OUT FOR BLOOD: BIOTECHNOLOGY TO ERADICATE HORSESHOE CRAB BLEEDING

BY GRACIE VENNEWITZ

Dating back 450 million years, the Atlantic horseshoe crab has almost nothing in common with our evolved human population. The primordial species is a living fossil that persisted through millions of years of global chaos and metamorphosis to simply exist alongside us today.[1,2] As a result, the ancient biology of the crab is not to be underestimated– in fact, research suggests it can even protect us from threats posed by our own immune system.

In the pharmaceutical industry, the horseshoe crab is a powerful weapon for combatting unwelcome microorganisms in parenteral products.[3,4] Horseshoe crab blood is used to test vaccines ranging from measles to COVID-19 for the presence of endotoxin: a byproduct of drug manufacturing that poses a major health threat to consumers.[5,6] Since the 1970s, this reliable, widespread practice has served as the sole framework for endotoxin detection.[3,4,7,8] However, biotechnological advancements in the 21st century continue to expand on this model in creative, unprecedented ways.

ENDOTOXIN: PUBLIC ENEMY NUMBER ONE

Endotoxins are core constituents in the cell membrane of gram negative bacteria that infect the human bloodstream.[5,9] When the human immune system recognizes endotoxin-containing bacteria in the blood, it directs white blood cells to attack and disintegrate the whole organism, thereby dispersing endotoxin fragments throughout the bloodstream.[9,10] This termination process is what makes endotoxin "toxic" in the first place, because these fragments cause symptoms like fever, shock, organ failure, and, in many cases, even death.[5,10,11] In order to prevent consumers from developing these symptoms, pharmaceutical companies require a lucid, foolproof diagnostic to confirm the absence of endotoxin in their products.

RUDIMENTARY BIOLOGY WITH REVOLUTIONARY CAPABILITIES

Conveniently, the simplistic design of the primitive horseshoe crab immune "system" enables it to be that ideal tool. Horseshoe crab immunity is regulated by unspecialized circulatory blood cells called amoebocytes.[12] Just like white blood cells, amoebocytes stimulate an immune response to endotoxin. But since these cells are not evolved to carry out complicated defense mechanisms, they take a more elegant approach: trapping.[5] This process begins when an enzyme called Factor C binds to bacterial endotoxin and initiates a lengthy signaling cascade involving the release of multiple enzymes into the blood by the amoebocyte cell. Notification of the threat passes from one molecule to the next until the message reaches coagulin, a molecule that surrounds the endotoxin and effectively traps it in a large blood clot. This mechanism is the framework for an innovative assay known as the Lysate Amoebocyte Limulus (LAL) test in which pharmaceutical products are screened for bacterial endotoxins by examining whether they stimu-

Figure 1: Picture of an Atlantic Horseshoe Crab, Limulidae.

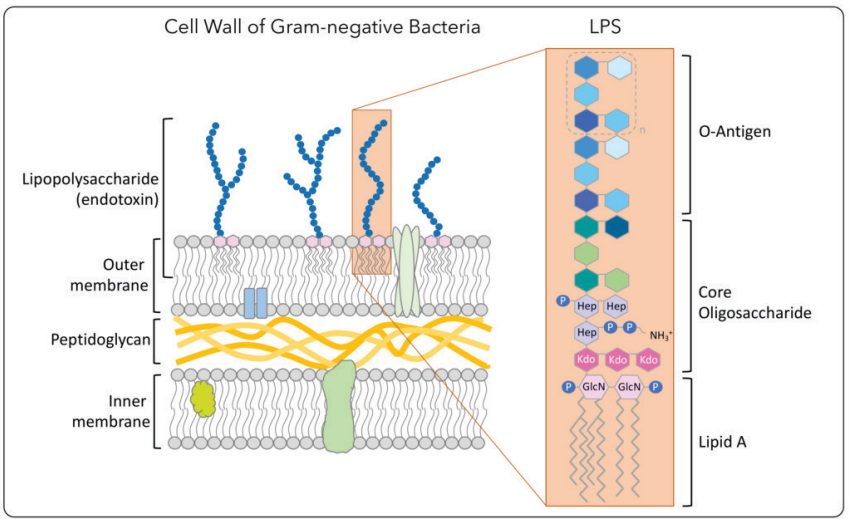

Figure 2: Schematic Diagram of the Cell Wall of Gram-Negative Bacteria. Enlarged on the right is the endotoxin constituent, Lipopolysaccharide (LPS). The Lipid A portion of LPS induces host immune response while the O-Antigen portion is targeted by host antibodies.

