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Proteomic and Cell Based Approaches for the study of the Intestinal Epithelial Response to *Helicobacter pylori* Soluble Components in an *Ex Vivo* Model of Ulcerogenesis

A thesis submitted to the University of Dublin, Trinity College

for the degree of

Doctor of Philosophy (Ph.D.)

by

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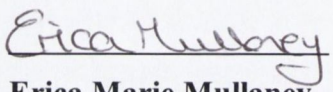
2007

DECLARATION

I hereby declare that this thesis has not been previously submitted for a degree at this or any other university. Except where otherwise stated the work described in this thesis is entirely my own. Part of the work described in Chapter V was carried out jointly with Dr. Sinead Smith and includes the unpublished and published work of others. Dr. Sinead Smith is duly acknowledged in the text where appropriate. I hereby give my permission to the library of Trinity College Dublin to lend or copy this thesis upon request for the purpose of study.



Erica Marie Mullaney

DEDICATION

THIS THESIS IS DEDICATED TO MY DAD, EUGENE MULLANEY WHO BELIEVED THAT SCIENCE IS THE ONLY TRUE RELIGION AND TO THE REST OF MY FAMILY WHO STILL BELIEVE THAT NOTHING WORTH DOING IS EVER EASY.

The sea is calm tonight
The tide is full, the moon lies fair upon the straits; on the French coast the light
Gleams and is gone; the cliffs of England stand,
Glimmering and vast, out in the tranquil bay.
Come to the window, sweet is the night air!
Only, from the long line of spray
Where the sea meets the moon-blanch'd land,
Listen! You hear the grating roar
Of pebbles which the waves draw back and fling'
At their return, up the high strand, Begin, and cease, and then again begin,
With tremulous cadence slow, and bring
The eternal note of sadness in.
Sophocles long ago heard it on the Aegean, and it brought
Into his mind the turbid ebb and flow
Of human misery; we find also in the sound a thought,
Hearing it by this distant northern sea.
The Sea of faith was once, too, at the full and round earth's shore
Lay like the folds of a bright girdle furl'd.
But now I can only hear its melancholy long withdrawing roar,
Retreating to the breath of the night-wind, down the vast edges drear
And naked shingles of the world.
Ah, love, let us be true to one another!
For the world which seems to lie before us like a land of dreams,
So various, so beautiful, so new,
Hath really neither joy, nor love, nor help for pain;
And we are here as on a darkling plain
Swept with confused alarms of struggle and flight,
Where ignorant armies clash by night.

Mathew Arnold (1867) Dover Beach

ACKNOWLEDGEMENTS

I would like to thank my supervisors: Professor Dermot Kelleher, Dr Henry Windle and Dr Ana Terres. Dermot, over the past 4 years you have been a wonderful teacher, mentor, supervisor and role model. Your enthusiasm for this project never waned and my gratitude and appreciation never will. Ana, you started me on this scientific path and Henry, you brought me to the finish line. Without you both it would never have been done.

I have learned by working in the very excellent Kelleher research group that a PhD is so much bigger than just one person is. Therefore, sincere thanks are due to so many of the following people.

Sincere thanks and gratitude are due to Dr Sinead Smith. Sinead, the work we did together was some of the most satisfying and enjoyable in all my time in the IMM. Being in the lab with you was always fun and I will really miss your patience, elegance and poise. You are the scientist I hope to be.

To the post-doc girls: Dr Deirdre Kelly, Dr Seonadh O'Leory and Dr Avril O'Riordan. Thank you for letting me be a part of Ladies That Lunch and thank you all for your individual advice and guidance over the past few years. It has made all the difference.

To Drs Mary O' Sullivan and Deirdre Ni Eidhin, I express extreme gratitude and thanks. You were both so selfless with your time and help from designing experiments with me to putting on the kettle and listening to me rant. A million thank yous!

To Drs Karen Brophy and Megan Dring – I know I will never have friends quite like you both again in my life. Things were never quite the same after you guys left.

To Miss Denise Cornally – girl. I would never have survived the last year without you. By December 2006, I could not move unless I had consulted you first! To all the gossiping and side-splitting laughter! Good luck missus!

To Miss Danijela Petrovic – my fellow cat fancier! Lola, keep at it. You've not long to go. You were a real breath of fresh air (and perfume) in the lab.

To Dr Anthony Davies – Tony, I never thought I would meet someone whom so accurately mirrored my thoughts back at me. "If you've nothing good to say about anyone, sit beside me!"

To all the new crew – Drs Michael Freeley, Stephen MacDonald, Eugene Dempsey, Peter Olwell and his beautiful white cells, Ang Seow Theng, Emma Lynch, Anne Marie Byrne, Jim Harris, Mr Ramon Vincente, Miss Stephanie Toetsch and Miss Jennifer Conroy and her confocal. You guys really filled up the place with laughter and fun!

To all the old crew, some gone, some remain – Drs Shane Duggan, Veronica Athie Morales, Siobhan Mitchell, Julie O' Brien, Denis Finn, Arun Chandran, Arun Mankan, Emma Cummins, Eileen Looby, Tony Ryan, Kaukab Azim, Basma El Shariff and Mr Graham Turner. To all the Immunology, Histo and T+H crew who were such good pals to me!! Thanks to Jodie Brunneau, Sarah Cooper, Sue Cahill, Stacey Kelly, Cathal O'Brien, Yvonne Williams and Karen Denning.

To Drs Aideen Long, Yuri Volkov and Ross McManus – thanks for everything!

To Dr Anne Murphy – thanks for all the blood taking and for all the tea time chats!

To Tharma – thanks for doing such a great job and for all the Christmas parties!

To all the doctors and nurses in the Endoscopy unit of St James's Hospital, especially the members of Dermot's team. Thank you for all your collaborations and your collective interest in the project. Thanks are especially due to Nasir, Ali and Zaheer.

To Drs Cathal Connolly and Cepta Duffy who knew me in a former life. Thanks for your continued support and encouragement.

To Olive, Paul and Pauline at the front desk for keeping us all safe!

To my dad, who didn't live to see this opus complete. "Your words in my memory are like music to me"

To my family, who felt every minute that I worked on this PhD. Thank you for staying the distance.

To Webbie – I love you babe! Thanks for caring…but not too much! Here's to us! "Nobody's listening!"

Finally, times of emotional upheaval, grief and loss can really bind you to a place and this is how I feel about the IMM. My work, the place and the people who worked alongside me helped me (unwittingly) through one of the darkest times of my life. Loss can really hollow you out but you all filled me back in. I'll never forget it.

SUMMARY

The aim of the present study was to investigate the effects of secreted components of the gastrointestinal pathogen *H. pylori* on the duodenal epithelium in humans. The effects of *H. pylori* are not only mediated by direct interaction with the organism, but also by released bacterial elements, known as outer membrane vesicles (OMV) entering the duodenum. The primary aim of this study was to proteomically and functionally characterize *H. pylori* OMV. The proteome of the OMV was identified using SDS-PAGE, LC-MS/MS and Western blotting techniques. The biological activity of specific identified proteins were assessed. The putative *H. pylori* virulence factor vacuolating cytotoxin A (VacA) present in the OMV was found to induce vacuolation in gastric epithelial cells. The neutrophil activating protein A (NapA) also identified within the OMV was solely responsible for neutrophil migration. Furthermore, exposing T84 and AGS epithelial cells to OMV induced a dose dependent increase in IL-8 secretion.

For this study T84 cells were grown in confluent monolayers, on semi permeable inserts, and apically stimulated with OMV in an *in vitro* model of infection. As an aim of this project was to look at the initial effects of OMV stimulation on duodenal biopsies in an *ex vivo* model of ulcerogenesis, this was an appropriate method by which this technique could be validated. Results from this study demonstrated that OMV, which are constantly shed from the surface of *H. pylori* in vivo, have a demonstrable effect on fully differentiated T84 cells. Internalisation of OMV was observed after 1 – 2 hours, changes at the protein level of the host cells after 24 hours and epithelial cell IL-8 production. However, neither apico-basal transfer of OMV, nor any effect on barrier function or dome formation in mature monolayers of T84 cells after 24 hours were not observed. These findings support the theory that OMV play a role in promoting host responses to support persistence of *H. pylori* in the human gastric mucosa.

With respect to *H. pylori*, the intestinal epithelial cell layer is thought to represent the first line of defence and the trigger for host innate and immune inflammatory responses. It has been demonstrated that intestinal epithelial cells constitutively express several Toll-like receptors (TLRs) in vitro and in vivo. It has been widely reported that epithelial cells respond to *H. pylori* infection at least in part via TLR2 and TLR5. It has not been reported to date as to which TLRs are involved in epithelial cell recognition of OMV. Outer membrane proteins (OMPs) present in all vesicles are all biologically active molecules that can activate immune cells via TLRs and induce neutrophil

migration. In this study the data demonstrates that infection of epithelial cells in vitro with *H. pylori* OMV induces IL-8 cytokine responses primarily via TLR2 and via TLR4 to a much lesser degree. This is the first study to investigate the role of TLRs in the response of epithelial cells to *H. pylori* OMV by examining cytokine responses at the mRNA and protein level of expression.

Finally, this study investigated the effects of *H. pylori* OMV on human duodenal explants using an ex vivo model to assess the acute response of the duodenal epithelium to OMV. Using this model, explant cytokine responses to OMV stimulation were measured. IL-8 was maximally secreted between 12 and 24 hours. In the explant culture model, background production of IL-8 was consistently detected in explants not treated with *H. pylori* OMV. However, the levels of IL-8 produced in the biopsies stimulated with *H. pylori* OMV were significantly higher at only 12 hours post stimulation. The data clearly show that *H. pylori* OMV can elicit an inflammatory response in duodenal biopsy specimens and that this response is not restricted to adherent *H. pylori*.

