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Enhancement of Phagocytosis of Macrophages in the Presence of Human Beta Defensin 2

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Abstract:- Antimicrobial peptides are natural peptides secreted by the epithelial cells and phagocytes. There are various types of antimicrobial peptides such as Defensins, Cathelicidens and Lysozymes. Defensins are of three main types, Alpha and Beta and Theta with alpha and beta defensins mainly found mainly in mammals. Defensins have a broad spectrum of activity against microorganisms which is achieved in various ways including distortion of the microbial membrane by forming pores or transient gaps leading to release of cellular contents and influx of water resulting in the lysis of the cell.

In the study, the opsonizing ability of the Beta defensin 2 was investigated. This was demonstrated by measuring the respiratory burst of two cell lines, Thp-1 and U937 which were activated with various stimulants including the Beta defensin 2 treated bacteria.

The respiratory burst was analyzed with the use of oxygen electrode apparatus. The study revealed a variety of results with Thp-1 cell line utilizing more oxygen than expected due to a possible early activation by contaminants in the sample or apparatus used. A slightly lower oxygen consumption was utilized by the U937 cell line although it was still higher than the other more potential cell line activating samples. The penicillin treated bacteria yielded a low oxygen consumption rate in both cell lines suggesting that the cell lines could have been partially dead or required more time for activation in the presence of the antibiotic treated bacteria. The Chloramphenicol treated bacteria in both cell lines consumed more oxygen indicating that the cell lines recognized the bacteria by possibly attaching to a surface protein on the bacteria. The heat killed bacteria which was only assessed in the case of Thp-1 cell line did not prove to stimulate a significant number of the cell line which could be due to the fact that macrophages phagocytose dead bacterial cells slowly compared to live bacterial cells. Finally, the cell line in the presence of beta defensin 2 were analyzed to have a respiratory burst rate higher than the rest suggesting a possible ability of beta defensin 2 to opsonize bacteria for the cell lines.

Keywords:- *Beta Defensin 2; Opsonin; Oxygen consumption; THP cell line.*

I. INTRODUCTION

The widespread presence of microorganism poses a threat to the health of many living things like humans, animals and more. Microorganisms such as Bacteria, Viruses, Fungi and Protozoans come into contact with humans and other species in various ways and once they enter the body are capable of causing illnesses such as Malaria, Tuberculosis, HIV and a lot more. The modes of entry for microorganisms can be through a wound or cut on the skin or through inhalation or ingestion. The illnesses caused in various ways which necessitates the existence of a defensive mechanism which is able to defend the body from such pathogens.

After an infection has been established within the host and the symptoms are apparent antimicrobial agents such as antibiotics to cure bacterial infections, anti-viral drugs to cure viral infections and anti-fungal to cure fungal infections are administered. At present there are a number of compounds

effective against pathogens of different kinds, however resistant pathogenic strains of bacteria, viruses, fungi and protozoa are appearing to exist which has necessitated the need for researching alternative therapeutics to treat patients with such resistant organisms.

In addition to the availability of antimicrobial compounds, the body also possesses natural defensive mechanisms responsible for the elimination of infections. The initial defense against pathogens is provided by the skin but in some case the microbes are able to breach the skin layer and enter the body where they as mentioned already cause infections leading to diseases. The innate immune system and adaptive immune system are two of the components of the immune system which offer protection to the host after the microorganisms enter the body.

A. Innate immunity

The innate immunity is the initial body response against pathogens that have breached the skin barrier.

The innate immune system mainly comprises of epithelial and mucociliary barriers but it also includes lymphocytes such as Neutrophils, Macrophages, Mast cells, Dendritic cells, Natural killer cells, Basophils and Eosinophils [1]. The epithelial layer offers protection by secreting antimicrobial peptides in response to an infection. The secretion of peptides occurs when the epithelial cells are activated by the recognition of microbial components by the pattern recognition receptor which includes Toll like receptors, Scavengers receptors, CD14, C-lectin receptors and Complement receptors [2]. The toll like receptors functions by signaling that the microbes have the barriers of the body.

