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Borrelia burgdorferi BB0172 (TTHc:PepB) derivative capacity for immunization: primer for “Enhanced protective efficacy of *Borrelia burgdorferi* BB0172 derived-peptide based vaccine to control Lyme disease”

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Abstract

Lyme disease is the most common vector-borne illness in the United States. Despite decades of research, no vaccine for Lyme disease is available for humans. However, new research conducted at Texas A&M University (Hassan et al., 2019) appears to be moving us closer to a Lyme disease vaccine in the foreseeable future. The new vaccine consists of a peptide derived from an outer surface protein of *Borrelia burgdorferi* (PepB) conjugated to either Tetanus Toxoid heavy chain (TTHc) or Cross-Reactive Material 197 (CRM197). When the vaccines were given to mice via subcutaneous injection, the data showed that the TTHc:PepB vaccine resulted in a 66% protection rate against Lyme disease. Furthermore, after being exposed to *B. burgdorferi* the number of bacteria cultured from immunized mice was reduced by up to 80% when compared to control groups. While the TTHc:PepB vaccine seems to be highly effective, more research is needed in the field to further understand the processes by which the mice were immunized.

Purpose

The Centers for Disease Control and Prevention (CDC) reports Lyme disease as the most prevalent tick-borne infection in the U.S., with 30,000 reported cases annually and many more cases unreported (CDC, 2011). This statistic makes the study of Lyme disease immunizations critical to human health. Success in attaining such an ambitious medical feat is dependent upon the availability of research and conclusions in the field. While research by Hassan et al. (2019) has made a breakthrough in the path to vaccination by using a peptide derived from *Borrelia burgdorferi* (PepB) conjugated to Tetanus Toxoid heavy chain (TTHc), the current material is formatted such that only experts in the field could appreciate the relevant information.

This primer's objective is to translate technical components and concepts from Hassan et al.'s article, "Enhanced protective efficacy of *Borrelia burgdorferi* BB0172 derived-peptide based vaccine to control Lyme disease" (2019), into language that is more accessible to the broader scientific community. This primer simplifies the original article's critical components while still preserving integral information. The most critical aspects of the original article are strategically organized and proportionally expanded upon based on relevance. This primer defines and elaborates on basic medical immunization terminology, as it also presents the logistical components of Lyme disease in a comprehensible manner. This primer also thoroughly analyzes the concept of vaccine conjugation and the importance of PepB and TTHc, much more than does the original article. Additionally, the primer explores previous research's findings and failings, with simplicity in mind. In so doing, this primer makes the original article's research and conclusions more accessible to a wider range of readers.

Introduction

Lyme Disease is a bacterial infection transmitted to humans through ticks. Symptoms start with fever, headaches, and often a characteristic red bullseye rash known as erythema migrans. When left untreated, Lyme Disease can spread to the joints, brain, or heart and elicit arthritis, Lyme neuroborreliosis, or Lyme carditis, respectively (Mayo Foundation, 2019; CDC, 2020a). As stated previously, there are 30,000 reported cases each year in the United States, but the CDC also estimates that approximately 270,000 or more cases go unreported (CDC, 2018; CDC, 2019).

In the United States, the ticks that cause Lyme disease are scientifically known as the species *Ixodes scapularis*, but are more commonly known as deer ticks, or black-legged ticks. These organisms facilitate the transmission of the bacteria known as *Borrelia burgdorferi*—the causative agent of Lyme disease. The bacterial agent is classified as a spirochete bacterium which characterizes its spiral shape.

According to the CDC, the spring and summer months are associated with more frequent reports of the disease (CDC, 2011). This increase can be attributed to a dense population of ticks in their nymphal stage. During this time, the young arachnids are in their smallest disease carrying stage (about 1 to 1.5 mm) and can be easily overlooked by anyone spending time outdoors. Fortunately, progress is being made to defend against Lyme disease through vaccination.

Background

Vaccines

There are many different types of vaccines for many different types of diseases, but all vaccines essentially work the same way and serve the same purpose. They introduce a pathogen to the body—or a substance related to a pathogen—to elicit an immune response. The immune response results in the formation of antibodies to prevent future infections. With some vaccines, the antibodies produced remain for 5 to 10 years, requiring "booster" vaccines to retain their effectiveness, whereas others—such as the measles vaccine—last a lifetime (Immunisation Advisory Centre, 2020).