In this study, the composition of *H. pylori* OMV has been characterized proteomically and the biological activity of some of their key constituents were evaluated. The effects of OMV on epithelial cell function and the role of TLRs in the epithelial response to stimulation via OMV have also been investigated. An ex vivo model of infection has also been developed to further evaluate the effects of OMV on the human duodenum. Such data may in the future be utilised to help elucidate the role played by OMV in the complete pathogenesis of *H. pylori* and hence, assist in the development of new effective treatments.

TABLE OF CONTENTS

CHAPTER I 1

GENERAL INTRODUCTION 1

1.1 Historical background of *H. pylori* 2

1.2 Epidemiology 5

1.3 Bacterial Pathogenesis 7

1.4 *H. pylori* Virulence Factors 9

1.4.1 Cag Pathogenicity Island and CagA 10

1.4.2 Neutrophil activating protein (NapA) 12

1.4.3 Outer inflammatory protein A (OipA) 12

1.4.4 Vacuolating cytotoxin A (VacA) 12

1.4.5 Outer Membrane Proteins 13

1.5 Proteomic Analysis of Secreted Proteins of *H. pylori* 15

1.6 Clinical Presentation 15

1.6.1 Acute Gastritis 16

1.6.2 Chronic Gastritis 16

1.6.3 Duodenal Ulceration 17

1.6.4 Atrophy and Gastric Cancer 18

1.6.5 MALT and Low-Grade B-Cell Lymphoma 18

1.7 Diagnosis 18

1.8 Treatment 20

1.9 *H. pylori* Vaccines 20

1.10 Epithelial Cell Lines as Models of *H. pylori* Infection 23

1.11 Introduction to Outer Membrane Vesicles (OMV) 26

1.12 Host Immune Response to Infection 29

1.12.1 Acquired Immunity 30
1.12.2 Innate Immunity 31
1.12.3 TLR Signalling to *H. pylori* 34
1.12.4 TLR Independent Recognition of *H. pylori* 35

1.13 Rationale and Objectives of the Study 36

CHAPTER II 37

MATERIALS AND METHODS 37

2.1 REAGENTS 38

2.2 ANTIBODIES 39

2.3*H. PYLORI* CULTURE 39
2.3.1 *H. pylori* Strains and Growth Conditions 39
2.3.2 *H. pylori* Extract (HPE) Preparation 40
2.3.3 *H. pylori* Outer Membrane Vesicle (OMV) Preparation 40

2.4CELL CULTURE 42
2.4.1 Source of Cell Lines and Cell Culture Reagents 42
2.4.2 Maintenance of Cell Cultures 42
2.4.3 Total Cell Lysate Preparation 43
2.4.4 Antigen Immunoprecipitation 43
2.4.5 Determination of Protein Concentration 44
2.4.6 Cell Freezing and Revival 44
2.4.7 Cell Enumeration and Viability 45
2.4.8 Co-culture of Cells with *H. pylori* OMVs and Other Stimuli 45
2.4.9 Neutral Red Assay 46
2.4.10 Immunofluorescence Staining 46
Introduction to Cellomics™ HCS Kineticscan Technology 48
2.4.11 Isolation of Polymorphonuclear leukocytes (PMNs) 49
2.4.12 Isolation of Mononuclear Cells from Peripheral Blood (PBMCs) 49
2.4.13 Chemotaxis Assay 49

2.5POLYACRYLAMIDE GEL ELECTROPHORESIS **50**
2.5.1Sample and Molecular Weight Standard Preparation 50
2.5.2 1D SDS-PAGE 50
2.5.3 Coomassie Blue Staining 51
2.5.4 Silver Staining of Protein or LPS 52
2.5.5 Western Blotting 53
2.5.6 Immunodetection and Development 53
2.5.7 Stripping of Western Blot Membranes 54
2.5.8 Sample Preparation for SDS-PAGE 54
2.5.9 Two – Dimensional Gel Electrophoresis (2-DE) 56
2.5.10 Scanning and Analysis of Gels 57
2.5.11 Peptide Mass Fingerprinting. 57
2.5.12 In-House Database Searching. 58
2.5.13 On-Line Database Searching. 58

2.6 RIBONUCLEIC ACID (RNA) EXTRACTION AND ANALYSIS **58**
2.6.1 RNA Extraction from cultured cells 58
2.6.2 Reverse Transcription (RT) 59
2.6.3 Polymerase Chain Reaction (PCR) 60
2.6.4 Agarose Gel Electrophoresis 61
2.6.5 Real Time PCR Analysis 62

2.7 ENZYME LINKED IMMUNOSORBENT ASSAYS **62**
2.7.1 Detection of Secreted IL-8 62
2.7.2 Detection of Secreted IL-12 64
2.7.3 Detection of RANTES 64
2.7.4 *H. pylori*ELISA 64
2.7.5 Cytokine and Growth Factors Array 64
Introduction to RANDOX evidence investigator ™ Technology 65

2.8 CLONING AND PURIFICATION OF NAPA FOR ANTIBODY PRODUCTION **66**
2.8.1 NapA Cloning and Purification 66
2.8.2 Antibody Production 66

2.9 CLINICAL SAMPLES **67**

2.9.1 Collection of Duodenal Biopsy Samples 67

2.9.2 Detection of Viable and Non-Viable Cells in Duodenal Explants 67

2.9.3 Culture of Human Duodenal Explants 67

2.9.4 Inoculation of Human Duodenal Explants 68

CHAPTER III **69**

PROTEOMIC AND FUNCTIONAL CHARACTERISATION OF *HELICOBACTER. PYLORI* OUTER MEMBRANE VESICLES **69**

3.1 INTRODUCTION **70**

3.2 OBJECTIVES **71**

3.3 RESULTS **71**

3.3.1 Isolation of *H. pylori* OMV. 71

3.3.2 Identification of Proteome of *H. pylori* OMV 73

3.3.3 Assessing the Biological Activity of Virulence Factors Identified 78

3.4 DISCUSSION **85**

CHAPTER IV **89**

THE EFFECTS OF *HELICOBACTER PYLORI* OUTER MEMBRANE VESICLES ON COLONIC EPITHELIAL CELLS: AN *IN VITRO* MODEL **89**

4.1 INTRODUCTION **90**

4.2 OBJECTIVES **91**

4.3 RESULTS **91**

4.3.1 Internalisation of OMV in Epithelial Cells 91

4.3.3 Effects of OMV on Tight Junctions and Barrier Function in Epithelial Cells 96

4.3.4 OMV Do Not Induce Dome Formation 99

4.3.6 Induction of Cytokine Expression in T84 Epithelial Cells by OMV 107

4.4 DISCUSSION **111**

CHAPTER V **115**

THE ROLE OF TOLL – LIKE RECEPTORS IN THE RESPONSE OF EPITHELIAL CELLS TO *HELICOBACTER PYLORI* OUTER MEMBRANE VESICLES **115**

5.1 INTRODUCTION **116**

5.2 OBJECTIVES **117**

5.3 RESULTS **117**

5.3.1 Toll-like Receptor mRNA Expression in AGS, T84 and HEK Epithelial Cell Lines 117

5.3.2 *H. pylori* OMV Induce IL-8 Expression in HEK Cell Lines 121

5.3.3 *H. pylori* OMV, *H. pylori* LPS and *E. coli* LPS Induce IL-8 Expression in Polarized Intestinal Epithelium (T84) 126

5.3.4 *H. pylori* OMV Induce IL-8 Expression in Gastric Epithelial Cells (AGS) 129

5.3.5 Pre-incubating *H. pylori* OMV with Polymyxin B / NapA anti-serum Fails to Inhibit Induction of IL-8 Expression in HEK TLR2 Cells 131

5.4 DISCUSSION **136**

CHAPTER VI **139**

IMMUNE AND EXPLANT TISSUE RESPONSES TO *HELICOBACTER PYLORI* OUTER MEMBRANE VESICLES **139**

6.1 INTRODUCTION **140**

6.2 OBJECTIVES **141**

6.3 RESULTS **141**

6.3.1 Biopsy Collection and Patient Criteria 141

6.3.2 Assessment of Viability of *Ex Vivo* Cultured Tissue 141

6.3.4 Induction of Cytokine Expression in Duodenal Explants by *H. pylori* OMV 144

6.3.5 Immunoblotting of *H. pylori* OMV Probed with Sera from *H. pylori* Infected and Uninfected Individuals 147

6.3.6 *H. pylori* OMV do not Stimulate IL-12 Production in Neutrophils or PBMCs149

6.4 DISCUSSION 151

CHAPTER VII 153

GENERAL DISCUSSION 153

7.1 Background 154

7.2 Proteomic and Functional Characterization of *H. pylori* OMV 157

7.3 Effects of OMV on Colonic Epithelial Cells: an *in vitro* model 157

7.4 The Role of Toll-like Receptors (TLRs) in the Epithelial Cell Response to OMV Stimulation 159

7.5 Immune and Duodenal Explant Responses to *H. pylori* OMV 160

7.6 Future Work 161

7.7 Concluding Remarks 165

APPENDICES 165

REFERENCES 177

LIST OF TABLES

Chapter 1

1.1 The story of the discovery of *H. pylori* 4

1.2 The number of patients attending for 13C-urea breath test at St James's Hospital in 2006 7

1.3 Putative virulence factors of *H. pylori* 10

1.4 Comparison of characteristics of primary cultures and cell lines as in vitro models of *H. pylori* infection 24

1.5 List of Toll-like receptors identified to date and their known ligands 33

Chapter 2

2.1 Composition of resolving gel for SDS-PAGE 50

2.2 Composition of stacking gel for SDS-PAGE 51

2.3 Composition of resolving gel for two dimensional gel electrophoresis 57

2.4 Reverse transcription PCR heat denaturing and annealing 59

2.5 Reverse transcription reaction master mix 59

2.6 PCR reaction mix 60

2.7 Primer sequences for PCR 60

2.8 Real time PCR reaction mix 62

Chapter 3

3.1 Outer membrane proteins identified within the proteome of OMV 75

3.2 Virulence factors identified within the proteome of OMV 76

3.3 Other proteins identified within the proteome of OMV 77

Chapter 4

4.1 Five host proteins differentially regulated by stimulation with OMV 106

LIST OF FIGURES

Chapter 1

1.1 *H. pylori* 2

1.2 Annual number of papers published on the subject of *H. pylori* 3

1.3 Incidence of *H. pylori* world-wide 6

1.4 Diagram of human stomach 8

1.5 Hypothetical model of *H. pylori* type IV secretion machinery encoded in Cag PAI 11

1.6 Interactions of host and bacterial factors in pathogenesis of *H. pylori* 14

1.7 Possible clinical outcomes following infection with *H. pylori* 16

1.8 Biopsy being taken during endoscopy 19

1.9 Increasing occurrence of antibiotic resistant strains of *H. pylori* in UK 22

1.10 Increasing occurrence of drug resistant strains of *H. pylori* in US 23

1.11 Simple Columnar epithelial cells 25

1.12 Diagram of a tight junction sealing the paracellular space between adjoining epithelial cells 25

1.13 Model of vesicle biogenesis 27

1.14 OMV being shed from the surface of *H. pylori* 28

1.15 Innate and acquired immunity 30

1.16 Toll-like receptors and their ligands 34

1.17 TLR dependent and independent recognition of microbial components 35

Chapter 2

2.1 *H. pylori* colonies following anaerobic culture on Columbia blood agar plate 41

