

## A New Live, Attenuated, With Enterotoxin Adjuvant, *V. Cholerae* Vaccine

### Literature Review:

Cholera is one of the most researched enteric diseases that continues to be a major cause of morbidity and mortality in many developing countries. Ninety four percent of the 101,383 cholera cases and 2345 related deaths registered with the World Health Organization (WHO) in 2004 occurred in sub-Saharan Africa (D. Sack et al., 2006). The statistics are misleading with the highest cases occurring in Asia, but many Asian countries do not report their cases due to effects on travel and trade. Cholera infects people around the world, but those in developing countries experience cholera in epidemic proportions. The cholera burden is shared among three primary continents Asia, Africa, and Latin America.

*Vibrio cholerae* is a Gram-negative motile bacterium which causes cholera, a highly contagious diarrheal disease. Cholera is transmitted through the ingestion of contaminated food, water, contact with feces, or vomit from an infected person. Symptoms appear within 1-5 days and death may result in just a few hours from severe loss of water and salt. The disease enters the body via the oral cavity where it then colonizes in the small intestine. In addition to diarrhea, other symptoms include: nausea, muscle cramps, shock, and severe dehydration. Many people infected are asymptomatic which can be deceiving to early detection. Some survivors are immune to a second infection showing that immunity can be acquired (Provenzano et al., 2006). Since the days of Dr. John Snow in 1854, when he demonstrated the cholera contagion at the Broad street pump in London researchers have relentlessly pursued the understanding of *V. cholerae* (Provenzano et al., 2006). Since then, the biology of cholera and of *V. cholerae* have become increasingly clear, but yet many questions about the disease still remain.

Two main serogroups of *V. cholerae* account for the majority of cholera. The serogroups, O1 (serotype Inaba or Ogawa) and O139 define the unique *V. cholerae* Lipopolysaccharide (LPS) structure. The cholera toxin produced by *V. cholerae* is needed for disease and attaches to villi of the small intestine using toxin co-regulated pillus (TCP). The toxin is comprised of two subunits, A and B. Subunit B binds to ganglioside GM1 receptors of intestinal cells facilitating the insertion and cleavage of the A subunit at the membrane. This increases cellular cAMP, over-activity of sodium pump resulting in secretion of chloride ions and water from intestinal epithelial cells leading to profuse diarrhea (Taylor et al., 2004).

Various studies have showed that seasonality is associated with cholera outbreaks. As with many other diseases' optimum conditions for rapid growth is favored by warm environmental temperatures. Resource deprived individuals, poor personal hygiene, weak or no primary health care, and substandard water purification practices are key factors in the epidemiology of cholera. The disease mostly affects those living in underdeveloped countries. Simple practices such as hand washing with soap after toilet use and before meals could have an impact on the spread of cholera (Izadi et al., 2006). The populations most at risk from cholera are adults and children living in rural communities in underdeveloped countries such as Asia, Africa, and Latin America. Usually, those affected by cholera outbreaks are people with limited or no access to healthcare and of poor socioeconomic status (R. Sack et al., 2003).

The simplest level of treatment for cholera involves aggressive rehydration therapy to restore circulating blood volume and antibiotics to shorten the course of illness and reduce the loss of fluid from diarrhea (D. Sack et al., 2006). Existing cholera vaccines are very effective in some settings, but are less so in others, especially in groups such as young children ranging from ages 2-5. Since the 1800's cholera has posed a challenge to mankind, the scientific community has worked relentlessly towards understanding this disease and developing a vaccine that works well in all cases. Cholera was chosen not only because it affects many under-represented communities, but also because an effective subunit cholera vaccine could provide protective immunity with one parental immunization. Clearly this would be a major advantage over the existing oral vaccines that require two doses for optimal protection.

There have been a number of cholera vaccines approved for human use. The first generation vaccines (1960's) were parentally (intramuscular) administered, killed, whole-cell vaccines. Although approved by the WHO years ago, they have been relegated to non use because they provide only short-term, partial protection that was in some cases associated with adverse reactions in immunized individuals. Oral, killed or live attenuated cholera vaccines were choice in the 1980's. Initially, promising results were obtained with up to 85% protection in the first 12 months that waned to 50% over 3 years (Taylor et al., 2004). Currently two vaccines are licensed for use in certain parts of the world, neither has been approved for use in the U.S. Current vaccines in use are delivered orally and consist either of killed whole cells or attenuated live bacteria. The LPS of *Vibrio cholerae* is the most consistently described antigen associated with protection of humans from cholera and the vaccine against vibrio O1 is not effective against O139 vibrio. Efforts continue in finding the most effective vaccine formulation to induce *V. cholerae* anti-LPS antibodies.