The microbial components which activate these receptors are Lipopolysaccharides, Lipoteichoic acid, Peptidoglycan, Lipoarabinomannan and Zymosan [1]. The epithelial layer upon activation by organisms secretes antimicrobial peptides Cathelicidin and human beta defensin [3]. An example of a defensin type released by the epithelial layer upon activation is the beta 2 defensin.

Human beta 2 defensin is produced when the pattern recognition receptors on the keratinocytes come into contact with Lipopolysaccharides, TNF-alpha or IL-1 Beta produced in response to infection [4]. TNF-alpha and IL-1 Beta are recruiter cytokines produced by macrophages upon their recognition of bacterial fragments. IL1-Beta is one of the first cytokine released by macrophages which causes other leukocytes such as Neutrophils to migrate to the injury site.

B. Antimicrobial peptides

The antimicrobial peptides are components of the innate immune system and form the first line of defense against invading pathogens. Antimicrobial peptides are produced in epithelial layers of the body as well as in phagocytes [5]. There are three families of defensive peptides namely Defensins, Lysozymes and Cathelicidins

C. Human Defensins

Defensins are cationic and contain cysteine and arginine residues and hydrophobic proteins. The positive charge of the defensins is mainly due to the presence of lysine and arginine residues.

There are three types of defensins, Theta defensin, Beta defensin and Alpha defensin discovered too date. The distribution of defensins into different groups is based on their pairing of the disulphide bridges and size. The alpha defensins have been reported to be 29-35 residues in length and the beta defensins 38-42 residues in size.

Antimicrobial peptides are characterized by the presence of an antiparallel beat sheet which is stabilized by three disulphide bridges [6]. The human defensins also contain cysteine residues which are present in different arrangements which distinguishes alpha defensins from beat defensins. In beta defensins, the disulphide bridges connect C1 and C5, C2 and C4 and C3 and C6 whereas the disulphide bridges connect C1 and C5, C2 and C6 and C3 and C4 [4]. The peptides are generally present in areas which frequently come in contract with pathogens in the environment. The defensins however are found to present more in specific areas than others.

The alpha defensin are found to exist in azurophil granules of Neutrophils and some macrophages [5]. Alpha defensins are also secreted by the paneth cells of the intestine. They function to kill the organisms which are phagocytosed by the phagocytes whereas the intestinal alpha defensins play a defensive role in protecting the intestine from infections.

Beta defensins on the contrary have been discovered to be secreted by the epithelial layer of most organs such as kidneys, trachea, uterus, salivary gland and skin.

Human beta defensins have a wide spectrum defensive activity against gram positive and gram negative bacteria and some fungal pathogens [7].

The beta defensive is effective in killing bacteria however they are individually more specific against particular bacteria. There are four types of this particular defensin human beta defensin 1, human beta defensin 2, human beta defensin 3 and human beta defensin 4. HBD2 is predominantly bactericidal towards bacteria of the gram-negative nature whereas HBD3 has a broader spectrum of antimicrobial activity which includes activity against Vancomycin resistance Enterococcus faecium and multi resistance Staphylococcus aureus.

D. Human beta defensin 2

Human beta defensin 2 is a microbial peptide 41 amino acids in size which is rich in cysteine. It is produced mainly by the epithelial cells of the skin, trachea, pancreas and salivary glands. HBD2 m RNA expression has also been studied to exist in phagocytes like macrophages suggesting another source for its secretion [8].

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Human beta defensins are reported to be expressed upon the recognition of the cytokines IL-1 Beta and TNF-alpha by the keratinocytes [4].

Apart from activation through cytokines, HBD2 have also been noticed to be activated through recognition of microbial components by to like receptors on epithelial cells. The beta defensins 2 like other mammalian peptides achieves such microbiocidity by two mechanisms which both result in the disintegration of the microbial membrane. Such distortion of the membrane increases its permeability which leads to increased water content within the microbial cell and release of potassium ions causing the cell to rupture and subsequently die. Pore formation is an example of the above stated defensin microbicidal mechanism which initiates by the electrostatic attraction between the positively charged defensin and negatively charged phospholipids of bacteria or other pathogens [4]. Moreover, lipopolysaccharide and Lipotechoic acid present on the surfaces of gram negative and gram-positive organisms give additional charge to the bacteria. Defensins apart from being cationic are also amphipathic which means that they contain hydrophobic and hydrophilic domains. The interaction of defensins occur in such a manner that the cationic domains settle near the head groups of the phospholipids while the hydrophobic domain is submerged within the hydrophobic domain of the fatty acid chains of the membrane [4].

