Kevin L. Williams *Editor*

Endotoxin Detection and Control in Pharma, *Limulus*, and Mammalian Systems

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bioMérieux Inc.
Durham, NC, USA

ISBN 978-3-030-17147-6 ISBN 978-3-030-17148-3 (eBook)
https://doi.org/10.1007/978-3-030-17148-3

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

The past 10 years have brought an increased intensity of progress in the sciences surrounding drug development, manufacturing, and endotoxin detection and control. A subtle change has been occurring since the advent of recombinant technology and the production of modern biologic drugs. As fit with increasing immunologically based understanding of disease causation, these drugs have transformed the therapeutic landscape. More and more, biologics make up the predominate bulk of drugs sold. However, they are costly to develop, expensive to produce, and have brought concerns that are of an immunological origin. The explosion of immunological knowledge has complicated the longstanding knowledge base of endotoxin detection and control.

For each generation, the modern zeitgeist proposes that only small gaps in knowledge remains, thus downplaying the bewildering complexity of nature so that it can be comfortably subsumed into various models. This provides many false handles that can be held onto past their utility. Consider how recently some very rudimentary knowledge has been established. It has been less than 30 years since Janeway proposed *the very idea* of the pattern recognition receptor (PRR) and today ten functional Toll-like receptors in humans have been thoroughly characterized (13 total in mammals) and the interaction of these receptors in immune responses have been elaborated along with additional families of PRRs as they detect a growing list of PAMPs that cover a wide range of the prokaryotic realm. This is the long list of prokaryotic artifacts that are viewed as "contaminants" from the metazoan perspective, not infectious but rather perceived as "*symbols of infection*." Endotoxin has always been at the crossroads of progress in that it is among the most potent stimulators of both innate and adaptive immune systems, by far the most prevalent, and resistant to deactivation, as well as, historically, in the vanguard of discovery in terms of mechanism of action in various immune milieu. But this utility should not stifle efforts to detect additional PAMPs to expand existing control capabilities.

Endotoxin has continued to be characterized in recent years from the initial knowledge as to how it is detected by the invertebrate (*Limulus*) serine protease cascade and mammalian (MD-2/TLR4) receptor complexes. Progress is being made toward the ability to turn off or block endotoxin's deleterious physiological effects

in sepsis, although this hope seems to rise and fall again every few years. And conversely, for vaccine purposes, detoxified forms of endotoxin have been made to utilize its ability to stimulate the immune system as an adjuvant. This has led to its being used as the first new (FDA approved) adjuvant in many decades. The growing knowledge of Toll-like receptors is feeding into vaccinology (so-called reverse vaccinology), immunology, as well as drug development and contaminant control. In this way, endotoxin, which has always been a bane to humans, is now routinely serving as another tool of progress.

Of course, endotoxin itself has not changed as a constituent of the Gram-negative bacterial (GNB) outer membrane (OM). But we will see that some perspectives here are also subtly changing. GNB have been among the most destructive of lifeforms to man in despoiling food, in contaminating water, and in bringing disease including plague, typhus, cholera, and various antibiotic-resistant infections. These bacteria are enabled by a thick outer membrane that contains endotoxin as a major and enduring functional component, and the bacteria work to maintain the structural asymmetry of this outer membrane for reasons that will become clear. Here, we will view new insights into the other important functional components of GNB membranes that have historically been overshadowed by endotoxin.

The integration of expanding scientific knowledge into highly regulated platforms is a challenging endeavor. Compliance, the meeting of current Good Manufacturing Practices (cGMP), is a strange topic to some scientists, a mixture of law and standardization and quality assurance that is a necessary jungle that must be traversed in order to float a new product upon the river of commerce. The concepts of "adulteration" and "483" bring fear of monetary ruin. After all, to submit a new drug application (NDA) or biologic license application (BLA) for review is to invite the most invasive form of scrutiny (regulatory). Compliance, therefore, is as integral to the practice of drug manufacturing as are the various sciences.

Laboratories all over the world today use a blood (hemolymph) reagent (*Limulus* amebocyte lysate or LAL) very much like the one developed initially by Dr. Jack Levin (Chap. 1) and new versions of a recombinant form of the *Limulus* biosensor molecule, Factor C, are also being used to ensure the safety of drugs, infusion solutions, and medical devices. New drug types include nanoparticles and increasingly new forms of biologics including antibodies, bispecific antibodies, half chain antibodies, Fab fragments, scFv, affibody, and other formats (Chap. 8). In terms of numbers of molecules and especially in dollar volume of sales, biologics are increasingly dominant.

The interaction of two parallel but overlapping immune systems (innate and adaptive) in terms of architecture and mechanism of action is still being elaborated. Yet, there is by far enough to know the generalized mechanisms of PRR detection and responses to microbial insult including cytokine production and lymphocyte clonal expansion. The modern view is that the older innate immune system signals control the adaptive immune response via costimulatory and coinhibitory receptors. The diversity of LPS structural variants and the variety of host responses provide any student of life sciences a detailed model for understanding the evolution of molecules (ancient to modern) in the context of immune system evolution. Historically, the adaptive immune system has not been included in microbiological control efforts; how-

ever, this is an emerging vantage of lipopolysaccharide (LPS) and other pathogen associated molecular patterns (PAMPs) as immune modulating substances that contrasts the singular historical view of them as *only pyrogens*.

The text includes chapters from world-renowned experts from a wide range of institutions where a significant focus includes endotoxin detection and control.

Part I, Chaps. 1 to 16, focuses on pharma systems, including LAL historical development, the history of drug regulation (often, as associated with various tragedies), LPS structure/function/heterogeneity, as well as the relevance of endotoxin in the microbiological context of the GNB cell. Many core topics of pharma detection and control include: disinfection and depyrogenation, practical *Limulus*-based testing, emerging immune context, the biologics drug revolution, endotoxin process removal, data integrity, low endotoxin recovery, nanoparticle testing, the recombinant factor C test, the monocyte activation test (MAT), and an advanced testing chapter that overviews some prospective test methods.

Part II, Chaps. 17, 18, and 19, covers *Limulus*-based mechanisms of detection and control, including the use of *Limulus* as a model organism, the detailing of its endotoxin-sensitive serine protease cascade, some comparisons of blood protein domain homology as shared between humans and other mammals with *Limulus* (as shared at the early protostome/deuterostome split), and the man-made use of *Limulus*-derived antimicrobial proteins for human therapeutic purposes.

