The Role of *Mycobacterium avium* ss *paratuberculosis* (MAP) in patients with Crohn’s Disease.

Jacqueline Turton

BSc (Hons)

Doctor of Philosophy (PhD)

December 2011
Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.
Acknowledgements

I would like to firstly thank my supervisor, Emeritus Prof. Robert Clancy, for his guidance and patience throughout my PhD project. His understanding of my personal circumstances and belief in me enabled me to complete this thesis. Thank you Robert.

I wish to thank Dr Zhigang Ren, for his help in the continued collection and processing of samples whilst I was on maternity leave. Also, thank you Ren for your technical support in the lab and also with the statistical analysis of my raw data.

Thank you to Dr Thomas Borody of the Centre for Digestive Diseases, Five Dock NSW, for his invaluable contribution. Without his passion for helping people with Crohn’s disease and other bowel disease, this thesis would not have happened. He was able to supply me biological samples from his patients and their clinical data. Thank you also to the staff of the Centre for Digestive Diseases which help prepare the ethics applications and provide all the samples for this project. A special mention to Mrs. Evelyn Vigano for her skillful technical assistance in the CDD laboratory.

Thank you to Professor John Herman-Taylor for supplying our positive PCR control, IS900 + plDL60.

A big thanks to my friend Kath Pink for her help in proof reading this thesis, you were a life saver Kath!

Thanks you to Mr Patrick McElduff, HMRI Biostatistician, for his expertise and advice concerning my statistical analysis.

Thank you to Dr Ira Shafran at Department of Molecular Biology and Microbiology and Center for Discovery of Drugs and Diagnostics, University of Central Florida, Orlando, Florida USA who
analysed serum samples for me using techniques he has developed to identify MAP infected patients using *M. paratuberculosis* antigens as serological markers (p35 and p36).

Thank you to the Mark Turner and Leslie Reddacliff from the Elizabeth Macarthur Agriculture Institute (Camden, NSW, Australia) for providing us with soluble MAP antigens.

Lastly, I must thank my family…my husband Richard, my beautiful children Flynn and Claire, and my parents Sandra and Ian Turton. They have put up with me over the course of this thesis and without them I wouldn’t have been able to continue…..Love you all xx

It hasn’t been smooth sailing for me during this thesis…I got married, gave birth to two beautiful, amazing kids who I love more then anything. In January 2006 we almost lost our son from undiagnosed Type 1 Diabetes at 14 months, which completely turned our world upside down. But I am proud to say I’ve finally finished!!
Dedication

I dedicate this thesis to my two beautiful children Flynn and Claire....I love them with all my heart.
List of publications


ABSTRACT

The cause of chronic inflammation in the gut of subjects with Crohn’s Disease (CD) is unclear; however most would agree that 3 interacting factors are critical to mucosal inflammation: genetic susceptibility, enteric microflora and the host immune system. The most controversial theory is whether or not a particular microbe(s) infects and maintains in intestinal tissues, resulting in chronic inflammation. The most discussed microbe is Mycobacterium avium ss paratuberculosis (MAP). The aim of this thesis was to better document the natural history of MAP infection in subjects with Crohn’s disease, IBS, “non-Crohn’s colitis” and normal subjects, correlating clinical status with parameters relating to MAP, to the mucosal T cell response and to the genetic susceptibility gene CARD15/NOD2.

MAP is an obligate intracellular pathogen, which causes chronic inflammation in the intestine of many species, including primates. MAP was first identified as the causative agent in Johnes Disease, a gastrointestinal disease in ruminants and primates. The infection of domestic livestock with MAP is now widespread, which has increased the risk of transmission of MAP to humans via milk products. The involvement of MAP in human patients with Crohn’s disease (CD) has been difficult to prove, due to the difficulties in isolating and detecting this organism.

By using nested PCR (Polymerase Chain Reaction) on DNA extracted from fresh human intestinal mucosal biopsy samples the presence of MAP can be verified. Bacteria contain unique insertion elements, which play a role in virulence, pathogenicity and antibiotic resistance. Within MAP a unique 1.4 kb insertion element was identified, IS900. By using DNA primers for PCR within this unique insertion element we were able to identify MAP in tissue. There was found to be no statistical significance among the disease groups tested.

The next objective was to determine whether the presence of MAP in gut biopsies was associated with a different cytokine secretion profile as observed in organ and whole blood culture, using an ELISA assay. Significantly higher levels of TNF-α
were found in culture supernatants from organ culture for Crohn’s Disease when compared to ulcerative colitis (p<0.05), irritable bowel (p<0.01) and controls (p<0.0001). When TNF-α levels were correlated with the presence of MAP, significantly greater concentrations were only found in MAP positive Crohn’s Disease patients (p<0.05). In whole blood culture significantly higher levels of IL-4 (p<0.05) and IL-2 (p<0.05) were found in MAP positive Crohn’s Disease patients compared to MAP negative CD, which is consistent with a skewed Th2 immune response. This data provided the first evidence of an abnormal macrophage handling of MAP and an altered T cell function linked to MAP infection.

