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Elke Mühlberger
Lisa L. Hensley
Jonathan S. Towner *Editors*

Marburg- and Ebolaviruses

From Ecosystems to Molecules

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Preface

Filoviruses have captivated the imagination of scientists and the public alike since Marburg virus was first isolated in Germany in 1967. Through the years, these viruses have gained much notoriety for the devastating nature of the outbreaks they cause and their repeated sensationalization by mainstream media and the entertainment industry. Over the last 50 years, we have seen tremendous advances in our understanding of these agents, and this book endeavors to capture the major areas of discovery but in no way expects to be an all-encompassing source. Perhaps it is appropriate that the completion of this book coincides with the 50-year anniversary of the discovery of the first filovirus.

The outline for this book was conceived in 2013, prior to the start of the West African Ebola virus outbreak. Many of the contributors to this book were among those who volunteered to respond to the outbreak, some for over a period of years, or set aside their normal work to help support outbreak response efforts.

A wide spectrum of renowned experts in the field worked together to make this book happen. They range from clinicians to virologists to biochemists, and we are incredibly grateful for their contributions. Some of the authors have worked on filoviruses since their discovery, while others are much newer to the field. Despite these differences, all of the authors have one common goal—to better understand how filoviruses work, and to use their knowledge to help prevent or mitigate the impact of future filovirus outbreaks.

We have separated this book into four parts. Part I covers filovirus ecology, outbreaks, and clinical management. It begins with a fascinating first-hand account of the challenges that faced researchers 50 years ago in a small German town when they encountered a highly virulent infectious agent of unknown origin. Chapter "Filovirus Research: How it Began" was written by one of the original filovirus discoverers and describes the first isolation of Marburg virus in 1967 during a time long before virologists had use of modern biocontainment facilities. We then move on to a global view of filovirus distribution and emergence in the chapter "Ecology

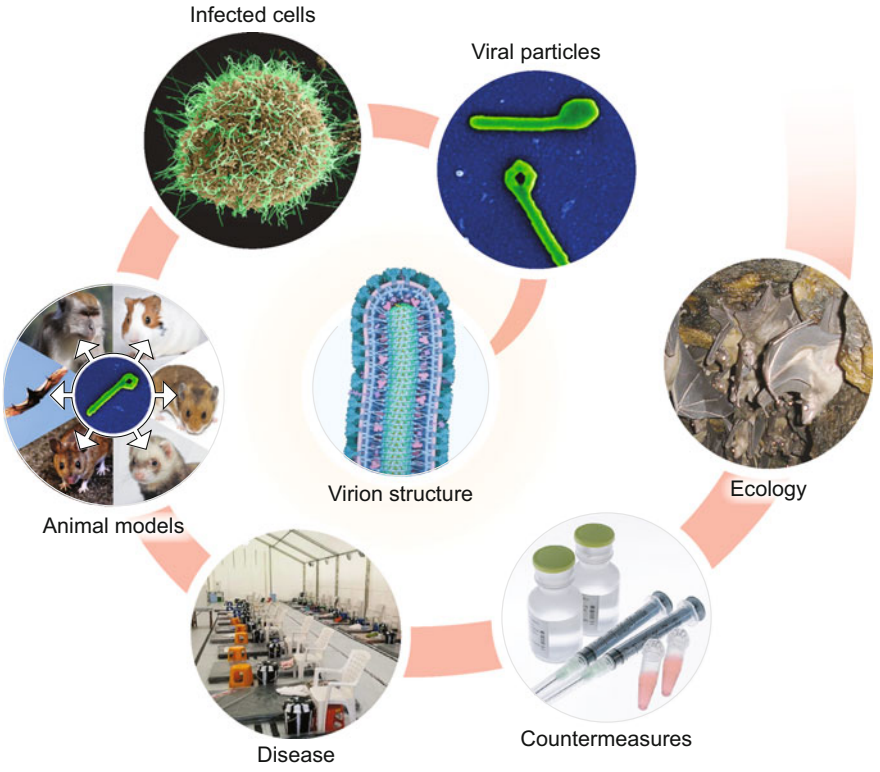
of Filoviruses" in which we learn about the natural origins of some enigmatic viruses, how they persist long term in nature, and what drivers might promote their spillover to other animals including humans. One of these spillover events led to the largest Ebola virus outbreak on record and is described in the chapter "West Africa 2013 Ebola: From Virus Outbreak to Humanitarian Crisis". This comprehensive account describes the devastating epidemic that not only brought Ebola virus directly to Europe and the USA, but overwhelmed the long-neglected public health infrastructures in Guinea, Sierra Leone, and Liberia and the international response alike. The next two chapters, "Clinical Management of Ebola Virus Disease Patients in Low Resource Settings" and "Clinical Management of Patients with Ebola Virus Disease in High Resource Settings", describe the challenges and risks facing clinicians when they treat patients infected with Ebola virus and how their approaches differ depending on the resource environment.

Part II of the book focuses on filovirus pathogenesis and protection. Chapter "Ebola Virus Disease in Humans: Pathophysiology and Immunity" provides a detailed review of the human disease, including fascinating new studies of the human immune response that resulted from the West African Ebola virus outbreak. Chapters "Nonhuman Primate Models of Ebola Virus Disease" and "Small Animal Models for Studying Filovirus Pathogenesis" summarize the vast body of work using animal models, big and small, to study filovirus disease and develop experimental treatments and vaccines. Part II concludes with the chapters "Accelerating Vaccine Development During the 2013–2016 West African Ebola Virus Disease Outbreak" and "Therapeutics Against Filovirus Infection" that each provide state-of-the-art summaries of current experimental countermeasures used to combat filovirus infections, including those deployed during the Ebola virus outbreak in West Africa.

