Disease Detectives: How Researchers Use Cell Culturing to Investigate Viruses

Abstract

Infectious viruses have a history of plaguing the population whether it's the common cold or a virus as complex as the coronavirus. The technique of cell culturing has allowed researchers to grow cells in a controlled environment in order to test potential vaccines that can be given to the population to combat these viruses. However, since each virus has a unique structure, they will activate different biochemical pathways within the host cell that contribute to infection. The interactions between the virus and the host cell and the resulting immune response must be studied for a researcher to create a vaccine that will work efficiently. The research presented demonstrates cell culturing as a technique for developing vaccines for viruses such as influenza and COVID-19. The way the virus enters a host cell and further replicates to cause infection is a main focal point for cell culturing models because this is what the vaccine will prevent from happening by providing the host cell with specific antigens and antibodies to prevent infection. This review delves into studies done by researchers where the interaction of the virus and the host cell is studied, and the immune response gives clues to how an effective vaccine can be made. The specific cell line that is chosen for each virus has an important role in mimicking the host cells within the human body which allows researchers to accurately determine whether a certain vaccine will be effective or not. The technique of cell culturing continues to grow as new methods come to light that provide even more accurate representations of virus and host cell interactions for a more effective vaccine. The work done by using cell culturing as a method of vaccine production can be used not only to produce an effective vaccine but to also mass produce vaccines when a pandemic situation occurs due to the ability of cell culturing to be scaled up.

Introduction

Cell culturing has been a reliable method for studying viral diseases in a laboratory setting. Although this process does not provide quick results and relies primarily on sterile techniques, it is still the most widely used method of diagnosing different strains of viral diseases [1]. When the method was first discovered in the early 1900s, it gave scientists a way to study viruses *in vitro* without using animal subjects [1]. This new advancement provided virologists with the ability to grow susceptible cells where viruses would reach high titers [1]. It also provided them with the ability to introduce many different variables because of the abundance of cells that could be grown in the controlled environment of culture tubes [1]. The overall technique of cell culturing continues to grow as there are more and more cell lines available for research. By using mammalian cell culture systems, researchers have the ability to combat public health crises as a result [1]. Advancements in cell culture systems have also changed the way virologists study virus isolation and the impact on the biochemical pathways within a specific host cell [1].

A virus's main target in the body once it enters is the host cell of the specific region it infects. For example, COVID-19 mainly infects lung cells. Therefore, it is important for researchers to understand the interaction between the virus and the host cells in order to understand how the virus enters and further replicates to cause infection. During the process of the virus entering the host cell and replicating, many of the host cell's biochemical pathways are affected. Selecting the cell to culture is very important for this reason in order to correctly mimic these pathways and for an effective pathway to be made. In this paper, I will be exploring the Madin-Darby Canine Kidney (MDCK) cell line and how it is used to investigate influenza virus and the resulting changes in the host cell that gives information about vaccine production. Additionally, the

Chinese Hamster Ovary (CHO) cell line will also be investigated to see how COVID-19 enters host cells and how these interactions also led to the production of an effective vaccine during pandemic situations. The goal of these investigations is to see how cell culturing methods are used for vaccine production and how these methods are modified in response to different pathways that are affected by the virus.

Cell Culture Use in Research

Before researchers started using mammalian cell lines to study viral infections, they used animal models such as chick embryos [1]. Using chick embryos was mainly an issue because the live vaccines, vaccines that are directly produced from the embryos, were not purified and this increases the risk of allergic reactions in people with an allergy to eggs [1]. Starting in the 1950s, the method of using cell cultures in order to isolate viruses has started expanding [1]. Once researchers understood the impact of manipulating the cell culture media in ways such as introducing antibodies or different chemicals, they were able to increase viral yield which allowed for effective vaccine development [1].

The overall process of isolating a virus in cell culturing first involves using a specific cell culturing tube. The standard cell culturing tube is a 16- by 125-mm glass or plastic round-bottom screw-cap tube which allows the cells to create a monolayer and adhere to the bottom and also holds the media to allow the cells to further proliferate and grow over time [1]. A virus has specific host cells that it infects, so to account for this, several cell lines are introduced along with the clinical sample in order to see which host cell is more susceptible to the virus [1]. Depending on the specific virus and where in the body the clinical sample was collected from,

the specific media concentration and what chemicals are present changes. Once these parameters are determined, the cells are plated with the appropriate media and allowed to incubate typically around 35 to 37℃ [1]. The cells remain under close observation under a microscope for the first week in order to make sure that there is proper cell growth and the virus is continuing to replicate [1]. Then, for the remainder of the incubation period, they are monitored more loosely until the virus reaches the end of its incubation period which is the period of replication for the virus within a host cell [1]. The presence of the virus within the host cell is determined by several factors that can be seen under the microscope such as swelling, shrinking, and syncytium formation [1]. Syncytium formation happens when a cell that is infected with the virus fuses with neighboring cells and the result is a multinucleated, enlarged cell [1]. All of these properties of the cells under the microscope allows the researcher to see that the cells are no longer normal host cells and they have been infected with the virus. This gives a loose determination that there is a virus present but it does not give specific information about which virus or which specific strain. This is determined by tests that incorporate specific information such as the cell line involved, the length of incubation, and the type of clinical samples that was used [1].

