillance approach is the consequences of false-positive activation. These systems must be designed for high sensitivity given the overlap of commonplace pathogens with potential bioterror agents. Unfortunately, this may often sacrifice the specificity of the systems. The false alarms may be due to technical malfunctions or to naturally occurring events, such as the detection of anthrax in areas with large concentrations of cattle [32]. The subsequent mobilization of significant resources is not only costly but can be very distracting and generate overwhelming public distress.

The acquisition of data to fuel a surveillance system, especially the syndromic and clinical-based ones, may be challenged by concerns for privacy of protected health information (PHI). Though the Health Insurance Portability

and Accountability Act (HIPAA) Privacy Rule allows for essential exchanges of health data during a public health emergency, the flow of PHI may be slowed by misunderstandings of the Privacy Rule's accounting requirement. This obstacle regarding HIPAA exceptions requires education of the necessary parties prior to the event of a bioterrorist attack [34].

9. Conclusions

Halting the spread of a bioterrorism attack will take a combination of the surveillance systems described above. It is only through active study, proper funding, and creative invention that we will be able to improve these systems.

References

- [1] D. M. Bravata, K. M. McDonald, W. M. Smith et al., "Systematic review: surveillance systems for early detection of bioterrorism-related diseases," *Annals of Internal Medicine*, vol. 140, no. 11, pp. 910–922, 2004.
- [2] M. Karwa, B. Currie, and V. Kvetan, "Bioterrorism: preparing for the impossible or the improbable," *Critical Care Medicine*, vol. 33, no. 1, pp. S75–S95, 2005.
- [3] C. Castillo-Salgado, "Trends and directions of global public health surveillance," *Epidemiologic Reviews*, vol. 32, no. 1, pp. 93–109, 2010.
- [4] D. J. Persell and C. H. Robinson, "Detection and early identification in bioterrorism events," *Family and Community Health*, vol. 31, no. 1, pp. 4–16, 2008.
- [5] T. K. Sell, "Understanding infectious disease surveillance: Its uses, sources, and limitations," *Biosecurity and Bioterrorism*, vol. 8, no. 4, pp. 305–309, 2010.
- [6] L. Uscher-Pines, C. L. Farrell, S. M. Babin et al., "Framework for the development of response protocols for public health syndromic surveillance systems: case studies of 8 US states," *Disaster Medicine and Public Health Preparedness*, vol. 3, no. 1, pp. S29–S36, 2009.
- [7] B. C. Pien, J. R. Saah, S. E. Miller, and C. W. Woods, "Use of sentinel laboratories by clinicians to evaluate potential bioterrorism and emerging infections," *Clinical Infectious Diseases*, vol. 42, no. 9, pp. 1311–1324, 2006.
- [8] V. Sintchenko and B. Gallego, "Laboratory-guided detection of disease outbreaks: three generations of surveillance systems," *Archives of Pathology and Laboratory Medicine*, vol. 133, no. 6, pp. 916–925, 2009.
- [9] E. R. Choffnes, "Improving infectious disease surveillance," in *Bulletin of Atomic Scientists*, University of Chicago, Chicago, Ill, USA, 2008, <http://www.thebulletin.org/print/web-edition/op-eds/improving-infectious-disease-surveillance>.
- [10] J. W. Buehler, R. L. Berkelman, D. M. Hartley, and C. J. Peters, "Syndromic surveillance and bioterrorism-related epidemics," *Emerging Infectious Diseases*, vol. 9, no. 10, pp. 1197–1204, 2003.
- [11] D. L. Buckeridge, "Outbreak detection through automated surveillance: a review of the determinants of detection," *Journal of Biomedical Informatics*, vol. 40, no. 4, pp. 370–379, 2007.
- [12] S. M. Borchardt, K. A. Ritger, and M. S. Dworkin, "Categorization, prioritization, and surveillance of potential bioterrorism agents," *Infectious Disease Clinics of North America*, vol. 20, no. 2, pp. 213–225, 2006.
- [13] J. A. Betancourt, S. Hakre, C. S. Polyak, and J. A. Pavlin, "Evaluation of ICD-9 codes for syndromic surveillance in the electronic surveillance system for the early notification of community-based epidemics," *Military Medicine*, vol. 172, no. 4, pp. 346–352, 2007.
- [14] A. Zelicoff, J. Brillman, D. W. Forslund et al., *The Rapid Syndrome Validation Project (RSVP)*, Sandia National Laboratories, Albuquerque, NM, USA, 2001.
- [15] "Real-time Outbreak and Disease Surveillance Laboratory at the University of Pittsburgh," 2011, <https://www.rods.pitt.edu/site/content/blogsection/9/69/>.
- [16] W. R. Hogan, F.-C. Tsui, O. Ivanov et al., "Detection of pediatric respiratory and diarrheal outbreaks from sales of over-the-counter electrolyte products," *Journal of the American Medical Informatics Association*, vol. 10, no. 6, pp. 555–562, 2003.
- [17] Z. F. Dembek, K. Carley, A. Siniscalchi, and J. Hadler, "Hospital admissions syndromic surveillance—Connecticut, September 2001–November 2003," *Morbidity and Mortality Weekly Report*, vol. 53, supplement, pp. 50–52, 2004.
- [18] R. Lazarus, K. Kleinman, I. Dashevsky et al., "Use of automated ambulatory-care encounter records for detection of acute illness clusters, including potential bioterrorism events," *Emerging Infectious Diseases*, vol. 8, no. 8, pp. 753–760, 2002.
- [19] A. T. Fleischauer, B. J. Silk, M. Schumacher et al., "The validity of chief complaint and discharge diagnosis in emergency department–Based syndromic surveillance," *Academic Emergency Medicine*, vol. 11, no. 12, pp. 1262–1267, 2004.
- [20] Early Aberration Reporting System (EARS), "Centers for Disease Control and Prevention," Atlanta, Ga, USA, 2010, <http://emergency.cdc.gov/surveillance/ears/>.
- [21] J. Ginsberg, M. H. Mohebbi, R. S. Patel, L. Brammer, M. S. Smolinski, and L. Brilliant, "Detecting influenza epidemics using search engine query data," *Nature*, vol. 457, no. 7232, pp. 1012–1014, 2009.
- [22] K. Wilson and J. S. Brownstein, "Early detection of disease outbreaks using the Internet," *CMAJ*, vol. 180, no. 8, pp. 829–831, 2009.
- [23] J. S. Brownstein, C. C. Freifeld, B. Y. Reis, and K. D. Mandl, "Surveillance sans frontières: internet-based emerging infectious disease intelligence and the HealthMap project," *PLoS Medicine*, vol. 5, no. 7, pp. 1019–1024, 2008.
- [24] J. S. Brownstein, C. C. Freifeld, E. H. Chan et al., "Information technology and global surveillance of cases of 2009 H1N1 influenza," *New England Journal of Medicine*, vol. 362, no. 18, pp. 1731–1735, 2010.
- [25] H. Rolka, D. Walker, and J. Heitgerd, "An Overview of CDC's OCIX BioPHusion Program," *Integrated Surveillance Seminar Series*, June 2009.
- [26] E. A. Wagar, M. J. Mitchell, K. C. Carroll et al., "A review of sentinel laboratory performance identification and notification of bioterrorism agents," *Archives of Pathology and Laboratory Medicine*, vol. 134, no. 10, pp. 1490–1503, 2010.
- [27] B. T. Kalish, C. A. Gaydos, Y. H. Hsieh et al., "National survey of Laboratory Response Network sentinel laboratory preparedness," *Disaster Medicine and Public Health Preparedness*, vol. 3, no. 1, pp. S17–S23, 2009.
- [28] M. Kosal, "The basics of chemical and biological weapons detectors: research story of the week," Centers for Non-proliferation Studies, November 2003, <http://cns.miiis.edu/stories/031124.htm>.

- [29] Institute of Medicine, “Biowatch and Public Health Surveillance: Evaluating Systems for the Early Detection of Biological Threats,” <http://www.iom.edu/Reports/2010/Bio-Watch-Public-Health-Surveillance-Evaluating-Systems-Early-Detection-Biological-Threats.aspx>.
- [30] J. F. Regan, A. J. Makarewicz, B. J. Hindson et al., “Environmental monitoring for biological threat agents using the autonomous pathogen detection system with multiplexed polymerase chain reaction,” *Analytical Chemistry*, vol. 80, no. 19, pp. 7422–7429, 2008.
- [31] Department of Homeland Security (DHS), “Testimony of Tara O’Toole Before the House Subcommittee on Homeland Security Appropriations, on Biosurveillance,” http://www.dhs.gov/news/testimony/testimony_1271436311919.shtm.
- [32] H. Schneider, “Protecting public health in the age of bioterrorism surveillance: is the price right?” *Journal of Environmental Health*, vol. 68, no. 5, pp. 9–13, 2005.
- [33] GAO report on biosurveillance, “Developing a collaboration strategy is essential to fostering interagency data and resource sharing,” US General Accounting Office, Washington, DC, USA, 2009, <http://www.gao.gov/new.items/d10171.pdf>.
- [34] J. G. Hodge Jr., E. F. Brown, and J. P. O’Connell, “The HIPAA privacy rule and bioterrorism planning, prevention, and response,” *Biosecur Bioterror*, vol. 2, no. 2, pp. 73–80, 2004.

Review Article

Advanced Development of the rF1V and rBV A/B Vaccines: Progress and Challenges

Mary Kate Hart,¹ George A. Saviolakis,¹ Susan L. Welkos,² and Robert V. House¹

¹DynPort Vaccine Company LLC, Frederick, MD 21702, USA

²Bacteriology Division, USAMRIID, Frederick, MD 21701, USA

Correspondence should be addressed to Mary Kate Hart, mhart20@csc.com

Received 29 April 2011; Revised 20 July 2011; Accepted 21 July 2011

Academic Editor: Kelly T. McKee

Copyright © 2012 Mary Kate Hart et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The development of vaccines for microorganisms and bacterial toxins with the potential to be used as biowarfare and bioterrorism agents is an important component of the US biodefense program. DVC is developing two vaccines, one against inhalational exposure to botulinum neurotoxins A1 and B1 and a second for *Yersinia pestis*, with the ultimate goal of licensure by the FDA under the Animal Rule. Progress has been made in all technical areas, including manufacturing, nonclinical, and clinical development and testing of the vaccines, and in assay development. The current status of development of these vaccines, and remaining challenges are described in this chapter.

1. Introduction

Certain highly pathogenic microorganisms and their products have the potential to be used as weapons against either military or civilian populations. The Centers for Disease Control and Prevention (CDC) classifies these agents into one of three categories (A, B, or C) according to seriousness of consequences following exposure (<http://emergency.cdc.gov/agent/agentlist-category.asp>; accessed April 7, 2011). The US Department of Defense (DoD) has a long history of developing therapeutics and prophylactics (vaccines) to protect the warfighter against offensive use of these agents. Until relatively recently, these countermeasures could be used under an investigational new drug application (IND) mechanism. Now, the DoD mandates that any such products administered to the US warfighters be licensed by the US Food and Drug Administration (FDA). Currently, DVC is developing two vaccines for DoD's Joint Vaccine Acquisition Program (JVAP); these include a recombinant vaccine to protect against fatal botulism following inhalational exposure to the A1 and B1 serotypes of botulinum neurotoxin (rBV A/B), as well as a recombinant vaccine to protect against pneumonic plague following inhalational exposure to *Yersinia pestis* (*Y. pestis*) (rF1V). The specific performance

and regulatory requirements, progress, challenges, and successes in each program are reviewed below.

2. Disease Characteristics

2.1. Botulism. Botulism is caused by neurotoxins produced by the bacterium *Clostridium botulinum* (*C. botulinum*), and disease presents in different forms including infant, wound, adult colonization, and foodborne botulism [1]. The clinical picture is due to cholinergic inhibition, and characteristic signs include a descending muscle weakness, dry mouth, difficulty swallowing, slurred speech, double or blurred vision, and drooping eyelids. The botulinum neurotoxin (BoNT) eventually causes paralysis of the respiratory muscles, which prevents unassisted respiration and leads to death in a short time. Exposure to aerosolized BoNT leads to another form of disease, known as inhalational botulism, which presents with similar symptoms [2]. Currently, the only points of reference for human lethality following inhalational intoxication are estimates based on DoD modeling and extrapolation from nonhuman primate (NHP) studies (both based on mass of BoNT/70 kg of body weight) and blood levels of BoNT achieved in foodborne cases of botulism. Estimates of human lethality of BoNT serotype A by intramuscular (IM)

exposure are within the range of 0.09 to 0.15 $\mu\text{g}/70\text{ kg}$ bodyweight and from 0.7 to 0.9 $\mu\text{g}/70\text{ kg}$ bodyweight by inhalation based on extrapolation from NHP studies [2, 3]. Published estimates of human lethality of BoNT serotype B are not available. The BoNT levels in the sera of patients with botulism are usually less than 10 mouse intraperitoneal lethal dose 50% (MIPLD₅₀)/mL [2].

Treatment of botulism cases in the USA usually consists of administration of equine antitoxin antisera and supportive care. The equine antitoxins include a licensed bivalent and monovalent antitoxin that contains neutralizing antibodies against BoNT types A/B and E, respectively, and an investigational heptavalent (ABCDEFG) antitoxin. The heptavalent botulinum antitoxin (HBAT, Cangene Corporation) is available through a CDC-sponsored IND protocol. Following expiration of the bivalent and monovalent products in March 2010, HBAT became the only botulinum antitoxin available in the USA for naturally occurring noninfant botulism. Cases of infant botulism are treated with the recently licensed BabyBIG which is derived from the blood of human donors vaccinated with a pentavalent (ABCDE) toxoid vaccine. In NHPs exposed to lethal challenge, treatment is only effective when administered within 18 to 36 hours of exposure.

2.2. Plague. Plague is a zoonotic infection with *Y. pestis* that is normally transmitted from rodents to humans when humans are bitten by infected fleas. There are three manifestations of the disease, with flea bites usually causing the bubonic form in which a painful swelling (bubo) of the draining lymph nodes occurs [4, 5]. Left untreated, the infection will cause sepsis and death in approximately half of the cases. Bubo formation is not present in the second form of disease, which leads directly to sepsis, and occurs in about a third of the cases. Bubonic and septicemic infections occasionally progress to secondary pneumonic infections. Infection with *Y. pestis* also rarely occurs through inhalation of the organism, and, after 1 to 6 days, the disease manifests in its pneumonic form, which is nearly always fatal unless the patient is treated with antibiotics within 20 hours of symptom onset [6], and spreads from person to person by respiratory droplets formed during coughing [7–11]. Clinical signs include fever with cough and dyspnea, and there may be production of bloody, watery sputum. Nausea, vomiting, abdominal pain, and diarrhea may also be present.

Other than early diagnosis and treatment of plague with antibiotics, which are essential to survival, there is no licensed plague vaccine available in the United States. The production of a killed, whole-cell plague vaccine (formalin-inactivated *Y. pestis*), previously licensed as *Plague Vaccine United States Pharmacopoeia*, was discontinued in 1999. Moreover, that vaccine was not effective in preventing against primary pneumonic plague.

3. DoD Vaccine Performance Requirements

There is a need for vaccines that can protect against respiratory exposure to botulinum neurotoxins and plague. Development of these vaccines by DVC is guided by the DoD

performance requirements and demonstration of efficacy in accordance with the Animal Rule (21 CFR 601.91 Subpart H, “Approval of Biological Products When Human Efficacy Studies Are Not Ethical or Feasible”).

The performance of DoD products is based on user requirements provided in a Capability Development Document (Table 1). For the two vaccines described in this paper, the key performance parameter is FDA licensure. All other requirements are characterized as either threshold (i.e., the absolute minimum acceptable level of performance) or objective (the characteristics that are considered to be ideal). Table 1 lists some of these characteristics for the rBV A/B and rF1V vaccines. These requirements form the basis for assessing the product during its development.

Given that FDA licensure is the ultimate key performance parameter for DoD’s medical countermeasures, the overall life cycle development plan is not dissimilar to products under development by the private sector. However, DoD products must also be developed in accordance with acquisition regulations (Federal Acquisition Regulation, Defense Federal Acquisition Regulation Supplement, and others). In addition, these programs are subject to defense funding which is not always as flexible as might be the case with commercial development.

4. Regulatory Strategy: The FDA Animal Rule

Licensure of the rBV A/B and rF1V vaccines will use the FDA Animal Rule. The Animal Rule was established by the FDA to allow development and licensure of products that could not otherwise be licensed using traditional efficacy testing. The Animal Rule may be used for licensure when efficacy studies are unethical due to high pathogenicity, and disease is rare enough in nature that field studies would be either impossible or at least impractical (<http://www.fda.gov/OHRMS/DOCKETS/98fr/053102a.htm>; accessed April 7, 2011).

The four major requirements of the FDA’s Animal Rule are as follows:

- (i) Requirement no.1: There is a reasonably well-understood pathophysiological mechanism of toxicity of the substance and its prevention or substantial reduction of this toxicity by the product.
- (ii) Requirement no.2: The effect is demonstrated in more than one animal species expected to react with a response predictive for humans, unless the effect is demonstrated in a single animal species that represents a sufficiently well-characterized animal model for predicting the response in humans.
- (iii) Requirement no.3: The animal study endpoint is clearly related to the desired benefit in humans, generally the enhancement of survival or prevention of major morbidity.
- (iv) Requirement no.4: The data or information on the kinetics and pharmacodynamics of the product or other relevant data or information, in animals or

TABLE 1: Required performance parameters for the rBV A/B and rF1V vaccines.

Performance attribute	Key Performance Parameter: FDA Licensure			
	Development threshold		Development objective	
	rBV A/B	rF1V	rBV A/B	rF1V
Efficacy	Protect 80% of immunized persons		Protect 90% of immunized persons	
Immune response	An immune response sufficient to meet threshold efficacy requirements for this vaccine within 210 days of the initial vaccine dose		An immune response sufficient to meet threshold efficacy requirements for this vaccine within 30 days of the initial vaccine dose	
Duration of protection	Protection for at least one year from completion of the primary vaccination series		Protection for five years after the administration of a single-dose vaccine	
Number of doses to achieve protection (primary series)	3		1	
Shelf life	1 year		5 years	

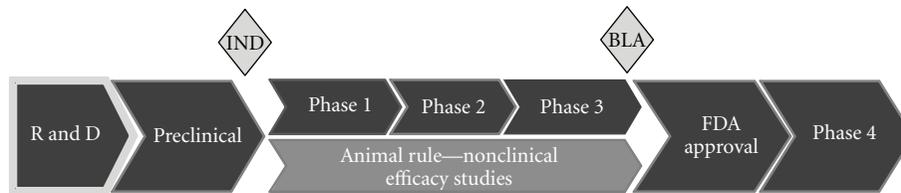


FIGURE 1: Stages of the advanced development of vaccines. The stages shown in dark grey are common to traditional and FDA’s Animal Rule licensure; the additional nonclinical studies are shown in light grey.

humans, allows selection of an effective dose in humans.

As of July 2011, no vaccine has been approved by the FDA under the FDA Animal Rule, so the length of time and costs associated with obtaining approval have yet to be determined. Traditional licensure may take 15 years from discovery to approval; approval of vaccines by the FDA Animal Rule may take longer due to the additional nonclinical requirements (Figure 1) and the need to develop and characterize the animal models and challenge systems.

5. Development of the rBV A/B and rF1V Vaccines

The rBV A/B and rF1V vaccine candidates were initially developed at the US Army Medical Research Institute for Infectious Diseases (USAMRIID). They were transferred to JVAP (and subsequently to DVC) in an early stage of development. The target indication of these vaccines is protection of adults 18 to 55 years of age from disease caused by inhalational exposure to BoNT/A1, BoNT/B1, or *Y. pestis*.

The rBV A/B vaccine candidate [12] comprises the recombinant 50kDa carboxy-terminal of the heavy chains of Antigen A and Antigen B expressed individually from *Pichia pastoris* using a methanol induction system. Antigen A is derived from the BoNT/A1 expressed by *C. botulinum* strain NCTC 2916 (group I, proteolytic), and Antigen B is derived from BoNT/B1 expressed by *C. botulinum* strain Danish (group I, proteolytic). Antigen A was modified to

prevent proteolytic cleavage at the N-terminus during gene expression by removing the codons coding for proteolytically susceptible amino acids. Each 0.5 mL dose of rBV A/B-40 consists of a 1 : 1 mixture of 20 µg Antigen A and 20 µg Antigen B adsorbed to Alhydrogel.

The rF1V vaccine candidate for plague [13] comprises the F1 capsular protein and the V virulence protein of *Y. pestis* Colorado 92 (CO92) fused into a single protein, which is produced in *Escherichia coli* (*E. coli*) and formulated with Alhydrogel.