Part III, Chaps. 20, 21, 22, and 23, focus on *mammalian* systems, including the body's canonical and non-canonical mechanisms of detection and control: the complement system, TLR4, and the partitioning of TLRs as used differentially in various tissues. The final three chapters cover sepsis diagnostic tools, pathogenesis, biomarker utility, and routine and advance sepsis treatment options.

One can feel the excitement today as scientific knowledge is coalescing (approaching the asymptote as Janeway said) and can be amassed from many sources for our interaction, not just with living nature, but with man's ideas and abstractions of her underlying mechanisms. Satisfaction comes from piecing together the whole, in viewing the interconnectedness of the various traditional and emerging sciences (genetics, evolutionary biology, immunology, microbiology, protein chemistry, etc.) as well as the evolutionary gradient of mechanisms of detection and control. Great scientific ideas have a kind of lingering resonance (like Beethoven's *fur Elise*), at first otherworldly, and then familiar and soothing as in providing a solid base for increasing personal understanding. The ideas of Pasteur, Pfeiffer, Darwin, Watson, Crick, Levin, Bang, Lewis, Nüsslein-Volhard, Janeway, Medzhitov, Beutler, Hoffmann, Burnet, and many others color this story as centered around one specific vantage: *the applied science of endotoxin detection and control*.

Durham, NC, USA Kevin L. Williams

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Author Biography

Kevin L. Williams graduated from Texas A&M University in 1982 with a BS degree in Microbiology. He worked for over 30 years at Eli Lilly and Company in Indianapolis developing microbiology and endotoxin assays and detection technology in the quality control laboratory. Since then, he has worked as a Senior R&D Scientist at Hospira (now Pfizer), Lonza, GE Water, and bioMerieux. He has authored two previous books on endotoxins (2nd and 3rd editions) and one book on parenteral manufacturing contamination control. In addition to publishing papers in industry journals, he is a frequent speaker at endotoxin-related pharmaceutical industry events.

Part I
Pharma

Chapter 1
Discovery and Early Development of the Limulus Test

Jack Levin

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1.1 Introduction

In 1956, Dr. Frederik B. Bang, a Professor at the Johns Hopkins University School of Hygiene and Public Health, reported his studies of the effects of bacterial infection in *Limulus polyphemus*, the horseshoe crab in the no longer published Bulletin of the Johns Hopkins Hospital [1]. Dr. Bang had undertaken these studies because of his observation that one horseshoe crab had become ill after it had been injected with bacteria present in sea water, which is known to contain significant numbers of Gram-negative bacteria. He noted that due to the infection the blood of the animal had subsequently become uncoagulable, in marked contrast to the coagulation that is uniformly observed when blood is removed from *Limulus*. He cultured the bacterium from this animal and when reinjected into other *Limuli*, this bacterium, which was shown to be a Gram-negative rod, caused intravascular coagulation and death. The same effect was produced by a heat stable extract of this bacterium, prepared by heating and disrupting the bacteria. This technique is the traditional method for the production of crude bacterial endotoxin. He also noted that the level of circulating amebocytes was reduced in these animals, and that the amebocytes were degranulated [1]. Pertinently, these effects were not produced by extracts of pneumococci,

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K. L. Williams (ed.), *Endotoxin Detection and Control in Pharma, Limulus, and Mammalian Systems*, https://doi.org/10.1007/978-3-030-17148-3_1

staphylococci or streptococci, all of which are Gram-positive bacteria. The similarity between the reaction observed in Limuli and the generalized Shwartzman reaction, which is caused by multiple injections of bacterial endotoxin, was obvious.

Bang's initial observations were put aside for almost 10 years, until 1963, when Dr. Bang discussed his data with Dr. C. Lockard Conley, then the director of the Hematology Division at The Johns Hopkins University School of Medicine and Hospital. Dr. Bang and Dr. Conley decided that if a hematologist carried out investigations with Dr. Bang, an effective collaboration would result. I was then a Research Fellow in Dr. Conley's hematology division, in the midst of carrying out experiments based on the Shwartzman reaction and also studying platelet function. Because of my interests in these two areas, and the effects of bacterial endotoxin on platelets and blood coagulation, Dr. Conley suggested that I join Dr. Bang for a Summer of research at the Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts, which I did in 1963, never having previously heard of, much less seen, a horseshoe crab. Experiments were initially designed to determine similarities between *Limulus* amebocytes, the only type of circulating blood cell in the horseshoe crab (Fig. 1.1), and human platelets.

1.2 Early Observations and Test Development

I was quickly able to demonstrate that cell-free plasma from *Limulus* would not clot [2], but learning more about amebocytes and coagulation initially proved difficult because samples of blood which were liquid when I left the laboratory in the evening were solidly clotted by the next morning. None of the standard anticoagulants used to prevent the coagulation of human blood, i.e., sodium citrate, EDTA, or heparin, were effective in preventing *Limulus* blood from clotting [2, 3]. Puzzled by this, I considered the possibility of contamination of the samples either by bacteria or a bacterial component, and when samples were drawn into sterile and pyrogen-free

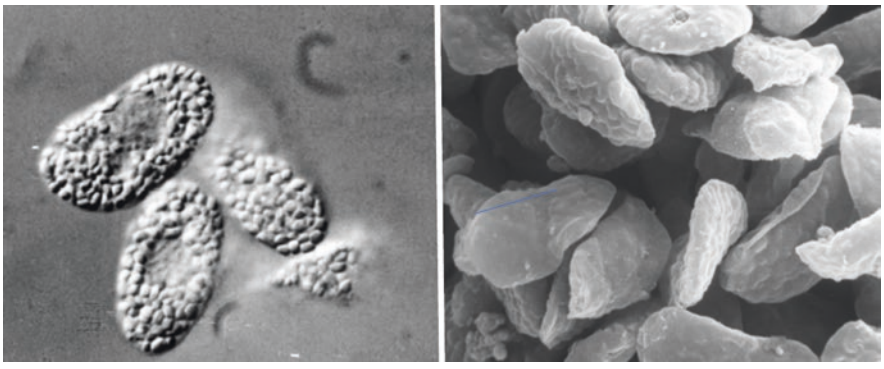


Fig. 1.1 Left: Three amebocytes, which are the size of macrophages (Nomarsky optics). Right: Amebocytes (scanning electron microscopy). Note that the nucleated amebocytes are flat discs, packed with granules

glass tubes, the latter being considered only because of my ongoing studies of the effects of bacterial endotoxin on blood coagulation, *Limulus* blood did not clot. We were then able to demonstrate that the coagulation system, or at least some of its components, were released from amebocytes [2]. Importantly, *Limulus* plasma which contained cellular components, i.e., factors derived from the circulating amebocytes, could be gelled by bacterial endotoxin, using either a heat stable component from the Vibrio species that Dr. Bang had isolated in 1957 or *E. coli* endotoxin. An extract of a pathogenic Gram-positive marine bacterium had no effect [2]. The rate of coagulation was dependent on the concentration of endotoxin, and we suggested that an enzymatic mechanism was involved [2]. Importantly, it was then demonstrated that the entire blood coagulation mechanism of *Limulus* was contained in the amebocytes [2]. Dr. Erik Murer and I subsequently determined that the granules of amebocytes contained the entire blood coagulation mechanism of the horseshoe crab [4].