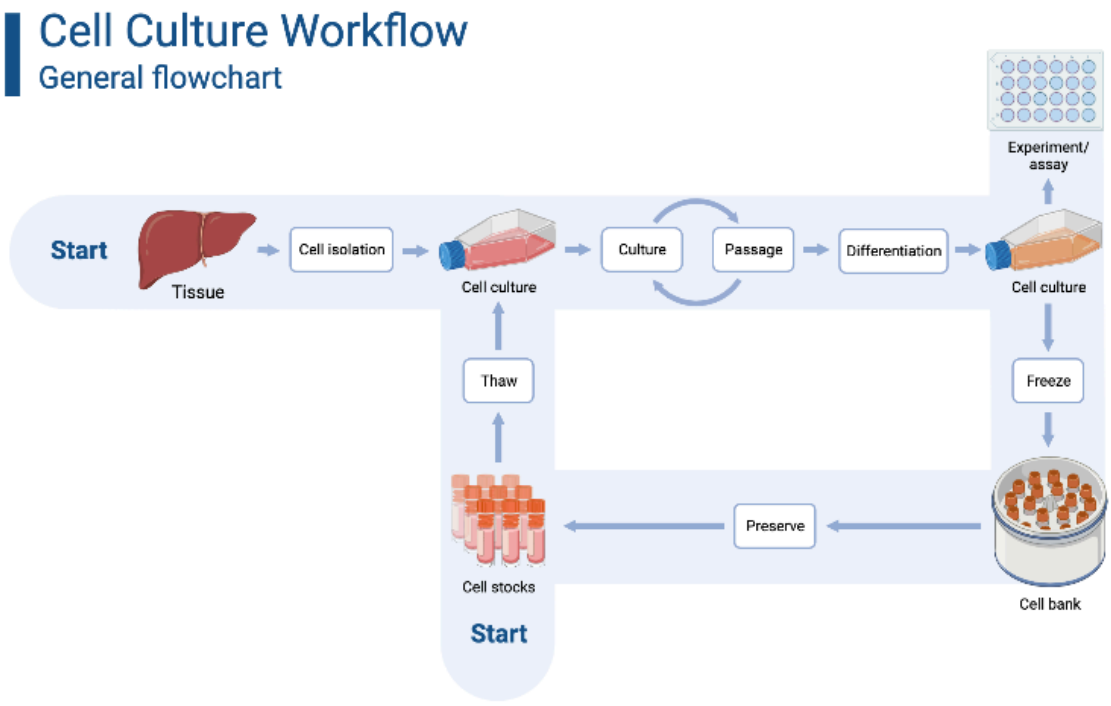

Figure 1. Schematic representation of the cell culturing process. *Cells isolated from a specific body tissue are cultured and passaged to allow the cells to grow. Differentiation allows the cells to have specific functions so they can mimic host cells. Culture cells are then divided for experimental use. [12]*

Viruses such as influenza and COVID-19 infect specific host cells within the body which not only causes severe symptoms in patients, but also causes a change in the biochemical processes within that specific cell. Cell culture models can replicate these different changes that the virus causes to specific cells and use the findings from the culturing to make medical applications that lead to vaccine production. Both influenza and COVID-19 enter host cells and replicate which triggers an immune response that throws off the entire equilibrium of the cell [6]. For example, in a normal host cell, there are processes such as transcription, translation and glycolysis that

occur, and the cell is well equipped to handle these metabolic processes. However, once the virus is introduced, this work load essentially doubles, meaning that more energy is required for transcription of the viral genes and then even more for the translations of proteins for the virus to replicate within the cell [6]. The virus is essentially feeding off the host cell which means that it has to work extremely hard to accommodate for this added load. In order for these cells to grow efficiently in culture without dying from the added stress of the virus, the media the cells are suspended in must be manipulated in order to keep both the host cell alive and the virus within it growing [6].

H1N1 and H5N1 Strains of Influenza Virus

Influenza is a virus that is highly contagious and can cause respiratory illness in the population. Influenza virus uses its nucleoprotein (NP) and its matrix protein (M1) to then be further broken into types A, B, and C [4]. Influenza types A and B are the most common types found in the population because they are responsible for most of the influenza infection in the population [5]. The two transmembrane proteins that are present on the surface of the virus, hemagglutinin (HA) and neuraminidase (NA), are the reason that influenza A is more complex than types B and C. Hemagglutinin works specifically to fuse the virus with the cell membrane of the host cell. Then, neuraminidase releases the virus into the host cell where it can replicate and cause infection. Since there are different types of these transmembrane proteins and each influenza virus can carry any combination of types, there are many different subtypes of influenza A. The specific strains that I will be discussing are the H1N1 and H5N1 strains.

The H1N1 virus is a swine flu pandemic that began in 2009 in the United States and Mexico and has been rapidly spreading worldwide [5]. This strain was able to spread rapidly within the population and led to respiratory illness in patients [5]. This is because the H1 subtype of the HA protein binds α-2,6-linked sialic acids that are in the upper respiratory tract of humans which means that H1N1 is easily spread between humans [8].

