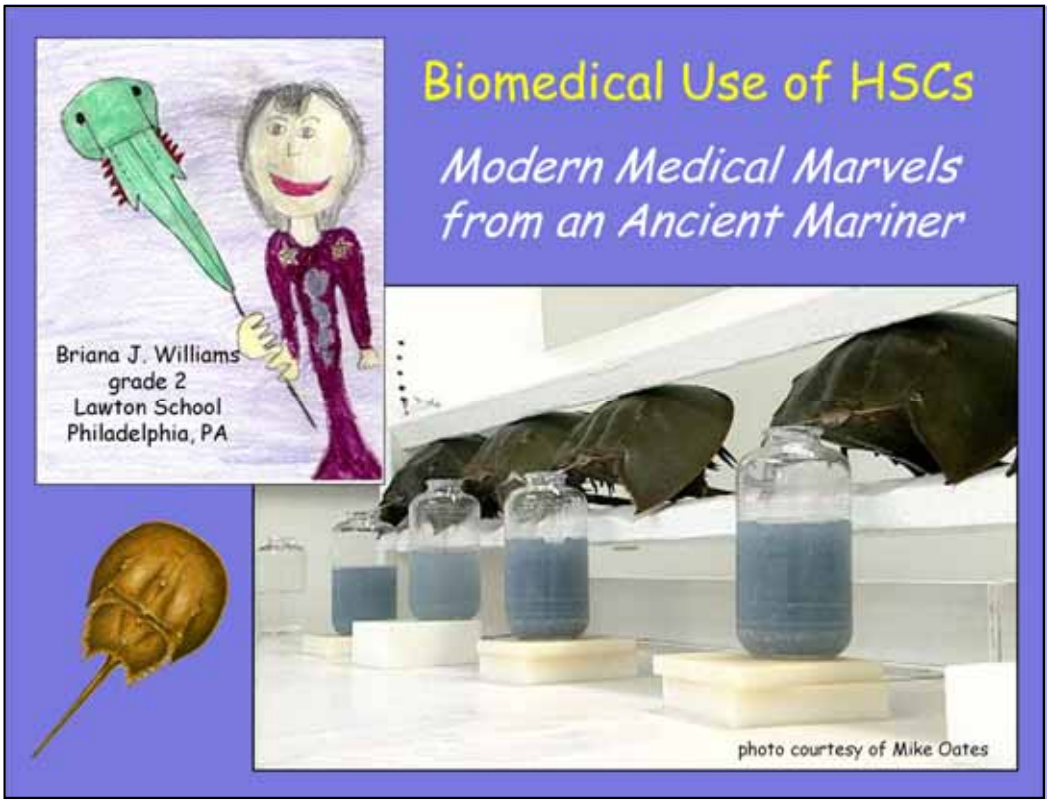


The image shows horseshoe crabs (HSCs) being bled in a biomedical laboratory. Notice that the needle has been inserted through the middle (hinge) area of the HSC and into its heart. [1]

The HSC heart doesn't pump like a human heart, so inserting the needle into it does not kill or cause serious harm to the crab. [2]

Reports vary widely on both the volume and percentage of blood (properly call hemolymph) that is drawn from a HSC in biomedical bleeding.[3] Typically, the larger the crab, the more blood it holds. Most accounts put the volume of blood taken to be about 100 ml on average (equivalent to a small coffee cup's worth), corresponding to about 25-30% of a HSC's total blood volume.

So it's important to point out that the amount of blood shown in each bottle in the picture above is not all from one HSC, but the product of bleeding several crabs.

Students will also notice the blue blood. That and other aspects of the discovery, processing, applications, and benefits of using HSC blood in biomedical testing will be covered in detail in the slides that follow.

You can almost guarantee that anyone watching this slide show has benefited in some way, or will benefit at some time from, the biomedical use of horseshoe crabs. Some may know that this has something to do with HSC blood, and a few may have heard that this involves making sure vaccines are safe to use, but most won't know about these other materials that also need to be tested with it.

In addition to the above-mentioned, required-by-the-FDA examples of medical materials that are tested with LAL, there are several other instances where LAL may be used to screen or assess other medicines for potential health threats to humans. An example of this is testing contact lenses or contact lens solutions that are suspect in causing bacterial eye infections, such as keratitis, an inflammation of the cornea[4] (which may also be caused by fungal infections or the *Herpes* virus).

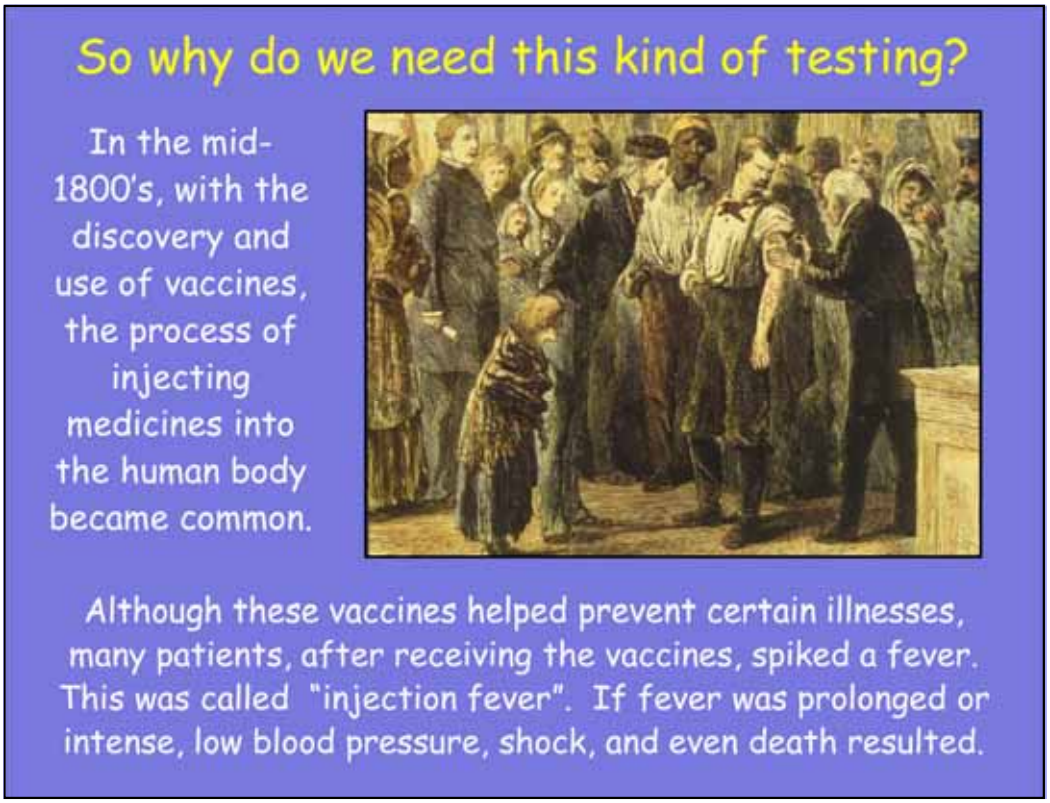
If you answered 'YES' to any of the above:

Then you owe a major debt of gratitude to this animal!

Since 1990, all medical products in the U.S. that come into contact with human blood, spinal fluid or mucous membranes, along with the needles, tubing, bags and other materials used to deliver them, must be tested with a product derived from horseshoe crab blood.

In addition to every man, woman and child near and dear to us, the health & well-being of our pets and many other domesticated animals also benefits from this test! As with medicines used in humans, all veterinary injectables and implantables in the U.S. are tested with HSC blood product to ensure that they are safe to use.

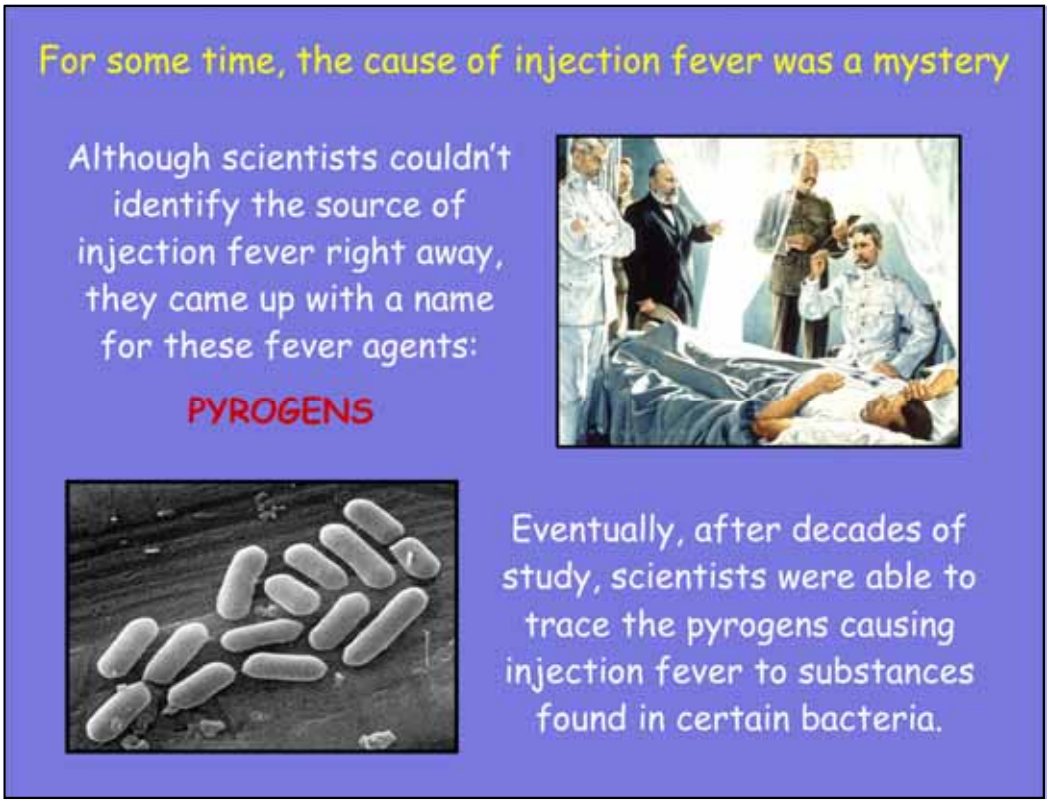
The reasons for this are the same as for humans – contamination of any veterinary medicines that come into direct contact with blood and tissue can cause the same kind of fever and illness reactions in dogs, cats, horses, etc. as they do in humans.



Up to the mid-1800's, diseases were thought to be caused by spontaneous generation, excess 'humors', or even demons, (the latter as punishment for a person's misdeeds). Eventually, thanks to the work of Louis Pasteur and others, the germ theory of disease, or the idea that microbes were causal agents for certain diseases, came to be accepted. In time, use of the microscope allowed for the association of various forms of bacteria with particular diseases, such as anthrax, smallpox, typhoid fever, the plague, etc.

