Studies of
*Pseudomonas aeruginosa* and its
virulence factors in a mouse model of
dermal infection

Peter Geerlings
Bsc (Hons) UWA

School of Veterinary and Life Sciences,
Molecular and Biomedical Sciences,
Murdoch University,
Perth, Western Australia

*This thesis is submitted in fulfillment of the requirement for the degree of*

*Doctor of Philosophy*

*2013*
Declaration

I declare that the research presented in this thesis, as part of the Doctor of Philosophy at Murdoch University, Western Australia, is original and my own work except where specific contributions of other persons are acknowledged. The work contained in this thesis has not been submitted for assessment, in full or part, within any other tertiary institution.

......................................................

Peter Michael Geerlings
April 2013
Experiments described in this thesis involved the use of animals. In accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* and the Animal Ethics Committee of Murdoch University, Western Australia, all animal work was approved under the following Murdoch University Animal Ethics permit numbers.

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1136/05
1147/05
2289/09
2344/10
Publications arising from this thesis

Abstract

*Pseudomonas aeruginosa* is an opportunistic pathogen of hosts with impaired immune function or concurrent disease, most notably patients with Cystic Fibrosis and those who have been severely burned. It has an extensive array of virulence factors (VFs) which enable it to colonise and penetrate host tissue. It is responsible for a high percentage of nosocomial infections and is becoming increasingly antibiotic resistant, so there is an immediate need for novel treatments of severe *P. aeruginosa* infections. Following severe traumatic injury to the skin, the dermis and epidermis may be destroyed, leaving the fascia and endothelium of the vasculature as the final structural barriers protecting the circulation and preventing bacteraemia. Little has been reported on the mechanisms used by *P. aeruginosa* to penetrate the fascia and enter the circulation, because conventional animal models of dermal injury do not allow for quantitative study of bacterial pathogenesis and host responses. To address this, a novel transdermal chamber was designed, manufactured and implanted into mice allowing for repeated sampling and quantitative analysis of bacterial replication, VFs, and host responses. Despite the bacterial load in the chamber exudate being similar to normal control mice, mice immunosuppressed using cyclophosphamide (CP) had significantly higher levels of Exotoxin A (ETA), *P. aeruginosa* elastase (PAE) and Phospholipase C (PLC), and developed fatal bacteraemia when compared to immunocompetent mice.

It was hypothesised that raised levels of ETA and PAE contributed to the pathogenesis of *P. aeruginosa* in CP treated mice by increasing the permeability of the fascia and endothelium allowing bacteria to enter the circulation. To test this, purified ETA and PAE were added to the chambers of immunocompetent mice, followed by fluorescently labelled dextran to assess efflux measured as a change in dextran concentration in the chamber exudate over time. ETA caused sustained local depletion of leukocytes and fibroblasts, reduction in exudate volume and weight loss, but did not change the dextran concentration in the chamber exudate compared to control mice. PAE was pro-inflammatory, increased exudate volume, damaged fascial tissue, induced local haemorrhage, and resulted in a decrease in dextran concentration in the chamber compared to controls. Combining ETA and PAE in the chamber
resulted in increased weight loss, haemoglobinaemia and increased accumulation of dextran in the local draining lymph nodes.

Preliminary trials using ovine anti-sera to *P. aeruginosa* antigens in infected chambers of CP-treated mice increased their survival. Collectively, these results show that the increased concentration of VFs, not bacterial load, facilitated bacterial translocation across the fascia and endothelium, and uncovers the underlying significance of the superficial fascia as a defensive barrier against infectious agents. Furthermore, this study suggests that neutrophils are crucial to reducing VF concentration, and that topically applied heterologous anti-*P. aeruginosa* antibodies may be useful in preventing fatal bacteraemia in severe dermal infections of susceptible patients.
List of Figures

Chapter 1 – Introduction

Figure 1.1   Common sites of *P. aeruginosa* infection in humans       5
Figure 1.2   Virulence factors of *P. aeruginosa*       9

Chapter 2 – Development of a transdermal chamber model of *P. aeruginosa* infection