In 1965, after having completed my two-year hematology research fellowship (1962–1964) and having been the Chief Resident in Medicine (1964–1965) at the Yale-New Haven Medical Center in New Haven, CT, I became a member of the faculty of the Hematology division at Hopkins. I returned to Woods Hole in the Summer of 1965, the second of many Summers at the MBL, where I subsequently served as member of the Board of Trustees (1988–1993). I then used N-ethylmaleimide (NEM) to prepare washed amebocytes, from which lysates, free of any plasma factors, could be prepared [5]. The conditions for this step were critical, since it was necessary to not only prevent the amebocytes from aggregating but to then be able to successfully wash and lyse them.

During this period, we described the preparation of *Limulus* amebocyte lysate and the basic characteristics of the Limulus amebocyte lysate (LAL) test for bacterial endotoxins [5]. Fig. 1.2 shows the author bleeding an adult horseshoe crab. Note the total absence of any of the now required conditions for the bleeding of horseshoe crabs for the preparation of GMP certified lysate. It should also be emphasized that bleeding was performed in a standard research laboratory, with the windows usually open, no temperature controls, and high humidity as the result of sea water continuously running into an open tank and splashing onto the laboratory floor. Despite these conditions, the author bled hundreds of animals without ever producing a contaminated batch of amebocytes, which provided the sole basis for a highly sensitive test for bacterial endotoxins.

The gel clot endpoint is shown in Fig. 1.3. The change from a clear liquid to an opaque gel is obvious. Interestingly, although this gel is very stable if undisturbed, if the tube is shaken, the gel will not reform but rather becomes a viscous mass of similarly opaque particulate material. Since the rate of the reaction between bacterial endotoxin and *Limulus* amebocyte lysate is dependent upon the concentration of endotoxin, one can quantitatively determine the concentration of endotoxin by measuring the rate of increase in optical density (Fig. 1.4) [5] or light scattering (Fig. 1.5) [6]. These three initially described endpoints remain the basis for the endpoints currently in commercial use. The insert in Fig. 1.4 established that the reaction between bacterial endotoxin and amebocyte lysate was enzymatic. Subsequent studies from our laboratory elaborated

Fig. 1.2 Dr. Jack Levin bleeding a horseshoe crab in his laboratory at the Marine Biological Laboratory, Woods Hole, MA (circa 1970). Blood flows via a needle directly from the cardiac sinus into an endotoxin-free siliconized flask which contains a warmed solution of N-ethyl maleimide (NEM). NEM prevents the amebocytes from aggregating. *Limulus* blood is blue because it contains a high concentration of hemocyanin, the oxygen carrying protein in the plasma

the enzymatic nature of the reaction [7–9]. Ours was the first report of an enzymatic blood coagulation mechanism in an invertebrate and it was established that serine proteases played an important role in the enzymatic reaction [10].

Critical to the acceptance of a new bioassay is the demonstration that the assay, e.g., the Limulus test, correlated with other established assays for endotoxin. Although this was eventually demonstrated (Table 1.1), some skepticism remained because the much greater sensitivity of the Limulus amebocyte lysate (LAL) test in comparison to the other available bioassays made it impossible to directly compare the results of the LAL test with other assays at the lowest concentrations of endotoxin which LAL could detect.

LAL was prepared by the vigorous disruption of washed amebocytes with unbuffered distilled water. We thoroughly investigated whether LAL was gelled by β-glucans and it was not [11]. The specificity of LAL as prepared in my laboratory is emphasized because subsequently, it was observed that LAL prepared commercially was sensitive to β-glucans. The commercial methods for preparation of amebocyte lysate involve the use of buffers and other additives, and the proprietary nature of the various commercial methods makes it impossible to learn which additives or perhaps

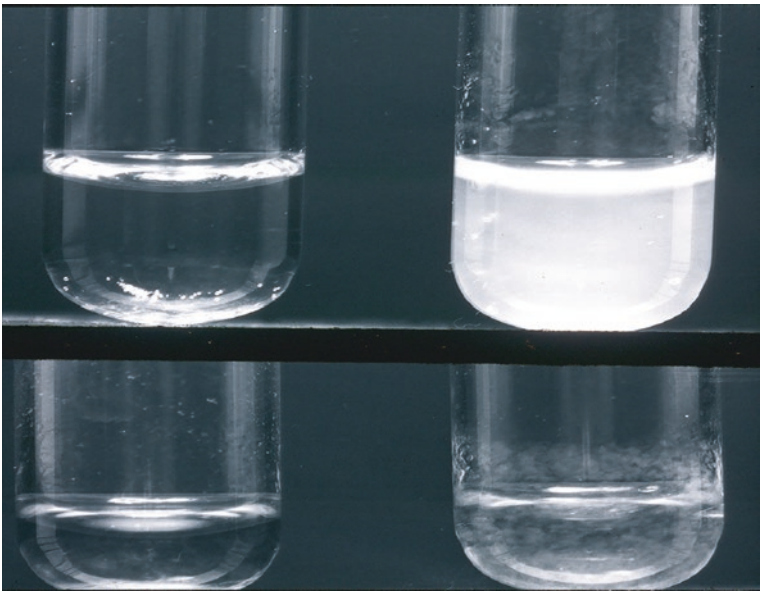


Fig. 1.3 Limulus amebocyte lysate (Gel clot endpoint). Left: Controls Right: + Endotoxin. The higher concentration of endotoxin (upper right) produced a solid gel, whereas the lower concentration produced only flocculation