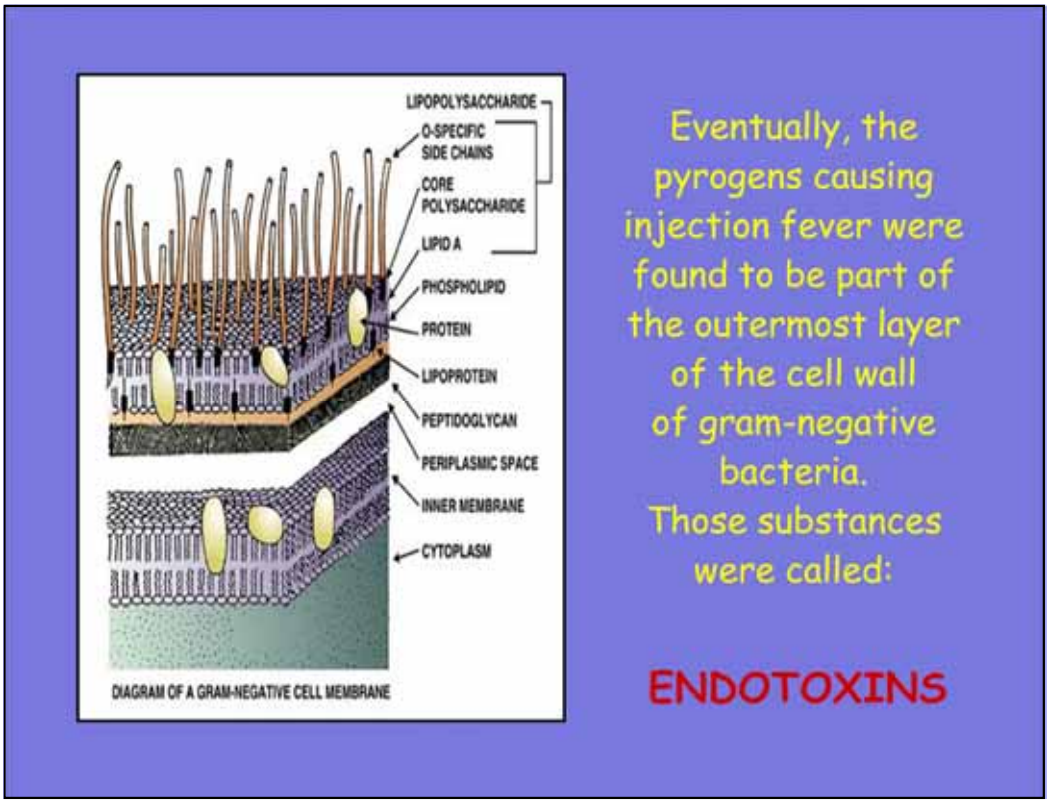
The first recorded cases of doctors injecting substances into their patients came out of Europe in the mid-1600's – including such things as ale, opium, wine, urine, and various putrid waste products. The development by Edward Jenner (1796) of a smallpox vaccine, made by taking the pus from the cowpox sores of a milkmaid and injecting it into an 8-year old boy (to confer immunity to smallpox) is an interesting side story to all this. [5]

In the 1800's, with the development and use of more vaccines, injections became more common. But doctors began noticing side reactions to these injections, including inflammation around the shot site, often followed by fever, low blood pressure, shock, and even death. Now if you're a physician – providing a treatment to make a sick person well or a vaccine to keep a well person from getting sick – then fever, shock, and death, would hardly be desired outcomes of your efforts!



For some time, the actual source or cause of injection fever remained a great mystery. Lacking a better explanation, the cause of injection fever was attributed to the body's response at being pricked by a needle.

In the meantime - as scientists sometimes do, when they don't have a clear answer for something - they concocted a fancy-sounding name for this mystery source. They called the unknown agents of injection fever 'PYROGENS' - from the Greek Pyros (for 'fire').



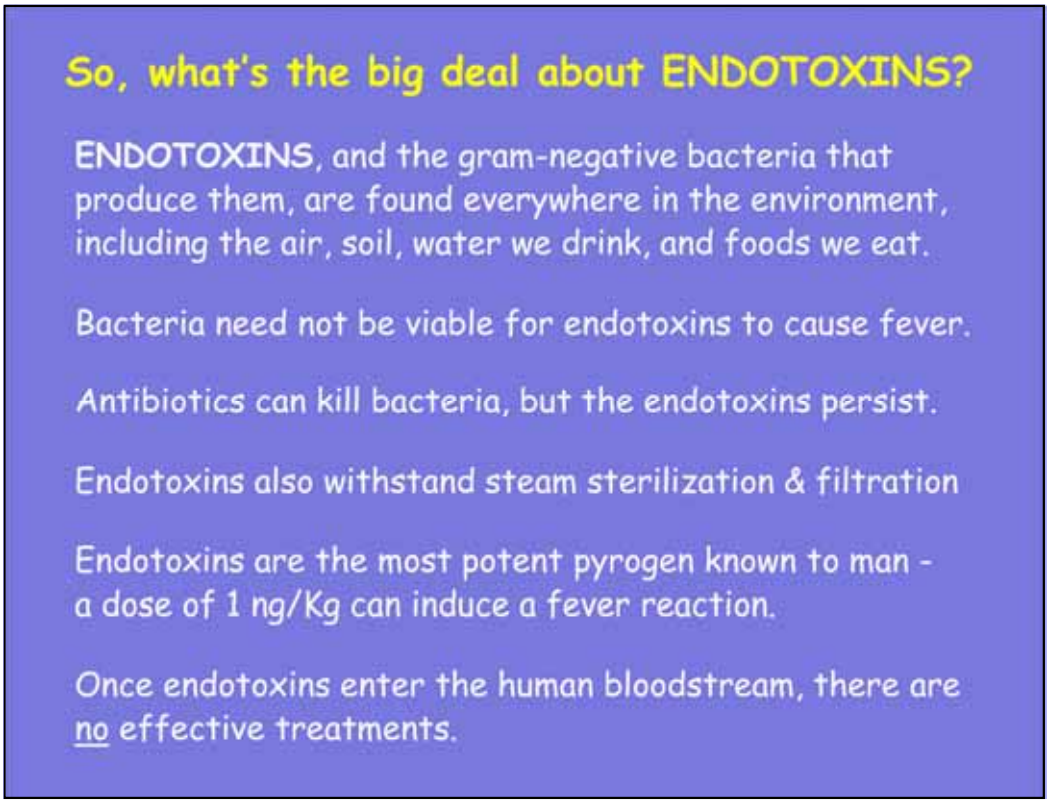
There's a fascinating history of scientific discovery that led to identifying the actual source of these pyrogens, including the work of Lister (1861), Koch (1880), Pfeiffer (1892), Hort & Penfold (1912) and Seibert (1923).

As a result of their efforts, the cause of injection fever was finally traced to the lipopolysaccharides (LPS) from the cell membrane of gram-negative bacteria (GNB).

GNB have been described as "thin-skinned". Just as humans routinely shed outer layers of skin, bits of GNB-LPS layer are sloughed off as they move. When GNB are killed, larger bits of LPS are released. When these endotoxins reach our blood, our immune system detects them, and raises our body temperature (as a way of killing the infection). Normally, in small doses, this is not a problem, but at higher levels and temperatures, fever is induced, and if intense or prolonged, it can be deadly. [6]

Because these toxic materials are derived from structural components of the bacterial cell (not substances that were produced by, and released externally from, the cell), scientists came up with another fancy name for them: endotoxins.

For an in-depth immersion in the significance of the LPS layer of gram-negative bacteria as an activator of various critical immune system pathways in animals from HSCs to humans, check out the article by Alexander and Rietschel (2001).[7]

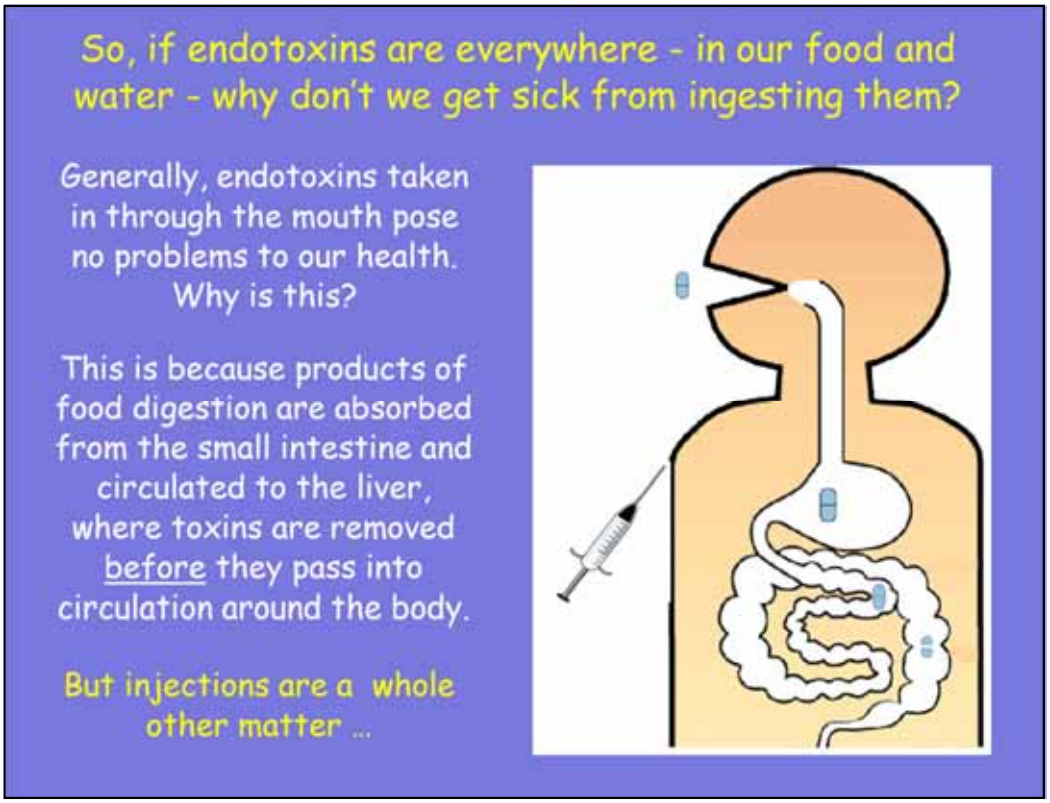


So what's the big deal about endotoxins? Go down the list. GNB are ubiquitous in the environment. We drink them, eat them, pick them up from things we touch, and even breathe them. And often, the very act of killing bacteria releases 'free' endotoxins to where they can do us harm. Antibiotics don't destroy them, nor does standard steam sterilization or other physical/chemical processes.

Endotoxins are also the most potent pyrogen known to man. So what's a 'nanogram'? 1 ng = 1-millionth of a milligram = 1 trillionth of a kilogram. This means it only takes very minute levels of endotoxin to produce a fever reaction in humans. Endotoxins can also cause profound inflammation of any exposed tissue, which if severe enough, can lead to impaired function of lungs, brain, kidneys, etc. If fever is prolonged, this can lead to tissue breakdown, shock and ultimately death – hardly a good outcome for medicines we're given!

