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Title: Effect of hydrogen peroxide produced by Streptococcus pneumoniae on bacterial survival in the presence of peroxidases released by immune cells.
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Introduction:
This project focuses on the interactions between the bacterium Streptococcus pneumoniae, neutrophil extracellular traps and the protein myeloperoxidase.

Streptococcus pneumoniae is a medically relevant gram-positive bacterium that is often carried asymptomatically in humans. However, it is also able to cause diseases such as pneumonia, meningitis, ear infections and sepsis. We are interested in S. pneumoniae as it produces hydrogen peroxide (H_2O_2) as a by-product of its normal metabolism. At low doses, hydrogen peroxide does not kill the bacteria, but in the presence of peroxidase enzymes produced by immune cells H_2O_2 may be converted to a more bacteriocidal compound. Myeloperoxidase is one of these enzymes. It utilises H_2O_2 as a substrate in order to produce hypochlorous acid (or bleach) in the presence of chlorine. Bleach is a very effective antimicrobial agent.

Myeloperoxidase is contained within a type of white blood cell called a neutrophil. Neutrophils are part of the innate immune system, which provide a non-specific immediate defence against infection. Neutrophils are able to kill microbes in a variety of ways such as engulfing bacteria into a compartment containing antimicrobial compounds, releasing antimicrobial proteins into the extracellular environment or through the formation of neutrophil extracellular traps (or NETs). NET formation is a recently discovered process by which neutrophils are able to trap and potentially kill bacteria. The process is not fully understood but essentially the neutrophils extrude their nuclear DNA, which proves a scaffold on which neutrophil bactericidal proteins are attached. Myeloperoxidase is one of the proteins found associated with NETs.

Aim:
The aims of this project were to identify whether S. pneumoniae are able to stimulate NET production, whether the bacteria are able to degrade pre-formed NETs and whether they are killed on NETs. If killing was found, we would examine if this killing is facilitated by myeloperoxidase and if myeloperoxidase used the hydrogen peroxide produced by S. pneumoniae to generate bleach.

Impact:
If neutrophils produce NETs in response to *S. pneumoniae* and the bacteria are killed on these NETs, therapeutic strategies supporting the recruitment of neutrophils and the generation of NETs may be helpful in diseases associated with *S. pneumoniae* infection.

Method:
Neutrophils were isolated daily from human blood of healthy donors with ethical approval and a standard laboratory strain of S. pneumoniae was used in this study.

NET formation assay: In this semi-quantitative assay, neutrophils were stimulated with a chemical stimulant called PMA, commonly used to produce NETs, or with S. pneumoniae then incubated for 4 hours in a 96 well plate. PMA was used as a control in all assays. After incubation, a fluorescent cell-impermeable dye was added to stain extracellular DNA which is a major component of NETs. When read in a fluorescent plate reader higher fluorescence can be an indicator of NET formation.

NET killing assays: These assays were used to quantify the amount of bacteria that were killed on NETs. NETs were pre-formed with PMA in 12 well plates before S. pneumoniae, that had been

incubated for an hour to allow production of hydrogen peroxide, were added. S. pneumoniae were incubated with the NETs for an hour to allow for NET-mediated killing to occur. As bacteria become trapped in NETs, an enzyme called DNase that breaks down DNA was used to degrade the NETs to release the bacteria. Bacteria were then scraped off the wells and plated onto agar, grown overnight then counted to determine how many had been killed. This assay was repeated with an inhibitor of the enzyme myeloperoxidase and agents that break down both hydrogen peroxide and bleach.

Results:
Results from the NET formation assay showed increased fluorescence associated with increasing concentrations of S. pneumoniae. These results could indicate that S. pneumoniae was inducing NET formation. We were also able to see that our strain of S. pneumoniae was not able to degrade NETs once they had formed.

My killing assays showed that S. pneumoniae were killed on NETs when they were pre-incubated in media to produce hydrogen peroxide. This killing was shown to be via a mechanism dependent on myeloperoxidase as an inhibitor of myeloperoxidase as well as an enzyme that breaks down bleach increased survival of the bacteria.

Conclusion:
Our strain of S. pneumoniae was able to increase neutrophil DNA fluorescence, which could indicate NET formation. Immunofluorescence and live cell microscopy is needed in the future to confirm that the increased fluorescence is due to NET formation. The killing of S. pneumoniae on NETs is interesting, as S. aureus has previously been shown to be killed on NETs only upon addition of hydrogen peroxide. However, S. pneumoniae is able to produce the hydrogen peroxide itself. Further repeats of killing assays with different concentrations and strains of S. pneumoniae as well as with different inhibitors would be a useful continuation of this research.