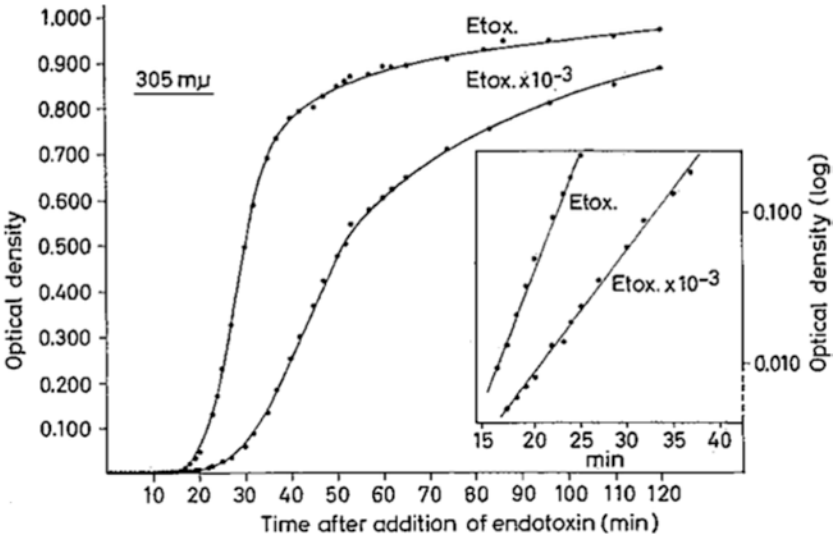


Fig. 1.4 Increase in optical density after addition of different concentrations of *E. coli* endotoxin. The rate of increase in optical density was dependent upon the concentration of endotoxin. The semi-log plot (insert) indicates that the reaction between Limulus amebocyte lysate and endotoxin is enzymatic. (From Ref. [5], with permission.)

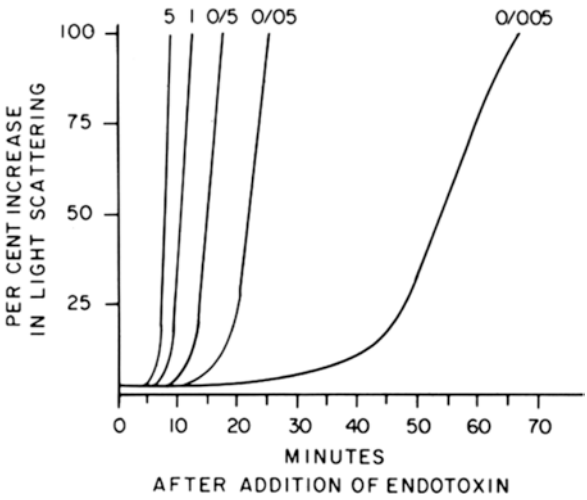


Fig. 1.5 Rate of increase in light scattering after addition of bacterial endotoxin from *E. coli*. The concentration of endotoxin is shown in μg/ml. As the concentration of endotoxin decreased, the rate of increase in light scattering decreased. (From Ref. [6], with permission.)

Table 1.1 Correlation of the Limulus test with other biological assays for bacterial endotoxins

Pyrogenicity
Mitogenicity
Complement activation
Chick embryo lethality
Dermal Shwartzman reaction
Tissue factor generation

pH adjustments resulted in Limulus amebocyte lysate becoming sensitive to β-glucans via active Factor G (Fig. 1.6). It is noteworthy that unbuffered LAL, as prepared in my laboratory, remains stable in liquid form for many years.

1.3 Official Approval of the Limulus Amebocyte Lysate Test by Federal Agencies

Since the rabbit pyrogen test (RPT) was the well-established official standard for the detection of bacterial endotoxins, a comparison of the LAL test with the RPT was mandatory if the Limulus test was going to be accepted. Thus, such a study was performed by James Cooper, then a graduate student at Hopkins, and myself [12]. This demonstrated that the results of a LAL test could be effectively compared with the RPT (Fig. 1.7). Cooper subsequently introduced the Limulus lysate assay to the FDA. An important major comparison of the Limulus and rabbit pyrogen tests by Mascoli and Weary clearly demonstrated the superiority of the Limulus test, which did not produce a single false negative in almost 30,000 tests, in addition to being much more sensitive than the RPT (Table 1.2). It is pertinent that the period during which these early studies were performed preceded the establishment of endotoxin

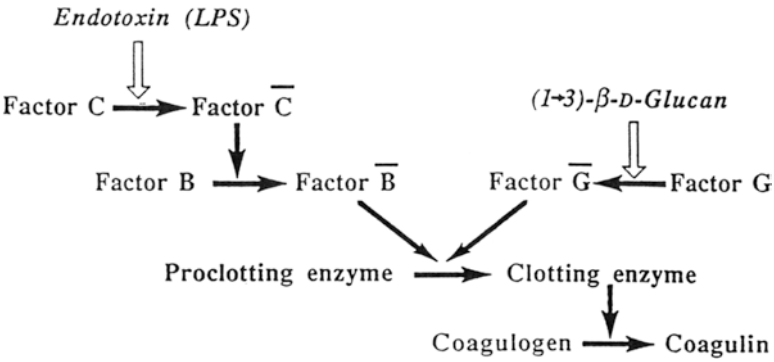


Fig. 1.6 Blood coagulation in Limulus is enzymatic. Serine proteases constitute the coagulation cascade. Factor C is remarkably sensitive to bacterial endotoxins. Factor G is activated by β-glucans

standards, and thus the official Endotoxin Unit (EU) did not come into existence until after the LAL test became available. In many respects, it was the LAL test which made necessary the establishment of an international endotoxin standard based on biological activity. Prior to this, endotoxin concentrations were described by their weight. Since the biological activity of a given weight of endotoxin depends at least in part on the intrinsic biological activity of the specific endotoxin and its method of preparation, it had been difficult to compare the results of experiments, performed in different laboratories, in which endotoxin was used. However, this problem had been partially managed by the common use of endotoxin from E. coli O55:B5 in many research laboratories.

Multiple steps were required to define the methods for the use of the LAL test for release of parenteral drugs and intravenous fluids. Extensive discussions of the approved use of the LAL test occurred at an international conference in Woods Hole, MA in 1981 [13–15]. Although the Limulus test was adequately described in 1965, it required almost 20 years for it to be formally announced as being validated as an end-product endotoxin test in the Federal Register (Fig. 1.8). Another more recent summary of the steps required for the validation of the LAL test was published in 2003 [16]. By 1984, there were 8 FDA licensed *Limulus* lysate manufacturers in the U.S. An amebocyte lysate made in Japan (TAL) from the Asian horseshoe crab, *Tachypleus tridentatus* was also licensed. However currently there are only 4 licensed commercial sources. A brief history of the commercialization of the Limulus test is available [17]. A detailed summary and overview of the sequence of these events is provided in Table 1.3.