For any product, successful progress towards licensure involves the integration of different functional groups to develop a scalable manufacturing process from the research and development (R&D) proof-of-concept, coordinate the logistics between the release of manufactured material suitable for testing, and stage the appropriate nonclinical, and clinical studies. The progress of the rBV A/B and rF1V vaccines is described below for manufacturing, nonclinical and clinical efforts.

5.1. Manufacturing Processes. The rBV A/B and rF1V vaccines were initially developed at USAMRIID and were transferred to advanced development at the stage indicated by the gray arrows in Figure 2. The black arrows indicate the stage of manufacturing development for each product. The products successfully overcame the following challenges to advance to this stage of manufacturing: (1) technology transfer to a contract manufacturing organization and process redesign due to equipment changes, (2) development of manufacturing methods that support the production of

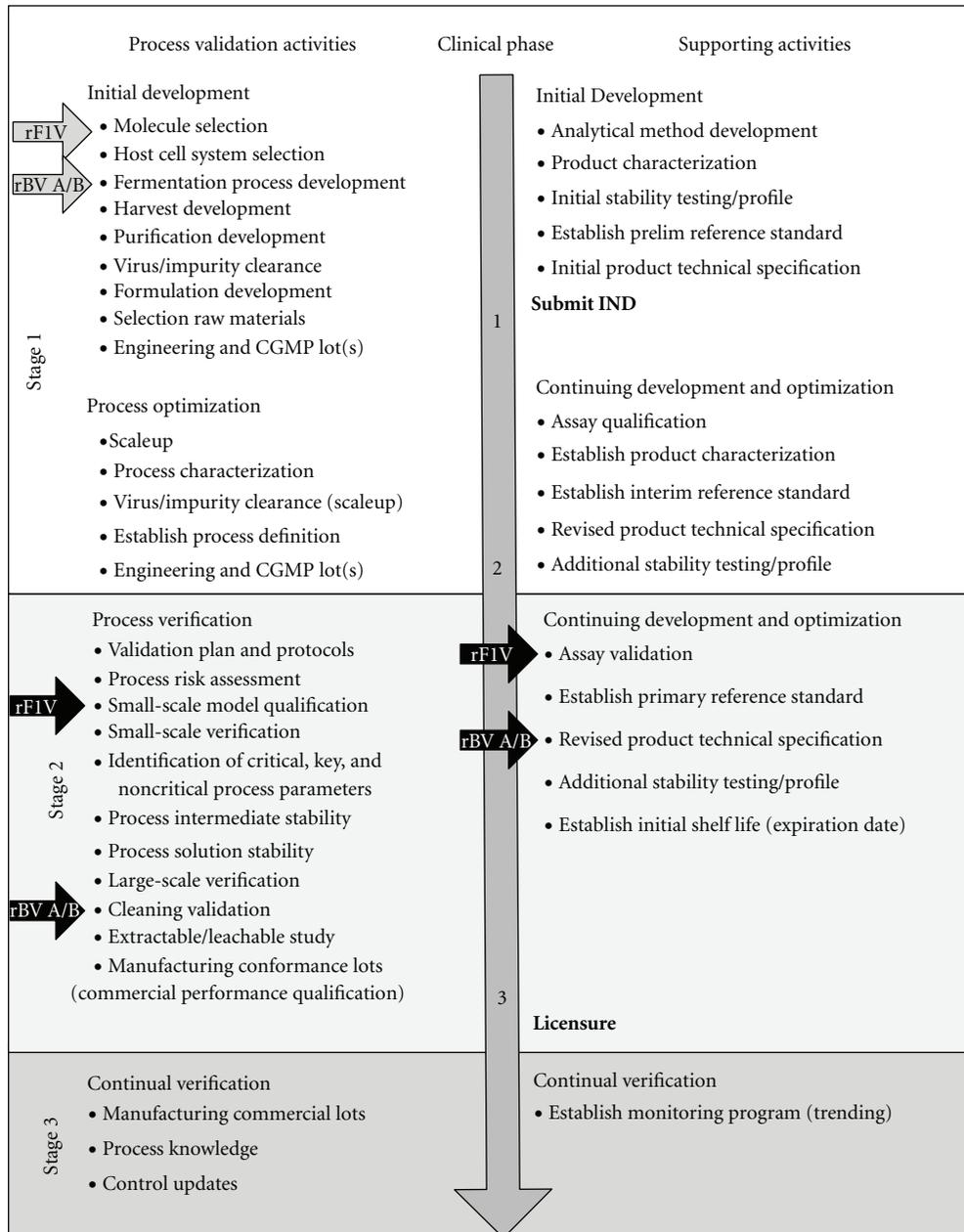


FIGURE 2: Stages of the manufacturing process, product transition to DVC (grey arrows), and current status (black arrows) of the development efforts for rF1V and rBV A/B vaccines. Some activities may be conducted concurrently.

clinical trial material and can be validated, (3) scale-up issues associated with either an increase in the number of required troop equivalent doses or non-scalable technological steps, (4) developing needed reagents and analytical methods for product quantitation, purity, and process impurities, and (5) determining conditions that support product stability throughout the manufacturing process.

The *Pichia pastoris* master and working cell banks for the Antigen A and Antigen B expression strains used to produce the rBV A/B vaccine were generated by expanding accession cell banks produced for each antigen and characterized for purity, identity, and suitability according to the FDA and International Conference on Harmonisation (ICH)

guidance. Antigen A and Antigen B are manufactured and adsorbed to Alhydrogel separately and then combined to form the final formulated bulk vaccine. The manufacturing process is at commercial scale (100 L for Antigen A and 600 L for Antigen B). Current Good Manufacturing Practices (CGMP) fill/finish activities were conducted at a 5,000 vial level to support the Phase 1B and Phase 2 clinical trials. Currently the formulated FDP manufacturing process is being scaled to the full commercial scale (approximately 300,000 vials/lot).

The fused rF1V protein comprising the F1 capsular protein and V virulence protein of *Y. pestis* is produced in *E. coli* and formulated with the adjuvant Alhydrogel.

The sequence encoding the rF1V antigen was derived from plasmid pPW731 produced at USAMRIID [13] and was initially expressed from pT5.F1V.1 cell banks but later transitioned to expression from pPW731 cell banks due to intellectual property constraints. Both expression systems use the same regulatory elements for gene expression and the same antibiotic resistance gene for plasmid maintenance. The cell banks were characterized for purity, identity, and suitability according to the FDA and ICH guidance. The manufacturing process was scaled to a final commercial process, which is 1,500 L (working volume for fermentation) and 500 L purification scale.

Clinical lots for each vaccine were manufactured and released for use in the completed Phase 1 and the ongoing Phase 2 trials, described below. A stability program is ongoing and was designed to establish, maintain, and execute a testing strategy that is compliant with the FDA and ICH guidance.

5.2. Nonclinical Studies to Support the Animal Rule Requirements. Animal models are critically important for FDA's Animal Rule licensure, in that they are used to assess vaccine efficacy, and the vaccine-induced animal immune responses are compared to human immune responses to predict clinical benefit. The "Draft Guidance for Industry-Animal Models-Essential Elements to Address Efficacy Under the Animal Rule" released in 2009 was followed to guide the design and execution of nonclinical studies. The nonclinical plans are integrated with the clinical development plans for each vaccine to support the comparison of immune responses across species and to enable the selection of the appropriate human dosage.

The animal studies described below were conducted at accredited facilities under the oversight of an assigned Study Director and attending veterinarian and performed according to the Institute- and the DoD-approved animal protocols. Every effort was made to minimize the suffering and distress of animals exposed to challenge agents or subjected to procedures, using approved anesthetics (1 to 6 mg/kg Telazol for macaques and isoflurane for mice). Biostatisticians were consulted in the study design phase to ensure the study used the proper number of animals needed to achieve interpretable data. Animals were observed multiple times per day for signs of clinical illness during the in-life phase. Macaques were anesthetized and humanely euthanized with an overdose of a euthanasia agent containing pentobarbital when meeting preapproved euthanasia criteria such as decreased body temperature to $<93.0^{\circ}\text{F}$, $>20\%$ loss of body weight from prechallenge weight, respiratory distress/failure, significant reduction in activity (e.g., unable to right itself, complete lack of activity, persistent prostration, or total paralysis), or signs of pneumonia. Mice were euthanized using CO_2 gas, and guinea pigs were euthanized by a barbiturate overdose injected intraperitoneally or directly into the heart after the animals were anesthetized.

Proof-of-concept studies conducted by USAMRIID using research material and pilot lots demonstrated the immunogenicity and efficacy of rBV A/B and rF1V vaccines in a variety of animal models including rodents and NHPs.

The nonclinical development plans for rBV A/B and rF1V continue the testing in stages: (1) performing initial toxicity studies to support the clinical development, (2) developing and characterizing aerosol challenge models, (3) identifying vaccination regimens that induce immune responses similar to the responses observed in clinical volunteers, (4) demonstrating efficacy in animal models, and (5) conducting final pivotal vaccination/aerosol challenge and reproductive toxicity studies.

Good Laboratory Practice- (GLP-) compliant nonclinical safety studies were conducted to support clinical testing of the rBV A/B and rF1V vaccines. These evaluated general toxicity following repeat-dose administration in mice and local reactogenicity of administration of a full human dose of vaccine in rabbits. An additional study to evaluate neurobehavioral toxicity was performed for the rBV A/B vaccine. The vaccines produced no apparent systemic toxicity and only mild inflammation at the injection site. The rBV A/B vaccine produced no apparent neurobehavioral toxicity. Together these nonclinical studies supported the initiation of Phase 1 clinical trials.

An important aspect of developing animal models is the requirement for well-characterized challenge agent. The challenge agents used in the nonclinical efficacy studies are classified as Category A Select Agents by the CDC, and all US facilities that possess or transfer the challenge agent must be registered with the CDC and/or the US Department of Agriculture. The BoNT/A1 and BoNT/B1 were fully characterized to confirm their identity, purity, and strength (biological activity or potency). Protein concentration and biological activity (in terms of MIPLD₅₀ units) of both BoNTs were verified using a micro-Bradford protein assay and mouse (toxin) potency assay, respectively. Testing protocols were established to monitor the real time and accelerated stability of the vialled BoNTs. The stability program includes annual testing to confirm the maintenance of strength and purity using the micro-Bradford protein assay, mouse (toxin) potency assay, SDS-PAGE, and size exclusion chromatography.

The rF1V challenge studies use the CO92 or C12 strains of *Y. pestis*. To ensure the quality and integrity of these strains, challenge material is grown, characterized, and stored in a three-tiered banking system. The banks are characterized by (1) purity on selective media, (2) titer, (3) phenotype, (4) Gram stain, (5) polymerase chain reaction (PCR) for presence of plasmids and chromosomal marker (pathogenicity island), (6) nitrate reduction, (7) antibiotic susceptibility, (8) growth curve, and (9) glycerol fermentation.

The first requirement of the Animal Rule relates to understanding the pathophysiologic mechanism of toxicity and demonstrating that the pathology is similar to that in humans. This is a significant challenge, especially when there is little information available for human disease (e.g., inhalational botulism). Usually studies in two animal species are required for this purpose, unless the disease is well characterized in one animal species and is an accepted model for the human disease. For the rBV A/B and rF1V vaccine development programs, rodent and NHP models

TABLE 2: Symptoms following aerosol exposure to BoNT/A1 or BoNT/B1.

Symptoms	Human ¹	NHP (rhesus macaque)	Guinea pig (Hartley)	Mouse (CD-1)	Inhalational BoNT/A1			Inhalational BoNT/B1		
Onset of symptoms: dose dependent	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Lethargy	Yes ¹	Yes	Yes	NR	Yes	Yes	Yes	Yes	NR	NR
Flaccid paralysis	Yes	NR	Yes	Yes	NR	NR	NR	NR	Yes	Yes
Ptosis (drooping eyelids)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Dysphagia (difficulty swallowing)	Yes ¹	Yes	NR	NR	Yes	NR	NR	NR	NR	NR
Symmetric, descending paralysis	Yes	Yes	NR	Yes	Yes	NR	NR	NR	NR	NR
Labored respirations	NR ²	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ataxia	Yes ¹	Yes	NR	NR	Yes	NR	NR	NR	NR	NR
Muscle weakness	Yes ¹	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Lateral recumbency	NR	Yes	NR	NR	Yes	NR	NR	NR	NR	NR
Nasal discharge	No	Yes	No?	NR	Yes	Yes	Yes	Yes	No	No
Constipation	Yes	Yes	NR	NR	NR	NR	NR	NR	NR	NR
Paresis	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NR	NR
Coughing	No	Yes	NR	NR	Yes	No	NR	NR	NR	NR
Piloerection	NR	NR	Yes	Yes	NR	Yes	Yes	Yes	Yes	Yes
Lethality	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Time to death: dose dependent	NR	Yes	Yes	Yes	NR	NR	NR	NR	NR	NR

¹ Specific observations in humans following inhalational exposure.

² NR, not reported.

were developed to evaluate disease pathophysiology following inhalational exposure of BoNTs and *Y. pestis*. The comparisons of the animal hallmarks of disease to what is known for humans are presented in Tables 2 and 3.

The lethality of BoNT/A1 and BoNT/B1 was determined in CD-1 mice and guinea pigs (unpublished) and recently in rhesus macaques [14]. A stage-wise approach was used to estimate the inhaled median lethal dose (LD₅₀) and exposure concentration (LC₅₀). The pathophysiologic responses to aerosol exposure were evaluated for each species to identify relevant endpoints for efficacy studies. The most relevant pathophysiological responses in mice and rhesus macaques were mortality and development of clinical signs of botulism. Clinical observations in all species were consistent with the recognized pattern of botulism disease progression in humans (Table 2). A significant dose response was observed with regard to lethality and the onset and duration of clinical signs in each species. No significant changes in clinical hematology and chemistry and gross and microscopic pathology were observed in mice or rhesus macaques. Changes in physiologic parameters measured by telemetry in rhesus macaques also did not correlate with mortality.

The pathology induced by *Y. pestis* CO92 was evaluated in animals through clinical chemistries, hematology, telemetry

cynomolgus macaques (CMs) only, and detailed histopathology in Swiss Webster mice and CMs. Exposed animals demonstrated multilobar pneumonia, bacterial infiltration of macrophages and lymphoid tissues, fever, sepsis, and death. Data collected from the mouse model development studies estimated the LD₅₀ of CO92 to be approximately 2,000 colony-forming units (cfu).

In the CMs, the inhaled dosage was calculated using the total accumulative tidal volume as measured by plethysmography [15]. The LD₅₀ was estimated to be 24 cfu by Probit analysis. Telemetry provided useful information on the clinical course of disease not captured by clinical observations. A rise in temperature routinely coincided with the loss of diurnal rhythm, while increased heart and respiration rate followed by inactivity strongly correlated with a lethal outcome. All CMs with *Y. pestis* positive blood cultures died from pneumonic plague. The pathology in the lungs of all CMs was consistent with the pathology observed in pneumonic plague described in humans. The significant findings are compared across the species in Table 3.

5.3. Identification of Vaccination Regimens and Demonstration of Efficacy in Animal Models. The second requirement of the Animal Rule relates to demonstrating that the responses to

TABLE 3: Clinical signs, gross pathology and histopathology associated with plague infection¹.

Symptom or lesion	Human	Historical CM	DVC SW mouse	DVC CM
Lymphadenopathy	Yes	Yes	Yes	Yes
Fever	Yes	Yes	ND	Yes
Malaise	Yes	Yes	Yes	Yes
Lethargy	Yes	Yes	Yes	Yes
Elevated pulse	Yes	Yes	ND	Yes
Cyanosis	Yes (late)	Yes	ND	ND
Pharyngitis	Yes	Yes	ND	ND
Cough	Yes	ND	ND	Yes
Rales	Yes	Yes	ND	ND
Sepsis	Yes	Yes	Yes	Yes
Gross pathology primary pneumonic plague				
Fibrinous pleuritis	Yes	ND	ND	Yes
Pneumonia	Yes	Yes	Yes	Yes
Mediastinal hemorrhage	Yes	Yes	ND	Yes
Congestion of trachea/bronchi	Yes	Yes	Yes	Yes
Histopathology primary pneumonic plague				
Pulmonary congestion	Yes	Yes	Yes	Yes
Necrohemorrhagic foci	Yes	Yes	Yes	Yes
Fibrinous pleuritis	Yes	Yes	ND	Yes
Disseminated intravascular coagulation	Yes	Yes	ND	ND
Neutrophil infiltration of lung	Yes	Yes	Yes	Yes
Bacteria in lung	Yes	Yes	Yes	Yes
Mediastinitis	Yes	Yes	Yes	Yes
Bacteria in spleen	Yes	Yes	Yes	Yes ²

¹ Modified from information contained in Adamovicz and Worsham [16].

CM: cynomolgus macaque, ND: not determined, SW: Swiss Webster.

² Bacterial burden not quantitated.

the countermeasures are similar in animals and humans. The third requirement is to demonstrate the same endpoint in animals that is expected for humans given the vaccine. For the rBV A/B and rF1V vaccines, that endpoint is survival.

The rBV A/B program vaccinated NHPs with the same material administered to healthy adults in clinical trials and followed the vaccination schedule used for humans. An abbreviated schedule was used for the mice. Neutralizing antibody responses to BoNT/A1 and BoNT/B1 in CD-1 mice and rhesus macaques were evaluated across various vaccine dosages and compared to the neutralizing antibody responses observed in the clinical trial volunteers. Dosages inducing similar antibody levels were identified for both animal models and will be used in pivotal animal studies using the Phase 3 clinical material.

Initial efficacy studies in rhesus macaques demonstrated protection from aerosol challenge using the identified vaccination regimen. The protective efficacy of the antibody levels induced in humans was assessed using passive transfer studies. A guinea pig passive transfer model was developed

and used to demonstrate the protective efficacy of purified immunoglobulin from human rBV A/B vaccinees [17].

A similar approach was used for the rF1V program. Vaccine dosage titration studies in CMs and mice are in progress using the material used in the Phase 2b clinical trial to assess the immune responses and efficacy. The first objective is to evaluate survival across five vaccine dosages to select vaccine dosages for use in follow-on studies. The secondary objective is to collect serum from animals in all groups for evaluation in passive transfer studies and to determine the antibody titers in Bridge ELISA (described in more detail below). The follow-on study is designed to confirm the minimum protective dosage of vaccine and estimate the minimum level of antibody in rF1V-vaccinated animals that correlates with surviving aerosol exposure to *Y. pestis* CO92.

A mouse passive transfer model was developed to assess the ability of immune sera to provide protection from an aerosol challenge. Sera from CMs or human volunteers vaccinated with rF1V were tested in the model and the results described [18]. A definitive correlation between survival in CMs and an antibody level remains to be determined.

TABLE 4: Vaccine program status for meeting the requirements of the FDA Animal Rule.

Animal Rule	rBV A/B	rF1V
Requirement 1: well understood pathophysiology and amelioration	<p>The published literature has shown that generation of neutralizing antibodies against BoNT provides protection against inhalational botulism.</p> <p>Pathophysiology following aerosol exposure of CD-1 mice and rhesus macaques is comparable to the pathophysiology of disease in humans.</p> <p>Vaccination with rBV A/B elicits a humoral immune response in mice and macaques that provides protection against exposure to aerosolized neurotoxins.</p>	<p>The published literature has shown that the F1 and V antigens from <i>Y. pestis</i> can provide protection from pneumonic plague.</p> <p>Pathophysiology following aerosol exposure of Swiss Webster mice and CMs is comparable to the pathophysiology of disease in humans.</p> <p>Vaccination with rF1V elicits a humoral immune response in mice and macaques that provides protection against exposure to aerosolized <i>Y. pestis</i>.</p>
Requirement 2: effect is demonstrated in more than one species	<p>The mouse and macaque models have immune responses to vaccination with rBV A/B that are similar to the response in humans. Data obtained to date indicate that vaccination induces neutralizing antibody titers believed to be protective in tested species.</p>	<p>The Swiss Webster mouse and cynomolgus macaque models have immune responses to vaccination with rF1V that are similar to the response in humans. Data obtained to date indicate that antibody titers to F1 and V are induced in tested species.</p>
Requirement 3: the animal study endpoint is related to the desired benefit in humans	<p>Nonclinical efficacy study endpoints measure survival against an aerosol challenge, which is the desired benefit in humans.</p>	<p>Nonclinical efficacy study endpoints measure survival against an aerosol challenge, which is the desired benefit in humans.</p>
Requirement 4: data allows selection of an effective dose in humans	<p>The mouse toxin-neutralizing antibody assay (MNA) provides a species-neutral assay for quantitating the level of neutralizing antibodies.</p> <p>The neutralizing antibody concentration (NAC) determined by the MNA is under evaluation as a correlate of protection.</p> <p>Passive transfer assesses the protective capacity of antibodies present <i>in vivo</i> at the time of aerosol challenge. This is under development as a model to assess the protective capacity of transferred immunoglobulin from human vaccines.</p>	<p>The Bridge ELISAs are in development as species-neutral assays that permit direct comparison across samples from different species.</p> <p>Bridge ELISA, macrophage cytotoxicity assays, and passive transfer studies are under evaluation for correlation with protection.</p> <p>Passive transfer assesses the protective capacity of antibodies present <i>in vivo</i> at the time of aerosol challenge. This is under development for consideration as a model to assess the protective capacity of transferred serum from human vaccines.</p>

The approach taken to meet the Animal Rule requirements is summarized in Table 4 for the rBV A/B and rF1V vaccines and described in more detail below. Briefly, the animal models with similar disease characteristics to those observed in exposed humans are being used to assess the immune responses and efficacy induced by vaccination. The status of progress to date for the specific requirements is indicated in Table 4. The designs and statistical analysis plans for the pivotal GLP nonclinical efficacy studies will need to be prepared and discussed with the FDA. The objective of these studies will be to generate data that supports the Animal Rule requirements to demonstrate efficacy and to extrapolate a dosage likely to produce clinical benefit in humans. These studies will use the Phase 3 clinical trial material, and reproductive toxicity will be assessed concurrently.