2.2 Cellomics™ Kineticscan HCS reader 48

2.3 Randox™ evidence investigator 65

2.4 Assembly of the insert cup (Transwell, Costar) and culturing of human explants 68

Chapter 3

3.1 Preparation and analysis of *H. pylori* outer membrane vesicles 72

3.2 Verification of presence of selected proteins in OMV by immunoblotting 78

3.3 OMV induce vacuolation in AGS cells 80

3.4 OMV stimulate IL-8 production in both gastric and colonic epithelial cells 82

3.5 OMV induce chemotaxis of human neutrophils through NapA 84

Chapter 4

4.1 (a) OMV internalized within epithelial cells 92
(b) Control image showing no non-specific binding 93
4.2 OMV are not associated with intercellular endosomes 94
4.3 DAKO *H. pylori*antibody recognises OMV 95
4.4 (a) Measurement of TER in T84 monolayers 97
(b) T84 monolayers stained with anti-ZO-1 (Control) 98
(c) T84 monolayers stained with anti-ZO-1 (OMV) 98
(d) T84 monolayers stained with anti-ZO-1 (HPE) 98
4.5 Tight junctions stained with a fluoescent cell label and analysed by Cellomics™ HCS Kineticscan Reader for changes at the molecular level. 99
4.6 OMV do not induce dome formation in T84 monolayers, in comparison with HPE 101
4.7 *H. pylori* OMV have no significant effect on TER of T84 monolayers after 24 hours stimulation. In comparison, HPE induces a dramatic decrease in TER after 1 hour but TER values are restored to normal levels after 24 hours. 102
4.8 (a) 2-DE gels of control and treated T84 monolayers 104
(b) 2-DE gels of control and treated T84 monolayers 105
4.9 Immunoblotting for proteins, seen to be differentially regulated inT84 cell monolayers in response to *H. pylori* OMV stimulation. 107
4.10 *H. pylori* OMV induce IL-8 expression in polarized T84 cells both apically and basally 109
4.11 RANTES expression in polarized T84 cells in response to *H. pylori* OMV 110
4.12 T84 cytokine response to stimulation with *H. pylori* OMV 111

Chapter 5

5.1 Toll-like receptor expression profile in HEK cell lines 119
5.2 Expression of Toll-like receptor mRNA in T84 cell lines 120
5.3 Expression of Toll-like receptor mRNA in AGS cell lines 121
5.4 (a) E.coli LPS induced IL-8 mRNA in HEK TLR4 cells 123
(b) *H. pylori* LPS induced IL-8 mRNA in HEK TLR2 cells 124
5.5 *H. pylori* OMV induced IL-8 in HEK cell lines 125
5.6 *H. pylori* OMV induced protein IL-8 expression in HEK cell lines 126

5.7 Transepithelial electrical resistance (TER) of the T84 monolayers did not significantly decrease in response to stimuli 127
5.8 mRNA IL-8 induction in polarized T84 monolayers in response to stimuli 128
5.9 Protein IL-8 induction in polarized T84 monolayers in response to stimuli 129
5.10 *H. pylori* OMV induce IL-8 expression in AGS cells 130
5.11 Protein IL-8 expression in AGS cells in response to *H. pylori* OMV 131
5.12(a) Polymyxin B inhibits induction of IL-8 in HEK TLR2 cells in response to *H. pylori* LPS 133
(b) Polymyxin B inhibits induction of IL-8 in HEK TLR4 cells in response to *E. coli* LPS 133
5.13 Polymyxin B fails to inhibit IL-8 induction in HEK TLR2 cells stimulated with *H. pylori* OMV 134
5.14 NapA antiserum fails to inhibit IL-8 induction in HEK TLR2 stimulated with *H. pylori* OMV 135
5.15 *H. pylori* OMV pre-treated with combination of Polymyxin B and NapA antiserum does not appear to inhibit IL-8 induction significantly 136

Chapter 6

6.1 Cryosection of duodenal explant stained with the fixable fluoroprobes CMFDA and ethidium homodimer-1 144
6.2 IL-8 expression in duodenal explants stimulated with *H. pylori* OMV, from 4 to 24 hours 146
6.3 RANTES expression in duodenal explants stimulated with *H. pylori* OMV, from 4 to 24 hours 147
6.4 Immunoblotting with human sera for recognition response to *H. pylori* OMV 149
6.5 IL-12 expression in human neutrophils in response to stimulation with *H. pylori* OMV 150
6.6 IL-12 expression in human PBMCs in response to stimulation with *H. pylori* OMV 151

ABBREVIATIONS

$Al(OH)_3$ Aluminium hydroxide

$AlPO_4$ Aluminium phosphate

ANOVA Analysis of variance

APS Ammonium persulphate

BHI Brain heart infusion

BSA Bovine serum albumin

CaCl Calcium chloride

CagA Cytotoxin associated

CFSE 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester

CFU Colony forming units

CMFDA Chloro methyl fluorescein diacetate

CTL Cytotoxic T lymphocytes

DC Dendritic cell

DMSO Dimethylsulphoxide

ECL Enhanced chemiluminescence

EDTA Ethylene diamine tetra-acetic acid

ELISA Enzyme linked immunosorbent assay

FAB Fastidious anaerobe broth

FCS Foetal calf serum

FHA Filamentous hemagglutinin

FSC Forward scatter

GFP Green fluorescent protein

HBSS Hanks buffered salts solution

HEPES N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid

HCS High content screening

HpaA

HRP Horseradish peroxidase

H_2SO_4 Sulphuric acid

IFN-γ Interferon gamma

IgA Immunoglobulin A

IgG Immunoglobulin G

IgM Immunoglobulin M

IgY Immunoglobulin Y

IL Interleukin

i.n.Intranasal

i.p. Intraperitoneal

kDa Kilodalton

LAL Limulus amoebocyte lysate

LB Luria-Bertani media

LPS Lipopolysaccharide

LT *E. coli* heat-labile toxin

MAP Mitogen activated protein

$MgCl_2$ Magnesium chloride

MHC Major histocompatibility complex

MOI Multiplicity of infection

MPL Monophosphoryl lipid A

MW Molecular weight

MYD88 Myeloid differentiation factor 88

NaCl Sodium chloride

NaOH Sodium hydroxide

NapA Neutrophil activating protein A

NK Natural killer

OipA Outer inflammatory protein A

OMP Outer membrane proteins

OMV Outer membrane vesicles

OPD 1,2-phenylenediamine dihydrochloride

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate buffered saline

PCR Polymerase chain reaction

P*I* Isoelectric point

PMA Phorbol 12-myristate 13-acetate

PMC Pseudomembranous colitis

PMN Polymorphonuclear leukocyte

PRR Pathogen recognition receptor

PVDF Polyvinylidene difluoride

REA Restriction endonuclease analysis

REK Recombinant enterokinase

RFLP Restriction fragment length polymorphism

RNA Ribonucleic acid

SCC Side scatter

SD Standard deviation

SDS Sodium dodecyl sulphate

SEM Standard error of the mean

S-IgA Secretory IgA

S-layer Surface layer

SLP Surface layer protein

TAE Tris-acetate EDTA

TBE Tris-boric EDTA

TBS Tris buffered saline

TE Tris-EDTA

TEMED N,N,N',N'-tetramethlyenediamine

TDM Trehalose dicorynomycolate

TGF-β Transforming growth factor beta

Th1 T-helper 1

Th2 T-helper 2

TLR Toll like receptor

TMB Tetramethylbenzidine

TMC N-trimethyl chitosan chloride

TNF Tumour necrosis factor

Tr T-regulatory

Tris Tris (hydroxymethyl) aminomethane

VacA Vacuolating cytotoxin A

PUBLICATIONS FROM THIS THESIS

Original Papers

- **Mullaney, EM**, Smith S, Botting, CH,, Yamaoka Y, Terres, AM, Kelleher, DP, Windle, HJ. Characterization of the Proteome of *H. pylori*Outer Membrane Vesicles. Manuscript submitted.
- **Mullaney, EM,** Smith, S, Windle, H, Kelleher, DP. The Role Of Toll-like Receptors in Epithelial Cell Response to *H. pylori* Outer Membrane Vesicles. Manuscript in Preparation.