The H5N1 strain is part of the avian influenza pandemic. This virulence of H5N1 is less than the H1N1 which means that its transmission is less prevalent [5]. This is because the H5 subtype of the HA protein binds α-2,3-linked sialic acids which is common in birds and the lower respiratory tract of humans which means that it is more rare for humans to become infected with this subtype, but the virus is extremely severe when it does [9]. The H5N1 strain causes more severe symptoms such as rapidly progressing primary viral pneumonia leading to acute respiratory distress syndrome (ARDS), multiple-organ dysfunction, lymphopenia, and hemophagocytosis [5].

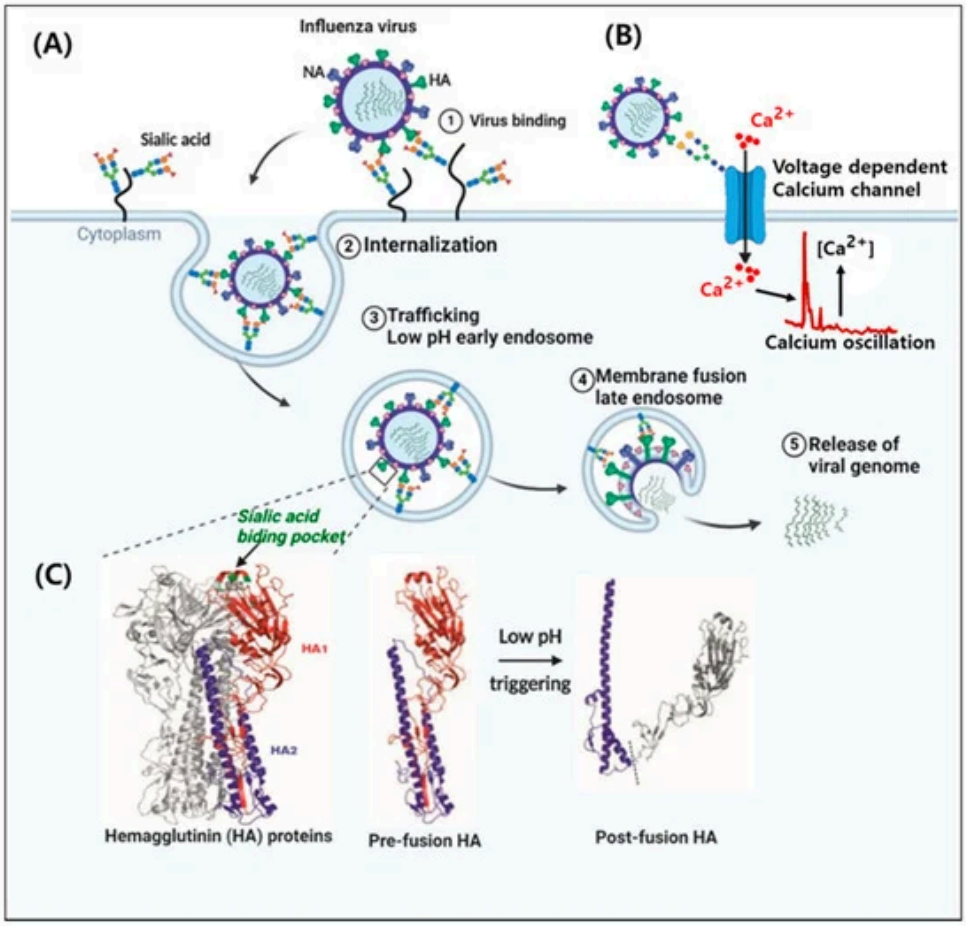

Figure 2. Influenza virus infecting a host cell. *Transmembrane hemagglutinin binds to sialic acid on the host cell which allows for influenza to enter through endocytosis. The endosome has a low pH which causes a conformational change in the HA protein that causes it to fuse with the membrane of the endosome and release the viral genome into the cytoplasm [13].*

Understanding how these two strains of the virus infect host cells will give researchers a solid foundation for culturing research and vaccine development. Both strains of the influenza virus, H1N1 and H5N1, are able to infect and replicate within alveolar epithelial cells [5]. However, H5N1 showed to induce more of a cytokine and chemokine response within host cells [5]. This means this strain causes an immune response in the form of inflammation which is what causes

such severe damage to the lungs [5]. Now that the pathogenesis of the virus has been determined, cell culture systems can be developed to produce vaccines that are specific to the interactions of these strains with the host cell.

Culturing Methods for Influenza Virus

The first step in preparing a culturing method in order to replicate a specific virus and study it further is to select a specific cell line that can mimic the interaction between the virus and the host cell within a patient's body. For influenza, based on the specific pathogenic behavior of the strains, the Madin-Darby Canine Kidney (MDCK) cells have been used since 1958 when they were first isolated from the kidney of a healthy cocker spaniel [11]. These cells have been determined as a model for polarized epithelial cells that are present in the human body [11]. Having a polarized cell model is important because these cells have distinct ends that have different structures that allow for different functions at either end of the cell [11]. By being able to mimic these types of cells that are present in the human body, MDCK cells are able to be infected by all the different subtypes of influenza and produce various strains of high titers [11].