An important early impact of the *Limulus* test was that for the first time, it was feasible for research laboratories to determine if their reagents had biologically significant concentrations of endotoxin. This was especially important when experimental models were susceptible to the effects of endotoxin. This was often the case [18, 19]. Thus, data from such experimental models were seriously flawed. Importantly, the availability of a highly sensitive and feasible test had an

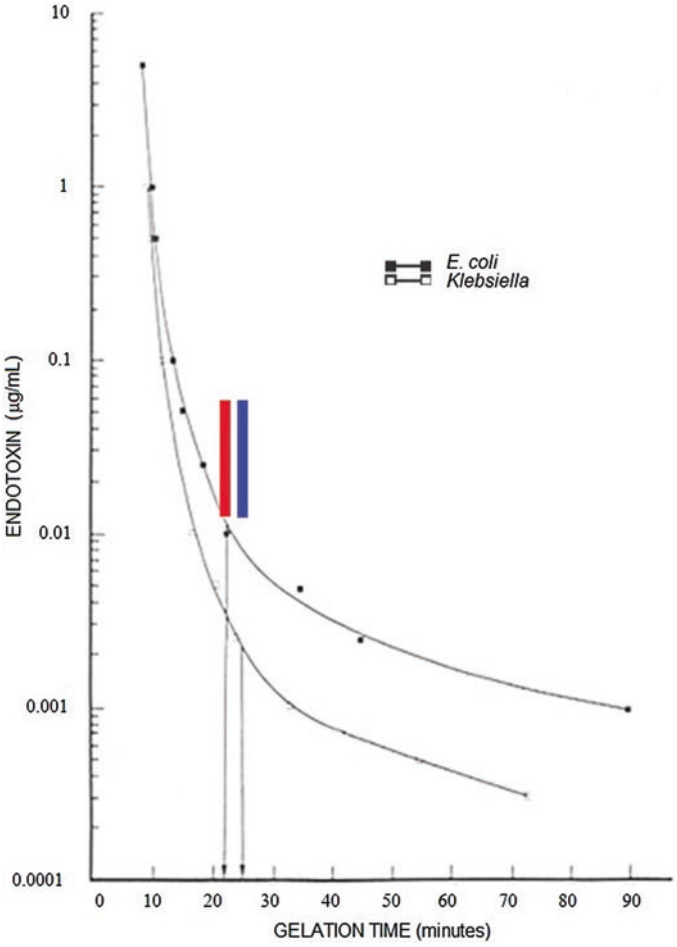
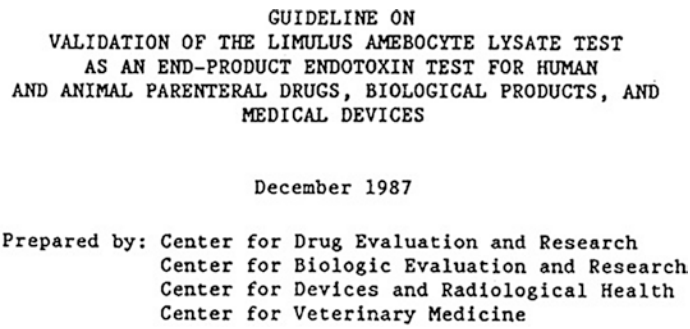


Fig. 1.7 Gelation of Limulus amebocyte lysate by *E. coli* or *Klebsiella* endotoxin vs. rabbit pyrogenicity. Gelation times increased as the concentration of endotoxin decreased. Arrows indicate gelation times of pyrogenic concentrations of endotoxin. Gelation times shorter than the red line correlated with rabbit pyrogenicity; gelation times longer than the blue line (to the right) correlated with non-pyrogenicity. (from Ref. [12], with permission.)

Table 1.2 Comparison of LAL and Rabbit Pyrogen tests for endotoxin. Note: There were no false negatives with the Limulus amebocyte lysate (LAL) test

	No. of tests
Rabbit Pyrogen tests	28,410
LAL (BET) tests	143,196
Positive tests detected by either procedure	37
Positive BET test	37
Positive USP Rabbit test	4
USP Rabbit Positive/BET Neg.	**0**

Modified from Mascoli and Weary [24]



GUIDELINE ON
VALIDATION OF THE LIMULUS AMEBOCYTE LYSATE TEST
AS AN END-PRODUCT ENDOTOXIN TEST FOR HUMAN
AND ANIMAL PARENTERAL DRUGS, BIOLOGICAL PRODUCTS, AND
MEDICAL DEVICES

December 1987

Prepared by: Center for Drug Evaluation and Research
Center for Biologic Evaluation and Research
Center for Devices and Radiological Health
Center for Veterinary Medicine

Fig. 1.8 Validation of the Limulus amebocyte lysate LAL test for bacterial endotoxin. Announcement in the Federal Register

impact on the safety of parenteral drugs and fluids. An early example was the effective clinical use of the Limulus Test to demonstrate that an outbreak of pyrogenic reactions to serum albumin infusions was due to contaminating bacterial endotoxin [20]. The Limulus test has also been used successfully to detect endotoxin in the blood of patients suspected of having endotoxemia [21], as well as in other body fluids.

1.4 End Points for the Detection of Endotoxin

The gel clot was the first endpoint officially approved for the Limulus test. For reasons unknown, it was decided that the gel clot assay had to be read at a single time point, i.e., 1 hour. A solid gel was required for a positive result. Since the reaction between endotoxin and Limulus lysate is enzymatic, it is obvious that a longer incubation time for the readout would result in a marked increase in the sensitivity. Unfortunately, my efforts to convince the FDA to have the gel clot endpoint serially read at multiple time points failed. However, when chromogenic and turbidimetric kinetic methods were introduced, this significant limitation of sensitivity was bypassed. Nevertheless, it should be pointed out that the gel clot end point technique, if not limited to 1 hour, is as sensitive as the chromogenic and turbidimetric kinetic assays (Fig. 1.9) [22].