Presentations at Scientific Meetings or Conferences

- **Mullaney, EM,** Windle, HJ, Zaheer, A, Webb, CS, Mahmmud, N, Alshareefy, A, Cretu, I, Jamal, S, Hamid, M, Terres, AM, Kelleher, DP. *H. pylori*Outer MembraneVesicles induce IL-8 secretion in cultured human gastric explants. Irish Society of Gastroenterologists, abstract, November 2006.
- **Mullaney, EM**, Botting, CH, Windle, HJ, Terres, AM, Kelleher, DP. Characterization of the Proteome of *H. pylori* Outer Membrane Vesicles. Gastroenterology Vol 130, Issue 4, Supplement S2, abstract, April 2006. Digestive Disease Week, Los Angeles, USA, May 2006.
- **Mullaney, EM**, Davies, AM, Williams, YE, Terres, AM, Kelleher, DP. Effects of secreted components of *H. pylori* on Gastric and Colonic Epithelial Cells. Society of General Microbiology, Oral Presentation, Dublin, April 2006
- **Mullaney, EM**, Terres, AM, Kelleher, DP. The Vacuolating Effects of *H. pylori* Outer Membrane Vesicles on Epithelial Cells. Irish Society of Gastroenterology, Oral Presentation, Coleraine, NI, September 2005
- **Mullaney, EM**, Terres, AM, Kelleher, DP. Development of systems suitable for proteomic analysis of duodenal biopsy tissue. The Faseb Journal, abstract, suppl.2, vol.19, Experimental Biology, San Diego, USA, March 200

CHAPTER I

GENERAL INTRODUCTION

1.1 Historical background of *Helicobacter pylori*

Gastric spiral organisms were first described in dogs in 1893 by Bizzozero, and Krienitz described similar organisms in the human stomach in 1906. These findings were viewed with some scepticism because most samples of these spiral organisms were obtained post mortem and thus contamination could not be ruled out (Axon, 1994). Until the culture of the microorganisms in 1982, physicians and microbiologists believed that the stomach was likely to be sterile, because of its acid 'milieu', and ignored the findings of previous animal and human studies. Real interest by the medical community in these 'curved bacilli' was aroused only in 1983-1984 after the publication by Marshall and Warren in the Lancet (Marshall and Warren, 1984). The isolation of *Camplyobacter pyloridis* (Marshall, 1988), as it was originally termed, and the attempt to fulfil Koch's postulates (Marshall et al., 1985), opened a new era in the understanding of gastroduodenal pathology, mainly of gastritis and peptic ulcer. In 1989, Goodwin et al., demonstrated that this bacteria did not belong to the genus *Camplyobacter* and renamed it *H. pylori* from its helical appearance *in vivo* (Fig 1.1) and the most common site of isolation, the pylorus of the stomach (Goodwin et al., 1989).

Figure 1.1 – *Helicobacter pylori*

The interest in *H. pylori* is reflected by the increasing number of articles listed in the medical database Pubmed/Medline (Fig 1.2).

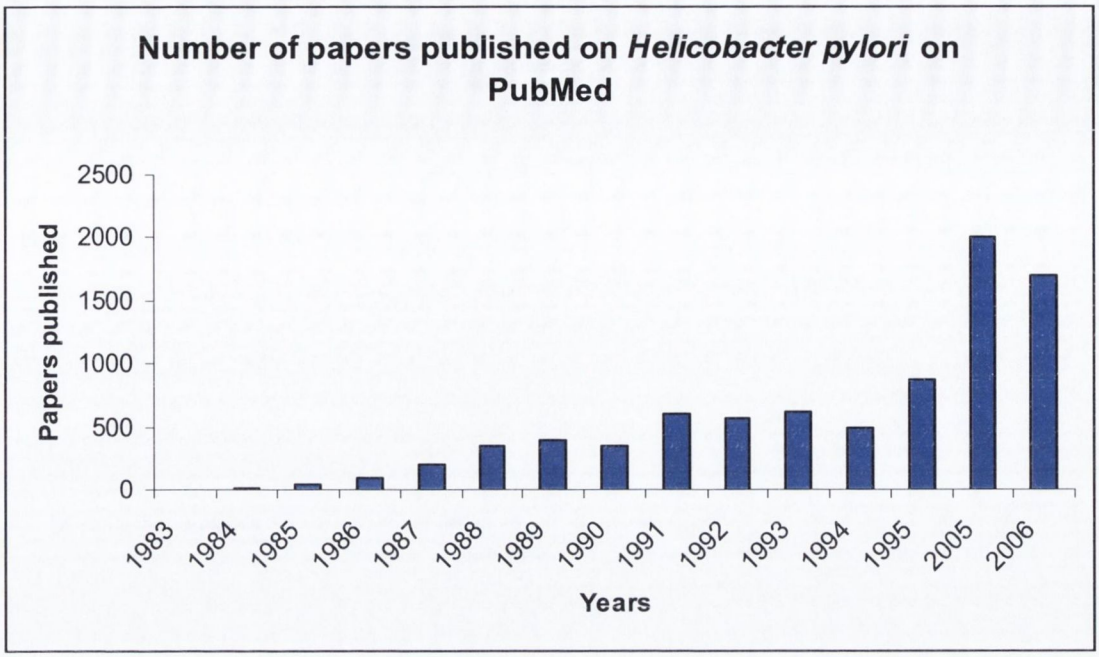


Figure 1.2 – The annual number of papers published on the subject reflects the rapid rise in interest in *H. pylori*

The pathogenesis of *H. pylori* in peptic ulcer and the effect of its eradication (Hentschel et al., 1993) has now been documented. The identification of a vacuolating cytotoxin (VacA) (Leunk et al., 1988) and the 120-128 kDa cytotoxin-associated protein (CagA) (Crabtree et al., 1992) have been important discoveries helping to distinguish differences in virulence between strains. In duodenal ulcer, the action of *H. pylori* on gastrin, somatostatin and acid secretion is established (Levi et al., 1989; Moss et al., 1992) , and the role of toxins and cytokines in the production of gastritis is being unravelled. Gastric ulcers are also part of the spectrum of *H. pylori* associated diseases, as is the influence of *H. pylori* on the development of mucosa-assocaited lymphoid tissue (MALT) lymphoid (Wotherspoon et al., 1993) and gastric cancer (IARC, Lyon, France, 1994). Table 1.1 is a historical timeline of the story of *H. pylori*.

Year	*Reference*	*Report*
1893	Bizzozero	Spiral organisms in dogs
1896	Salomon	Spiral organisms in dogs and cats
1906	Balfour	Spirochaetes in gastric ulcers
1906	Krienitz	First description in a human with gastric cancer
1919	Kassi and Kobayashi	Stomach spirochaete occurring in mammals
1954	Luck and Seth	Presence of urease activity in stomach
1938	Doenges	Spirochaetes in stomachs (autopsies)
1940	Freedburg and Barron	Spirochaetes in stomachs with ulcer or carcinoma
1951	Allende	Treatment of gastric ulcer with penicillin
1954	Palmer	Bacteria in stomachs believed to be contaminants
1975	Steer and Colin-Jones	Bacteria on gastric ulcer patients
1979	Fung et al	Bacteria in gastric biopsies from patients with chronic gastritis
1983	Marshall and Warren	First culture of *H. pylori*
1984	Marshall and Warren	Correlation between the organism and gastroduodenal ulcers
1987		Formation of European *H. pylori* study group
1993	Wotherspoon et al	Regression of MALT lymphoma after *H. pylori* eradication
1994	National Institute of Health	NIH consensus statement – *H. pylori* eradication in peptic ulcers
2005	Nobel Commission	Warren and Marshall win Nobel prize for work on *H.pylori*

Table 1.1 – The story of the discovery of *H. pylori* (Reproduced from *Helicobacter plori* An Atlas, SP publishers, 1996)

1.2 Epidemiology

H. pylori infection is the most common gastrointestinal bacterial disease worlwide. It is the principal cause of chronic gastritis, and many of the diseases associated with gastritis are also associated with *H. pylori*. Infection is found in 95 % of duodenal ulcer patients and in 70-80 % of those with gastric ulcer. In non-ulcer dyspepsia about 50 % of cases in hospital practice are associated with *H. pylori*. Individuals who are *H. pylori* positive have at least a fourfold higher risk of developing a peptic ulcer than those who are not infected. Both prospective(Forman et al., 1994) and retrospective (Kikuchi et al., 1995) studies have demonstrated pre-existing *H. pylori* infection in 90 % of cases of gastric cancer, including gastric lymphoma. The risk for gastric cancer attributable to *H. pylori* has been estimated at 70% in industrialized regions and 80 % in developing countries (Forman, 1995) .

Great variation exists in the prevalence of *H. pylori* infection between different countries and age groups. Prevalence reflects the balance of the frequency and the duration of infection in a community. These events are related to the rate of acquisition, i.e. the incidence, and to the rate of loss of infection. Once established, *H. pylori* infection in humans can last for decades and spontaneous eradication is rare. Incidence cannot be determined directly, however, as acute *H. pylori* infection has few, if any, characteristic symptoms. Thus incidence can only be estimated from prospective follow-up studies of uninfected subjects, from repetitive surveys within a population, or from follow up studies of patients treated for eradication (Cammarota et al., 1995).

Two patterns of *H. pylori* infection can be identified. The first is characteristic of developing countries, where a large proportion of children are infected in early life and almost all adults in different age groups have a chronic *H. pylori* infection. This scenario has been documented in Algeria, the Ivory Coast, Tailand, Saudi Arabia, Chile, Peru, Southern India, South Africa and Brazil, with figures ranging from 13 to 70 % in the 0-20 year old age group and from 70 – 94 % in those over 30 years old. Incidence varies from 5% in Thailand to 9% in Brazil. The second pattern is typical of western developed countries, where the prevalence of *H. pylori* increases from age 20 years onwards. Several studies performed in England, France, Scandinavia, Italy, San Marino (Gasbarrini et al., 1995), Belgium and the USA have demonstrated that *H. pylori* seropositivity ranges from 5 to 15% in childhood and from 20 to 65% in people aged 30 to 75 years. Interestingly, incidence rates in

industrialized regions are lower than those in developing countries, ranging from 0.5 to 2%, and incidence in children seems to be lower now than it was in the past (Pretolani et al., 1997). Figure 1.3 is a map showing the incidence of *H. pylori* infection world-wide.