5.4. Clinical Testing and Human Safety. The rBV A/B and rF1V vaccine candidates were (or are being) tested in the

Phase 1 and the Phase 2 clinical trials. All clinical trials were evaluated by a Scientific Merit Review Board consisting of independent experts and approved by an independent Investigational Review Board and the US Army's Human Research Protections Office. The studies were managed by a contracted clinical research organization. Safety was monitored continuously by independent physicians and overseen by a Data Safety Monitoring Board (DSMB). All studies were conducted in accordance with the current Good Clinical Practice as required by applicable US federal regulations (21 CFR Parts 50, 56, and 312) and the ICH guidelines.

Male and female volunteers were recruited and assessed for eligibility after signing an informed consent form. Subjects had to be healthy, as determined by standard screening assessments including medical history, physical examination, and laboratory tests (hematology, chemistries, and urinalysis). For rBV A/B trials, subjects with a history of neurological disorders, immunological disorders or prior

therapy with botulinum toxin were excluded. In trials for both vaccines, subjects with a history of use of immunosuppressive drugs, including glucocorticoids, and recent vaccinations were excluded from the study. Study vaccine was administered as a 0.5 mL IM injection in the deltoid muscle.

Safety monitoring consisted of collection of injection site and systemic reactogenicity data in a volunteer diary via an interactive voice response system after vaccination, and assessment of treatment emergent adverse events (TEAEs) at scheduled and *ad hoc* visits, if needed, throughout the study. Injection site reactions (local reactions) were defined as pain, tenderness, pruritus, redness/erythema, other rash, and swelling or induration. Prespecified systemic reactions included fever, fatigue, myalgia, headache, nausea, vomiting, and diarrhea. Any other system organ manifestation was also to be recorded and evaluated as an adverse event (AE). Grading of AEs was performed by study-specific adaptation of the most up-to-date FDA guidelines for toxicity grading in preventive vaccine clinical trials. Any abnormal laboratory value, abnormal vital sign, or abnormal physical finding that was considered clinically significant by the investigator or met the grading criteria for toxicity of Grade 1 or higher was reported as an AE.

5.5. Clinical Trials for rBV A/B. The rBV A/B vaccine was evaluated in adults (18–45 years) in two Phase 1 (rBV A/B-01 and rBV A/B-01B) and one Phase 2 (rBV A/B-02) clinical trials (unpublished). Blood was collected to determine the NAC to BoNT/A1 and BoNT/B1 using the MNA at predetermined intervals during the study and for calculation of the seroconversion rate. Blood was also collected for passive transfer studies for evaluation of efficacy in animals.

The first trial, rBV A/B-01, was a single-center, open-label, dosage-escalation study designed to evaluate the safety, tolerability, and immunogenicity of a two-dose regimen (Days 0 and 28) of rBV A/B given at three ascending dosages (10 μ g, 20 μ g, and 40 μ g total immunizing protein with adjuvant) and an unadjuvanted antigen-only formulation at the 40 μ g total immunizing protein. Forty-four volunteers participated in this study, with 11 in each of the 4 treatment cohorts. The second study, rBV A/B-01B, was a follow-on study to evaluate formulated vaccine administered at two dosages (40 μ g and 100 μ g total immunizing protein) using four different three-dose schedules (Days 0, 28, 56, Days 0, 28, 112 or Days 0, 28, 182, or Days 0, 56, 182). The addition of a third vaccine dose in the Phase 1B study was expected to increase the level and duration of the immune response. Eighty volunteers participated in this study (10 volunteers per vaccination cohort, 40 volunteers per dosage group). Dose escalation to the higher dosage occurred after a review by the DSMB of all safety data in both studies.

The majority of volunteers experienced at least one TEAE, and most AEs were mild to moderate in intensity and self-limited in both Phase 1 clinical trials. About 30% of the TEAEs were considered related to vaccination, and these generally consisted of injection site reactions, with pain being the most prevalent. Pruritus, erythema, and swelling were reported much less frequently. The most common related systemic reactions included headache, diarrhea, and

malaise. Sporadic abnormalities in laboratory test results, most commonly hemoglobin changes, were reported after vaccination in most volunteers but were not considered clinically significant, and there were no notable changes from baseline through the end of each study (6 months after last vaccination) within or across cohorts. There were no serious adverse events (SAEs). The overall incidence of TEAEs and the incidence of administration site reactions were higher in the cohorts that received rBV A/B vaccine compared to the cohorts that received antigens only in the rBV A/B-01 study. No apparent dosage relationship was seen across cohorts that received adjuvanted rBV A/B in either study.

In the rBV A/B-01 trial, at least 80% of volunteers vaccinated with the two highest dosages (20 μ g and 40 μ g total immunizing protein) of rBV A/B developed sustained NAC above the lower limit of quantitation for anti-BoNT/A1 and anti-BoNT/B1 antibodies. The antigen-only formulation was not immunogenic. In the rBV A/B-01B trial, administration of three doses of either 40 μ g or 100 μ g of rBV A/B vaccine elicited detectable levels of neutralizing antibody for both BoNT/A1 and BoNT/B1 in all volunteers. Longer vaccination schedules (third vaccination given at Day 182) elicited a greater NAC than shorter schedules (third vaccination given at Day 56 or Day 112). Based on maximum NAC and antibody kinetics, the vaccination schedule of Days 0, 28, and 182 elicited the highest NAC levels, and there were no significant differences among dosages.

The rBV A/B-02 Phase 2 trial was a multicenter, blinded, randomized study designed to evaluate the safety, tolerability, and immunogenicity of a three-dose regimen (Days 0, 28, 182 and Days 0, 56, 182) of rBV A/B given at a single dosage of 40 μ g total immunizing protein compared to saline placebo. There were 440 volunteers in this study allocated to 2 cohorts of 165 subjects each that received rBV A/B and 2 cohorts of 55 subjects each that received saline. Subjects were followed up to 12 months after the last vaccination. Interim data to 4 weeks after the last vaccination were analyzed; final data analysis is not yet complete.

Nearly all volunteers experienced at least one AE, with approximately the same number among vaccine-treated and placebo-treated volunteers. The majority of AEs were mild or moderate in intensity, and there was no difference in the overall incidence of TEAEs among treatment cohorts. Three subjects were discontinued because of AEs that could be related to vaccination (allergic dermatitis, erythema, and swelling, all at the injection site). There were no SAEs related to study vaccine. More volunteers treated with rBV A/B reported injection site reactions compared to those treated with placebo, the most common being pain, tenderness, swelling, erythema, pruritus, and axillary pain. The most common systemic reactions were headache, myalgia, arthralgia, feeling abnormal, fever, anxiety, malaise, and nausea. Most TEAEs, however, were laboratory values outside the normal range reported as AEs per the protocol. These occurred in about 96% of subjects treated either with rBV A/B or placebo. The most frequently reported laboratory AEs were hemoglobin decrease from baseline or increase from baseline, with no significant difference between treatment cohorts. Most laboratory-related TEAEs were considered

mild or moderate in severity and not clinically significant and resolved without treatment.

The highest neutralizing antibody rates for both anti-BoNT/A1 and anti-BoNT/B1 were observed at Day 210, 28 days after the last vaccine dose and were similar for both vaccination schedules. Final data will evaluate the immune response to one year after last vaccination.

5.6. Clinical Trials for rF1V. The rF1V vaccine was evaluated in one Phase 1 (rF1V-01) and one Phase 2 (rF1V-02a) clinical trial. A second Phase 2 (rF1V-02b) clinical trial is ongoing. Male and female volunteers, age 18 to 40 years in the rF1V-01 trial and 18 to 55 years in the rF1V-02a trial, were recruited and assessed for eligibility after signing an informed consent form. These studies were conducted, monitored, and reviewed as described for rBV A/B. The immune response to the vaccine was evaluated by measurement of the concentration of antibodies to rF1, rV, and rF1V by the Bridge ELISA at predetermined intervals during the study and calculation of the seroconversion rate. Blood was also collected for passive transfer studies for evaluation of efficacy in animals.

The first trial, rF1V-01, was a single-center, open-label, dosage-escalation study designed to evaluate the safety, tolerability, and immunogenicity of a two-dose regimen (Days 0 and 28) of rF1V given at four ascending dosages (20 μg , 40 μg , 80 μg , and 160 μg total immunizing protein). Forty-four subjects participated in the study, with 11 per cohort. Based on analysis of the immunogenicity data, an extension study evaluated the effect of a third dose of 160 μg administered about 230 days following the first dose in 8 of 11 subjects who had previously received the same vaccine dosage. All volunteers were followed for 180 days after the last vaccination.

All volunteers experienced at least one TEAE, and the majority were either mild or moderate in intensity. Injection site reactions were the most frequent related TEAEs and were generally mild or moderate and more frequent at the two highest dosages after two or three vaccinations. The most common injection reactions were pain, swelling, and erythema. Systemic reactions considered related to vaccination were headache, fatigue, nausea, and diarrhea. Most of these reactions were also mild or moderate and were not considered clinically significant. There were no clinically significant or related laboratory changes. After allowance for the different length of time between the vaccinations, there was no apparent increase in the frequency of TEAEs after the second or third vaccination compared to the first vaccination. Serial electrocardiograms were recorded after the first two doses in all cohorts, and no clinically significant abnormalities were observed.

The rF1V vaccine was immunogenic after two doses of 20 μg , 40 μg , 80 μg , or 160 μg of vaccine. The antibody response was markedly increased in volunteers who received three doses of 160 μg of vaccine, compared to their response after two 160 μg doses. Peak GMCs of all three antibodies tested (anti-rF1, anti-rV and anti-rF1V) occurred 14 days after the third dose. The administration of a third dose also increased the rate of detectable antibody to all three antigens. Based on this study, the two highest dosages (80 μg and

160 μg) were selected for evaluation in the Phase 2a study in a three-dose regimen.

The rF1V-02a Phase 2 trial was a multicenter, blinded study designed to evaluate the safety, tolerability, and immunogenicity of a three-dose regimen (Days 0, 28, and 182 and Days 0, 56, and 182) of rF1V given at two dosages (80 μg and 160 μg total immunizing protein). There were 400 subjects (100 per cohort) in the study. Vaccinated subjects were followed for 12 months after the last vaccination.

All subjects experienced at least one TEAE, and no statistically significant difference in overall incidence across groups was observed. A total of six volunteers discontinued due to a TEAE, two because of injection site reactions. The majority of TEAEs were mild or moderate in intensity. There were no SAEs related to study vaccine. Most volunteers had TEAEs that occurred within 28 days following a vaccination, and these were primarily injection site reactions. Most of these reactions were mild or moderate in intensity, and the most common were pain, swelling, erythema, and pruritus. The most common related systemic reactions were headache, malaise, nausea, and diarrhea. Most of these reactions also were mild or moderate. The most common laboratory abnormalities reported as AEs were increased blood glucose and protein present in the urine and decreased hemoglobin. These TEAEs were sporadic, not associated with other clinical abnormalities, and resolved without treatment. In general, no clinically meaningful trends were noted in changes to laboratory parameters in any vaccination group, dosage, or schedule. Overall, the 80 μg dosage had a slightly better safety and tolerability profile than the 160 μg dosage, and the Days 0, 56, and 182 schedule had a slightly better safety and tolerability profile than the Days 0, 28, and 182 schedule. In addition, there did not appear to be an increase in the rate of either local or general TEAEs within 28 days after vaccination with subsequent vaccinations.

The immunogenicity data indicate that GMCs for anti-rF1, anti-rV, and anti-rF1V antibodies were much higher after the third vaccination than after the second vaccination, and almost all subjects had evidence of seroconversion 7 to 14 days after the last vaccination. The seroconversion rate was indistinguishable among the selected dosage and schedules following vaccination 3. Both groups that received vaccination on the Days 0, 56, and 182 schedule showed higher anti-rF1, anti-rV, and anti-rF1V GMCs and seroconversion rates from 7 days after the second vaccination to the prevaccination 3 assessment than groups that received vaccination on the Days 0, 28, and 182 schedule. Based on the results of this study, the 80 μg dosage and the Days 0, 56, 182 vaccination schedule were selected for further testing in the Phase 2b clinical trial. In addition, a shorter vaccination schedule is being evaluated in the Phase 2b clinical trial to assess whether or not equivalent immunogenicity is achieved earlier by administering the third vaccination sooner.

In completed human trials, the rF1V vaccine was safe and well tolerated in the dosages and schedules used and elicited an immunological response to the vaccine recombinant antigen (rF1V) and to each of its components (rF1 and rV).

6. Plan for Bridging Animal Responses to Predict Human Efficacy

One of the most difficult challenges of licensing vaccines under the FDA Animal Rule is to bridge the animal and human immune responses, demonstrating that the qualitative and/or quantitative immune responses generated in the animal studies are relevant to those observed in humans and can be used to predict clinical benefit and establish an effective dose. This requires a validated assay(s) that serves as a correlate of protection to bridge animal and clinical data. The challenges associated with this become significant if the mechanism(s) of immunity is not well understood, as is the case for plague.

6.1. rBV A/B Program. The protective capacity of antibodies specific for the BoNT was demonstrated in animals [19–23]. Much of the groundwork for elucidating neutralizing antibodies as a correlate of protective immunity was performed by Iakovlev in the 1950s, who demonstrated that passive immunity provided to mice and guinea pigs was sufficient to protect these animals from inhalational challenge of serotype-specific BoNT [24]. The BoNT A/B NACs in sera are assessed in a mouse (toxin) neutralizing antibody assay (MNA) based on the Cardella method [25]. The NACs are generally accepted as a measure of protection from exposure to BoNT.

The results of human studies to date indicate that the rBV A/B vaccine is safe, well tolerated, and immunogenic. The anti-BoNT NACs observed to be protective in animals will be compared to the NACs observed in vaccinated humans to predict clinical benefit in humans (bridging). As noted above, animal vaccine dosages were selected based on their ability to induce NACs that are similar to those obtained in the clinic, and those vaccine dosages and regimens will be used to assess efficacy in pivotal studies. Passive transfer studies designed to be representative of the observed human responses will be an important means of demonstrating the ability of the rBV A/B vaccine to provide protection to humans and for bridging the clinical and nonclinical information.

6.2. rF1V Program. In contrast, there is no accepted assay that correlates with protection from *Y. pestis*. Animals were protected from pneumonic plague following vaccination with the *Y. pestis* F1 and V antigens [26, 27]. F1 is a 17 kDa protein that forms a capsule and may interfere with complement-mediated opsonisation [26, 28]. The 37 kDa V virulence factor is a component of the Type 3 secretion system known as the “Yop virulon.” The Yop virulon induces apoptosis in host phagocytes through the injection of effector proteins from the bacterial cell. The V antigen is secreted in response to environmental stimuli and is critical for virulence [27, 29] independently and as a consequence of its role in facilitating Yop effector translocation.

The F1 and V antigens induce humoral and cell-mediated immune responses in mice, NHPs, and humans [7, 13, 30–38]. The ability of the humoral responses to contribute to

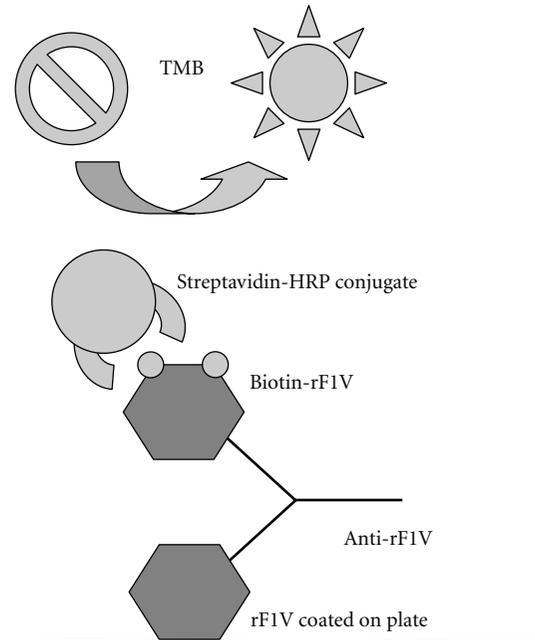


FIGURE 3: Bridge ELISA schematic.

protection from bubonic and pneumonic plague was demonstrated with the passive transfer of F1-specific monoclonal antibodies to mice. Likewise, V-specific antibodies protected mice from aerosol challenge with F1+ and F1- strains of *Y. pestis* [39, 40]. More recently, polyclonal and monoclonal antibodies to rF1, rV, or rF1V protected naïve mice from subcutaneous, intranasal, and aerosol exposure to *Y. pestis*, [41, 42] providing additional evidence of a protective role for antibodies.

Less is known about the role of cell-mediated immune responses in providing protection from plague. Cytokines secreted by T cells, including IFN- γ and TNF- α , which are believed to activate phagocytes, restrict intracellular *Y. pestis* replication, and facilitate the killing of intracellular bacilli are being studied [37]. In addition, these cytokines appear to contribute to protection by the humoral response, as the neutralization of these cytokines in mice receiving sub-optimal doses of F1- or V-specific antibody significantly reduced survival in these mice [38]. Blocking the cytokine activity with cytokine-specific neutralizing antibodies also interfered with protection in actively or passively vaccinated mice challenged by the respiratory route with *Y. pestis* CO92 [43]. A role for IL-17 in protection from *Y. pestis* is also being investigated [44].

Bridging the rF1V immune responses to predict clinical benefit is, therefore, more challenging. As humoral responses are known to be involved in the protection from *Y. pestis*, several antibody-based assays are being considered to support bridging. These include a Bridge ELISA (Figure 3), passive transfer of antibodies, and a macrophage cytotoxicity assay.

6.3. Bridge ELISA. The Bridge ELISAs use rF1, rV, and rF1V antigens to evaluate the humoral immune response to rF1V

vaccination, using a single standard curve that allows direct comparison across clinical and nonclinical samples. The assays are based on the capture of reactive antibodies in immune sera using plates coated with his-F1, his-V, or his-F1V antigen. The bound antibodies are detected by adding diluted biotinylated antigen (rF1, rV, or rF1V) followed by horseradish peroxidase-conjugated streptavidin solutions and tetramethylbenzidine substrate. A chicken IgY standard curve is used to quantitate the levels of antibody to rF1, rV, and rF1V in the tested serum. A representation of the Bridge ELISA for rF1V is presented in Figure 3, and the same format is used for the rF1 and rV assays.

The key advantage of the Bridge ELISA over a standard direct ELISA is that the same reagents are used across various assays in a manner that has the capacity to be species neutral. The advantage of species neutrality is that it avoids the bias introduced by species-specific secondary reagents. A limitation of the Bridge ELISA is that it is not a functional assay that assesses the protective antibodies in the immune sera but measures any antibody capable of binding the antigen regardless of its protective capacity.

Titers measured in the Bridge ELISA are evaluated statistically for correlation with survival data from direct challenge studies in mice and CMs. Data obtained to date indicate a continuing trend towards correlation with survival, but the data from additional studies will be needed to fully assess the utility of this assay as a correlate. Similarly, the immunogenicity data from the Phase 2b trial will be needed to analyze the human responses in light of the animal titers and efficacy.

6.4. Passive Transfer Studies. Passive transfer studies assess the combined protective capacity of antibodies to F1 and V. A mouse passive transfer system was used previously to support a decision in 1941 to vaccinate military personnel under serious threat of exposure to bubonic plague with killed plague bacilli. The readout of the mouse passive transfer assessed both percent mortality and time to death as a ratio termed the Mouse Protection Index (MPI). Based on animal data, MPI values were adopted as a reliable indicator for predicting survival in nonclinical bubonic plague vaccine efficacy studies and to determine when booster vaccinations were required in humans [45, 46].

