THE EFFECT OF MV-II-065 ON THE PHAGOCYTOSIS OF *STAPHYLOCOCCUS AUREUS*

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Maurice Royal

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The Effect of MV-II-065 on The Phagocytosis of *Staphylococcus aureus*

Maurice Royal

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Maurice Royal, Student Date

Approvals:

_____

Diana Fagan, Ph.D., Thesis Advisor Date

_____

David Ash, Ph.D., Committee Member Date

_____

Jonathan Caguiat, Ph.D., Committee Member Date

_____

Peter J. Kasvinsky, Dean of School of Graduate Studies and Research Date

Abstract:

Staphylococcus aureus is a major source of many clinical problems and is associated with an increase in the cost of health care. The bacteria's ability to be resistant to antibiotics remains ever constant. *S.aureus'* cell wall is composed of a capsule that hinders the human body's immune system by preventing phagocytosis of the bacteria. The ineffectiveness of antibiotics and the body's immune system creates the need for alternative methods of treatment for *S.aureus* infections. A potential alternative form of treatment involves the use of mimetics, modified capsular carbohydrates. The goal of these studies was to test the ability of a mimetic, MV-II-065, to block the synthesis of *S. aureus* capsule. To ascertain the effectiveness of the mimetics, they were administered in different concentrations and incubated with *S.aureus* overnight. A decrease in capsule production (26% of solvent control) was indicated by decreased binding of monoclonal antibodies to the bacteria during an ELISA assay. Results of phagocytosis assays were variable, however the mimetics had no effect on bacterial growth rates. Colony counts were performed on bacteria treated with mimetics. These studies demonstrated that incubation with the MV-II-065 altered the light absorbance of the bacteria. Bacteria concentrations standardized by absorbance at 550 nm gave falsely low values in bacteria treated with the mimetics, resulting in higher numbers of bacteria in mimetic treated populations compared to controls. In future studies examining the functional effect of MV-II-065, the bacterial concentrations will have to be verified using colony counts prior to performing phagocytosis assays.

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List of Abbreviations

Abs antibodies (Abs)

Bovine serum albumin (BSA)

Capsule (CP)

Carbohydrate (CHO)

Cationic antimicrobial peptides (CAMP)

Colony forming unit (CFU)

Columbia Salt Agar (CSA)

Deoxyribonucleic acid (DNA)

Ethylenediaminetetraaceticacid (EDTA)

Enzyme linked immuno assay (ELISA)

Erythomycin recombinate protein A (EPA)

Erythromycin resistant gene (erm)

Fetal calf serum (FCS)

Fragment (Fc)

Human complement type AB plasma (hAB)

Hypoxyanthine aminopterine thymidine (HAT)

Intracellular adhesion molecules (I-CAM)

Macrolis (MLS)

Phosphate bufferd saine (PBS)

Polyethylene glycol (PEG)

Open reading frame (ORF)

Ribonucleic acid (RNA)

RNA polymerase resistant gene (rpoB)

Tetracyclin resistant gene (tet K)

Tetramethylbenzidine (TMB)

Transfer ribonucleic acid (tRNA)

Type 5 (T 5)

Type 8 (T 8)

Introduction

Staphylococcus aureus is a major source of many clinical problems and infections are associated with an increase in the cost of health care. The bacteria is rapidly developing resistance to most available antibiotics, making treatment difficult. The *S. aureus* cell wall is surrounded by a capsule that hinders the human body's immune system from being effective by preventing phagocytosis of the bacteria. The ineffectiveness of antibiotics and the body's immune system in eliminating these infections creates the need for an alternative method of treatment. A possible alternative form of treatment involves preventing the development of the *S. aureus* capsule using carbohydrate mimetics. Mimetics are chemicals designed to resemble modified forms of the carbohydrates incorporated into the capsule as it is synthesized. It is hoped that these carbohydrate mimetics may be used to inhibit capsule synthesis. The goal of these studies was to test the ability of mimetics to block the synthesis of *S. aureus* capsule. Capsule synthesis was demonstrated by the ability of monoclonal antibodies to bind to the capsule. A phagocytosis assay was also conducted to determine any functional effect of decreased capsule production.

There are many disorders and diseases associated with *Staph aureus* infections that can cause morbidity, mortality, and rising cost in health care (Cunnion et al., 2005). The most serious threat is posed in people who have weakened or undeveloped immune systems, such as infants or people who have influenza, chronic pulmonary disorders, chronic skin disorders, neoplasms, transplants, surgical incisions, prostheses, burns, or diabetes mellitus. Infections of many types can be seen, such as breast abscess, neonatal infections, endocarditis, osteomylitis, and post operative infections (Volk et al., 1996).

S. aureus infections are difficult to control because virulent strains are carried asymptomatically in the nasopharynx of 10%-50% of adults (Volk et al., 1996). Staphylococci can also be present in the human body as normal flora which means they are natural residents of certain areas. When barriers are broken through breaches in the skin or mucosal surfaces, this allows *S. aureus* to become an invasive infection. Under these circumstances infections can arise in bones, endocardium, the bloodstream, and at other metastatic sites. Peripheral barriers are needed to maintain an asymptomatic host bacterial relationship.

S. aureus also produces many extra cellular enzymes and toxins that are associated with disease (Mitchell et al., 1985). An example is the production of coagulse. Coagulase allows for clots to form in plasma. Clot formation isolates the bacterium protecting it from the immune response (Volk et al., 1996). Coagulase production is also an important contributor to the formation of abcesses, which are often seen in staphylococcal infections.

Some diseases are caused by staphylococcal toxins rather than the actual organism. Food poisoning can be attributed to the ingesting of preformed heat stable enterotoxin. Enterotoxins can also cause severe diarrhea along with vomiting. At least one third of all clinical staphyloccocal isolates secrete enterotoxin. One key factor that makes enterotoxin problematic is that even when the food is heated to kill the bacteria the entrotoxin is not destroyed. Enterotoxins can also cause an expansion of T cells by binding to class II major histocompatibility complexes. Exfoliating toxins cause exfoliation of the skin and are associated with staphylococcal scalded skin syndrome (Gotz, 2004).

Several additional toxins are produced by *S. aureus*. Hemolysins are toxins that are categorized into four classes, α, β, γ, and δ hemolysins. The α hemolysins damage smooth muscle and kill skin cells. They can also be lethal to macrophages, platelets and cause degranulation of Polymorphonuclear cells (PMNs) by disrupting lysomsomes. Destruction of the eukaryotic cell by α hemolysins result from the formation of a 2-3 mm ring shaped hexamer in the membranes of cells. Alpha hemolysins cause PMNS to release leukotriene B4 (Volk et al., 1996). Leukotrienes are lipid mediators of inflammation which are products of arachidonic acid (Kuby 2000). Beta toxins react with sphingomyelin to split off phophorylcholine resulting in the lysis of erythrocytes. Cells that have abundant sphingomyelin are vunerable to this action. Little is known about the mechanism of γ toxin, except that it can cause the lysis of erythrocytes, and that its action is stopped in the presence of certain phospholipids. It has been suggested that phospholipids may be its target. The δ toxin can lyse several types of cells such as red blood cells, PMNs, macrophages, lymphocytes, and platelets through its hydrophobic amino acids interacting with phospholipids in the cell membranes (Volk et al., 1996).

Other enzymes of *S. aureus* are hyaluronidase (a spreading factor), penicillinase (destroys penicillin), and staphylokinase (causes the lysis of fibrin clots). The staphylococcal decomplementation antigen causes the consumption of complement components in human serum. This allows for the suppression of the opsonization role of host complement by causing its destruction (Volk et al., 1996). Opsonization is the binding of complement or antibodies to antigens, such as bacteria. The importance of opsonization is that it facilitates phagocytosis of bacteria by macrophages. Macrophages

have complement and antibody receptors, which enable them to bind to and phagocytose antigens or bacteria easier.

Staph infections usually lead to an acute inflammatory response with immigration of PMNs to the site. PMNs are the first line of defense against many bacteria. However, *S. aureus* has an ability to resist phagocytosis resulting in extra cellular antibacterial infections (Weiss et al., 2000). One virulence determinanat that prevents phagocytosis of *S. aureus* is the production of a capsule.

More than 90% of *S. aureus* clinical isolates produce capsular polysaccharides. In many bacteria the capsule allows the evasion of the host immune system. The *S. aureus* capsule has been divided into 11 serotypes which fall into two classes, mucoid and microencapsulated. The mucoid strains produce thick capsules, while microencapsulated produce thin capsules. Mucoids account for serotypes 1 and 2. The rest of serotypes fall under microencapsulated. *S. aureus* types 5 and 8 account for 70% of infections that cause *S. aureus* disease (Mitchell et al., 1985). Serotypes 5 and 8 are in similar in structure and differ only in the linkages between the amino sugars and the O acetylation. Type 5 structure is as follows →4-β-DManNAc (1→ 4)α-L-FucNAc(3OAc)-β-DFucNAc(→1 and type 8 structure is →3)-β-DManNAc(4OAc)-(1→3)-α-L-FucNAc(1→3)-α-DFucNAc(→1 . Type 5 and type 8 have 16 linked genes in the *cap* 5 and *cap* 8 loci. Twelve of these 16 are practically identical. Sequence comparison shows 61%-71% homology (C. Lee and J. Lee, 2000).

The role of the microcapsule remains unclear. There is also uncertainty about how the mode of infection affects *S. aureus* virulence. Previous studies of capsular antibodies that induce type specific phagocytosis of *S. aureus* by polymorphonuclear

leukocytes have suggested microcapsules resist phagocytic killing and that type specific antibodies bind to capsules *in vitro*. However, this has been refuted due to studies of type 5 and type 8 showing that they are no more virulent than non encapsulated strains. The presence of type 5 capsule results in decreased *S. aureus* virulence in rats exposed to catheter induced endocarditis when the mode of infection is intravenous. Furthermore, capsule specific antibodies do not protect against endocarditis when given *S. aureus* is given intravenously. In contrast to these studies, rats immunized with type 5 and 8 capsules that are conjugated to protein are protected against a lethal dose of type 5 and 8 when they are given intraperitoneally. *S. aureus* administered interperitoneally allows for a gradual entry into the blood and seeding of the organs. It is noted that this may be more clinically relevant than the events that following intravenous route (C. Lee and J.Lee 2000).

There are several immune mechanisms which may be employed to prevent staph infections. PMN migration from the blood to the site of infection is one such mechanism. The exact mechanism of how *S. aureus* causes PMN migration is not fully understood, but is thought to encompass proinflammatory action of derivatives or fragments of peptiyloglycan matrix of *S. aureus* cell wall along with wall associated bacterial products such as lipoteichoic acid (Weiss et al., 2000).

PMN migration can be stimulated by chemotaxins which cause changes in the cell surface of PMNs and endothelium. These changes allow for the adherence of PMNs to endothelial cells by increasing the amount of I-CAM (immunoglobulin-like cell adhesion molecule) that is expressed on PMNs and endothelial cells. Mice that have genetic

deficiencies in I-CAM have an increased chance of infections or disease (Weiss et al., 2000).

Opsonization is another mechanism that is utilized by the immune system to combat *S. aureus* infections. PMNs can clear bacteria present through the interaction with bacterial cell wall associated proteins and fibronectin. Opsonins are molecules that bind to antigens on *S. aureus* and fibronectin receptors on PMNs to aid in the phagocytosis of the bacteria. The most effective opsonin is C3b, which can be generated from the classical pathway of complement activation (Weiss et al., 2000). The classical pathway involves the activation of complement through antigen antibody complexes or binding of antibody to antigen on a target such as bacterial cell walls. Antibodies IgG and IgM can activate this pathway (Kuby 2000).

Resident *S. aureus* in the nasopharyngeal cavity is known to give rise to naturally occurring antibodies to staphylococcal cell walls. These naturally occuring antibodies are found in plasma and can promote phagocytosis by participating in opsonization through their Fc region which binds to Fc receptors on phagocytes. These natural occurring antibodies can also promote phagocytosis through the activation of complement. However, the presence of capsule can lower the action of these opsonins by masking the complement deposited on the bacteria. This hinders the ability of the receptors on phagocytes to recognize complement (Weiss et al., 2000).

Phagocytosis of bacteria triggers a respiratory burst in the phagocyte granules, causing the rapid release of superoxide radicals and hydrogen peroxide which are toxic to the bacteria. These events also lead to the formation of acidic vacuoles in which bacteria are digested by activated enzymes. Myeloperoxidase or defensins are also contained in

PMN granules (Weiss et al., 2000). Myeloperoxidase is responsible for the formation of hypochlorous acid from hydrogen peroxide and chloride anions. Defensins are proteins that form small pores in bacterial cell membranes. The importance of these proteins can be seen in host who suffer from chronic granulomas disease (Weiss et al., 2000). This disease occurs when phagocytes are unable to kill bacteria within phagosomes, resulting in grauloma (walling of infections in various organs) formation.

Pathogenic staphylococci are regarded as antibiotic-resistant super bugs (Gotz, 2004). Before antibiotics, *S .aureus* bacteremia was fatal 90% of the time. The discovery of penicillin led to a reduction in mortality rates. With the use of penicillin, resistant strains evolved almost immediately. Even with the wide range of antimicrobials available today, *.S aureus* still poses a problem due to its ever increasing resistance to antibiotics. The growing resistance to antibiotics in *S. aureus* limits treatments available. Death rates have risen for patients infected with methicillin-resistant *S. aureus* (MRSA) when compared to patients infected with methicillin-susceptible *S. aureus*. Nosocomial isolates become more resistant as they are transferred from patient to health care worker or from colonized health care worker to patient (Projan, 2000).

The mechanisms of antibiotic resistance stem from three things: antibiotics are pumped out by an efflux action specific for the antibiotic or along with other molecules; there is a mutation in the target of the antibiotic; or the bacteria has developed a mode of inactivation of the antibiotic. There are numerous antibiotics that are becoming ineffective against *S. aureus*, such as penicillin, methicillin, glycopeptides, rifampin, fluoroquinolone, aminoglycosides, tetracyclines, and macrolides (Projan, 2000).