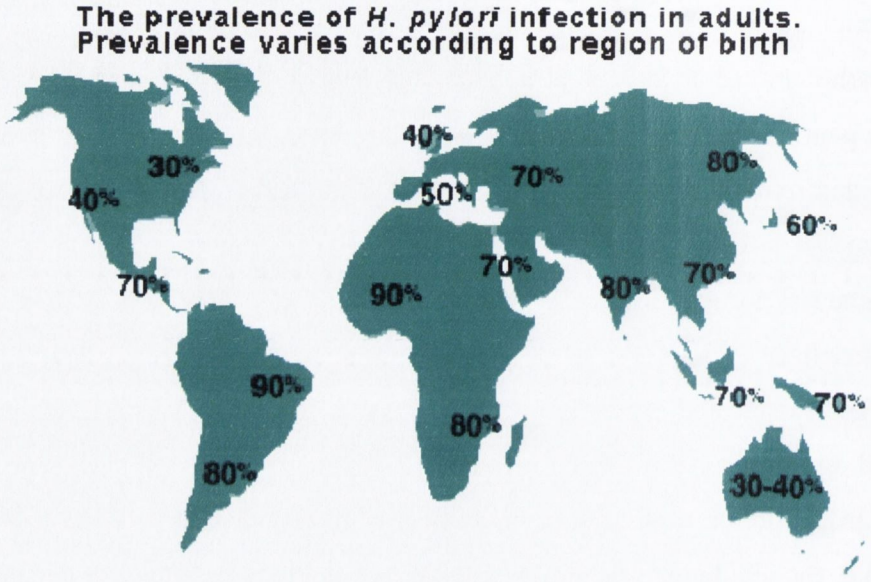


Figure 1.3 – Incidence of *H. pylori* world-wide

Recent health statistics from the USA state that *H. pylori* accounted for the following:

(i) peptic ulcer prevalence: 14.5 million (2003)

(ii) mortality: 4079 deaths (2002)

(iii) Hospitalizations: 403,000 (2002)

(iv) Ambulatory care visits: 876,000 million (1999-2000)

(v) Prescriptions: 2 million(2003)

(**Kozak LJ, Owings MF, Hall MJ.** National Hospital Discharge Survey: 2002 annual summary with detailed diagnosis and procedure data. *National Center for Health Statistics. Vital Health Stat.* 2005, **Lethbridge-Cejku M, Vickerie J**. Summary health statistics for U.S. adults: National Health Interview Survey, 2003. *National Center for Health Statistics. Vital Health Stat.* 2005.)

The Health Protection Surveillance Centre, Ireland, does not collect data on the incidence of *H. pylori* infection, therefore relevant data was acquired from St James's

Hospital, Dublin 8 of patients attending the hospital who tested positive for *H. pylori* infection with the ^{13}C-urea breath test (Table 1.2).

H. pylori *Infection*	*Number*
Positive	465
Negative	1092
Total	1557

Table 1.2 – Numbers of patients attending for ^{13}C-urea breath test at St. James's Hospital Dublin 8 in 2006. (Figures from Gerald Cox, Dept of Biochemistry, SJH)

Risk factors for *H. pylori* acquisition are low social class in adults and exposure to poor living conditions in childhood (Mendall et al., 1992), such as bed sharing and overcrowded living conditions. *H. pylori* can be transmitted between spouses, but sexual activity is not a risk factor and this type of person to person transmission is probably rare. Clustering of the infection occurs within families, and the presence of an infected child in the family could be a source of transmission. Gastroenterologists and other healthcare workers who have contact with secretions of the upper gastrointestinal tract may be at increased risk (Lin et al., 1994). All of these findings suggest an oral-oral mode of transmission of *H. pylori*. In the developing world however, culture of bacterium from faeces (Thomas et al., 1992) and the higher prevalence of infection in populations without sewage facilities support the faecal –oral route of infection (Klein et al., 1991).

1.3 Bacterial Pathogenesis

The gastric pathogen *H. pylori* exhibit a specific host tropism and a detailed tissue tropism by colonising the mucus layer and the gastric epithelial cell lining. It has adapted to the hostile and acidic environment of the human stomach (Falk et al., 1993). In this hostile environment, competition with other microbes for nutrients and space is limited. After the initial contact with the host tissue, the microbes must be prepared to face and adapt to a multitude of host responses and antimicrobial defence systems. Peristalsis and the high turnover rate of epithelial cells and mucus can pose a serious threat to microorganisms (Clyne and Drumm, 1993). However, *H. pylori* can establish a protected niche for survival and long-term colonisation of the human gastric mucosa and this can persist for a lifetime (Blaser, 1993). *H. pylori* has developed efficient

features such as flagella and spiral morphology and adhesive properties. Together, these traits make *H. pylori* highly motile and capable of avoiding the continuous clearance processes in the gastric mucosa. *H. pylori* motility has been shown to be essential for colonisation (Eaton et al., 1992) and is also dependent on its chemo-sensing of host chemotaxins (Logan et al., 1995), including urea and sodium bicarbonate which will direct the organism towards the epithelial cell lining.

H. pylori exhibits both host and tissue tropism. It is a primate specific pathogen so natural infections are limited to humans and monkeys (Solvnick et al., PNAS 2004). *H. pylori* is normally found in the lower part of the human stomach, the antrum (Fig 1.4).

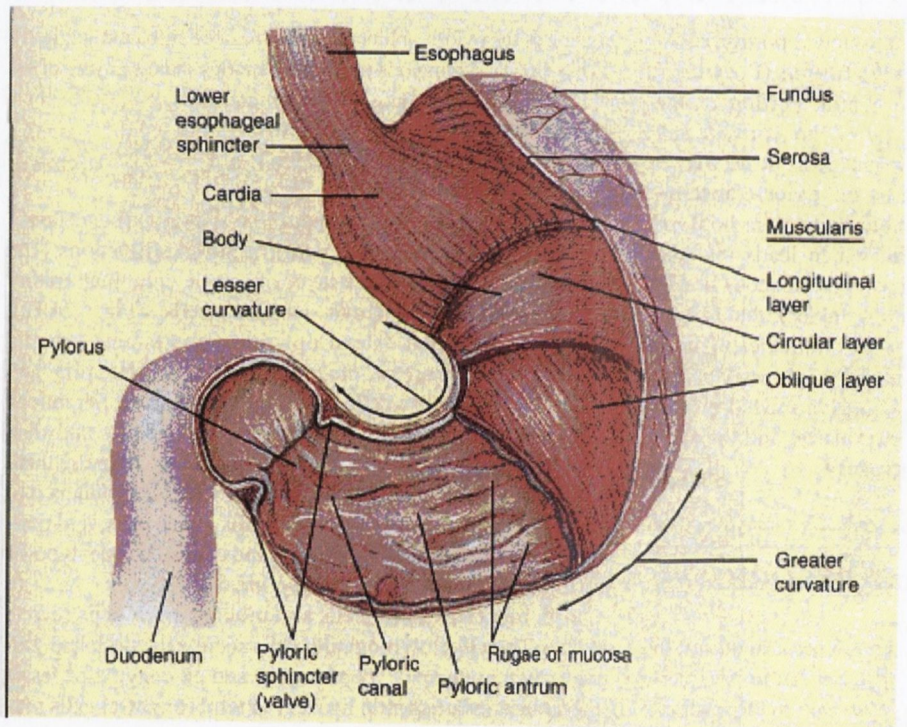


Figure 1.4 – Diagram of human stomach detailing the specific areas of interest, specifically the antrum

In this region, the pH is less acidic than in the corpus, where the acid generating parietal cells are abundant. *H. pylori* does not naturally colonise duodenal tissues, colonisation will occur in patches of gastric metaplasia, whereby gastric surface mucous cells appear in the duodenal tract (Wyatt et al., 1990). Gastric metaplasia is a disruption in the normal cell differentitaion of the duodenum induced by hypersecretion of acid from the corpus region. This hypersecretion is due to the activation of the gastrin producing G-cells in the antrum in response to an established *H. pylori* infection.

In vivo, only 2-20 % of the *H. pylori* population demonstrates adherence to epithelial cell surfaces, whereas the bulk of the population remains in the protective mucus layer (Hessy, SJ et al., Gut 1990). Flagella, Lipopolsaccharide (LPS), urease and outer membrane proteins (OMP) serve as putative adhesin molecules. The fucosylated blood group antigen Lewis^b and sialylated receptors expressed on the host epthelial cell function as receptor molecules (Clyne and Drumm, 1996; Logan, 1996; Monteiro et al., 1998) . Lewis^b, together with Lewis^x, is also expressed on the pathogens surface, thus mimicking human cell surface glycoconjugates and glycolipids and downregulating an anti-*H. pylori* T-cell response (Sherburne and Taylor, 1995). The adherence abilities of *H. pylori* appear to be directly linked to its pathogenesis. While non-adherent *H. pylori* strains are rapidly removed by the constant shedding of surface epithelial cells, adherent strains are also able to survive in the gastric mucus layer and can re-colonize gastric epithelial cells (Hayashi et al., 1998). However, it remains to be determined if *H. pylori* adherence is the main feature of its persistence (Heczko et al., 2000).

1.4 *H. pylori* Virulence Factors

Virulence factors are related to the ability of an organism to induce disease. Important virulence factors are defined as those that either have a disease association or have an in-vivo correlate with disease such as increased mucosal inflammation (Lu et al., 2005b). Criteria for an *H. pylori* virulence factor is as follows:

(i) The virulence factor should have a disease or other *in vivo* correlation

(ii) There should be epidemiologic consistence across populations and regions

(iii) Biological activity should be reduced or eliminated by gene deletion

(iv) Biological activity should be restored by complementation

The identification of *H. pylori* virulence factors is a controversial area at present. Some researchers believe that disease associations that arise from predictions based on *in vitro* studies are spurious and highly suspicious. Only a few putative virulence factors have been confirmed to be associated with clinical out comes such as peptic ulcer and gastric cancer (Lu et al., 2005b). Some of these are listed in Table 1.3. Some of these candidates are only supported by in-vitro data. For the purpose of this work some of these virulence factors will now be considered in greater detail.