One of the challenges of preparing injectable medicines for safe use in humans is in finding endotoxin-free sources of the raw water (called Water for Injection or WFI) for preparing the meds. These waters are derived from natural sources (surface or ground water) having varying endotoxin levels. Distillation is the preferred method for making endotoxin-free WFI. But problems can arise via transfer or contamination of storage containers and delivery devices. And GNB can grow/survive in distilled water at low ambient temperatures (some for well over a year); even if bacteria are killed off, endotoxins persist to cause problems.[8]

Note: Some cases of patients getting ill from meds that had passed the LAL test were found to be caused by contaminated storage containers or pumps used to deliver WFI that was mixed in with (otherwise safe) meds administered in IV systems.[9]

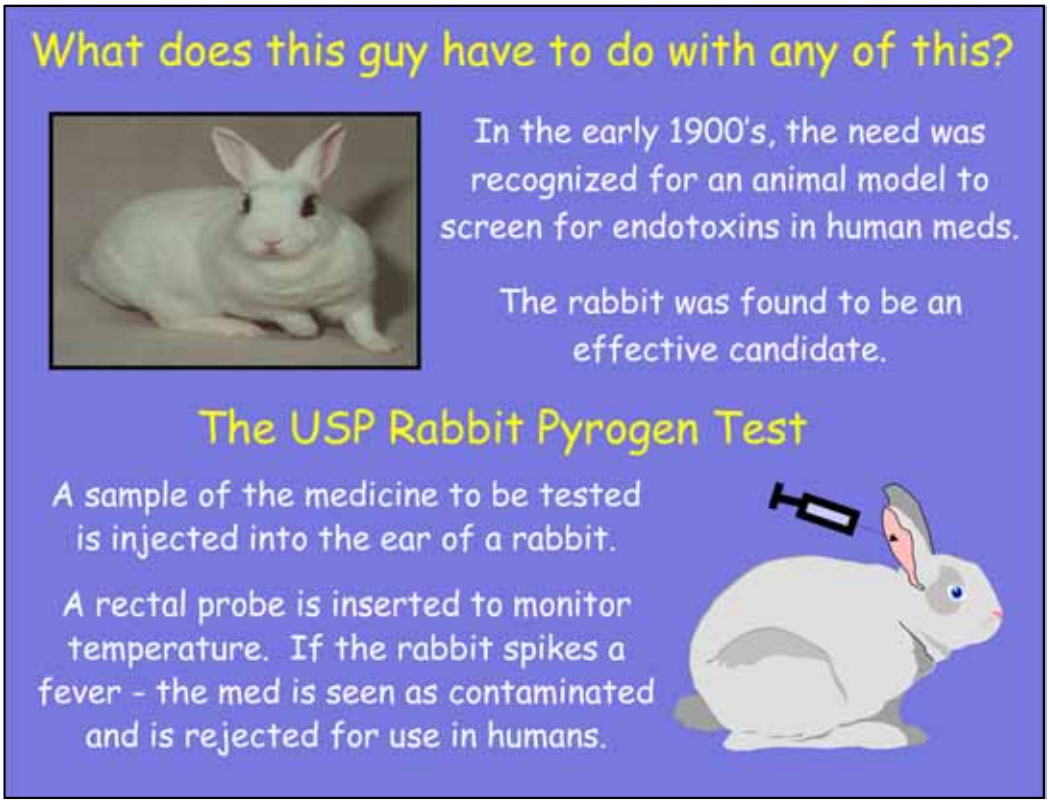


We already know the bad news about endotoxins - if they get into our blood, they can make us sick. The good news is that it's OK to drink them and eat them - inside our closed digestive system they are not a problem. In fact, endotoxins are present in our food and water, and are even produced by bacteria in our mouth and intestines.

There are several ways a healthy body deals with food and drink delivered endotoxins. Acids in the stomach kill many bacteria, minimizing production of further endotoxins. Then there's the physical barrier offered by the stomach lining and intestinal mucosa. And any endotoxins that manage to cross these barriers are inspected by, and typically removed by, cells and proteins of the human immune system. But the body's main defense against endotoxins reaching the blood from the digestive system is the liver. The products of food digestion are absorbed from the small intestine by capillaries that deliver them to the liver via the hepatic portal vein. The liver acts as 'gatekeeper' filtering and detoxifying any digestive endotoxins before they pass into the blood. [10]

Problems with endotoxins from foods can arise when any breakdown in any of these levels of protection occurs, such as stomach ulcers or ulcerative colitis (impacting the intestinal lining), and hepatitis or cirrhosis (impairing liver's detoxifying function). [11]

But what happens when we receive and injection? (click to advance slide animation) That blood goes to the heart and circulation before it can be screened by the liver, and thus gets pumped and delivered to cells and tissues of the brain, kidneys and various other parts of the body, where the endotoxins can cause fever and other reactions.



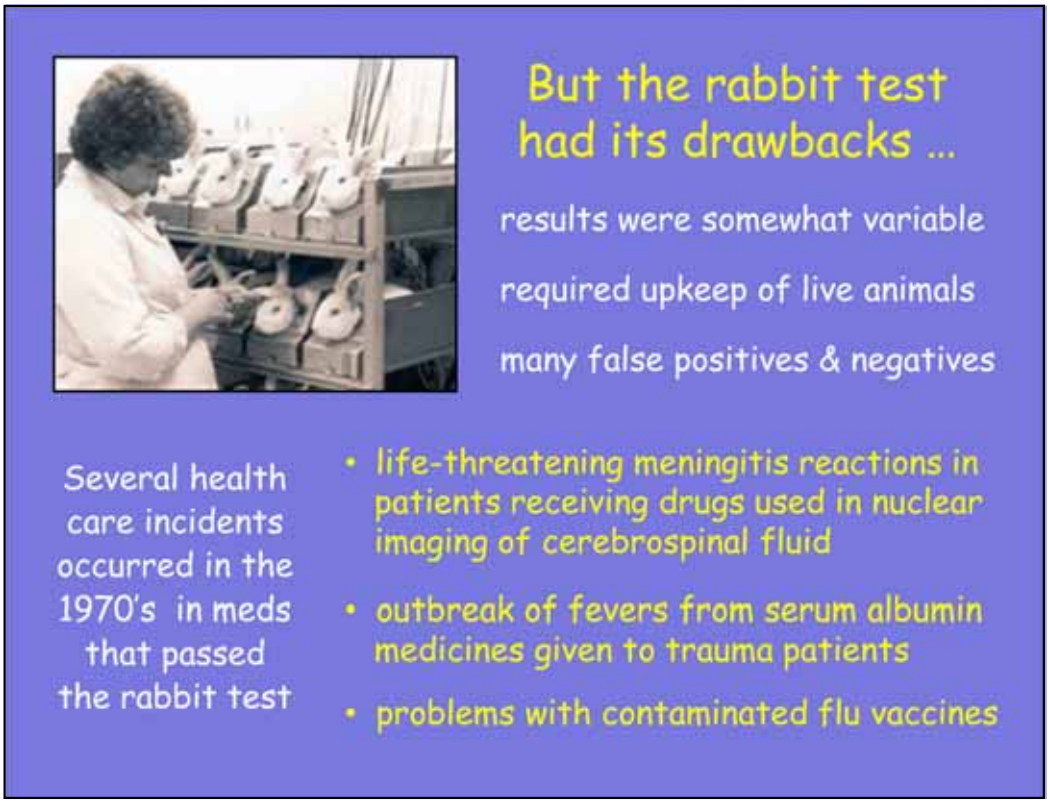
With increasing use of injectable medicines in the 1900's – and especially with the introduction of intravenous drugs in treating battlefield-wounded soldiers in WW1 - the need for a test to screen medicines for endotoxins intensified.

In looking for an animal model, lab mice and rats were not especially sensitive. Dogs and horses were sensitive, but not the most desirable lab animals.

The rabbit proved best – they were easy to keep, care for, and work with, and pound per pound, showed very similar response to endotoxins as humans.

The rabbit pyrogen test thus became the gold standard for pyrogen testing in 1942, when it was introduced into the USP (United States Pharmacopoeia).

The rabbit was chosen by Seibert (1925), who is credited with discovery of the pyrogenic principle (involving a measurement in body temperature after the application of not more than 10 ml/kg body weight of substance to be tested).[12]

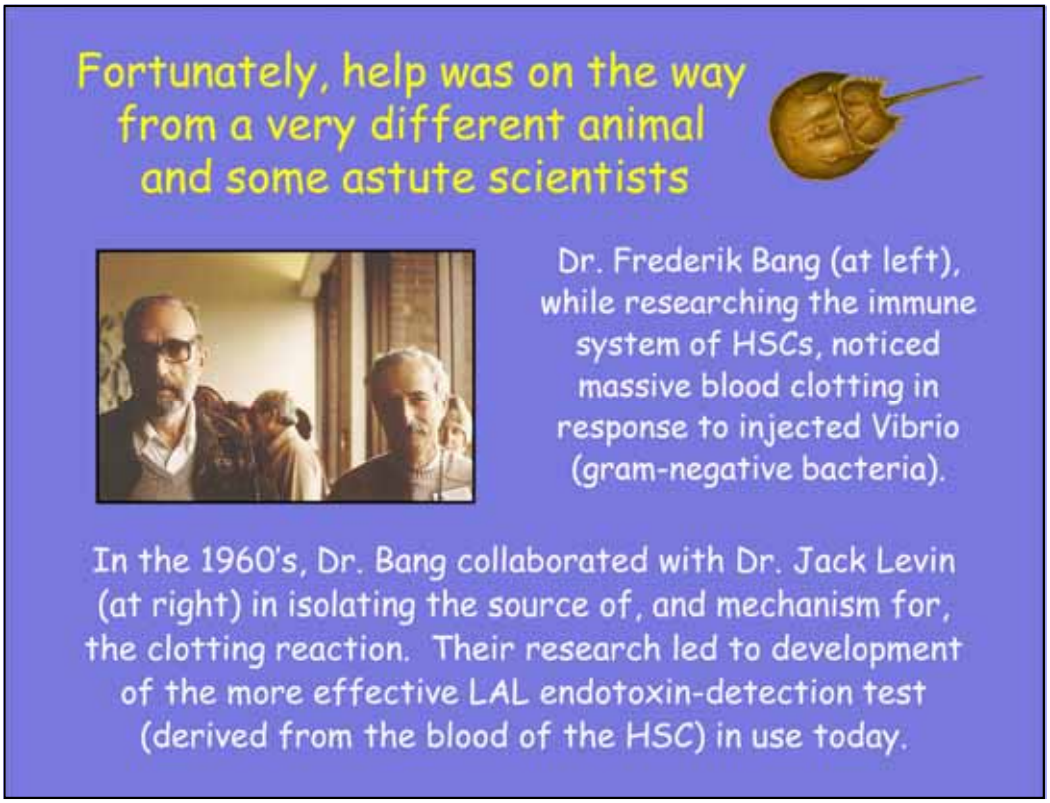


But the rabbit test had its problems. Not only were conditions difficult to control – due to individual differences in rabbit excitability factors, and other variables – but the tests also yielded many false positives (thinking the medicine failed the test, when it was actually safe to use) and false negatives (meds passing the test, when they weren't safe for use). The latter results were especially troublesome, including several health care incidents in the 1970's from meds that had passed the rabbit test:

1) In 1972, 39 cases of life-threatening aseptic meningitis occurred in patients receiving radiotracer injections for assessing flow of cerebrospinal fluid. Cooper and Harbert found endotoxins to be the cause, and that endotoxins were at least 1000x as toxic when administered intrathecally (into sheath surrounding the spinal cord) as compared to typical bloodstream pathways. They concluded that the rabbit test was not sensitive enough to pick up these lower endotoxin levels (but the LAL test was). [11]

2) In 1974, an outbreak of endotoxin-induced fever-reactions occurred in patients receiving rabbit-test-approved serum albumin for trauma & severe disease therapies. Such reactions were subsequently minimized by using LAL to test albumin supplies.[8]

3) In 1976, after receiving swine flu vaccine, 30 people died and several hundred developed Guillain-Barré syndrome [13] (a rare condition in which the immune system attacks the nerves causing temporary muscle weakness or paralysis). Later testing w/LAL indicated high levels of endotoxins in the vaccine that (had this been known) might have prevented use of these vaccines, and the adverse health probs resulting.[9]