Figure 2.1   Components of the moulding device       41
Figure 2.2   Stages in the manufacture of the chamber       42
Figure 2.3   The direction of suture placement around the flange       43
Figure 2.4   Cross sectional diagram of the chamber in situ       43
Figure 2.5   The transdermal chamber implanted in an anaesthetised C57BL/J6 mouse       44
Figure 2.6   Instruments used during routine sampling from the chamber       44
Figure 2.7   The original chamber prototype       45
Figure 2.8   Chamber implantation in deceased mice       45
Figure 2.9   Chamber implantation in anaesthetised mice       46
Figure 2.10   A typical example of a chamber that has been partially dislodged by the mouse, or other mice       47
Figure 2.11   Prototype o-ring to protect sutures       47
Figure 2.12   The transdermal chamber components       48
Figure 2.13   Male C57BL/J6 strain mice one day after chamber implantation       48
Figure 2.14   Average change in weight of male C57BL/J6 following implantation with the transdermal chamber       49
Figure 2.15   Chamber persistence *in-situ*       50
Chapter 3 – *Experimental Pseudomonas aeruginosa* infection studies in chamber implanted mice

Figure 3.1. Change in mouse weight after chamber implantation, CP treatment and infection 61

Figure 3.2. *P. aeruginosa* growth in the chamber of control and CP treated mice 62

Figure 3.3. Viable leukocytes in chamber exudates of normal control and CP treated mice following *P. aeruginosa* inoculation 63

Figure 3.4. Total and differential leukocytes analysis in chamber exudate of *P. aeruginosa* infected control mice 64

Figure 3.5. Chamber exudate volume and protein concentration following *P. aeruginosa* infection 65

Figure 3.6. Histopathology of chamber floor tissue of normal and CP treated mice infected with *P. aeruginosa* 66

Figure 3.7. Liver pathology of CP treated mice 9 days after infection by *P. aeruginosa* 68

Figure 3.8. Comparison of virulence factors in chamber exudate of normal control and CP treated mice infected by *P. aeruginosa* 69

Chapter 4 – *In vivo* studies of *Pseudomonas aeruginosa* virulence factors

Figure 4.1. Measurement of fluorescent dextrans using appropriate light filters 84

Figure 4.2. Comparative PAE production from *P. aeruginosa* strains 85

Figure 4.3. Chromatogram of concentrated *P. aeruginosa* culture Supernatant on DEAE sepharose 86

Figure 4.4. Elastase activity of fractions from DEAE sepharose chromatography 87

Figure 4.5. Chromatogram of pooled elastase positive fractions from DEAE sepharose separation on Sephacryl S-200 88

Figure 4.6. Elastase activity of selected fractions from Sephacryl S-200 chromatography 88

Figure 4.7. SDS-PAGE of PAE purification 89

Figure 4.8. Confirmation of purity of ETA 91
Figure 4.9.  Cytotoxicity of ETA

Figure 4.10.  Reduction in dextran concentration over time

Figure 4.11.  Measurement of 500 KDa FLD in organs

Figure 4.12.  Measurement of 3 KDa, 40 KDa and 70 KDa FLD in organs

Figure 4.13.  FLD concentration in chamber exudate in response to duration of treatment and PAE dose

Figure 4.14.  Exudate volume in the chamber in response to duration of treatment and PAE dose

Figure 4.15.  Concentration of FLD in chamber exudate of PAE treated mice

Figure 4.16.  Exudate volume following PAE treatment

Figure 4.17.  Exudate recovered from the chamber of mice on day 9 contained RBC

Figure 4.18.  Haemoglobin in chamber exudate following PAE treatment

Figure 4.19.  Viable leukocytes in the chamber exudate following PAE treatment

Figure 4.20.  Proportion of leukocytes in chamber exudate following PAE treatment

Figure 4.21.  Fluorescence in organs of PAE treated mice

Figure 4.22.  Histology of fascia and muscle following PAE treatment

Figure 4.23.  Change in mouse weights following ETA treatment

Figure 4.24.  FLD concentration in chamber exudate following ETA treatment

Figure 4.25.  Comparative concentration of 40KDa FLD in the brachial lymph nodes of ETA treated mice

Figure 4.26.  Total exudate in chamber following ETA treatment

Figure 4.27.  Viable leukocytes in the chamber exudate, 12 hours after ETA treatment