Putative Factors	*Originally associated with a disease outcome*
AlpAB (adherence associated lipoprotein)	No
BabA (Blood group antigen binding adhesion)	Yes
CagA (Cytotoxin associated gene)	Yes
DupA (Duodenal ulcer promoting gene)	Yes
Cag PAI (Cag pathogenicity island)	Yes
IceA (Induced by contact with epithelium)	Yes
LPS (Lipopolysaccharide)	No
NapA (Neutrophil activating protein)	No
OipA (Outer inflammatory protein)	Yes
SabA (Sialic acid binding adhesin)	No
VacA (Vacuolating cytotoxin)	Yes

Table 1.3 – Putative virulence factors of *H. pylori* (Reproduced from Lu et al., 2005, Current Opinion in Gastroenterology, 21: 653 - 659)

1.4.1 Cag Pathogenicity Island and CagA

The major disease associated genetic difference in H. pylori strains is the presence of a pathogenicity island (PAI) containing 28 putative genes which code for proteins involved in a specialised type four secretion system (TFSS) (Censini et al., 1996; Akopyants et al., 1998; Azuma et al., 2004). A constituent of the 35-40 kb cag PAI

encodes the 120 – 140 kDa cytotoxin associated gene product A (cagA) that contains tyrosine phosphorylated (EPIYA) motifs. The cag pathogenicity island (PAI) qualifies as a true virulence factor in that its presence is associated with an increased risk of peptic ulcer and gastric cancer. It is associated with enhance mucosal inflammation both *in vivo* and *in vitro* (Lu et al., 2005b). The cag PAI region is associated with increased virulence of strains, and severe clinical outcome, such as severe gastritis, duodenal ulceration and gastric adenocarcinoma (Censini et al., 1996; Akopyants et al., 1998). The cag PAI encoded type IV secretion system (Fig 1.5), is a molecular syringe that injects the CagA protein and other bacterial products into eukaryotic cells (Lehours et al., 2004).

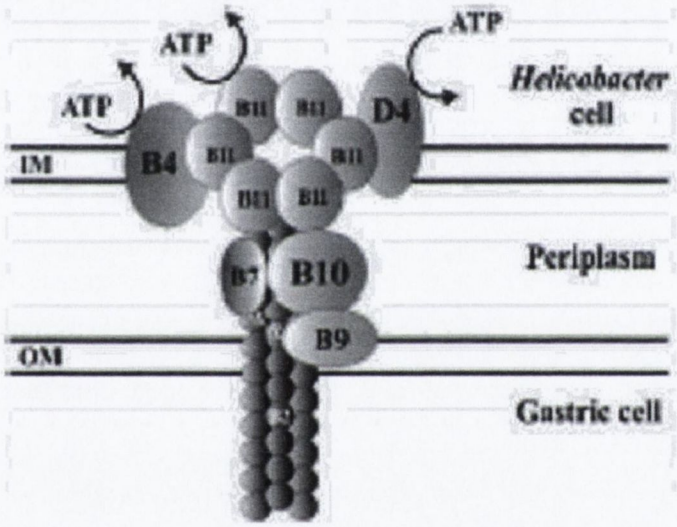


Figure 1.5- Hypothetical model of *H. pylori* type IV secretion machinery encoded in Cag PAI (Reproduced from S. Backert et al., 2000)

Although the risk of a clinical outcome is increased in association with the presence of Cag PAI and cagA, it is not required for either peptic ulcer or gastric cancer. The CagA protein has been a major focus of very detailed molecular studies. It is the general consensus currently, the *H. pylori* strains expressing CagA are not associated with gastric MALT lymphoma but are associated with diffuse large cell lymphoma (Lehours et al., 2004). After CagA is translocated from the bacteria into the cytoplasm of host cells, its tyrosine residues become phosphorylated. Phosphorylated CagA then interacts with several host signaling molecules and activates protein kinase cascades, SHP-2, Src and C-terminal Src kinase (Argent et al., 2004). If virulence is associated with the number of phosphorylation sites, the result would be an acceleration of disease.

However, no data exists to support this theory or indeed CagA in the pathogenesis of *H. pylori* associated disease (Lu et al., 2005b).

1.4.2 Neutrophil activating protein (NapA)

Neutrophil activating protein, NapA (Evans et al., 1995), was so named because of its ability to mediate neutrophil adhesion to endothelial cells (Yoshida et al., 1993) and to bind both mucin and neutrophil glycosphingolipids (Namavar et al., 1998). Recently, NapA was also shown to bind iron *in vitro* (Tonello et al., 1999) and to protect cells against oxidative stress induced lethality (Cooksley et al., 2003). More recently NapA has been shown to be a potent immunomodulatory molecule which functions through TLR2 (Amedei et al., 2006).

From the data currently available it seems clear that NapA has a protective role for *H. pylori* but it seems unlikely that it facilitates the pathogenicity of the organism as it was not originally associated with a disease outcome (Lu et al., 2005b). However, NapA is known to induce activation of human neutrophils involved in the inflammatory response linked with *H. pylori* associated gastritis. Additionally, Amedei et al. (2006) showed that NapA was a TLR2 agonist able to stimulate neutrophils to increase their expression of IL-12, which acts as a key cytokine for the differentitation of naïve Th cells into the Th1 phenotype. They suggest that NapA could be used as an adjuvant for promoting Th1 immune responses (Amedei et al., 2006).

1.4.3 Outer inflammatory protein A (OipA)

The OipA protein is an important virulence factor associated with enhanced IL-8 secretion, increased inflammation and pronounced neutrophil infiltration *in vivo* as well as *in vitro*, validated by the clinically important presentation of peptic ulcer (Kudo et al., 2004; Yamaoka et al., 2005). However more recent data suggests that its effect on epithelial cells may be limited and that its main function is to induce genes within the CagA PAI. The induction of IL-8 is normally associated with a functional cag PAI and / or the presence of OipA.

1.4.4 Vacuolating cytotoxin A (VacA)

Among several virulence factors produced by *H. pylori*, the vacuolating cytotoxin VacA, plays a potentially pivotal role in pathogenicity (Ricci et al., 2005). VacA is a

protein toxin which induces cytoplasmic vacuoles in eukaryotic cells in culture (Ricci et al., 2000). Virtually all the cell vacuolation caused by Vac+ strains is accounted for by free soluble VacA released by the organism (Ricci et al., 2005). It has also been suggested that the only important *in vivo* effect of VacA is related to its ability to selectively increase the permeability of the epithelial paracellular pathway (Lu et al., 2005a) . There are still no data that VacA has any effect beyond the surface cells. This hypothesis is consistent with VacA having an important role in the initial establishment of the infection (Salama et al., 2001).

1.4.5 Outer Membrane Proteins

Most virulence factors are located on the cell surface (Lu et al., 2005b). There are at least 32 outer membrane proteins (OMP) already identified in *H. pylori*, many of which are involved in bacterial adhesion. OMP are incorporated into the outer membrane of *H. pylori* and are important for ion transport, adherence, structural and osmotic stability, virulence but may also be antigenic due to their surface exposure (Voland et al., 2003). Important OMP include the blood group antigen-binding adhesin (BabA), sialic acid-binding adhesin (SabA), adherence-associated lipoprotein, *H. pylori* adhesin A (HpaA) and the outer inflammatory protein, OipA (previously discussed) (Lu et al., 2005b). Functional receptors for *H. pylori* adherence include fucosylated ABO blood group antigens and sialyl-Lewis x/a antigens. These receptors are recognised by BabA, which binds Lewis b antigen (Leb) and related ABO antigens, and SabA (Unemo et al., 2005). SabA expression has also been shown to be increased in strains isolated from patients with gastric cancer, intestinal metaplasia and gastric atrophy which suggests that there may be a relation between SabA and the antigens/receptors expressed in an altered epithelium (Yamaoka et al., 2005). HpaA is a surface located lipoprotein (O'Toole et al., 1995) that was initially described as a sialic acid binding adhesin but evidence to support this claim is lacking. It is recognized by antibodies from *H. pylori* infected individuals (Mattsson et al., 1998) and the expression of the HpaA protein is highly conserved among *H. pylori* isolates (Bolin et al., 1995). It has also been demonstrated that HpaA is essential for bacterial colonization in the gastric mucosa of mice, establishing a very relevant physiological role for HpaA *in vivo* (Carlsohn et al., 2006).

There is now evidence to suggest that virulence as assessed by disease presentation is actually a host-dependent factor. Considering *H. pylori* on a global basis, there is interaction between the organism, the host and the environment. The virulence of the strain itself acts only as an accelerator in the disease process and not as a predictor of outcome (Graham and Yamaoka, 2000). Future research should continue to look for disease associated virulence factors. Figure 1.6 is a hypothetical diagram depicting the interactions of the host and *H. pylori* virulence factors.