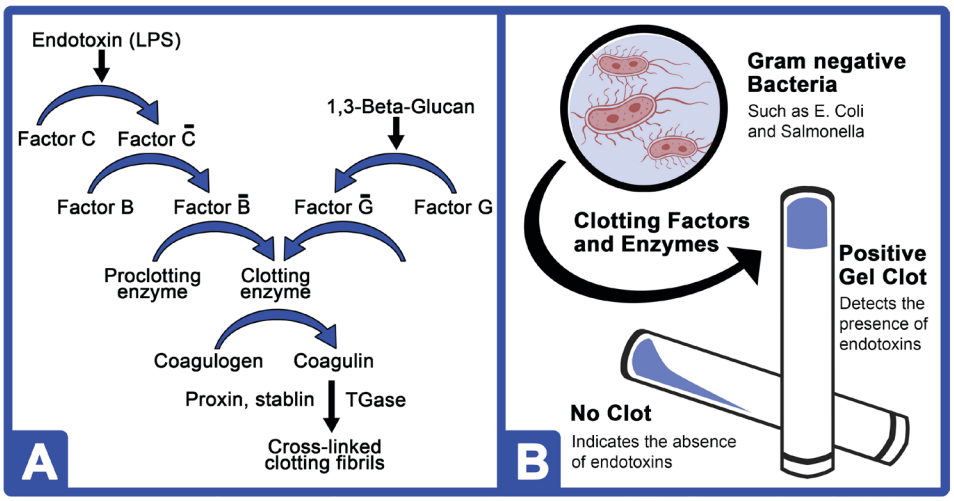

Figure 3: Diagrams of the Lysate Amoebocyte Limulus (LAL) qualitative gel clot test. (A) Flowchart illustrating the amoebocyte enzyme signaling cascade utilized by the LAL gel clot test. Note that this image displays interference by "1,2-Beta-Glucan" molecules, a common occurrence to be explained later in this article. (B) Schematic of LAL gel clot assay.

late clot formation in horseshoe crab blood.

The primary challenge of LAL is that it requires raw amebocyte samples extracted from live horseshoe crabs. In a process aptly referred to as "bleeding," scientists extract anywhere from 50 to 400 mL of blood from wild horseshoe crabs harvested directly from the Atlantic Ocean.[3] Since these crabs can support up to 40% blood loss, the bled population is returned to its environment within days of the procedure at a survival rate of 85%.[13] However, this estimate is hotly, *hotly* contested. The increased crab behavioral and health issues associated with unregulated handling and transportation practices have led many sources to theorize that the mortality rate is actually pushing 30%—roughly 150,000 crabs per year.[3,14,15]

BLEEDING ENDANGERS MORE THAN JUST CRABS

This number is alarming for ecologists and conservationists concerned about the impact of a diminishing horseshoe crab population on the Atlantic ocean ecosystem.[3,8,14] Horseshoe crab eggs are an indispensable mid-migration snack for shorebirds passing through the Delaware Bay.[16] The removal of gravid female crabs from the bay has resulted in the threatened species status of the Rufa Red Knot and a decrease in the number of birds passing through the region.[17] In an effort to prevent these problems from compounding, earnest conservationists advocate for the abolishment of horseshoe crab bleeding.[8] As a result, scientists are in pursuit of a creative, viable alternative to LAL testing.

RFC TESTING: THE LIGHT AT THE BOTTOM OF THE TEST TUBE?