### **Description of Vaccine:**

*Vibrio cholerae* has several vaccines already produced. However, all of the vaccines currently in use or in testing apparently use some combination of subunit and killed bacteria or killed and live attenuated. While they are partially effective, some experiments demonstrate that a live attenuated bacterium, with enterotoxin as an adjuvant, produces the most effective secondary immune response (Bhattacharya 2003). Particularly, the O-antigen, the antigen of the outer membrane of *V. cholerae*, will be used as the main activator of the immune system function, with the enterotoxin amplifying the response.

*V. cholerae* primarily affects the small intestines, which is a mucosal membrane, thus will to be treated differently than a blood infection. The biggest difference will be activating the manufacture of IgA by B-cells rather than IgG. Since the infection is taking place in the intestines, there must be a way to access the immune system's cells which are underneath the mucosal epithelial. M Cells are the body's way of transferring the bacteria so the immune system can be activated by it. This is easy with a real infection because the infection will try to colonize as much as possible. However, to ensure the safety of the patient, usually a killed bacteria has been used to activate the immune system. The problem with this is the lack of way for the bacteria to not be pushed down the gut track. Normally, *v. cholerae* can use its flagella to fight against the "current", then use its fimbriae to attach to the intestine walls (Bhattacharya 2003). Killed bacteria can not do this. Thus, it relies on the M cells and searching dendritic cells

to pull antigen through the mucosal membrane. While in great enough quantities, this would be successful, the activation of the immune system normally requires multiple vaccinations to have that effect. A live vaccine would eliminate the extra immunizations required. By attenuating the bacteria so that it does not produce enterotoxin, the cause of the diarrhea and fluid loss, but still giving it the ability to colonize would seem to provoke the best response with minimal side effects. It would continue to survive for some time, reproducing and being a ready source of antigen, specifically the O-antigen serotype, but not cause the same debilitating damage.

There are specific actions that immune system should take upon being exposed to any antigen. In the case of this vaccine, the entire immune system should be activated. Initially, the macrophages will be activated. This is important because their secretions, mainly the cytokines, will cause inflammation to take place as well as bring in complement. Complement has a dramatic effect on *v. cholerae* when there is O-antigen in the area. Complement can cause it to lyse, effectively lowering the load of infection before the cellular immunity has even started. Enterotoxin is very effective in activating macrophages. Macrophages have many receptors for this particular toxin, which allows for very strong reactions. This increases the production of complement by the body and inflammation of the area. The cytokines released by the macrophage also attract leukocytes to the nearest Peyer's Patches. Peyer's Patches are a lot like lymph nodes in that there are numerous T and B cells. Antigen-presenting cells drain into the patches, where they will activate the T and B cells. B cells are important to activate because IgA is needed to most effectively counteract the infection, since it does occur in a mucus membrane. B cell receptors bind specific epitopes. In this case, the receptors can bind any O-antigen or the enterotoxin epitope. Since, the bacteria is alive, there should be numerous *V. cholerae* available for binding. Activated B cells need to go through isotype switching from the typical IgM to IgA so it can specifically take care of the mucosal area. Isotype switching occurs when the B cell's switch regions are activated by antigen or cytokines. This changes the heavy region from the "M" to whatever is required, in this case "A". T helper cells are also useful, because they can help activate even more B cells, as well as activate any macrophages that have bacteria inside them they haven't destroyed any cells in a vesicle. Cytotoxic T cells are not necessary since *V. cholerae* is not an intracellular bacteria. The B cells will be made into memory B cells so they will respond to a real infection at a later date. Overall, this should be repeated faster on the second infection (Janeway 2005).

Problems could arise if the bacteria's enterotoxin causes the macrophages to overproduce their cytokines. There is a fine balance to how much adjuvant needs to be used in the vaccine. Too much will cause the immune system to go haywire and injure the patient. Too little, and the immune system might not respond to the attenuated *V. cholerae*. That is the other problem lacking with the vaccine. There is the possibility that the bacteria will not provoke the response of the immune system because it is not doing anything other than colonizing the intestines. However, the adjuvant should take care of that.

The main purpose of this vaccine is to produce antibodies to the O-antigen of *V. cholera*'s outer membrane. While there is enterotoxin present, it's only main use is to activate the immune system. Thusly, there won't be much in the way of antibodies produced to the toxin. Therefore, testing for antibodies should focus almost exclusively on the O-antigen.

## **Immunity Assessment**

### Trial

The vaccine will be first tested on primates, due to their immune system being the most similar to humans. Once these trials have lapsed and shown positive results then human trials can begin.

Testing of this vaccine will call for 25 people from India who have not been previously exposed to the pathogen. The subjects are to be from India as they will be the main recipients for this vaccine due to the prevalence of the disease there. Fecal-oral transmission is a problem in India because not all places are developed to have plumbing and clean fresh water.

The vaccine of choice will be attenuated. *Vibrio cholerae* will be grown lacking the ability to make the endotoxins that cause diarrhea. These bacteria will contain the specific O1 antigen that is found on the bacteria's cell wall. This allows the bacteria to colonize the epithelial lining of the intestines without causing the disease. These cells will therefore colonize the intestines and an immune response to the O1 antigen will follow. This will create memory T and B Cells and lead to immunity against future infections by *Vibrio cholerae*.