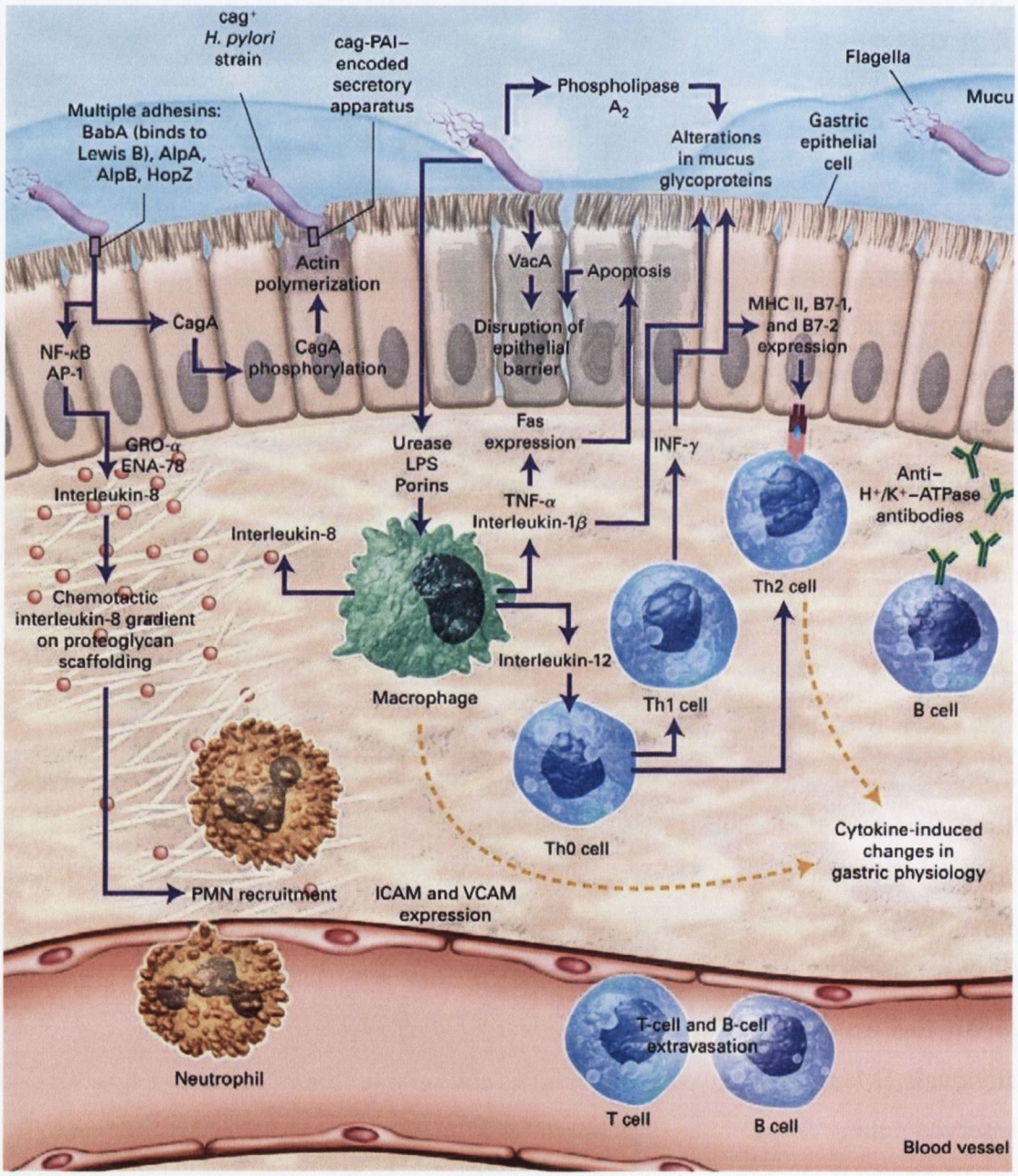


Figure 1.6 – Interactions of host and bacterial factors in pathogenesis of *H. pylori* infection (Reproduced from Suerbaum and Michetti, 2002)

1.5 Proteomic Analysis of Secreted Proteins of *H. pylori*

To date, sequencing of the complete genomes of two strains of *H. pylori*, 26695 (Tomb et al., 1997) and J99 (Alm et al., 2000), have taken place. The focus has now turned to investigating the functional part of the genetic information containing macromolecules, the proteome. Two-dimensional gel electrophoresis (2-DE) of proteins allows the seaparation of up to 10,000 protein species in a single run (Klose and Kobalz, 1995). The combination of 2-DE with mass spectrometry enables the identification of proteins on 2-DE patterns on a large scale. Clinical interest in understanding the distribution of virulence factors in *H. pylori* membranes for the potential development of vaccines and therapeutic targets has driven numerous studies to explore the membrane and secreted proteins of *H. pylori* (Backert et al., 2005b). Membrane surface proteins can mediate important pathogen-host interactions that are essential for survival, colonization, adherence and virulence of pathogenic organisms. Secreted proteins (the secretome) of *H. pylori* may be of special importance as these proteins come into direct contact with host compartments (Bumann et al., 2002). In recent years the proteomic studies of *H. pylori* have included: the subproteomes of soluble and structure bound *H. pylori* proteins (Backert et al., 2005b), the sarcosine insoluble outer membrane fraction of *H. pylori* 26695 (Baik et al., 2004), the cellular proteins and antigens from *H. pylori* 26695 (Krah et al., 2003), the secreted proteins of *H. pylori* (Bumann et al., 2002) and the surface proteins of *H. pylori* (Sabarth et al., 2002).

Like the majority of Gram-negative bacteria, *H. pylori*, constitutivley secrete outer membrane vesicles (OMV) from their surface. To date their have been proteomic studies carried out on the OMV of: Leptospira interrogans (Nally et al., 2005), Neisseria meningitidis (Ferrari et al., 2006; Uli et al., 2006), Neisseria lactamica (Vaughan et al., 2006) and Pseudomonas aeruginosa (Bauman and Kuehn, 2006).

1.6 Clinical Presentation

H. pylori is aetiologically linked to several major gastroduodenal diseases but the outcome in an infected individual is largely unpredictable (Cover et al., 1990). Some infected individuals can be assymptomatic. Others can suffer from acute or chronic gastritis, inflammation and peptic ulceration, atrophy and gastric cancer, MALT and low grade B-cell lymphoma (Fig 1.7).

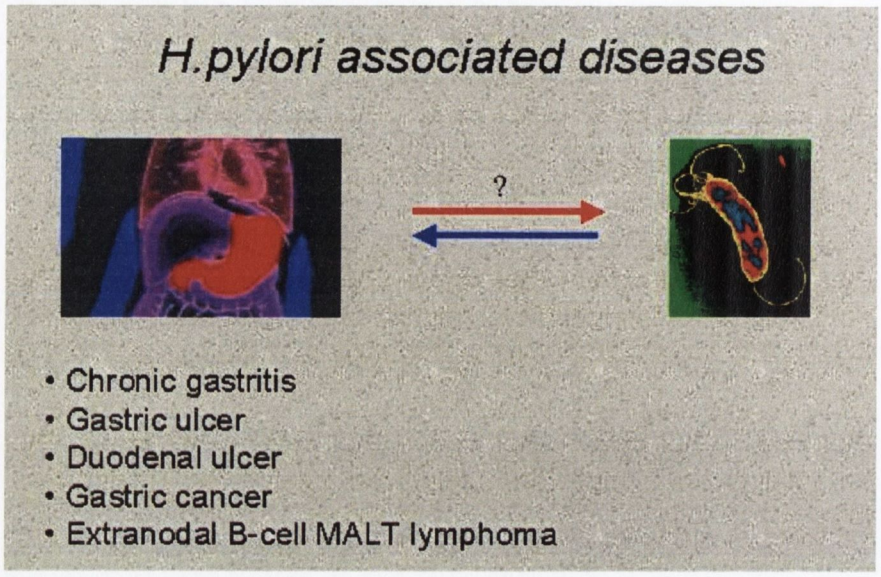


Fig 1.7 – Possible clinical outcomes following infection with *H. pylori*

1.6.1 Acute Gastritis

H. pylori is normally acquired by the oral route and following ingestion the organism penetrate through the viscid mucus layer and multiplies in close proximity to the surface of epithelial cells. The epithelium responds by mucin depletion, cellular exfoliation and synctial regenerative changes. The invading bacteria release chemotactic moieties, which penetrate through the epithelium and induce migration of polymorphs and neutrophils into the lamina propria and epithelium (Crabtree and Lindley, 1994). Released bacterial products also activate mast cells and their degranulation releases other acute inflammatory mediators, which increase vascular permeability. *H. pylori* stimulates the gastric epithelium to produce a potent neutrophil chemokine, IL-8, whose production is up-regulated by tumour necrosis factor - α (TNF-α) and IL-1 released by macrophages in response to bacterial lipopolysaccharide (Graham et al., 1994). This acute phase is accompanied by profound hypochlorhydria and a failure of ascorbic acid secretion into gastric juice (Sobala et al., 1991). This acute phase is short lived. In a small minority of people the infection is cleared naturally but the majority of sufferers go on to accumulate chronic inflammatory cells.

1.6.2 Chronic Gastritis

The arrival of lymphocytes and plasma cells in the mucosa signals augmentation of the acute inflammatory response by the induction and expression of cytokines and specific anti–*H. pylori* antibodies (Anthony P. Moran, 1997). Proliferation of B-cells and

plasma cell differentiation results in the synthesis of IgM opsonising and complement-fixing which amplify the inflammatory reaction. This vigorous response fails to eliminate the infection and the continued presence of the organism leads to the development of a second arm of the immune response becoming activated. This second-line response initiates the recruitment of primed B-cells into lymphoid follicles with the production of plasma cells committed to the synthesis of protective IgA antibodies. This arm of the immune response is aimed at preventing the damaging effects of intraluminal pathogens (Anthony P. Moran, 1997). However, this is still insufficient to eradicate the infection. The antigenic stimulation continues and follicle formation becomes a distinctive feature of chronic *H. pylori* gastritis (Ernst et al., 1994). Th1 cells direct the acute inflammatory response by promoting inflammation and by activating CD8+ T-cells, which lead to autoantibody formation and cell-mediated epithelial damage. Th2 cells are responsible for the secretory immune response (Enno et al., 1995).