The passive transfer system involves the administration of immune sera from vaccinated animals or humans to *Y. pestis*-naïve mice. Based on completed pharmacokinetic studies, mice are exposed to aerosol challenge when serum titers plateau. Results of completed CM-to-mouse passive transfer assays demonstrate an association between humoral immunity and protection against pneumonic plague. Results of the survival analysis showed associations of antibody levels with survival although no specific level of circulating antibody (anti-rF1, anti-rV, or anti-rF1V) in either mouse or donor serum has yet been defined as providing a specific level of protection. Mean survival time in the groups of mice that received immune sera was greater than median survival time in the control mice. The survival of the recipient mice, the time to death, and the MPI are being evaluated

statistically for correlation with survival in direct aerosol challenge studies in mice and CMs. Human sera from the clinical trials are also being tested and evaluated statistically in both the Bridge ELISA and in the passive transfer system to bridge to the animal data.

6.5. Macrophage Cytotoxicity Assay. *Y. pestis* and the other pathogenic species of *Yersinia* are cytotoxic for macrophages and resistant to phagocytosis by cultured macrophages, but the cytotoxicity and resistance can be neutralized by anti-V antibodies. Several macrophage-based assays of immunity to infection by *Y. pestis* were reported [47–51].

Y. pestis induces macrophage cell death through a caspase-3-dependent apoptotic pathway. One test under active investigation examines the ability of immune serum from rF1V-vaccinated individuals to neutralize *Yersinia*-induced macrophage cytotoxicity by measuring reduction in caspase-3 levels. The key assay components include a mouse macrophage-like cell line, J774A.1, *Y. pseudotuberculosis* (*Y. ptb* [V]), where the endogenous V gene has been replaced with the V gene from *Y. pestis*, serum samples from immunized individuals, and the EnzChek caspase-3 II kit. The kit uses a microtiter fluorometric assay and a caspase-3-specific substrate, Z-DEVD-R110, which is cleaved by active caspase-3 to release the highly fluorescent R110. This test is being evaluated for its feasibility as a potential correlate assay.

Sera from rabbits vaccinated with V or rF1V were first evaluated to verify the ability of immune sera to neutralize macrophage cytotoxicity and to quantitatively detect differences in serum cytotoxicity-neutralizing activity. The cytotoxicity assay was performed at USAMRIID as described in detail previously [48], and a serum neutralization value (NT₅₀) determined. The NT₅₀ values correspond to the reciprocal of the serum dilution resulting in a 50% decrease in caspase-3 levels. A good dose response was observed in twofold titration assays as determined by regression analysis, and the rabbit antisera yielded NT₅₀ values ranging from 157 to 1384; there was no correlation between serum anti-rV ELISA titers and NT₅₀ values ([48], data not shown). The results of preliminary evaluations in the macrophage assay of sera from rF1V-vaccinated mice, macaques, and human volunteers were highly suggestive of protection in the animal or passive mouse transfer models.

6.6. Remaining Challenges for the rBV A/B and rF1V Development Efforts. The pivotal nonclinical studies and the Phase 3 clinical trials that will be conducted following manufacture of the conformance lots have significant risks associated with the ability to satisfy the FDA's requirements for licensure by the Animal Rule. The most significant technical challenges to be overcome prior to these pivotal studies are successful manufacturing at commercial scale, validation of processes and assays, and identifying a suitable means for predicting clinical benefit for rF1V. In addition to the licensure requirements, the data generated during the advanced development programs will need to satisfy the DoD's performance requirements.

7. Summary

Protection of the Nation's warfighters and civilians is crucial to the defense of the United States. One of the threats faced by the warfighter and civilians is the offensive use of biological agents intended to either kill or incapacitate. Advanced development efforts for vaccines against botulinum neurotoxin and pneumonic plague are well on the way toward achieving the performance and efficacy objectives required to advance to pivotal testing prior to applying for FDA licensure.

Acknowledgments

These advanced development efforts on rBV A/B and rF1V were funded by the Chemical Biological Medical Systems-Joint Vaccine Acquisition Program (CBMS-JVAP), the Department of Defense (DoD) Contract DAMD 17-98-C-8024. The initial development of the macrophage cytotoxicity study was supported in part by the Medical Biological Defense Research Program, the US Army Medical Research and Materiel Command, Projects 5.10047-05-RDB and 1.1A0008-08_RDB (USAMRIID - S. Welkos) and by the Interagency Agreement no. A151 Work Plan A.18 between USAMRIID and CBMS-JVAP. The authors thank the rBV A/B and rF1V team members for their assistance in preparing this paper. DVC acknowledges and thanks the numerous subcontractors involved in developing the manufacturing, testing, nonclinical, and clinical studies.

Opinions, interpretations, conclusions, and recommendations are those of the authors and do not reflect official policy or position of the Department of the Army, the Department of Defense, or the US Government.

References

- [1] Centers for Disease Control, "Botulism in the United States, 1899–1996: handbook for epidemiologists, clinicians, and laboratory workers," Public Health Service, U.S. Department of Health and Human Services, Atlanta, Ga, 1998, http://www.cdc.gov/nczved/dfbmd/disease_listing/files/botulism.pdf.
- [2] S. S. Arnon, R. Schechter, T. V. Inglesby et al., "Botulinum toxin as a biological weapon: medical and public health management," *Journal of the American Medical Association*, vol. 285, no. 8, pp. 1059–1070, 2001.
- [3] J. L. Middlebrook and D. R. Franz, "Botulinum toxins," in *Medical Aspects of Biological Warfare*, M. K. Lenhart, D. E. Lounsbury, J. W. Martin, and Z. F. Dembek, Eds., pp. 643–654, US Department of the Army, Office of the Surgeon General and the Borden Institute, Washington, DC, USA, 2007.
- [4] B. J. Hinnebusch and D. L. Erickson, "Yersinia pestis biofilm in the flea vector and its role in the transmission of plague," *Current Topics in Microbiology and Immunology*, vol. 322, pp. 229–248, 2008.
- [5] V. Vadyvaloo, C. Jarrett, D. Sturdevant, F. Sebbane, and B. J. Hinnebusch, "Analysis of Yersinia pestis gene expression in the flea vector," *Advances in Experimental Medicine and Biology*, vol. 603, pp. 192–200, 2007.
- [6] M. Daya and Y. Nakamura, "Pulmonary disease from biological agents: anthrax, plague, Q fever, and tularemia," *Critical Care Clinics*, vol. 21, no. 4, pp. 747–763, 2005.
- [7] T. V. Inglesby, D. T. Dennis, D. A. Henderson et al., "Plague as a biological weapon: medical and public health management," *Journal of the American Medical Association*, vol. 283, no. 17, pp. 2281–2290, 2000.
- [8] J. Koirala, "Plague: disease, management, and recognition of act of terrorism," *Infectious Disease Clinics of North America*, vol. 20, no. 2, pp. 273–287, 2006.
- [9] M. B. Prentice and L. Rahalison, "Plague," *Lancet*, vol. 369, no. 9568, pp. 1196–1207, 2007.
- [10] T. W. McGovern and A. M. Friedlander, "Plague," in *Medical Aspects of Chemical and Biological Warfare*, F. R. Sidell, E. T. Takafugi, and D. R. Franz, Eds., pp. 479–502, US Department of the Army, Office of the Surgeon General, and Borden Institute, Washington, DC, USA, 1997, Textbook of Military Medicine.
- [11] R. D. Perry and J. D. Fetherston, "Yersinia pestis-Etiologic agent of plague," *Clinical Microbiology Reviews*, vol. 10, no. 1, pp. 35–66, 1997.
- [12] M. P. Byrne and L. A. Smith, "Development of vaccines for prevention of botulism," *Biochimie*, vol. 82, no. 9–10, pp. 955–966, 2000.
- [13] D. G. Heath, G. W. Anderson, J. M. Mauro et al., "Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine," *Vaccine*, vol. 16, no. 11–12, pp. 1131–1137, 1998.
- [14] D. C. Sanford, R. E. Barnewall, M. L. Vassar et al., "Inhalational botulism in rhesus macaques exposed to botulinum neurotoxin complex serotypes A1 and B1," *Clinical and Vaccine Immunology*, vol. 17, no. 9, pp. 1293–1304, 2010.
- [15] R. Warren, H. Lockman, R. Barnewall et al., "Cynomolgus macaque model for pneumonic plague," *Microbial Pathogenesis*, vol. 50, no. 1, pp. 12–22, 2011.
- [16] J. J. Adamovicz and P. L. Worsham, "Plague," in *Biodefense Research Methodology and Animal Models*, J. R. Swarengren, Ed., pp. 107–135, CSC Press, Boca Raton, Fla, USA, 2006.
- [17] J. D. Shearer, M. L. Vassar, W. Swiderski, K. Metcalfe, N. Niemuth, and I. Henderson, "Botulinum neurotoxin neutralizing activity of immune globulin (IG) purified from clinical volunteers vaccinated with recombinant botulinum vaccine (rBV A/B)," *Vaccine*, vol. 28, no. 45, pp. 7313–7318, 2010.
- [18] P. Fellows, J. Adamovicz, J. Hartings et al., "Protection in mice passively immunized with serum from cynomolgus macaques and humans vaccinated with recombinant plague vaccine (rF1V)," *Vaccine*, vol. 28, no. 49, pp. 7748–7756, 2010.
- [19] Z. F. Dembek, L. A. Smith, and J. M. Rusnak, "Botulinum toxin," in *Medical Aspects of Biological Warfare*, M. K. Lenhart, D. E. Lounsbury, J. W. Martin, and Z. F. Dembek, Eds., pp. 337–353, US Department of the Army, Office of the Surgeon General and the Borden Institute, Washington, DC, USA, 2007.
- [20] S. S. Arnon, R. Schechter, S. E. Maslanka, N. P. Jewell, and C. L. Hatheway, "Human botulism immune globulin for the treatment of infant botulism," *New England Journal of Medicine*, vol. 354, no. 5, pp. 462–471, 2006.
- [21] D. R. Franz, L. M. Pitt, M. A. Clayton, M. A. Hanes, and K. J. Rose, "Efficacy of prophylactic and therapeutic administration of antitoxin for inhalation botulism," in *Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects*, B. R. Das Gupta, Ed., pp. 473–477, Plenum, New York, NY, USA, 1993.

- [22] L. W. Cheng, L. H. Stanker, T. D. Henderson, J. Lou, and J. D. Marks, "Antibody protection against botulinum neurotoxin intoxication in mice," *Infection and Immunity*, vol. 77, no. 10, pp. 4305–4313, 2009.
- [23] T. R. Gelzleichter, M. A. Myers, R. G. Menton, N. A. Niemuth, M. C. Matthews, and M. J. Langford, "Protection against botulinum toxins provided by passive immunization with botulinum human immune globulin: evaluation using an inhalation model," *Journal of Applied Toxicology*, vol. 19, 1, pp. S35–S38, 1999.
- [24] A. M. Iakovlev, "Significance of antitoxic immunity in protection of the organism in respiratory penetration of bacterial toxins. I. Role of passive immunity in protection of the organism from respiratory lesions by B. botulinum toxins," *Zhurnal Mikrobiologii Epidemiologii i Immunobiologii*, vol. 29, no. 6, pp. 63–68, 1958.
- [25] M. A. Cardella, "Botulinum toxoids," in *Proceedings of the Symposium on Botulism*, K. H. Lewis and K. Cassel, Eds., pp. 113–130, U.S. Department of Health, Education and Welfare, Cincinnati, Ohio, USA, 1964.
- [26] A. P. Anisimov and K. K. Amoako, "Treatment of plague: promising alternatives to antibiotics," *Journal of Medical Microbiology*, vol. 55, no. 11, pp. 1461–1475, 2006.
- [27] G. I. Viboud and J. B. Bliska, "Yersinia outer proteins: role in modulation of host cell signaling responses and pathogenesis," *Annual Review of Microbiology*, vol. 59, pp. 69–89, 2005.
- [28] G. P. Andrews, D. G. Heath, G. W. Anderson Jr., S. L. Welkos, and A. M. Friedlander, "Fraction 1 capsular antigen (F1) purification from *Yersinia pestis* CO92 and from an *Escherichia coli* recombinant strain and efficacy against lethal plague challenge," *Infection and Immunity*, vol. 64, no. 6, pp. 2180–2187, 1996.
- [29] J. Heesemann, A. Sing, and K. Trülsch, "Yersinia's stratagem: targeting innate and adaptive immune defense," *Current Opinion in Microbiology*, vol. 9, no. 1, pp. 55–61, 2006.
- [30] S. T. Smiley, "Current challenges in the development of vaccines for pneumonic plague," *Expert Review of Vaccines*, vol. 7, no. 2, pp. 209–221, 2008.
- [31] E. D. Williamson, S. M. Eley, K. F. Griffin et al., "A new improved sub-unit vaccine for plague: the basis of protection," *FEMS Immunology and Medical Microbiology*, vol. 12, no. 3-4, pp. 223–230, 1995.
- [32] G. W. Anderson, D. G. Heath, C. R. Bolt, S. L. Welkos, and A. M. Friedlander, "Short- and long-term efficacy of single-dose sub unit vaccines against *Yersinia pestis* in mice," *American Journal of Tropical Medicine and Hygiene*, vol. 58, no. 6, pp. 793–799, 1998.
- [33] E. D. Williamson, S. M. Eley, A. J. Stagg, M. Green, P. Russell, and R. W. Titball, "A single dose sub-unit vaccine protects against pneumonic plague," *Vaccine*, vol. 19, no. 4-5, pp. 566–571, 2000.
- [34] S. R. Morris, "Development of a recombinant vaccine against aerosolized plague," *Vaccine*, vol. 25, no. 16, pp. 3115–3117, 2007.
- [35] V. L. Motin, R. Nakajima, G. B. Smirnov, and R. R. Brubaker, "Passive immunity to yersiniae mediated by anti-recombinant V antigen and protein A-V antigen fusion peptide," *Infection and Immunity*, vol. 62, no. 10, pp. 4192–4201, 1994.
- [36] E. D. Williamson, H. C. Flick-Smith, E. Waters et al., "Immunogenicity of the rF1+rV vaccine for plague with identification of potential immune correlates," *Microbial Pathogenesis*, vol. 42, no. 1, pp. 11–21, 2007.
- [37] M. A. Parent, L. B. Wilhelm, L. W. Kummer, F. M. Szaba, I. K. Mullarky, and S. T. Smiley, "Gamma interferon, tumor necrosis factor alpha, and nitric oxide synthase 2, key elements of cellular immunity, perform critical protective functions during humoral defense against lethal pulmonary *Yersinia pestis* infection," *Infection and Immunity*, vol. 74, no. 6, pp. 3381–3386, 2006.
- [38] L. W. Kummer, F. M. Szaba, M. A. Parent et al., "Antibodies and cytokines independently protect against pneumonic plague," *Vaccine*, vol. 26, no. 52, pp. 6901–6907, 2008.
- [39] G. W. Anderson, S. E. C. Leary, E. D. Williamson 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*, vol. 64, no. 11, pp. 4580–4585, 1996.
- [40] A. Roggenkamp, A. M. Geiger, L. Leitritz, A. Kessler, and J. Heesemann, "Passive immunity to infection with *Yersinia* spp. mediated by anti-recombinant V antigen is dependent on polymorphism of V antigen," *Infection and Immunity*, vol. 65, no. 2, pp. 446–451, 1997.
- [41] G. W. Anderson Jr., P. L. Worsham, C. R. Bolt et al., "Protection of mice from fatal bubonic and pneumonic plague by passive immunization with monoclonal antibodies against the F1 protein of *Yersinia pestis*," *American Journal of Tropical Medicine and Hygiene*, vol. 56, no. 4, pp. 471–473, 1997.
- [42] J. Hill, C. Copse, S. Leary, A. J. Stagg, E. D. Williamson, and R. W. Titball, "Synergistic protection of mice against plague with monoclonal antibodies specific for the F1 and V antigens of *Yersinia pestis*," *Infection and Immunity*, vol. 71, no. 4, pp. 2234–2238, 2003.
- [43] J. S. Lin, S. Park, J. J. Adamovicz et al., "TNF α and IFN γ contribute to F1/LcrV-targeted immune defense in mouse models of fully virulent pneumonic plague," *Vaccine*, vol. 29, no. 2, pp. 357–362, 2010.
- [44] S. Lin, L. W. Kummer, F. M. Szaba, and S. T. Smiley, "IL-17 contributes to cell-mediated defense against pulmonary *Yersinia pestis* infection," *Journal of Immunology*, vol. 186, no. 3, pp. 1675–1684, 2011.
- [45] K. F. Meyer and L. E. Foster, "Measurement of protective serum antibodies in human volunteers inoculated with plague prophylactics," *Stanford Medical Bulletin*, vol. 6, no. 1, pp. 75–79, 1948.
- [46] K. F. Meyer, "Effectiveness of live or killed plague vaccines in man," *Bulletin of the World Health Organization*, vol. 42, no. 5, pp. 653–666, 1970.
- [47] J. Bashaw, S. Norris, S. Weeks, S. Trevino, J. J. Adamovicz, and S. Welkos, "Development of in vitro correlate assays of immunity to infection with *Yersinia pestis*," *Clinical and Vaccine Immunology*, vol. 14, no. 5, pp. 605–616, 2007.
- [48] S. Welkos, S. Norris, and J. Adamovicz, "Modified caspase-3 assay indicates correlation of caspase-3 activity with immunity of nonhuman primates to *Yersinia pestis* infection," *Clinical and Vaccine Immunology*, vol. 15, no. 7, pp. 1134–1137, 2008.
- [49] S. Weeks, J. Hill, A. Friedlander, and S. Welkos, "Anti-V antigen antibody protects macrophages from *Yersinia pestis*-induced cell death and promotes phagocytosis," *Microbial Pathogenesis*, vol. 32, no. 5, pp. 227–237, 2002.
- [50] E. D. Williamson, H. C. Flick-Smith, E. Waters et al., "Immunogenicity of the rF1+rV vaccine for plague with identification of potential immune correlates," *Microbial Pathogenesis*, vol. 42, no. 1, pp. 11–21, 2007.
- [51] A. Zauberman, S. Cohen, Y. Levy et al., "Neutralization of *Yersinia pestis*-mediated macrophage cytotoxicity by anti-LcrV antibodies and its correlation with protective immunity in a mouse model of bubonic plague," *Vaccine*, vol. 26, no. 13, pp. 1616–1625, 2008.

Review Article

The Role of Immune Correlates and Surrogate Markers in the Development of Vaccines and Immunotherapies for Plague

E. D. Williamson

Biomedical Sciences Department, Defence Science & Technology Laboratory, Porton Down, Salisbury Wilts SP4 0JQ, UK

Correspondence should be addressed to E. D. Williamson, dewilliamson@dstl.gov.uk

Received 7 June 2011; Accepted 8 August 2011

Academic Editor: Phillip R. Pittman

Copyright © 2012 E. D. Williamson. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

One of the difficulties in developing countermeasures to biothreat agents is the challenge inherent in demonstrating their efficacy in man. Since the first publication of the Animal Rule by the FDA, there has been increased discussion of potential correlates of protection in animal models and their use to establish surrogate markers of efficacy in man. The latter need to be relatively easy to measure in assays that are at least qualified, if not validated, in order to derive a quantitative assessment of the clinical benefit conferred. The demonstration of safety and clinical benefit is essential to achieve regulatory approval for countermeasures for which clinical efficacy cannot be tested directly, as is the case for example, for biodefence vaccines. Plague is an ancient, serious infectious disease which is still endemic in regions of the modern world and is a potential biothreat agent. This paper discusses potential immune correlates of protection for plague, from which it may be possible to derive surrogate markers of efficacy, in order to predict the clinical efficacy of candidate prophylaxes and therapies.

1. Plague

The ancient disease of plague is still present in endemic regions of the modern world and results in approximately 3,000 reported cases each year [1]. Plague is a flea-vectored infection caused by the Gram-negative bacterium *Yersinia pestis*, a potential biothreat agent. Originally an enteric pathogen, *Y. pestis* is thought to have evolved from the enteropathogen *Y. pseudotuberculosis* [2] as a flea-vectored, enzootic infection. Fleas feed on infected rodents and then transmit bacteria to a susceptible mammal by flea bite. Man is an accidental host in this cycle, but if bitten can contract bubonic plague, a serious infection if not treated promptly before the individual becomes symptomatic. A secondary pneumonic plague can develop in an individual suffering from bubonic plague, and this is of even greater concern, since *Y. pestis* bacteria are highly transmissible in aerosolised form between unprotected individuals in close contact, with the potential for epidemic spread [3].

