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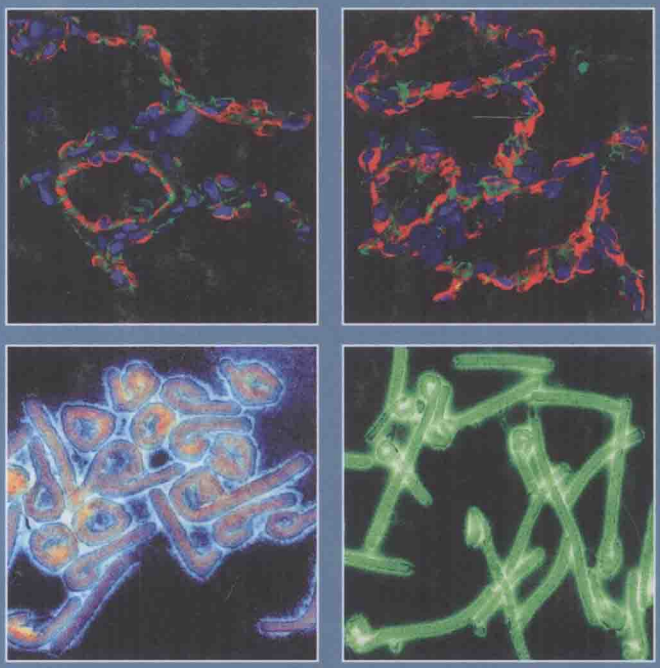

BIODEFENSE RESEARCH METHODOLOGY AND ANIMAL MODELS

EDITED BY
JAMES R. SWEARENGEN

CRC Press
Taylor & Francis Group

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In the world of biodefense research, there exists a cadre of men and women who have dedicated their lives to protecting the world from those who would use infectious biological organisms and toxins for nefarious purposes. The scientific community has banded together across many organizational lines to bring new technology, information, and countermeasures into the biodefense portfolio to better prepare against these threats. In addition to the devoted scientists, I want to acknowledge the people whose critical contributions made these advances possible. These are the professionals who maintain the facilities, make sure the research is done safely, oversee the use of animals and ensure they are used humanely in accordance with regulatory requirements; and the laboratory and veterinary technicians who are the heart and soul of this research. The vigilance and remarkable talents of these teams of professionals are our best defense.

Preface

The evolution of biodefense research has made significant advances in animal model development since the publication of the first edition of this book in 2006. The Food and Drug Administration's (FDA) Animal Efficacy Rule (read more about this in Chapter 3) has begun to mature in both understanding by the scientific community and the expectations of the FDA. Like the first edition, this edition continues to span the spectrum from basic research to advanced development of medical countermeasures. The return reader will most likely notice an increase in discussions about the FDA animal efficacy rule as it applies to animal model development and research directions for the various biological agents and toxins. As we all know, redundant efforts often waste more than just time and fiscal resources—they also result in the unnecessary use of animals. Animals have been and will continue to be an invaluable and absolutely necessary part of infectious disease research, but we all have the ethical and moral obligation to ensure that each animal is used in the most humane manner possible and to obtain the maximum benefit in advancing science and human health. It should be understood that much work precedes moving to the use of animal models, and the models presented in this book were developed in conjunction with many *in vitro* techniques including computer modeling, cell culture systems, hollow fiber systems, and other *in vitro* laboratory procedures. All of these techniques have replaced or reduced the use of animals for certain purposes, but as questions arise that require an intact, more complex biological system to answer, animal use becomes essential. The primary aims of this edition remain true to the first edition in an effort to share science, to advance science, and to minimize the number of animals required for use by reducing unnecessary duplication of effort in animal model development and use. The participation of all the chapter authors and coauthors is a testament to their belief in these values and dedication to advancing science, and protecting the health of our world's population.