Figure 4.28.  Differential leukocytes in the chamber exudate of ETA treated mice
Figure 4.29. Fascia following ETA treatment

Figure 4.30. FLD concentration in chamber exudate following ETA/PAE treatment

Figure 4.31. Fluorescent 40 KDa dextran in BLN following ETA/PAE treatment

Figure 4.32. Total exudate in the chamber following ETA/PAE treatment

Figure 4.33. Viable leukocytes in the chamber following combination ETA/PAE treatment

Figure 4.34. Differential leukocytes in the chamber exudate of ETA/PAE treated mice

Figure 4.35. Fascia of ETA/PAE treated mice

Figure 4.36. Change in mouse body weights following VF treatment

Figure 4.37. Relative free Hb concentration in serum following VF treatment

Figure 4.38. Change in mouse body weight following infection with PAE negative P. aeruginosa strain PA103

Figure 4.39. Superficial and deep fascia of CP treated mouse infected by P. aeruginosa ETA+/PAE− strain PA103

Figure 4.40. Illustration of the influences driving the net filtration and reabsorption pressure across endothelial vessels

Figure 4.41. Potential sources of error and interference in fluorometry

Figure 4.42. Illustration of the influence that ETA and PAE can have on host immunity and healing

Chapter 5 – Passive immunotherapy of experimental P. aeruginosa infections in chamber implanted mice

Figure 5.1. Electron micrographs of flagella preparation

Figure 5.2. Chromatogram of sheep IgG purification on thiophilic resin

Figure 5.3. Purified IgG from sheep serum

Figure 5.4. Sheep antibodies to P. aeruginosa antigens

Figure 5.5. Mouse survival following P. aeruginosa infection and treatment with dilutions of normal sheep serum
Figure 5.6. Change in body weight following infection and treatment with dilutions of normal sheep serum 153

Figure 5.7. Replication of *P. aeruginosa* in chamber fluid treated with normal or anti-Pseudomonas sheep serum 155

Figure 5.8. Change in body weight of mice infected with *P. aeruginosa* and treated with 1:20 dilutions of sheep serum 155

Figure 5.9. Mouse survival following *P. aeruginosa* infection and treatment with 1:20 dilutions of sheep serum 156

Figure 5.10. Replication of *P. aeruginosa* in chamber fluid treated with 1:50 dilutions of sheep serum 157

Figure 5.11. Change in body weight of mice infected with *P. aeruginosa* and treated with 1:50 dilutions of sheep serum 158

Figure 5.12. Mouse survival following *P. aeruginosa* infection and treatment with 1:50 dilutions of sheep serum 159

Figure 5.13. *In vitro* neutralisation of PAE by purified sheep IgG 160

Figure 5.14. *In vitro* neutralisation of ETA by purified sheep IgG 161

*Chapter 6 – General discussion*

Figure 6.1. Hypothetical pathogenic mechanisms of ETA and PAE on the fascia 172

*Appendix B – Purification of Phospholipase C*

Figure B.1. Activity of SMM fractionated culture supernatant 219

Figure B.2. Activity of SMM (with glucose) fractionated culture supernatant 220

Figure B.3 Activity of SMM (with choline) fractionated culture supernatant 221

Figure B.4. Activity of SMM (with glucose and choline) fractionated culture supernatant 222
List of Tables