The first three chapters of Part III take us deep into the cellular level of filovirus infection. Chapter "Filovirus Strategies to Escape Antiviral Responses" provides a comprehensive account of mechanisms used by filoviruses to counteract antiviral responses. In the following two chapters, "Mechanisms of Filovirus Entry" and "Inside the Cell: Assembly of Filoviruses", we learn how filoviruses make their way into cells and which strategies they use to replicate their genomes and assemble to new particles. Finally, we reach the atomic level in "Filovirus Structural Biology: The Molecules in the Machine" which focuses on the structural analysis of filovirus proteins through the use of stunning images of these structures.

The book ends with a description of research tools used to study filoviruses. Chapter "Reverse Genetics of Filoviruses" summarizes the use of reverse genetics as a powerful tool to investigate virus replication and pathogenesis. The last chapter, "Guide to the Correct Use of Filoviral Nomenclature", is meant as a useful tool to help guide virologists through the sometimes confusing, and recently evolved, world of filovirus taxonomy.

Last but not least, we wish to thank all the authors who have contributed their work to this book. We are grateful to Jens Kuhn, who volunteered to critically read and edit almost all of the chapters, and to Jiro Wada for designing the preface figure. We also wish to state up front that any views or opinions expressed in the book do not necessarily reflect those of the editors, authors, or their respective institutions.



Preface Figure Marburg- and Ebolaviruses: From Ecosystems to Molecules. Figure designed by Jiro Wada, NIH/NIAID, Integrated Research Facilities. The watercolor of the virion structure was kindly provided by David S. Goodsell, RCSB Protein Data Bank. Bat photo provided by Chris Black, WHO.

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Part I
Maintenance in Nature and Spillover: Ecology, Outbreaks and Clinical Management

Filovirus Research: How it Began

Werner Slenczka

Abstract The first reported filovirus outbreak occurred in August 1967, when laboratory workers in Marburg and Frankfurt, Germany, and Belgrade, Yugoslavia (now Serbia) became infected with an unknown highly pathogenic agent. The disease was characterized by high fever, malaise, rash, hemorrhagic and tetanic manifestations, and high lethality, amounting to 25%. The disease was introduced to Europe by grivets (*Chlorocebus aethiops*), which were used for biomedical research and vaccine production. The causative agent, Marburg virus, was isolated and identified by scientists of the University of Marburg, Germany in cooperation with specialists for viral electron microscopy at the Bernhard Nocht Institute in Hamburg, Germany. In this chapter, Dr. Slenczka, who was involved in the first isolation of Marburg virus in 1967, describes the desperate hunt of the causative agent of this first filovirus disease outbreak in the center of Europe, its successful isolation, the likely route of transmission from a monkey trading station to vaccine production facilities in Germany and Yugoslavia, and the consequences of this outbreak, including a shortage in the production of poliomyelitis vaccine In addition, this chapter provides insight into some of the peculiarities of filovirus infection, such as sexual virus transmission several months after recovery and the role of Ca^{2+}-loss in Marburg virus pathogenesis, which were already observed during this first well-documented Marburg virus disease outbreak.

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1 Introduction

> One can think of the middle of the twentieth century as the end of one of the most important social revolutions in history, the virtual elimination of the infectious disease as a significant factor in social life (Sir Macfarlane Burnet) (Burnet and White 1962).

Burnet's optimistic view on the future of infectious diseases is perfectly in accordance with parts of the published opinion, which during the 60s of the past century was prevalent. Representatives of scientific organizations for oncology and for psychiatric diseases postulated that research funds for infectious disease should be cut and the money devoted to their own scientific interests. In a decade during which many insect-borne diseases had vanished due to large-scale distribution of DDT, during which bacterial infections were controlled with antibiotics, and during which the eradication of smallpox, poliomyelitis, measles, and other viral diseases had progressed from an utopian dream to a realistic prophecy, nobody was prepared to face the advent of a completely unknown infectious disease with an extremely high lethality in the center of Europe.

The story of the 1967 Marburg virus disease (MVD) outbreak has often been told and it is not the aim of this article to repeat all the known details. Instead, the intention is to restore some forgotten or neglected details and to give a personal view on some events.

2 An Unknown Disease

The summer of 1967 was very hot in Marburg, Germany, and whoever was able to leave the town went to the seaside or the mountains. In August 1967, 20 people, who lived in small villages surrounding Marburg, fell ill with fever, malaise, headache, vomiting, rash (Fig. 1), and conjunctivitis, the tentative diagnosis was "summer diarrhea" (often caused by enteroviruses or by coliform bacteria) or dysentery. Initially, despite the gravity of the symptoms, these patients were treated in their homes for up to a week. The patients were admitted, on average, on day 5 after the onset of symptoms to the hospital of the University of Marburg which had an isolation ward for the treatment of infectious diseases (Fig. 2). In total, fourteen men and six women were admitted between August 15 and the end of the month.