Finally, the co-existence of MAP infection and NOD2/CARD15 mutation status was investigated. SNP analysis of the three most common NOD2/CARD15 variants: missense mutations R702W (2104C→T, SNP8) and G908R (2722G→C, SNP12) and the frameshift mutation 1007fs (3020insC, SNP13); was performed on trial participants and compared to MAP status to determine if genetic susceptibility to CD predisposes them to MAP infection. Analysis of CD patients NOD2 status and cytokine profile found no correlation. Given that there is no clear link between NOD2 gene mutation, presence of MAP and cytokine secretion in our CD patients the suggestion that defective handling of MAP is due to NOD2/CARD15 gene mutations is not relevant to our group.

We can conclude that

1. MAP is not essential for CD,
2. That MAP is present in IBD and non-IBD patients,
3. There is a defect in the cellular handling of MAP in CD and
4. MAP in CD has the capacity to enhance drive a Th2 response, which in turn down regulates the protective Th1 response and enhances mucosal permeability which leads to increased inflammation.
Table of Contents

CHAPTER ONE: Literature Review.
1.1 Crohn’s Disease 1
1.2 Pathogenesis of Crohn’s Disease 2
1.3 *Mycobacterium avium* ss *paratuberculosis* – MAP 3
1.4 Immune response in Crohn’s Disease 14
1.5 CARD15/NOD2 gene mutations 16
1.6 Hypothesis 19

CHAPTER TWO: MAP Detection and Culture from Mucosal Biopsies.
2.1 Introduction 21
2.2 Materials and Methods 23
  2.2.1 Mucosal Biopsy Specimen Collection 23
  2.2.2 DNA Extraction from Mucosal Biopsies 24
  2.2.3 IS900 + pDIL60 Plasmid Preparation 26
  2.2.4 IS900 Nested PCR 27
  2.2.5 DNA Sequencing 30
  2.2.6 Culture of Mucosal Biopsy Specimens 33
  2.2.7 Analysis of Sera by Recombinant p35 and p36 34
2.3 Results 35
  2.3.1 IS900 PCR Results 35
  2.3.2 IS900 Detection in Biopsy Specimens 37
  2.3.3 Mucosal Biopsy Culture 37
2.3.4 Analysis of Sera by p35 and p36 Antigens

2.4 Discussion

CHAPTER THREE: Pattern of Cytokine Secretion in Crohn’s Disease Patients

3.1 Introduction

3.2 Materials and Methods

3.2.1 Organ Culture

3.2.2 Preparation of MAP Antigen for Culture Stimulation

3.2.3 Whole Blood Culture

3.2.4 Whole Blood Culture and IL-4 Cytokine ELISA

3.2.5 Cytokine ELISA – IL-2, IFN-γ, IL-10, IL-2 and TNF-α

3.2.6 Statistical Analysis

3.3 Results

3.3.1 Organ Culture

3.3.2 Cytokine Secretion from Organ Culture

3.3.3 Whole Blood Culture

3.3.4 Cytokine Secretion in Whole Blood Culture

3.3.5 Effect of MAP Antigen on Cytokine Secretion

3.4 Discussion
CHAPTER FOUR: Does the Presence of MAP Alter the Cytokine Secretion Profile.

4.1 Introduction 68
4.2 Materials and Methods 69
4.2.1 Whole Blood Culture and IL-4 ELISA 70
4.2.2 Statistical Analysis 75
4.3 Results 76
4.3.1 IL-4 Secretion in Whole Blood Culture 76
4.3.2 IL-2 Secretion from Whole Blood Culture 78
4.3.3 IFN-γ and TNF-α Secretion in whole blood culture 78
4.3.4 Effect of MAP Antigen on Cytokine Secretion 78
4.3.5 Cytokine secretion in organ culture in relation to MAP status 80
4.4 Discussion 83

CHAPTER FIVE: Analysis of CARD15/NOD2 Polymorphisms by Real-Time PCR.

5.1 Introduction 86
5.2 Materials and Methods 90
5.2.1 Preparation of DNA 90
5.2.2 CARD15/NOD2 SNP Analysis 93
5.2.3 Allelic Discrimination Analysis Procedure using ABI Prism7900HT Sequence Detection System 95
5.2.4 Statistical Analysis 98
### 5.3 Results

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3.1 CARD15/NOD2 Mutation Analysis</td>
<td>100</td>
</tr>
<tr>
<td>5.3.2 CARD15/NOD2 SNPs and MAP Status</td>
<td>103</td>
</tr>
<tr>
<td>5.3.3 CARD15/NOD2 and Cytokine Analysis</td>
<td>105</td>
</tr>
</tbody>
</table>

### 5.4 Discussion

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER SIX: FINAL DISCUSSION</td>
<td>114</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>122</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>153</td>
</tr>
<tr>
<td>1. Solutions</td>
<td>153</td>
</tr>
<tr>
<td>2. Published papers</td>
<td>162</td>
</tr>
</tbody>
</table>