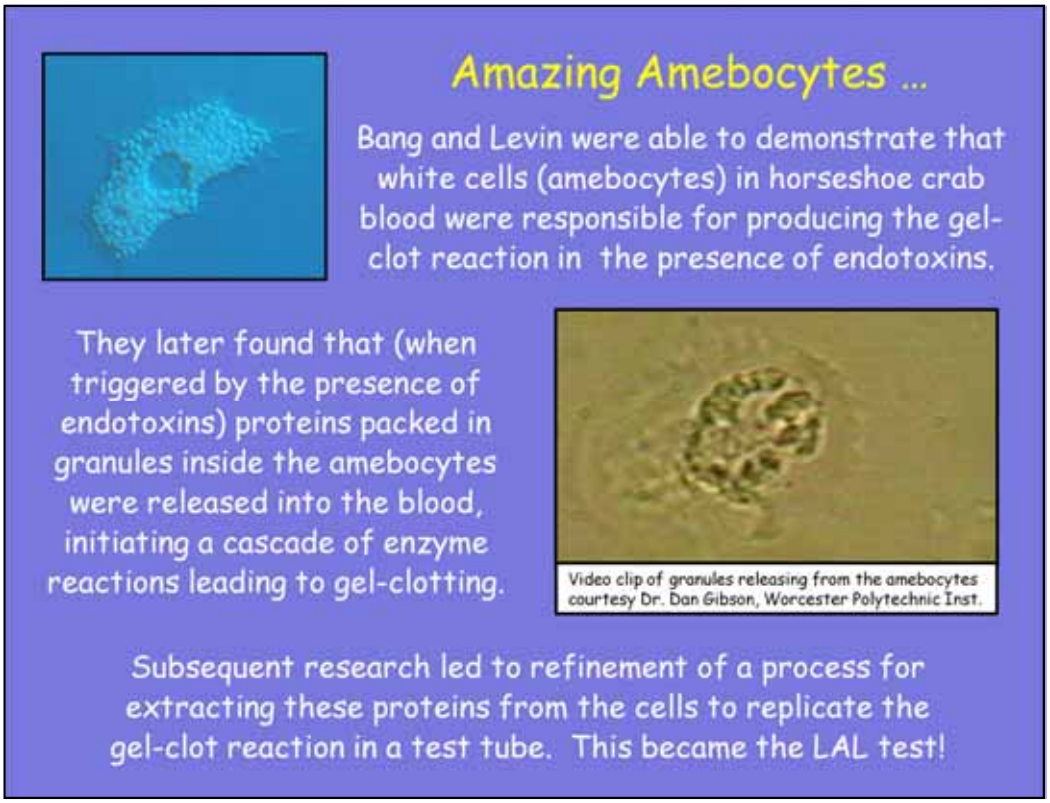


Such problems prompted health care officials to seek out an alternative, more reliable way of screening for endotoxins. Enter *Limulus* and Doctors Fred Bang & Jack Levin! As with certain other scientific discoveries, this involved some serendipity, along with a measure of that old "chance favors the prepared mind" adage.

In 1950, Dr. Frederick Bang, a researcher at Woods Hole Oceanographic Institute on Cape Cod, was asking a basic scientific question: If an animal like *Limulus* had been living in a seawater soup of pathogens for millions of years, what was its immune system like? How did it keep from getting sick? Due to its ancient lineage, he considered the HSC a good candidate for revealing primitive immunological functions.

He started injecting HSC blood w/various bacteria, and hit the jackpot with one called *Vibrio*, observing massive clotting of the blood in one of the test crabs.

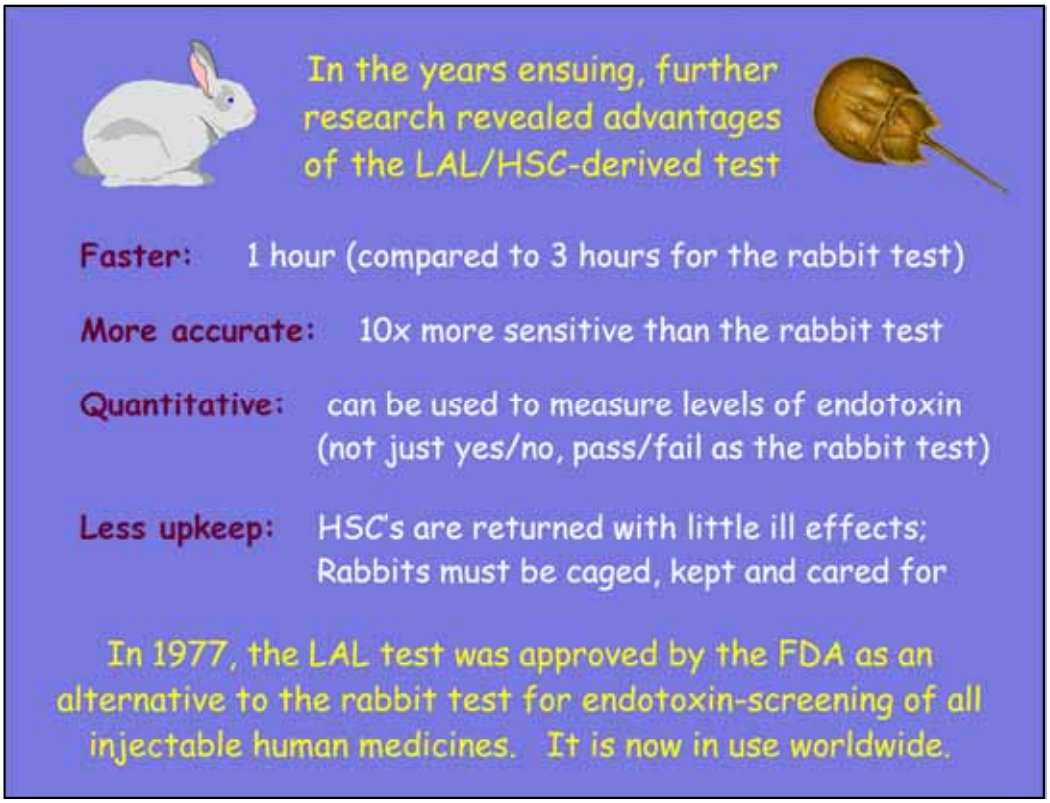
Upon further testing, Bang was able to show that heat-killed extracts of these bacteria also induced clotting. Although these were not the kind of results he expected to find, like all good scientists, Dr. Bang was smart enough to see that he was on to something significant. He was also wise enough to see that he could use some collaboration, so he hooked up with Dr. Jack Levin, a specialist in hematology from Johns Hopkins, and together they started to unravel the mystery that ultimately led to the development of the LAL test.[14]



But these discoveries hardly happened overnight either. Many year of research and levels of experimentation ensued. In one of the experiments, Bang and Levin separated out the HSC blood cells (amebocytes) from the plasma (hemolymph). They found that plasma without cells would not clot in the presence of endotoxins. This clued them that the clotting factors were derived from within the amebocytes (the only kind of blood cell HSCs have). [12]

Eventually, they were able to trace those clotting factors to proteins found in granules within the amebocytes. When exposed to harmful bacteria, these amebocytes change shape (developing long pseudopod-like processes), and the granules "pop out' or are released out of the cell.

Further investigation revealed that proteins released from the granules triggered a cascade of enzymatic reactions that caused the blood of the HSC to form a clot that engulfs and immobilizes the bacteria and/or endotoxins. Subsequent research enabled them to isolate and refine this biochemical process and duplicate it inside a test tube. Development of the LAL test was the end product of Bang & Levin's outstanding research and collaboration. [12]

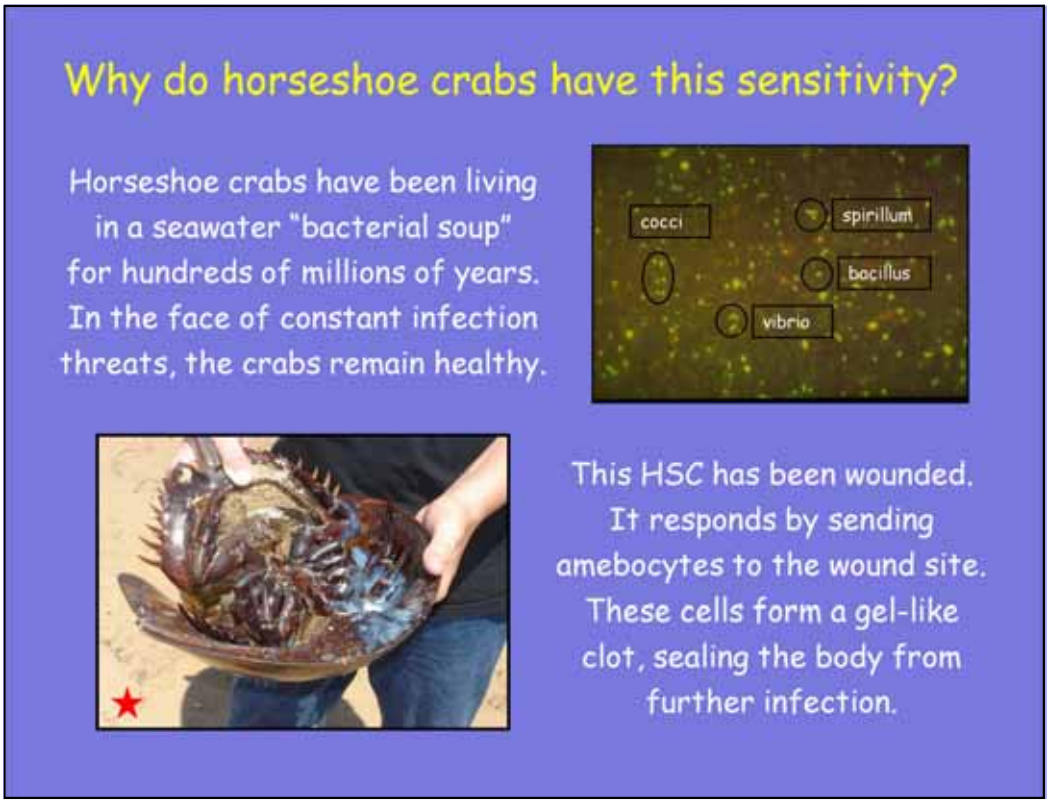


Though LAL was shown to offer a much more reliable and sensitive tool for endotoxin screening, it took some time for the test to become accepted, used and approved as the standard for pharmaceutical testing. Although developed by the 1960's, and in use by the medical community by the early 1970's, it wasn't until 1977 that the FDA officially approved its use (as an alternative to the rabbit test) for testing injectable medicines and until 1979 that it was approved for testing implantable medical devices.

There were several reasons for the delay. The reluctance of pharmaceutical companies to make a major shift in testing procedures was one issue. Highly variable results with early LAL tests didn't help - though this was later attributed to bleeding of juvenile HSCs (which have fewer amebocytes than adults)[2] and was subsequently corrected.

A big breakthrough occurred in 1990, when a conference of U.S. Pharmacopeia (USP) aimed at minimizing use of animals in pharmaceutical testing, removed the rabbit test from the USP, making the LAL test the only viable tool for endotoxin screening. [15]

Although a major improvement over the rabbit test, LAL did not solve all problems and is not considered foolproof. One of the major drawbacks of LAL is that it only detects levels of endotoxins. It cannot identify which kind of bacteria releases the endotoxins. This greatly limits its value as a diagnostic tool. Also contaminations of drugs with gram-positive bacteria (e.g. *Staphylococcus* species or *Bacillus cereus*, common contaminants in pharmaceutical manufacturing), are not an unlikely event. [8]



So, why do HSCs have this extraordinary sensitivity? HSCs have been living in a microbial soup for hundreds of millions of years, exposed to a myriad of microbes that could infect them and even kill them. A single teaspoon of seawater may contain billions of bacteria!