Table 2.1. Summary of the total live mouse chamber experiments during this project 51
Table 3.1. Systemic *P. aeruginosa* in CP treated, infected mice 68
Table 3.2. Systemic ETA in CP treated, infected mice 70
Table 4.1. Compatibility of FLD with organ autofluorescence 96
Table 4.2. Bacteria recovered from blood and liver of mice infected with *P. aeruginosa* strain PA103 122
Table A.1 Bacterial strains used in this project 209
Table B.1 Preliminary culture media and incubation conditions trialled to produce PLC-H 214
Table B.2 Constituents of media used to produce PLC-H for fractionation on celite 215
Table B.3. Protein concentration, haemolytic and relative enzymatic activity of culture media trialled to produce PLC-H 217
Table B.3 Concentration of SMM (with choline) bacterial culture supernatant by ultrafiltration on 10KDa MWCO membrane 223
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_2$-M</td>
<td>Alpha 2-macroglublin</td>
</tr>
<tr>
<td>$\alpha_2$-MR/LRP</td>
<td>Alpha 2-macroglublin receptor/low density related protein</td>
</tr>
<tr>
<td>AbR</td>
<td>Antibiotic resistant</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AI</td>
<td>Autoinducer</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherin junction</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ARC</td>
<td>Animal Resource Centre (Murdoch Western Australia)</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BLN</td>
<td>Brachial lymph node</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CARS</td>
<td>Compensated anti-inflammatory response syndrome</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CP</td>
<td>Cyclophosphamide</td>
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<tr>
<td>CPK</td>
<td>Creatine phosphokinase</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<tr>
<td>DAB</td>
<td>Diethylaminoethanol</td>
</tr>
<tr>
<td>DEAE</td>
<td>Dendritic cell</td>
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<td>DF</td>
<td>Deep fascia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>diH₂O</td>
<td>Deionised water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dubbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
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<td>EL-2</td>
<td>Elongation factor-2</td>
</tr>
<tr>
<td>Em</td>
<td>Emission wavelength of light</td>
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<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>ETA</td>
<td>Exotoxin A</td>
</tr>
<tr>
<td>Ex</td>
<td>Excitation wavelength of light</td>
</tr>
<tr>
<td>Fc</td>
<td>Fraction crystallisable (of immunoglobulin molecule)</td>
</tr>
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<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FL</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>FLD</td>
<td>Fluorescently labeled dextran</td>
</tr>
<tr>
<td>g</td>
<td>Force of gravity</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Haematoxylin and eosin (stain for histopathology)</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>HbA</td>
<td>Haemoglobinaemia</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrogen chloride</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HPA</td>
<td>Heterologous polyclonal antibody</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IC</td>
<td>Immunocompetent</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>ID</td>
<td>Internal diameter</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgG-AP</td>
<td>Immunoglobulin G – alkaline phosphatase (conjugate)</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>Dose that results in death to 50% of test subjects</td>
</tr>
<tr>
<td>LF</td>
<td>Lethal factor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Mab</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MC</td>
<td>Mast cell</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney cells</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSB</td>
<td>Martius, scarlet and blue (stain for histopathology)</td>
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<tr>
<td>MV</td>
<td>Membrane vesicles</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
</tr>
<tr>
<td>NA</td>
<td>Nutrient agar</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NETs</td>
<td>Neutrophil extracellular traps</td>
</tr>
<tr>
<td>NPPC</td>
<td>p-nitrophenylphosphorylcholine</td>
</tr>
<tr>
<td>OD</td>
<td>Outer diameter</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>PAE</td>
<td><em>P. aeruginosa</em> elastase</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PAP</td>
<td>Pseudomonas alkaline protease</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine (molecule of lipid)</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PLA</td>
<td>Phospholipase A</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>Plc-H</td>
<td>Phospholipase C-haemolytic</td>
</tr>
<tr>
<td>Plc-N</td>
<td>Phospholipase C- non haemolytic</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PNPP</td>
<td><em>p</em>-nitrophenylphosphate</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine (molecule of lipid)</td>
</tr>
<tr>
<td>QS</td>
<td>Quorum sensing</td>
</tr>
<tr>
<td>RAP</td>
<td>Receptor associated protein</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SF</td>
<td>Superficial fascia</td>
</tr>
<tr>
<td>SIR</td>
<td>Systemic inflammatory response</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline and Tween 20</td>
</tr>
<tr>
<td>TDTAB</td>
<td>Tetradecyltrimethylammonium bromide</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor-alpha</td>
</tr>
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<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
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<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight-junction</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMR</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>TR</td>
<td>Texas Red&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptone Soya Broth</td>
</tr>
<tr>
<td>TTSS</td>
<td>Type III secretory system</td>
</tr>
<tr>
<td>U</td>
<td>Units of enzymatic activity</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
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To my supervisors, Dr Phil Stumbles and Professor John Penhale. Thank you, John - my colleague, mentor and friend. You have taught me so much about research, animal handling and surgery. Phil, your dedication to my study was unwavering, and your professionalism and attention to detail is inspirational. Thank you, for always making yourselves available to discuss my concerns and progress.