There are two different methods of culturing that researchers use, laboratory spinner or fermenter [4]. Growing a virus in a laboratory spinner involves suspending the specific cell line in media that fits the need of the specific cell line [4]. Then, this cell suspension is spinned continuously and the concentration of the media is closely monitored in case there is a need for any chemical additives to promote cell growth [4]. Once the amount of cells has grown significantly, the virus is introduced and the amount of replication once it enters host cells is monitored over a period of time [4]. Using a fermenter involves placing the cell suspension into a culture vessel that keeps

the concentration of the media constant [4]. This means that the media concentration and the different ions that are present are held constant. Once the cells have replicated to a maximum density, the virus is introduced into the cells [4]. Then, just like the laboratory spinner method, the amount of replication is monitored over a period of time [4]. Figures 3 and 4 show the difference between the methods of using a laboratory spinner and a fermenter. Researchers lean more towards using a fermenter when using cell culturing to study viral replication because the conditions within a fermenter can be changed so the virus replicates at an even faster rate which is something that cannot be achieved when a laboratory spinner is used [4].

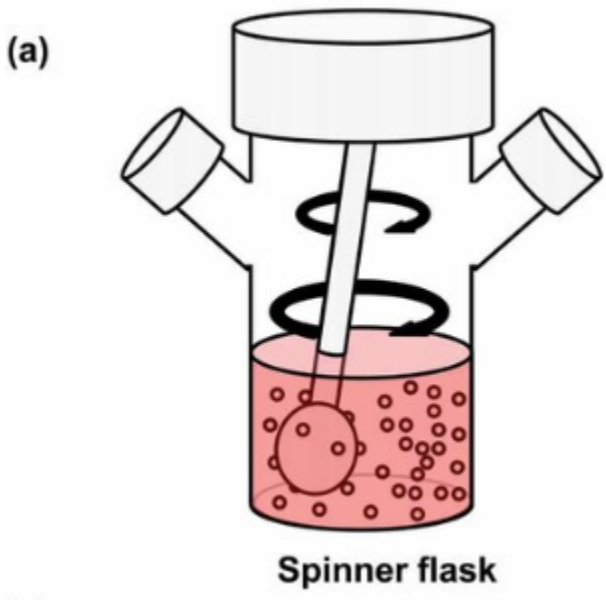

Figure 3. Cell culturing in a laboratory spinner. *Cells are suspended in media and then placed in the laboratory spinner where the suspension is spinned continuously until the cells grow to the desired amount for further experimentation [14].*

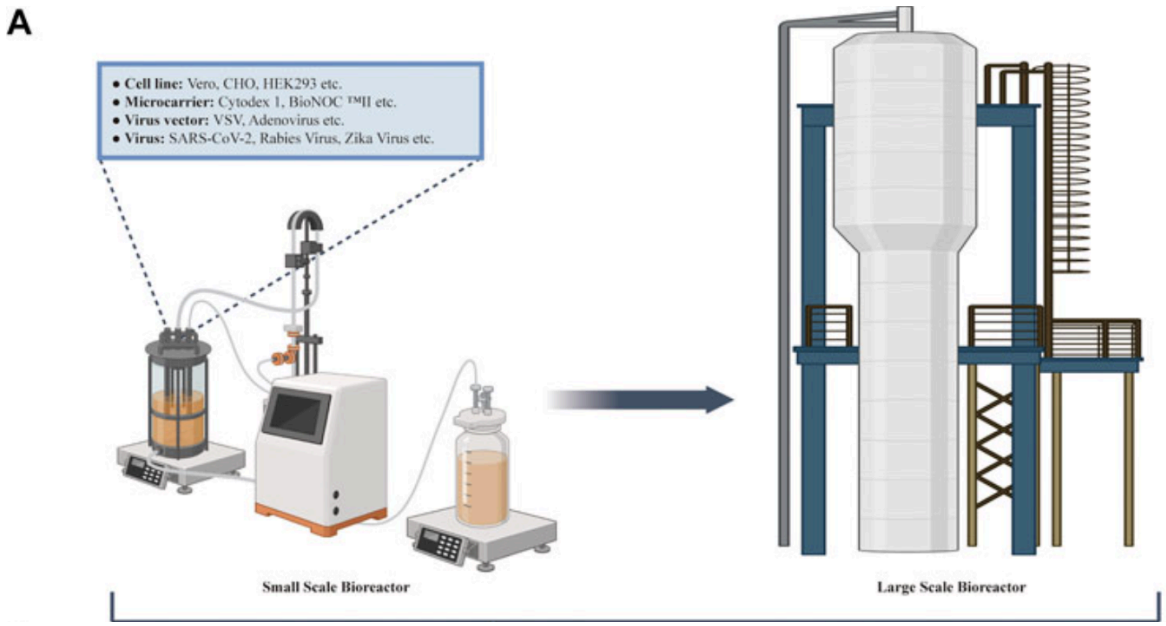

Figure 4. Cell culturing in small scale and large scale bioreactors (fermenters). *The cell suspensions begin the growing process in the small scale bioreactor. Then, the virus is introduced and can remain in the small scale bioreactor or it can be scaled-up to the large scale bioreactor [15].*

