**Hemocyanin, a vaccine adjuvant. The search for an alternative source**

**Background:**

Hemocyanin is a large oxygen carrying molecule from 4-8 million Dalton consisting of 10 subunits, commonly found in arthropods, mollusks, and occasionally other invertebrates. [9][10] Hemocyanin is somewhat analogous to hemoglobin. While hemoglobin uses cell contained iron to bind oxygen for transport, hemocyanin is freely diffused in the hemolymph (invertebrate blood) and uses copper to bind and transport oxygen. [1]

Hemocyanin and specifically Mollusc hemocyanins have been studied because of their directed Th1 activation when used as an adjuvant. [2][9] An adjuvant is an immunostimulant that, when administered in tandem with a vaccine, elicits a stronger immune response than the vaccine alone, resulting in a stronger and longer lasting immunity [3]. The most widely used adjuvants are aluminum derivatives, which generate a strong humoral response but induce a poor cellular immune response. [8] As the intent of a vaccine is to produce a specific T cell (Th1/Th2) response and immune memory, if the adjuvant used doesn’t illicit a strong cellular immune response the vaccine is sub-optimal and could be improved with a better adjuvant.

In a recent 2019 study by Roman et al. they compared the immunotherapeutic potentials of different mollusc hemocyanins in a mouse model of oral squamous cell carcinoma: Keyhole Limpet Hemocyanin (KLH), *Concholepas concholepas* hemocyanin (CCH), and *Fissurella latimarginata* hemocyanin (FLH) in combination with alum, AddaVax and QS-21 (a plant extract). Their main discoveries were that KLH and FLH-QS-21 formulations showed a reduction in tumor development and increased survival. Hemocyanins had no cytotoxic effect on cancer cells indicating that the anti-tumor effects are associated with their immune modulating characteristics. [9]

Unfortunately, current production of hemocyanin consists of harvesting blood from Keyhole Limpets and purifying hemocyanin from their blood. Keyhole limpets are found worldwide, but as you can imagine, they do not produce much blood. As a result, Keyhole Limpet Hemocyanin (KLH) is extremely expensive, from $5,000 to more than $150,000 per gram. [4][5][6]

Because of its use in research, continuous harvesting of hemocyanin from this species may lead to widespread ecological repercussions. Unfortunately, recombinant KLH is difficult to produce because of its massive size and extensive post translational modifications, specifically glycosylation. To this date, I am only aware of one source of recombinant hemocyanin.and with a purity of 95% it is not suitable for Good Manufacturing Practice (GMP) or Good Clinical Practice (GCP) use and is likely not comparable to the native protein [6]. With a global pandemic and wait for a vaccine, it seems advantageous to have multiple sources of an adjuvant to avoid breakdown of the vaccine supply chain.

Four species of mollusk have been reported to express similar forms of hemocyanin: Concholepas concholepas, Fissurella latimarginata, Haliotis tuberculata, and Rapana thomasiana. Hemocyanin is estimated to be at least 540 million to 600 million years old, first emerging at the same time as the divergence of crustaceans and chelicerates [7]. This should leave us with a wide variety of organisms to investigate as alternative sources of hemocyanin.

For a preliminary investigation, a python script was written to discover organisms with conserved regions similar to KLH1 and KLH2. For each sub-unit, the script uses the KLH-A and KLH-B protein IDs to pull fasta sequences from uniprot and blasts the returned sequences against the NCBI Swiss-prot database. The swiss-prot databasse was used because of its quality annotations and identification of hemocyanin sub-units. Entrez protein IDs for hits with an E-value of less than 1e-7 are compiled and their corresponding protein sequences are fetched from the Entrez protein database. For each sub-unit, multiple sequence alignment is performed with ClustalW2 and the alignments are viewed as phylogenetic trees for an easy to understand graphical representation of the similarities of the protein sequences.

Helix pomatia

Rapana venosa

Enteroctopus dofleini

Sepia officinalis

<This study will focus on the immunological significance of native keyhole limpet hemocyanin, not on optimization of purification protocols>

**Questions/Aims:**

Overarching goal:

Can we find an alternative organism to source hemocyanin for purification?

1. Test novel hemocyanin proteins for immune response
2. Can we identify the functional subunits of Keyhole Limpet Hemocyanin that elicit an immune response?
   1. If immunostimulatory sub-units can be isolated, do other organisms express similar hemocyanin subunits?

**Methods:**

Once organisms expressing similar hemocyanin are found, the immunological effects of their hemocyanin will be characterized for comparison. Organisms with a larger blood volume and fast growth phases would be prioritized over smaller organisms like Keyhole Limpets. Also, organisms that can be easily farmed will be considered as a replacement for Keyhole Limpets. Immune responses for both innate and adaptive immune systems will be characterized with a focus on developing memory T cell populations in response to vaccination.

This opens the possibilities of future studies characterizing the structure and sub-units of various organism's hemocyanin. If we can better understand why this protein elicits such a strong immune response, we can create and produce recombinant proteins at a fraction of the price that it currently costs to harvest and purify from limpets.

**Expected Results:**

**Potential Difficulties/Backup Plan:**

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