Penicillins are beta lactam antibiotics. There are several classes, penams, penems, cephamycins, cephalosporins, carbapenes, and monobactams. All of these contain the beta lactam ring. The target of these drugs is the transpeptidase of the cell wall. The synthesis of peptidoglycan is inhibited, which leads to the formation of autolysins, and ultimately lysis of the cell wall. Bacteria that are resistant possess beta lactamase, an enzyme which hydrolyzes the beta lactam ring. Over 90% of clinical isolates produce beta lactamase. The gene that encodes beta lactamase is found on a transposn TN552 on a plasmid. Methecillin was developed following the development of penicillin resistance. It was effective for a while, but eventually became ineffective due to alterations in the specificity of the beta lactamase (Projan, 2000).

Glycopeptides are one of the last effective antibiotics against beta lactam resistant bacteria. Vancomycin and teicoplanin work by blocking the polymerization of the cell wall. *S. haemolyticus* is the only species of staphylococci that appears to have resistance to glycopeptides. However, there has been indication of clinical isolates of *S. aureus* that have a reduced susceptibility to glycopeptides (Projan, 2000). It is noted that patients who suffer from infections caused by *S. aureus* with reduced susceptibility to glycopeptides have been on long term use of vancomycin. It has been proposed that step wise mutations attribute to lowering the minimal inhibitory concentration of vancomycin and are responsible for resistance to vancomycin (Projan, 2000).

Rifampin is an alternative antibiotic that targets bacteria using a different mechanism. Rifampin inhibits transcription by attacking the β subunit of RNA polymerase. RNA polymerase is required for RNA synthesis, making this an excellent target. Although rifampin is effective at inhibiting the growth of *S. aureus* at a very low

concentrations *in vitro*, resistance has been detected. Resistance can be seen in *S. aureus* due to a mutation in the *rpo* B gene which encodes the β subunit of RNA polymerase. Resistance creates a lower affinity of the drug. As a result, rifampin must be used in combination with other antibiotics, such as fusidic acid (Projan, 2000).

Flouroquinolones can also be used to treat *S. aureus* infections and include the antibiotics ciprofloxacin, levofloxacin, and trovafloxin. These drugs are broad spectrum. They work by preventing DNA replication which occurs when they target the DNA gyrase and topoisomerase proteins. These proteins are responsible for the breaking and joining of double stranded DNA. A major contributor to resistance to these drugs is the mutation in the efflux pump allowing for flouroquinolones to be pumped out of the cell. Another contribution to the resistance to fluoroquinolones in staphylococcus are the mutations in the genes *gyr* A, *gyr* B, and *grl* A and *grl* B that code for the topiosomerase and the gyrase (Projan, 2000).

Tetracyclins mediate control of *S. aureus* by inhibiting protein synthesis. The antibiotic consist of 6 interlocking rings that enable it to bind to the 30 S subunit ribosome hence blocking the entrance of aminoacyl tRNA (Projan, 2000). Some *S. aureus* strains are susceptible to some forms of tetracycline where some are resistant to all forms. Resistance depends on the genes that code for the resistance and where it is found. The *tet* K gene is found on plasmid $_{P}$T181 and it encodes for an efflux pump for tetracycline. Even though $_{P}$T181 allows antibiotic resistance to tetracycline, it does not include all tetracyclines such as minocycline and doxycycline. *Tet* M however, affords *S. aureus* with resistance to all forms of tetracycline. The *tet* M is found on constant chromosome region in *S. aureus* (Projan, 2000).

There is a disadvantage to using traditional methods such as antibiotics to treat *S. aureus* infections. Some antibiotics are effective, but can only be used in the most extreme cases. Chloramphenical is one such antibiotic that is only used in emergency situations. Chloroamphenical is very effective, because there are limited numbers of clinical isolates that are resistant to the drug. The drug works by binding to the 50S ribosomal subunit and, like tetracycline, blocks the binding of the aminoacly tRNA. The downside of the drug is severe risk of developing aplastic anemia, which is fatal. The mechanism of resistance is through the inactivation of acetyltanseferases encoded by genes found on plasmids pC223 and pUB112 (Projan, 2000).

Another downside to using antibiotics is that some antibiotics can actually cause selection for resistant strains. Macrolids –lincosamides are categorized as streptogramin B and demonstrate this property. They inhibit the peptidyl transferase activity of *S. aureus*. Three related genes are known to encode resistance to this antibiotic, *erm* A, *erm* B, and *erm* C. Resistance to erythromycin, which is a macrolide, can be induced when *S. aureus* is exposed to small amounts of the antiobiotic. Even though the bacteria can develop resistance to erythromycin, it is still susceptible to clindamycin, which is a form of lincosamide. Mutations that occur in the leader sequence upstream of *erm* C cause constitutive resistance. This allows the bacteria the means to form resistance against erythromycineven when exposed to low amounts. Because of this, reducing the use of erythromycins will not necessarily reduce antibiotic resistance (Projan, 2000).

The overwhelming ability of *S. aureus* to develop resistance to antibiotics, along with the side effects caused by the remaining effective antibiotics, leaves researchers with the daunting task of developing new forms of therapeutic treatment in the form of

vaccines or drugs. The slow development of a feasible vaccine relates back to the physiology and serological detection of the *S. aureus* capsule. The capsules of clinical strains were hard to detect at first, because few staphylococci express mucoid capsular polysaccharide that can be detected by direct observation (Fattom and Naso, 1996). Environmental factors also play a role in capsule production. Type 8 capsule seems to be produced more when *S. aureus* is grown on agar instead of in broth (C.Lee and J.Lee, 2000). Further work revealed that capsule can be produced in late logarithmic and stationary growth phase. These capsules were present in *S. aureus* grown in media with low amounts of phosphate. Type 5 and 8 capsule polysaccharides are produced in small amounts compared to capsular polysaccharides of other bacteria that are used for vaccines. However, with the discovery of serologically distinct capsular antigens comes the possibility of new treatments (Fattom and Naso, 1996).

Capsular polysaccharide conjugate vaccines are currently being investigated. An advantage to developing type 8 and type 5 capsule vaccines is that they would be very effective in treating most *S. aureus* infections. When first investigated, clinical isolates, including those from bacteremia, appeared to produce no capsule. However *S. aureus* isolated from several locations in hospitals from different countries have been shown to have type 5 or type 8 capsules. Previous studies indicate that type 5 and 8 capsule can be used to activate an immune response. When conjugated to protein, these capsules have the ability to stimulate high antibody titers in immunized animals. Fattom and coworkers (Fattom and Naso, 1996) bound capsule (CP) type 5 and type 8 to pseudomonas recombinant exoprotein A (rEPA). These types account for the majority of infections caused by *S. aureus*. The vaccines haven been proven to be safe and are effective in

eliciting an antibody response in healthy individuals and some immunocompromised individuals. The antibodies generated from these vaccines evoke type specific phagocytosis and can protect against a lethal challenge of *S. aureus* in animal models. Type 8 CP-rEPA and type 5 CP-rEPA have been tested in healthy humans and are well tolerated. Since *S. aureus* is an opportunistic pathogen, vaccines would not be given to the mass public, but would be used for high risk patients, including renal disease patients on dialysis, HIV infected patients, and high risk surgery patients. These patients are capable of mounting an immune response that will help protect them against *S. aureus* infections (Fattom and Naso, 1996). One problem with this vaccine is that carbohydrate antigens usually do not stimulate a long lasting memory response. This vaccine may require booster shots to maintain high antibody levels. Some high risk patients, such as dialysis patients and AIDS patients, may also have decreased ability to respond to these vaccines.

Several other forms of vaccines for *S. aureus* are being considered or have been considered. One such vaccine is the α toxoid from the *S. aureus* exotoxin. The α toxoid was shown to reduce mastitis, but not to prevent infection. Recently there has been an attempt to use *S. aureus* adhesins (proteins produced by bacteria to adhere to host surfaces) as vaccines, but the effectiveness of this vaccine was dependent on the infection model. Adhesin vaccines appear to show protection when the infection models are endocardits, mastitis, and septic arthritis (Lee, 1998).

Another approach is to use a vaccine that would target peptide autoinducers (Lee, 1998). Autoinducers are short peptides involved bacterial cell-cell communication. They are exported out of the cell and interact with receptors bound on external membranes

causing signal transduction, regulating virulence factor expression. During signal transduction phosphorylation occurs activating a DNA binding protein that controls target proteins (Bassie, 2002). Sar A is a *S. aureus* DNA binding protein specific for P2 and P3 promoter regions of arg. The P2 contains *agr* C, which serves as a membrane associated signal receptor, and *arg* A, which is the response regulator of typical bacterial signal transduction pathway. P3 is the up regulator of transcription for staphylococcal exoproteins and represses the transcription of proteins expressed on the surface *S.aureus* (Lee, 1998). The vaccine would stimulate antibodies to the secreted autoinducers, resulting in decreased signal transduction through these pathways. Inhibition of signal transduction would result in less bacterial virulence.

Genes responsible for teichoic acid biosynthesis have also been proposed as potential targets of *S. aureus* drug therapy. Teichoic acids are components of *S. aureus* bacterial cell walls. Highly charged teichoic acids are involved in the control of cell shape, autolytic enzymes, and the magnesium ion concentration within the cell envelope, making these biosynthetic enzymes prospective targets for an antibacterial compound. The drug could possibly work by inactivating genes responsible for the esterfication of teichoic acids with D-alanine (dlt operon). When dlt is inactivated, it causes the bacteria to be very sensitive to cationic antimicrobial peptides. These antimicrobial peptides are found in the innate human immune system such as defensins, cathelicidins, and thrombocidins, all of which protect the skin and epithelia against microorganisms (Gotz, 2004).

Staph aureus continues to develop new resistances to antibiotics. It has been noted that natural antibiotics are limited in their effectiveness. The limitation in their

ability to combat infections stems from the fact that the antibiotics are produced by bacteria that are immune to actions of the drug they produce and later on pass this resistance on to pathogenic bacteria (Projan, 2000). *S. aureus* causes many health problems and its resilience towards antibiotics requires a new form of treatment.

A possible alternative form of treatment involves preventing synthesis of the *S. aureus* capsule. As indicted earlier, the capsule is a virulence factor that prevents phagocytosis. The goal of these studies was to test the ability of carbohydrate mimetics to block the synthesis of *Staphylococcus aureus* capsule. Capsule synthesis was measured by the ability of monoclonal antibodies to bind to the capsule. The antibodies were produced by the fusion of myeloma cells with B cells from mice that were immunized with *S. aureus*. The hybridomas were cloned to create individual monoclonal antibodies. An ELISA was developed using these monoclonal antibodies that tested the ability of mimetics to block capsule synthesis. Inhibition of capsule synthesis by mimetics was also tested by measuring the ability of white blood cells to phagocytose *S. aureus*. Development of mimetics that inhibit capsule synthesis may lead to new therapeutic treatments for antibiotic resistant *S. aureus* infections.

Materials

Phorbol myristate acetate, Sodium bicarbonate, trypsin EDTA (ethylene diamine tetraacetic aid), polyethylene glycol (PEG), bovine serum (BSA), sodium phosphate dibasic & monbasic sodium chloride (PBS), peroxidase conjugate anti mouse immunoglobin, polyoxyethylene sorbitan monolaurate (tween – 20), tetramethylbenzidine, OPI, and phorbol myristate acetate were purchased from Sigma Chemicals Company (St. Louis, Mo). Minimal Essential Media, L-glutamate, hypoxyanthine aminopterine thymidine (HAT), and RPMI media 1640 were purchased from Gibco BRL Life Technologies (Grand Island, New York). The cell lines MRC-5, P-3X, *S.aureus*, and U937 were purchased from American Type and Culture (Rockville Maryland). Fetal calf serum (FCS) was purchased from Cell culture laboratories (Cleveland, Oh). Columbia Broth and microtitre plastic (tissue culture treated plate) were purchased from Becton Dickinson (Sparks, Md)

Methods

Media for hybridoma preparation

MRC-5

Media for MRC -5 cells were prepared by combining Minimal Essential Media containing Earl's salts and nonessential amino acids (Sigma Chemicals 3050 Spruce street St.Louis Missouri)with 17 mM sodium bicarbonate (tissue culture tested) per liter at pH 7.2-7.4. To the media we added 2mM L-glutamate,1 ml of 1 mM sodium pyruvate along with fetal calf serum (heat inactivated at 55-60 °C for 45 min) was added to the media. (Harlow et al 1988)

P3X and hybridoma

Media for P3X cells was prepared by combining 1640 RPMI media (Sigma Chemicals 3050 Spruce street St.Louis, Missouri) with 24 mM sodium carbonate (tissue culture tested) per liter at pH 7.2-7.4, 2m M l-glutamine, and 20% fetal calf serum heat inactivated at 55-60 °C for 45 min.

All media was sterilized by filtration through a .22 μm filter before use. (Harlow et al 1988)

Passage of MRC-5 cells

Media was removed and cells were washed with serum free media. The cells were treated with trypsin-EDTA (ethylene diamine tetraacetic acid .5g tyrpsin and .2g EDTA) to detach cells from the flask. The amount of trypsin used was determined by the

size of the flask, 2 ml/T25 or 5 ml/T75. Excess trypsin-EDTA was then removed. The cells were allowed to incubate at 37°C for 5-10 minutes. The flask was then tapped to dislodge the cells when they start to round up. Finally the cells were suspended in media plus serum. (Harlow et al 1988)

Fusion of spleen and myeloma cells

Animals must be healthy in order to have viable clones if they are infected with a parvovirus this will not occur. The animals were well rested 3 weeks after their last immunization and they were immunized intravenously 4 days before fusion (Harlow et al 1988). The P3X cells were fed the morning of the fusion and allowed time (a few hours) to become logarithmic growth. Spleen cells were removed from mice and released by pressing the spleen with a syringe plunger. The cells were then washed twice with RPMI plus antibiotics without fetal calf serum (FCS). The spleen cells were collected in 50 ml centrifuge tubes and allowed to clump and settle for 5-10 minutes. Next, the supernatant was removed until 5 ml of supernatant with clumps were left in the tube. The supernatant containing cells was saved. Cells were then washed with three times with RPMI at 37°C with no FCS, to get rid of FCS and centrifuged at 250 x g for 8 min. After washing the cells were counted. Myeloma cells were harvested and washed 3 times (centrifuge at 250 x g for 8 min) in RPMI (medium at 37°C) without FCS. A count was then performed on the myeloma cells and spleen cells and myeloma cells were mixed at a ratio of 5-8 spleen cells: 1 myeloma cell. Cells were pelleted at 250 x g for 8 min at room temperature. Remove supernatant, tap on the tube to loosen the cell pellet. To the pellet add 50 % polyethyleneglycol (PEG). Two to three drops were added to the side of the tube, and

swirl the tube for about 5 seconds to mix the contents. Continue adding the PEG slowly until all the contents have been added over 1 minute. Add 8 ml of RPMI (warm without FCS) over 5 minutes. Next the cells were centrifuged for 5 minutes at 250 x g and the supernatant removed. Complete RPMI (RPMI + 20 FCS =L-glut) 25 ml was then added to the cell pellet. The cells were resuspended by pipetting gently, as cells were fragile at this point. The suspension was then dispersed into a 48 well plate at .5 ml/well. The plate was incubated overnight with a feeder layer (MRC-5), the media in the plate was then aspirated off before the cell mixture was added to the plate. The plate containing hybridomas were then incubated overnight. After overnight incubation .5 ml of 2 X hypoxyanthine, aminopternine, thymidine (HAT) in complete RPMI was added into the wells. Every other day, remove .5 ml (without disturbing the cells) and add .5 ml of RPMI with 1x HAT. The media with 2x HAT was only used for the first feeding before switching to 1x HAT media. After 14 days, add HAT medium (in complete RPMI) at each feeding. Continue to feed the cells three times per week. After two weeks the cells were fed with complete media. Once the cells were confluent they were transferred to bigger wells or culture plates. Hybridoma cells were split no more than a 1:4 dilution and fed every other day. After the cells became 2/3 confluent they were collected and screened for antibodies (Morgan et al 1993).