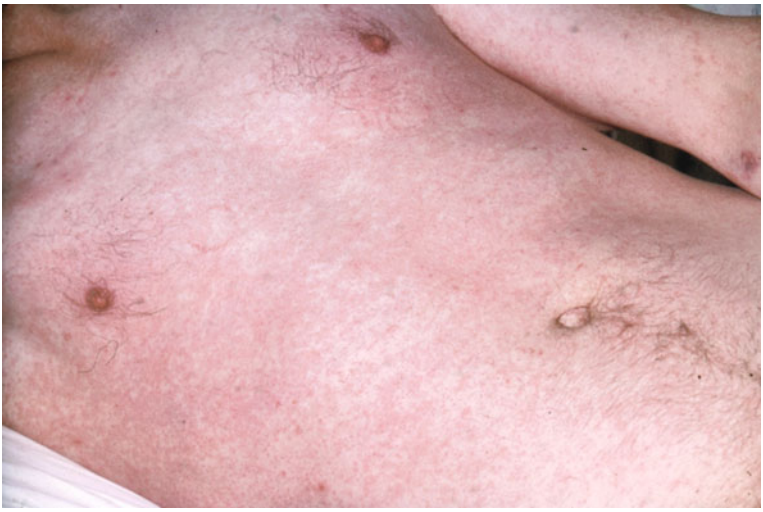

Fig. 1 Characteristic rash which was seen with all Marburg virus disease patients during the 1967 epidemic. Kindly provided by Gerhard Baltzer, Marburg, 1967

Fig. 2 Isolation ward of the hospital of the University of Marburg in 1967

It soon became clear that they all were employees of Behringwerke AG, a pharmaceutical company founded in 1904 by Emil von Behring. All of these patients had been involved in the production of poliomyelitis vaccine which relied on the use of primary simian cell cultures for propagation of the attenuated vaccine strains (Sabin 1–3). It soon became evident that concurrently four patients with similar

clinical signs were being treated at the university hospital in Frankfurt, Germany (Siegert et al. 1967, 1968; Martini et al. 1968a, b; Stille et al. 1968). These patients were employees of the Paul Ehrlich Institute, a governmental institution responsible for the approval of sera and vaccines. The Frankfurt patients had also been working with monkeys and with simian cell cultures that were needed for safety control procedures and standardization of poliomyelitis vaccines. In addition to these primary cases, four members of the hospital staff, two each in Frankfurt and in Marburg, acquired nosocomial infections. In another case of secondary infection, the spouse of one of the primary cases fell ill 67 days after her husband's disease resolved and, intriguingly, sexual transmission was supposed to be the route of infection (Slenczka et al. 1968; Siegert et al. 1968; Martini and Schmidt 1968). This is the first case described in the literature of a virus causing a systemic infection that was transmitted by the sexual route even several months after convalescence. The seminal fluids of nine other convalescents were tested for the presence of Marburg virus at the same time, but infectious virus or viral antigen was not found (Siegert and Slenczka 1971). Two female convalescents gave birth to healthy children about 18 months after they had survived the disease. No virus or virus antigen was found in the placentae or umbilical cords. Antiviral IgG, but not IgM, was found in the blood (Siegert and Slenczka 1971).

During the course of the disease, most patients developed hemorrhages varying in intensity from discrete petechiae to bleeding from needle puncture sites and massive bleedings from the gastrointestinal, respiratory, and urogenital tracts. A 39-year-old patient died of massive intraventricular hemorrhage. Eventually, five of the primary cases in Marburg and two of the cases in Frankfurt succumbed to the disease. The onset of massive hemorrhagic disease proved to predict an unfavorable outcome; none of the survivors developed severe hemorrhages (Martini et al. 1968a).

In addition to the cases in Marburg and Frankfurt, two cases occurred in Belgrade, Yugoslavia (now Serbia). The two patients in Belgrade, a veterinarian and his wife, worked at the Torlak Institute, an institution devoted to the production and safety control of poliomyelitis vaccines. The female patient was infected while caring for her husband. Both patients survived the infection (Todorovic et al. 1969; Stojkovic et al. 1971).

In 1982, we tested the serum of a man who claimed that he had the disease in 1967. Although he had been severely ill, he had been treated at home. He, too, worked in the cell culture lab at Behringwerke AG and had been exposed to infected simian cell cultures. Out of more than 120 contact persons who were retrospectively tested for Marburg virus antibodies, he was the only one who was seropositive, indicating that he had indeed been infected with Marburg virus. In summary, there were 26 primary and 6 secondary cases during the MVD outbreak in 1967. None of the secondary cases succumbed to the disease, but seven of the primary cases were fatal, amounting to an overall case fatality rate of 21.9% (26.9% for the primary cases only) (Slenczka and Klenk 2007).

In all three institutions, the common epidemiological denominator of the primary cases was contact with grivets (*Chlorocebus aethiops*), or handling monkey organs and cell cultures derived from these. The monkeys had been imported from Uganda and were used for the production of kidney cell cultures, to be used for the production of vaccines against measles and poliomyelitis (Hennessen et al. 1968).

3 Role of Ca^{++}-Loss in the Pathogenesis of Marburg Virus Disease

In the treatment of patients, the hemorrhagic disease was neither mitigated by blood transfusions, nor by treatment with coagulation factors (frozen plasma) or by applying thrombocyte concentrates. However, Martini noted that the drop in the concentration of plasmatic coagulation factors was not so remarkable as to explain the severe hemorrhagic diathesis (Martini 1971).