1.5 Conclusion

Serendipity has been defined in a variety of related ways. The term is often used incorrectly to indicate a purely accidental observation or discovery. It is more than that, as illustrated by the definitions shown (Table 1.4). The Limulus test would

Table 1.3 Endotoxin timeline

1885	First recorded scientific observation of the coagulation of *Limulus*' blood. Observations upon the chemical composition and coagulation of the blood of *Limulus polyphemus*, *Callinectes hastatus*, and *Cucumaria sp*. Johns Hopkins Univ Circ 5:4.
1953	Frederik B. Bang describes the effects of injecting a marine bacterium into *Limulus polyphemus*. His results indicate this causes intravascular clotting; other Gram-negative bacteria could cause similar results; the clotting did not require a living bacteria, the bacterial component that caused clotting was not heat labile; and, Gram-positive bacteria did not produce this effect. This finding was the foundation which ultimately lead to the discovery of LAL many years later. Bang, FB (1953) The toxic effect of a marine bacterium on *Limulus* and the formation of blood clots. Biol Bull 105:447–448.
1956	Renewed interest in *Limulus polyphemus* as a biological model for the study of disease mechanisms. Bang, FB (1956) A bacterial disease of *Limulus polyphemus*. Bull Johns Hopkins Hosp 98:325.
1964	First detailed modern description of cellular coagulation in *Limulus*. Levin, J, and Bang, FB (1964) A description of cellular coagulation in *Limulus*. Bull Johns Hopkins Hosp 115:337.
1964	Discovery that endotoxin is the key factor in clotting of Limulus blood. Levin, J, and Bang, FB, (1964) The role of endotoxin in the extracellular coagulation of Limulus blood. Bull Johns Hopkins Hosp 115:265.6.
1968	Discovery that bacterial endotoxin (pyrogen) was responsible for the clotting of Limulus blood and that the mechanism was located in the amebocyte granules. Levin, J. Bang, FB (1968) Clottable protein in Limulus: Its localization and kinetics of its coagulation by endotoxin. Thromb Diathes Haemorrh (Stuttg) 19:186.
1969	James F. Cooper begins a study under the direction of Jack Levin and Henry N. Wagner to explore the use of LAL as an alternative to using the rabbit pyrogen test to detect endotoxin in pharmaceuticals.
1970	First application of LAL to the diagnosis of human disease. Levin, J, Tomasulo, PA, and Oser, RS (1970) Detection of endotoxin in human blood and demonstration of an inhibitor. J Lab Clin Med 75:903.
1971	LAL shown to correlate well with other assays for endotoxin, e.g. Pyrogen (rabbit) Test. Cooper, JF, Levin, J, and Wagner, HN Jr. (1971) Quantitative comparison of *in vitro* and *in vivo* methods for the detection of endotoxin. J Lab Clin Med 78(1):138.
1972	LAL shown it could be applied to the detection of endotoxin in pharmaceutical drugs. Cooper, JF, Hochstein, HD, Seligmann, EB Jr. (1972) The Limulus test for endotoxin (pyrogen) in radiopharmaceuticals and biologicals. Bull Parenter Drug Assoc 26(4):153.
1973	Food and Drug Administration first proposes guidelines for the manufacture of LAL. Federal Register January 1973. Federal Register Vol. 38, No. 8 p. 1404.
1973	Food and Drug Administration proposes standards for the manufacture of LAL. September 18, 1973. United States Federal Register Vol. 38, No.180. Limulus Amebocyte Lysate: Additional standards. p. 26103–26132.
1973	Cooper, Hochstein, and Seligmann Drafted "Return To Sea" Policy.

(continued)

Table 1.3 (continued)

1974	Travenol Laboratories, Inc. establishes a lysate production laboratory at their Kingstree, South Carolina plant and is using their LAL to test pharmaceuticals both domestically and in some international plants by 1974. Mascoli, Carmine C, and Marlys E. Weary. (1979) Applications and advantages of the Limulus amebocyte lysate (LAL) pyrogen test for parenteral injectable products. *In* Progress in Clinical and Biological Research, Vol. 29, Biomedical Applications of the Horseshoe Crab (Limulidae), Elias Cohen, et al. eds. Alan R. Liss, Inc., New York (Proceedings of a Symposium Held at the Marine Biological Laboratory, Woods Hole, Massachusetts, October, 1978)
1977	Beginning with Associates of Cape Cod, Inc., Woods Hole, MA, in September 1977, by October 1978 there were three additional LAL manufacturers licensed by the FDA. These were, in order of licensing: Mallinckrodt Inc., St. Louis, MO, Microbiological Associates, Walkersville, MD, and Travenol Laboratories, Morton Grove, IL
1977	FDA allows substitution of LAL for the official rabbit pyrogen test when testing biological products and medical devices providing approval is first obtained from the appropriate bureau of the FDA. Federal Register, November 4, 1977, Vol. 42. No. 213, p 57749.
1978	FDA proposal for the live release of horseshoe crabs back to their native environment after only one blood collection. (This regulation was rescinded in 1996 under the Federal Reinvention of Government (REGO) Initiative.) United States Federal Register (1978) 43:35731–35734
1979	Worthington Laboratories, Freehold, NJ joins the group of laboratories manufacturing LAL (Pyrostat™ brand). Teller, Joseph D., and Kristine M. Kelly. (1979) A turbidimetric Limulus amebocyte lysate assay for the quantitative determination of gram negative bacterial endotoxin. *In* Progress in Clinical and Biological Research, Vol. 29, Biomedical Applications of the Horseshoe Crab (Limulidae), Elias Cohen, et al. eds. Alan R. Liss, Inc., New York (Proceedings of a Symposium Held at the Marine Biological Laboratory, Woods Hole, Massachusetts, October, 1978)
1980	In the FEDERAL REGISTER of January 18, 1980 (45 FR 3668), FDA announced the availability of a draft guideline that set forth procedures for use of the LAL test as an end-product testing method for endotoxins in human and animal injectable drug products. This draft guideline was made available to interested parties to permit manufacturers, especially those who had used the LAL test in parallel with the rabbit pyrogen test, to submit data that could be considered in the preparation of any final guideline.
1980	The United States Pharmacopeial Convention (USP) publishes General Chapter <85> Bacterial Endotoxins Test in USP XX, making the LAL test a Compendial method to detect bacterial endotoxin in pharmaceutical products and medical devices.
1987	The United States Food and Drug Administration publishes Guideline on Validation of the *Limulus* Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices, describing FDA's opinion regarding the appropriate methods for validation and use of LAL for detecting the presence of endotoxin in medical products.

