Chapter 1
Discovery and Early Development of the Limulus Test

Jack Levin

Contents

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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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

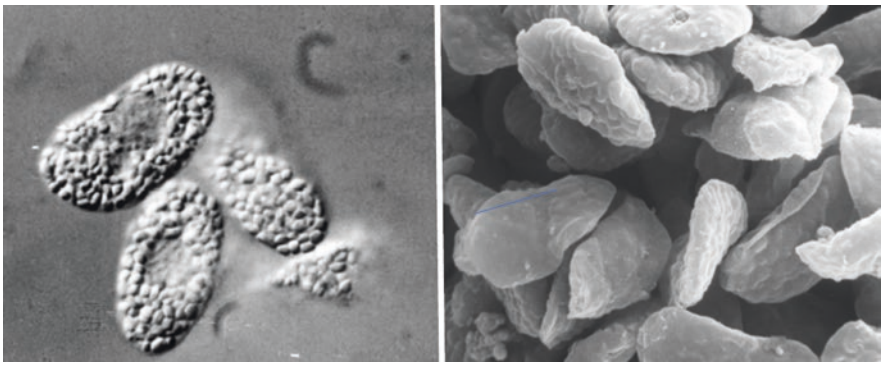


Fig. 1.1 Left: Three amebocytes, which are the size of macrophages (Nomarsky optics). Right: Amebocytes (scanning electron microscopy). Note that the nucleated amebocytes are flat discs, packed with granules

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

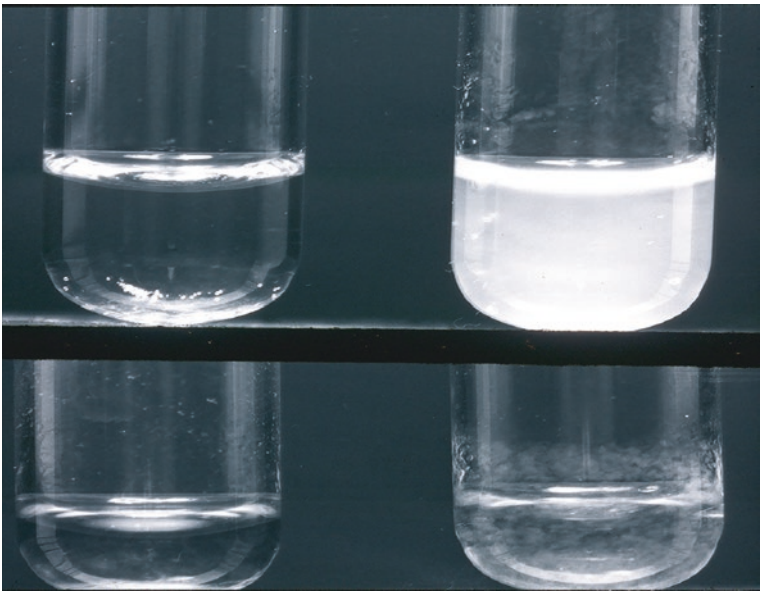


Fig. 1.3 Limulus amebocyte lysate (Gel clot endpoint). Left: Controls Right: + Endotoxin. The higher concentration of endotoxin (upper right) produced a solid gel, whereas the lower concentration produced only flocculation