The second mode of killing is by forming gaps within the membrane which occurs after the interaction of defensin and microbe. The peptides in this case aggregate into positively charged patches that possess the ability to neutralize the lipid head groups of membrane. The outcome of such aggregation is that the integrity within the lipid bilayers is disrupted causing gaps to form and ions to leave the cell [9].

Apart from the above stated mechanisms of achieving microbicidity, beta defensins are also able of stimulating an inflammatory response. The ability of Beta defensins to cause inflammation is mainly due to their affinity for a chemokine receptor CCR6 which is expressed on immature dendritic cells, B lymphocytes and memory T cells which are components of the innate and adaptive immune response [10]. The binding of beta defensins to these cells allow the recruitment of other lymphocytes to the traumatized site and it also links the innate and adaptive immune response. Although some studies have shown the inflammatory characteristic of beta defensin 2, this remains unclear and cannot be fully established as there are other reports contradicting it.

Beta defensin 2 has also been researched to possess a unique quality to cause wound healing. In a study undertaken to examine the presence of beta defensin 2 after ocular injury, it was reported that an increase in the m RNA expression of beta defensin 2 was detectable in the re-epithelialization suggesting a possibly role in healing of the traumatized corneal epithelium [11].

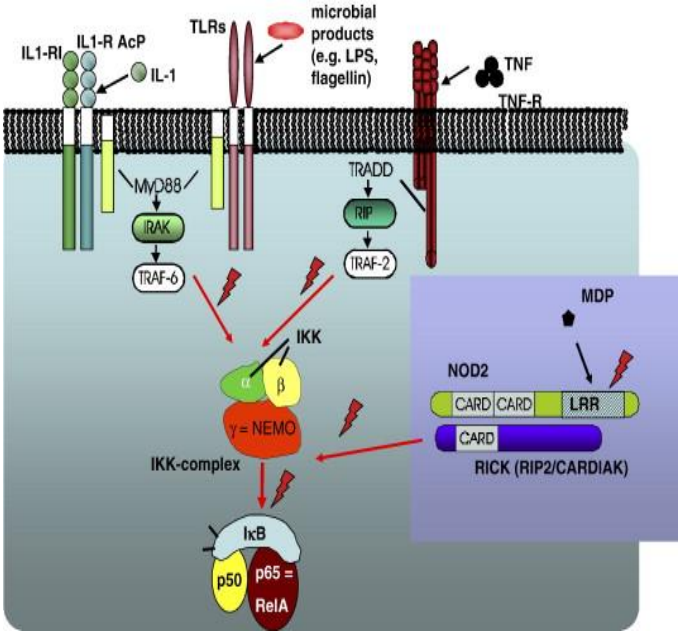

Fig. 1. The production of HBD2 from epithelial cells upon recognition of microorganisms by pattern recognition receptors and cytokines [4].

II. MATERIALS AND METHODS

A. Materials

1) Cell line

The cell lines chosen from the study were THP-1 and U937 cells which are both human acute monocytic leukaemia cell line.

2) Bacteria used.

The NCTC6353 strain of *Streptococcus pneumonia* was the microorganism used for the research.

3) Penicillin and Chloramphenicol

Penicillin and Chloramphenicol were the two antibiotics used to treat bacteria as required for part of the research

4) D. Beta defensin 2

Beta defensin 2 was the antimicrobial peptide type used to demonstrate the opsonizing ability of defensins. The defensin was consumed in a concentration of 10^{-6} cells/ml.

5) Oxygen electrode apparatus

The oxygen electrode apparatus, digital model 10 manufactured by Rank brother limited, was used for demonstrating the experiment. The volume D in the experiment was 0.5ml of the cell immersed in medium and bacteria for the series of experiment. The beta defensin 2 was used in a volume of 1.5µl for 0.5ml of the bacterial cells.