Staphylococcus aureus **stock prep**

This procedure was used when bacteria were lyophilized. The bacteria were brought up in 1 ml of nutrient broth to dissolve the frozen bacteria. One drop of the mixture was placed on a nutrient agar plate. The plate was then streaked and place in the

incubator at 37°C overnight. The plate was then taped and stored at 4°C for up to one month. The remainder of the bacteria was inoculated into Columbia broth containing 2% NaCl. The bacteria were grown 3 hours in a shaking in incubator at 37^{o}C. Ten ml of bacteria was transferred to a centrifuge tube. The remainder of the bacteria was stored at 4°C up to 1 month. The centrifuge tube was centrifuged at 6000 rpm for 5 min at 4°C. The supernatant was discarded. A glycerol nutrient mixture was created by mixing together 300 µl of glycerol and 1.7 ml of nutrient broth. The mixture was then sterile filtered using a syringe and the pellet was resuspended into the glycerol nutrient mixture. The mixture was then transferred to a cryogenic vial. The cryogenic vial was then placed in a box and taped shut and stored at -70°C. Once frozen, the vial was transferred to liquid nitrogen. (Maniatas et al 1982)

Elisa (trypsin method)

S. aureus was grown overnight in Columbia broth containing 2% NaCl at 37°C. The cells were then centrifuged at 13,000x g at 4°C for 30 min. The cells were washed with PBS (phosphate buffered saline sodium chloride, sodium phosphate dibasisc, and monobasic) 3 times. Cells were then treated with 3% formalin overnight at 4°C. Following formalin treatment the cells were washed with PBS 3 times. The cells were then treated with 1µg/ml Trypsin at 37°C overnight, then washed 3 times and brought up to optical density of 1.0 at 550. The cell suspension (100 ul/well) was added to 96 well microtitre plate (tissue cultured treated) and incubated overnight at 37°C. The plate was then centrifuged at 400 x g for 15 min. The cells were then washed 3 times with PBS containing 0.05% tween-20. After washing, the cells were incubated in the wells of the

plate with 1% BSA in PBS at room temperature for 1 hour. The wells were coated with serial dilutions of mouse sera (serial 2-fold dilutions of 1:1000 stock) and incubated at 37°C for 2.5 hours. The plates were then washed with 0.01 M PBS + 0.05% tween-20 three times. Peroxidase conjugate anti-mouse immunoglobulin (1:1000 dilution) in PBS containing 0.05% Tween-20 + 0.1% BSA at pH 7.2 was added to the plate and incubated at 37°C for 2 hours. The plates were then washed with PBS + 0.05% Tween -20. The substrate tetramethylbenzidine (TMB) was added (100ul) to each well. The reaction was stopped after 20 min by adding 50µl of 2N sulfuric acid. The absorbance was read at 450nm. (Thakker et al 1998)

Columbia salt broth

A 35% Columbia broth solution was created containing 20g NaCl with total volume 300-400 ml of deionized water. The solution was mixed until slightly dissolved. The mixture was then poured into 1000 ml graduated cylinder. The mixture was then divided into 200-300 ml and placed into 1.0 L erlenmeyer flask. Next to ensure sterility of the broth, the flasks were then plugged with cotton, covered with foil and autoclaved at 121°C for 15 minutes. The broth was then allowed to cool before using.

Cloning by Limiting dilution

Cloning of hybridomas were performed through the process of dilutions by adding 50 µl of media and 2X OPI to each well of a 96 well plate. A volume of 50 µl of feeder cells (MRC-5) in the concentration of 1 x 10^5 /ml were added to every well. Next 100 µl of hybridoma cells were added to the top left hand well (A-1). Serial dilutions

(1:2) were preformed down the left hand row (row 1). Serial dilutions were then performed across the plate using an eight well multipipettor. Wells were later examined for clones. If this is the first subclone screen all wells with multiple clones as well as wells with single clones. If this is the second subclone screen only the wells with single clones. The best wells were then selected and cells were grown or subcloned. To ensure purity subcloning was performed twice. (Harlow et al 1988)

U937 Opsonization

U937 Differentiation

U937 cells were counted and pelleted by centrifugation at a speed of 200 X g for 10 min. The cells were then brought up to 10^6/ml in the media containing 100 ng/ml phorbol myristate acetate. Next 250ul of cells (2.5 x 10^5/well) were plated onto a 48 well plate and incubated 48 hrs at 37^{o}C in 5% CO_2. The media along with non adherent cells were then aspirated off and 500ul of RPMI with 10% FCS was added. The plate was incubated for another 24hrs and 450ul MEM with 1% BSA. (Wood et al 2001)

Bacteria preparation

Bacteria were grown on Colombia salt agar or in nutrient broth (+/- mimetics) ON at 37^{o}C. The bacteria were then harvested in PBS and serial two fold dilutions were performed in a microtitre plate. Next the OD of the cells was checked at 550 nm. Cells were then diluted to an OD of 0.5 in PBS.

Opsinization

Done when adding antibody or complement to phagocytosis assay

In a 500 µl reaction mixture 4.5 µl of 0.5 OD *S. aureus* along with hybridoma supernatant (15%/75µl) or antisera (2%/10µl) with or without 2% human AB plasma or guinea pig complement. The solution was then incubated at for 30 min at 37^{o}C with shaking (100 rpm). (Fattom et al 1988)

Phagocytosis

Concentrations of 1x10^{5} were then aliquoted (50µl) to the U937 cells. After mixing, 100ul of supernatant and 100ul of 1:100 dilutions (PBS) were then pipetted onto Columbia Salt Agar plates, spread and incubated at 37^{o}C (0 hr/starting CFU). Next incubate the microtitre plate for one hr at 37^{o}C with rocking. After one hr mix supernatant and 1:100 dilutions and plate and spread on Columbia Agar plates then incubate at 37^{0}C overnight. Colony counts were performed the following day. The data was then calculated as the percentage of the number at 0 hr divided by the number at one hr. (Xu et al 1992)

Serial dilutions colony counts

Bacteria that have been treated under the four conditions mimetics were aseptically transferred (100µl) from broth culture to a dilution tube DF 10^{1} that contains 900µl of PBS and mixed. Next 100µl of the 10^{1} tube was aseptically transferred to a dilution tube DF 10^{2} that contains 900µl of PBS and mixed. Dilutions were carried out in

this manner until a diution of 10^7 was achieved. Aseptically 100µl from the following dilutions 10^4,10^5,10^6 and 10^7 were transferred to a petri plate and spread. After spreading, the plates were placed in the incubator at 37°C and incubated 24-48 hrs. Following incubation the plates with countable colonies (1500 or less) were set aside to have their colonies counted. If more than one plate was used for the same dilution factor then the average was taken of the colony counts. (Pierce et al 1999)

Growth curves

The bacteria (50µl) were grown in side arm flasks under the following four conditions, methanol (MEOH), 1µg/ml, 0.1µg/ml, and 0.01µg/ml. The absorbance readings were taken under 550nm. A side arm flask containing only broth was used as a blank at 0% transmittance on the spectrophotometer. At 0 hr MEOH was read followed by 1ug/ml,0.1ug/ml, and 0.01ug/ml. After readings the flask were placed in the shaking incubator at 200 rpm with a temperature at 37°C for 30 min. After 30 min the absorbance readings were taken. The absorbance readings were taken in 30 min intervals for 5 hours. (Pierce et al 1999).

Results

Previous studies in our lab have shown the ability of the mimetics (MV-II-065) to prevent capsule formation in *S. aureus* bacteria was investigated using an ELISA performed on type 5 bacteria (T5). Type 5 bacteria were tested under four conditions, methanol (MEOH 1µl/ml) and three different concentrations of mimetics (1 µg/ml, 0.1 µg/ml, and 0.01 µg/ml). Methanol served as a control to ensure it had no affect on the T5 bacteria since the mimetics were dissolved in MEOH. In Figure 1 (N=1) the T5 bacteria treated with only MEOH 1µl/ml had higher absorbance readings than did T5 bacteria that had been treated with MV-II-065. Higher absorbance readings can be attributed to more anti-T5 antibody binding to the capsule of *S. aureus* type5. *S. aureus* type5 treated with mimetics had less binding to anticapsular antibody, as suggested by the lower absorbance values.

If MV-II-065 has the ability to prevent capsule formation in T5 bacteria then it should enhance the capabilities of macrophages to phagocytize T5. This was investigated using a phagocytosis assay. Bacteria were grown on agar plates containing MEOH or the different concentrations of mimetics. Growing *S. aureus* on agar plates facilitates production of capsule being produced. As mentioned earlier the capsule of *S. aureus* retards the ability of the body's immune system to phagocytize the bacteria. If mimetics prevents capsule formation, then T5 bacteria treated with mimetics should be phagocytized more than T5 treated only with MEOH.

The phagocytosis assay in Figure 2 (N=3) shows entirely different results than were expected. T5 treated with MEOH 1µl/ml was at 70% of 0 hr and was the lowest

Figure 1. MV-II-065 ELISA (N=1)

Staphylococcus aureus type 5 bacteria were treated with the control MEOH or different concentrations of the MV-II-065, 1µg /ml, 0.1 µg/ml, and 0.01 µg/ml. The bacteria were allowed to incubate in flasks with shaking at 37^{o}C overnight. After incubation, the bacteria were washed with PBS and adjusted to an optical density of 1.0 at 550 nm. The bacteria were then pipetted into wells of a culture plate. Serial dilutions of anti-*S.aureus* mouse sera(serial 2-fold dilutions of 1:1000 stock) were pipette into the wells and incubated at 37°C for 2.5 hours. The plates were then washed with PBS containing 0.05% tween-20 three times. Peroxidase conjate anti-mouse immunoglobulin (1:1000 dilution) was added to the wells and the plate was incubated at 37°C for 2 hours. The plates were then washed with PBS + 0.05% Tween -20 three times. Tetramethylbenzidine substrate solution was added to all wells. The reaction was stopped before 20 min by adding 50µl of 2N sulfuric acid. The absorbance was read at 450 nm.

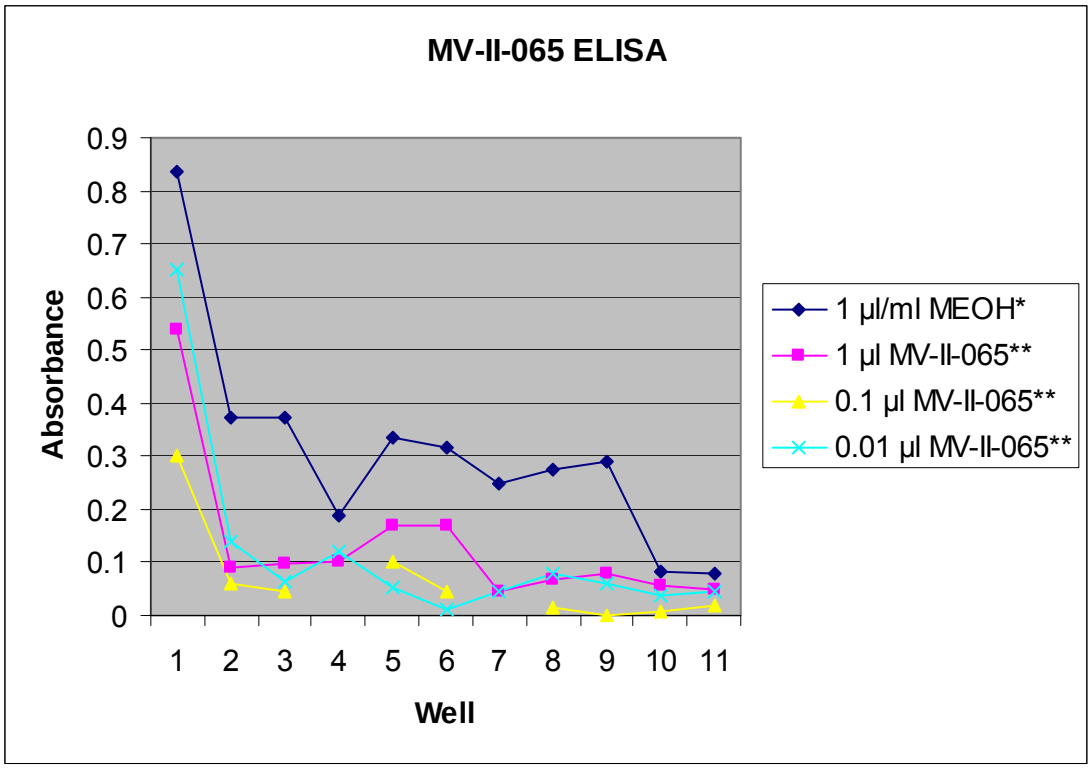
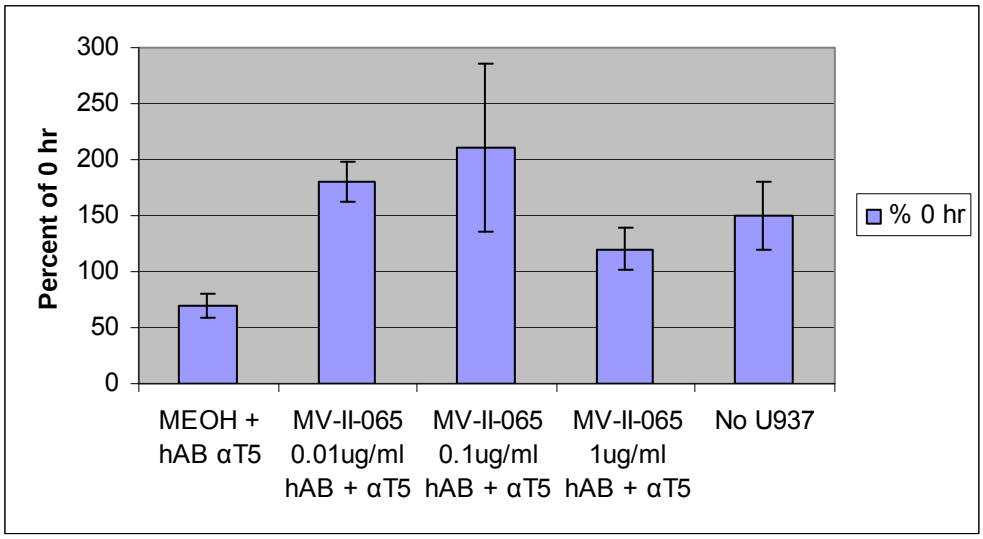