Modified from http://www.horseshoecrab.org/med/timeline.html

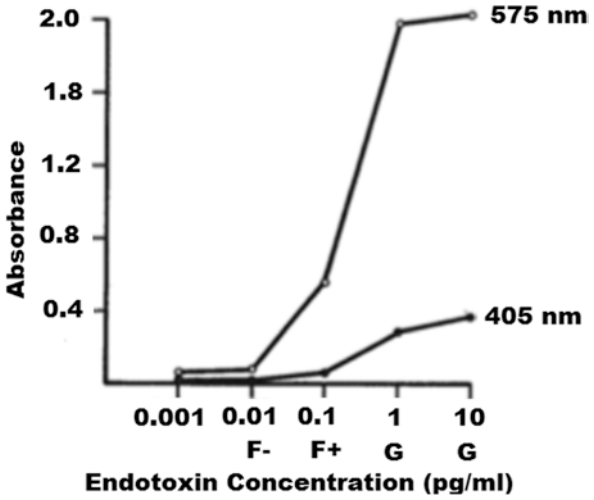


Fig. 1.9 End points for performance of Limulus test for bacterial endotoxin. Comparison of amidolytic activity quantified by measurement of PNA absorbance or of PNA-DACA Schiff base absorbance with visual gelation method. Amidolytic activity was generated by incubation of *Limulus* lysate with various concentrations of *E. coli* endotoxin and quantified with the chromogenic substrate, S-2222. Measurements of absorbance were: free PNA at 405 (•); and PNA-DACA complex at 575 (O). Additional samples also were scored visually after incubation for I day for presence of borderline flocculation(F-), heavy flocculation(F+), or gelation(G). Results of representative experiment are shown. (From Ref. [22], with permission)

Table 1.4 Definitions of serendipity

(ser'en-dip'i-tē), A knack for discovery involving a combination of **accident** and **wisdom** while pursuing something else; in science, finding one thing while looking for something else, as in Fleming's discovery of penicillin. [coined by Horace Walpole and relates to The Three Princes of Serendip.]
Serendipity is the way to make discoveries, by accident but also by sagacity, of things one is not in quest of. Based on experience, knowledge, it is the creative exploitation of the unforeseen**.** *Adrian Bejan*
Serendipity. Look for something, find something else, and realize that what you've found is more suited to your needs than what you thought you were looking for. *Lawrence Block*

never have been conceived and then established if during my initial struggles with *Limulus* blood and its very biologically active amebocytes I had not also been studying the effects of bacterial endotoxin on blood coagulation and platelets in rabbits. Lacking the experience and knowledge of the literature gained from my rabbit experiments, I would never have considered the possibility that the spontaneous aggregation and disruption of Limulus amebocytes, with activation of blood coagulation despite the blood being anticoagulated, was due bacterial endotoxin. In 1960, A. V. Hill wrote "By the methods of comparative physiology, or of experimental

biology, by the choice of a suitable organ, tissue or process, in some animal far removed in evolution, we may often throw light upon some function or process in the higher animals, or in man." [23]. The "basic research" that unexpectedly led to the discovery of the sensitivity of the blood coagulation mechanism in *Limulus* to bacterial endotoxin and subsequent development of the *Limulus* test validates Hill's thoughtful and wise statement.

Acknowledgment The author wishes to thank Dr. Thomas Novitsky for sharing his insights into the years during which *Limulus* amebocyte lysate transitioned from the research laboratory into commercial development as the Limulus amebocyte lysate (LAL) test for bacterial endotoxins.

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Chapter 2
Origins and Evolution of Drug Regulation

Kevin L. Williams

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Abstract It is easy, in this modern age, to forget the fear and trepidation surrounding infectious disease that has ruled humanity from antiquity. Diseases that were epidemic such as flea-borne bubonic plague, as well as water-borne ailments including typhoid, mosquito-borne malaria, viral-borne small pox and polio were highly feared. Infections that occurred sporadically as endemic in nature included rabies, lockjaw, botulism, and anthrax and were terrifying prospects that could arise from relatively minor scrapes (a dog bite, stepping on a nail, eating contaminated food, or from breathing spores, respectively). Improved sanitation practices allowed people to avoid contracting many illnesses and, eventually, vaccination greatly reduced the worst microbial and viral diseases. By the turn of the twentieth century, the public began to expect that the federal government should provide regulation of food and drugs and otherwise contribute to the public welfare via legislation. In a reactionary manner, these regulatory efforts proceeded in fits and starts, usually as a counter to specific tragedies.

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K. L. Williams (ed.), *Endotoxin Detection and Control in Pharma, Limulus, and Mammalian Systems*, https://doi.org/10.1007/978-3-030-17148-3_2

2.1 Drug Regulations Origin in Tragedy

We think of many of the worst infectious diseases as largely "solved" and things of the past, yet, in some parts of the world they are on-going concerns. China, for example, had almost 1000 cases of rabies in 2014 [1]. The increasing acceptance and command of practical "germ theory" as well as a rudimentary understanding of immunology furthered a second great round of disease eradication in vaccination (the first being sanitation), although a true understanding of the principles of vaccination would remain elusive for many decades after its initial use in alleviating the scourges of small pox and polio. Smallpox vaccination brought about a unique form of injection. See Fig. 2.1.

> The (bifurcated needle) technique was used on a very wide scale during the final years of the (smallpox) Eradication Campaign and it was estimated that 200 million vaccinations per year were done with the device [2]. It is appropriate that those closely involved with the Campaign were awarded 'The Order of the Bifurcated Needle' and presented with a small lapel badge formed from the device. [3]

Interestingly, the bifurcated needle has found use in more modern times as well:

> …with a weak vaccine, the improvement in the take rate from 2 insertions varied from 17.9%–31.9% with the bifurcated needle technique to 27.4%–100% with the rotary lancet technique. In areas where storage facilities for vaccine are inadequate, and particularly in rural field conditions where the potency of vaccines is likely to be slightly reduced, the use of 2 insertions with the bifurcated needle technique should be seriously considered. [4]

It is a testimony to modern medical progress that some methods used in the past seem almost barbaric by today's standards.

The deep-seated fear of infectious disease may be a part of the underlying archetypes in the pop culture preoccupation with zombies and "living dead" [5], which is

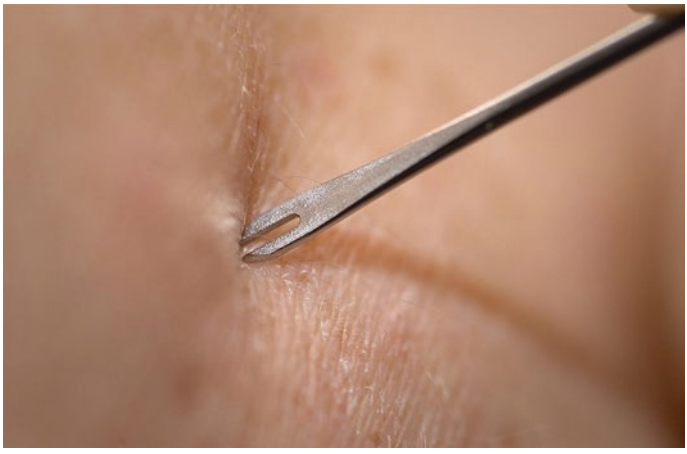

Fig. 2.1 A bifurcated needle used in lieu of the syringe to inoculate for small pox. The space between the forks allowed for a single "dose" after puncturing the skin multiple times. (CDC)

what some became upon infection in previous times (rabies and lockjaw being fast and slow versions with the modern additions of "mad cow" and AIDS reaching hysterical levels for some short periods of time). For us, in modernity, the fear of infection is restricted to fears of antibiotic resistance (MRSA), "flesh-eating" bacteria, AIDS, as well as other viral and prion-based infections which are viewed primarily as rare lurking factors. More recently, the underlying cause of disease has sometimes surprisingly pointed to microbial roots, with *H. pylori* (stomach ulcers and cancer [6–8]), dental hygiene (associated with poor cardiovascular prognosis [9]), and several types of virally induced cancers [10] all having underlying microbial roots. Endotoxin, though highly scrutinized in drugs, has a role in disease causation that continues to elude researchers in sepsis and related morbidity (see Section III) [11].