There are vaccines to prevent diseases caused by both bacterial and viral pathogens. Some vaccines contain a live-attenuated (weakened) form of the pathogen, that when injected causes a response similar to a natural infection. These vaccines usually generate a strong immune response that lasts a long time—often providing lifelong protection from a particular disease (Nat'l Institute of Allergy and Infectious Diseases [NIAID], 2019). Other vaccines use an inactivated (killed) form of the pathogen. These vaccines generally cause a weaker immune response and may require subsequent booster shots to retain their effectiveness (US Dept. of Health & Human Services [DHHS], 2020). Toxoid vaccines, such as the tetanus shot, use the toxin produced by the microorganism to elicit an immune response rather than the microorganism itself. Toxoid vaccines often also require booster shots (DHHS, 2020).

The focus of the study by Hassan et al. (2019) is a type of vaccine called a conjugate vaccine. Conjugate, subunit, polysaccharide, and recombinant vaccines all use a part of the pathogen to elicit an immune response (DHHS, 2020). For example, they may use something like a peptide derived from a protein found on the outer surface of the microorganism, like the conjugated vaccine in the original article by Hassan et al. (2019). They usually cause a very strong immune response to a targeted part of the microorganism but may require booster shots (DHHS, 2020).

Immune Response

The microorganism that causes a disease is called a pathogen. The part of the pathogen that the body recognizes and makes antibodies for is called the antigen. Each antibody has binding sites that are highly specific to a type of antigen. When a foreign invader enters the body, specialized white blood cells called Helper T cells and B cells lead to the production of antibodies. Antibodies mark the antigen to destroy it.

These antibodies—also called immunoglobulins (shortened to Ig)—are differentiated based on the type of heavy chain they contain. The heavy chains are the longer of the polypeptide subunits that form the "Y" shape of an antibody. Each antibody usually consists of two heavy chains and two of the shorter light chains. The five subclasses of immunoglobulins in mammals are IgG, IgM, IgA, IgD, and IgE. (Immune Deficiency Foundation, 2019).

No matter the antigen, the first antibody subclass to respond is IgM because it will bind indiscriminately. IgG is produced after IgM and specializes in attacking specific antigens.

When first exposed to an antigen, IgM and IgG have about the same response level, with IgG lagging behind IgM. After exposure, either through the environment or vaccination, IgG will respond much faster than before and at much higher levels. The level of antibodies measured in the body is referred to as its titer. Titers can help determine which immune cell is responding, such as the category of Helper T Cell—Type 1 (TH1) or Type 2 (TH2).

Further subclasses of antibodies can be found, such as IgG1 and IgG2. IgG1 is the most common IgG subclass found in the body whereas IgG2 is specific to bacterial

infections (Vidarsson, Dekkers, & Rispens, 2014). In mice, TH1 leads to the production of IgG2a, and TH2 leads to the production of IgG1 (Stevens et al., 1988). The response level of TH1 has been found to be faster and more effective in clearing Lyme disease (Jarefors et al., 2006; Sjöwall et al., 2011).

The immune response in the study by Hassan et al. (2019) was generated using a conjugate vaccine, as mentioned previously. Using a conjugate vaccine is one way to increase the immune response by taking an antigen that elicits a strong immune response and pairing it with an antigen that does not elicit a strong response (Creative Biolabs, n.d.; Goldblatt, 2000). The antigen that elicits the strong response is called a carrier protein. It's the part of the vaccine that causes the Helper T cells to activate B cells to make antibodies. In the past, Helper T cell-independent vaccines didn't elicit as strong of a response as the more modern conjugate vaccines. There were five carrier proteins licensed for use in conjugate vaccines as of 2013 (Pichichero, 2013).

In this study, the antigen that elicits the weaker response is PepB, which is the peptide taken from the outer surface of the *B. burgdorfer*i pathogen. Without conjugating PepB to a carrier protein such as TTHc, the body may not generate a strong enough immune response due to lack of Helper T cell involvement. This can lead to not enough antibodies being produced to fight future infections. This is especially true in infants and children, or those with immune system deficiencies (NIAID, 2019; Rappuoli, Gregorio, & Constantino, 2018).