Editor

Dr. James R. Swearengen, following retirement from the U.S. Army after 21 years of service, served for 4 years as the senior director at the Association for Assessment and Accreditation of Laboratory Animal Care International before joining the National Biodefense Analysis and Countermeasures Center as their comparative medicine veterinarian in 2009. Dr. Swearengen obtained his DVM degree from the University of Missouri-Columbia in 1982 and joined the Army after 2 years of private practice. After tours in Texas and Germany, Dr. Swearengen completed a residency in laboratory animal medicine at the Walter Reed Army Institute of Research from 1990 to 1994, during which period he attained board certification in the specialties of both Laboratory Animal Medicine and Veterinary Preventive Medicine and is a past-president of the American College of Laboratory Animal Medicine.

He began working at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) in 1994 as the assistant director, and then director, of the Veterinary Medicine Division. He gained extensive experience in providing veterinary and husbandry support to infectious disease animal research at all levels of biocontainment and spent many hours working under biosafety level 3 and 4 conditions. Dr. Swearengen became intimately involved with the existing animal models used in biodefense research, provided veterinary expertise in the development of new models, and coauthored publications utilizing animal models for Ebola virus and monkeypox virus infections. In 1996, he was selected to serve on the United Nations Special Commission (Biological Group) and spent 3 months in Iraq performing monitoring and verification functions of Iraq's former biological weapons program. Since 2007, Dr. Swearengen has served on the National Academies of Science National Research Council Standing Committee on Biodefense for the U.S. Department of Defense and the National Academies of Science Institute for Laboratory Animal Research Committee on Animal Models for Assessing Countermeasures to Bioterrorism Agents.

In 1997, Dr. Swearengen provided part-time support for a Defense Threat Reduction Agency program by evaluating and modernizing animal care and use programs in infectious disease research institutes in the former Soviet Union. His expertise was recognized in 2003 as he was selected as the Laboratory Animal Medicine Consultant to the Surgeon General of the U.S. Army. Dr. Swearengen's military career culminated in 2003 as he was chosen to serve as the Deputy Commander of USAMRIID, a position he held until his retirement from the U.S. Army in 2005.

Contributors

Jeffrey J. Adamovicz
Midwest Research Institute
Frederick, Maryland

Arthur O. Anderson
U.S. Army Medical Research Institute
of Infectious Diseases
Fort Detrick, Maryland

Jaime B. Anderson
U.S. Army Medical Research and
Material Command
Fort Detrick, Maryland

Donald L. Fine
Dynport Vaccine Company LLC
Frederick, Maryland

David L. Fritz
Bacteriology Division
U.S. Army Medical Research Institute
of Infectious Diseases
Fort Detrick, Maryland

Thomas W. Geisbert
Galveston National Laboratory
and
Department of Microbiology and
Immunology
University of Texas Medical Branch
Galveston, Texas

Stephen B. Greenbaum
BAI, Inc.
Alexandria, Virginia

Mary Kate Hart
DynPort Vaccine Company LLC
Frederick, Maryland

Peter B. Jahrling
Integrated Research Facility
National Institute of Allergy and
Infectious Diseases
Fort Detrick, Maryland

Teresa Krakauer
U.S. Army Medical Research Institute
of Infectious Diseases
Fort Detrick, Maryland

Frank J. Lebeda
U.S. Army Medical Research Institute
of Infectious Diseases
Fort Detrick, Maryland

Elizabeth K. Leffel
Director of Non-clinical Sciences
PharmAthene, Inc.
Annapolis, Maryland

James W. Martin
DiLorenzo Healthcare Clinic
Walter Reed Army Medical Center
Washington, District of Columbia

Shannon S. Martin
Dynport Vaccine Company LLC
Frederick, Maryland

M. Louise M. Pitt
U.S. Army Medical Research Institute
of Infectious Diseases
Fort Detrick, Maryland

William D. Pratt
U.S. Army Medical Research Institute
of Infectious Diseases
Fort Detrick, Maryland

Bret K. Purcell
U.S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Nelson W. Rebert
Division of Laboratory Sciences
U.S. Army Public Health Command Region-Pacific
Camp Zama, Japan

Douglas S. Reed
Center for Vaccine Research
University of Pittsburg
Pittsburg, Pennsylvania