2. Virulence Factors in *Yersinia pestis*

Y. pestis produces a range of antigens and virulence factors, three of which have known protective efficacy as candidate

subunit vaccines: F1-antigen [4], V-antigen [5], and *Yersinia* secretory factor F (YscF) [6]. These three proteins are virulence factors when secreted by *Y. pestis* during infection. F1 antigen is a capsular protein with antiphagocytic properties [7], whilst the V-antigen is a regulatory protein in the type three secretion system (TTS) utilised by the bacterium to gain access to and deliver other cytotoxic and antiphagocytic *Yersinia* outer proteins (Yops) to host cells [8]. V-antigen occurs both within the bacterium, where it has some regulatory function in the initiation of the TTS process, and also at the tip of the injectisome [9]. The columnar structure of the injectisome is comprised of YscF [6].

Many other factors have been evaluated as potential vaccine candidates, including for example plasminogen activator (Pla), which during infection facilitates the delivery of the blood meal from the infected flea into mammalian host cells, by the degradation of physical barriers such as endothelium and connective tissue [10, 11]. However, Pla was found to be poorly immunogenic and provided no protection against lethal plague in a mouse model [11]. YscF has also been evaluated for efficacy [6], as has ph6 antigen [12] and many of the Yop's [13, 14]. Whilst some of these factors confer partial protection in animal models of plague, full protective efficacy against *Y. pestis* has been

achieved only with F1- or V-antigens and was optimum when these antigens were used in combination, or as a genetic fusion [15, 16]. The protective efficacy of the combined recombinant F1 and V (rF1 + rV) subunits against *Y. pestis* has now been reported by a number of laboratories and in a range of laboratory animal models (reviewed in [17]). Immunisation with rF1/V has been shown to protect animal models against flea-vectored plague [18] as well as against experimental exposure to *Y. pestis* [19, 20].

3. Vaccination to Protect against Plague

Vaccination and postexposure therapy are both options to protect individuals against exposure to *Y. pestis*. There has been a series of killed whole cell vaccines (KWCVs) for plague, starting with Haffkine's vaccine in the late 1800s [21], through to the currently available KWCV produced by the Central Serum Laboratories (CSL), which comprises a suspension of heat-killed *Y. pestis* ($>10^9$ /mL). Whilst KWCVs are efficacious against bubonic plague, epidemiological evidence suggests that they have little protective efficacy against pneumonic plague [15, 20, 22, 23]. F1 appears to be the key protective antigen in KWCV formulations, which thus do not protect against F1⁻ *Y. pestis* [4, 5]. Unlike the KWCVs, the rF1-/V-antigen combination has been demonstrated to protect both mice and macaques against pneumonic plague [24–27], representing a significant advance in candidate countermeasures for plague infection. Different presentations of F1/V have been studied including DNA vaccines [28], oral formulations [29], and live vaccine-vectored expression from, for example, salmonella strains [30].

A naturally attenuated live vaccine strain, EV76, has been demonstrated to protect mice and macaque models against pneumonic plague [31, 32]. Recently, a strain of *Y. pestis* KIM, mutated to stimulate TLR4 responses in the vaccinee, has been mooted as a vaccine candidate, protecting 80% of vaccinated mice against pneumonic plague [33]. This differential between live attenuated and killed vaccines in efficacy against pneumonic plague has been attributed to lack of the V-antigen in the KWCV formulations which contain effective quantities of the F1-antigen only [15, 26]; by comparison, live attenuated vaccines contain both F1- and V-antigens [15]. However, live attenuated vaccines such as EV76, have caused morbidity in nonhuman primates (NHP) [34], raising safety concerns over their use in man.

4. Postexposure Therapy

The early detection and administration of antibiotic therapy within 18–24 hours following suspected exposure to *Y. pestis* and before the appearance of symptoms, is critical for the successful treatment of plague. The recommended antibiotic regimen comprises a high dose of gentamicin intravenously (5 mg/kg intravenously once a day) or the equivalent dosage of streptomycin, ciprofloxacin, gentamicin, or doxycycline for 10 days [35]. Chloramphenicol may also be used if plague meningitis is suspected [36]. As the patient responds to treatment, it may be possible to change to the oral route

of administration of the preferred antibiotic. It is essential that antibiotic treatment is adjusted dependent on the antibiotic susceptibility of the infecting organism in culture, particularly if deliberate use of an antibiotic-resistant strain is suspected.

In animal models, the administration of monoclonal antibodies (Mab's) with specificity for F1 and V, has been shown to protect mice infected with *Y. pestis*, even when the Mab's were administered at 48 h post-exposure [37]. However, the protective effect of the anti-V Mab 7.3 was abrogated by the coadministration of anti-TNF α and anti-IFN γ indicating that a cellular proinflammatory response is also contributing to protection [38]. There is scope for combining immuno- and antibiotic therapy after exposure to *Y. pestis*, in order to shorten the duration of antibiotic therapy required.

5. Bridging between Nonclinical and Clinical

Since standard Phase III clinical efficacy studies are not feasible to carry out with plague and other serious human diseases, on both ethical and practical grounds (too few naturally occurring cases as well as outbreaks which are spasmodic), it is essential to establish satisfactory animal models of the disease. These, in turn, can be used to assess the efficacy of candidate vaccines and therapies and to identify correlates of protection. Robust animal models of plague infection which authentically represent the human disease syndrome are the objective and models have been established in standard laboratory animal species (mouse, rat, rabbit, and macaque), as well as nonstandard species such as the black-footed ferret (reviewed in [17]).

The rF1/V combination is potentially immunogenic in the mouse, guinea pig, macaque, and human [15, 39–41] and has been shown to be efficacious in nonclinical models against either injected [15], aerosolised [16, 20], flea-vectored [18], or ingested [42] exposure to *Y. pestis*. From these nonclinical studies, there is a need to identify the immune correlates of protection to facilitate the progression of candidate countermeasures through the clinical phase.

Bridging the gap between the nonclinical and clinical phases of the development process for a countermeasure is arguably the most risky element of the entire R&D cycle and has previously been termed “the valley of death” [43]. Many candidate prophylaxes and therapies have foundered at this interface, possibly because of the difficulty in comparing nonclinical and clinical datasets in terms of protective efficacy. This highlights the need to understand the immunological mechanisms required to achieve protective efficacy against such agents and to derive immune correlates of protection in animal models. Identification of the latter, based on immunological readouts which have been found to correlate statistically with protective efficacy in appropriate animal models, should lead to the derivation of surrogate markers of efficacy (Figure 1). Surrogate markers need to be measurable and quantitative endpoints for clinical trial volunteers which predict efficacy. If several surrogate markers are used, collectively these may be used to predict the degree of efficacy that can be achieved. Thus the nomination of

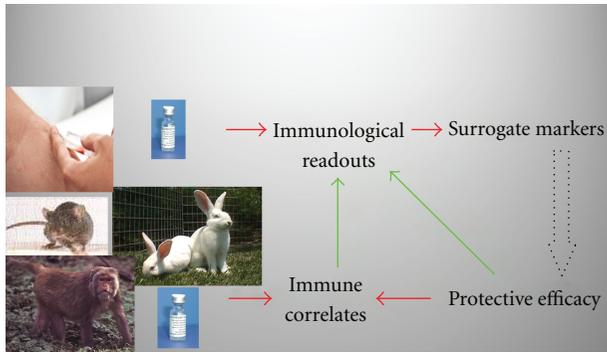


FIGURE 1: Integrating immunological readouts from nonclinical and clinical studies to identify surrogate markers of efficacy.

surrogate markers of efficacy effectively bridges the gap between the nonclinical and clinical phases of R&D.

Depending on how closely the animal model mimics the human infection, more than one animal model of the infection may be required to provide immune correlates, concepts embodied in the Animal Rule by the Food and Drug Agency in the USA [44]. In summary, the Animal Rule requires the following:

- (1) there is a well-understood pathophysiological mechanism operating and of its prevention or substantial reduction by the product;
- (2) the effect is demonstrated in one or more animal species expected to react with a response predictive for humans, unless the effect is demonstrated in a single animal species that represents a sufficiently well-characterized animal model for predicting the response in humans;
- (3) the animal study endpoint is clearly related to the desired benefit in humans, generally the enhancement of survival or prevention of major morbidity;
- (4) the data or information on the kinetics and pharmacodynamics of the product in animals and humans, allows selection of an effective dose in humans.

Having identified immune correlates of protection, there are various mathematical approaches to extrapolate these nonclinical data to man in order to predict degrees of protection [45].

6. Immune Correlates of Protection in Plague

Immunisation of mice with either [4, 5] or both F1 and V proteins [15, 16] was protective against plague and a titre of specific antibody correlated with protection. Whilst the development of an IgG titre to these proteins correlates with protection as observed in mice [46], guinea pig [39], nonhuman primate (NHP) [40] and inferred from passive transfer studies with clinical trial serum [41], neutralizing antibody alone does not describe the entire mechanism of protection against this virulent pathogen [17]. Researchers

from several groups have reported a strong CMI response to be operating [38, 47, 48] and in response to an alhydrogel-adsorbed formulation of the rF1 + rV vaccine, this generally has been observed to be a CD4+ Th2-biased CMI response [46]. However, alternative formulations of the rF1 + rV vaccine in which different adjuvants have been substituted for alhydrogel have also been demonstrated to induce protective immunity in a CD4+ Th1-biased setting [49, 50]. Additionally, strains of mice with targeted gene deletions affecting antibody production by B cells (μ MT B cell knockouts or SCID/beige) or the nature of the Th cell response including Stat 4/Stat 6 knockouts and IL4/IL10 knockouts have been studied [17, 49–51]; rF1 + rV-immunised Stat-4-deficient mice, which have low levels of IFN γ production, were found to be poorly protected from *Y. pestis* challenge, despite producing similar antibody titres to rF1 + rV as the intact controls [49]. Moreover, the rF1 + rV vaccine was able to induce protective immunity in IL4 knockout mice despite a Th1-biased environment operating in these animals [50]. Indeed, Stat-4-mediated immune mechanisms leading to a Th1 response were found to be essential for protection, whereas Stat 6/Th2-mediated responses were not [49]. Thus for the rF1 + rV vaccine, the induction of specific antibody neutralising the F1 and V antigens is a significant immune correlate of protection; however the supporting CMI response is not necessarily Th2-polarised and indeed the operation of Th1 mechanisms during infection appears to be essential for full protection and recovery [17].

Whilst the measurement of total Ig indicates that an immune response has been induced by a candidate vaccine, this alone cannot indicate that protective immunity has been achieved. The assay of the functionality of the induced antibody may be more instructive. If protection can be demonstrated in the selected animal models and related to the presence of a neutralising antibody response, then the identification of the same neutralising antibody within serum samples from human clinical trial volunteers indicates an immune correlate of protection and potential surrogate marker of efficacy. Thus immune macaque [40] and human Phase 1 trial volunteers [41] sera have been demonstrated to compete with the plague-protective monoclonal antibody (Mab 7.3) for binding to the V-antigen on solid phase *in vitro*; these sera (results not shown) as well as Mab 7.3 protected J774 cells *in vitro* from the cytotoxic effect of V-antigen secreted by *Y. Pseudotuberculosis* (Figure 2); passively protected naive mice from *in vivo* challenge with *Y. pestis*. The passive transfer of protective immunity in human serum into mice also correlated significantly with the total IgG titre in the human donors to rF1 + rV at days 21 ($P < 0.001$) and 28 ($P < 0.03$) [41].

Subsequently however, competitive ELISA has not been shown to be consistent between laboratories as a correlate of protection assay [52], likely due to the existence of more than one protective B-cell epitope on the V antigen [53]. Thus a pragmatic approach towards assays showing a correlation between immunological readouts in relevant animal models and man needs to be taken to thoroughly test such assays for consistency and utility.

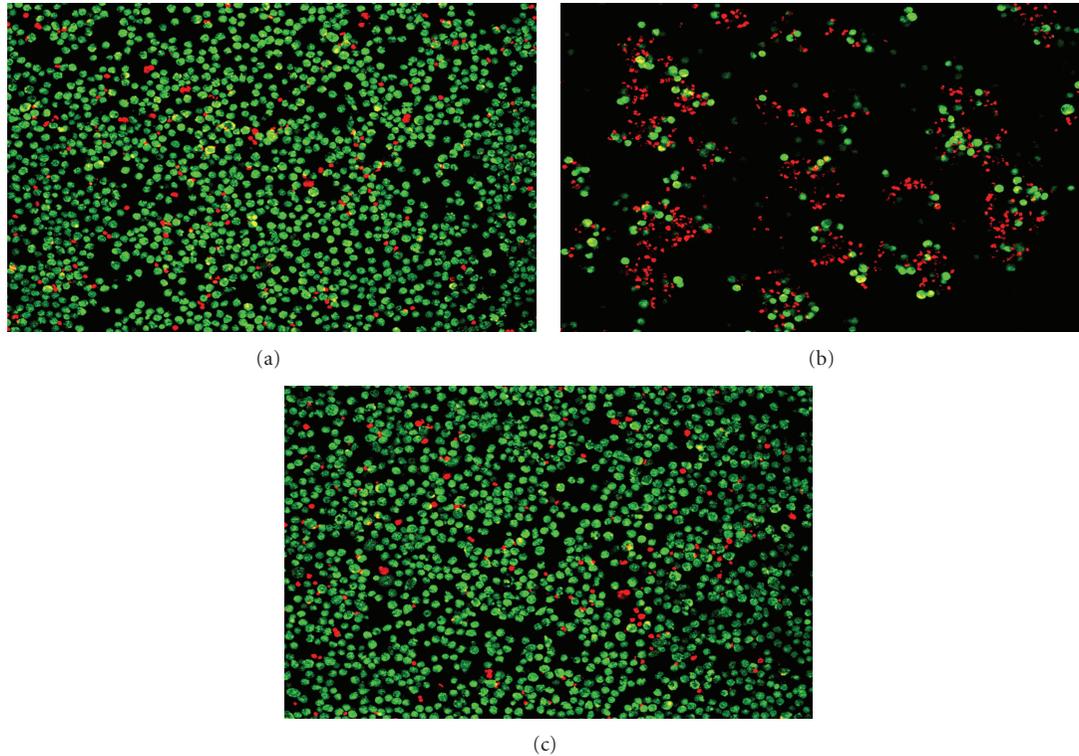


FIGURE 2: The cytotoxicity of *Y. pestis* V antigen expressed from *Y. pseudotuberculosis* for J774 cells was inhibited by pretreatment with anti-V Mab 7.3. Cells were stained with ethidium bromide/acridine orange to identify live cells (green) and dead cells (red). (a) Uninfected J774 cells, (b) J774 cells infected with *Y. pseudotuberculosis* expressing V antigen were killed, shown by the preponderance of dead cells. (c) J774 cells pretreated with Mab 7.3 prior to exposure to *Y. pseudotuberculosis* expressing V antigen were protected, with no significant difference in appearance, compared with uninfected cells.

7. Potential Surrogate Markers of Efficacy for Countermeasures to Plague

Based on these data on immune correlates and on the immunoanalysis data published to date on samples from clinical trial volunteers immunised with the rF1/V subunit vaccine [41], it is possible to identify several serological surrogate markers of efficacy. These may include the inhibitory-activity of human immune serum on the cytotoxicity of V-antigen secreted from *Y. pseudotuberculosis*. Qualitative data from this assay have been published [40], however, the assay has subsequently been improved and made quantitative [54]. It has been demonstrated that decreased caspase-3 activity in macrophages exposed to immune NHP serum correlated with increased survival of those NHP to *Y. pestis* infection.

Passive transfer of human serum from volunteers enrolled in a Phase I clinical trial has been demonstrated to protect naive mice against plague infection, in a dose-related manner [41]. The passive transfer of protective immunity into mice also correlated significantly with total IgG titer to rF1 plus rV at days 21 ($r^2 = 98.6\%$; $P < 0.001$) and 28 ($r^2 = 76.8\%$; $P < 0.03$).

Assays for cellular surrogate markers of efficacy have traditionally been more challenging, particularly in a clinical

setting, since they have required fresh whole blood samples and relatively prompt analysis. However recent advances in flow cytometry have simplified this, allowing the assay of T-cell responses and the quantitative analysis of lymphocyte subsets in whole blood. Nevertheless, sample size is important: attempts to analyse changes in cell surface markers on peripheral blood mononuclear cells (PBMC) by flow cytometry during the course of a small Phase I clinical trial for rF1V did not reveal any significant trends, due to the large variation in response between individuals [41]. The demonstration of a cellular recall response to rF1/V has been reported in *ex vivo* splenocytes from immunised mice [55]. A more practical alternative may be to use an ELISpot assay, where for example, IFN γ secretion from splenocytes restimulated *in vitro* with vaccine antigens is detected [48]. More specifically, CD4+ T-cell epitopes for F1 and V have been identified in mice [56, 57] and an H-2^d-restricted murine T-cell epitope in F1 has been shown to be essential for protection in Balb/c mice [58]. Similarly, HLA-restricted T-cell epitopes have been mapped in F1 [59] and are being sought in V-antigen using HLA transgenic mice. These data may in the future provide functional targets for human T-cell memory responses, recognition of which by immune PBMC could provide a cellular surrogate marker of efficacy.

8. Conclusions

Much work is ongoing to identify statistically valid immune correlates of protection for plague, particularly since a clinical demonstration of efficacy is not possible. This has required the development of nonclinical models which authentically represent the human infection. As far as possible, the immune correlate should be demonstrated in more than one nonclinical model. Whilst the immune correlate(s) may not describe all the immune mechanisms operating in protection against a pathogen, they should be reproducibly consistent between the selected nonclinical models and the clinic and should be quantitative, to assess the likely benefit to be conferred on the vaccinee. With an increasing understanding of the molecular basis of pathogenicity and of the innate and adaptive immune response mechanisms required to counter *Y. pestis*, immune correlates of protection are being identified and reported and this in turn will expedite the development of next-generation vaccines and immunotherapies.