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Contents

Declaration i
Ethics ii
Publications arising from this thesis iii
Abstract iv
List of Figures vi
List of Tables xi
List of Abbreviations xii
Acknowledgements xvii

Chapter 1 – Introduction

1.1 Historical perspectives 1

1.2 P. aeruginosa 2

1.2.1 Description 2
1.2.2 Habitat 3

1.2.3 Pathogenicity 3

1.2.3.1 Influence of Age 4
1.2.3.2 Hospitalisation 5
1.2.3.3 Organ transplantation and AIDS 6
1.2.3.4 Cystic Fibrosis 6
1.2.3.5 Severe burns and dermal infection 7

1.3 Pseudomonas aeruginosa virulence factors 8

1.3.1 Structural VFs 8

1.3.1.1 Flagella 8
1.3.1.2 Type IV pili 10
1.3.1.3 Type III Secretory Systems- Exoenzymes PcrV 11
1.3.1.4 Lipopolysaccharide 11

1.3.2 Secreted VFs 12

1.3.2.1 Exotoxin A 12

1.3.2.2 Elastase 14

1.3.2.3 Phospholipases 16

1.3.2.3.1 Phospholipase C-Haemolytic 16

1.3.2.3.2 Phospholipase C-Non Haemolytic 18

1.3.2.3.3 Phospholipase C-A, & Phospholipase C-B 18

1.3.2.4 Alginate 19

1.3.2.5 Pyocyanin 19

1.3.2.6 Pyoverdine & pyochelin 20

1.3.2.7 Alkaline protease 21

1.3.2.8 Rhamnolipid 22

1.3.3 Quorum sensing & regulation of virulence factors 23

1.4 Structure, function & immunity of skin 25

1.4.1 Epidermis 25

1.4.2 Dermis 26

1.4.3 Fascia 27

1.4.4 Healing 28

1.4.4.1 Inflammation phase 28

1.4.4.2 Proliferation phase 29

1.4.4.3 Maturation and re-modeling phase 30

1.4.5 Immune surveillance 30

1.5 Current treatment of P. aeruginosa dermal infection 31

1.5.1 Topical antimicrobial solutions 31

1.5.2 Antibiotics 31
1.5.3 Immunotherapy

1.5.3.1 Immunisation

1.5.3.2 Passive antibody therapy

1.6 Animal models of dermal infection

1.7 Summary

1.8 Project aims

Chapter 2 – Development of a transdermal chamber model of P. aeruginosa infection

2.0 Introduction

2.1 Materials and Methods

2.1.1 Development of the chamber

2.1.1.1 Design prerequisites

2.1.1.2 Prototypes

2.1.1.3 The ultimate version

2.1.2 Mice

2.1.3 Chamber implantation

2.1.4 Routine chamber sampling

2.2 Results

2.2.1 Chamber development

2.2.1.1 Original chamber body and flange, and implantation

2.2.1.2 Modification to the flange and implantation procedure

2.2.1.3 Addition of a protective o-ring and circlip

2.2.1.4 The ultimate version of the transdermal chamber

2.2.2 Mouse response to chamber implantation

2.2.3 Chamber persistence in vivo
2.2.4 Summary of overall chamber usage during development and subsequent experimentation

2.3 Discussion

Chapter 3 – Experimental *Pseudomonas aeruginosa* infection studies in chamber implanted mice