So how do they keep the harmful microbes at bay? Like all invertebrates, HSCs are cold-blooded/ectotherms (so they can't use fever to heat up and kill off bacteria like we do), and they lack antibodies (to detect, recognize and confer immunity to foreign invaders). HSCs also have an open circulatory system, meaning that harmful bacteria, upon entry into their bloodstream, can quickly gain access to many of the HSCs' internal organs and tissues. In contrast to humans, HSCs also are limited to just one kind of blood cell (called an amebocyte) in their immune system defense arsenal.[16]

So how <u>do</u> they do it? Despite its apparent simplicity, over those millions of years of exposure to all sorts of microbes, the HSC has evolved a highly efficient and sensitive system for detecting and dealing with them. So when an HSC is wounded (as in picture above), the amebocytes react quickly by releasing enzymes from granules (found in the cytoplasm) into the blood around the wound site. This triggers the gel-clot reaction, which not only seals off the wound site from further invasion, but also acts to neutralize the bacteria & endotoxins present, thus buying time for other immune system weapons (peptides and other proteins found in the blood) to activate and destroy them. [17]

Comparing human blood to horseshoe crab blood

Human blood	HSC haemolymph
flows in a closed system	bathes tissues in open system
has iron-based hemoglobin, turns red when oxidized	has copper-based hemocyanin, turns blue when oxidized
oxygen is carried in cells	oxygen not carried in cells
has several kinds of blood cells	has only one kind of blood cell
antibody-based immune system	lacks antibodies
has platelets to induce clotting	amebocytes provide clotting

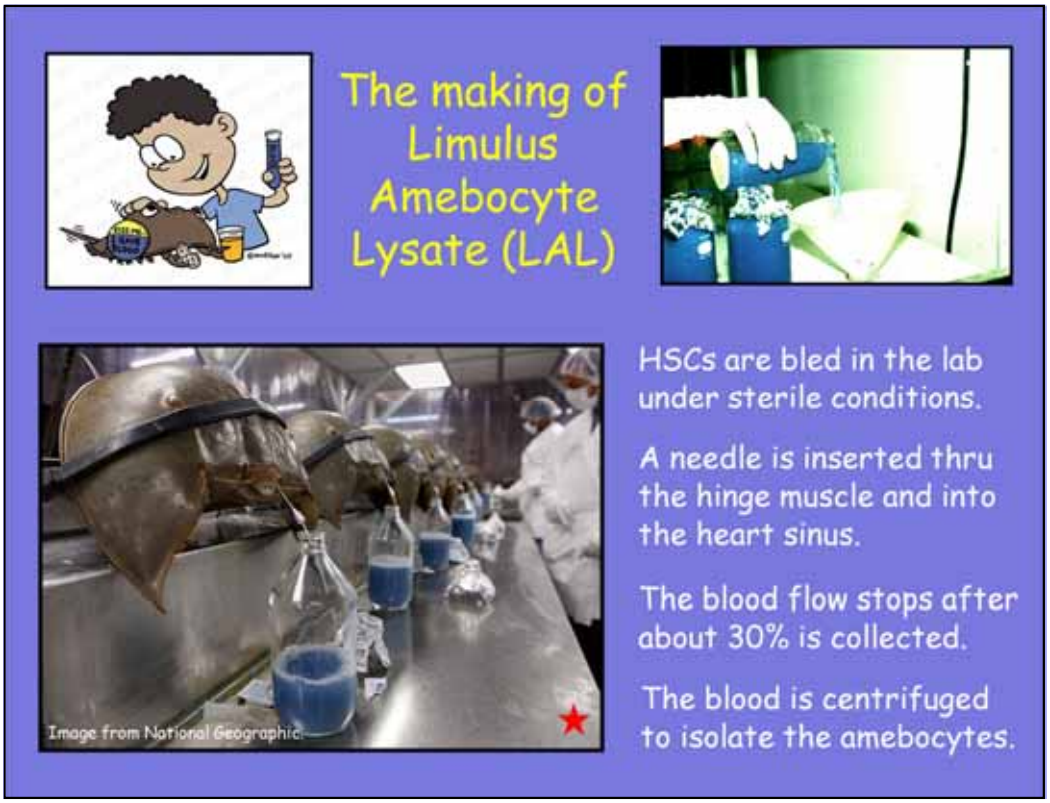
Please note: The color of HSC blood has nothing to do with its endotoxin-detecting properties!

This slide in offered as a point of reference. Go over the list row-by-row. Note: some of the information included in the notes section of slide 14 could apply here as well.

Although complex, antibody-based, immune systems are only found in vertebrates, this doesn't mean that the immune systems of invertebrates are poor or ineffective. As we've seen with the HSC, these animals have evolved other weapons and strategies! Anyone who sees the HSC as primitive, should read the chapter on the HSC immune system in The American Horseshoe Crab (Shuster, Barlow, and Brockmann, 2003). [17]

Also this is as good a place as any to demystify the blue blood part of the HSC story. Firstly, the blood of the HSC inside the animal is actually more of a straw color. Only when blood is exposed to air does the copper pigment in it cause it to oxidize blue. And the blue color has nothing to do with endotoxin-detecting capacities of its blood. HSCs are also not the only blue bloods. Molluscs (clams, snails, squid and octopi), crustaceans (lobsters, shrimp, blue crabs, etc.) and scorpions also have blue-blood.

Questions often arise as to why blood in human veins appears blue under our skin. The answer has to do with how the light spectrum is reflected or refracted by skin. Veins appear blue because only the high-energy, low-frequency blue wavelengths of light are reflected by the veins, while the other colors are absorbed by these vessels. In reality, venous blood tends to be a dark, dirty red because it is unoxidized and carries wastes. Arterial blood, having been freshly oxygenated in the lungs, appears bright red, because its iron-based pigment, hemoglobin, oxidizes red like rust.



Due to the design of the HSC circulatory system, bleeding only removes blood from its large pericardial sinus (heart) and adjacent large arteries. Most of the remaining hemolymph (roughly 70% of the total blood volume) remains in the spongy tissues of the prosoma and diffuses slowly into the large vessels.[12]

One may notice in these and other video clips or images of HSCs being bled that it is done in a sterile lab environment with technicians wearing masks, lab coats and other protective equipment. Might be interesting to ask students why they think this is the case. Is it to protect the humans or protect the crabs?

Actually it is neither. It's all about protecting the blood product that is to be used in making the LAL. Why would this be important? If conditions were not sterile, blood drawn from the HSC would be exposed to bacteria/endotoxins, causing amebocytes to release clotting proteins into the blood and induce a clot.

Think about it! If the blood is already clotted before or while it is being turned into a product that relies on a clotting reaction to be useful as a test - well that just wouldn't work out, would it? That's why the bleeding process takes place in sterile chambers where ambient air, water, surfaces, etc., as well as the humans entering or leaving them, are carefully monitored and controlled.

In China - where crabs are also bled for biomedical use and then used for food and shell products - the product is called TAL, for *Tachypleus* Amebocyte Lysate. In India, where the Indian HSC is used, it is referred to as CAL for *Carcinoscorpius* Amebocyte Lysate. The more generic term "Lysate" is also often used to describe any of these products.

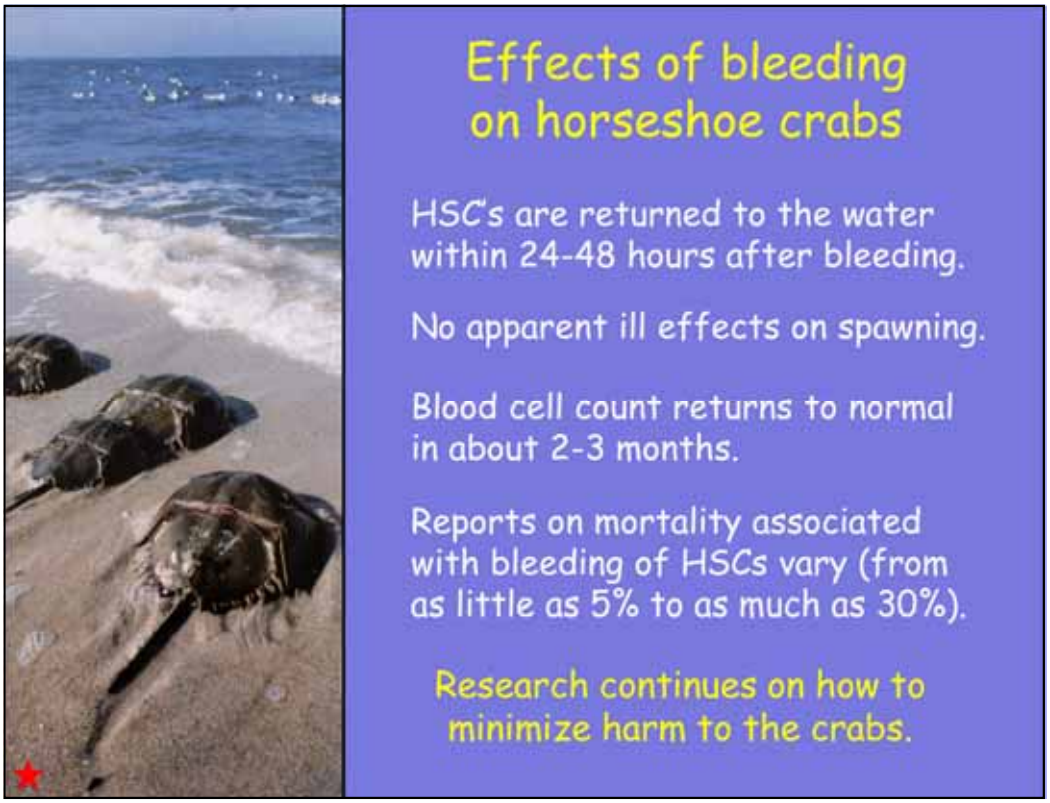
In recent years, realization of the economic potential of biomedical use of HSCs has prompted concerns about potential overexploitation in parts of the world (e.g. India and Southeast Asia) where HSC populations are not nearly as robust and regulated as in North America, where production of LAL is a multi-million dollar industry. [18]

One of the questions teachers often ask is: "How many vials of LAL are produced from the bleeding of one HSC?" Another is: "How much is that product worth?" Thanks to information provided by Dr. Ron Berzofsky (personal communication),[15] we took a stab at answering those questions, as follows:

Firstly, we assume an average volume of 100 ml of blood product per HSC bleeding.

Then, according to Dr. Berzofsky, 100 ml of HSC blood, upon centrifuging, yields 5 ml of packed amebocytes, and this amount yields 425 LAL single (gel-clot) test vials (in case your wondering, each single test vial uses 0.1 mg of LAL material).

Now to the economics. In 2015, single test LAL gel-clot vials market commercially in the U.S. for around $5 each; [19] so (doing the math), the total value of LAL product derived from the bleeding of one HSC would translate to: 425 x $4.44 = $1887.



Nearly all of the 500,000 HSCs that are collected annually in the U.S. for biomedical bleeding are returned to the habitat where they were harvested within 24-48 hours after collection. An exception to this occurs in Massachusetts, where regulations allow that animals bled for biomedical purposes can be passed on to commercial fishermen for use as bait in catching conch and eel. [20] The rationale behind this is that if a certain number of crabs are going to be harvested and used for bait anyway, they might as well use the biomedically-bled crabs twice (getting dual use out of one crab), as opposed to exposing still more crabs to human use.

The other important thing to know about biomedical use is that the large majority of crabs do survive the bleeding process. Variation in reported mortality rates (2-30%) from bleeding are attributed in large part to how they were collected, and to a lesser extent, on conditions under which they are kept and bled. Crabs collected by dredge or trawl show higher mortality rates than those collected by hand harvest.[3]

The level of stress horseshoe crabs are exposed to during handling also factors in. One study found that crabs bled under low stress conditions, had 0% mortality, compared to 8.3% dying when exposed to high-stress handling conditions. Another study found mortality to be as high as 29% in females bled to 40% of their blood volume (more than normal amounts) under high stress conditions. [21]

Biomedical companies are constantly looking for ways to reduce negative impacts on the HSCs they use and depend on for the life-saving products they provide.

Factor C is already available[22], but will take some time to replace LAL – due to it being so well entrenched in pharmaceutical protocol and confirmed reliability.