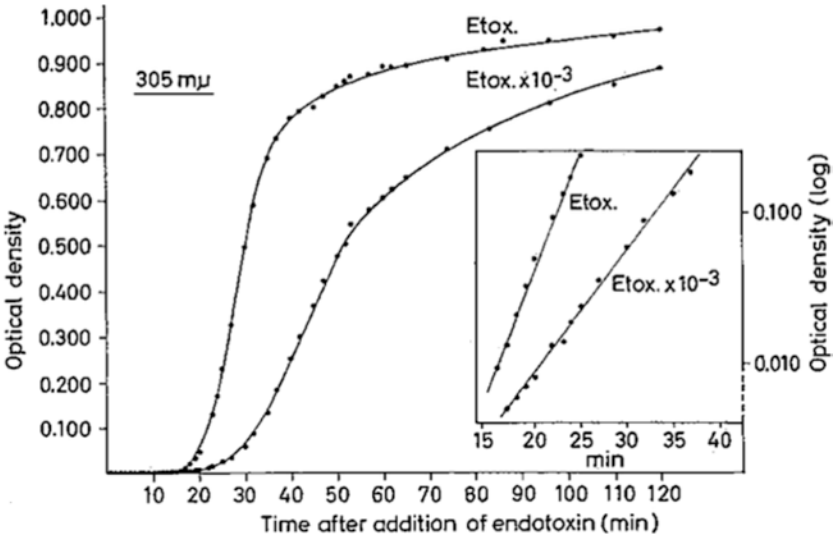


Fig. 1.4 Increase in optical density after addition of different concentrations of *E. coli* endotoxin. The rate of increase in optical density was dependent upon the concentration of endotoxin. The semi-log plot (insert) indicates that the reaction between Limulus amebocyte lysate and endotoxin is enzymatic.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

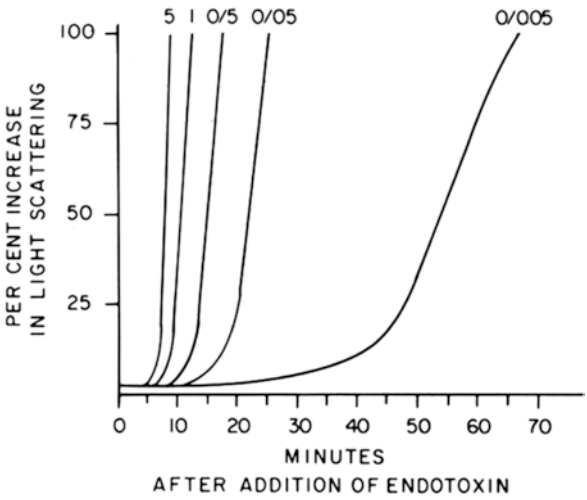


Fig. 1.5 Rate of increase in light scattering after addition of bacterial endotoxin from *E. coli*. The concentration of endotoxin is shown in μg/ml. As the concentration of endotoxin decreased, the rate of increase in light scattering decreased. (From Ref. [6], with permission.)

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

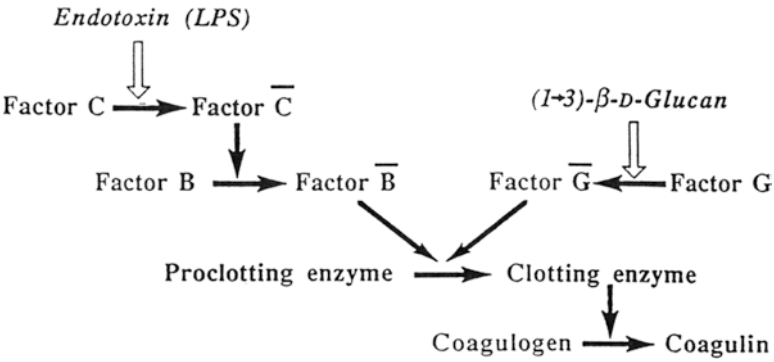


Fig. 1.6 Blood coagulation in Limulus is enzymatic. Serine proteases constitute the coagulation cascade. Factor C is remarkably sensitive to bacterial endotoxins. Factor G is activated by β-glucans