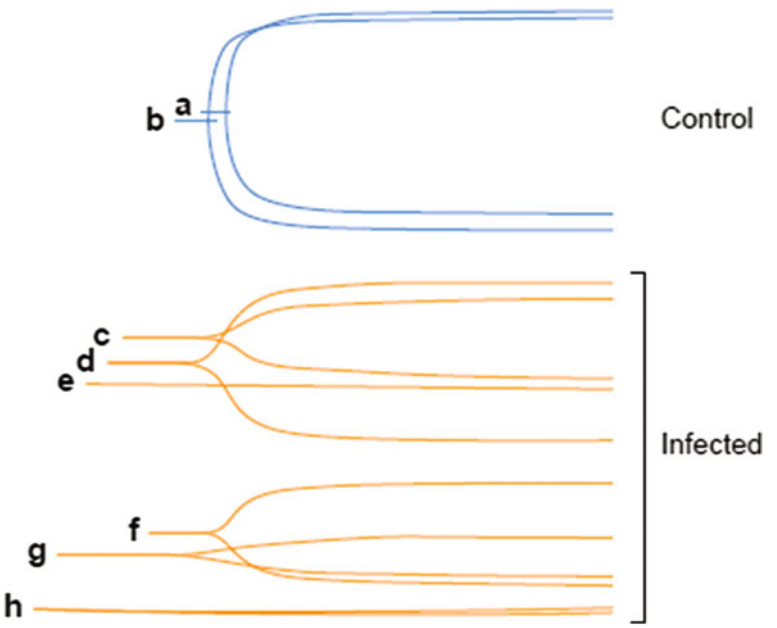

Fig. 3 Thromboelastograms (TEGs) performed with blood from one noninfected (*top*) and two Marburg virus-infected guinea pigs. *a* + *b* citrate plasma TEGs from a noninfected guinea pig. *c* − *h* TEGs from Marburg virus-infected guinea pigs on day 4 after onset of fever. *e* + *h* unmodified plasma TEGs without additional substances. *c* + *d* unmodified plasma TEGs with 0.1 ml (*c*) or 0.2 ml (*d*) of 0.1 mM solution of $CaCl_2$ per ml plasma; *f* + *g* citrate plasma TEGs with 0.1 ml (*f*) and 0.2 ml (*g*) 0.1 of mM solution of $CaCl_2$ per ml plasma. *Source* Egbring et al. (1971)

In case descriptions of the 1967 MVD outbreak, the reported symptoms include generalized paraesthesia, restless legs, sleeplessness in spite of fatigue, hyperesthesia of the skin and the feeling to "lie on crumbs". These symptoms are typical for tetany, a diagnosis, which was not verified and not even suspected at that time (Martini et al. 1968b). Tetany results when the concentration of Ca^{2+} in plasma is reduced to less than 50% of the normal value. Since ionized calcium is an important clotting factor (factor IV), tetany can be associated with spontaneous bleeding. But, where did the Ca^{++} go? Using von Kossa stain, Zlotnik (1969) and Korb et al. (1971) observed extravascular deposits of calcium in necrotic tissues of Marburg virus-infected guinea pigs and MVD patients.

In an early study on the coagulopathy in Marburg virus-infected guinea pigs, Egbring, Slenczka, and Baltzer found that on days 4 and 5 after onset of fever, coagulation was no longer detectable using thromboelastography. However, 50% of the coagulation capacity could be restored when ionized calcium ($CaCl_2$) at a final concentration of 10 mM was added directly into the reaction vessel (Fig. 3) (Egbring et al. 1971). It should be emphasized that restitution of clotting capacity by substituting calcium was performed in vitro using plasma from infected animals that contained less than 20% of clotting factors and platelets. Based on these results, MVD is the first disease in the literature for which it was shown that a consumption coagulopathy can be caused by loss of ionized calcium (Egbring et al. 1971).

4 Search for the Etiologic Agent

Diagnostic laboratory tests in search of the etiologic agent of the unknown disease were conducted in the clinical microbiology laboratories at the university hospitals in Frankfurt and Marburg. In Marburg, all this work was carried out by technicians using classical microbiology techniques and—at least initially—without awareness of the high risk. Masks and gloves were only used by laboratory scientists/technicians after the first patients had died. Laboratory infections did not occur and no seroconversions were found in these technicians.

The initial diagnostic arsenal included tests for salmonellosis, shigellosis, rickettsiosis, chlamydiosis, yellow fever, and many other infectious diseases. The negative results of these tests eventually convinced the medical staff that this was not a domestic but rather an exotic disease. Therefore, serum specimens were sent to 12 international laboratories known to be experienced in the diagnosis of zoonotic or tropical diseases. The focus of these investigations was predominantly on arboviruses and on bacterial and viral agents known to cause hemorrhagic fever. Serologic tests for antibodies against more than 80 arboviruses and other tropical viruses were negative (Siegert et al. 1968).

The serological tests lead to a single trace for a tentative diagnosis: Dr. L. Popp at the *Staatliches Medizinaluntersuchungsamt* (Governmental Medicinal Office of Investigations) in Braunschweig, Germany, an expert in leptospirosis, detected anti-leptospiral antibodies in some of the patient sera. Although this finding was not

unexpected in serum specimens from laboratory staff working with wild-caught animals, it served as an incentive to start a search for leptospira. Since the guinea pig model was routinely used for isolating leptospira, Dr. W. Mannheim, who was in charge of the bacteriology laboratory at the University of Marburg, inoculated guinea pigs with blood from one of the patients on August 22, 1967. The animals did not develop overt clinical disease but showed a moderate increase in temperature for 2–3 days. However, neither leptospira nor any other microorganisms were detected in the blood of these animals (Siegert et al. 1967; Slenczka et al. 1968).