References

- [1] WHO, "Plague, Democratic Republic of the Congo," *The Weekly Epidemiological Record*, vol. 80, pp. 86, 2005.
- [2] M. Achtman, K. Zurth, G. Morelli, G. Torrea, A. Guiyoule, and E. Carniel, "*Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 24, pp. 14043–14048, 1999.
- [3] R. D. Perry and J. D. Fetherston, "*Yersinia pestis*—etiologic agent of plague," *Clinical Microbiology Reviews*, vol. 10, no. 1, pp. 35–66, 1997.
- [4] G. P. Andrews, D. G. Heath, G. W. Anderson, S. L. Welkos, and A. M. Friedlander, "Fraction 1 capsular antigen (F1) purification from *Yersinia pestis* CO92 and from an *Escherichia coli* recombinant strain and efficacy against lethal plague challenge," *Infection and Immunity*, vol. 64, no. 6, pp. 2180–2187, 1996.
- [5] G. W. Anderson, S. E. C. Leary, E. D. Williamson 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*, vol. 64, no. 11, pp. 4580–4585, 1996.
- [6] J. S. Matson, K. A. Durick, D. S. Bradley, and M. L. Nilles, "Immunization of mice with YscF provides protection from *Yersinia pestis* infections," *BMC Microbiology*, vol. 5, article 38, 2005.
- [7] Y. Du, R. Rosqvist, and A. Forsberg, "Role of fraction 1 antigen of *Yersinia pestis* in inhibition of phagocytosis," *Infection and Immunity*, vol. 70, no. 3, pp. 1453–1460, 2002.
- [8] G. R. Cornelis, "The *Yersinia* Yop virulon, a bacterial system to subvert cells of the primary host defense," *Folia Microbiologica*, vol. 43, no. 3, pp. 253–261, 1998.
- [9] C. A. Mueller, P. Broz, S. A. Müller et al., "The V-antigen of *Yersinia* forms a distinct structure at the tip of injectisome needles," *Science*, vol. 310, no. 5748, pp. 674–676, 2005.
- [10] B. J. Hinnebusch, E. R. Fischer, and T. G. Schwan, "Evaluation of the role of the *Yersinia pestis* plasminogen activator and other plasmid-encoded factors in temperature-dependent blockage of the flea," *Journal of Infectious Diseases*, vol. 178, no. 5, pp. 1406–1415, 1998.
- [11] T. J. Easterbrook, K. Reddin, A. Robinson, and N. Modi, "Studies on the immunogenicity of the Pla protein from *Yersinia pestis*," *Contributions to Microbiology and Immunology*, vol. 13, pp. 214–215, 1995.
- [12] R. W. Titball and E. D. Williamson, "Vaccination against bubonic and pneumonic plague," *Vaccine*, vol. 19, no. 30, pp. 4175–4184, 2001.
- [13] S. E. C. Leary, K. F. Griffin, E. E. Galyov et al., "*Yersinia* outer proteins (YOPS) E, K and N are antigenic but non-protective compared to V antigen, in a murine model of bubonic plague," *Microbial Pathogenesis*, vol. 26, no. 3, pp. 159–169, 1999.
- [14] G. P. Andrews, S. T. Strachan, G. E. Benner et al., "Protective efficacy of recombinant *Yersinia* outer proteins against bubonic plague caused by encapsulated and nonencapsulated *Yersinia pestis*," *Infection and Immunity*, vol. 67, no. 3, pp. 1533–1537, 1999.
- [15] E. D. Williamson, S. M. Eley, K. F. Griffin et al., "A new improved sub-unit vaccine for plague: the basis of protection," *FEMS Immunology and Medical Microbiology*, vol. 12, no. 3–4, pp. 223–230, 1995.
- [16] D. G. Heath, G. W. Anderson, J. M. Mauro et al., "Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine," *Vaccine*, vol. 16, no. 11–12, pp. 1131–1137, 1998.
- [17] E. D. Williamson, "Plague in: vaccines for biodefense, supplement to vaccine," *Vaccine*, vol. 27, pp. D56–D60, 2009.
- [18] C. O. Jarrett, F. Sebbane, J. J. Adamovicz, G. P. Andrews, and B. J. Hinnebusch, "Flea-borne transmission model to evaluate vaccine efficacy against naturally acquired bubonic Ppague," *Infection and Immunity*, vol. 72, no. 4, pp. 2052–2056, 2004.
- [19] B. S. Powell, G. P. Andrews, J. T. Enama et al., "Design and testing for a nontagged F1-V fusion protein as vaccine antigen against bubonic and pneumonic plague," *Biotechnology Progress*, vol. 21, no. 5, pp. 1490–1510, 2005.
- [20] E. D. Williamson, S. M. Eley, A. J. Stagg, M. Green, P. Russell, and R. W. Titball, "A sub-unit vaccine elicits IgG in serum, spleen cell cultures and bronchial washings and protects immunized animals against pneumonic plague," *Vaccine*, vol. 15, no. 10, pp. 1079–1084, 1997.
- [21] K. F. Meyer, "Effectiveness of live or killed plague vaccines in man," *Bulletin of the World Health Organization*, vol. 42, no. 5, pp. 653–666, 1970.
- [22] D. C. Cavanaugh, B. L. Elisberg, C. H. Llewellyn et al., "Plague immunization. V. Indirect evidence for the efficacy of plague vaccine," *Journal of Infectious Diseases*, vol. 129, supplement, pp. S37–40, 1974.
- [23] R. J. Cohen and J. L. Stockard, "Pneumonic plague in an untreated plague-vaccinated individual," *Journal of the American Medical Association*, vol. 202, no. 4, pp. 365–366, 1967.
- [24] J. A. Chichester, K. Musiychuk, C. E. Farrance et al., "A single component two-valent LcrV-F1 vaccine protects non-human primates against pneumonic plague," *Vaccine*, vol. 27, no. 25–26, pp. 3471–3474, 2009.
- [25] S. B. Mizel, A. H. Graff, N. Sriranganathan et al., "Flagellin-F1-V fusion protein is an effective plague vaccine in mice and two species of nonhuman primates," *Clinical and Vaccine Immunology*, vol. 16, no. 1, pp. 21–28, 2009.
- [26] E. D. Williamson, S. M. Eley, A. J. Stagg, M. Green, P. Russell, and R. W. Titball, "A single dose sub-unit vaccine protects against pneumonic plague," *Vaccine*, vol. 19, no. 4–5, pp. 566–571, 2000.
- [27] E. D. Williamson, P. J. Packer, E. L. Waters et al., "Recombinant (F1 + V) vaccine protects cynomolgus macaques against

- pneumonic plague," *Vaccine*, vol. 29, no. 29-30, pp. 4771-4777, 2011.
- [28] H. Yamanaka, T. Hoyt, X. Yang et al., "A parenteral DNA vaccine protects against pneumonic plague," *Vaccine*, vol. 28, no. 18, pp. 3219-3230, 2010.
- [29] M. Domingos, D. Lewis, T. Jansen, D. Zimmerman, E. D. Williamson, and R. New, "The use of an oil-based delivery formulation for both oral and parenteral vaccination," *The Open Drug Delivery Journal*, vol. 2, pp. 52-60, 2008.
- [30] S. E. C. Leary, K. F. Griffin, H. S. Garmory, E. D. Williamson, and R. W. Titball, "Expression of an F1/V fusion protein in attenuated *Salmonella typhimurium* and protection of mice against plague," *Microbial Pathogenesis*, vol. 23, no. 3, pp. 167-179, 1997.
- [31] P. Russell, S. M. Eley, S. E. Hibbs, R. J. Manchee, A. J. Stagg, and R. W. Titball, "A comparison of Plague vaccine, USP and EV76 vaccine induced protection against *Yersinia pestis* in a murine model," *Vaccine*, vol. 13, no. 16, pp. 1551-1556, 1995.
- [32] Y. Qiu, Y. Liu, Z. Qi et al., "Comparison of immunological responses of plague vaccines F1 + rV270 and EV76 in Chinese origin rhesus macaque, *Macaca mulatta*," *Scandinavian Journal of Immunology*, vol. 72, no. 5, pp. 425-433, 2010.
- [33] W. Sun, D. Six, X. Kuang, K. L. Roland, C. R.H. Raetz, and R. Curtiss, "A live attenuated strain of *Yersinia pestis* KIM as a vaccine against plague," *Vaccine*, vol. 29, no. 16, pp. 2986-2998, 2011.
- [34] K. F. Meyer, G. Smith, L. Foster, M. Brookman, and M. Sung, "Live, attenuated *Yersinia pestis* vaccine: virulent in nonhuman primates, harmless to guinea pigs," *Journal of Infectious Diseases*, vol. 129, pp. S85-12, 1974.
- [35] EMEA/CPMP, Guidance document on use of medicinal products for treatment and prophylaxis of biological agents that might be used as weapons of bioterrorism.
- [36] CDC, Facts about Pneumonic Plague, October 2001, <http://www.bt.cdc.gov/agent/plague/factsheet.asp>.
- [37] J. Hill, C. Copse, S. Leary, A. J. Stagg, E. D. Williamson, and R. W. Titball, "Synergistic protection of mice against plague with monoclonal antibodies specific for the F1 and V antigens of *Yersinia pestis*," *Infection and Immunity*, vol. 71, no. 4, pp. 2234-2238, 2003.
- [38] J. S. Lin, S. Park, J. J. Adamovicz et al., "TNF α and IFN γ contribute to F1/LcrV-targeted immune defense in mouse models of fully virulent pneumonic plague," *Vaccine*, vol. 29, no. 2, pp. 357-362, 2010.
- [39] S. M. Jones, K. F. Griffin, I. Hodgson, and E. D. Williamson, "Protective efficacy of a fully recombinant plague vaccine in the guinea pig," *Vaccine*, vol. 21, no. 25-26, pp. 3912-3918, 2003.
- [40] E. D. Williamson, H. C. Flick Smith, E. L. Waters et al., "Immunogenicity of the rF1+rV vaccine with the identification of potential immune correlates of protection," *Microbial Pathogens*, vol. 42, no. 1, pp. 12-22, 2007.
- [41] E. D. Williamson, H. C. Flick-Smith, C. LeButt et al., "Human immune response to a plague vaccine comprising recombinant F1 and rV antigens," *Infection and Immunity*, vol. 73, no. 6, pp. 3598-3608, 2005.
- [42] J. S. Mencher, S. R. Smith, T. D. Powell, D. T. Stinchcomb, J. E. Osorio, and T. E. Rocke, "Protection of black-tailed prairie dogs (*Cynomys ludovicianus*) against plague after voluntary consumption of baits containing recombinant raccoon poxvirus vaccine," *Infection and Immunity*, vol. 72, no. 9, pp. 5502-5505, 2004.
- [43] National Research Council. Committee on Accelerating Technology Transition, *Accelerating Technology Transition: Bridging the Valley of Death for Materials and Processes in Defense Systems*, The National Academies Press, Washington, DC, USA, 2001.
- [44] US FDA HHS, "New drug and biological drug products; evidence needed to demonstrate effectiveness of new drugs when human efficacy studies are not ethical or feasible," *Federal Register*, vol. 67, no. 105, pp. 37988-37998, 2002.
- [45] E. D. Williamson, M. G. Duchars, and R. Kohberger, "Predictive models and correlates of protection for testing biodefence vaccines," *Expert Review of Vaccines*, vol. 9, no. 5, pp. 527-537, 2010.
- [46] E. D. Williamson, P. M. Vesey, K. J. Gillhespy, S. M. Eley, M. Green, and R. W. Titball, "An IgG1 titre to the F1 and V antigens correlates with protection against plague in the mouse model," *Clinical and Experimental Immunology*, vol. 116, no. 1, pp. 107-114, 1999.
- [47] M. A. Parent, K. N. Berggren, L. W. Kummer et al., "Cell-mediated protection against pulmonary *Yersinia pestis* infection," *Infection and Immunity*, vol. 73, no. 11, pp. 7304-7310, 2005.
- [48] S. T. Smiley, "Cell-mediated defense against *Yersinia pestis* infection," *Advances in Experimental Medicine and Biology*, vol. 603, pp. 376-386, 2007.
- [49] S. J. Elvin and E. D. Williamson, "Stat 4 but not Stat 6 mediated immune mechanisms are essential in protection against plague," *Microbial Pathogenesis*, vol. 37, no. 4, pp. 177-184, 2004.
- [50] S. J. Elvin and E. D. Williamson, "The F1 and V subunit vaccine protects against plague in the absence of IL-4 driven immune responses," *Microbial Pathogenesis*, vol. 29, no. 4, pp. 223-230, 2000.
- [51] D. Reithmeier-Rost, J. Hill, S. J. Elvin et al., "The weak interaction of LcrV and TLR2 does not contribute to the virulence of *Yersinia pestis*," *Microbes and Infection*, vol. 9, no. 8, pp. 997-1002, 2007.
- [52] D. M. Anderson, N. A. Ciletti, H. Lee-Lewis et al., "Pneumonic plague pathogenesis and immunity in brown Norway rats," *American Journal of Pathology*, vol. 174, no. 3, pp. 910-921, 2009.
- [53] J. Hill, S. Leary, S. Smither et al., "N255 is a key residue for recognition by a monoclonal antibody which protects against *Yersinia pestis* infection," *Vaccine*, vol. 27, no. 50, pp. 7073-7079, 2009.
- [54] S. Welkos, S. Norris, and J. Adamovicz, "Modified caspase-3 assay indicates correlation of caspase-3 activity with immunity of nonhuman primates to *Yersinia pestis* infection," *Clinical and Vaccine Immunology*, vol. 15, no. 7, pp. 1134-1137, 2008.
- [55] E. D. Williamson, A. J. Stagg, S. M. Eley et al., "Kinetics of the immune response to the (F1 + V) vaccine in models of bubonic and pneumonic plague," *Vaccine*, vol. 25, no. 6, pp. 1142-1148, 2007.
- [56] J. A. Musson, M. Morton, N. Walker et al., "Sequential proteolytic processing of the capsular Caf1 antigen of *Yersinia pestis* for major histocompatibility complex class II-restricted presentation to T lymphocytes," *The Journal of Biological Chemistry*, vol. 281, no. 36, pp. 26129-26135, 2006.
- [57] H. K. Shim, J. A. Musson, H. M. Harper et al., "Mechanisms of major histocompatibility complex class II-restricted processing and presentation of the V antigen of *Yersinia pestis*," *Immunology*, vol. 119, no. 3, pp. 385-392, 2006.
- [58] D. A. Chalton, J. A. Musson, H. C. Flick-Smith et al., "A functional monomeric plague vaccine created by circular permutation," *Infection and Immunity*, vol. 74, pp. 6624-6631, 2006.

- [59] J. A. Musson, R. Ingram, G. Durand et al., “Repertoire of HLA-DR1-restricted CD4 T-cell responses to capsular Caf1 antigen of *Yersinia pestis* in human leukocyte antigen transgenic mice,” *Infection and Immunity*, vol. 78, no. 10, pp. 4356–4362, 2010.

Review Article

FDA Experience with Medical Countermeasures under the Animal Rule

Paul Aebersold

Global Regulatory Affairs, Quintiles, Inc., 1801 Rockville Pike, Rockville, MD 20852, USA

Correspondence should be addressed to Paul Aebersold, paul.aebersold@quintiles.com

Received 4 May 2011; Revised 14 July 2011; Accepted 21 July 2011

Academic Editor: Kelly T. McKee

Copyright © 2012 Paul Aebersold. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The Food and Drug Administration issued a final rule in May 2002 to permit the Agency to approve drugs or license biological products on the basis of animal efficacy studies for use in ameliorating or preventing serious or life-threatening conditions caused by exposure to lethal or permanently disabling toxic biological, chemical, radiological, or nuclear substances. Only two drugs were approved in the first nine years of the “Animal Rule” despite massive investment by the federal government since 2001 to stimulate development of medical countermeasures to biological threats. This article therefore examines the Food and Drug Administration reviews made public after approval of those two drugs and the public discussion at the Agency’s Anti-Infective Drugs Advisory Committee of one biological product under development under the Animal Rule. Despite the paucity of approved drugs or licensed biological products as medical countermeasures, several investigational drugs have been placed in the National Strategic Stockpile for use as medical countermeasures, if needed.

1. Introduction

There is a risk in the post-September 11, 2001, world of chemical, biological, radiological, or nuclear attack on the United States, either on civilian populations or on military forces. To support the development of medical countermeasures (MCM) against such threats, the government has invested an estimated \$54 billion in civilian spending from fiscal year 2001 through fiscal year 2010 [1]. Yet despite this massive investment, the United States lacks the range of MCM listed in the Health and Human Services Public Health Emergency MCM Enterprise Implementation Plan [2].

In 2002, the Food and Drug Administration (FDA) issued what has become known as the “Animal Rule,” intended to expedite the development of new drugs and biologic products as MCM to chemical, biological, radiological, and nuclear threats. The Animal Rule applies only to new drug or biologic products for which definitive human efficacy studies cannot be conducted because it would be unethical to deliberately expose healthy human volunteers to a lethal or permanently disabling toxic biological, chemical, radiological, or nuclear substance.

Since the Animal Rule was issued, only two drug products for humans have been approved by the FDA on the basis of efficacy studies in animals. This paper summarizes the context in which the Animal Rule came into being, the regulatory provisions of the Animal Rule, and the publically-available information on how FDA has reviewed animal efficacy studies in support of drug or biologic product applications for use in humans.

2. The Medical Threats

In September 1980, Iraq invaded Iran in a war that lasted until August 1988. During the war, Iran reported that Iraq used chemical weapons on Iranian soldiers. One of the chemical-warfare instances reported by Iran, at Hoor-ul-Huzwaizeh on March 13, 1984, was verified by an international team of specialists dispatched to Iran by the United Nations Secretary General. The evidence adduced in the report by the UN team lends substantial credence to Iranian allegations of Iraqi chemical warfare on at least six other occasions during the period from February 26 to March 17 of that year [3].

A decade later in August 1990, Iraq invaded Kuwait. An international coalition of countries deployed troops to Saudi Arabia in preparation to liberate Kuwait. At that time, Iraq was thought to possess biological as well as chemical weapons.

In the decade following the ensuing Gulf War, the United Nations passed 16 Security Council resolutions calling for the complete elimination of Iraqi weapons of mass destruction. The United Nations Special Commission (UNSCOM) was established to oversee Iraq's compliance with destruction of chemical, biological, and missile weapons facilities [4]. UNSCOM learned that Iraq had ordered tons of growth media from the British company Oxoid, whereas Iraq's hospital consumption of growth media was only 200 kilograms a year. Rihab Rashid Taha, an Iraqi microbiologist educated in England, admitted to UNSCOM inspectors that she had grown 19,000 liters of botulism and 8,000 liters of anthrax, as well as smaller amounts of other dangerous organisms. She also had conducted research with camel pox virus, which raised fears that Iraq had planned to weaponize smallpox virus.

On September 18, 2001, apparently unrelated to events in Iraq, a letter was mailed from Trenton, New Jersey, to NBC news anchor Tom Brokaw in New York City. The letter contained a brown granular material that turned out to be anthrax spores. The staff member who opened the letter developed cutaneous symptoms around September 29 and saw a doctor on October 1. Another letter containing anthrax was mailed on September 18 to the New York Post, where three employees developed cutaneous anthrax. Although never recovered, three other letters are thought to have been mailed at the same time to ABC News and CBS News in New York and to the National Enquirer at the offices of AMI in Boca Raton, Florida. A photo editor at AMI developed inhalation anthrax and died on October 5 [5].

On October 9, 2001, two more letters were mailed from Trenton to Senate Majority Leader Tom Daschle and Senator Patrick Leahy in Washington, DC. Both senators had expressed concerns about the administration's proposed "anti-terrorism" Patriot Act. These October 9 letters contained anthrax spores in the form of a highly refined, dry white powder. Two workers at the Brentwood postal facility near Washington died of inhalational anthrax on October 22. In all, from the four recovered and three suspected letters, five people died from inhalational anthrax and at least 22 others were infected but recovered from either cutaneous or inhalational anthrax. Thousands of other people thought to have been exposed to anthrax were placed on antibiotic therapy [5].

More than a year later, on February 5, 2003, in remarks to the United Nations Security Council, Secretary of State Colin Powell noted that it had taken less than a teaspoon of dry anthrax to shut down the United States Senate [6].

In 2003, Al Qaeda issued a fatwa authorizing the use of biological, chemical, and nuclear weapons against infidels [7]. In March 2005, the Robb-Silverman Report on Weapons of Mass Destruction documented that Al Qaeda had a major bioweapons effort in Afghanistan as of 2003 [8].

3. Impasse at FDA on Medical Countermeasures

During the 1980s, the time of the Iraq/Iran war, the Army was investigating pyridostigmine bromide (PB) as a potential protection against the nerve gas soman. The Army discussed its promising animal results with FDA, which explained to the Army in 1988 that it had no regulatory pathway to approve the drug on the basis of animal studies.

In October 1990, in preparation for the Gulf War to liberate Kuwait, the Department of Defense (DoD) requested of FDA that it establish authority to waive its requirement for informed consent for use of investigational drugs [9]. In less than two months, FDA announced an interim final rule, Informed Consent for Human Drugs and Biologics: Determination that Informed Consent is Not Feasible [10]. One week later, DoD requested waivers for PB and anthrax vaccine; 18 days after the request, FDA granted both waivers [9]. Operation Desert Storm began 11 days after the waivers were granted. Under orders, soldiers in combat took PB as an experimental protection against the nerve gas soman. After Kuwait was liberated, a number of veterans suffered from undiagnosed illnesses which collectively came to be known as "Gulf War Syndrome." Veteran groups alleged that the investigational drugs had caused these illnesses, and a presidential advisory committee on gulf war veterans' illnesses was established in 1995 [11].

In December 1992, FDA promulgated new regulations to facilitate the development of treatments for acquired immuno-deficiency syndrome (AIDS) based on surrogate markers [12]. A surrogate marker, such as CD-4 white blood cell counts, might respond rapidly to a new antiviral treatment, whereas it could take years to evaluate the effect of the new antiviral drug on long term survival of AIDS patients. These "Accelerated Approval" regulations required that a surrogate marker must be reasonably likely to predict clinical benefit. The Army saw in this new regulation a path forward for licensure of PB as a protection against Soman nerve gas and filed a New Drug Application (NDA) with FDA in May, 1996.

Ten months later in March, 1997, Dr. Paul Leber, director of the Division of Neuropharmacological Drug Products at FDA, disputed the Army's claim that the surrogate marker of their clinical trials was reasonably likely to predict clinical benefit for the intended military use. He argued that the predictive power of the surrogate must be shown in humans before any "reasonable person" could predict clinical benefit. He considered that extrapolation of clinical benefit in humans based on animal experiments was unjustified [13].

Dr. Robert Temple, director of the Office of Drug Evaluation I at FDA, did not agree with what he read as Dr. Leber's essentially absolute conclusion that animal data linking inhibition of blood cholinesterase by PB to protection against nerve agents, together with human evidence of inhibition of blood cholinesterase, could *never* be taken as evidence of effectiveness under regulations for Accelerated Approval of New Drugs for Serious or Life-Threatening Illnesses. In his memorandum of August 1998, Dr. Temple noted: "As I wrote that part of the preamble, I agree with it." General principles aside, however, he did agree that

cholinesterase inhibition had not been shown by the Army to be a reasonable surrogate for survival benefit [13].

Dr. Leber replied by memorandum in September 1998, noting that Dr. Temple was incorrect in his assertion that he, Dr. Leber, wrote that animal data could “never” be used as a basis for reaching a conclusion under the Accelerated Approval regulations. Dr. Leber noted that his memorandum spoke to why, from an epistemological and scientific perspective, it would be imprudent to extrapolate from an effect observed in an animal model to a conclusion about the effectiveness of the intervention in humans [13].