Ghendon et al. studied the MDCK cell line by infecting them with cold-adapted (CA) strains of a live influenza A vaccine to study the replication [4]. CA strains of the influenza virus are specific strains that replicate better in cooler conditions of around 33℃. Cells were grown and infected using both methods of a laboratory spinner and in a fermenter. Then, the results of the titer were graphed to analyze which CA reassortant had the highest multiplicity of infection [4]. Figure 1 shows that the reassortants in the laboratory spinner reproduce well with the highest titer at 10^{9} to $10^{9.5}$ EID_{50}/mL with a multiplicity of infection equal to 0.001 EID_{50}/cell. The reassortants in the fermenter also showed around the same values when titrated, 10^{9} to $10^{9.7}$ EID_{50}/mL. The parameter EID_{50} is the 50% embryo infectious dose [4]. This parameter is used to describe how

much of a virus is needed to infect 50% of a group of cells. A higher value indicates that there are more cells that have been infected by that specific dose which is what is needed when producing a vaccine because it will be able to infect the host cells quickly and combat severe infection from the influenza virus. The figure shows a comparison between using fetal serum or the serum-free medium which highlights how the culture media that the cells are suspended in is directly related to how the cells grow and thrive in order to maximize viral replication and antigen yield [4]. The timing in which cells are able to proliferate is also important when producing a vaccine because that is directly related to how the vaccine can multiply for production. In the experiments, the cells were able to be proliferated by a factor of 8 to 10 within 3 to 4 days which means the amount of vaccine can be increased 10 times within less than a week [4].

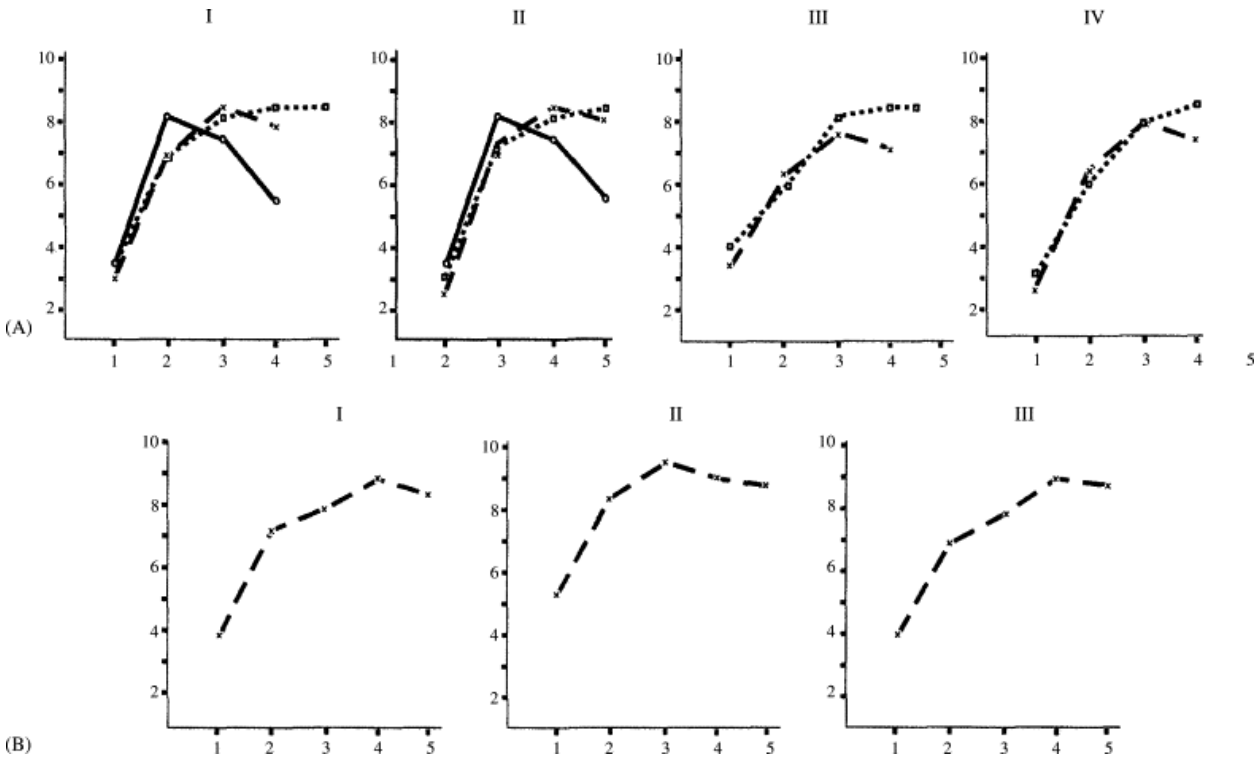

Figure 5. Results of the CA reassortant influenza virus in MDCK cells. *Results of the experiment are graphed with the x-axis showing the days after they have been infected with the*

CA reassortant influenza virus and the y-axis showing the EID_{50}/mL. The serum-free medium (b) showed higher titers which shows that CA reassortant influenza virus replications well under these conditions rather than with fetal serum medium [4].