Meanwhile, on August 24, four of the hospitalized patients, two in Frankfurt and two in Marburg, succumbed to the disease after showing signs of severe hemorrhagic shock. None of the patients had responded to any therapeutic measures. At this point, it had become clear that the patients suffered from an unknown agent causing severe hemorrhagic fever and that Dr. Mannheim had most probably transmitted this agent to guinea pigs. However, it also had become clear that none of the agents which were, at that time, known to cause hemorrhagic fever were involved (Siegert et al. 1967). In 1967, various bacteria, including members of the *Borrelia*, *Rickettsia*, and *Leptospira* genera, were known to cause hemorrhagic disease. The concept of viral hemorrhagic fevers was first introduced in 1948 by the virologist Čumakov (1948). Later, Daniel Carleton Gajdusek, who worked on the etiology of Korean hemorrhagic fever, postulated that Omsk hemorrhagic fever, Crimean Congo hemorrhagic fever, and hemorrhagic fever with renal syndrome were caused by at least three different viruses (Gajdusek 1962).

The death of four patients within 2–3 weeks after onset of the disease was a shock and raised concerns that an unknown agent with high pathogenicity might be distributed in the general population. Considering the high pathogenicity of the agent, the inappropriate biosafety conditions of the diagnostic laboratories in Frankfurt and in Marburg, and their location in the centers of these towns, it was decided that further diagnostic work in search of the etiologic agent should not be conducted in these laboratories but rather in institutions which had more experience and were better equipped for work with agents of extreme pathogenicity. Diagnostic specimens were sent to the Institute Pasteur in Dakar, Senegal, the Microbiological Research Establishment in Porton Down, Salisbury, UK, the CDC in Atlanta, GA, USA, the Middle America Research Unit, Balboa Heights, Canal Zone, Panama, the Poliomyelitis Research Foundation in Johannesburg, South Africa, and the Poliomyelitis Institute Moscow, Soviet Union (now Russia) (Siegert et al. 1968).

All these institutions had offered their help in search of the causal agent and were informed on the details of the clinical presentation and results of diagnostic tests, which up to that time had been carried out. Specifically, they were informed that guinea pigs developed a fever in response to infection. The material destined for the Microbiological Research Establishment was taken to London by Dr. Dick and then shipped to Porton Down.

Inclusion of a Soviet institute among those to receive specimens raised some concerns. At this time, there was a deep distrust between western and eastern countries due to the Cold War. Newspapers in the Eastern Bloc had already claimed that the events in Marburg and in Frankfurt were due to an accident in western

research facilities working on biological weapons. Shipping patient specimens to Moscow was meant as a confidence-building measure to show that there was nothing to conceal. Some years later, the strain isolated from this material, the Popp strain—the name refers to a Frankfurt patient—was used by Russian scientists for developing biological weapons (Leitenberg et al. 2012).

Three weeks later, in mid-September, the intensity and spread of the disease had dissipated. Sadly, two patients in Frankfurt and five in Marburg had succumbed to the disease, but the majority of the hospitalized patients had survived. Most of them had recovered and had been dismissed from the infection wards in a rather good physical condition. Transmission in the general population had not occurred and was no longer to be feared.

At this point, the guinea pig experiments were resumed to identify the unknown agent. In Marburg, Prof. Dr. R. Siegert, (head of the Institute of Hygiene and Medical Microbiology at Marburg University) and a research assistant in his laboratory, Dr. H.L. Shu started to inoculate guinea pigs with diagnostic material. Body temperature was monitored daily, and when the animals developed a fever, they were bled by cardiac puncture. Guinea pigs that were infected with the original material developed a fever on day 3 after inoculation without showing any other symptoms and survived the disease. However, guinea pigs, which were treated with passaged material (passage 3–5), became severely ill and had clinical signs similar to those observed in patients, fulfilling one of the Henle–Koch postulates (Koch 1890), later modified by Rivers for viral diseases (Rivers 1937).

Animals that had survived the disease were resistant to exposure with high passage material. Moreover, guinea pigs were protected from the disease by administration of convalescent sera from patients (Siegert et al. 1968).

These experiments were a step in the right direction, but the agent remained unidentified. To facilitate identification, blood from infected animals, taken at the climax of the disease, was mixed with glutaraldehyde and formaldehyde to inactivate and preserve the unknown agent and was sent to the electron microscopy (EM) laboratories at the University of Marburg and the Bernhard Nocht Institute for Tropical Medicine in Hamburg, Germany for analysis: Dr. D. Peters, head of the Virology Department at the Bernhard Nocht Institute, was a renowned virological electron microscopist and highly experienced in analyzing viral structures.

Of course, EM-based search for an unknown pathogen in biological materials can be extremely fatiguing and—in case of a negative result—very frustrating.

In the case of the guinea pig material, an additional obstacle became evident soon. A serious complication, often encountered in the search for unknown pathogens, is contamination by organisms unrelated to the disease. These “pick-up” contaminants may interfere with the etiological agent or may even cause disease themselves. Contamination may occur as a result of a preexisting infection. During passage to new animals, contaminants might be transferred with a higher efficiency than the unknown etiological agent. The risk of cultivating a contaminating agent may be reduced by using animals from an SPF (specific pathogen free) breed. In 1967, SPF guinea pigs could not be afforded in Marburg. Instead, the animals were

purchased from local breeding stations which did not control for infections. Therefore, in the experiments carried out by Drs. Siegert and Shu, it happened that microbial contaminations, including pseudomonads, pasteurellae, and in some cases paramyxoviruses were present in the guinea pigs. Although it was quite clear that these well-known organisms were not the etiological pathogen, the presence of these contaminants complicated data interpretation.