MV-II-065 ELISA
Absorbance
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 2 3 4 5 6 7 8 9 10 11
Well
1 µl/ml MEOH*
1 µl MV-II-065**
0.1 µl MV-II-065**
0.01 µl MV-II-065**

Figure 2. MV-II-065 T5 Phagocytosis assay (with human AB serum) (plus mouse anti-*S.aureus* Type5 sera). (N=3)

U-937 cells were pretreated with 1mM PMA for 48 hrs at 37^{o}C+ 5%CO_2. Four Columbia salt agar plates containing MEOH or one of three concentrations of MV-II-065 (1 μg/ml, 0.1 μg/ml, and 0.01 μg/ml) were inoculated with *S.aureus* type 5. The agar plates were incubated overnight at 37^{o}C. The plates were then washed with PBS and the T5 *S.aureus* were broht up to an optical density of 1.0. Bacteria were opsonized with human complement (human type AB plasma) plus anti-T5 mouse serum by incubation at 37^{o}C for 30 mins. Following opsonization of bacteria a phagocytosis assay was performed. T5 *S.aureus* (100ul) was pipetted into wells containing 1 X 10^5 U-937 cells. Cell culture supernatants containing bacteria were then plated on Columbia salt agar plates at 0hr and 1hr following incubation at 37^{o}C. Wells that did not contain U-937 cells served as a negative control for bacterial growth in the absence of phagocytes. Plates were incubated at 37^{o}C overnight and colony counts were performed. Bacterial growth is expressed as % of zero hour = (number of bacteria at one hour / number of bacteria at zero hour) X 100.


Percent of 0 hr
300
250
200
150
100
50
0
MEOH + hAB αT5
MV-II-065 0.01ug/ml hAB + αT5
MV-II-065 0.1ug/ml hAB + αT5
MV-II-065 1ug/ml hAB + αT5
No U937
% 0 hr

percentage of all the four conditions under which the bacteria were treated. The percentage values for the different concentrations of MV-II-065 were as follows, 1 µg/ml 120%, 0.1 µg/ml 210%, and 0.01 µg/ml 180%. The control which contained no U937 cells was at 150% of 0 hr. Human serum and anti-T5 were used as opsonins in Figure 2. It is possible that the anti-T5 antibodies or the human serum could be responsible for the increase in phagocytosis of the bacteria treated with MEOH. The anti-T5 antisera may have effectively opsonized bacteria with capsule resulting in phagocytosis, while bacteria without capsule were less easily phagocytized. In addition most people are exposed to *S. aureus*, resulting in the formation of naturally occurring antibodies to *S. aureus*. These naturally occurring antibodies could have skewed the results by being able to bind to the MEOH treated bacteria more than the bacteria treated with MV-II-065, allowing accelerated phagocytosis of MEOH treated bacteria.

To test this hypothesis, bacteria were opsonized with serum complement as the only. To compensate for the possible presence of naturally occurring antibodies, the serum was absorbed against *S. aureus* prior to being used in the phagocytosis assay (Figures 3 & 4 N=3). The results displayed in Figure 3 show a slight increase in phagocytosis of MV-II-065 treated bacteria (102% of zero hour) when compared to the MEOH treated control (116% of zero hour). However, in Figure 4 the data is very similar to Figure 2. The MV-II-065 treated bacteria grew better (157% of zero hour) than the MEOH control (81% of zero hour).

In Figure 5 (N=3) adsorbed human serum was used again, however the assay was performed with *S. aureus* type 8 instead of *S. aureus* type 5. The structures of the two types of bacteria are slightly different and may respond differently to opsonization with

Figure 3. MV-II-065 T5 Phagocytosis assay hAB. (N=3)

Bacteria were grown on CSA plates as in Figure 2. Opsonization and phagocytosis assay performed as described in Figure 2. In this assay human type AB plasma serum was absorbed against *S.aureus* type 5 bacteria for 1 hr at 4^{o}C to remove any naturally occurring antibodies to T5 *S.aurues*. Human complement (human type AB plasma) was used in opsonization by incubation at 37^{o}C for 30 mins. Bacterial growth is expressed as % of zero hour = (number of bacteria at one hour / number of bacteria at zero hour) X 100

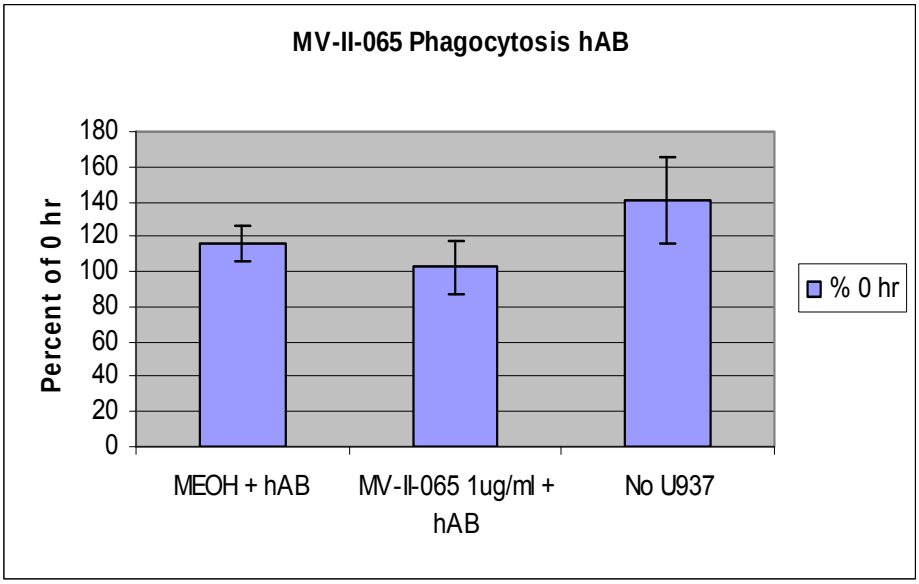

MV-II-065 Phagocytosis hAB
Percent of 0 hr
180
160
140
120
100
80
60
40
20
0
MEOH + hAB
MV-II-065 1ug/ml + hAB
No U937
% 0 hr

Figure 4. MV-II-065 T5 Phagocytosis assay hAB. (N=3)

Bacteria were grown on CSA plates as in Figure 2. Opsonization and phagocytosis assay performed as described in Figure 2. In this assay human type AB plasma serum was absorbed against *S.aureus* type 5 bacteria for 1 hr at 4^{o}C to remove any naturally occurring antibodies to T5 *S.aurues*. Human complement (human type AB plasma) was used in opsonization by incubation at 37^{o}C for 30 mins. Bacterial growth is expressed as % of zero hour = (number of bacteria at one hour / number of bacteria at zero hour) X 100.

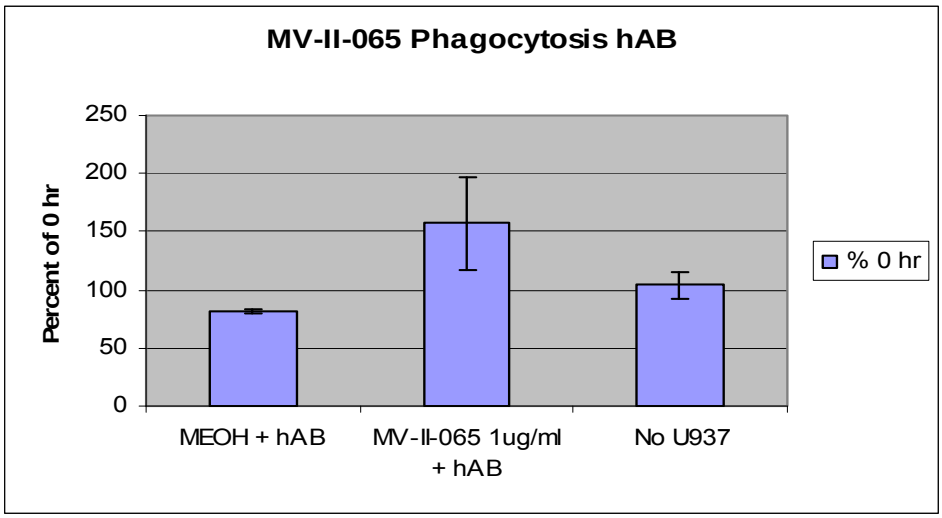
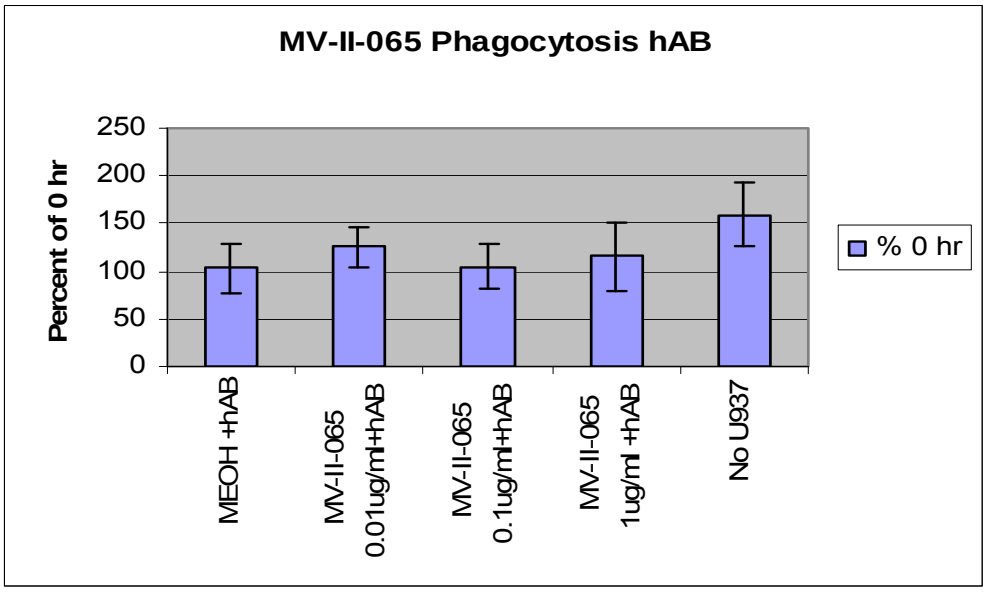

MV-II-065 Phagocytosis hAB
Percent of 0 hr
250
200
150
100
50
0
MEOH + hAB
MV-II-065 1ug/ml + hAB
No U937
% 0 hr

Figure 5. MV-II-065 T8 Phagocytosis assay hAB. (N=3)

Bacteria were grown on CSA plates as in Figure 2. The pretreatment of U937 cells phagocytosis assay was performed as in Figure 2, but with type 8 S. aureus instead of type 5. Human type AB plasma serum was absorbed against *S.aureus* type 5 bacteria for 1 hr at 4^{o}C to remove any naturally occurring antibodies to T5 *S.aurues*. Human complement (human type AB plasma) was used in opsonization by incubation at 37^{o}C for 30 mins. Bacterial growth is expressed as % of zero hour = (number of bacteria at one hour / number of bacteria at zero hour) X 100.


MV-II-065 Phagocytosis hAB
Percent of 0 hr
250
200
150
100
50
0
MEOH +hAB
MV-II-065 0.01ug/ml+hAB
MV-II-065 0.1ug/ml+hAB
MV-II-065 1ug/ml +hAB
No U937
% 0 hr

complement. The results (Figure 5) were very similar to the previous data using *S. aureus* T5. The percent of 0 hr for MEOH and the mimetics were as follows, MEOH 1µl/ml 103%, 1 µg/ml 115%, 0.1 µg/ml 105%, 0.01 µg/ml 125%, and no U937 159%.

The assay in Figure 6 (N=3) was conducted using adsorbed human serum and anti-type 5 mouse serum as opsonins. The results in figure 6 depict a slight difference between the MEOH 1µl/ml treated bacteria and the bacteria treated with MV-II-065 1ug/ml. The percentages of 0 hr were as follows, MEOH 1µl/ml 140% and MV-II-065 1ug/ml 124%.

In Figures 7-9 (N=3) bacteria were grown in broth instead of on Colombia salt agar plates. Colony counts performed after the phagocytosis opsonization assays were lower for *S. aureus* when broth was used. It is uncertain if lower colony counts were due to increased phagocytosis or poor growth of bacteria. In Figures 7-8 anti-T 5 mouse serum was used as an opsonin. The values for 0% hr in Figure 7 were as follows, MEOH 1 µl/ml 0%, MV-II-065 1 µg/ml 855%, MV-II-065 0.1 µg/ml 324%, MV-II-065 0.01 µg/ml 400%, no U937 100%.

Due to low numbers of colonies data is expressed as colony forming units rather than as % of zero hour in Figures 8a& 8b (N=3). The results for figure 8a at 0hr were MEOH 1µl/ml 0.3, MV-II-065 1 µg/ml 260, MV-II-065 0.1 µg/ml 138, MV-II-065 0.01 µg/ml 2, noU937 0.6. For Figure 8b at 1 hr results were MEOH 1µl/ml 0.6, MV-II-065 1 µg/ml 198, MV-II-065 0.1 µg/ml 94, MV-II-065 0.01 µg/ml 0.3, noU937 0.6.