PepB

Peptides, most simply put, are small subunits of proteins formed by linking together chains of amino acids. Proteins are the components of a cell that provide structure and are important to cell function. Hassan et al. (2019) targeted a specific peptide from a highly conserved protein (BB0172) in the *B. burgdorferi* bacteria called PepB. A conserved protein is a protein that is so essential for cell function that it has remained virtually unchanged throughout evolutionary history. PepB was chosen because it has demonstrated superior protection against Lyme disease in low doses. Further, because PepB is from a highly conserved protein it is likely to be found in many strains of Borrelia, increasing the likelihood of broad-spectrum protection.

TTHc and CRM197

As stated previously, conjugate vaccines use a combination of strong and weak antigens (see Vaccines). Two common strong antigens, or carrier proteins, used are TTHc and Cross-Reactive Material 197 (CRM197) conjugated to PepB. TTHc can activate the Helper T cells that coordinate immune response at high levels making it an ideal antigen to use. CRM197 is a nontoxic mutant version of the diphtheroid toxin with a similar high use in vaccine development. (Shinefield, 2010). The conjugated vaccines are referred to as TTHc:PepB and CRM197:PepB.

The Experiment

"Enhanced protective efficacy of *Borrelia burgdorferi* BB0172 derived-peptide based vaccine to control Lyme disease"

Experiment Design

The study by Hassan et al. (2019) was conducted at Texas A&M University, using a well-planned procedure to determine which conjugated vaccine is the most effective for preventing Lyme disease. The study used mice as the subjects to contract Lyme disease. Hassan et al. believed the best method would be to conjugate a carrier protein like TTHc or CRM197 to PepB. That way they could elicit a strong enough antibody response to *B. burgdorferi* to make a viable conjugate vaccine without the mice contracting Lyme disease. They knew the experiment would need to monitor several different things such as the effect on the mice's tissues, antibody levels in the mice's blood, and the amount of *B. burgdorferi* bacteria present in the mice's blood and tissues. They also knew any differences in these things being monitored would need to be analyzed using appropriate statistical methods.

First, the mice were divided into four different groups. Two groups of mice were used as a control and given unconjugated carrier proteins (TTHc or CRM197). The other two groups were each given one of the two different conjugate vaccines (TTHc:PepB or CRM197:PepB). By separating the mice into groups like this, the researchers were able to see which conjugate vaccine was most effective. This way, they could also see if there is a significant difference between giving the mice a conjugated vaccine or just giving the mice the TTHc or CRM197 carrier proteins alone. Figure 1 below shows how the groups of mice were separated in the experiment.

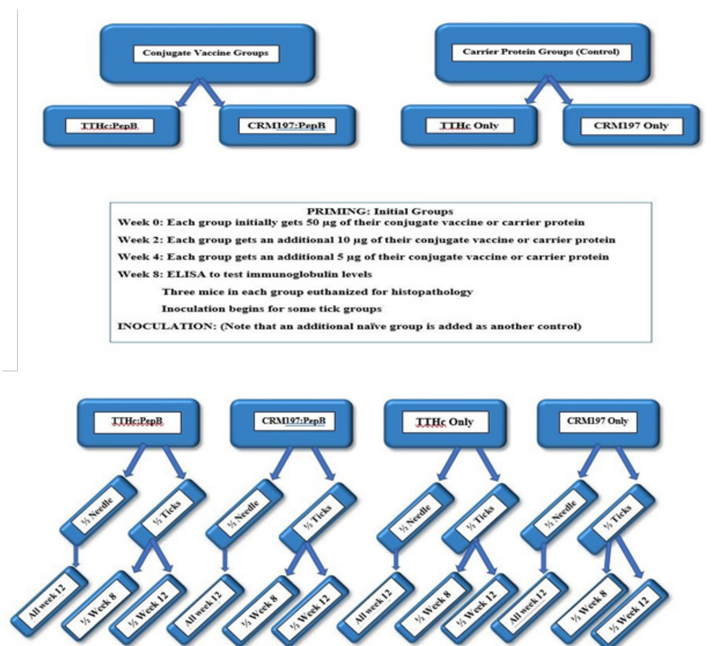

Figure 1:
(Top): The mice were divided into two groups that either received a conjugate vaccine or a carrier protein alone. They were then divided further into four groups based on whether they were getting TTHc or CRM197
(Middle): The middle part of the figure explains the priming procedure
(Bottom): The four groups were split further into groups being infected via a needle challenge or infection via live ticks. The groups infected via needle challenge were all infected 12 weeks post-priming, and the groups infected via live ticks were infected either 8 weeks or 12 weeks post-priming.