Robert Rivard
U.S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Chad J. Roy
Tulane National Primate Research Center
Tulane School of Medicine
Covington, Louisiana

Bradley G. Stiles
U.S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

James R. Swearengen
National Biodefense Analysis and Countermeasures Center
Fort Detrick, Maryland

Kenneth Tucker
Tauri Group
Alexandria, Virginia

David M. Waag
Bacteriology Division
U.S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Victoria Wahl-Jensen
Integrated Research Facility
National Institute of Allergy and Infectious Diseases
Fort Detrick, Maryland

Kelly L. Warfield
Vaccine Development
Integrated Biotherapeutics, Inc.
Gaithersburg, Maryland

Patricia L. Worsham
U.S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Contents

1 History of Biological Agents as Weapons

*James W. Martin**

CONTENTS

The earliest use of biological weapons in warfare resulted from the use of corpses first to contaminate water sources and subsequently as a terror tactic, hurling bodies over the wall of fortified cities. From these crude beginnings were to develop national programs for biological weapons development, stockpiling, and deployment that would rival all other weapons systems in scope and magnitude as well as potential to cause human harm. Recent unveiling of these programs as well as recognition of the failure of the Biological Weapons Convention to prevent some countries from engaging in biological weapons development has made the public aware, if not frightened, of the possibilities. Ergo, use of biological agents as weapons of warfare, methods of terrorism, or means for engaging in criminal activity has come to the forefront of public attention in recent years. Widespread understanding of the biological threat in terms of biological agents' historic use is vital for those who endeavor to find ways to protect society from those who intend to use these agents. It is important to have some common agreement of definitions of terminology used in this discussion. *Biological agent* refers to any living organism or substance produced by an organism that can be used as a weapon to cause harm to humans. Broadly speaking, this includes any living organism or biologically derived substance, but in practical terms (for the classical biological warfare agents), this list is limited to viruses, bacteria, and toxins. *Biowarfare* in its broadest sense refers to any use of these agents to harm others. However, *biowarfare* in more common usage ascribes a narrower definition—use in the context of war, that is, it refers to the use of a biological agent by a nation-state as an act of war. *Bioterrorism* refers to the use of biological agents by a political group, religious group, or cult (group not otherwise recognized as an extension of the government of a state) to achieve some intended political or ideological objective. However, even this definition is fraught with confusion because it does not preclude use by an organization with state sponsorship which can be covert. The term *biocrime* refers to the use of biological agents in the perpetration of criminal activity in which the perpetrator's

* The views expressed in this chapter are those of the author and do not reflect the official policy of the Department of the Army, Department of Defense, or the U.S. Government.

motivation appears to be personal in nature, as opposed to some broader ideological, political, or religious objective. Although specific circumstances and events can blur the distinction, it is helpful to keep these three definitions in mind as we review the world's experience with biological agent use.

De Mussis provides a dramatic record of the use of plague victims in an attempt to engage in biological warfare. After war broke out between the Genoese and the Mongols in 1344 over control of access to the lucrative caravan trade route from the eastern shores of the Black Sea to the Orient, the Mongols laid siege to the Genoese port city of Caffa. The plague, which was later to become known as the Black Death, was spreading from the Far East and reached the Crimea in 1346. The Mongols besieging the city were severely affected and had come close to lifting their siege when they changed their tactics and hurled bodies of plague victims over the city wall, probably with the use of a trebuchet. Eventually, plague did spread to the city, though more likely from rats fleeing the Mongol encampment than as a consequence of the spread of the disease by contamination of the city with plague-infected corpses. After plague struck, the residents of Caffa, who had been successfully withstanding the siege, abandoned their defense and fled to ports in Italy, carrying the plague on board the ships with them. As a consequence, the Black Death began its scourge across Europe [1].

Along with contamination of water sources, another ancient tactic was to allow the enemy to take sanctuary in an area endemic for an infectious agent in anticipation that the enemy force would become infected and weakened by the resulting disease. Most prominent examples were the allowance of unimpeded access to malarious areas, where disease transmission was highly likely to occur [2].