When Drs. Siegert and Shu were unable to identify the unknown agent, I came into play. Up to that point in time I had not been involved in the search for the causative agent for two reasons. First, I was a research assistant receiving my salary from the German Research Foundation for conducting research and not for performing diagnostic work. Second, I had a family at home with three little children and it was an accepted policy not to expose parents to the dangerous agent. During my time as a postdoctoral fellow in Dr. F. Lehmann-Grube's laboratory, I had developed a diagnostic assay for lymphocytic choriomeningitis virus based on immunofluorescence. Today, the reagents needed for this technique can be easily obtained from commercial suppliers in excellent quality. However, in 1967, although it was possible to purchase secondary antibodies, these reagents generally led to unsatisfactory results due to insufficient quality. Therefore, researchers usually prepared their own antibody reagents to fractionate immunoglobulins and to conjugate them with fluorescein isothiocyanate (FITC). This was not a simple task, as it was not easy to find a supplier offering coupling agent of satisfactory quality. I had spent several months adapting these techniques and was thus prepared to make use of immunofluorescence analysis as a tool to identify the unknown agent.

Taking convalescent sera from humans and from infected guinea pigs, as well as the corresponding negative controls, I prepared IgG-fractions, coupled the antibodies to FITC, and purified them from unbound FITC and from nonspecific binding substances to improve serologic specificity.

Organs from infected and noninfected guinea pigs, especially livers and spleens, were used to make imprint preparations on microscopic slides, which were then air-dried and fixed with ice-cold acetone. Since we did not know if the unknown agent would be killed by acetone, we handled these slides with extreme caution. It took 3 weeks of hard work before we had the first results. I found brilliantly fluorescent cytoplasmic inclusions in liver cells from an infected guinea pig. Since all the controls were negative, I was sure I had found antigenic structures of the unknown pathogen (Fig. 4). At this time, it was not yet possible to tell whether these inclusions, which resembled the Negri bodies found in rabies virus-infected cells (Goldwasser et al. 1959), were indicative of a viral or bacterial infection. However, it was clear that I had detected something that nobody had seen before; structures of an unknown agent causing a deadly disease (Slenczka et al. 1968).

Using this assay, it was now possible to identify those animals which were infected with this agent to select material for EM investigations. Once again, guinea pig blood treated with glutaraldehyde and formaldehyde was sent to the Bernhard Nocht Institute in Hamburg for EM analysis. Dr. D. Peters, together with a technician, analyzed negative stained material for more than a day but did not observe anything reminiscent of a viral structure. On the second day of his search, Dr. Peters

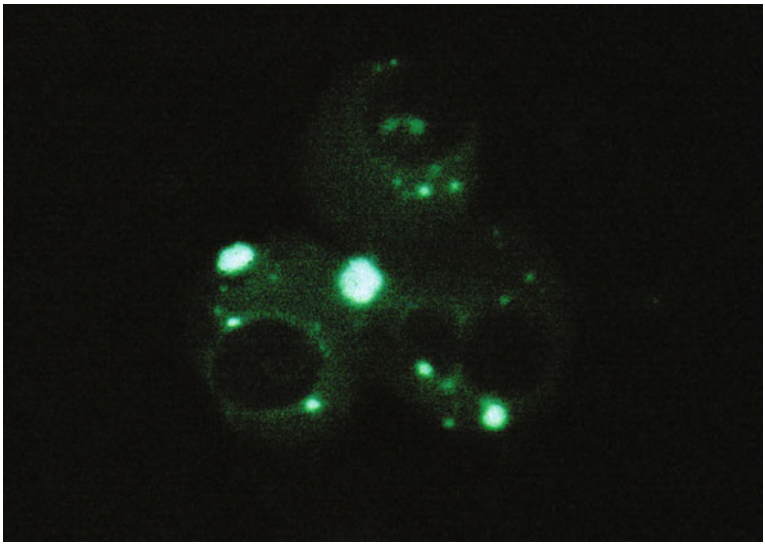

Fig. 4 Immunofluorescence analysis showing liver cells from a guinea pig infected with Marburg virus. Immunofluorescence analysis was performed using FITC-conjugated immunoglobulins derived from a Marburg virus disease survivor. Marburg virus forms large inclusions in the cytoplasm of the infected cells. W. Slenczka, October 1967 (Siegert et al. 1967, 1968)

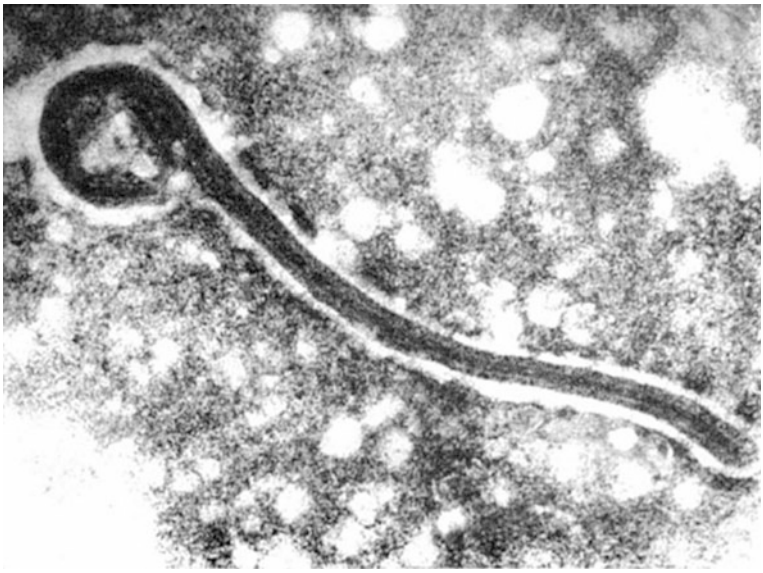

Fig. 5 First electron micrograph of a Marburg virus particle. Picture taken on November 20, 1967 by G. Müller, Bernhard Nocht Institute, Hamburg, Germany at 60,000 magnification (Siegert et al. 1967, 1968; Brauburger et al. 2012)

left the laboratory for a lunch break and handed the specimen to his coworker, Dr. G. Müller, asking him to continue the search. In less than an hour, Dr. Müller had succeeded in finding viral particles that, due to their sizes and unique morphologies, were identified as the products of an unknown virus (Fig. 5). When Dr. Peters returned from his lunch break, Müller showed him the new virus. It is not clear why Dr. Peters had not found the viral particles when he examined the samples. The most probable explanation seems to be that the particles had spontaneously sedimented to the bottom of the tube and Dr. Peters took material from the top only.