3.0 Introduction

3.1 Materials and Methods

3.1.1 Chamber implantation

3.1.2 General methods

3.1.3 Estimation of VF concentration in mouse samples

3.1.3.1 ETA concentration in chamber exudate and organs

3.1.3.2 PAE concentration in chamber exudates

3.1.3.3 PLC enzymatic activity in chamber exudates

3.2 Results

3.2.1 General condition of mice following infection

3.2.2 *P. aeruginosa* replication in the chamber of normal and CP treated mice

3.2.3 Local cellular & physiological responses to *P. aeruginosa*

3.2.3.1 Total leukocytes in chamber exudate of normal and CP treated mice

3.2.3.2 Total & differential leukocytes in chamber exudate of normal mice infected with *P. aeruginosa*

3.2.3.3 Chamber exudate volume and protein concentration in normal mice infected with *P. aeruginosa*

3.2.3.4 Tissue pathology of normal and CP treated mice infected with *P. aeruginosa*

3.2.4 *P. aeruginosa* bacteraemia in CP treated mice

3.2.4.1 Viable *P. aeruginosa* from internal organs

3.2.4.2 Liver abscesses
3.2.5 Virulence factors in organs and chamber exudate of mice 67

3.2.5.1 ETA in organs of CP treated mice 67

3.2.5.2 VFs in the chamber exudate 69

3.3 Discussion 71

3.3.1 General health of normal control and CP treated mice following sub-dermal infection by P. aeruginosa 71

3.3.2 Normal immune response to P. aeruginosa infection 72

3.3.3 Cellular response by CP treated mice to P. aeruginosa infection 74

3.3.4 Histopathology of normal control and CP treated mice infected by P. aeruginosa 74

3.3.5 P. aeruginosa bacteremia in CP treated mice 75

3.3.6 ETA in organs of CP treated mice 75

3.3.7 P. aeruginosa & VF concentration in the exudate of CP treated mice 76

3.3.8 Anatomical and immunological functions of the fascia 77

3.3.9 Limitations of the study 78

3.3.10 Conclusion 78

Chapter 4 – In vivo studies of Pseudomonas aeruginosa virulence factors

4.0 Introduction 80

4.1 Materials and Methods 83

4.1.1 Transdermal chamber implantation and sampling 83

4.1.2 Dextran preparations 83

4.1.3 Fluorometric analysis 83

4.1.3.1 Selection of excitation and emission filters 83

4.1.3.2 Chamber exudate analysis 83

4.1.4 Purification of PAE 85

4.1.4.1 Selection of optimal PAE producing strain 85

xxiii
4.1.4.2 Purification of PAE from culture

4.1.4.2.1 Protein fractionation on DEAE sepharose

4.1.4.2.2 Protein separation on Sephacryl S-200

4.1.4.2.3 SDS- PAGE analysis of purified PAE

4.1.4.3 Removal of pyrogens

4.1.5 ETA

4.1.5.1 Purity

4.1.5.2 Cytotoxicity

4.1.6 PLC

4.2 Results

4.2.1 Fluorescently labelled dextrans; studies in control mice

4.2.1.1 Chamber fluid

4.2.1.2 Tissues

4.2.3 The effect of PAE on superficial fascia

4.2.3.1 Establishing PAE dose and duration of treatment

4.2.3.1.1 Fluorescently labelled dextrans in chamber fluid

4.2.3.1.2 Exudate volume

4.2.3.2 Analysis of chamber exudate following PAE treatment

4.2.3.2.1 Fluorescently labelled dextrans in chamber fluid

4.2.3.2.2 Exudate volume

4.2.3.2.3 Cellular content of chamber exudates

4.2.3.3 FLD in organs and local draining lymph nodes

4.3.3.4 Histological examination

4.2.4 The effect of ETA on superficial fascia

4.2.4.1 General health of mice
4.2.4.2 Tracking of fluorescently labelled dextrans

4.2.4.2.1 Chamber fluid

4.2.4.2.2 Brachial lymph nodes

4.2.4.3 Analysis of chamber exudate

4.2.4.3.1 Exudate volume

4.2.4.3.2 Cellular content

4.2.4.4 Histological examination

4.2.5 The effect of ETA and PAE applied in combination to the fascia

4.2.5.1 Tracking fluorescently labelled dextran

4.2.5.1.1 Chamber fluid

4.2.5.1.2 Brachial lymph nodes

4.2.5.2 Analysis of chamber exudate

4.2.5.2.1 Volume

4.2.5.2.2 Cellular content

4.2.5.3 Histological examination

4.2.5.4 General health of mice

4.2.5.4.1 Weight

4.2.5.4.2 Haemoglobinemia

4.2.6 Pathogenesis of ETA⁻/PAE P. aeruginosa strain PA103

4.3 Discussion

4.3.1 Selection and use of fluorescent dextrans

4.3.2 PAE treatment

4.3.3 ETA treatment

4.3.4 Effect of combined ETA and PAE treatment on host tissue and cells

4.3.5 Significance of fascia during P. aeruginosa infection
4.3.6 Conclusion

Chapter 5 – Passive immunotherapy of experimental *P. aeruginosa* infections in chamber implanted mice