The Carthaginian leader Hannibal is credited with the first use of biological toxins in warfare, in the naval battle of Eurymedon in 184 BC. He ordered earthen pots filled with serpents hurled onto the decks of the Pergamene ships, creating panic and chaos. The Carthaginians exploited the situation, with Hannibal defeating King Eumenes of Peragamum in the battle that ensued [2].

Smallpox was particularly devastating to the Native Americans. Cortez's introduction of smallpox to the Aztecs, whether intentional or not, played a major role in allowing for their defeat and subjugation by the Spanish conquistadors. Sir Jeffery Amherst, British commander of forces in the American colonies during the French and Indian War, provided Indians loyal to the French with blankets and other articles contaminated by smallpox. Native American Indians defending Fort Carillon (subsequently named Fort Ticonderoga) experienced an epidemic of smallpox that contributed to their defeat and the loss of the fort to the British. Subsequently, a smallpox epidemic broke out among the Indians in the Ohio River valley [3].

During the American Revolutionary War, successive smallpox epidemics affected major Continental Army campaigns early in the conflict and resulted in the aborted attempt to capture Quebec City early in the war. The British forces, which were immune to the disease because of their exposure to the natural infections endemic in much of Europe, were relatively protected from smallpox, whereas the colonists, living in more rural and isolated settings, were nonimmune. Because of his recognition of the consequences of this disparity of immunity between the two forces, General George Washington ordered the variolation (inoculation with smallpox) of all

nonimmune recruits in 1778. This was a controversial procedure that predated vaccination and carried a potential mortality of 1–3%; it was the first time in world military history that such a measure had been ordered by a commander and it set the precedence for military immunization programs of today [2].

The Germans undertook a covert biological campaign in the United States in the first part of World War I, before the United States had entered the war. The Allies had been purchasing draft animals from the United States for use by their military forces. German operatives infected animals awaiting shipment overseas with glanders and anthrax organisms [4]. The Germans also conducted similar operations in Romania, Russia, Norway, Mesopotamia, and Argentina, with varying levels of success. Attempts were also made to infect the grain production in Spain with wheat fungus, but without success [5].

An international protocol, known as the 1925 Geneva Protocol [for the Prohibition of the Use in War of Asphyxiating, Poisonous, or Other Gases, and Bacteriological (*Biological*) Methods of Warfare], was created in response to the use of chemical agents during World War I. The 1925 Geneva Protocol created by the League of Nations' Conference for the Supervision of the International Trade in Arms and Ammunition concerned use only between nation-states. It has no verification mechanism and relies on voluntary compliance. Many of the original signatory states held reservations to the protocol for the right to retaliatory use, making it effectively a no-first-use protocol [2]. After the Japanese defeat of Russia in the 1905 Russo-Japanese War, Japan had become the dominant foreign power in Manchuria. The Kwantung Army was created to maintain Japanese economic interests in the region. During the 15 months from September 1931 to the end of 1932, the Japanese military seized full control of all of Manchuria, setting the stage for its complete exploitation. It was in 1932, just as Japan obtained military control, that Major Ishii Shiro, a Japanese Army physician with a confirmed interest in biological agents, came to Harbin to exploit Manchurian human resources in the name of research. He established his initial laboratory in the industrial sector of Harbin known as the Nan Gang District, but he soon came to realize that his more controversial involuntary human research could not be conducted without scrutiny there and moved the human research to a secret facility at Beiyinhe, which was 100 km south of Harbin. Unobserved by the outside world, Major Ishii began human experimentation on a more dramatic scale. Each victim, once selected for study, continued to be a study subject until his or her death as part of the study—or through live vivisection. There were no survivors among the research study subjects. These studies continued until the occurrence of a prisoner riot and escape, which resulted in closure of the facility in 1937. Not to be deterred, the closure of the Beiyinhe facility was followed by the creation of even larger, more extensive facilities [6].