4. FDA’s Response to Chemical, Biological, Radiological and Nuclear Threats

4.1. *The Animal Rule.* In July 1997, FDA requested comments on its proposed rule for Accessibility to New Drugs for Use in Military and Civilian Exigencies When Traditional Human Efficacy Studies Are Not Feasible [14]. Specifically, FDA asked the following questions.

- (1) Should its rule permitting waiver of informed consent in very limited circumstances involving military exigencies be revoked or amended?

This question soon became moot.

- (2) When, if ever, is it ethical to expose volunteers to toxic substances for testing antidotes?

FDA received nine comments. One comment suggested that the developers of these drugs, if they are confident that the drugs are both safe and effective, should offer themselves for final testing of safety and efficacy. DoD strongly opposed testing of toxic substances and also stated that testing of sublethal doses of the toxic substances would be uninformative.

- (3) What evidence of efficacy, other than from human trials, would be appropriate to demonstrate the safety and efficacy of products that may provide protection against toxic chemical and biological substances?

FDA received nine comments, most of which did not mention specific types of information that would be needed for approval. The Public Citizen Litigation Group rejected as illegal, without elaboration, the idea that animal data could serve as the basis of approval of an antidote.

This rulemaking was rendered moot by the *National Defense Authorization Act for Fiscal 1999*, which authorized the president to waive FDA’s informed consent requirements in certain military situations. Consequently, in October 1999, FDA revoked its 1990 interim final rule and issued a new interim final rule, which established criteria and standards for the president to apply [15].

Also in October 1999, FDA issued a proposed rule, *New Drug and Biological Products; Evidence Needed to Demonstrate Efficacy of New Drugs for Use Against Lethal or Permanently Disabling Toxic Substances When Efficacy Studies in Humans Ethically Cannot Be Conducted* [16].

The proposed rule would also apply when field trials after accidental or hostile exposure are not feasible. Human safety studies would be needed to support licensure.

FDA received comments on the proposed rule from only two pharmaceutical companies, one physician affiliated with a university, and the National Institutes of Health. The final rule was issued in May 2002, with the revised title, *New Drug and Biological Drug Products; Evidence Needed to Demonstrate Effectiveness of New Drugs When Human Efficacy Studies Are Not Ethical or Feasible* [17].

This rule has become known as the Animal Rule. The scope of the rule and the standards for effectiveness are reproduced below from the Code of Federal Regulations, as the author, who formerly worked at FDA, studiously avoids paraphrasing regulations:

TITLE 21—FOOD AND DRUGS

CHAPTER I—FOOD AND DRUG ADMINISTRATION

DEPARTMENT OF HEALTH AND HUMAN SERVICES

SUBCHAPTER D—DRUGS FOR HUMAN USE

PART 314 APPLICATIONS FOR FDA APPROVAL TO MARKET A NEW DRUG

Subpart I—Approval of New Drugs When Human Efficacy Studies Are Not Ethical or Feasible. (i) Sec. 314.600 Scope. This subpart applies to certain new drug products that have been studied for their safety and efficacy in ameliorating or preventing serious or life-threatening conditions caused by exposure to lethal or permanently disabling toxic biological, chemical, radiological, or nuclear substances. This subpart applies only to those new drug products for which definitive human efficacy studies cannot be conducted because it would be unethical to deliberately expose healthy human volunteers to a lethal or permanently disabling toxic biological, chemical, radiological, or nuclear substance; field trials to study the product’s effectiveness after an accidental or hostile exposure have not been feasible.

(ii) Sec. 314.610 Approval based on evidence of effectiveness from studies in animals.

(a) FDA may grant marketing approval for a new drug product for which safety has been established and for which the requirements of 314.600 are met based on adequate and well-controlled animal studies when the results of those animal studies establish that the drug product is reasonably likely to produce clinical benefit in humans. In assessing the sufficiency of animal data, the agency may take into account other data, including human data, available to the agency. FDA will rely on the evidence from studies in animals to provide substantial evidence of the effectiveness of these products only when

- (1) there is a reasonably well-understood pathophysiological mechanism of the toxicity of the substance and its prevention or substantial reduction by the product;

- (2) the effect is demonstrated in more than one animal species expected to react with a response predictive for humans, unless the effect is demonstrated in a single animal species that represents a sufficiently well-characterized animal model for predicting the response in humans;
- (3) the animal study endpoint is clearly related to the desired benefit in humans, generally the enhancement of survival or prevention of major morbidity;
- (4) the data or information on the kinetics and pharmacodynamics of the product or other relevant data or information, in animals and humans, allows selection of an effective dose in humans.

To date, nine years after establishing the Animal Rule, no biologic product has been licensed using this regulatory pathway [18]. The two approvals under the Animal Rule were for new indications for drugs that had previously been approved for other indications. An analysis in 2008 of the costs and likelihood of success for medical countermeasures estimated the failure rate at more than 85% [19].

FDA's budget request for FY 2012 includes the following for medical countermeasures [18]:

FDA MCM Objective (1)—Enhance the Review Process for MCM by Establishing Public Health and Security Action Teams (PHSATs) (+\$24,199,000/85 FTE);

FDA MCM Objective (2)—Advance Regulatory Science for MCM Development and Evaluation (+\$36,903,000/60 FTE);

FDA MCM Objective (3)—Modernize the Legal, Regulatory, and Policy Framework for Effective Public Health Response (+\$5,267,000/20 FTE).

These requests for funding to enhance the review process and advance the regulatory science for MCM at FDA suggest that an ill-prepared FDA has somehow been responsible for the slow process of bringing MCM to approval or licensure, rather than that the chemistry, manufacturing and quality control of MCM, and the efficacy requirements of the Animal Rule are themselves inherently difficult.

4.2. FDA Background on Anthrax. FDA has been proactive about the development of vaccines and therapeutics for anthrax since the attacks of 2001:

- (i) April 2002: FDA, the National Institute for Allergy and Infectious Diseases, and the Department of Defense sponsored a workshop on Anthrax Vaccines: Efficacy Testing and Surrogate Markers of Immunity Workshop;
- (ii) June 2004: FDA, the Centers for Disease Control, and the National Institutes of Health sponsored a workshop on Strategies for Developing Therapeutics that Directly Target Anthrax and its Toxins;
- (iii) November 2007: FDA, the National Institute of Allergy and Infectious Diseases, and the Department

of Health and Human Services Office of Biomedical Advanced Research and Development Authority sponsored a workshop on Anthrax Vaccines: Bridging Correlates Of Protection In Animals To Immunogenicity In Humans;

- (iv) November 2010: FDA's Vaccines and Related Biological Products Advisory Committee considered the Pathway to Licensure for Protective Antigen-based Anthrax Vaccines for a Postexposure Prophylaxis Indication Using the Animal Rule.

The 2004 workshop on therapeutics included sessions on

- (i) pathogenesis of *B. anthracis*;
- (ii) in vitro characterization;
- (iii) animal studies;
- (iv) human testing; and
- (v) challenges and opportunities in product development.

The session on animal studies included the following.

- (i) The Animal Rule applied Pyridostigmine for Nerve Gas Exposure and Gentamicin for Plague.
- (ii) GLP issues.
- (iii) Animal efficacy:
 - (a) species: mouse, rat, hamster, guinea pig, rabbit, and nonhuman primate (*cytomolgus rhesus*),
 - (b) technical methods for animal studies.
- (iv) Panel discussion.

FDA did not present its current thinking on animal efficacy studies in a prepared talk but rather addressed questions to the panel. The FDA moderator recognized that antibiotic therapies would likely be used in conjunction with antibodies and perhaps other classes of agents; therefore, he asked the panel members how they envisioned designing efficacy studies when more than one agent was being studied, in order to identify the correct timing and dosing of the investigational therapeutic for (a) the postexposure prophylaxis indication and (b) the treatment indication.

The first point made was that each agent needs to be understood individually, before studying combinations, in order to have a better idea about timing for the combination protocol. The moderator responded by asking if it would be sufficient to study each agent individually, to show that each is better than a control, and the reply to that was it will depend on what the product is expected to do. An FDA representative on the panel noted that it could be a complicated and resource-intensive matter to determine whether an agent is synergistic or additive when used in combination. An FDA representative not on the panel agreed that in the more complicated case of treatment, as opposed to prophylaxis, particularly if there is the potential of using the agent in combination rather than individually, the developer would need to talk with the review division before starting

to design the studies. He said that FDA was looking forward to interacting with those who are developing interventions to try to make the process as efficient as possible.

With regard to timing of treatment in a therapeutic study, an audience member asked if it would be reasonable to treat cohorts of animals at successively later times, rather than monitoring animals around the clock for fever. An FDA representative on the panel replied that it depends on what the study is trying to prove—if the developer wants to say that an intervention should be used upon evidence of fever, they would have to monitor their animals and design the experiment contingent upon fever, because the instructions for use would be based on evidence of fever. Another audience member noted that he had heard nothing about the actual clinical manifestations occurring in animals, nothing to help him design studies in animals that would help to treat symptomatic anthrax. He noted that for some of the models that had been discussed, after the animals are infected with the agent, the next thing that is clinically apparent is death, which is a little late to treat. He wanted to hear about data from animal models that could be used for designing studies for the treatment of symptomatic anthrax infection. (Applause noted in the transcript at this point.) An FDA representative agreed that there was a lot of information that was just not there.

Perhaps the most salient comment in all these public venues with respect to the Animal Rule was at the 2007 workshop in the form of the following quotation from statistician George Box.

Essentially, all models are wrong, but some are useful [20].

5. Approvals under the Animal Rule

5.1. Pyridostigmine Bromide (PB). PB was approved in the United States in 1955 for the treatment of myasthenia gravis, a rare neurological disorder of too few acetylcholine (ACh) receptors resulting in muscle weakness. Acetylcholinesterase (AChE) is widely present in the body and breaks down ACh. PB is a reversible inhibitor of AChE and thus allows ACh to remain present longer, resulting in improved muscle strength. Too much PB, however, can result in too much ACh, leading to chronically stimulated muscles that quickly result in paralysis.

Soman is an irreversible inhibitor of AChE and can similarly result in paralysis. When Soman initially binds to AChE, it can be displaced by pralidoxime, but within minutes the binding becomes irreversible. The benefit of PB in *pretreatment* for Soman poisoning stems from its reversible binding to AChE, protecting some of the AChE from inactivation by Soman. Pralidoxime and atropine must be administered within minutes of Soman exposure. When a PB molecule leaves an AChE molecule in the free state, any residual Soman that might initially bind to the free AChE can be displaced by pralidoxime.

On 3 January 2003, barely seven months after the final Animal Rule was issued, the Army resubmitted its NDA for PB to establish that the elements of the rule had been met.

On 5 February 2003, FDA approved the application with indications and usage as follows.

Pyridostigmine bromide is indicated for prophylaxis against the lethal effects of Soman nerve agent poisoning. Pyridostigmine is intended to be used in conjunction with protective garments, including a gas mask and immediate atropine and pralidoxime therapy at the first sign of nerve agent poisoning. Pyridostigmine should be stopped at the first sign of nerve agent poisoning.

The evidence for the effectiveness of pyridostigmine as prophylaxis against Soman-induced toxicity was derived from animal studies alone.

There was nothing slow about FDA's review and approval of the Army's application under the new Animal Rule. Dr. Temple's memorandum supporting approval noted that there had been discussion and, to a degree, disagreement about whether the expectations of the rule had been met and the relevance of those data to humans.

Dr. Temple noted that there are good explanations, supported by considerable data, carried out by many investigators, of the apparent differences between animal species in their protective ratio to PB (and their different sensitivities to Soman) that mitigate the concern arising from the apparent minimal protection in some species. The difference in protective ratios between monkeys/guinea pigs and rodents/rabbits can be explained by different levels of a Soman-binding enzyme, carboxylesterase, in different species that protects these species (rodents in particular) from Soman poisoning. Based on human carboxylesterase levels, the response of humans to PB is thus far more likely to be similar to that seen in monkeys and guinea pigs (a substantial protection for Soman lethality) than to the smaller protective ratio effect in rabbits and rodents.

Dr. Temple noted that the data strongly support the proposed mechanism of action of PB in protecting against Soman toxicity (requirement 1) and lead to a conclusion that the results in monkeys and guinea pigs will be predictive of the results in humans (requirement 2). He thus concluded that we do understand "reasonably well" the pathophysiology of the protective effect of PB and that the effect seen in monkeys and guinea pigs is in fact expected to be predictive of an effect in humans [21].

5.2. Cyanokit. Cyanokit is a lyophilized formulation of hydroxocobalamin for use as an antidote in treating patients with known or suspected cyanide poisoning. Cyanide displaces the hydroxyl group in hydroxocobalamin, resulting in the formation of cyanocobalamin, which is vitamin B12. Hydroxocobalamin has been an approved drug for decades for treatment of vitamin B12 deficiency, albeit at thousands of fold lower doses than used in Cyanokit. The currently marketed generic form of hydroxocobalamin was approved in 1978.

Cyanokit was granted marketing authorization in France in May 1996 based on one prospective study and several

retrospective studies in victims of smoke inhalation that included blood sampling prior to hydroxocobalamin treatment for measurement of cyanide levels, along with one retrospective study in subjects who had been exposed to cyanide by other than fire or smoke. None of the studies were placebo controlled, as expected when testing an antidote anticipated by its mechanism of action to be effective. The study results indicated that many of the treated subjects survived what would otherwise have been lethal doses of cyanide. FDA considered that the lack of a comparator limited the interpretation of these findings, concluding only that the levels of cyanide in humans in the French studies were similar to cyanide levels that are lethal in dogs. Survival in the French studies was very low for subjects who presented in cardiac arrest, but quite high for subjects who were not in cardiac arrest.

FDA requested that Merck Santé consider seeking approval of Cyanokit in the United States. In March 2001, FDA met with Orphan Medical, Inc., which had entered into a letter of intent with the Merck subsidiary. FDA's medical review of Cyanokit describes that the requirements for an NDA were established at that meeting and several following interactions and included efficacy studies in animals conducted in accordance with 21 CFR 314 Subpart I. Orphan Medical undertook the animal studies and submitted an NDA with the expected results demonstrating efficacy. Cyanokit was approved in the United States in December 2006, based primarily on a single placebo-controlled study in a single species (dogs) [22].

6. An Almost-Approved Anthrax Antidote

6.1. Commercial Development of an Anthrax Antidote. With regard to developing medical countermeasures for treatment of anthrax disease, Human Genome Sciences (HGS) announced the following in press releases or in the briefing package for the October 2009 meeting of the Vaccines and Related Biological Products Advisory Committee [23, 24].

- (i) October 2002: pre-IND meeting between HGS and FDA;
- (ii) February 2003: use of protein and antibody drug development capabilities to develop therapeutic candidates to address microbial targets including anthrax;
- (iii) March 2003: discovery of a human monoclonal antibody drug that is effective in protecting against anthrax in multiple experimental models in animals;
- (iv) May 2003: submitted IND to FDA;
- (v) June 2003: clearance from the Food and Drug Administration (FDA) to begin human trials of raxibacumab;
- (vi) July 2003: initiation of clinical development of raxibacumab for the prevention and treatment of anthrax infections;
- (vii) August 2003: designation from the FDA for raxibacumab as a Fast Track Product;

- (viii) November: orphan drug designation for raxibacumab granted by FDA;
- (ix) March 2004: finding in the Phase 1 trial that raxibacumab is safe and well tolerated in healthy volunteers and achieved the blood levels predicted by relevant animal models as necessary to afford significant protection from the lethal effects of the anthrax toxin;
- (x) 2004 to 2007: a series of meetings with FDA regarding the additional animal efficacy studies needed to support licensure and/or use in the Strategic National Stockpile. These interactions established;
 - (a) the requirement to demonstrate efficacy in two species (rabbits and monkeys);
 - (b) that the animals had to have evidence of systemic anthrax disease at the time of raxibacumab administration for an indication in therapeutic treatment;
 - (c) that serum protective antigen (PA) could be used as a trigger for therapeutic treatment;
 - (d) that the antibiotic exposure in animals in the raxibacumab/antibiotic combination studies should approximate the exposure achieved by the recommended dose in humans.

Agreement on the division of studies between those needed to support submission of an IND by the CDC to use raxibacumab in the Strategic National Stockpile and those additional studies needed for licensure was also achieved. During 2007, HGS submitted the protocols and analysis plans for the rabbit and monkey efficacy studies. The protocols for the rabbit and monkey raxibacumab/antibiotic combination studies were completed in the summer of 2008.

- (i) July 2005: publication of Phase 1 study results in *Clinical Infectious Diseases*;
- (ii) October 2005: award for a two-phase contract to supply raxibacumab to the United States Government. Under the first phase of the contract, HGS would supply ten grams of raxibacumab to the Department of Health and Human Services for comparative *in vitro* and *in vivo* testing. Under the second phase of the contract, the Government had the option to order up to 100,000 doses of raxibacumab for the Strategic National Stockpile;
- (iii) June 2006: government exercised option to purchase 20,000 treatment courses of raxibacumab for the Strategic National Stockpile;
- (iv) October 2008: Pre-Biologics License Application meeting with FDA;
- (v) February 2009: commencement of delivery of 20,000 doses raxibacumab to the Strategic National Stockpile;
- (vi) May 2009: submission of a Biologics License Application (BLA) for raxibacumab;

- (vii) July 2009: publication in *The New England Journal of Medicine* of the results of two pivotal animal efficacy studies, which showed the life-saving potential of raxibacumab in the event of life-threatening inhalation anthrax disease;
- (viii) July 22, 2009: Government exercised option to purchase additional 45,000 doses of raxibacumab for the Strategic National Stockpile;
- (ix) October 2009: consideration of the BLA for raxibacumab at FDA's Anti-Infective Drugs Advisory Committee;
- (x) November 2009: FDA issued Complete Response Letter requesting additional information relating to the BLA for raxibacumab;
- (xi) March 2010: \$180.2 million in sales of raxibacumab to the Strategic National Stockpile in 2009 (the first product sales for the company);
- (xii) January 2011: HGS working closely with FDA to obtain approval of raxibacumab for the treatment of inhalation;
- (xiii) February 2011: \$47.2 million in sales of raxibacumab to the Strategic National Stockpile in 2010.

6.2. FDA Advisory Committee. In October 2009, FDA's Anti-Infective Drugs Advisory Committee convened to consider the Biologics License Application from Human Genome Sciences for raxibacumab injection, a monoclonal antibody product for treatment of inhalation anthrax [25]. At the outset of the meeting, FDA indicated that results of inspections of bioanalytical assays for raxibacumab and ciprofloxacin raised questions about the reliability of the clinical pharmacology data. Therefore, FDA would discuss neither pharmacokinetic or pharmacodynamic results nor the selection of a human dose.

6.3. Human Genome Sciences' Perspective. Dr. Sally Bolmer of Human Genome Sciences presented results of anthrax challenge studies in New Zealand white rabbits and cynomolgus monkeys, both well-characterized models of inhalational anthrax. Raxibacumab doses of 20 and 40 mg/kg achieved concentrations that were at least as high as the highest PA levels in anthrax-infected animals. The pivotal animal efficacy studies had parallel groups, with animals randomized to raxibacumab or placebo. To mimic the human clinical situation, other studies were conducted in which animals in some of the treatment groups received antibiotics at the onset of symptoms after anthrax challenge. Dr. Bolmer noted, however, that the preamble to the Animal Rule describes the need for a wide range of therapeutic options for the treatment of bioterror pathogens and specifically cites anthrax. She noted further that the preamble states that there is no requirement for new therapies to provide meaningful therapeutic benefits to subjects over existing therapies, nor does it require the toxic agent to be without a proven treatment. Finally she reported that concomitant administration of raxibacumab with antibiotics in animals

did not alter the antibiotic efficacy or pharmacokinetics. In other words, raxibacumab was effective in the animal models, and antibiotics were effective in the animal models, and there was no decrease in effectiveness when raxibacumab was administered with antibiotics. Dr. Bolmer concluded her remarks by introducing Dr. Daniel Lucey, who was chief of the Infectious Disease Service at the Washington Hospital Center in Washington, DC, during the anthrax attacks in 2001.

Dr. Lucey noted that in controlled experimental settings, antibiotics can achieve up to 100% survival but that in real world clinical use antibiotics are not as successful. He noted that in the United States in the 20th century, mortality from inhalational anthrax was 90% with antibiotic susceptible strains of anthrax [26]. In the anthrax attacks in 2001, survival was 55% in the 11 patients with inhalational anthrax, and Dr. Lucey considered that more rapid blood culture results, use of two or more antibiotics (ideally at least one which crosses the blood-brain barrier in order to prevent or treat anthrax meningitis), and pleural fluid drainage have improved survival. Nevertheless, the mortality rate of 45% was highly unacceptable, and he considered that an antitoxin is needed as an additional treatment modality, because the toxins still exert deleterious effects after control of bacterial replication with antibiotics.