These advancements in cell culture systems for influenza virus provided the foundation for researchers to be able to develop a COVID-19 vaccine at such a pace that matched the rapidness of its spread within the population. Both influenza and COVID-19 are RNA-based viruses that depend on the makeup of the host cell's pathways for replication. However, COVID-19 introduced a new challenge at the biochemical level because of its different virus structure [7]. Previous knowledge from MDCK systems such as media nutrient adaptations, optimization of viral yield, and overall purification were able to be directly applied to development of a stable and effective vaccine for COVID-19 [7].

Innate and Adaptive Immune Response to COVID-19

The coronavirus disease began in 2019 and continues to be a global health concern today. Once the virus is contracted, the innate immune response is the first to respond to try to combat it in the body [7]. The virus enters the host cell through endocytosis and the spikes of the virus bind to the angiotensin-converting enzyme 2 (ACE2) which is a receptor on epithelial cells of the airway [7]. The binding of the spike proteins to ACE2 on the membrane of host cells is completed by transmembrane serine protease 2 (TMPRSS2) which causes proteolytic cleavage and allows for membrane fusion [6]. After binding to the receptor, the virus enters and its positive-sense RNA genome and its nucleocapsid are released into the cytoplasm of the host cell [6]. The innate immune response recognizes viral RNA through Toll-like receptors (TLR3 and

TLR7) and pattern recognition receptors (PRRs) which triggers a cascade of intracellular signaling pathways that cause the production of pro-inflammatory cytokines and type I interferons (IFNs) [7]. Traditionally, these interferons would induce apoptosis in infected host cells in order to limit viral replication, but COVID-19 contains open reading frame 6 (ORF6) which functions by blocking the signaling pathway between the host cells and interferons [7]. Without these interferons inducing apoptosis, two viral polyproteins (pp1a and pp1b) are encoded but ORF1a and ORD1b genes are processed by proteases and this produces 16 non-structural proteins (NSPs) [6]. These NSPs form the transcriptional complex that replicates the viral genome and this causes subsequent control over the translational machinery in the host cell in order for the virus to produce its own proteins [6].

The adaptive immune response is the next line of defense against the virus. It activates cytotoxic T lymphocytes ($CD8^+$) and B-cells to generate neutralizing antibodies [7]. Strong T-cell and B-cell responses can clear the virus in mild cases [7]. The B-cell and T-cells together produce antibodies that bind to the spike glycoprotein which prevents it from attaching and entering the host cell [7]. However, severe infections that are able to pass the innate immune response show lymphopenia and weakened adaptive immunity. Almost all patients with mild and moderate disease severity demonstrate that the immune response is primarily led by T-cells and B-cells even several months after primary infection [7]. Since this is such a complex disease that affects each individual differently, the levels of the immune cells will vary depending on the severity of infection [7]. In patients with severe symptoms, the level of these cells will be lower meaning there is an inadequate anti-viral response against infection [7]. On the other hand, there will be more of these immune cells allowing for a more stronger defense against the virus in patients

with a more moderate disease severity [7]. This complexity shows the need for cell culturing systems that produce vaccines that can help combat the effects of the virus on the immune cellular responses.