B. Methods

1) THP-1 cell culture

The cell line of choice THP-1 was cultured in T-75 flask using DMEM/F12 medium. The initial cell line used was THP-1. The cells were viewed and sub-cultured using

DMEM/F12 medium over a 6-week interval in T- 75 flask. The addition of the antibiotics Amnphoterich B, penicillin and streptomycin to the flask was introduced after the appearance of bacteria was observed within the flasks. The final volume of the flasks was kept at 20ml throughout the project and 200µl of each Amnphoterich B and a mixture of penicillin and streptomycin were then added to the flask.

2) U937 Cell line

The second cell line U937 was introduced after the THP-1 cells were noticed to be contaminated with a different type of cells three weeks after its initial subculturing. It was then decided to introduce a different cell line U937 for the experiments to be performed on that day and to culture a new set of THP-1 cells for future experiments.

The U937 cell line was cultured in an exact similar way to the THP-1 cell line along with the addition of similar antibiotics to avoid any microbial contamination which would hinder the demonstration of experiments required to be carried out.

3) THP-1 and U937 Cell count

In order to achieve the actual cell number required the contents of the flasks were poured into universal tubes for centrifugation at a speed of 400* g for 5 minutes. The medium was aspirated post centrifugation and cells were re-suspended in PBS to clear the antibiotics used to avoid contamination. The universal tubes were filled to the top with PBS to ensure efficient clearance of antibiotics. The cells after centrifugation were once again suspended in 3 ml of fresh DMEM/F12 medium. The contents of all the tubes were poured into one a single tube to allow the yielding of the maximum cell number from all the incubated flasks. A 96 well plate was used to obtain a 2-dilution factor solution by mixing a 20µl of medium containing the suspended cells and 20µl of Trypan blue in order to ease the counting of live cells by staining the dead cells blue. 10µl of the Trypan blue and cell mixture was loaded onto a haemocytometer for counting. The cells within the square and the ones touching the middle line of the top and left side of the square were considered for counting and the cells touching the bottom and left side of the square were ignored. After counting the cells the volume with the required cell number was extracted from the re-suspended volume of cells and was then further centrifuged and the accurate volume of medium was used to re-suspend the cells to obtain the cell number in a specific volume.

4) Subculture of THP-1 and U937 cell lines

Once the desired cell number was obtained the remaining cells were sub-cultured with fresh medium reaching a final volume of 20 ml for growing cells required for future planned experiments.

5) Bacteria culture

Streptococcus pneumonia was the bacteria chosen for the study which was of the NCTC 6353 strain. The bacteria were originally cultured on blood agar plates prior to extracting the cultures for storage. A broth culture was prepared of the organism by growing it in Todd Hewitt broth incubated over night at 37 C anaerobically.

6) Bacteria count

The bacteria was counted using a haemocytometer after centrifugation at a speed of 3000*g for 10 minutes. The pellet obtained was then re-suspended in 1 ml of Ringer's solution and mixed well. 10µl of the mixture of bacteria pellet and Ringer's solution was then poured into a cuvette and 0.99 ml of Ringer's solution was pipetted into the cuvette to yield a 1 ml solution of bacteria and Ringer's solution. 10 µl of the solution was the loaded onto a haemocytometer and the cells in one of the small squares within the one big square were counted. This cell number was then multiplied by the number of squares in one big square and then by the number of big squares within the counting grid. The above-mentioned calculation was then multiplied by the volume of the haemocytometer and the dilution factor to yield cell number 1 ml.

7) Chloramphenicol and Penicillin treated bacteria

The bacteria was grown as previously. Penicillin G and Chloramphenicol were ordered by Sigma and 6µg/ml of penicillin and 8µg/ml of chloramphenicol was prepared. 0.1 ml of the 6µg/ml of penicillin was poured into one of the broth solutions to obtain a penicillin treated bacteria culture. 1ml of the chloramphenicol was then extracted to obtain 8µg/ml of the antibiotic.

III. RESULTS

TABLE. I. OXYGEN CONSUMPTION PER MINUTE OF SAMPLE WITH THP-1 CELL LINE OBTAINED FROM THE AVERAGE OF TWO EXPERIMENTS PER SAMPLE.