In August 1936, Lt. Col. Ishii was made Chief of the Kwantung Army Boeki Kyusui Bu (Water Purification Bureau). That autumn, the Japanese appropriated 6 km^2 of farmland, which encompassed 10 villages located 24 km south of Harbin, displacing 600 families from their ancestral homes. It was here that Ishii built the massive Ping Fan research facility, where 200 prisoners were always on hand to become the expendable subjects of further experimentation. A minimum of 3000 Chinese prisoners were killed and cremated consequent to these experiments, but

most of the evidence was destroyed at the end of the war—in all likelihood the actual number of victims of this ghastly research was much greater [6].

The Unit 100 facility at Changchun was run by an equally ruthless veterinary officer, Major Wakamatsu Yujiro. In 1936, the Japanese appropriated 20 km^2 of land near Mokotan, a small village just 6 km south of Changchun, the capital of Japanese-occupied Manchuria. Unit 100 was a predominantly veterinary and agricultural biowarfare research unit—a completely independent operation from Unit 731 at Ping Fan. The principal focus of Unit 100 was to develop biological weapons useful in sabotage operations. Although animals and crops were the focus of most of the research, a tremendous number of human studies were also conducted that were very similar in nature to those conducted at Ping Fan by Unit 731 [6].

In April 1939, a third major research facility, known as Unit Ei 1644, was established in an existing Chinese hospital in Nanking under the command of one of Ishii's lieutenants, Lt. Col. Masuda. On the fourth floor of the hospital were housed prisoners, many of them women and children, who became the subjects of grisly experimentation. The human experimental subjects were cremated after the studies in the camp incinerator, usually late at night. A gas chamber with an observation window was used to conduct chemical warfare experiments. Unit Ei 1644 supported the research efforts of Unit 731, with support responsibilities that included production of bacterial agents as well as cultivation of fleas [6]. At the end of the war, in a move that has now become controversial, Ishii, then a lieutenant general, and his fellow scientists were given amnesty in exchange for providing information derived from their years of biological warfare research [2].

In contradistinction to Japanese efforts during World War II, German interest seemed to be more focused on developing an adequate defense against biological agents. Although German researchers experimentally infected prisoners with infectious agents, there were no legal actions taken after the war, and no German offensive biological warfare program was ever documented. The Germans, however, accused the British of attempting to introduce yellow fever to the southern Asian subcontinent as well as of an Allied introduction of Colorado beetles to destroy the German potato crops. These claims were never substantiated [5].

During the Korean conflict, numerous allegations of use of biowarfare by the United States were made by North Korean and Chinese officials. Many of the allegations appear to be based on experiences that the Chinese had in Manchuria with the "field testing" done by Unit 731. Polish medical personnel were sent to China to support the Communist war effort, accompanied by Eastern European correspondents. Numerous allegations based on anecdotal accounts of patients came from these correspondents and other sources. These accounts were not supported with scientific information. Some of the accounts, such as the use of insects for vectors of cholera and the spread of anthrax with infected spiders, had dubious scientific validity [7].

After World War I, Major Leon Fox, Medical Corps, U.S. Army, wrote an extensive report in which he concluded that modern improvements in health and sanitation made use of biological agents unfeasible and ineffective. Some mention was made of the ongoing Japanese offensive biological program in his report, but it was, ironically, his erroneous concerns about German biological weapons' development that led to serious U.S. interest in the subject. In the autumn of 1941, before U.S. entrance

into World War II, opinions differed as to the validity of biological warfare potential: "Sufficient doubt existed so that reasonable prudence required that a serious evaluation be made to the dangers of a possible attack" [8, p. 1]. As a consequence, the Secretary of War asked the National Academy of Sciences to appoint a committee to study the question. The committee concluded in February 1942 that biowarfare was feasible and that measures were needed to reduce U.S. vulnerability [2].