Dr. Lucey went on to say that prior to the development of symptoms, antibiotics were very effective for post-exposure prophylaxis in 2001. Even in the first one to five days of prodromal flu-like symptoms, prompt treatment with antibiotics may have great benefit. In an inhalational anthrax infection, the prodromal phase is followed by an intermediate progressive phase characterized by bacteremia and/or pleural effusions and/or mediastinal adenopathy. Lastly, progression to the late fulminant stage can occur rapidly and lead to death within 6 to 24 hours. At this late fulminant stage, there has been no demonstrated benefit from antibiotics alone, and all five patients in 2001 who died had reached this stage. Finally, Dr. Lucey noted that the treatment paradigm of using an antitoxin in combination with antimicrobials is well established for tetanus.

Dr. Thi-Sau Mignone of Human Genome Sciences described the pivotal animal studies with raxibacumab in detail. The challenge dose of anthrax was 200 times the LD₅₀ (the dose that causes lethality in 50% of the animals). At this dose, the majority of rabbits died between days 3 and 5 whereas the majority of monkeys died between days 4 and 6. Positive bacteremia and anthrax PA in blood occurred at 24 hours in the rabbits and at 36 hours in the monkeys. A significant rise in temperature was also seen in rabbits at 24 hours, but monkeys have a strong diurnal rhythm, and temperature rise was not observed at 36 hours. When raxibacumab was administered at 40 mg/kg at the onset of these symptoms, 44% of the rabbits and 64% of the monkeys survived.

The studies in which antibiotics and raxibacumab were coadministered upon onset of symptoms, as would be expected in clinical practice in humans, were conducted to test whether raxibacumab would interfere with levofloxacin or ciprofloxacin. The studies were not designed to detect

superiority or noninferiority of the combined treatment to treatment with the antibiotic alone but rather to detect superiority of the combined treatment to placebo. In both rabbits and monkeys, survival was statistically significant higher with combined raxibacumab and levofloxacin (rabbits) or ciprofloxacin (monkeys) than with placebo, but survival was not different between combined treatment and treatment with the antibiotic alone.

In contrast to the animal models, in humans in the anthrax attacks of 2001, symptoms developed 4 to 6 days after exposure, and death occurred 5 to 8 days after the appearance of symptoms, for a total time to death in the range of 9 to 14 days.

Dr. Dan Hanfling, special advisor for emergency preparedness and response for the Inova Health System and clinical professor of emergency medicine at George Washington University, reviewed the risk-benefit profile of raxibacumab. He had been integrally involved in the emergency management response during the 2001 attacks in the national Capitol region, during which time two postal workers were successfully diagnosed and treated. He noted that over 33,000 people were treated with prophylactic antibiotics after the 2001 attack and reported that the Commission on the Prevention of Weapons of Mass Destruction had recently warned that anthrax spores released by a crop duster could kill more Americans than died during all of World War II. Although Human Genome Sciences had not tested antibiotic-resistant strains of anthrax, he pointed out that PA is highly conserved in all known strains of anthrax and that raxibacumab would thus as a single agent be expected to be effective against antibiotic-resistant strains. He concluded that it is reasonable to predict that the benefit to risk profile in humans is strongly positive for raxibacumab as a treatment for anthrax infection.

6.4. FDA's Perspective. Dr. Yuliya Yasinskaya, a medical officer in the Division of Special Pathogens and Transplant Products at the Center for Drug Evaluation and Research, presented an FDA perspective on the raxibacumab animal studies. She pointed out that the animal studies were subject to several limitations in predicting response in humans, among which is the time of intervention, which could be different between animals in a controlled research environment and humans in a clinical setting. Antibiotics were very effective in the animal studies, with close to 100% survival.

FDA was also quite concerned about the unexpected finding of a greater incidence and severity of central nervous system (CNS) lesions in raxibacumab-treated animals that died compared to placebo animals that died. Dr. Yasinskaya noted that although only a single rabbit and a single monkey died in the combined treatment groups, there were high grade CNS lesions in 100% of those animals, whereas in the placebo group the incidence of high grade lesions was low. Dr. Yasinskaya concluded that the question remains whether in humans the addition of raxibacumab added to antibiotics will provide either additional benefit or additional risk. Due to limitations of the animal studies, it remained uncertain how long antibiotic treatment can be delayed and thus how

late raxibacumab might be able to provide improved survival in these models.

6.5. Advisory Committee's Perspectives. After much preliminary discussion, the FDA questions were read and the advisory committee voted as follows.

- (1) Does the evidence from the animal models evaluating raxibacumab at 40 mg/kg IV predict response for treatment of humans with inhalational anthrax disease? And if not, what additional studies should be conducted?

One committee member interpreted the question to refer to monotherapy. Another committee member noted that the question could be taken to mean response at any time postexposure or at any stage of clinical illness.

The committee voted 16 in favor, 7 opposed, with 1 abstention. Several of the committee members, whether they voted in favor or opposed, expressed a desire to see data from animal studies in which antibiotic treatment was not 100% successful, either because doses would be more in keeping with human doses or because timing of administration would be delayed, so that additional benefit of raxibacumab could be observed if additional benefit indeed exists. It was noted that in a disaster situation, humans would probably show up a week or more after their exposure, after they probably have been PA positive for a while, a very different situation than the animal studies.

One committee member remarked that the wording of the question was almost Talmudic, and the people who voted in favor or opposed often had all the same reasons and all the same concerns but wound up coming down on one side or the other of the fence. One committee member who voted in favor commented she thought elements 1 through 3 of the Animal Rule were met and that we should move forward with this kind of drug. Several other committee members who voted in favor stated that they narrowly interpreted the question as referring to monotherapy with raxibacumab. One committee member explained his opposition as due to the wording of the question with respect to anthrax disease, pointing out that appearance of PA in the blood of animals is not anthrax disease.

- (2) Does the evidence provided in these studies support the conclusion that raxibacumab will not diminish the anticipated efficacy of antimicrobials in inhalational anthrax? And if not, what additional studies should be conducted?

One committee member immediately noted that FDA had instructed the committee to ignore the pharmacokinetic data, because of questions about their reliability. Therefore, he asked how is the committee to answer this question if ignoring the pharmacokinetic data? FDA suggested that he should vote as he believed he could vote and then provide his perspective as to why he took that vote and how he might have voted depending on what kind of clinical data were available.

The committee voted 10 in favor, 11 opposed, with 3 abstentions. The first committee member called upon to

explain his vote noted that there was a major control arm missing, raxibacumab alone, so that it was very difficult for him to make that comparison. Another who voted in opposition said that the antibiotics had been given in doses so far above their therapeutic thresholds that if there was a detrimental effect of raxibacumab, it might not have been seen in these studies. One committee member voted in favor because he knew of no precedent for a protein therapeutic like an antibody to alter the behavior of a small molecule antimicrobial. Another who voted in favor said that there was no evidence to the contrary.

The statistician committee member calculated that it would take about 400 animals to detect a statistically significant difference between 95% survival in one group versus 85% in the other. He considered that a study with that number of animals sounded like a nonstarter. One member said he abstained because he could not decide which way to vote and that the committee vote had validated his feelings. Eventually, one member said that he voted in opposition because of the narrow construction of what was presented and that he voted in favor (laughter noted in the transcript at this point) because of the narrow construction that the percentages were the same. Finally one member said that the last time he looked in a biochemistry textbook, antibiotics can be protein bound and that there was a serious matter here and that the animals should be treated with antibiotics at doses that we would use to treat a human being.

- (3) Should evidence be requested that raxibacumab makes a contribution to the efficacy over the antimicrobial alone in rabbit and monkey models? If yes, what types of additional studies should be requested and then conducted?

The committee voted 17 in favor, 6 opposed, with 1 abstention. A common sentiment was that in additional studies, the animals should be given the study material at a time that is truly therapeutic and not prophylactic. One member who voted in favor noted that the question did not say who would conduct the studies and he was not sure that the “poor sponsor” who had the misfortune of developing this compound should have to do all those studies. Another member who voted in favor thought that it might be a difficult bar for the sponsor to meet. A member who voted in opposition pointed out that raxibacumab may have benefit as monotherapy if it is dosed correctly, although it would be good to know if it could help rescue patients that had delayed antibiotic therapy. Another member who voted in opposition said it may be an unreasonable burden to say raxibacumab is going to benefit every patient who receives it. He thought there was every reason to think raxibacumab is safe and efficacious and would benefit a significant fraction of those who receive it.

- (4) Do you have any recommendations how this (the CNS findings) might be further evaluated?

Despite a number of committee members advocating further studies in a meningitis model, one member pointed out that their understanding was biased by the fact that the

only data were from nonsurviving animals. He noted that the only way to determine whether there was causality would be to look at survivors as well to see if they have any CNS toxicity. A couple of committee members were concerned about antibody complexes as potentially a source of toxicity.

- (5) Are there additional comments or further recommendations for safety evaluation in humans? If yes, what are these recommendations?

One committee member recommended that some work needs to be done to make sure that raxibacumab would be safe for use in children, and another recommended that the same level of assurance was needed for elderly patients.

7. Discussion

7.1. Pyridostigmine Bromide (PB). In 2008, the congressionally appointed Research Advisory Committee on Gulf War Veterans’ Illnesses reported that roughly 25% of the 697,000 veterans were afflicted. Exposure to PB and pesticides that were heavily used during the war was implicated as contributing to the illnesses [27].

A National Academy of Sciences committee, however, reviewed the evidence in the Research Advisory Committee’s report and published in 2010 that human epidemiologic evidence was not sufficient to establish a causative relationship between any specific drug, toxin, plume, or other agent, either alone or in combination, and Gulf War illness [28].

The Animal Rule was born of the controversy over administration of investigational drugs and biologics to soldiers under military orders without informed consent, under an administrative rule for waiver of informed consent in certain military exigencies. Now that PB has been licensed by FDA under the Animal Rule, should there be future military exigencies in which the use of Soman is anticipated, informed consent would no longer be an issue in ordering soldiers to take the licensed drug as a pretreatment against possible attack. Thus the legal situation for use of PB has changed, but the safety profile of PB has of course not changed at all because of its regulatory transition from an investigational to a licensed drug.

7.2. Cyanokit. FDA considered the approval of Cyanokit to have been based primarily on the dog study, despite the fact that one prospective and several retrospective studies had been conducted in humans. Apparently FDA was more willing to extrapolate efficacy from dogs to humans than it was to make a medical decision about the efficacy of Cyanokit based on the open label studies in France. FDA appeared reluctant to stray from its gold standard of randomized, blinded, and placebo-controlled trials, apparently giving more credence to statistical analyses of data from dogs than to medical evidence from humans.

FDA could have compared the cyanide levels in the French patients who were in cardiac arrest and died to the cyanide levels in patients who were not in cardiac arrest at the time of treatment and lived. It would not have been difficult to evaluate whether a number of patients not in cardiac arrest

had baseline cyanide levels that were fatal in subjects who had progressed to cardiac arrest before treatment, but FDA would have had to have wanted to see what could be learned from the French data. As noted above, FDA concluded only that cyanide levels in the French patients were similar to cyanide levels that are lethal in dogs and therefore was apparently unwilling to make a medical decision based on evidence that did not come with a gold star of statistical significance. In the end, more than five years after Merck Santé was solicited to seek approval and conducted a randomized, placebo-controlled animal study, FDA approved Cyanokit as effective, as the French regulatory authority had already done on the basis noncontrolled human studies.

7.3. Raxibacumab. Regarding the specific matter of CNS toxicity, since raxibacumab does not cross an intact blood-brain barrier, the basis for FDA's concern is not completely clear. After all, if raxibacumab is effective in other tissues in preventing damage from anthrax toxins, one might expect delayed progression of disease in those tissues—and indeed time to death was longer in the raxibacumab-treated animals that died compared to placebo-treated animals that died—thus leaving more time for lesions to develop in the brain. In fact, in raxibacumab-treated rabbits in the pivotal study, the incidence and severity of histopathology in nonsurvivors were the same or lower than in placebo-treated rabbits for all tissues except for the brain. In the lungs in particular, the pathology was severe in the placebo-treated animals but lower in the raxibacumab-treated animals. In the words of Dr. Mignone, the CNS lesions were more likely due to a difference in the site of benefit for raxibacumab than to an actual deleterious effect. Even if the blood-brain barrier is compromised as anthrax disease progresses and raxibacumab then does enter the CNS, it is not clear why some advisory committee members were concerned about a monoclonal antibody forming immune complexes with the PA protein, since raxibacumab binds only to a single epitope on PA and would not be expected to cross-link the PA proteins in brain.

Regarding the specific matter of antibiotic dosing in the combination studies, the HGS briefing package notes that 50 mg/kg in the rabbit produces similar exposure to that reported for the approved human doses of 500 or 750 mg levofloxacin. Since many humans weigh between 50 kg to 75 kg, the human doses are thus in the range of 10 mg/kg. HGS was unable to rebut the advisory committee's criticism that they had used doses in the animals that were in excess of the human doses, because FDA had taken any discussion of the pharmacokinetic data off the table. Taking the briefing package statement about similar antibiotic exposure at face value and recognizing that HGS and FDA had concurred that the antibiotic exposure in animals should approximate that of the recommended dose for humans, the advisory committee's criticism appears to have been misplaced.

Regarding the general matter of FDA providing guidance to HGS during the entire course of clinical development and specifically on the protocols for the animal studies, it appears to be an awkward regulatory posture for FDA to have prospectively concurred with the design of the

combination raxibacumab-antibiotic studies but then to have retrospectively required that HGS needed to evaluate whether or not raxibacumab provides additional benefit beyond that provided by antibiotics alone. The question as to whether additional benefit had to be demonstrated for use of a new therapeutic intervention in combination with antibiotics had been raised during the workshop in 2004, so FDA was very well aware of the matter. Further, HGS had conducted a pilot study of levofloxacin in rabbits challenged with anthrax, with levofloxacin dosing triggered by rise in temperature. Levofloxacin was quite effective at 10 mg/kg, 25 mg/kg, and 50 mg/kg, the latter of which in rabbits was found to be equivalent in exposure to the approved human dosing. The results of this study had been submitted to FDA and had predicted that survival would be very high in the proposed combination study of levofloxacin and raxibacumab, meaning that an additional benefit of raxibacumab could not be detected, only as a detriment. One can only assume from the outside that the FDA review team decided that a showing of additional benefit for raxibacumab beyond the benefit of antibiotics alone was not necessary, at the same time that they reviewed the HGS protocol designed to evaluate whether raxibacumab had a detrimental effect on antibiotic efficacy. The FDA recommendation at the 2004 workshop for a developer to talk with the FDA review division before commencing animal studies had years later appeared in the January 2009 Draft Guidance for Industry: Animal Models—Essential Elements to Address Efficacy Under the Animal Rule.

FDA strongly encourages sponsors to submit a development plan and to communicate frequently with the agency when developing products under the Animal Rule. The protocols for the animal efficacy studies should be discussed with FDA, with sufficient time for FDA review and comment, prior to the study being conducted.

From HGS's description of its interactions with FDA from 2004 to 2007 on the protocols for animal efficacy studies, it would appear that HGS comported with what is now FDA guidance. On FDA's side, after the workshop in 2004 on the very topic of Strategies for Developing Therapeutics that Directly Target Anthrax and its Toxins, the review team should have been better prepared for providing guidance to HGS for development of raxibacumab than they appear to have been.

8. Conclusions

In its FY 2012 budget request, FDA requested \$36.9 million for advancing regulatory science for MCM development and evaluation, which amounts to funding for 60 full-time equivalents. However, it was not lack of FDA personnel that led to nonlicensure of raxibacumab, but rather lack of forethought by FDA personnel as to whether HGS needed to demonstrate that their antidote to anthrax toxin provides additional benefit to antibiotics for treatment of inhalational anthrax disease.

Tens of thousands of vials of raxibacumab now reside in the Strategic National Stockpile, as investigational drugs, and no doubt would be used in the event of future cases of patients with advanced inhalational anthrax disease. Clearly there have been different standards for national stockpiling on one hand and for FDA approval of drugs or licensure of biologics on the other as medical countermeasures for biothreats. At least two other investigational biologic drugs, Anthrax Immune Globulin and Botulism Antitoxin Heptavalent, also reside in the Strategic National Stockpile [29].

The Animal Rule was developed to provide a basis for approving certain drugs or licensing certain biologic products as medical countermeasures to biological threats without efficacy data in humans, the alternative being to leave these products languishing as investigational drugs that require informed consent from human subjects prior to use. Personally and for the sake of his children, the author is relieved that promising medical countermeasures do not need FDA approval or licensure in order to be produced in large quantities and stockpiled for ready availability in the event of a mass bioterror attack on the United States.

References

- [1] C. Franco, "Billions for biodefense: federal agency biodefense funding, FY2009-FY2010," *Biosecurity and Bioterrorism*, vol. 7, no. 3, pp. 291–309, 2009.
- [2] HHS Public Health Emergency Medical Countermeasure Enterprise, "Implementation Plan for Chemical, Biological, Radiological and Nuclear Threats," April 2007, https://www.medicalcountermeasures.gov/BARDA/documents/phemce_implplan_041607final.pdf.
- [3] J. P. Robinson and J. Goldblat, "Chemical Warfare in The Iran-Iraq War 1980 1988," SIPRI (Stockholm International Peace Research Institute) Fact Sheet, May 1984, http://www.iranchamber.com/history/articles/chemical_warfare_iran_iraq_war.php.
- [4] Resolution 687 of April 1991, United Nations Security Council, <http://www.un.org/Depts/unscom/General/basic-facts.html>.
- [5] L. A. Cole, *The Anthrax Letters: A Medical Detective Story*, Joseph Henry Press, 2003.
- [6] C. Powell, "Remarks to the United Nations Security Council," <http://www.guardian.co.uk/world/2003/feb/05/iraq.usa>.
- [7] R. Mowatt-Larssen, "Al Qaeda's Pursuit of Weapons of Mass Destruction," *Foreign Policy*, January 2010, http://www.foreignpolicy.com/articles/2010/01/25/al_qaedas_pursuit_of_weapons_of_mass_destruction?page=full.
- [8] Report to the President of the United States, "U.S. Commission on the Intelligence Capabilities of the United States Regarding Weapons of Mass Destruction," March 2005.
- [9] R. Rettig, "Military Use of Drugs Not Yet Approved by the FDA for CW/BW Defense: Lessons from the Gulf War," Rand Corporation, http://www.rand.org/pubs/monograph_reports/MR1018.9/index.html.
- [10] *Federal Register*, December 1990.
- [11] *Executive Order* 12961, May 1995.
- [12] *Federal Register*, December 1992.
- [13] "Administrative documents on FDA website under Drugs@FDA," <http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.DrugDetails>.
- [14] *Federal Register*, July 1997.
- [15] *Federal Register*, October 1999.
- [16] *Federal Register*, October 1999.
- [17] *Federal Register*, May 2002.
- [18] "FDA FY 2012 Budget Request," <http://www.fda.gov/downloads/AboutFDA/ReportsManualsForms/Reports/BudgetReports/UCM243370.pdf>.
- [19] J. Matheny, M. Mair, and B. Smith, "Cost/success projections for US biodefense countermeasure development," *Nature Biotechnology*, vol. 26, no. 9, pp. 981–983, 2008.
- [20] G. Box and R. Draper, *Empirical Model-Building and Response Surfaces*, Wiley, New York, NY, USA, 1987.
- [21] "Administrative documents on FDA website under Drugs@FDA," <http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.DrugDetails>.
- [22] "Administrative documents on FDA website under Drugs@FDA," http://www.accessdata.fda.gov/drugsatfda_docs/nda/2006/022041_cyanokit.toc.cfm.
- [23] <http://www.hgsi.com/>.
- [24] <http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/Anti-InfectiveDrugsAdvisoryCommittee/UCM187312.pdf>.
- [25] <http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/Anti-InfectiveDrugsAdvisoryCommittee/UCM191757.pdf>.
- [26] J. E. C. Holty, D. M. Bravata, H. Liu, R. A. Olshen, K. M. McDonald, and D. K. Owens, "Systematic review: a century of inhalational anthrax cases from 1900 to 2005," *Annals of Internal Medicine*, vol. 144, no. 4, pp. 270–280, 2006.
- [27] "Gulf War Illness and the Health of Gulf War Veterans, Research Advisory Committee on Gulf War Veterans' Illnesses," November 2008, http://www.va.gov/RAC-GWVI/Committee_Documents.asp.
- [28] "Gulf War and Health: Volume 8: Update of Health Effects of Serving in the Gulf War," 2010, Institute of Medicine, http://www.nap.edu/catalog.php?record_id=12835#orgs.
- [29] http://cangene.com/biodefense_products.shtml#aig.