Sample	Oxygen consumption/ min
Thp - 1	3.135%
Thp - 1 & Heat killed bacteria	1.1%
Thp - 1 & Live bacteria	2.3%
Thp - 1 & Penicillin treated bacteria	1.09%
Thp - 1 & Chloramphenicol treated bacteria	1.25%
Thp - 1 & Beta Defensin 2 treated bacteria	1.625%

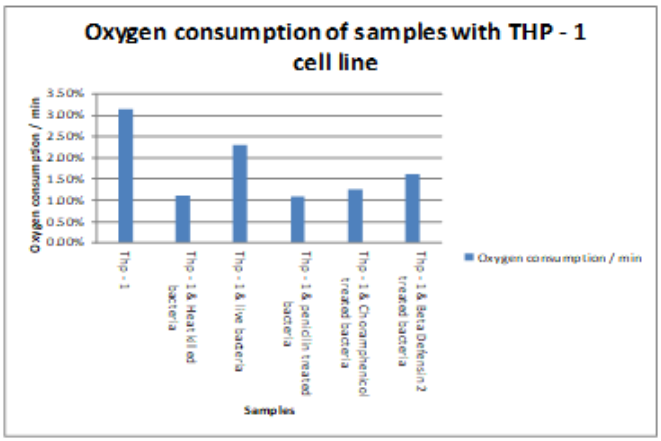

Fig. 2. Bar chart showing the oxygen consumption in various sample with the Thp-1 cell line. The results are the average of 2 experiments performed for each sample shown above.

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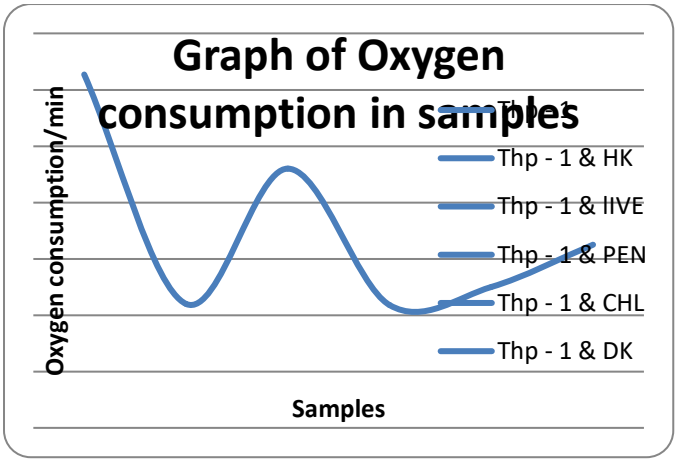

Fig. 3. Graph of the oxygen consumption per minute of the samples with THP-1 Cell line obtained from the average of two experiments.

TABLE. II. OXYGEN CONSUMPTION OF THE SAMPLE IN µM BASED ON THE AVERAGE OF TWO EXPERIMENTS PER SAMPLE.

Sample	Oxygen Consumption/min
U937	2.45%
U937% Live bacteria	3.94%
U937 & Penicillin treated bacteria	1.65%
U937 & Chloramphenicol treated bacteria	1.98%
U937 & Beta Defensin 2 treated bacteria	7.47%

TABLE III. OXYGEN CONSUMPTION IN SAMPLE WITH U937 CELL LINE OBTAINED FROM AVERAGE

Sample	
Thp -1	0.0039 µM/min
Thp -1 & Live bacteria	0.0013 µM/min
Thp -1 & Heat killed bacteria	0.0028 µM/min
Thp -1 & Penicillin treated bacteria	0.0013 µM/min
Thp -1 & Chloramphenicol treated	0.0015 µM/min
Thp -1 & Beta Defensin 2 treated bacteria	0.0011 µM/min

TWO EXPERIMENTS PER SAMPLE.