In 2018, the FDA approved the first possible replacement for LAL: a fully synthetic recombinant Factor C (rFC).[18] Cloned entirely from horseshoe crab DNA, rFC is simply an *in vitro* version of the amebocyte Factor C enzyme. Hence, rFC's endotoxin response is *just like* LAL's–except instead of clotting, it glows. That is, rFC skips the tedious signaling cascade of LAL and instead enzymatically converts a single chemical substrate into a fluorescent product.[19,20,21] The simplistic, unambiguous, and self-evident nature of this fluorescent assay makes rFC a concise and standardized identification method.

To confirm the reliability of rFC, numerous studies of its sensitivity have been conducted using controls ranging from natural spring water to dust in dairy barns.[22,23] Astoundingly, these studies have all drawn the same controversial conclusion: rFC is entirely comparable to LAL.[20,22,23,24] In fact, some have indicated that the true simplicity of rFC might make it even *better*.

Third-party molecules can falsely trigger enzymes in the clotting cascade of crab amoebocytes, making LAL assays susceptible to errors (see Figure 6).[19] As rFC testing does not require these enzymes, it evades such cross-reactions and significantly reduces the likelihood of false positive results.[19,25] Consequently, rFC methodology is generally considered to be more consistent than that of LAL, which also varies from batch to batch due to labile factors like habitat, crab

Figure 4: Horseshoe crab bleeding procedure performed by pharmaceutical labs to acquire amoebocyte samples for the LAL assay.

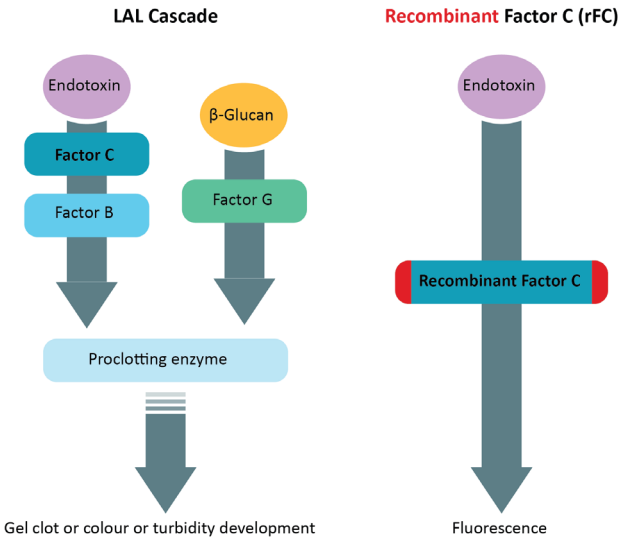

Figure 5: Delineative comparison of LAL and rFC enzymatic cascades, demonstrating the increased simplicity, absence of "B-Glucan" (third party) interference, and fluorescent final product of rFC testing.

gender, seasonality, and unstandardized harvest practices.[19,26] Predominantly, poor maintenance of crab harvest practices might produce even *less* accurate LAL results by causing crab blood to become unhealthy and deprived.[3,13,15] As a result, the adoption of a new, standardized rFC model is substantiated by its capacity to eliminate all variability.

RELIABILITY OR REFORMATION?

Despite these advantages, the implementation of rFC testing has been almost entirely nonexistent. This is due to the unwillingness of pharmaceutical companies to upend the industry's status quo by replacing a method with over 50 years of reliability.[19,20] Motivated by a desire to guarantee safe products and to save money and energy, their pushback against rFC remains unwavering. As growing experimental evidence of rFC's accuracy does not seem to add to its case, manufacturers are left wondering what it will take to convince skeptics of their product's credibility.

The endotoxin testing debate sits at the intersection of many different subsections of scientific interest. These intensely contrasting perspectives position pharmaceutical distributors, biochemical engineers, and conservationists at constant odds with each other. The result is an indefinite standstill, making the adoption of an rFC model appear very far on the horizon. Can the Atlantic ecosystem stand to wait? Is sustainability worth risking unforeseen health hazards of rFC? Regardless of standpoint, it is undeniable that this creative invention holds the potential to usher in a new era for the biomedical industry and stamp out a practice as ancient as the animal used to execute it.

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