5 History of Publication

On November 27, 1967, press conferences were held in Marburg and Hamburg to announce the identification of the etiological agent, which had caused the "monkey disease". At this time, the virus was named "Marburg virus" as a reference to the town, where the greatest number of cases had occurred and to the place where the causative agent had been isolated and identified.

The first scientific communication on the isolation and identification of the Marburg virus was made at the IV Congreso Latinamericano de Microbiologia in Lima, Peru held from November 26 to December 2, 1967. This paper was published in the proceedings of this congress (Siegert, R., Shu H.L., Slenczka, W., Peters, D., and Müller, G. Detection of the so-called green monkey agent.). Three weeks later, on December 21, 1967, the groups in Marburg and Hamburg published a detailed report on the discovery of the Marburg virus in a German medical journal (Siegert et al. 1967) with the English translation following on January 1, 1968 (Siegert et al. 1968). Reprints of these papers containing the first electron micrographs of the new virus were sent to all the groups and institutions that had taken part in the efforts to isolate this agent and also to WHO (1968).

On November 29, 1967 the group in Porton Down published their results in a Lancet paper (Smith et al. 1967) in which they suggested that a member of the *Rickettsia* or *Chlamydia* genera was the etiologic agents of the "vervet monkey disease". In addition, it was stated in this paper that "our virological findings were negative" (Smith et al. 1967). Although Smith and colleagues did not identify the causative agent of MVD, their paper unfortunately has been frequently cited as the first report describing the isolation of Marburg virus.

During the first 6 months of 1968, six groups published confirmations of our findings (Kissling et al. 1968; Kunz et al. 1968; Strickland-Cholmley and Malherbe 1970; May et al. 1968). While some acknowledged the first description of Marburg virus by Siegert and colleagues, others did not.

The researchers who deserve credit for isolating and identifying Marburg virus are Walter Mannheim, University of Marburg for successful transmission to guinea pigs, Werner Slenczka, University of Marburg for detecting and identifying the Marburg virus antigen by immunofluorescence analysis, and Gerhard Müller,

Bernhard Nocht Institute for identifying the virus by EM. Walter Mannheim, a bacteriologist, was uninterested in co-authoring publications despite his involvement in the virus isolation.

6 The Monkeys

Originally, during the first decades of the nineteenth century, Indian rhesus monkeys (*Macaca mulatta*) were the preferred nonhuman primates (NHPs) used in biomedical research. However, when monkey kidney cells were needed for vaccine production, it was clear that these monkeys were not convenient because the members of the *Macaca* genus are natural hosts of herpes B virus (Sabin and Wright 1934). Therefore grivets, which were believed to be free from viruses pathogenic to humans, were used as a source to prepare kidney cell cultures for vaccine production. The attenuated vaccine strains of poliovirus (Sabin 1–3) could only be propagated in primary monkey kidney cells. Therefore, grivets were imported in great numbers from Eastern Africa to be used for the production and also for the safety testing of poliovirus vaccines.

Grivets are part of a group colloquially often referred to as "African green monkeys". African green monkeys were previously classified as a single species (*Cercopithecus aethiops*). This classification has since then been revised repeatedly and the species was split into at least six species (Groves 2001). Grivets (*C. aethiops*) are endemic in Ethiopia, Eritrea, and the Sudans. In 1962, about 25,000 grivets were exported from Uganda to a number of countries for biomedical purposes, mainly for vaccine production. Due to loss of habitat and being hunted as a source of meat, they are now endangered. In 1967, there were no restrictions or regulations for the export or import of these monkeys.

The grivets which played a role in the 1967 MVD outbreak were exported from Entebbe, Uganda by F.R. Mann, a relative of the famous German novelist Thomas Mann. They were imported to Germany by the animal transportation company Samen-Eckers, Viersen, Germany. Normally, these animals were transported by Lufthansa on a direct flight from Entebbe to Frankfurt or to Düsseldorf, Germany. Before leaving Entebbe, a certificate of good health from the Ugandan veterinary office was needed. In 1967, however, due to the Six Days War (June 5–10, 1967), the direct Lufthansa flight was discontinued and therefore the monkeys had to be transported via London Heathrow to their German destinations. Usually, the shipments to Germany included approximately 100 monkeys. Due to technical problems, the animals stayed in London for 9–46 h before they were shipped to Germany. During this time the monkeys were transported to an animal house of the Royal Society for Prevention of Cruelty to the Animals (RSPCA) outside the airport area. This was allowed under condition that they would not have contact with other animals and especially not with rhesus monkeys. However, on one occasion the grivets were kept overnight in a room together with two Hanuman langurs (*Semnopithecus* sp.) from Ceylon (now Sri Lanka). One of the langurs became sick

and died soon after, but the cause of its sickness and death was not disclosed. During another transport, grivets were caged in a room together with finches of non-European origin and with other animals. The circumstances of these stays in London and the contacts with animals from other continents caused considerable difficulties in the search for the geographic provenance of the agent of disease. On one occasion, two monkeys escaped during the transport to Germany on June 28, 1967. Luckily, they monkeys were captured quickly and arrived at Düsseldorf airport on June 29. Several journalistic conspiracy theories arose from this event.