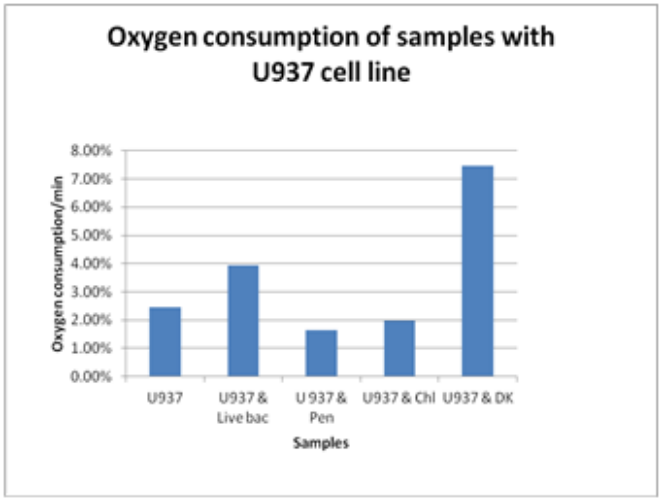

Fig. 4. Bar chart showing oxygen consumption in different samples with U937 cell line based on the average of two experiments performed per sample.

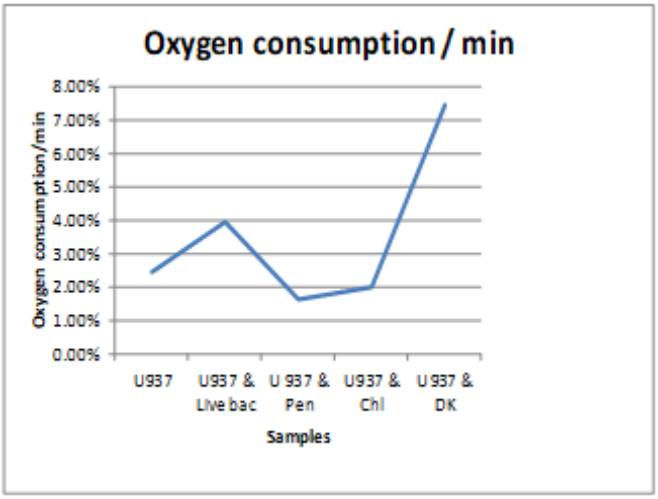

Fig. 5. Graph of oxygen consumption per minutes of sample obtained from the average of two experiments per sample.

TABLE IV. OXYGEN CONSUMPTION OF THE SAMPLE IN µM BASED ON THE AVERAGE OF TWO EXPERIMENTS PER SAMPLE.

Sample	
U937	0.0030 µM/min
U937 & Live bacteria	0.0049 µM/min
U937 & Penicillin treated bacteria	0.0020 µM/min
U937 & Chloramphenicol treated bacteria	0.0024 µM/min
U937 & Beta Defensin 2 treated bacteria	0.0093 µM/min

IV. DISCUSSION

The study was performed to investigate the activity of phagocytosis of monocytes in the presence of beta defensin 2. Two cell lines were chosen, and their results were compared in order to confirm the accurate functioning of the cell lines as previous researchers have shown a need for the comparison when using cell lines. These two cell lines were then used to investigate for the induction of a respiratory burst with various stimuli.

The respiratory burst was measured by a standard technique using oxygen electrode. The various stimuli used were human Beta defensin 2, bacteria alone, Penicillin and Chloramphenicol treated bacteria and heat killed bacteria. The organism chosen for the study was Streptococcus pneumoniae.

After analyzing the results from the experiments, a number of different outcomes were observed. To begin with the THP-1 cell line, an increase in oxygen consumption in the absence of any added stimulants was noticed. The unexpected result could be due to the activation of the cell line prior to its investigation in the oxygen electrode apparatus. The early activation could be due to the presence of contaminants such as bacteria in the oxygen electrode apparatus which could have been left uncleaned after usage by some other laboratory worker or possibly due to the use of a contaminated pipette tip used for extracting the cell line from the tube it was stored into the oxygen electrode apparatus.

Macrophages are also capable of activating themselves when place under stress by altering their environment. The change in environment in the case of Thp-1 cell line could have occurred by removing the cell line from the T-75 flasks containing the medium and placing them in smaller containers such as cuvettes or universal tubes which were used for the experiments. The accumulation of Thp-1 cells after being placed in tubes could have caused disturbances amongst the cells leading to an increase in activation of the cells causing more oxygen to be consumed. In the respiratory burst demonstration of the bacteria and the Thp-1 cell line, a slight increase in the respiratory burst rate was identified as compared to other experiments, but lower rate compared to the Thp-1 cell line alone. Although despite a similar way of organism recognition a higher respiratory burst rate in this case suggests that Thp-1 cell line was activated as previously by contaminants present in the electrode apparatus or by being put under stress.