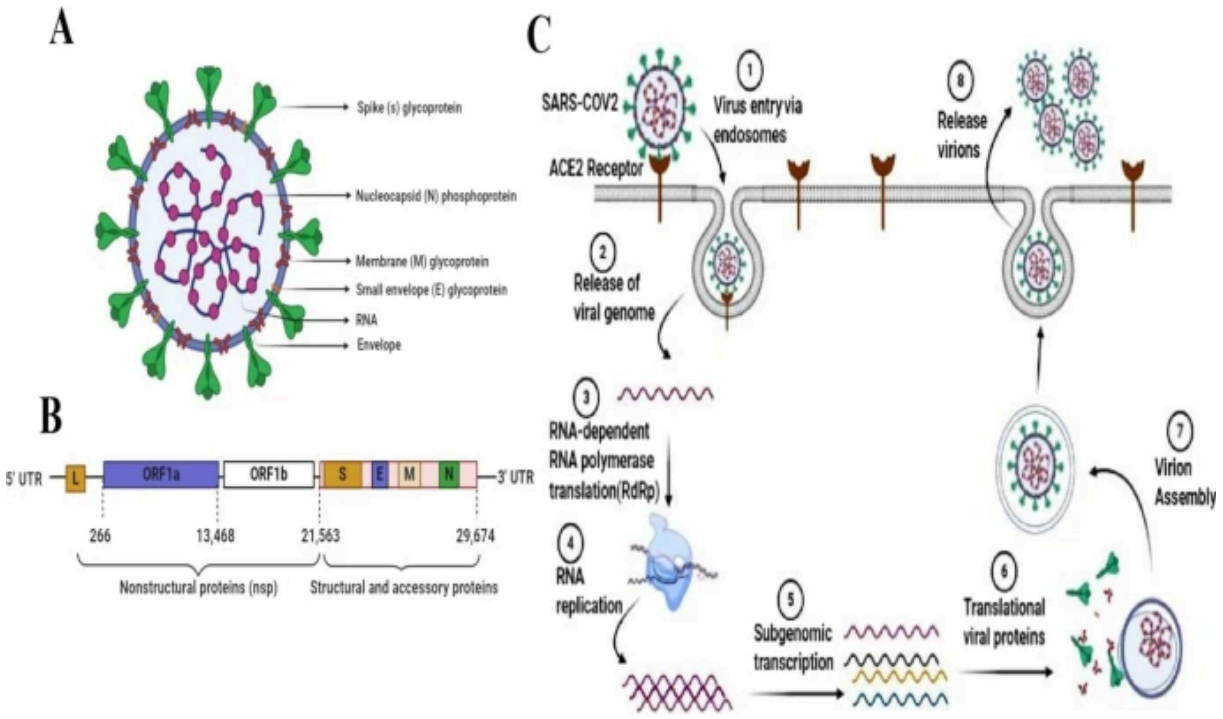

Figure 6. COVID-19 virus structure and infection mechanism. *Binding of the spike glycoprotein with the ACE2 receptor is what fuses the viral and cellular membranes and releases the viral genome into the host cell. The genome that codes for the ORF1a and ORF1b is shown and this is what permits the replication of the virus within the host cell [16].*

Culturing Methods for COVID-19

As with influenza virus, the first step of developing a culture method for vaccine development is to choose a cell line that will mimic the host cells within the body. Chinese Hamster Ovary (CHO) cells are a central cell line when analyzing viruses with recombinant proteins [10].

Specifically, CHO cells are genetically stable and capable of complex post-translational modifications [10] One of these modifications is glucosylation, which is critical for antibody stability and activity [10]. This cell line was used to express antibodies that neutralize the spike protein that is characteristic of the COVID-19 virus [10]. Since the CHO cell line has a long history of producing products it enabled accelerated chemistry, manufacturing, and control methods which aided in reducing timelines from years to months for both development and clinical supply [10].

In a study done by Liu et al., CHO cells were used to produce a recombinant COVID-19 subunit vaccine. CHO cells were grown in a bioreactor until they reached peak production levels of around 500 mg/L [10]. After studying the vaccine entry mechanism, the spike glycoproteins that form a trimer on the surface of the virus and the receptor binding domain (RBD) are the most reasonable vaccine target due to the abundance of antibodies [10]. Expression vectors were transfected into CHO cells so the cells will produce both of these factors in order to allow these cells to produce neutralizing antibodies (nAbs) [10]. These nAbs have the ability to block the virus from infecting cells which is why it was important that the CHO cells express the factors to produce them [10]. To make sure the vaccine antigen was pure, the protein was purified through multi-step chromatography [10]. This removes host cell DNA, host cell proteins, and any other impurities [10]. To have a maximum immune response, a dual adjuvant system containing aluminum hydroxide and CpG 7909 was used [10]. Aluminum salts were used to absorb and stabilize the antigens within the vaccine [10]. CpG 7909 adds additional protection against infection by inducing the T helper type 1 (Th1) cellular and humoral immune responses [10]. The candidate vaccine was tested in mice, nonhuman primates, and hamsters [10]. After

comparing results among the different animal systems, the candidate vaccine triggered a strong immune response against the COVID-19 virus [10]. Specifically, the figure shows that in mice, the candidate vaccine combined with the adjuvants produced higher titers of nAbs than the amount produced by RBD with the adjuvants [10]. Additionally, the candidate vaccine paired with the adjuvants produced a higher antibody and T cell response than when it was paired with Aluminum or CpG alone [10]. These results support the use of a dual adjuvant system to maximize the effectiveness of a vaccine [10] They also show an immune response that is long-lasting [10].