For Figures 9a & 9b (N=3) averages of colony counts were also taken at 0hr and 1hr instead of the 0 percent hour. Human serum was used as the opsonin. The average of colony counts for Figure 9a were as follows MEOH 1µl/ml 0, MV-II-065 1 µg/ml 426,

Figure 6. MV-II-065 T5 Phagocytosis assay hAB+ anti T5 ms. (N=3)

Bacteria were grown on CSA plates as in Figure 2. In this phagocytosis assay serum containing complement and anti T5 was used. Complement was absorbed as previously described in Figure 3. The pretreatment of U937 cells, opsonization, and steps to the phagocytosis assay were carried out as described previously in Figure 2. Bacterial growth is expressed as % of zero hour = (number of bacteria at one hour / number of bacteria at zero hour) X 100.

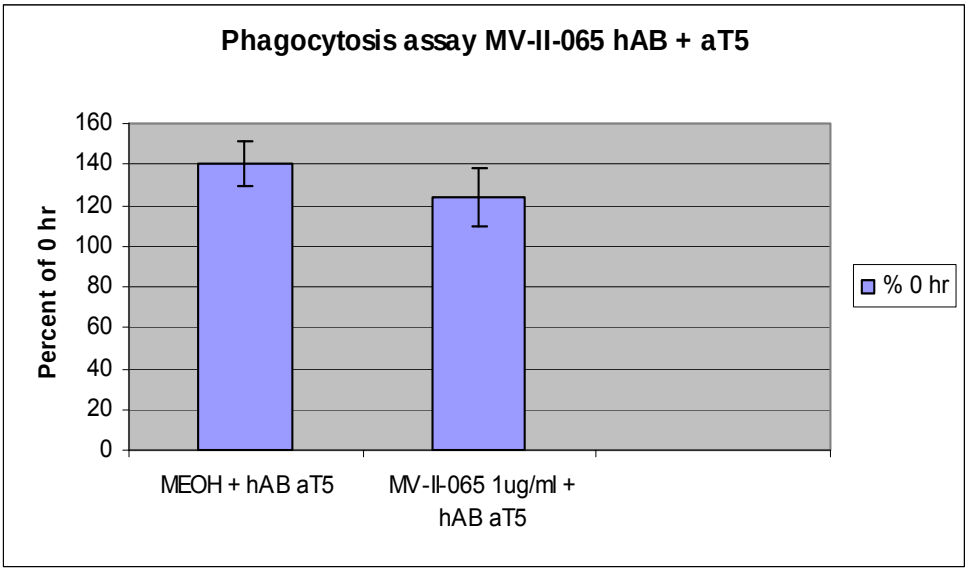

Phagocytosis assay MV-II-065 hAB + aT5
Percent of 0 hr
160
140
120
100
80
60
40
20
0
MEOH + hAB aT5
MV-II-065 1ug/ml + hAB aT5
% 0 hr

Figure 7. MV-II-065 T5 Phagocytosis assay anti T5 ms. (N=3)

In this phagocytosis assay bacteria were grown in mimetics broth with the same concentrations as the CSA plates as in Figure 2. Only anti T5 mouse serum was used as an opsonin source. The pretreatment of U937 cells, opsonization, and steps to the phagocytosis assay were carried out as described in Figure 2. Bacterial growth is expressed as % of zero hour = (number of bacteria at one hour / number of bacteria at zero hour) X 100.

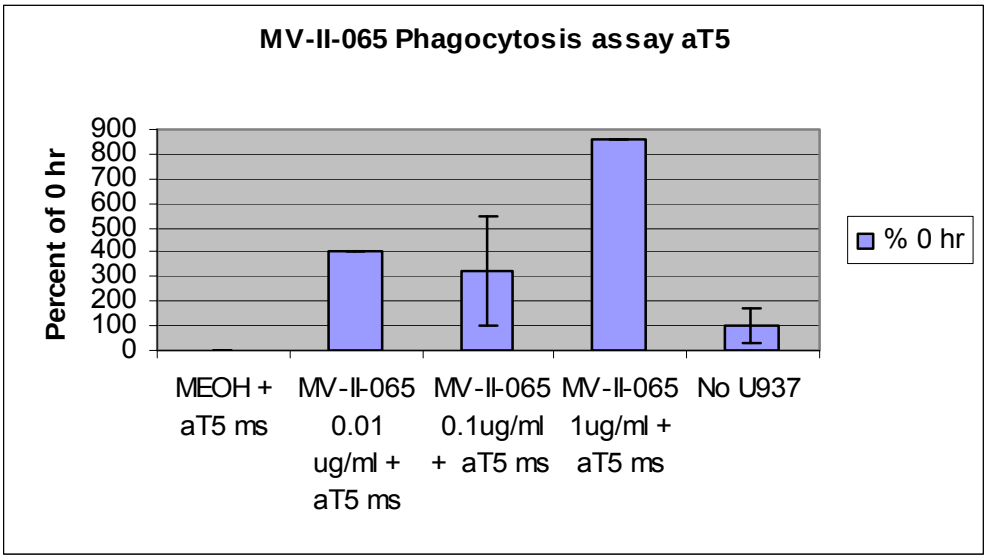
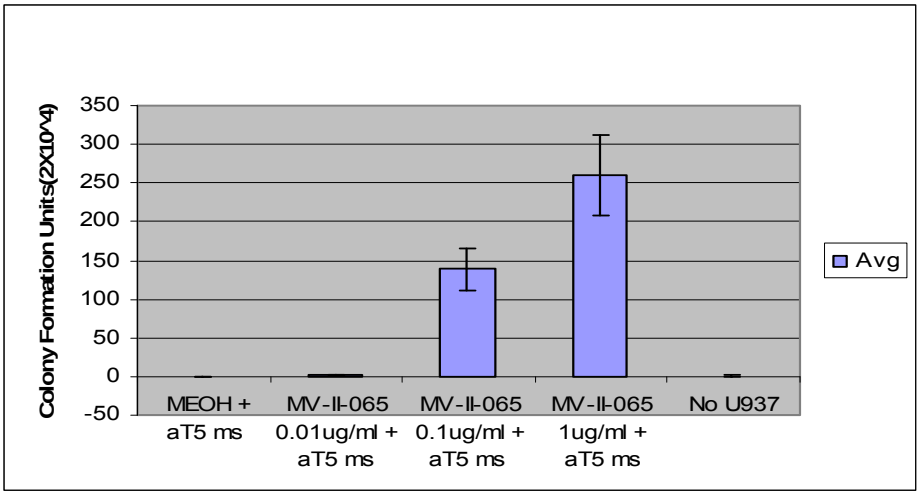

MV-II-065 Phagocytosis assay aT5
Percent of 0 hr
900
800
700
600
500
400
300
200
100
0
MEOH + aT5 ms
MV-II-065 0.01 ug/ml + aT5 ms
MV-II-065 0.1ug/ml + aT5 ms
MV-II-065 1ug/ml + aT5 ms
No U937
% 0 hr

Figure 8a. MV-II-065 T5 Phagocytosis assay anti T5 ms. (N=3)

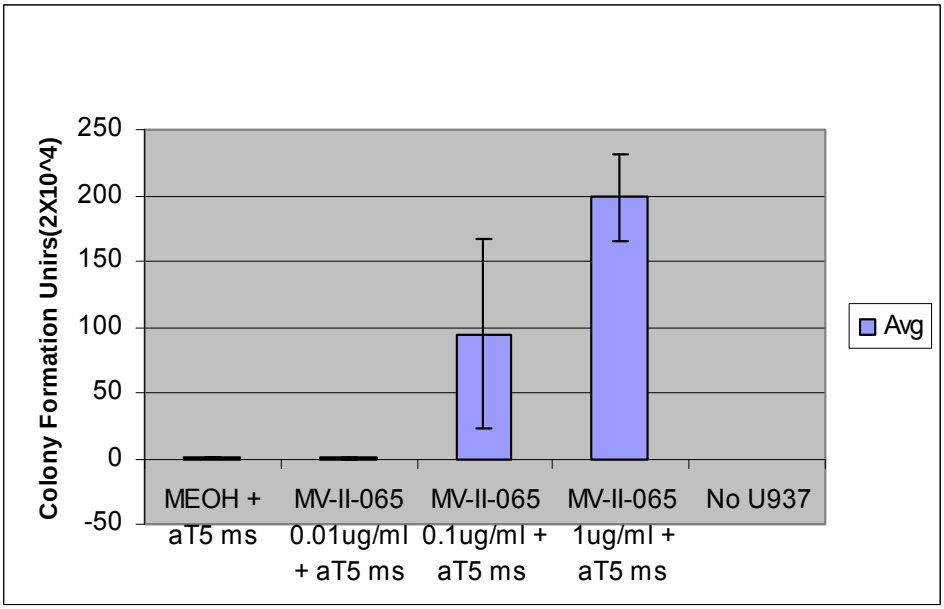
Figure 8a represents the average number of CFU bacteria at zero hour. In this phagocytosis assay bacteria were grown in mimetics broth with the same concentrations as the CSA plates as in Figure 2. Only anti T5 mouse serum was used as an opsonin source. The pretreatment of U937 cells, opsonization, and steps to the phagocytosis assay were carried out as described in Figure 2.

Figure 8b. MV-II-065 T5 Phagocytosis assay anti T5 ms. (N=3)

Figure 8b represents the average number of CFU bacteria at one hour. In this phagocytosis assay bacteria were grown in mimetics broth with the same concentrations as the CSA plates as in Figure 2. Only anti T5 mouse serum was used as an opsonin source. The pretreatment of U937 cells, opsonization, and steps to the phagocytosis assay were carried out as described in Figure 2.


Colony Formation Units(2X10^4)
350
300
250
200
150
100
50
0
-50
MEOH + aT5 ms
MV-II-065 0.01ug/ml + aT5 ms
MV-II-065 0.1ug/ml + aT5 ms
MV-II-065 1ug/ml + aT5 ms
No U937
Avg

8a.


Colony Formation Unirs(2X10^4)
250
200
150
100
50
0
-50
MEOH + aT5 ms
MV-II-065 0.01ug/ml + aT5 ms
MV-II-065 0.1ug/ml + aT5 ms
MV-II-065 1ug/ml + aT5 ms
No U937
Avg

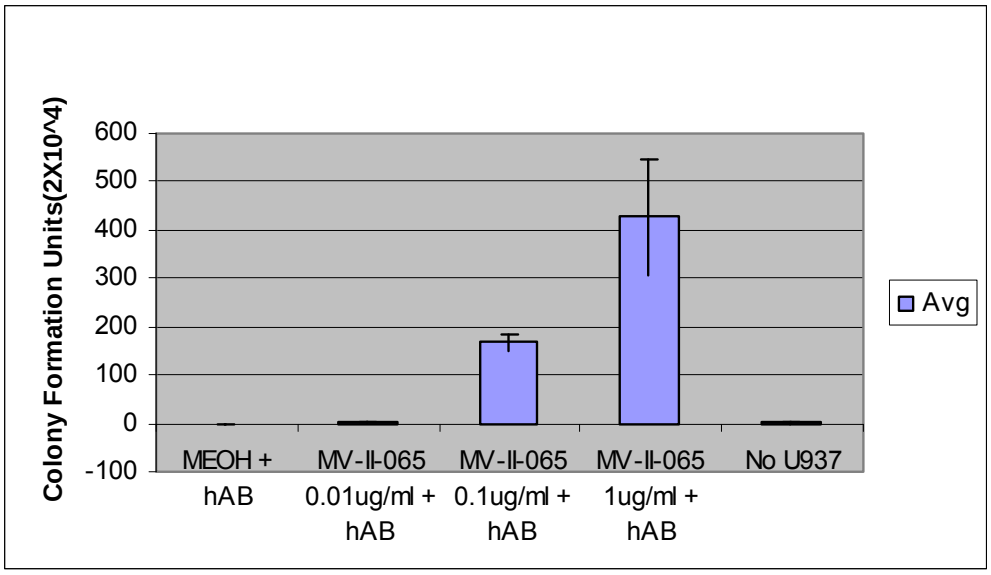
8b.

Figure 9a. MV-II-065 T5 Phagocytosis assay hAB. (N=3)

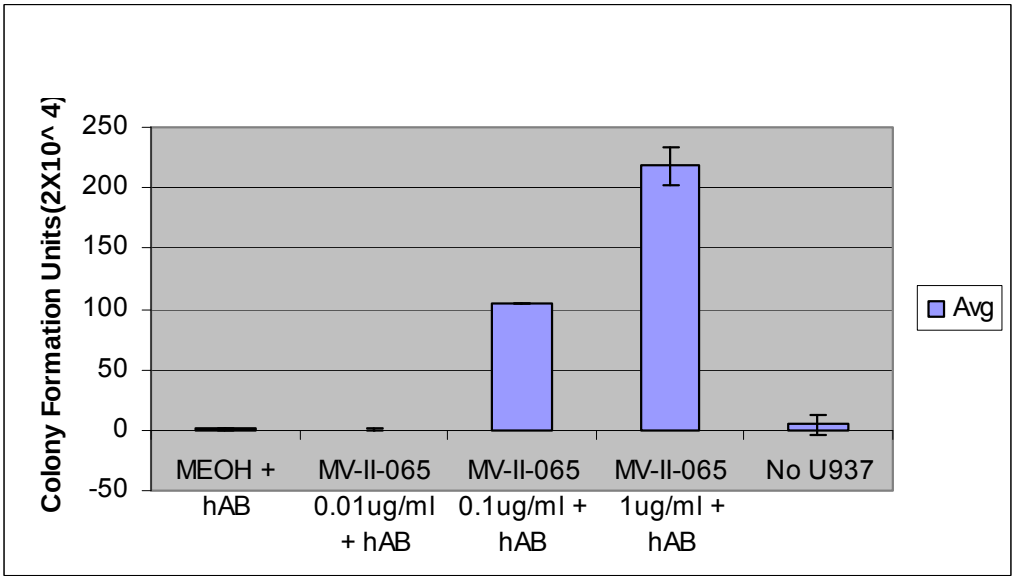
Figure 9a represents the average number of CFU bacteria at zero hour. In this phagocytosis assay bacteria were grown in mimetics broth with the same concentrations as the CSA plates in Figure . Only human complement (human AB plasma) was used as an opsonin source and absorbed as previously described in Figure 3. The pretreatment of U937 cells, opsonization, and steps to the phagocytosis assay were carried out as described in figure 2.