Likewise with whatever comes of this latest promising alternative offered up by researchers on the African clawed frog.[23] Like the HSCs, these frogs have also evolved a strong antibacterial defense system. In the wild, the African clawed frog produces antibacterial peptides - small chains of amino acids - on its skin to protect it from infection. Princeton researchers have found a way to attach these peptides, which can be synthesized in the lab, to a small electronic chip that emits an electrical signal when exposed to harmful bacteria, including pathogenic E. coli and salmonella.

The African frogs, which are common in laboratories and pet stores, are not harmed in the process, and the peptides can be synthesized. McAlpine and Manu Mannoor, a Princeton graduate student who worked on the project, hope that technology based on their electronic chip will eventually replace LAL as the standard for contamination testing, obviating the need for HSC blood. [23]

Not so good news on biomedical use of HSCs

The number of HSCs bled for LAL in the U.S. has more than doubled in the last decade (to >500,000/yr.)

Global demand has ramped up the bleeding of Asian HSCs for TAL

In many parts of Asia where HSCs are bled, best husbandry practices are not well established, and after bleeding the HSCs are harvested for their eggs, meat and chitin.

Clandestine bleeding of HSCs for TAL at a shark finning warehouse in Hong Kong (http://horseshoecrabwatch.blogspot.com/2013/03/catastrophic-declines-in-tachypleus.html)

Although efforts are now underway to introduce better management measures on such uses in Asia, the challenges are many and major, and meantime populations will continue to suffer.

A special effort is being made by ERDG to help (working with & through the pharmaceutical industry) – through a soon-to-be released update to the biomedical section of their popular www.horseshoecrab.org webite

The challenges in Asia are complicated. Unlike *Limulus*, for which something like 99% of its population is confined to the borders of one country (the U.S.), the three Asian HSC species are distributed across numerous countries, all with varying economic, political and cultural priorities and practices that make a unified approach to management a major challenge. And in many of these places, research on HSCs has not been as directed, so data is lacking on status of populations and level of harvest.

The practice of treating the HSC as an "all-parts-use-animal" (as is the case currently in China) - though laudable from the standpoint of making the most use of an animal being sacrificed for human benefit - means that all HSCs bled for TAL in this way are lost from the population (in contrast to bleeding of HSCs for biomedical in the US, where HSCs are returned to the wild after use).

On the good news front, a group of scientists from North America and Asia are now working as a Horseshoe Crab Species Specialist Group (SSG) under the International Union for the Conservation of Nature (IUCN) to promote global conservation efforts.

Another encouraging pathway for mitigating this problem is underway through ERDG's efforts to promote use of best management practices for HSC bleeding and use of the synthetic LAL/TAL alternative by the pharmaceutical industry. To learn more about this initiative, check out the new "Protecting Health" section of the ERDG website at: http://www.horseshoecrab.org/med/med.html[24]

So, what does all this mean to us?

Thanks to the HSC and LAL test, endotoxins can be detected before they cause human health problems.

The risk of infection from injected or implanted medicines is minimized.

So are the risks of developing gram-negative **sepsis** - a disease that kills thousands of people each year.

Though the LAL test cannot tell us what species of bacteria are present, it has been used in diagnosing a gram-negative bacterial cause to certain illnesses - including spinal meningitis, certain eye and urinary tract infections, and endotoxemia - thus enabling a speedy and effective course of treatment.

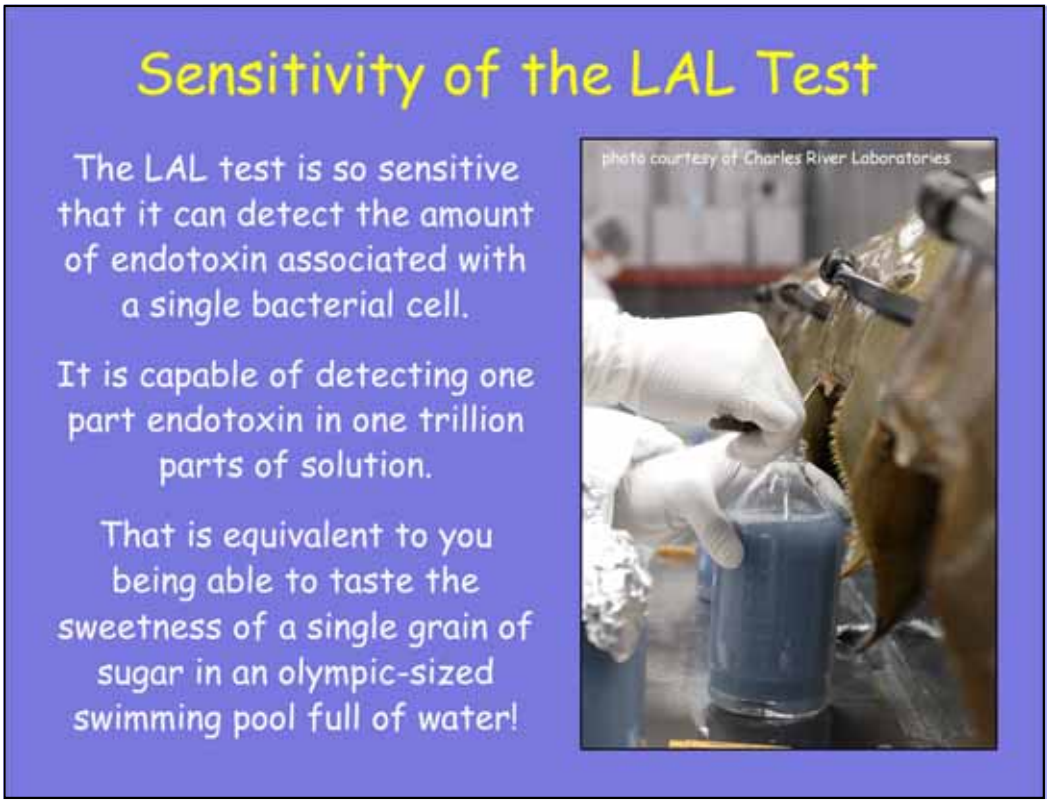
Using LAL to ensure that lab-cultured skin tissue is safe for use in burn victims (photo by Mike Oates).

So – bottom line – what does all of this mean to you and me? The answer is "a lot!" In addition to ensuring that our vaccines, insulin, allergy shots, IV's, and other injectable and implantable meds are free from contaminants that could make us sick, LAL has also greatly improved the speed of diagnosis and treatment for certain bacterial diseases, including spinal meningitis and some urinary tract infections. [9]

Bacterial sepsis - a condition that kills thousands of hospital patients each year - also has potential to be greatly reduced through use of LAL for rapid diagnosis. As with meningitis, LAL cannot identify the kind of bacteria causing sepsis, just that it's bacterial (not viral), so antibiotic treatment can be promptly administered. LAL has also been used to screen anti-endotoxin compounds for potential life-saving sepsis therapies. Interestingly enough, one of the compounds showing therapeutic potential was isolated from *Limulus* hemolymph![9]

And many other specialized applications are also in use. Reference picture above: In the LAL video segment (that comes with the GE&S curriculum), there's a piece at the end on using LAL to ensure that lab-cultured skin tissue for burn victims is safe. There's a rather tight window of time for use of these tissue-cultured skin samples. Before LAL, they would have had to use a petri dish approach to test for bacterial contamination, taking at least 24 hours. By using LAL, tissues can be screened in an hour, greatly speeding up the healing and recovery prospects for burn patients.

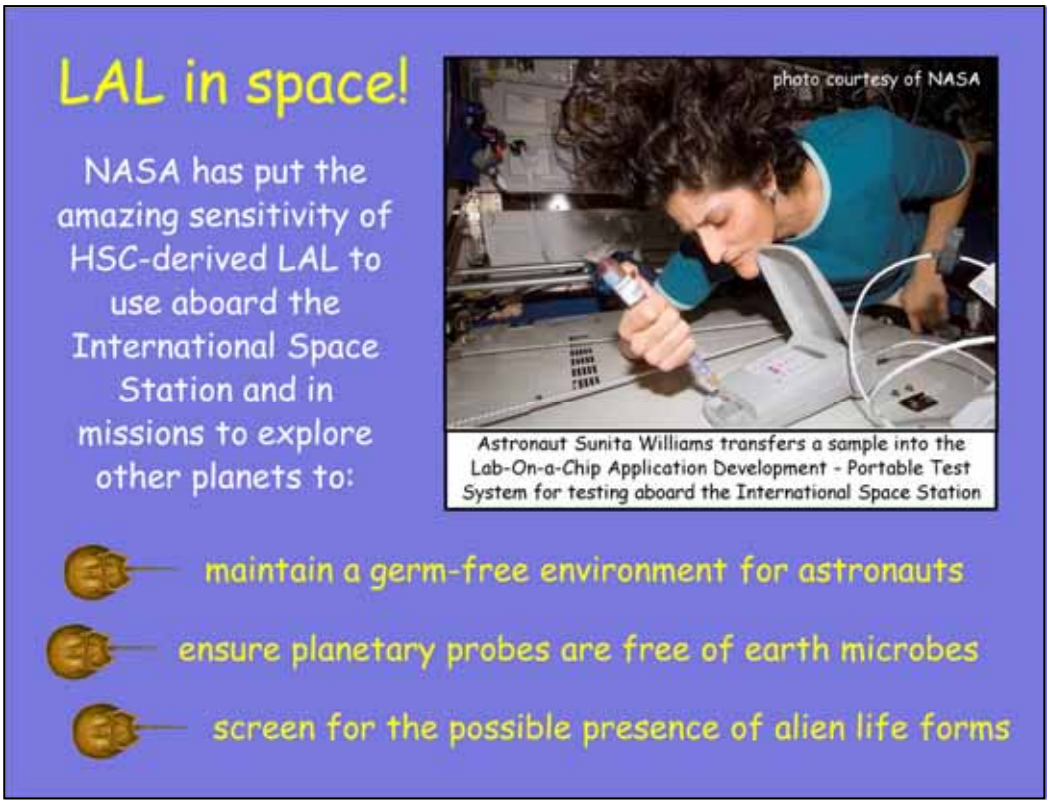
Another special application relates to artificial kidneys and the water used to prime dialysis machines, both of which can be contaminated in use/reuse w/endotoxins.[9]



So how sensitive is LAL to endotoxin? Read the two blurbs on the slide. It's also been noted that the the LAL test is so sensitive that it can pick up the tiny bit of endotoxin produced from a single *E. coli* bacterium in a ml of water!