IgA will be the most important antibody immunoglobulin isotope in this immune response. The bacteria will enter the body via the oral cavity and infect and invade through the lining of the intestines, which is a mucosal lining. IgA's function is to provide protection in secreted mucous. This will be the main immune response we will be searching for to show immunity is created for *Vibrio cholerae*.

Blood will be drawn from each subject. IgA can be tested for in mucosal samples in addition to the previously mentioned drawn blood in efforts to detect IgA. The amount of IgA is likely to be less substantial than IgM within the blood (amount of IgA seen that provides immunity?). Samples will be taken once every month for 6 months to determine if a response is made. ELISA will be used to find how much IgA is produced each month. These samples will be tested for specific antibody to the O1 antigen.

### ELISA

First antigen will be used from *Vibrio cholerae*, the same as the attenuated vaccine form, to coat the plate. These will be allowed to sit for one hour and then unbound protein will be washed out. The serum samples taken will be added into the wells at different dilutions. Likely these dilutions will begin with 1:1 (no dilution), 1:10, 1:50, 1:100, 1:500, and 1:1000. Positive and negative controls will also be added. A positive control will be done with unknown serum instead of known positive serum. A negative control will be done by not adding the antigen to the well and not allowing anti-human immunoglobulin (Fernandez-Miyakawa et al., 2006).

Once the antibodies from the added serum bind to the antigen, rabbit anti human alpha chain will be added to the wells. This binds to the IgAs Fc region. The IgAs are already bound to

the antigen. The Fc region of the anti-Ig is linked to an enzyme and when the chromogenic substrate is added, a color change will take place in presence of the enzyme linked to the anti-Ig. HorseRadish Peroxidase (HBR) will be the substrate to be used for this assay (Shen et al., 2007). When reacted with the enzyme it turns blue yielding a positive result for the antibody. The more intense the color of the well the more antibodies were in the sample dilution. In this case a color change at the 1:100 or higher dilution will be adequate immunity for *Vibrio cholerae*.

### Flow Cytometry

The final test of immunity will be to measure the Helper T Cells, or Th Cells. This is a measure of cellular immunity, and will be done by flow cytometry. In flow cytometry cells from serum are differentiated using light and a substrate for coloring. Fluorochromes are used to stain specific membrane molecules (in the case of Th Cells, CD4).

The stained cells pass through a sensor one cell at a time. A laser excites the fluorochromes and the differences in spectrum given off can be measured. The different measures of spectrum from cells can be graphed on a histogram. The cells are differentiated and CD4 cells can be quantified giving the number of CD4 cells in the sample. From this a measure of T-Cell immunity (memory against future infection) can be determined (Zhang 2003).

## Information Sources

- Bhattacharya SK, National Institute of Cholera and Enteric Diseases (2003). "An evaluation of current cholera treatment". *Expert Opin Pharmacother* 4 (2): 141-6.
- Izadi S., Shakeri H., Roham P., Sheikhzadeh K. Cholera outbreak in Southeast of Iran: routes of transmission in the situation of good primary health care services and poor individual hygienic practices. *Japanese Journal of Infectious Diseases* 59:174-178, 2006.
- Fernandez-Miyakawa M.E., Brero M., and Mateo N.A. Cholera Toxin Modulates the Systemic Immune Responses against *Vibrio cholerae* Surface Antigens after Repeated Inoculations. *Microbiology and Immunology* 50 (8): 607-619, 2006.
- Janeway, C.A., P. Travers, M. Walport and M. J. Schlomchik, IMMUNOBIOLOGY The Immune System in Health and Disease (2005). Sixth Edition, Garland Publishing.
- Zhag G., Chen Y. ELDKWA-epitope-specific monoclonal antibodies inhibit HIV env-mediated Provenzano D., Kovac P., and Wade F.W. The ABCs (antibody, b cells and carbohydrate epitopes) of cholera immunity: considerations for an improved vaccine. *Microbiology and Immunology* 50 (12): 899-927, 2006.
- Sack D.A., Sack R.B., and Chaignat C-L. Getting serious about cholera. *The New England Journal of Medicine* 355 (7): 649-651, 2006
- Sack R.B. et al. A 4-year study of the epidemiology of *Vibrio cholerae* in Four rural areas of Bangladesh. *Journal of Infectious Diseases* 187: 96-101, 2003.
- Shen S., Wang S., Britt W.J., Lu S. DNA vaccine expressing glycoprotein complex II antigens gM and gN elicited neutralizing antibodies against multiple human cytomegalovirus (HCMV) isolates. *Vaccine* 25 (17): 3319-3327, 2007
- Taylor R.K., Kirn T.J., Bose N., Stonehouse E., Tripathi S.A., Kovac P., and Wade W.F. Progress towards development of a cholera subunit vaccine. *Chemistry and Biodiversity* 1: 1036-1057, 2004.