Figure 9b. MV-II-065 T5 Phagocytosis assay hAB. (N=3)

Figure 9b represents the average number of CFU bacteria at one hour. In this phagocytosis assay bacteria were grown in mimetics broth with the same concentrations as the CSA plates in figure 3. Only human complement (human AB plasma) was used as an opsonin source and absorbed as previously described in Figure 3. The pretreatment of U937 cells, opsonization, and steps to the phagocytosis assay were carried out as described in Figure 2.


Colony Formation Units(2X10^4)
600
500
400
300
200
100
0
-100
MEOH + hAB
MV-II-065 0.01ug/ml + hAB
MV-II-065 0.1ug/ml + hAB
MV-II-065 1ug/ml + hAB
No U937
Avg

9a.


Colony Formation Units(2X10^ 4)
250
200
150
100
50
0
-50
MEOH + hAB
MV-II-065 0.01ug/ml + hAB
MV-II-065 0.1ug/ml + hAB
MV-II-065 1ug/ml + hAB
No U937
Avg

9b.

MV-II-065 0.1 µg/ml 167, MV-II-065 0.01 µg/ml 1.6, noU937 0.6. For figure 9b the results were MEOH 1µl/ml 0.6, MV-II-065 1 µg/ml 218, MV-II-065 0.1 µg/ml 105, MV-II-065 0.01 µg/ml 0.5, noU937 4.3.

Figure 10a (N=3) compares the averages of colony counts of *S. aureus* in the presence of U937 cells at 0hr as opposed to those that were not. These bacteria were also grown on Colombia salt agar plates under the previous four condition of MEOH and mimetics. The results for Figure 10a were as follows for cells in the presence of U937, MEOH 1µl/ml 1142, MV-II-065 1 µg/ml 1153, and MV-II-065 0.1 µg/ml 984. For bacteria that were not in the presence of U937 the results were MEOH 1µl/ml 1442, MV-II-065 1 µg/ml 1425, MV-II-065 0.1 µg/ml 1272. Figure 10b (N=3) displays the comparison of the averages of colony counts of *S. aureus* in the presence of U937 cells at 1 hour opposed to those that were not. The results for Figure 10b were as follows for cells in the presence of U937, MEOH 1µl/ml 1046, MV-II-065 1 µg/ml 704, and MV-II-065 0.1 µg/ml 805. For bacteria that were not in the presence of U937 the results were MEOH 1µl/ml 1317, MV-II-065 1 µg/ml 1129, MV-II-065 0.1 µg/ml 1081.

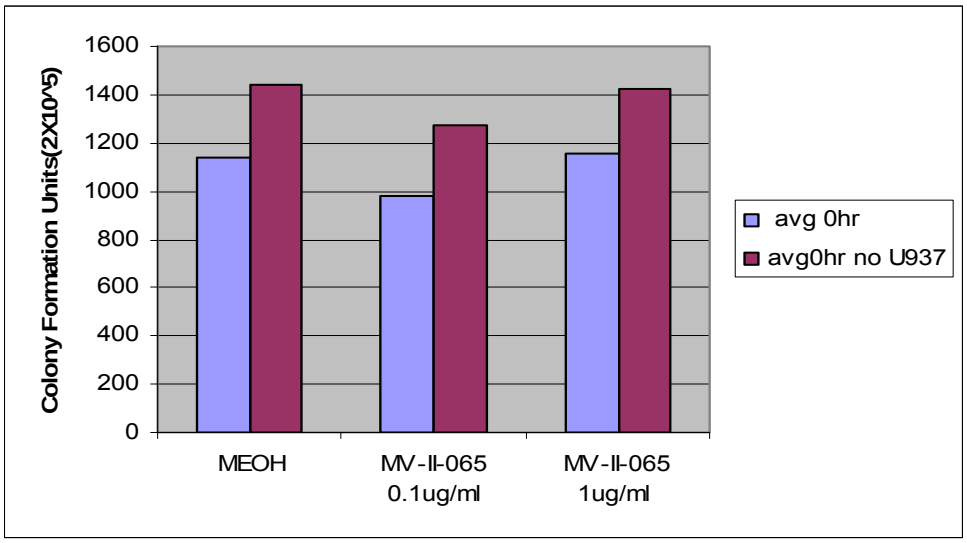
In Figure 11 (N=1) a growth curve assay was performed to investigate if growth rates were a contributing factor in the varying colony counts of phagocytosis assays of *S. aureus*, type 5 treated with MEOH and different concentrations of mimetics. Absorbance readings for the experiment were taken in increments of 30 minutes. The growth curve assay revealed the bacteria were growing at the same rate. The absorbance values for the *S. aureus* treated under the four conditions (MEOH 1µl/ml, MV-II-065 1 µg/ml, MV-II-065 0.1 µg/ml, MV-II-065 0.01 µg/ml) were identical as displayed by the superimposed

Figure 10a. MV-II-065 T5 Phagocytosis assay U937 vs no U937 0hr. (N=3)

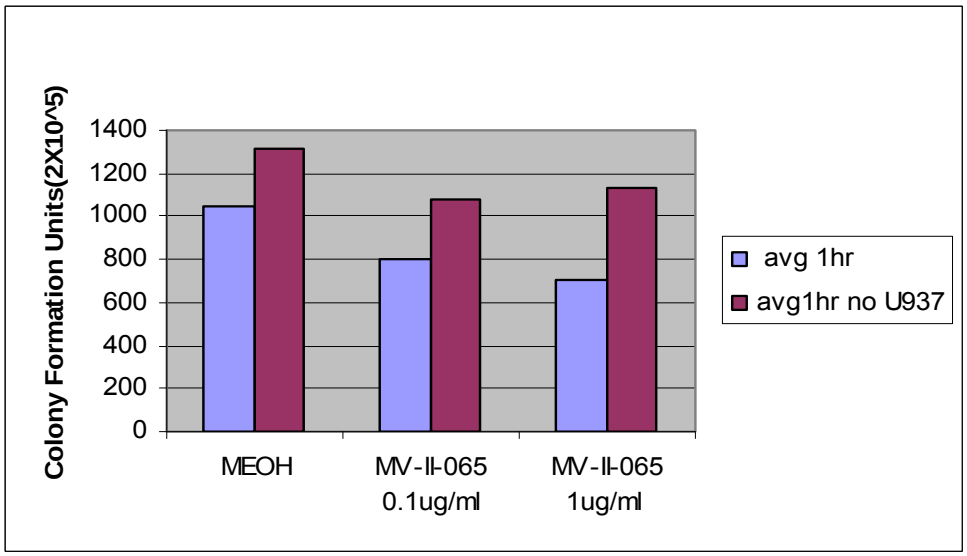
Figure 10a represents the average number of CFU bacteria at zero hour. In this phagocytosis assay three CSA agar plates were inoculated with *S.aurues* type 5 containing MEOH and two concentrations of MV-II-065 (1 μg/ml and 0.1μg/ml). MEOH served as control to ensure it had no affect on the T5 since the mimetics were dissolved in MEOH. The pretreatment of U937 cells and steps to the phagocytosis assay were carried out as described in Figure 2.

Figure 10b. MV-II-065 T5 Phagocytosis assay U937 vs no U937 1hr. (N=3)

Figure 10b represents the average number of CFU bacteria at one hour. In this phagocytosis assay three CSA agar plates were inoculated with *S.aurues* type 5 containing MEOH and two concentrations of MV-II-065 (1 μg/ml, and 0.1μg/ml). MEOH served as control to ensure it had no affect on the T5 since the mimetics were dissolved in MEOH. The pretreatment of U937 cells and steps to the phagocytosis assay were carried out as described in Figure 2.


Colony Formation Units(2X10^5)
1600
1400
1200
1000
800
600
400
200
0
MEOH
MV-II-065 0.1ug/ml
MV-II-065 1ug/ml
avg 0hr
avg0hr no U937

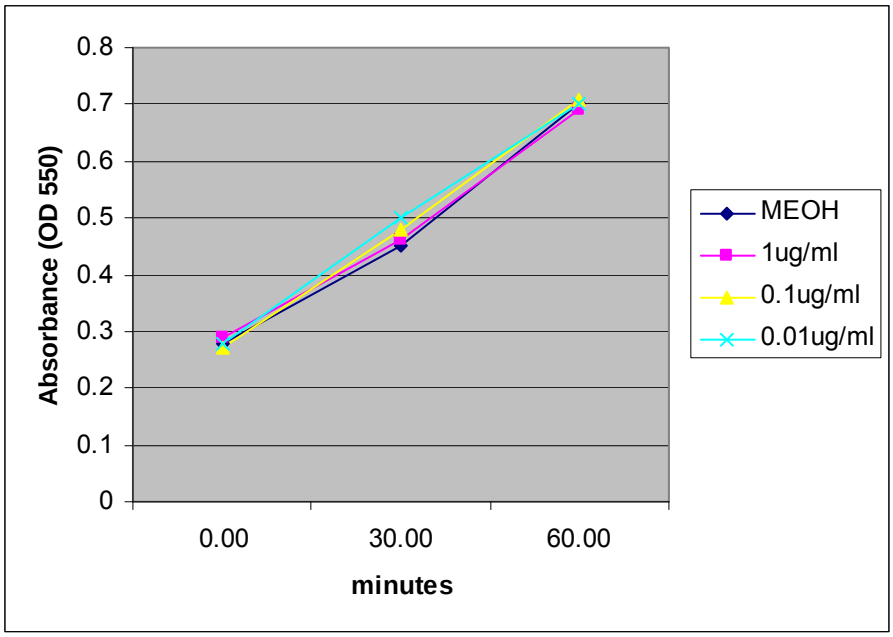
10a.


Colony Formation Units(2X10^5)
1400
1200
1000
800
600
400
200
0
MEOH
MV-II-065 0.1ug/ml
MV-II-065 1ug/ml
avg 1hr
avg1hr no U937

10b.

Figure 11. Growth curves for bacteria incubated with MV-II-065 (N=1)

S.aureus type5 bacteria were incubated at 37 ^{o}C with shaking with MEOH or three different concentrations of MV-II-065 (MEOH, 1 μg/ml, 0.1 μg/ml, and 0.01 μg/ml) in side arm flask. Absorbance readings at 550 nanometers were taken every 30 minutes. A flask containing only Columbia salt broth was used as a blank.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance (OD 550)
0.00
30.00
60.00
minutes
MEOH
1ug/ml
0.1ug/ml
0.01ug/ml

curves plotted according from the data of each condition. In Figure 11 the absorbance were as follows according to the time, 2pm 0.3, 2:30 pm 0.45, 3pm 0.7.

The bacterial counts at zero hour in Figures 8 & 9 suggest differences in absorbance may result in the addition of higher numbers of bacteria in MV-II-065 treated samples. Therefore, colony counts of serial dilutions were executed to examine if the decrease in capsule caused by mimetics leads to differences in absorbance of the bacteria. In Figures 12-15 (N=1) *S. aureus* type5 were treated under the four conditions (solvent control MEOH 1µl/ml, MV-II-065 1 µg/ml, MV-II-065 0.1 µg/ml, and MV-II-065 0.01 µg/ml). After treatment serial ten fold dilutions 10^3-10^7 were performed on the cultures.

In Figure 12 bacteria treated with the solvent control contained the following colony forming units per milliliter 2.5 X 10^7. In this assay, the addition of MV-II-065 to the cultures appeared to influence the colony counts found. When all samples were diluted to the same absorbance, samples treated with MV-II-065 1ug/ml and 0.01ug/ml had higher colony counts. The colony count for MV-II-065 1 µg/ml was 2.9 X 10^7 cfu/ml and for MV-II-065 0.01 µg/ml was 3.4 X 10^7 CFU/ML. Bacteria treated with MV-II-065 0.1 µg/ml contained 1.3X10^7 cfu/ml.

The experiment in Figure 13 depicts *S. aureus* diluted to the same absorbance treated with MV-II-065. All samples treated with mimetics had higher bacterial counts than the solvent control. The concentration of 0.01 µg/ml had the greatest affect on the colony counts; colony forming units per milliliter was a value of 2.5 X 10^8 cfu/ml. Samples treated at 1 µg/ml and 0.1ug/ml appeared to be close to the value for bacteria

Figure 12. colony count serial dilutions. (N=1)

T5 bacteria were treated with the control MEOH or different concentrations of mimetics, 1 μg/ml, 0.1 μg/ml, and 0.01 μg/ml. The bacteria were allowed to incubate at 37^{o}C overnight. After incubation bacteria were adjusted to an optical density of 1.0 at 550 nm. Serial dilutions were performed on the bacterial suspensions and cell numbers were quantified by spread plate colony counts.