Priming Procedure

Before infecting the mice with bacteria, Hassan et al. needed to prime the mice with whichever vaccine/antigen group they belonged to. Priming is an important process in vaccination that gradually builds up antibodies to fight against a specific pathogen. We see the same thing in human medicine when patients are given a series of shots.

To begin, the mice received 50 µg of their respective antigen via subcutaneous injection, as seen in Figure 1. After 2 weeks they were given another 10 µg booster of their respective antigen, and after 4 weeks they were given another 5 µg booster of their respective antigen. That way, Hassan et al. could ensure the mice had enough antigens in their blood to trigger a response to build up antibodies.

ELISA

As described earlier in the section about immune response, the two main antibodies of interest for Hassan et al. were IgM and IgG. After the researchers primed the mice with their respective antigens, the mice should have all developed these two antibodies in response over time. The best, and most common way, to test for IgM and IgG antibody levels is to use the laboratory procedure "enzyme-linked immunosorbent assay," or ELISA.

An ELISA test is performed by allowing antibodies and antigens to interact with each other on a plate with 96 wells (BosterBio, n.d.). First, the wells are filled with a medium that contains antibodies. Some of the antibodies are adsorbed onto the plate's wells, and the excess is washed off. Next, the wells are filled with a solution containing a protein that isn't an antigen or an antibody. This neutral protein is adsorbed onto the plate's wells that haven't already had antibodies adsorbed onto them. Then the plate is washed again to remove any excess neutral proteins, and the entire area of the wells is covered with antibodies or the neutral protein. After that, an enzyme conjugated to antigens is applied to the plate to allow the antigens and the antibodies that were adsorbed onto the plate previously to interact. A substrate is added that reacts with the enzyme portion of the enzyme/antigen conjugate so that the antibody levels in the original medium can be interpreted.

In the ELISA test performed by Hassan et al., the scientists took blood samples from all the mice in every group 8 weeks post-priming. They separated the blood serum from the whole blood so the serum could be used as the medium containing the antibodies to be interpreted. The presence of IgM antibodies in the serum alone would indicate that the subject had been recently introduced to the antigen and was actively working on fighting it. The presence of IgG antibodies in higher quantities than IgM would indicate that the subject had already fought the antigen. In that case, the subject's immune system had "remembered" the antigen, and the mice already had the antibodies needed to attack the specific antigen from the *B. burgdorferi* pathogen. If IgG antibody levels were higher, then the priming was a success.

Pre-inoculation Histopathology

In addition to taking blood samples from all the mice, the researchers euthanized three of the mice from each antigen group 8 weeks post-priming, as described in Figure 1 above. Tissue samples from different organs were observed by a board-certified pathologist to examine what effects, if any, the priming had on the tissues. The reason for observing the tissues is because previous potential Lyme disease vaccinations had caused inflammation in certain joints and tissues (Wormser, 1999). Arthritic inflammation is also a symptom of Lyme disease.

By examining the tissues before the mice were inoculated, the scientists could be sure that the inflammation was caused by the immunization, and not a symptom of contracting Lyme disease after inoculation. The pathologist ranked the amount of inflammation observed on a scale of 0-4, with 0 being no inflammation, and 4 being 30% or more of the area inflamed.

Inoculation Method

After the mice were primed, they were infected with *B. burgdorferi* via two different methods. One method was directly injecting the mice with the bacteria via subcutaneous needle injection. The other method was to let live deer tick nymphs feed on the mice's blood to infect them. This setup meant the mice were not only split into groups according to which antigen they received, but further split into groups according to which mode of infection they received.

In addition to splitting the mice into groups according to how they would be infected, the mice that were to be infected with ticks were split into two different groups according to when they would be infected. Some of the mice were infected via live ticks 8 weeks post-priming, and some were infected 12 weeks post-priming. The entire process is illustrated in Figure 1 above.

The researchers infected the mice after different periods of time because Hassan et al. had found the antibody titers to be the highest after 8 weeks and to be declining back to normal levels after 12 weeks. The researchers needed to see if the vaccination would still be effective against live tick infection when antibody titers were past their peak.

Mice that hadn't received any of the priming treatments the other mice received, or any other type of experimental treatment, are referred to as naïve. To create another control group for comparison, the researchers selected a group of mice to serve as naïve subjects. The naïve mice were infected via live ticks at the same time as the groups being infected via live ticks 12 weeks post-priming.