President Roosevelt established the War Reserve Service, with George W. Merck as director, with the initial task of developing defensive measures to protect against a biological attack. By November 1942 the War Reserve Service asked the Chemical Warfare Service of the Army to assume the responsibility for a secret large-scale research and development program, which included the construction and operation of laboratories and pilot plants. The Army selected the small National Guard airfield at Camp Detrick, Frederick, Maryland, as a site for new facilities in April 1943. By the summer of 1944, the Army had a testing site at Horn Island, Mississippi, which was subsequently moved to Dugway Proving Grounds, Utah, and a production facility in Terre Haute, Indiana, which was soon closed. The War Reserve Service was disbanded and the Research and Development Board established under the War Secretary to supervise the biological research programs. An assessment of the biological warfare situation was provided to the Secretary of War by George Merck in January 1946. The report concluded that the United States clearly needed to have a credible capability to retaliate in kind if ever attacked with biological weapons [7].

Only after the end of World War II did the United States learn of the extent of Japanese biological weapons research. Gradually, in the late 1940s, the scope of the Japanese program became known, along with an awareness of Soviet interest in the program. War broke out on the Korean peninsula in June 1950, adding to concerns about Soviet biological weapons development, and the possibility that the North Koreans, Chinese, or Soviets might resort to biological weapons use in Korea. The Terre Haute, Indiana, production facility, which was closed in 1946, was replaced with a large-scale production facility in Pine Bluff, Arkansas. During the 26 years of biological weapons development, the United States weaponized eight antipersonnel agents and five anticrop agents [9].

Field testing was done in the United States in which the general public and the test subjects themselves were uninformed, and these studies have unfortunately tainted the history of the offensive biological warfare program. The first large-scale aerosol vulnerability testing was the San Francisco Bay study conducted in September 1950. *Bacillus globigii* and *Serratia marcescens* were used as stimulants for biological agents. Unfortunately, a number of *Serratia* infections occurred subsequently in one of the hospitals in the study area, and although none of the infections was ever documented to be the 8UK strain, many people held on to their perceptions that the U.S. Army study had caused the infections [10].

Serratia marcescens, then known as *Chromobacter*, was thought to be a nonpathogen at the time. Several controversial studies included environmental tests to see whether African Americans were more susceptible to fungal infections caused by *Aspergillus fumigatus*, as had been observed with *Coccidioides immitis*, including the 1951 exposure of uninformed workers at Norfolk Supply Center, in Norfolk, Virginia, to crates contaminated with *Aspergillus* spores. In 1966, in New York City

subways, the U.S. Army conducted a repeat of studies that had been done by the Germans on the Paris Metro and some of the forts in Maginot Line to highlight the vulnerability of ventilation systems and confined spaces. Light bulbs filled with *Bacillus subtilis* var. *nigeri* were dropped into the ventilator shafts to see how long it would take the organisms to spread through the subway system [11]. The Special Operations Division at Camp Detrick conducted most of the studies on possible methods of covert attack.

After 1954, the newly formed Medical Research Unit conducted medical research separately from the studies done by the Chemical Corps. This research began using human volunteers in 1956 as part of a congressionally approved program known as "Operation Whitecoat." This use of human volunteers set the standard for ethics and human use in research. The program used army active-duty soldiers with conscientious objector status as volunteers to conduct biological agent-related research. All participation was voluntary and was performed with the written informed consent of each volunteer. The program concluded in 1973 with the end of the draft, which had been the source of conscientious objectors [9]. In July 1969, Great Britain issued a statement to the Conference of the Committee on Disarmament calling for the prohibition of development, production, and stockpiling of bacteriological and toxin weapons [12].

In September 1969, the Soviet Union unexpectedly recommended a disarmament convention to the United Nations General Assembly. In November 1969, the World Health Organization of the United Nations issued a follow-on to an earlier report by the 18-nation Committee on Disarmament, on biological weapons, describing the unpredictable nature, lack of control once released, and other attendant risks of biological weapons use. Then, President Nixon, in his November 25, 1969, visit to Fort Detrick, announced new U.S. policy on biological warfare, renouncing unilaterally the development, production, and stockpiling of biological weapons, limiting research strictly to the development of vaccines, drugs, and diagnostics as defensive measures. The 1972 Biologic Weapons Convention, which was a follow-on to the 1925 Geneva Protocol, is more properly known as the "1972 Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and their Destruction." Agreement was reached among 103 cosignatory nations and went into effect in March 1975. "The convention prohibits the development, production, stockpiling or acquisition by other means or retention of microbial or other biological agents toxins whatever their origin or method of production of types and in quantities that have no justification of prophylactic, protective or other peaceful purposes, as well as weapons, equipment or means of delivery designed to use such agents or toxins for hostile purposes or in armed conflict"[13].