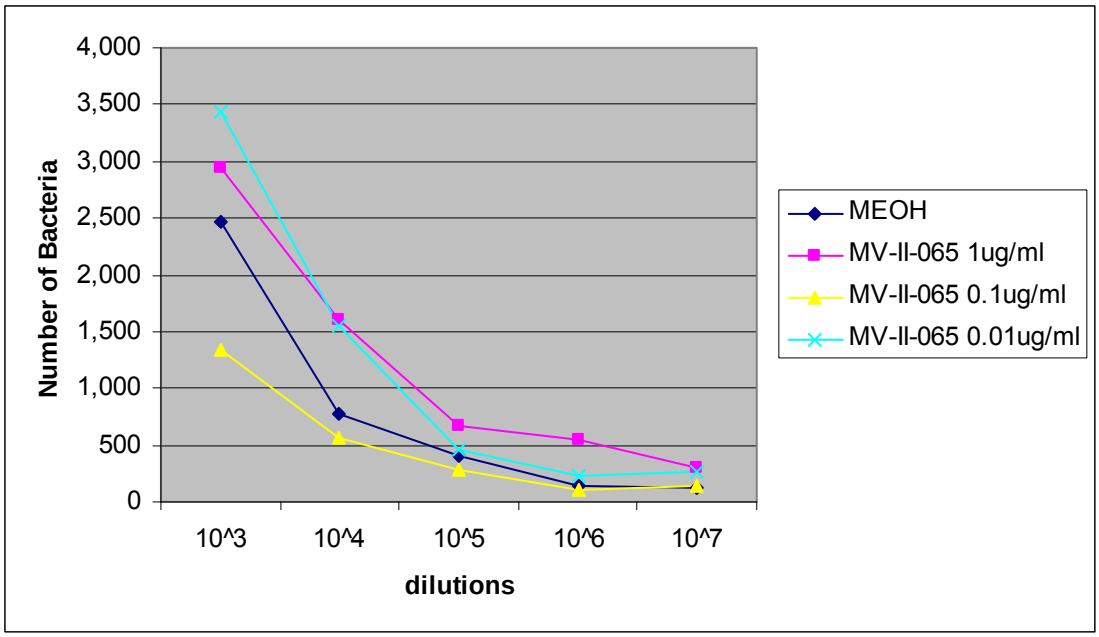
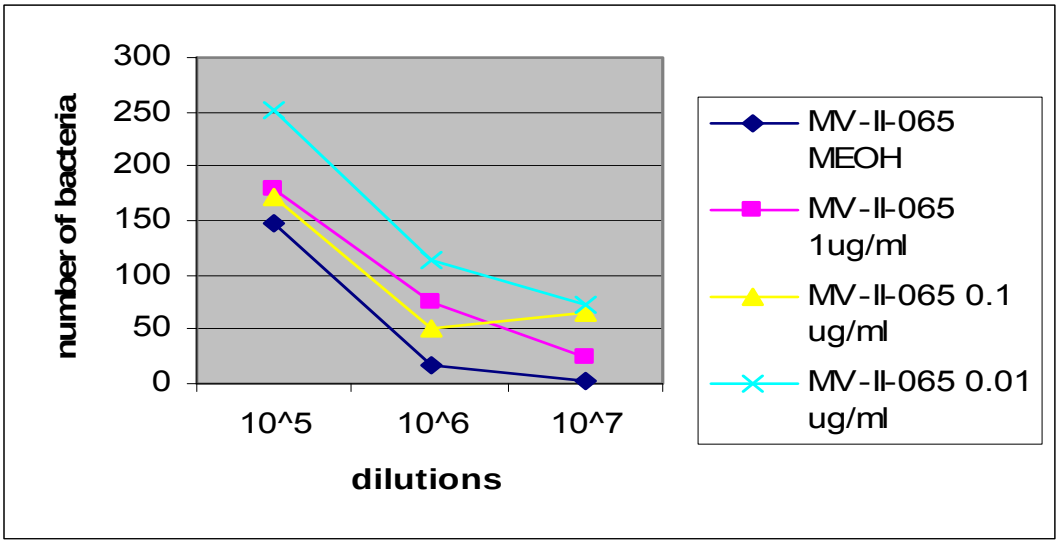

4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
Number of Bacteria
10^3
10^4
10^5
10^6
10^7
dilutions
MEOH
MV-II-065 1ug/ml
MV-II-065 0.1ug/ml
MV-II-065 0.01ug/ml

Figure 13. colony count serial dilutions. (N=1)

T5 bacteria were treated with the control MEOH or different concentrations of mimetics, 1 μg/ml, 0.1 μg/ml, and 0.01 μg/ml. The bacteria were allowed to incubate at 37^{o}C overnight. After incubation bacteria were adjusted to an optical density of 1.0 at 550 nm. Serial dilutions were performed on the bacterial suspensions and cell numbers were quantified by spread plate colony counts.


300
250
200
150
100
50
0
number of bacteria
10^5
10^6
10^7
dilutions
MV-II-065 MEOH
MV-II-065 1ug/ml
MV-II-065 0.1 ug/ml
MV-II-065 0.01 ug/ml

treated with the control solvent. The values were as follows for these conditions, solvent control ($1.5X10^8$ cfu/ml), 1 μg/ml ($1.8X10^8$ cfu/ml), and 0.1 μg/ml ($1.7X10^8$ cfu/ml).

All three MV-II-065 treated samples for the assay in Figure 14 had higher concentrations than solvent control. Colony counts on the samples treated with mimetics were as follows, 1 μg/ml ($5.3X10^9$cfu/ml), 0.1 μg/ml ($1.5X10^9$ cfu/ml), and 0.01 μg/ml ($1.4X10^9$ cfu/ml). The sample treated with the solvent control had a value of $2.8X10^8$ cfu/ml.

The assay in Figure 15 displays results of colony counts that resemble the previous Figures 12-14 when dealing with cultures treated with MV-II-065. Although the samples were diluted to the same absorbance, *S. aureus* treated with MV-II-065 has higher numbers of colony forming units than do those treated with the control solvent. The colony counts for MV-II-065 1 μg/ml ($1.3X10^9$cfu/ml) and MV-II-065 0.01 μg/ml ($1.4X10^9$ cfu/ml) were closer together than MV-II-065 0.1 μg/ml ($9.9\ X10^8$cfu/ml), but all three had a far greater amount of colony forming units than did MEOH 1μl/ml ($4.8X10^8$cfu/ml)

The data from the 10^6 dilution in Figures 12-15 are shown in Table 1. The data from table four were averaged. The values for the three concentrations were as follows, MV-II-065 0.01 μg/ml (587.5), MV-II-065 0.1 μg/ml (421), and MV-II-065 1 μg/ml (1034.5). The fold increases for the three concentrations were, MV-II-065 0.01 μg/ml(4.4), MV-II-065 0.1 μg/ml (3.1), and MV-II-065 1 μg/ml (7.7).

Figure 14. colony count serial dilutions. (N=1)

T5 bacteria were treated with the control MEOH or different concentrations of mimetics, 1 μg/ml, 0.1 μg/ml, and 0.01 μg/ml. The bacteria were allowed to incubate at 37^{o}C overnight. After incubation bacteria were adjusted to an optical density of 1.0 at 550 nm. Serial dilutions were performed on the bacterial suspensions and cell numbers were quantified by spread plate colony counts.

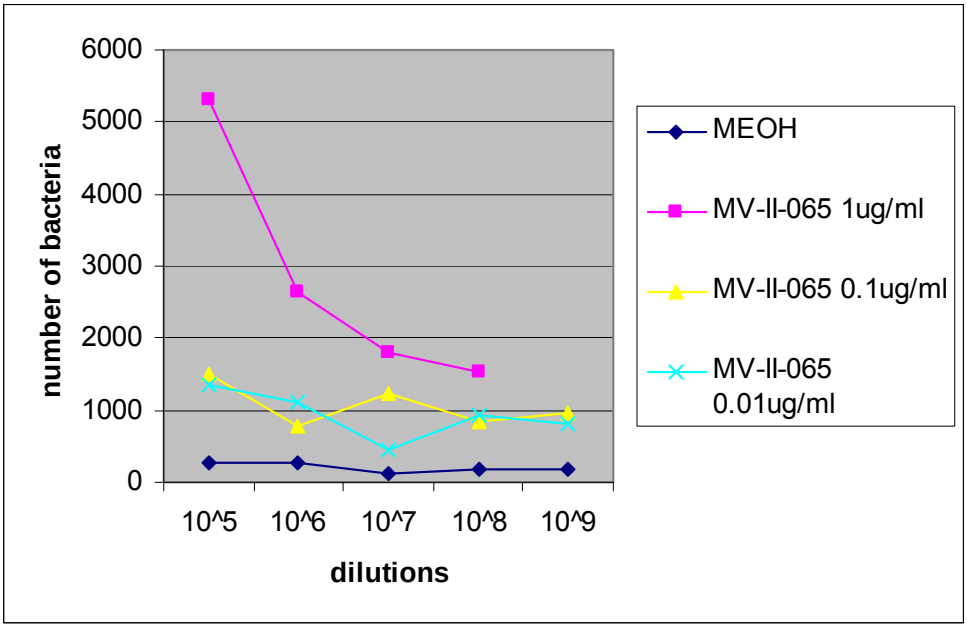

6000
5000
4000
3000
2000
1000
0
number of bacteria
10^5
10^6
10^7
10^8
10^9
dilutions
MEOH
MV-II-065 1ug/ml
MV-II-065 0.1ug/ml
MV-II-065 0.01ug/ml

Figure 15. colony count serial dilutions. (N=1)

T5 bacteria were treated with the control MEOH or different concentrations of mimetics, 1 μg/ml, 0.1 μg/ml, and 0.01 μg/ml. The bacteria were allowed to incubate at 37^{o}C overnight. After incubation bacteria were adjusted to an optical density of 1.0 at 550 nm. Serial dilutions were performed on the bacterial suspensions and cell numbers were quantified by spread plate colony counts.

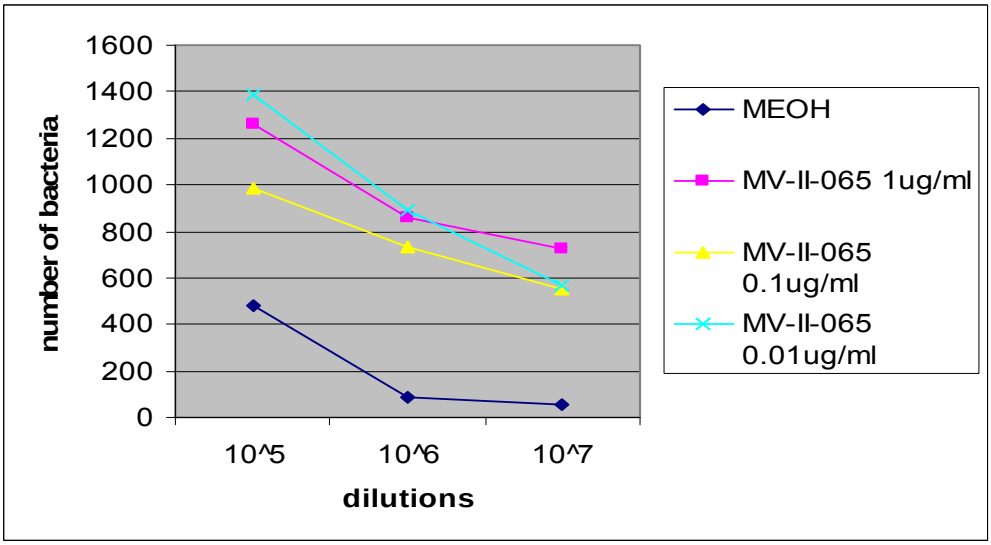

1600
1400
1200
1000
800
600
400
200
0
number of bacteria
10^5
10^6
10^7
dilutions
MEOH
MV-II-065 1ug/ml
MV-II-065 0.1ug/ml
MV-II-065 0.01ug/ml

Table 1 Averages of Serial Dilutions

Dillutions 10^6 exp dates	7.06.07	7.23.07	8.29.07	9.12.07	[a]CFU X 10^6	fold increase
MEOH	147	284	17	86	133.5	
MV-II-065 0.01	231	1112	113	894	587.5	4.400749064
MV-II-065 0.1/ml	112	788	52	732	421	3.153558052
MV-II-065 1/ml	548	2653	76	861	1034.5	7.74906367

[a]The averages of colony counts at 10^6 dilutions were calculated from four experiments in which bacteria were treated under the four conditions (MEOH , 0.01 μg/ml, 0.1 μg/ml, and 1 μg/ml). A fold increase of the MEOH percentage was also calculated of the bacteria treated with different concentrations of MV-II-065. Fold increases = (average experimental colony count/average MEOH colony count)

Discussion

S. aureus is rapidly becoming antibiotic resistant. There is an increasing problem in the hospital and community with bacteria having capsular types 5 (T5) and type 8 (T8) being responsible for 85% of infections. In this study we tested carbohydrate (CHO) mimetics for their ability to improve the immune defense against *S. aureus* infections. We propose that CHO mimetics resembling carbohydrates similar to those found in *S. aureus* capsules may be used to inhibit capsule production. Compounds that decrease capsule production could be used to treat infections, as a decrease in capsule synthesis should increase phagocytosis of the bacteria by white blood cells

The effect on capsule production was investigated in bacteria incubated with the newly synthesized compound MV-II-065. This compound, an n-glucosyl 1,2,3-triazole, was incubated with bacteria prior to Elisa analysis. This laboratory previously, and as part of this thesis, developed monoclonal antibodies against *S. aureus* Type 5 that were used to measure capsule presence in the Elisa Assay. Decreased binding of anti-capsule antibody should indicate decreased capsule production or a change in the antigenic binding site on the surface of the bacteria.

To investigate the ability of the mimetic (MV-II-065) to prevent capsule formation, *S. aureus* type 5 (T5) bacteria were treated under four conditions (MEOH, 1ug/ml, 0.1ug/ml, and 0.01ug/ml) fixed with formalin, trypsin treated to remove protein A, then used as the antigen in an ELISA. In Figure 1 the ELISA shows that there was a decrease in the amount of capsule being produced by the T5 bacteria treated with mimetic MV-II-065. The T5 bacteria treated with only MEOH have higher absorbance readings than do T5 bacteria treated with MV-II-065. Higher absorbance readings can be

attributed to more anti-T5 antibody binding to the capsule of *S. aureus* type 5. This data suggest that MV-II-065 was effective at inhibiting capsule synthesis.

In our study the method employed was the ELISA trypsin method, which has also been used by Thakker and coworkers (Thakker, *et al.,*. 1998). This method allows for the determination of high capsule or low capsule production depending on the absorbance reading in the assay. A drawback to the ELISA trypsin method is that it is only semiqauntitative and lacks the ability to determine how much capsule is being produced. Thakker and coworkers utilized an ELISA inhibition method in which antiserum was first adsorbed against the bacteria being tested. After adsorption of the serum, the antiserum was added to an ELISA which was used to calculate the amount of antibody left in the serum. These results can be compared to absorbance readings of known capsule amounts and used to determine how much capsule is produced. The advantage to this method is that it is quantitative and allows for determination of the actual amount of capsule produced. The disadvantage is that this method requires significant amounts of purified capsule to produce the standard curve. Future studies in our lab may utilize this method as our laboratory produces more purified capsule.

S. aureus poses a threat to hospitalized patients with weakened immune systems and people with normal immune sytems (Nilsson *et al*., 1997). The capsule of *S. aureus* inhibits the ability of the body's immune system to phagocytize the bacteria. This is accomplished by the capsule's ability to shield the C3b complement deposited on the cell wall from C3b receptors on phagocytes (Luong *et al*., 2002). The ability of *S. aureus* capsule to prevent phagocytosis has been demonstrated in a study performed by Luong and coworkers (Luong *et al*., 2002). In the study, *S. aureus* type 8 was genetically

altered to produce more capsule than the normal type 8 bacteria. Bacteria with high levels of capsule were more resistant to phagocytosis. Their results indicate that capsule does play a major role in white blood cell's ability to phagocytize *S. aureus*. Macrophages are able to ingest and kill non-encapsulated *S. aureus* better than capsulated *S. aureus* (Thakker *et al*., 1998). The decrease in capsule production we may be seeing in the mimetic treated bacteria should enhance the capabilities of macrophages to phagocytize the bacteria and greatly benefit patients with *S. aureus* infections.