If time allows, this could be a good place to mention the more quantitatively precise variations of the LAL test that have been developed. This includes: [12,25]

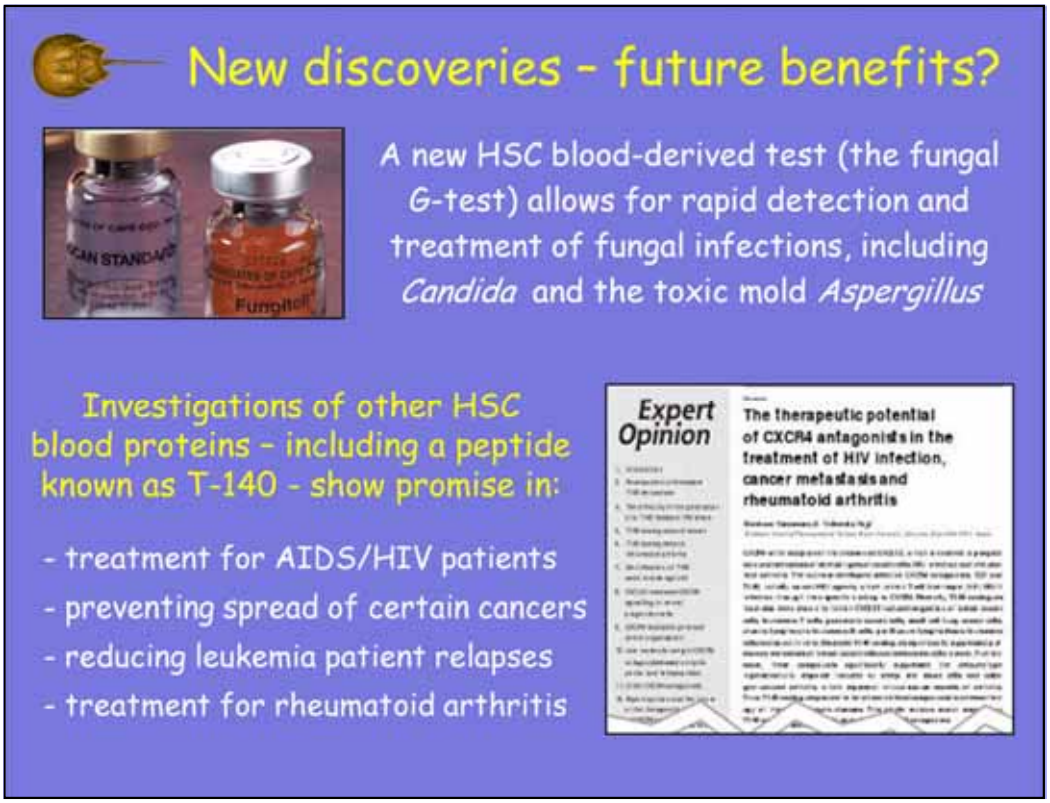
1) Turbidimetric systems measure (using a spectrophotometer) the level of cloudiness or turbidity in the coagulogen that is produced by the gel clot reaction after incubation at a fixed temperature over a fixed time period (the higher the turbidity, the greater the concentration of endotoxins).
2) Chromogenic assays utilize a chromogen, or chemical that changes color in response to the amount of endotoxin (again at fixed temp. and time period). The higher the endotoxin concentration, the more chromagen that is released (as measured by an optical reading device tuned to chromagen wavelength).
3) Kinetic assays measure the rate of change in turbidity or color during the assay (based on the premise that the higher the concentration of endotoxin, the more rapidly the reaction being measured will take place). Kinetic assays can provide a greater sensitivity over a wider range than turbidimetric or chromagenic endpoint assays, but require more elaborate instrumentation.
4) Portable, hand-held electronic versions of the LAL test are now available that allow for simplified, fast (15-minute) and accurate testing in the lab or field.



If it's not already doing enough for us on Earth, LAL is now being used in outer space! NASA , with help from Charles River Laboratories, has developed a mini-LAL-lab called LOCAD-PTS (Lab-On-a-Chip Application Development–Portable Test System) for use on the International Space Station, the space shuttle and other space missions. Thanks to clever engineering and the rapid sensitivity of HSC enzymes, this device is small, sleek, and fast (not unlike the 'tricorder' device of recent Star Trek films!). [26]

Use in space involves swabbing the spacecraft instrument panels and other surfaces, and inserting the swab contents into one of several narrow channels in LOCAD-PTS. Each channel contains dried LAL powder and a colorless liquid, which turns green in the presence of bacteria and fungi (darker the green, the greater the contamination). Results are available in 15 minutes - much faster than the old (3-day) petri dish culture method. Astronaut health is similarly monitored with this device, requiring only tiny amounts of body fluids to be tested for bacterial infection. In a recent space station mission, Astronaut Sunita Williams (note HSC earrings above) tested this technology. [26]

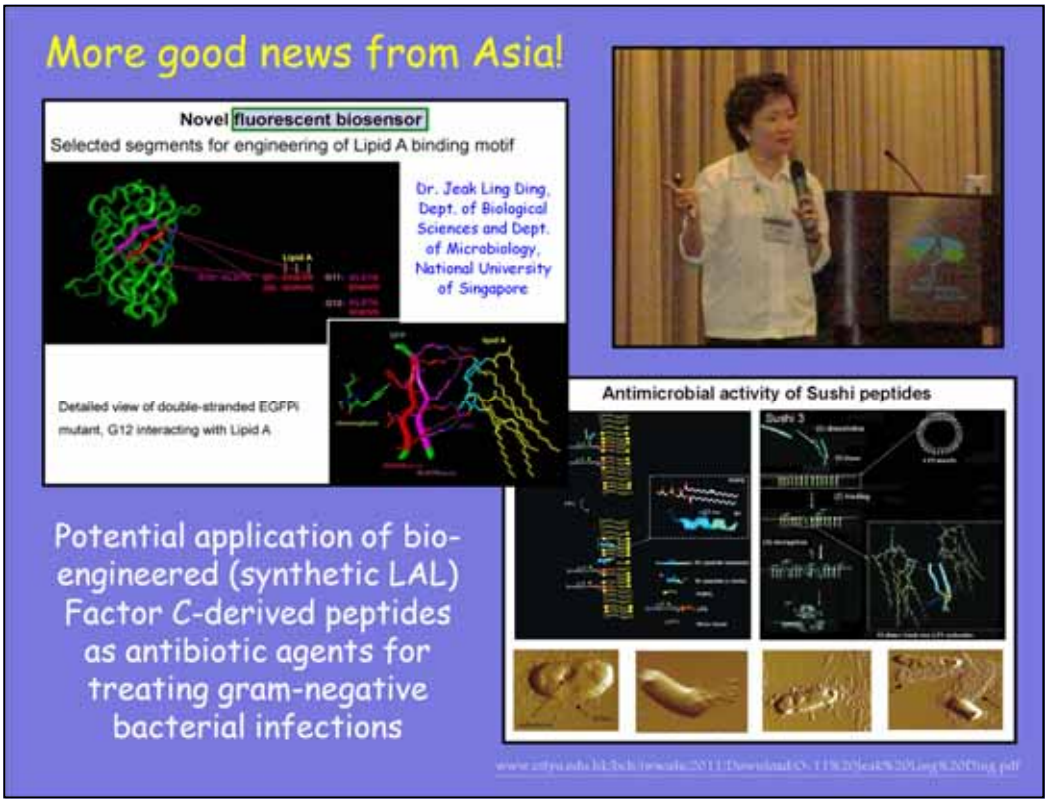
NASA also plans to use this technology to analyze samples from spacecraft surfaces before launch to verify that they are free of microbes and other organic material. The rigorous cleaning process is required by the Planetary Protection Act, established by NASA to ensure spacecraft don't contaminate pristine environments on other planets with Earth microbes. And given the incredible sensitivity of LAL to microbial life, NASA is also exploring whether the PTS gizmo could be sent into space for use in testing soils scooped up by probes from other planets for the presence of microbial life.



Given the wonders of LAL, researchers are looking to the HSC for a wealth of other biomedical applications, and they are finding them!

HSC blood has also proven sensitive to glucans, a component of the fungal cell wall, resulting in a variation of LAL (called the Fungal G-test) that is now being used as a diagnostic tool for fungal infections in humans. This test has been found especially useful in early detection of Candida (yeast) and Aspergillus (toxic mold) infections,[9] both of which are widespread and can cause a wide array of human health problems.

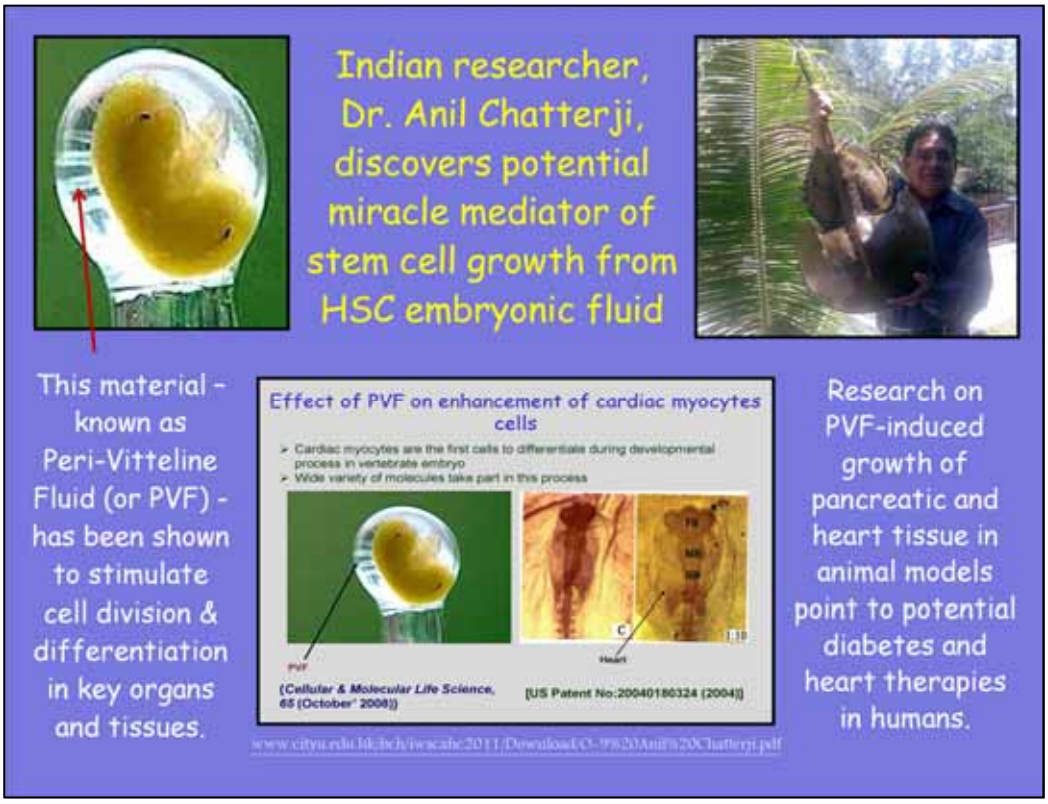
In other research over the last decade, other proteins in HSC blood have been identified and investigated for their promise in various human health arenas. One of these, a peptide known as T-140, has been synthesized in the lab and found to be an effective candidate for potential use in treatment of AIDS, Leukemia, rheumatoid arthritis and several forms of cancer.[17,27,28]



In 2011, an International Conference on the Horseshoe Crab was held in Hong Kong. There were some amazing presentations there, including a few on biomedical use of HSCs. One of those was offered by Professor Jeak Ling Ding of Singapore University, the researcher responsible for development of LAL alternative Factor C. [29]

Based on new research presented by Dr. Ding at the conference, they are taking Factor C in some impressive new directions. One of them (point to upper left screen-shot) is in development of a highly sensitive new flourescence-based biosensor that allows for rapid quantitative detection of endotoxins in samples.