5.0 Introduction

5.1 Materials and Methods

5.1.1 Bacteria

5.1.2 Antigens

5.1.2.1 Lipase and ETA

5.1.2.2 PAE preparation

5.1.2.3 Flagella preparation

5.1.2.4 Protease

5.1.3 Animals

5.1.3.1 Mice

5.1.3.1.1 Transdermal chamber implantation

5.1.3.1.2 Immunosuppression

5.1.3.1.3 Serum protection experiments

5.1.3.2 Sheep

5.1.3.2.1 Immunisation

5.1.4 Anti-sera and whey preparation for mouse protection experiments

5.1.4.1 Serum preparation

5.1.4.2 Whey preparation

5.1.4.3 Analysis of sheep serum/whey for confirmation of antibodies

5.1.4.4 Heat inactivation of complement in immune serum

5.1.4.5 Combination sheep anti-sera for mouse protection experiments

5.1.5 In vitro neutralisation of ETA and PAE by sheep IgG
5.1.5.1 Purification of sheep IgG
5.1.5.2 Neutralisation of ETA
5.1.5.3 Neutralisation of PAE

5.2 Results

5.2.1 Combination anti-sera for preliminary protection experiments
5.2.2 Confirmation of sheep antibodies to P. aeruginosa antigens
5.2.3 Protection by natural antibodies in sheep serum
5.2.4 Protection by anti P. aeruginosa sheep serum

5.2.4.1 Serum treatment 1:20 dilution
5.2.4.1.1 Bacterial replication in the chamber
5.2.4.1.2 Weight loss
5.2.4.1.3 Survival

5.2.4.2 Serum treatment 1:50 dilution
5.2.4.2.1 Bacterial replication in the chamber
5.2.4.2.2 Weight loss
5.2.4.2.3 Survival

5.2.6 In vitro neutralisation of PAE and ETA by purified sheep IgG

5.3 Discussion

5.3.1 Heterologous Polyclonal Antibodies to P. aeruginosa infection
5.3.2 Neutralising anti-ETA/PAE IgG
5.3.3 Concluding remarks

Chapter 6 – General discussion

6.1 Introduction
6.2 Fascia
6.2.1 Fascia as part of a systemic defence network 167

6.2.2 Fibroblast sensitivity to Exotoxin A 168

6.3 Primary function of neutrophils during *P. aeruginosa* dermal infection 169

6.4 Biofilm in the transdermal chamber model of infection 170

6.5. *P. aeruginosa* elastase (PAE) activity on components of fascia 170

6.6 Exotoxin A intoxication of cells within the fascia 171

6.7 Combinatorial effect of Exotoxin A and elastase on fascia 173

6.8 Protection from *P. aeruginosa* by heterologous polyclonal antibodies 174

6.9 Comments on Phospholipse C-H 175

6.10 Conclusion 176

Chapter 7 – References 177

Appendix A – General materials and methods

Part 1 General Methods

A.1 Animals 208

A.2 Transdermal chamber implantation & exudate sampling 208

A.3 Immunosuppression 208

A.4 Bacterial preparations 208

A.5. Leukocytes in chamber exudate- viable and differential 209

A.5.1 Viable leukocytes 209

A.5.2 Differential cell counts 209

A.6 Histology 210

A.7 Estimation of protein concentration 210

A.8 Statistical analysis 210

Part 2 Buffers and solutions 211
Appendix B – Purification of Phospholipase C

B.1 Introduction 213

B.2 Materials and Methods 214
  B.2.1 Bacteria 214
  B.2.2 Media and incubation 214
  B.2.3 Preparation of broth supernatant analysis & fractionation 215
  B.2.4 Fractionation on celite 215
  B.2.5 PLC Activity 216
    B.2.5.1 PLC enzymatic activity 216
    B.2.5.2 PLC haemolytic activity 216

B.3 Results 217
  B.3.1 Activity of Stinson’s Minimal Media with supplements 217
  B.3.2 Fractionation and PLC-H activity of SMM with supplements 217
  B.3.3 Concentration of culture supernatant 218

B.4 Discussion 224