Another logical reason for high oxygen consumption could be due to the fact that *Streptococcus pneumoniae* was also consuming oxygen leading to a high oxygen consumption rate as previously by contaminants present in the electrode apparatus or by being put under stress.

In the other sample where the bacteria were treated with penicillin (0.06µg/ml) in an overnight culture a lower oxygen consumption rate was yielded after the analysis of the graphs obtained from the experiment. Penicillin achieves bactericidity by inhibiting the transpeptidase enzyme which functions to strengthen the cell wall of bacteria by cross linking their peptidoglycan [12]. This inhibition of the transpeptidase results in the weakening of the cell wall and eventually its lysis releasing its components within the blood stream activating lymphocytes through interaction with their receptors such as TLR2 on macrophages which recognize Lipopolysaccharides.

In the penicillin treated bacterial sample, instead of achieving a high level of Thp-1 activation on the rate of oxygen consumption a lower rate was observed possible due to the fact that the Thp -1 cells were not activated in the time provided for them to react with the bacteria. This could establish another fact about Thp -1 cells that they're activation is a slow process and that in the future experiments a longer period of interaction with bacteria should be allowed in order to achieve significant data. The chloramphenicol (8µg/ml) treated bacteria on the other hand revealed a higher rate of oxygen consumption as compared to penicillin. Chloramphenicol is a bacteriostatic which suppresses the protein synthesis of the organism that is treated with it [13]. This increase in the utilization of external oxygen implied that the Thp-1 cells were being activated more in the absence of any release of the microorganism's cellular components such as Lipopolysaccharides which would activate the Thp-1 cells. A possible explanation for this could be that there are proteins expressed on the surface of the bacteriosatically treated bacteria which allowed the monocytes to recognize it and in turn phagocytose it.

The monocyte response to denatured protein was also studied by denaturing the protein of *Streptococcus pneumonia* by heating it in the water bath for 10 minutes. The same method was used for measuring the oxygen consumption of the heat killing bacteria which was found to be slightly higher than the penicillin treated bacteria's consumption rate lower than chloramphenicol treated bacteria. Neutrophils have been reported to engulf dead bacteria cells at a slower rate as compared to live bacteria and due to the similarity in the phagocytosis are activated by dead cellular components in some way possible by the binding of killed lipids in the cellular membrane to the monocyte pattern recognition receptors. In the final demonstration of the oxygen uptake by the bacteria treated with beta defensin 2which was left to act on it's for 10 minutes, a comparatively higher level of oxygen consumption was observed compared to some of the other studies. As stated in the introduction beta defensin 2 are capable of forming pores in the bacterial membrane by attaching to the phospholipids, it would be result in the secretion of membranous components of the bacteria such as Lipopolysaccharides or Lipoteichoic acid which are recognized by the receptor of Thp-1 cells. The secretion of such particles would also be the result of penicillin action on the microbial membrane which would activate the Thp-1 cells in a similar way, however despite of the similarity in the activation of the monocytes a increased oxygen consumption rate is observed in the beta defensin 2 containing sample. The difference in the oxygen uptake by the monocytes in this particular study indicates a possibility of the existing of another mechanism which is assisting the macrophages in phagocytosing the bacteria. Opsonization is a process which

enhances the phagocytosis of the bacteria by making the bacteria seems more presentable resulting in its easy uptake.

V. CONCLUSION

Beta defensin 2 could possibly be acting as an opsonin causing more Thp-1 cells to be activated apart from their activation thorough the cellular particle. U937 cell line alone consumed more oxygen than the penicillin and Chloramphenicol treated bacteria with the same cell line suggesting a similar occurrence of early activation by the reason stated for Thp-1 cell line which also had shown a high oxygen consumption rate when investigated individually. Higher cell line and bacteria and bacteria oxygen consumption suggests that U937 recognizes bacteria easily as compared to Thp-1 cell line or that prior activation again resulted in more oxygen utilization. The ability of beta defensin to opsonize pathogens in humans is unclear and requires more research, however from the outcome from the study can conclude that the beta defensin 2 could possibly possess the ability to opsonize bacteria.

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