Growing *S. aureus* on agar plates facilitates the production of capsule. Thakker and coworkers demonstrated that Reynolds strain (*S. aureus*, type 5) grown on Columbia salt agar (CSA) produces 100-fold more capsule than cells grown in Columbia salt broth (Thakker *et al*., 1998). To test the ability of mimetics to aid in the phagocytosis of *S.aurues*, we developed a phagocytosis assay in which the bacteria were grown on agar plates containing MEOH or the different concentrations of mimetics. The method involved incubating *S. aureus* and U937 cells (a human macrophage cell line) together and then performing colony counts on the remaining bacteria. Prior to incubation of S. aureus with U937 cells, the bacteria were opsonized by incubating them with human complement (human AB serum), *S. aureus* mouse antiserum, or both. U937 cells can be induced into a state of differentiation into human macrophage like cells by subjecting them to the chemical phorbol 12-myristate 13-acetate (PMA). After differentiation into macrophages, PMA was removed and the cells were incubated in culture media for 48 hours prior to the phagocytosis assay (Verhoeckx *et al*., 2004). Following incubation of treated or untreated bacteria with the phagocytes, aliquots of the bacteria were plated and

colony counts performed. Bacterial counts were expressed as percentages of zero hour, calculated from colony counts of 1 hour plates divided by colony counts of 0 hour plates.

To determine if human AB plasma (complement) and anti-T5 mouse serum aids in phagocytosis of *S. aureus* type 5 by U937 cells we added them as opsonins in the phagocytosis assay shown in Figure 2. Opsonins bind to bacterium and to white blood cell receptors, facilitating phagocytosis. The anti-T5mouse serum was obtained from mice immunized with killed *S. aureus* type 5 and should contain antibodies to *S. aureus*, type 5. Using complement in combination with T5 antisera was expected to enhance the phagocytic ability of the U937 cells. However, the phagocytosis assay in Figure 2 displayed entirely different results than expected. T5 treated with MEOH appeared to have been phagocytized the most. It was thought that the use of human serum could be responsible for the high levels of phagocytosis seen in the assay of the bacteria treated with MEOH. Everyone is exposed to *S. aureus*, which causes naturally occurring antibodies to *S. aureus* to be formed. These naturally occurring antibodies could have skewed the results by being able to bind to the encapsulated, MEOH-treated bacteria more than the less capsulated bacteria treated with MV-II-065. Evidence of naturally occurring Abs has been supported by work in the labs of Xu and coworkers (Xu *et al*., 1992). Xu and coworkers have demonstrated phagocytosis of *S. aureus* type 5 even when normal nonimmune human serum is heat inactivated to destroy complement, indicating the presence of Abs to *S. aureus*.

Since prior exposure to *S. aureus* was not accounted for in Figure 2, the human serum was absorbed against *S. aureus* before being used in the phagocytosis assays in Figures 3 and 4. The conditions for the phagocytosis assays were the same in both

experiments. Both assays were performed against T5 with adsorbed human AB plasma as the opsonin. The results of both experiments resemble Figure 2 in having the highest bacrterial killing in the control and the lowest bacterial killing in bacteria treated with chemical. Once again, even in the absence of anti-T5 antibody, our hypothesis that the chemical would increase phagocytosis was not supported. In Figure 6 the opsonophagocytic assay was performed against type 5 *S. aureus* using anti-type5 mouse antiserum and adsorbed human AB plasma (complement source) as opsonins. The results of Figure 6 show a slight decrease of percent 0 hr of *S. aureus* treated with mimetics compared to S. aureus treated with MEOH, however the values were insignificant. Previous studies in other labs have shown that Ab plus complement in combination yielded increased phagocytosis (Thakker *et al*., 1998). The results of an opsonophagocytic assay performed by Thakker and coworkers, demonstrate that when antisera to the capsule in combination with complement is used there is an increase in phagocytic activity compared to using complement or antisera alone. Thakker and coworkers used rabbit antiserum, guinea pig serum as complement, and neutrophils as phagocytes. When compared to our studies there is a difference in animal models used, which could be responsible for the difference in results seen.

S. aureus type 8 was used instead of type 5 (Figure 5) to determine if the slight difference in the two types of *S. aureus* would be a factor in the ability of MV-II-065 to prevent capsule formation. Antigenic specificity is important in antibody binding induction in vitro of neutrophil phagocytosis of type 8 organisms (Karawaka *et al*., 1988). As there is a difference in the structure of the capsules for these two types of bacteria, there was uncertainty on the results expected. The results displayed in Figure 5 appear to

be similar to those in Figures 2-4. MEOH treated bacteria were more easily phagocytized and mimetic treated bacteria showed decreased phagocytosis.

We attempted to modify the amount of capsule production by growing the bacteria in broth instead of on plates (Fig 7-9). In Figures 7-9 the control and minetics were administered by inoculating *S. aureus* into broth which contained MV-II-065 or MEOH. This was done because there may have been too much capsule being produced to see the effects of the mimetics taking place. As mentioned earlier, *S. aureus* produces less capsule when grown in broth as compared to being grown on agar. In these experiments anti-T5 mouse serum was used as the only opsonin; the mouse serum was expected to be enough to enable phagocytosis to take place since it contains antibodies to the T5 bacteria. Even though *S. aureus* were inoculated in broth, both experiments followed the trends of previous experiments Figures (2-5). In Figure 7, bacteria treated with MEOH had the lowest number of bacteria. In Figures 8 & 9 due to low colony counts, averages were taken at 0hr and 1hr instead of data being expressed percent of 0 hr. The data is similar to the results of Figure 7 in which MEOH treated *S. aureus* had less colonies than did those treated with mimetics. Several possibilities may explain the outcomes of Figures 7-9, such as the absence of complement, the size of the capsule produced, and time.

The absence of complement is known to have an affect on phagocytic activity because phagocytosis of *S. aureus* appears to take place better when both complement and Abs are used. In a study done by Peterson and coworkers (Peterson *et al*., 1978), antisera against type 5 *S. aureus* alone only allowed for phagocytosis to take place against *S. aureus* Smith compact (unencapsulated) strain, but not against *S. aureus* M

(encapsulated) strain. However, phagocytosis uptake of *S. aureus* Smith diffuse (encapsulated) was close to the uptake of *S. aureus* Smith compact when incubation time was increased using antiserum alone.

A difference in the strains of bacteria could lead to differences in capsule sizes, which might be responsible for the outcome of dissimilar results when comparing ours to the Peterson lab results. Our lab used type 5 *S. aureus* NCTC 6133 Peterson and coworkers used type 5 *S. aureus* Smith diffuse. Peterson and coworkers noted in their study that the Melly lab (Peterson *et al*., 1978) had witnessed greater virulence of the M strain compared to the diffuse strain, which is linked to its larger capsule size. Furthermore, the results of Figure 8 and 9 can not be compared as a whole to the results of previous experiments that were taken as a percentage of 0 hr. Although there was an increase in colony forming units as the concentration of mimetics increased in Figures 8 and 9, when both Figures 8 and 9 were compared there was a decrease in colony forming units over time.

In Figures 10 & 11 the colony count averages of 0hr and 1hr had to be taken due to low colony counts. *S. aureus* were inoculated in broth to administer control or mimetics. The phagocytosis assay was performed using human AB plasma (complement) as an opsonin to determine its effectiveness against bacteria grown in broth. Thakker and coworkers (Thakker *et al*., 1998) have demonstrated that complement is an effective opsonin when *S. aureus* is grown in broth. However, results of Figures 10 & 11 bear resemblance to our previous experiments in which bacteria treated with MEOH has lower colony counts than do bacteria treated with mimetics. There is an increase in colony forming units as the concentration of mimetics increases,

but when compared to Figure 11 there is a decrease in the overall colony forming units. Comparison of Figures 8 and 9 to Figures 10 and 11 reveal less colony forming units in Figures 10 and 11 which could be due to opsonization of bacteria with antiserum.

Since the cultivation of *S. aureus* in CSA broth did not seem to have an influence on the outcome of the previous experiments, *S. aureus* was inoculated onto CSA plates containing control or different concentrations of mimetics in Figures 12 & 13. Neither antisera nor human AB plasma was used as an opsonin. Phagocytic ability of U937 cells should be decreased without opsonin being used. The results of Figures 12& 13 show opposite of what was expected, there was an increase in phagocytosis of *S. aureus* by the U937 cells. There also seem to be a reverse in the trends seen in earlier experiments. *S. aureus* treated with mimetics seemed to be more easily phagocytized than MEOH treated S. aureus, but the MV-II-065 (1ug/ml) treated bacteria at 1 hr was the only significant value. Although there was only one significant change between the four conditions of S. aureus, when compared the assays that contained U937 cells had a noticeably lower amount of colony forming units than did the assays that did not contain U937 cells.

Lacking significant data from experiments and contrary trends occurring such as an increase in bacteria with increase in concentration of mimetics gave rise to questions. Does the treatment of *S. aureus* with MV-II-065 cause the bacteria to grow at a slower rate as the concentration of the chemical increases or does the treatment of *S. aureus* with MV-II-065 cause the bacteria to absorb light less affecting the starting concentration of bacteria that is used?

To investigate the first question we performed a growth rate curve of *S. aureus* represented by Figure 14, which was performed to determine if treatment of *S. aureus*

with mimetics affects its growth. The data of Figure 14 revealed that bacteria treated with mimetic and control have identical growth rates. The results of Figure 14 give the indication that mimetics do not affect the growth rate of the bacteria.

The possibility of mimetics influencing the amount of light adsorbed by the bacteria was investigated next. Treatment of *S. aureus* with mimetics is expected to cause a decrease in the amount of capsule being produced by the bacteria. If this is true then there is the possibility of *S. aureus* producing less capsule to absorb less light when placed in the spectrophotometer. Less light being absorbed could require more bacteria to achieve the same absorbance as those treated with control. Furthermore this would offset the attempt to achieve plating of equal concentrations of bacteria when performing phagocytosis assays.

The plating of serial ten fold dilutions 10^3-10^7 of bacteria treated with either MEOH or mimetics was performed to examine if the decrease in capsule could affect the amount of bacteria being plated and cause the unexpected results seen in the phagocytosis assays. To achieve equal amounts bacteria being pipetted into the plate, T5 bacteria were originally adjusted to an optical density of 0.5 at 550 nm. Figures 15-18 represent these serial dilutions. The serial dilutions revealed that *S. aureus* treated with mimetics had higher colony forming units than did those treated with control at the same dilution factor when plated. A table examining the data of 10^6 serial dilutions from Figures 15-18 was composed to show how MV-II-065 has an effect on the amount of bacteria plated after treatment with mimetics. After analyzing the serial dilutions of 10^6 it became apparent that bacteria treated with mimetics plated at higher bacterial counts than those treated with MEOH.

Results from the ELISA suggest that there is a decrease in the amount of capsule being produced by MV-II-065 treated *S. aureus* when compared to the bacteria treated with MEOH, which meets our goal of determining if MV-II-065 prevents capsule formation. The indication of a decrease in capsule led to the expectation of U937 cells having the ability to phagocytize mimetic treated bacteria more than MEOH treated bacteria. Results of phagocytosis assays seem to dispute this assumption showing just the opposite. MEOH treated *S. aureus* were phagocytized more easily. However, when averages of 0 hr were compared to averages of 1 hr there was indication of phagocytosis of the mimetic and control treated bacteria. Phagocytosis has also been demonstrated in Figures 12 and 13 as indicated by the differences in colony forming units between plates that had U937 cells and those that did not. In all of the phagocytosis assays, except for Figures 12 and 13, MEOH treated bacteria had the lowest colony forming units followed by decreasing concentrations of MV-II-065. This led to the investigation of MV-II-065's affect on *S. aureus*' growth rate. Growth rate curves performed revealed that the bacteria grew at the same rate under all four conditions. This then caused us to investigate how MV-II-065 influences the standardization of bacterial concentrations using absorbance values. It was learned through serial dilutions that mimetics affect the standardization of the bacterial concentrations. Knowing that mimetics has an effect on standardization of bacterial concentrations, it is impossible to tell if *S. aureus* treated with MV-II-065 is more readily phagocytized than MEOH treated *S. aureus*. The goal of determining this using starting concentration based upon absorbance values is not feasible; unequal amounts of *S. aureus* are pipetted during phagocytosis assays. Gaining insight on how mimetic treated *S. aureus* affects the method of standardization, it has been proposed that

future phagocytosis experiments achieve equal concentrations of bacteria by basing them upon colony counts.

Other laboratories have used phagocytosis assays to measure *S. aureus* killing in the absence of capsule. Their methods have differed from ours in cells used. Phagocytosis assays performed by Thakker and coworkers (Thakker *et al*., 1998) were more successful in obtaining results. The strains of bacteria and phagocytes used in the assays along with methods were slightly different. Thakker *et al*., used human PMN's as phagocytes as opposed to a macrophage cell line. Although both are capable of phagocytizing opsonized bacteria, they each possess different receptors for binding to these complexes. Also rabbit antiserum and guinea pig complement were used in Thakkers experiment. Ours used mouse antiserum and human complement. These could bind differently to the receptors of PMNs and macrophages. Thakker and coworkers lysed the PMNs before plating the aliquot, our lab did not. Lysing of the phagocytes allows for the measurement of an increase in intracellular bacteria, which enables the amount bacteria phagocytized but not yet dead to be seen. The advantage to this is that it allows for small changes to be detected. The disadvantage is that if bacteria are dead it can not be observed. The method we employed allows for a measure of a decrease in the number of extracellular bacteria.

In future studies we will try to develop better methods to more effectively measure our results. These methods include adjusting bacteria concentrations so that the same amounts are added to assays and measuring bacteria using an intracellular method instead of an extracellular method to determine the effectiveness of phagocytosis. With the development of better methods for measuring functional effect of the mimetics, we

hope to find mimetics useful in treating *S. aureus* infections. The need for other forms of treatment for *S. aureus* infections becomes ever more important as the bacterium becomes more antibiotic resistant.

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