All the mice being infected via subcutaneous needle injection were infected 12 weeks post-priming only. They were infected with a dose of *B. burgdorferi* 1000 times greater than the dose needed to infect a healthy mouse half the time, which is called the ID50 (infectious dose 50:50).

Post-Inoculation Histopathology

Four weeks after being infected by ticks, the mice from the tick inoculation groups were euthanized, and blood and tissue samples were evaluated by a board-certified pathologist. This time, the pathologist was looking for signs that the *B. burgdorferi* bacteria had spread into the blood and tissues, as well as signs that the antigens had caused further tissue inflammation.

The mice that were infected via subcutaneous needle injection were treated a little differently. Instead of being euthanized four weeks after infection, they were euthanized after just three weeks. The reason for premature euthanasia isn't clear, but it may have been that since such a high dose of *B. burgdorferi* was used, the researchers didn't want to risk all the mice being killed by Lyme disease before they could be euthanized and inspected. Just like with the mice infected by ticks, the pathologist was looking for the spread of *B. burgdorferi* bacteria, as well as inflammation.

qPCR

The method the pathologist used to measure bacteria in the tissues is called quantitative real-time polymerase chain reaction (qPCR). This is a method in which the mouse's DNA is separated from the mouse's cells obtained from its tissues. Before performing qPCR, the researchers analyze a piece of *B. burgdorferi* DNA, deeming a specific sequence as the "target sequence".

In one of the first parts of qPCR, the mouse's DNA is "unzipped," and a single strand of DNA is used as a template. Then, a short sequence of DNA is laid down that's complementary to a sequence of the mouse's single stranded DNA template. This short sequence of DNA acts as a primer for DNA polymerase, the enzyme that synthesizes DNA. The DNA polymerase moves along the DNA, adding the nucleotides that are complementary to the corresponding nucleotide in the template, and the DNA is "zipped" back up.

This copying of DNA is done several times, which is called amplification. Once in a while, the "target sequence" from the *B. burgdorferi* DNA is copied, indicating that that part of the DNA taken from the mouse didn't belong to the mouse, but rather to the bacteria. Hassan et al. measured how many times the "target sequence" was copied in real time. The more times the "target sequence" was copied, the more bacteria there were present in the mouse's tissues.

Statistical Analysis

To see if the levels of bacteria in each group were significantly different, the scientists used a statistical test called two-way analysis of variance (two-way ANOVA). This is the same statistical test they used to determine if the levels of IgG and IgM were significantly different from one another in each antigen group. The statistical analysis is two-way, because two independent variables were being analyzed simultaneously: The amount of *B. burgdorferi* bacteria in the tissues, and the number of antibodies in the blood serum samples.

“Conjugate Vaccine” Results

Hassan et al. used the methods described above to examine the effect of their new conjugated vaccine on mice. The mice’s immune response to the vaccine and later to *B. burgdorferi* was quantified and used to determine the efficacy of the new vaccine. High antibody titers as well as low numbers of *B. burgdorferi* bacteria in the tissues indicate a robust immune response.

In order to trigger an infection, some mice were injected with *B. burgdorferi* bacteria with a needle. In the needle inoculation challenge, mice injected with an infectious dose of *B. burgdorferi* were evaluated for the protection gained from the conjugate vaccine candidates. The protection provided by the TTHc:PepB vaccine showed particular promise. Later, in order to more realistically simulate tick infection, live Borrelia-carrying ticks were allowed to feed on a second group of TTHc:PepB vaccinated mice. They were then compared to the mice in the needle inoculation group for any changes in vaccine efficacy.

In the needle inoculation challenge, Hassan et al. examined antibody titers following vaccination but prior to infection. IgG titers were high in the conjugated vaccine groups and at base levels in the unconjugated groups. Following infection with *B. burgdorferi*, *anti-Borrelia* specific IgG levels were high in all groups, conjugated and unconjugated. This observation is contrary to what Hassan et al. expected because IgG titers should be lower in unvaccinated groups. Based on the IgG2a and IgG1 titers, TH1 and TH2 response was high in both conjugated vaccine groups and at base levels in the unconjugated groups. Hassan et al. expected this response, since the carrier proteins of conjugated vaccines are what attracts Helper T cells to the Borrelia specific antigen.