It is good to look back before looking forward. History is important, if for no better reason, such that the tragic parts of it should not be repeated. Hillman said: "For us, individuals, makeweights[1] that may tip the scales of history, our task is to discover the psychic connection between past and future, otherwise the unconscious figures within us who are as well the archaic past will shape the historical future perhaps, disastrously." [12] Historically, regulatory laws arose in response to tragedy and subsequent public outcry. Before the twentieth century there was no federal regulation of drugs and other purported remedies of affliction. Tragedy in the form of contaminated smallpox vaccines and diphtheria antitoxins struck in 1901 resulting in tetanus outbreaks and the deaths of several children. In the U.S., such sporadic tragedies lead to the **Biologics Act of 1902** that required federal preapproval for every biological drug produced as well as oversight of the process and facility producing it. Similar authority was enacted for animal biologics in the **Virus, Serum and Toxin Act of 1913**. It is interesting and informative to view the state of the art of biologics therapy in 1915. From the 1915 book for general practitioners: "*Modern Biologic Therapeusis"* [13] one can see some sophisticated attempts to treat infectious disease existed then. See Fig. 2.2.

Upton Sinclair's novel, *The Jungle*, in 1906 exposed the putrid conditions of the meatpacking industry. The novel follows the travails of a poor working-class Chicago immigrant that worked in a meatpacking plant. Below Sinclair decries the foulness of the product itself.

> It seemed that they must have agencies all over the country, to hunt out old and diseased and crippled cattle to be canned. These were cattle which had been fed on "whiskey-malt", the refuse of the breweries, and had become what men called "steerly"- which means covered with boils. It was a nasty job killing these, for when you plunged your knife into them they would burst and splash foul-smelling stuff into your face; and when a man's sleeves were smeared with blood, and his hands steeped in it, how was he ever to wipe his face, or to clear his eyes so that he could see? It was stuff such as this that made the "embalmed beef" that had killed several times as many United States soldiers as all the bullets of the Spaniards; only the army beef, besides was not fresh canned, it was old stuff that had been lying for years in the cellars.

[1] Refers to Jung's question: "Does the individual know that he is the makeweight that tips the scales?".

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Fig. 2.2 Table of contents excerpts from "*Modern Biologic Therapeusis*" of 1915

A vivid description of a coworker falling into meat being processed without so much as his fellows being able to pluck out his dead carcass brought an outcry for better conditions surrounding food production.

> …as for the other men, who worked in tank rooms full of steam, and in some of which there were open vats near the level of the floor, their peculiar trouble was that they fell into the vats; and when they were fished out, there was never enough of them left to be worth exhibiting—sometimes they would be overlooked for days, till all but the bones of them had gone out to the world as Durham's Pure Leaf Lard!

The most visible beginnings of biologics as a drug class included vaccinology, specifically Pasteur's efforts to prevent anthrax and treat rabies in miraculous fashion. Without the knowledge of virus particles or immune system components who could have felt comfortable dabbling in such a manner? But Pasteur had seen the diminishment of microbial virulence after repeated passage of the rabies virus in rabbits. They were injected, infected, and their spinal cords were removed and dried out. Progressive passages gave more diminished (attenuated) infections until the killer virus had lost its blood lust altogether. However, surprisingly, also upon administration the concoction was able to prevent the subsequent occurrence of

Table 2.1 Chronology of first human vaccines developed per affliction

Vaccine	Date
Rabies	1885
Diphtheria	1888
Typhoid	1896
Plague	1897
Scarlet fever	1924
Tuberculosis	1927
Yellow fever	1936
Measles	1963
Mumps	1967
Rubella	1969
Varicella	1995
Rotavirus	1998

disease in man and animals recently infected. Attenuation is less used today as many vaccines are the product of subunit proteins combined with an adjuvant that presents an acceptable immune stimulation with no risk of infection. Smith [14] describes Pasteur's attenuation method in detail:

> Spinal cords taken from rabbits newly dead of rabies were suspended in flasks open to the air that contained potassium hydroxide as a desiccant, which Pasteur introduced to prevent the cords from putrefying. It appeared to Pasteur that each day of desiccation gradually led to an attenuation of virulence, such that after 14 days, if a portion of the dried cord was emulsified and injected into either rabbits or dogs, it had lost its virulence…
>
> Here, Pasteur assumed that the virus in the desiccated jars remained alive but had lost its virulence, and thus was attenuated. However, obviously Pasteur had no way to identify the rabies organisms, or to tell whether they were alive or dead…
>
> The rabies vaccines, which were produced and sent worldwide[2] initially consisted of desiccated nervous tissue, which was used as the vaccines for ~10 years until 1895, when carbolic acid-inactivated nervous tissue-derived vaccines were introduced, followed by phenol-inactivated nervous tissue-derived vaccines in 1915 [15]. These vaccines were then used for the next 40 years until the mid-1950s when tissue culture-derived inactivated rabies virus was first used for the rabies vaccine, which is still in use today. However, ironically, live, but replication-deficient rabies virus vaccines are in development now, and they provide the hope that single-dose human live rabies vaccines will replace the current inactivated vaccines, with their associated toxicity and complicated repetitive dosing regimens.

Table 2.1 shows the successful application of vaccinology to human disease.

Modern criticisms that Pasteur misunderstood the mechanism of action behind his rabies vaccine discovery seem misplaced. The scientific background for such an understanding was missing in 1885. There was virtually no knowledge of immunology or virology. Pasteur had only just shown (in 1859) that microbes didn't arise by

[2] Some references claim the vaccines were sent worldwide while others maintain that people were sent to Europe for treatment.