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

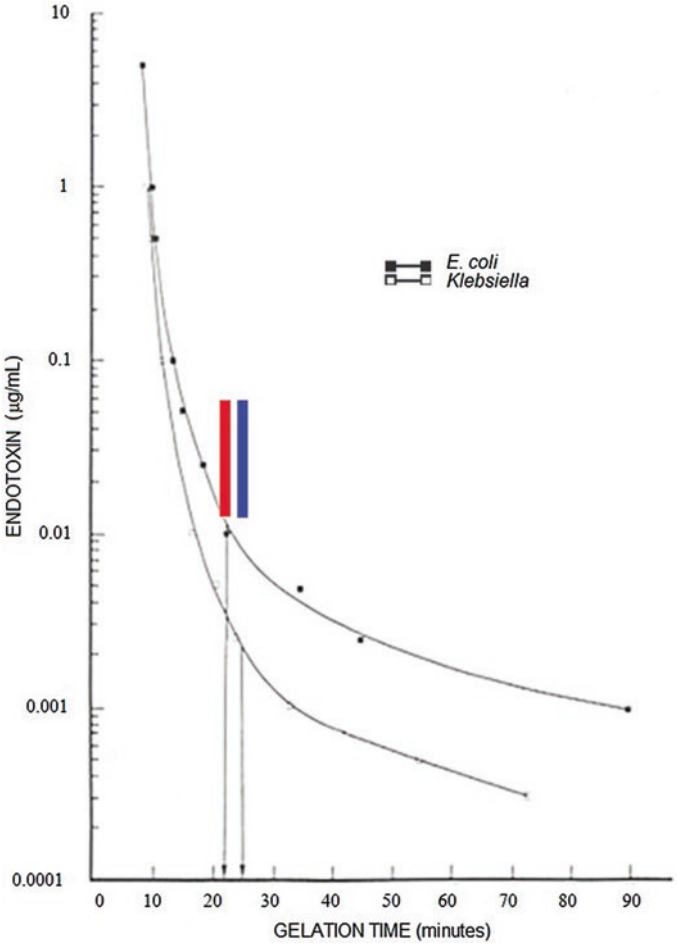
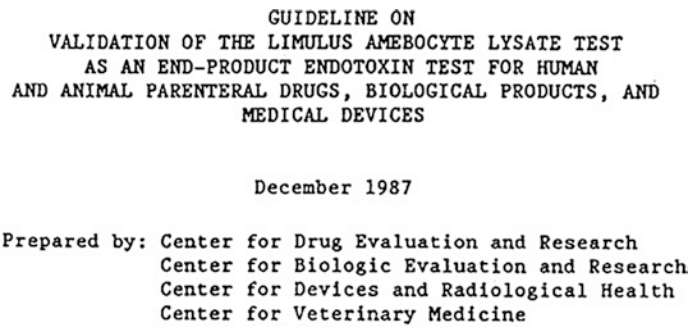


Fig. 1.7 Gelation of Limulus amebocyte lysate by *E. coli* or *Klebsiella* endotoxin vs. rabbit pyrogenicity. Gelation times increased as the concentration of endotoxin decreased. Arrows indicate gelation times of pyrogenic concentrations of endotoxin. Gelation times shorter than the red line correlated 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

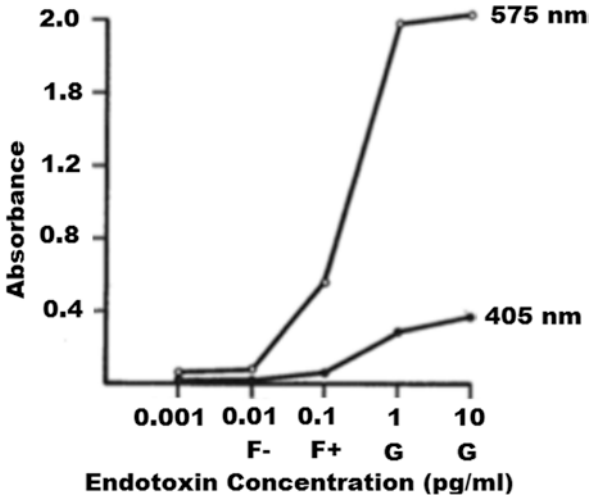


Fig. 1.9 End points for performance of Limulus test for bacterial endotoxin. Comparison of amidolytic activity quantified by measurement of PNA absorbance or of PNA-DACA Schiff base absorbance with visual gelation method. Amidolytic activity was generated by incubation of *Limulus* lysate with various 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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