The researchers used qPCR to determine the number of *B. burgdorferi* bacteria in select tissues after infection. The results of the qPCR from the skin, spleen, lymph nodes, and tibiotarsal joints showed that the TTHc:PepB mice showed a significant reduction in the number of B. burgdorferi compared to the TTHc-only group. The CRM197:PepB mice showed only a moderately reduced number of B. burgdorferi compared to the CRM197-only group.

The protection rate of the TTHc:PepB vaccine was 66%. This rate was found by adding up the number of TTHc:PepB mice that contained B. burgdorferi recovered in culture, and then dividing that number by the total number of TTHc:PepB mice. B. burgdorferi was cultured out of 33% of the TTHc:PepB mice. The other 66% of mice did not have B. burgdorferi recovered in culture, so they were protected by the TTHc: PepB vaccine.

The histopathology results showed that both TTHc:PepB and CRM197:PepB mice had low rates of inflammation, which has been an issue in previous vaccines. This low inflammation rate was true for the histopathology analyses performed both 8 weeks post-priming and 4 weeks post-infection. However, inflammation rates were slightly higher in the CRM197:PepB group. In the CRM197:PepB mice there was minimal to mild inflammation of the heart, kidney, and tibiotarsal joint. The TTHc:PepB mice only showed minimal to mild inflammation in the liver.

In the tick challenge, only the TTHc:PepB and TTHc groups were analyzed. Antibody titers were examined following vaccination but prior to infection. The results mirrored the needle inoculation challenge with high IgG titers in the conjugated vaccine groups post vaccination and high anti-B. burgdorferi specific antibody levels in both the conjugated and unconjugated groups after inoculation of B. burgdorferi.

Like the results for the needle inoculation challenge, the results of the qPCR from the skin, spleen, and tibiotarsal joints showed that the TTHc:PepB mice had a significant reduction in the number of B. burgdorferi bacteria compared to the TTHc group. In contrast to the needle inoculation challenge, B. burgdorferi was recovered in culture out of almost all the mice in both the conjugated and unconjugated groups in the tick challenge.

Implications

The goal of the work performed by Hassan et al. was to determine if conjugating PepB (a peptide derived from a highly conserved protein in B. burgdorferi) to a carrier protein (TTHc or CRM197) would effectively protect against B. burgdorferi infection that leads to Lyme disease. The goal was to create a vaccine that could be administered to healthy individuals before they're infected. Success in this research could provide critical information that may lead to a commercially available Lyme disease vaccine in the near future.

Hassan et al.'s work resulted in a 66% protection rate and as much as an 80% reduction in bacterial burden in the TTHc:PepB conjugated vaccine. The protection rate may seem low; however, the flu vaccine has a protection rate of 40% to 60% (CDC, 2020b). On the other hand, the flu vaccine is a function of herd immunity. That is, the less people able to catch a disease, the lower the chance the disease can spread. This herd immunity works very well in diseases spread from person to person, but non-contagious, vector-based diseases work best with a higher protective rate. Another important perspective on this protection rate can be found examining canine vaccines for Lyme disease. These vaccines vary widely in their protection rates; as much as 50% to 100% according to a metanalysis by Vogt et al. (2018).

There are two main categories for a vaccine's failure to protect: vaccine-based failure or host-based failure. Vaccine-based failure arises from issues with administration, or flaws in the vaccine's design. Host-based failure is due to some issue with the subject receiving the vaccine, perhaps due to genetics or other personal factors (Wiedermann, Garner-Spitzer, & Wagner, 2016). Between the protection rate being on par with other vaccines and the vaccine being administered in a lab setting, it is hard to say which category may be impacting the vaccine's protective rate. With all of this in mind, the new vaccine will require more research before it can be synthesized.

Conclusion

Lyme disease is an often-overlooked condition that can result in lifelong health issues including chronic joint pain and fatigue. Considering its low rate of detection and the increased risk of chronic issues due to delayed treatment, prevention is the optimal

strategy in preserving human health. This situation makes research for a commercially available vaccine for Lyme disease vital. Overall, Hassan et al.'s study is a promising step towards that vaccine. While the TTHc:PepB immunized mice were not all free from infection, the significant reduction in bacterial presence and improvement of symptoms are a testament to its capacity as an immunization. This research is an excellent advancement towards a vaccine for Lyme disease.

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