Mr. Mann from Entebbe insisted on the validity of the Ugandan health certificate: "presumably a document of doubtful quality" (Hennessen). He also stated that a similar disease had never occurred among the 500 Ugandans he employed as monkey trappers. Later, however, it became known that there had been an epizootic event with many dead monkeys in the trapping areas around Lake Kyoga. The monkeys were captured in three places: Namasale, Kidera, and Ndolwa, prior to transport to Entebbe. With rare exceptions, the monkeys were held in individual cages during their stay in Uganda. As a rule, animals that would not thrive or showed other signs of sickness were shipped to a "Monkey island" in Lake Victoria where they were set free to live or to die. Whenever there were not enough monkeys to complete an order, the trappers would go to the "Monkey island" and would capture some healthy looking monkeys and bring them to Entebbe.

The final proof that Africa was the origin of Marburg virus was not obtained until 1975, when a 20-year-old Australian man was admitted to a hospital in Johannesburg, South Africa, where he was diagnosed with MVD and later died. His 19-year-old female travel companion and a 20-year-old nurse caring for both patients acquired secondary infections. Both women survived the infection and recovered (Gear et al. 1975). The Australian travelers had been hitchhiking in Rhodesia (now Zimbabwe). The source of their infection was never determined.

The number of monkeys imported to Germany was negligible before 1960. Beginning that year, air transportation facilitated the import of up to 5,000 animals per year for biomedical research and for vaccine production, mainly rhesus monkeys and grivets. At the Paul-Ehrlich Institute in Frankfurt monkeys were euthanized on two days per week, at Behringwerke AG in Marburg, on five days. On August 21, 1967, 18 monkeys were euthanized at on two days per week Behringwerke AG for diagnostic purposes. Postmortem specimens were collected and sent to the Institute of Hygiene in Freiburg, Germany, to the Institute of Hygiene in Vienna, Austria, to the Poliomyelitis Research Foundation in Johannesburg, South Africa, and to the Institute Pasteur in Dakar, Senegal. During this activity, a 39-year-old veterinarian was infected in Marburg and succumbed to the infection on September 3.

On August 25, 1967, per governmental order all the remaining monkeys at Behringwerke AG were killed: 235 rhesus monkeys and 266 grivets. Out of the latter group, 110 were being kept in a quarantine station outside the facility and 156 animals were held in a house in the area of the facility. None of the individuals who cared for the monkeys and cleaned the cages got the disease. The animal care takers used gloves and face masks resembling those which are worn for welding work and these are not aerosol-tight. Only animal care takers and laboratory workers who had

contact with blood or organs of the monkeys were at risk of getting the disease. Three men who opened the skulls of killed monkeys to remove the brains were infected. This work was done without personal protective equipment (PPE) since the monkeys were believed to be healthy. Animal handling included to capture the monkeys with a net and to anesthetize them by electric shock, to exsanguinate the animals by opening the carotid artery, to fix the monkeys on the table, to perform ventral nephrectomies, to perform necropsies, to remove the cadavers from the table, and to transport them to the incinerator. PPE was mandatory for all these activities. For capturing and anesthetizing the monkeys as well as for the nephrectomies protective gowns and sterile masks were used in addition. Other employees were most likely exposed to the virus when they transported the kidneys to the cell culture lab or during the necropsies. On August 29, it was suspected that kidney cell cultures derived from these monkeys were infectious and therefore all remaining cell cultures, including 1000 glass vessels and 9000 glass tubes were inactivated by autoclaving. A 19-year-old student who worked at Behringwerke AG during the summer break accidentally broke one of the tubes, was infected with Marburg virus, and died on September 10. Three employees, one male, two females, were infected by contact with cell cultures. Most of the female primary cases were infected by cleaning the glassware used in the lab.

Summarizing the use of protective measures, it can be concluded that PPE was exclusively directed toward protection of monkeys, monkey organs, and cell cultures from contamination. Since the monkeys were regarded to be healthy, PPE was not used when skulls were opened, when cadavers were discarded, or when glass vessels were cleaned (Hennessen et al. 1968). Hennessen and coworkers published a thorough analysis of the sources of infection for the employees in Frankfurt and in Marburg and came to the conclusion that a small number of animals from transports that had arrived on July 21 and July 28 must have carried the virus (Hennessen et al. 1968) (Table 1). The last case that occurred in Frankfurt, however, must have been infected by contact with a monkey that had arrived on August 10.

Table 1 Human cases resulting from direct or indirect contact with monkeys from two shipments

Shipment arrival	7/28	Day 11	Day 19	Day 20	Day 21	Day 22	Day 24	Day 25	Day 31
First day of illness		8/8	8/16	8/17	8/18	8/19	8/21	8/22	8/28
Direct contact		1	3	1	2	1	2	2	1

Shipment arrival	7/21	Day 32	Day 38	Day 41	Day 49
First day of illness		8/22	8/25	8/28	9/5
Direct contact			1	2	
Indirect contact		1		2	1

The time between arrival of the shipments and the first day of reported illness of patients is indicated. Direct contact indicates contact with monkey blood or organs. Indirect contact indicates exposure by handling cell cultures or cleaning contaminated glassware. Table 1 was compiled based on data published in Hennessen et al. (1968)