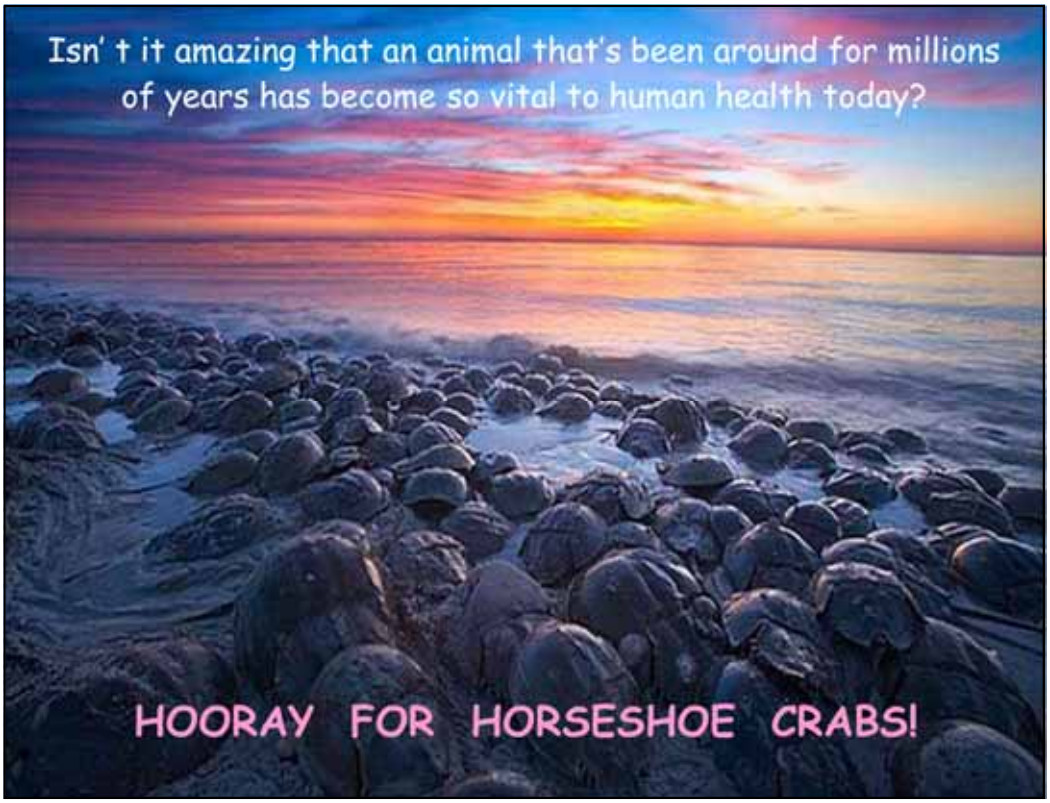
Even more intriguing for their potential medical value are (point to lower right graphic) is Dr. Ding research on special genetically-engineered, factor C-derived peptides that are proving effective at binding and killing gram-negative bacteria pathogens. The potential for use as a tool in both rapid assessment of gram-negative bacterial caused diseases and as a powerful antibiotic for treatment of life-threatening gram-negative sepsis is huge. [29]



Meanwhile, Dr. Anil Chatterji has been investigating amazing medical applications arising from the embryonic peri-vitteline fluid (PVF) inside the HSC egg. He found PVF to be a particularly viable medium for promoting and prolonging stem cell culture, with potential human health applications to heart, pancreas and other organ/tissue regeneration therapies.[30,31]

In one avenue of research, researchers found that the molecule lectin, from HSC PVF, had a dramatic influence on cardiac development in chicks and mice, resulting in an increase in number of cells and size of the heart. Similar results were observed with PVF stimulating production of beta cells in pancreatic tissue (where insulin is produced), suggesting potential future therapies for diabetes. Through similar use of these HSC peptides to induce naïve stem cells to take a particular developmental pathway, scientists may be able to target additional repair/regeneration therapies for other organs and tissues.[32]

Biomedical benefits of HSC PVF have also been forecast relative to its observed anti-angiogenic activities. This includes potential applications to treatment of cardiac disorders and cancer therapies, using inhibition of angiogenesis as a means of “suffocating” tumors, by depriving them of vascular irrigation and oxygen supply.[30]



Do any of you still see horseshoe crabs as useless, ugly, primitive, unevolved animals? OK, so maybe they're not the most beautiful creatures. But maybe not so primitive, arguably more evolved than credited for, and definitely far, far from useless! And we've hardly touched on the contributions of research on HSC eyes to human vision!

If you think about it, this animal's been around for a long, long time, withstanding ice ages and other major climatic changes, adapting to major shifts in continent formation and location, and surviving mass extinctions that wiped out as many as 90% of the species it shared the planet with at the time (including its trilobite relatives, the great dinosaurs, giant stone age mammals, and many many others).

Along the way, it's probably come across, and been exposed to, about every kind of microbial pathogen that's come down the pike, and - as we've learned with LAL, and are learning more and more from closer looks at other parts of its immune system – the HSC has had lots of time to develop systems & strategies for dealing with them. And as Peter Armstrong points out, the fact that these "primitive" immune system proteins (tracing back hundreds of million years) have been preserved and remained functional in HSCs (and in so many other higher animals from insects to humans), speaks to their biological significance, and to the benefits of using a relatively large, simple, easily studied animal like the HSC to decipher their relevance to humans. [33]

The horseshoe crab is not only – as Glenn Gauvry, one of its major human friends and supporters has called it - "a survivor on the grandest scale", but an animal that has given, and is still giving, so much to the health and well-being of us humans.

LAL PowerPoint Credits

Developed by: Gary Kreamer, Tricia Cosbey & Melissa Pierce,
DE Aquatic Resources Education (ARE) Center

Inspired by: Glenn Gauvry, Ecological Research Development Group

Video clips and images supplied by: Michael Oates of Anew, Inc.

Additional images & info courtesy of: John Dubczak, Charles River Laboratories
Dr. Mick Dawson, Cape Cod Associates

Special thanks to: Dr. Ron Berzofsky of Wako Chemicals USA for sharing his ideas, images, expertise & wonderful way of making complex concepts comprehensible!

References for "Modern Medical Marvels from an Ancient Mariner" presentation 2015

(numbers correspond to those provided in the presenter notes portion of the PowerPoint)

1. ERDG (2010) The horseshoe crab: anatomy, circulatory system (http://horseshoecrab.org/anat/circ.html)
2. Shuster CN Jr. (2003) A blue blood: the circulatory system. In: Shuster, CN Jr., Barlow RB, Brockmann HJ (eds) The American Horseshoe Crab. Harvard University Press, Cambridge, MA. pp 276-287
3. Hurton L, Berkson J, Smith J (2005) Estimation of total hemolymph volume in the horseshoe crab *Limulus polyphemus*. Marine and Freshwater Behaviour and Physiology, June 2005; 38(2): 139–147
4. Prior RB (ed) (1990) Clinical applications of the *Limulus* amoebocyte lysate test. CRC Press, Boca Raton, FL
5. Wikipedia: Edward Jenner (http://en.wikipedia.org/wiki/Edward_Jenner)
6. Marine Biological Laboratory (2010) The Horseshoe Crab: Blue Blood, Bacteria Basics (http://www.mbl.edu/marine_org/images/animals/Limulus/blood/index.html)
7. Alexander C, Rietschel ET (2001) Bacterial lipopolysaccharides and innate immunity. Journal of Endotoxin Research 7(3): 167-202. (www.biologia.uniroma1.it/didattica/att/8448.7167.file.pdf)
8. Williams KL (ed) (2007) Endotoxins: Pyrogens, LAL Testing and Depyrogenation (3rd ed). Informa Health-care, New York, NY, 440 pp
9. Novitsky TJ (2009) Biomedical applications of *Limulus* amebocyte lysate. In: Tanacredi JT, Botton ML, Smith DR Biology and Conservation of Horseshoe Crabs. Springer Science, New York, NY, pp 315-330
10. Jacob AI, Goldberg PK, Bloom N, Degenshein GA (1977) Detection of endotoxins in human blood. Gastroenterology 72(6): 1268-70
11. Novitsky TJ (1994) *Limulus* amebocyte lysate (LAL) detection of endotoxin in human blood. Journal of Endotoxin Research Vol. 1(4): 253-263
12. Levin J, Hochstein HD, Novitsky TJ (2003) Clotting cells and *Limulus* amebocyte lystate: an amazing analytical tool. In: Shuster, CN Jr., Barlow RB, Brockmann HJ (eds) The American Horseshoe Crab. Harvard University Press, Cambridge, MA. pp 310-340
13. Wikipedia: 1976 Swine Flu Outbreak. (http://en.wikipedia.org/wiki/1976_swine_flu_outbreak)
14. Bang F (1955) A bacterial disease of *Limulus Polyphemus* (http://www.mbl.edu/marine_org/images/animals/Limulus/Bang/index.html)
15. Berzofsky R (personal communication) Wako Chemicals USA, Inc. Richmond, VA
16. Marine Biological Laboratory (2010) The Horseshoe Crab: Blue Blood, Blood Cells (http://www.mbl.edu/marine_org/images/animals/Limulus/blood/bang.html)
17. Armstrong PB (2003) Internal defense against pathogenic invasion: the immune system. In: Shuster, CN Jr., Barlow RB, Brockmann HJ (eds) The American Horseshoe Crab. Harvard University Press, Cambridge, MA. pp 288-309

18. Gauvry G (2008) The American Horseshoe Crab *Limulus polyphemus*: signs of recovery. LAL Users Group Newsletter 2(2): 1-4 (http://horseshoecrab.org/press/2008/10/lal_news_v2n3.pdf)

19. Lonza (for ordering LAL) (http://www.lonza.com/group/en/products_services/products/lal/endo.html)

20. Massachusetts Department of Fish and Game (www.mass.gov/dfwele/dmf/spotlight/hc_pleasant_bay.htm)

21. Leschen AS, Correia SJ (2010) Mortality in female horseshoe crabs (Limulus polyphemus) from biomedical bleeding and handling: implications for fisheries management. Marine and Freshwater Behaviour and Physiology, 43: 2, 135 — 147 (http://dx.doi.org/10.1080/10236241003786873)

22. Chen L (2006) Reckoning recombinant factor C. (www.lonza.com/group/en/products_services/products/catalog_new.ParSys.0007.File0.tmp?path=eshop/Technical_Library/PFQ_25_web.pdf)

23. New sensor derived from frogs may help fight bacteria and save wildlife. Science Daily: Oct. 20, 2010 (www.sciencedaily.com/releases/2010/10/101019171819.htm)

24. The horseshoe crab and public health (2015) (http://www.horseshoecrab.org/med/med.html)

25. Endotoxin detection methods for pharmaceuticals and medical devices (www.rapidmicrobiology.com/test-methods/Endotoxin.php)

26. Hsu J (2009) 'Star Trek' Tricorder scans for life on space station. Live Science (http://horseshoecrab.org/press/2009/07/lal-in-space.pdf)

27. Tamamura H, Tsutsumi H, Nomura W, Tanaka T, Fujii N (2008) A future perspective on the development of chemokine receptor CXCR4 antagonists. Expert Opinion on Drug Discovery, Oct. 2008, 3(10): 1155-1166 (http://informahealthcare.com/doi/abs/10.1517/17460441.3.10.1155)

28. Burger JA, Peled A (2009) CXCR4 antagonists: targeting the microenvironment in leukemia and other cancers. Leukemia (2009) **23:** 43–52; (www.nature.com/leu/journal/v23/n1/pdf/leu2008299a.pdf)

29. Ding, JL, Ho B (2011) Conservation of horseshoe crab –genetic engineering of Factor C for pyrogen testing & beyond. Departments of Biological Science & Microbiology, National University of Singapore (www.cityu.edu.hk/bch/iwscahc2011/Download/O-11%20Jeak%20Ling%20Ding.pdf)

30. Chatterji A, Shaharom F (2011) Present and future of horseshoe crab in biomedical research. Institute of Tropical Aquaculture, University Malaysis Teregganu. (www.cityu.edu.hk/bch/iwscahc2011/Download/O-9%20Anil%20Chatterji.pdf)

31. Chatterji N, Ismail N (2008) Horseshoe crab – a valuable marine creature. Prospect Malaysia pp 50-56 (http://horseshoecrab.org/press/2009/01/horseshoearticle.pdf)

32. Mascarenhas A (2008) A bigger and better heart, thanks to a crab. Originally published at ExpressIndia.com (http://horseshoecrab.org/press/2008/12/a-bigger-and-better-heart-thanks-to-this-crab-express-india.pdf)

33. Standing the Test of Time. (www.mbl.edu/publications/pub_archive/labnotes/4.3/time.html)