The U.S. Army, in response to the 1969 presidential directive, did not await the creation of the 1972 Biological Warfare Convention or its ratification. By May 1972, all personnel-targeted agents had been destroyed and the production facility at Pine Bluff, Arkansas, converted to a research facility. By February 1973, all agriculture-targeted biological agents had been destroyed. Fort Detrick and other installations involved in the offensive weapons program were redirected, and the U.S. Army Medical Research Institute of Infectious Diseases was created in place of the U.S. Army Medical Unit, with biosafety level 3 and 4 laboratories dedicated strictly to the development of medical defensive countermeasures [2].

Although a signatory to the 1925 Geneva Convention, the Soviet Union began its weapons development program at the Leningrad Military Academy in Moscow under the control of the state security apparatus, the GPU. Work was initially with typhus, with what was apparently human experimentation on political prisoners during the prewar era conducted at Slovetsky Island in the Baltic Sea and nearby concentration camps. This work was subsequently expanded to include work with Q fever, glanders, and melioidosis, as well as possibly tularemia and plague. Outbreaks of Q fever among German troops resting in Crimea and outbreaks of tularemia among the German siege forces of Stalingrad are two suspected but unconfirmed Soviet uses of biological warfare during World War II [14].

During World War II, Stalin was forced to move his biological warfare operations out of the path of advancing German forces. Study facilities were moved to Kirov in eastern European Russia, and testing facilities were eventually established on Vozrozhdeniya Island on the Aral Sea between the Soviet Republics of Kazakhstan and Uzbekistan. At the conclusion of the war, Soviet troops invading Manchuria captured the Japanese at the infamous Unit 731 at Ping Fan. Through captured documents and prisoner interrogations, the troops learned of the extensive human experimentation and field trials conducted by the Japanese. Stalin put KGB chief Lavrenty Beria in charge of a new biowarfare program, emboldened by the Japanese findings. The production facility at Sverdlosk was constructed using Japanese plans. When Stalin died in 1953, a struggle for control of the Soviet Union ensued. Beria was executed during the struggle to seize power, and Khruschev emerged as the Kremlin leader and transferred the biological warfare program to the Fifteenth Directorate of the Red Army. Colonel General Yefim Smirnov, who had been the chief of army medical services during the war, became the director [14].

Smirnov, who had been Stalin's minister of health, was a strong advocate of biological weapons. By 1956, Defense Minister Marshall Georgi Zhukov announced to the world that Moscow would be capable of deploying biological in addition to chemical weapons in the next war. By 1960, there existed numerous research facilities addressing every aspect of biological warfare scattered across the Soviet Union [14].

The Soviet Union was an active participant in the World Health Organization's smallpox eradication program, which ran from 1964 to 1979. Soviet physicians participating in the program sent specimens back to Soviet research facilities. For the Soviets, participation in the program presented an opportunity not only to rid the world of smallpox but also obtain, as source material for biological weapons development, virulent strains of smallpox virus that could be used subsequently for the more sinister purpose of releasing it as a weapon of war. In 1980 the World Health Organization announced the eradication of smallpox, and the world rejoiced at the elimination of a disease that had caused more human deaths than any other infection. However, the Soviets had another reason to celebrate: Elimination of natural disease meant that, over time, vaccination programs would terminate, and neither natural nor vaccine-acquired immunity would exist for the majority of the world's population [2].

In 1969, President Richard Nixon announced unilateral disengagement in biological warfare research [12]. As mentioned previously, research came to an abrupt halt; production facilities and weapon stockpiles were destroyed. The 1972 Biological