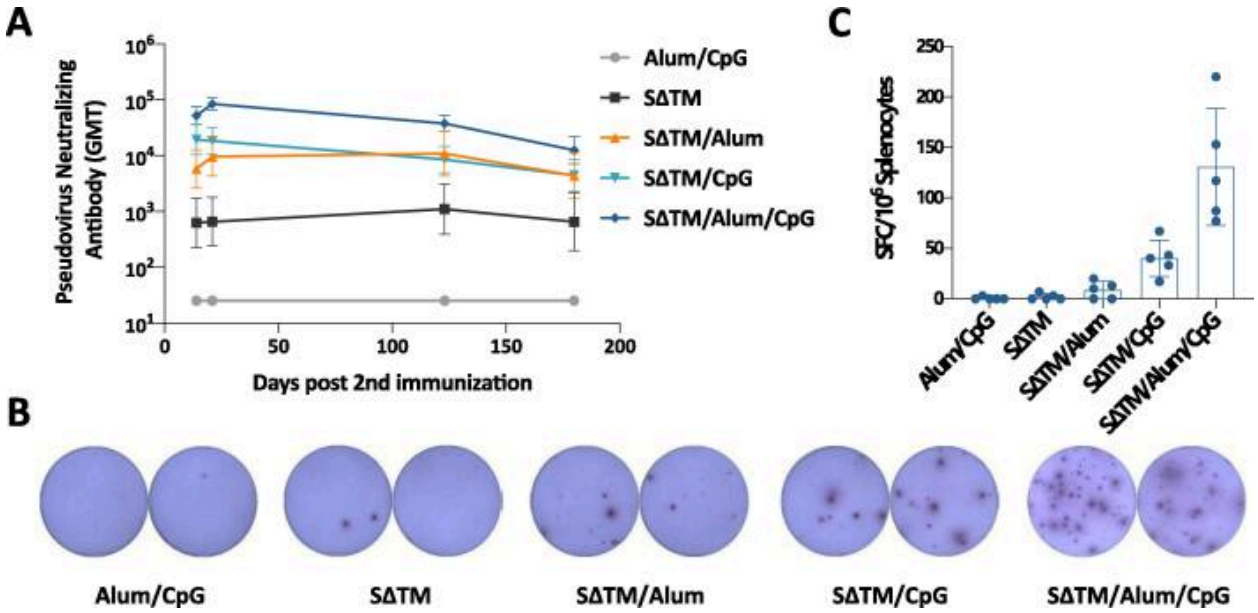

Figure 7. Results of the candidate vaccine with different combinations of the dual adjuvant system. *The candidate vaccine (SΔTM) with Alum and CpG showed the highest amount of neutralizing antibodies after the second immunization. B shows a specific B cell ELISPOT experiment where the dual adjuvant system showed the most density of B cells. Then, C shows the memory B cells present 180 days after the second immunization where the dual adjuvant system produced the most compared to the other systems [10].*

Limitations of Cell Culturing

For cell culturing methods, the main concern is contamination of the cells in culture. The method depends on sterile techniques that allow the cells to grow in culture so the vaccine can be tested and results can be properly obtained. Additionally, there are also commercial challenges to using cell culture as a method of vaccine production [11]. Multiple tests need to be performed to make sure there are no adventitious agents such as bacteria, viruses, fungi, and mycoplasma present for both human and mammalian pathogens [11]. For influenza specifically, the virus has high lytic activity, meaning that the virus uses the machinery within the host cell to rapidly replicate which ends in lysis of the cell membrane [11]. In order to extract the desired product from this mixture, the proteins derived from the host cell and its DNA need to be carefully removed to meet regulatory requirements and decrease unwanted immune responses [11]. Furthermore, production of the vaccine per unit dose is high and the technology that is necessary to perform this method is not as easily accessible [11]. However, it is noted that with recent investments in new facilities that will aid in the research and development of cell culturing will increase the use of this method to produce vaccines [11].

Future Directions

Recent studies show that conventional single-cell culture is not adequate for measuring the immune response to a virus . This is because immunogenicity to vaccines is a process that involves several factors such as cytokines, immunological components, and specific cells [17]. 3D cell culture technologies have been developed to allow researchers to view pathogen-host cell interactions with more specificity than a single-cell culture. *In vitro* imitation of the organ that

needs to be studied also allows for the prediction of undesired side effects along with insufficient immune responses in vaccines which saves time and money by avoiding unnecessary *in vivo* research [17]. Studies that use organoids and 3D cell culture are mostly focused on these pathogen-host cell interactions and tumor research for cancer patients. In a study done by Wagar et al., healthy tonsils were harvested and divided into cells that were maintained in optimal conditions for them to grow [17]. Organoids were developed using transwell membranes and in order to protome the growth of the organoids under the membranes, they used recombinant human B cell activating factor [17]. These tonsil organoids were used to see the immune response of live attenuated influenza vaccination along with the measles, mumps and rubella vaccine [17]. It was reported that the influenza vaccine stimulated follicular dendritic cells and follicular helper T cells. 14 days after the vaccine had been administered, there was an increase in CD8+ T cell activation which aided in the immune response to the virus [17]. This revealed 3D cell culturing methods and organoid growth to be a helpful tool in assessing the immune response to vaccines [17].

Conclusion

The studies of using cell culturing methods for both influenza and COVID-19 show that cell culturing is a reliable technique that can be used to produce vaccines. Cell culturing is a technique that relies on the use of a specific cell line that can accurately mimic the virus interacting with the host cell. Using the specific cell lines that have been proven to give results provides the mechanism for researchers to be able to view these interactions and the immune response they elicit. By shifting away from the use of egg based systems, vaccines have been able to be produced faster and are more safer to the population.

Although this research showed successful results, cell culturing is still not a method that is widely used today for vaccine production. This is because the method primarily relies on having a completely sterile environment in order for the cells to grow. Without this, it is unknown whether change in the cell structure or even cell death is due to the lack of sterility or from the virus. Additionally, most labs do not have the facilities that can support this technique. However, as time and technology advances, so will the facilities that can be used for this technique. The use of 3D culture mechanisms opens a window of opportunity for researchers to have even more accurate results. Being able to see the virus and host cell interactions not only gives insight to how the virus infects the host cell but also what type of immune response it causes. Understanding the immune response can help researchers understand what needs to be done to keep the virus from taking over the host cell machinery and infecting the patient. With this knowledge, a vaccine can be produced that is more effective at combating the virus.

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