1.6.3 Duodenal Ulceration

Most *H. pylori* positive individuals present with diffuse chronic inflammation which involves both antrum and corpus mucosa. A small number of individuals develop marked gastritis in the antrum, with very little inflammation in the corpus mucosa. This is known as antral-predominant chronic gastritis and it is these sufferers who are likely to develop duodenal ulceration (DU). It has been previously proposed that the acid production in this area will determine the colonisation and virulence of *H. pylori* and the degree of concomitant inflammation (Dixon, 1991). It is likely that the higher the acid output, the more infection and inflammation will be concentrated in the antrum. There is a school of thought that suggests that *H. pylori* does not cause DU but can lead to resistance in its healing (Hobsley et al., 2006). They report that in countries with a high incidence of *H. pylori* infection, there is no evidence of an overall higher prevalence of DU than in countries with a low *H. pylori* prevalence. After healing following eradication of *H. pylori* DU can still recur. Hobsley et al believe that the relationship between DU, *H. pylori* and gastric acid secretion is most likely to be:

Acid Excess > DU > Treatment > *H. pylori* infection > Failure to heal

1.6.4 Atrophy and Gastric Cancer

Atrophy is the key pathological change, underlying the increased risk of gastric cancer in patients which chronic gastritis (Correa, 1992). Atrophy could result from direct effects of *H. pylori* or indirect consequences of the host response. Direct injury to epithelial cells by released bacterial products is likely to be an important mechanism. The degree of atrophy and the extent of the host response may well be influenced by strain variation among *H. pylori* (Anthony P. Moran, 1997). Once atrophy has been established, the associated hypochlorhydria and consequent bacterial proliferation favours the formation of intraluminal N-nitroso compounds (Farinati et al., 1996). The lack of ascorbic acid in the gastric juice encourages persistence of damaging oxidative and potentially carcinogenic agents (Drake et al., 1996). The increased cell turnover elicited by a *H. pylori* infection also has a part to play in carcinogenesis (Lynch et al., 1996). High cell turnover will stress DNA repair mechanisms and combined with defects in p53 and other protective systems could lead to the perpetuation of mutations through cell division (Goldstone et al., 1996). This can, in combination with other events lead to diffuse gastric cancers.

1.6.5 MALT and Low-Grade B-Cell Lymphoma

The acquisition of lymphoid follicles in chronic *H. pylori* gastritis is an expression of MALT acquired in accordance with a Th2 response. MALT is the precursor of low-grade B-cell lymphoma and a great majority of such stomach lymphomas have their origins in *H. pylori* gastritis (Anthony P. Moran, 1997). The nature of the abnormal response to antigenic stimulation responsible for lymphomagenesis is speculative. It has been suggested that T-cell activation of a growth-promoted B-cell could bring about its uncoordinated proliferation with clonal expansion of centrocyte-like cells (Crabtree and Spencer, 1996). In low grade lymphomas, this monoclonal proliferation is still antigen dependent and eradication of *H. pylori* infection can lead to complete remission (Morgner et al., 2001). Transformation to a high-grade (large cell) lymphoma reflects antigen independence and proliferation is no longer influenced by *H. pylori* eradication.

1.7 Diagnosis

Numerous tests, both invasive and non-invasive, are available for diagnosis *of H. pylori* infection. The development of reliable tests has led to an increased understanding of the epidemiology of *H. pylori* infection and its role in the pathophysiology of gastric and

duodenal ulcers. A combination of tests are generally used, but this may depend on the reason for testing. In clinical trials, accurate detection of *H. pylori* before and after eradication therapy has proved of critical importance for evaluating the effects of different treatment regimens (Megraud, 1996).

Invasive techniques are of most use in patients who, for clinical reasons, have been referred for endoscopy (Fig 1.8). Urease tests are rapid and quite specific, but sensitivity is low in the post-treatment period (Marshall et al., 1987). Histological examination is the only method that can show both the extent of *H. pylori* infection and the degree of mucosal damage. DNA techniques, specifically the polymerase chain reaction (PCR), can also be used to detect *H. pylori* in biopsy specimens. PCR derived techniques have been used for molecular typing. They show the great genomic diversity of *H. pylori* strains among different individuals and the frequent occurrence of similar genotypes within families indicating intrafamilial transmission.

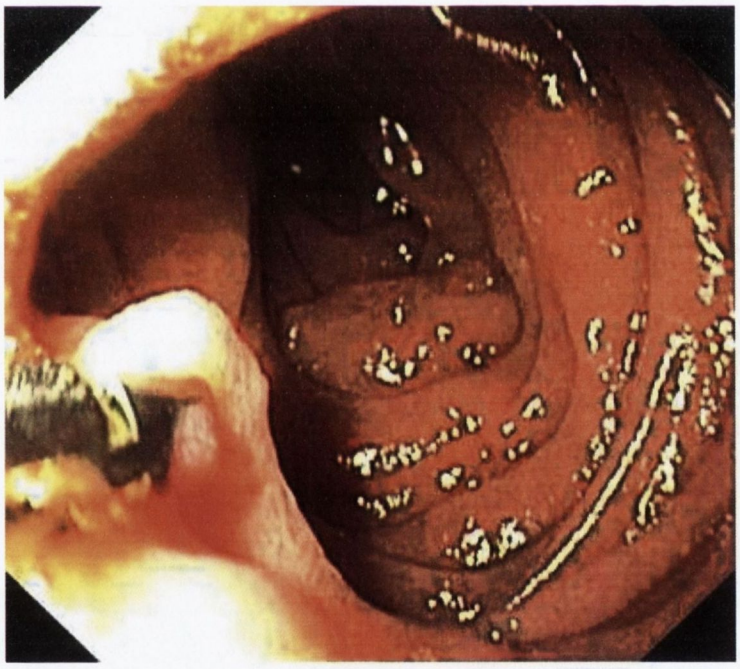


Figure 1.8 – Biopsy being taken during endoscopy

Non-invasive tests, mainly based on samples of blood or expelled air, are particularly valuable because they provide a rapid diagnosis. The use of the urea breath test has been standardized and assays based on the detection of ^{13}C- or ^{14}C-carbon dioxide in expired air have been developed (Logan et al., 1991). They are useful for determining whether a treated patient has been cured. Several serological tests have been validated

for diagnostic purposes. Monitoring of *H. pylori* eradication after antimicrobial treatment is also possible by serology, if the test is performed not sooner than 6 months after the end of treatment and is compared with the pretreatment sample.

Two different tests performed on multiple biopsy specimens (Histology and another test) yield optimum results. Such a procedure is mandatory in clinical trials, whereas in patients who receive established treatment regimens, diagnosis and follow up can be performed with non-invasive tests.

1.8 Treatment

All patients with a peptic ulcer, who have *H. pylori* infection, should receive treatment aimed at eradication of the bacterium. The ideal treatment regimen should achieve consistently high eradication rates, be well tolerated, safe, inexpensive and should heal the ulcer (Borody et al., 1995b). The classic treatment for the infection consists of a bismuth compound with a nitromidazole and tetracycline (or amoxycillin). This triple therapy combination consistently eradicates *H. pylori* in approximately 80 % of patients treated. Drug related side-effects occur in one-third of patients and the resulting decreased compliance significantly affects eradication rates. Primary nitroimidazole resistance decreases the efficacy of this triple therapy to 60%. The addition of a proton pump inhibitor to bismuth-based triple therapy increases the eradication rate to more than 90% (Borody et al., 1995a). Today most countries prefer a 7 to 10 day regimen containing a proton pump inhibitor (PPI), clarithromycin and amoxicillin as first line treatment. An alternative (or second line) treatment contains a PPI, bismuth, tetracycline and metronidazole (Treiber et al., 2007).

1.9 *H. pylori* Vaccines

In 1992, Chen et al, provided proof of principle that stimulation of the immune system can protect a mammalian host against infection by *Helicobacter* species (Chen et al., 1992). Since this early observation, much work has been performed, information gained, and hypotheses regarding the mechanisms of protective immunity have come and gone (Sutton and Lee, 2000). Various animal models are now in use from mice infected with *H. pylori*, through gnotobiotic pigs and primates to ferrets naturally infected with their own *Helicobacter*, *H. mustelae* (Cuenca et al., 1996). A persistant problem is the requirement for a suitable mucosal adjuvant. Detoxofication or the use of low doses of adjuvants already available, like Alum or Cholera Toxin, may provide a

solution and new immune stimulating compounds have been tested with some success (Guy et al., 1998; Sutton and Lee, 2000). New approaches are currently being developed which aim to remove the necessity for the addition of a biochemical adjuvant to the vaccine formulation. These include the delivery of *Helicobacter* antigens by DNA immunization (Corthesy-Theulaz et al., 1996a; Ulivieri et al., 1996), microparticles for oral delivery of encapsulated antigens (Kim et al., 1999) and the use of live vectors such as attenuated salmonella (Corthesy-Theulaz et al., 1998).

Much recent work has focused on examining various routes of vaccine administration. Due to the localization of *H. pylori* in the stomach, most attention has focused on the delivery of potential vaccines via the oral or intragastric routes (Sutton and Lee, 2000). Much of the common mucosal immune system is linked, with memory lymphocytes re-circulating between different mucosal surfaces so although the stomach is the target organ for immunity against *H. pylori*, immunization at other mucosal surfaces, could potentially induce protective immunity in gastric tissue. There is clear evidence that protection in the mouse model can be induced following vaccination via most mucosal routes including rectal (Kleanthous et al., 1998), nasal (Ermak et al., 1998) or directly into the Peyers patches (Dunkley et al., 1999). However, it is also becoming apparent that subcutaneous immunization is also capable of inducing protection (Guy et al., 1999).

There is increasing concern and awareness that under certain circumstances, immunization of mice followed by *Helicobacter* challenge can lead to a more severe form of gastritis than in non-infected control mice. This is known as post-immunization gastritis. This is an important observation because if immunization can exacerbate *Helicobacter*-induced gastritis, then the application of these vaccines to humans could be potentially harmful. However, no similar observation have been reported in other animal models and the solution may lie in the time at which the mice were challenged (Sutton and Lee, 2000). A major area of interest is the mechanism by which immunization actually influences *Helicobacter* colonization. Antibodies appear to be unimportant (Ermak et al., 1997), whereas CD4+ T-cells are essential (Mohammadi et al., 1997; Pappo et al., 1999).

A main advantage of an effective vaccine would be that once an individual is immunized, protection should be long lasting. *H. pylori* infection is the major cause of gastric adenocarcinoma (Pritchard and Crabtree, 2006). There are currently no predictors for gastric cancer, which remains a major killer and has been projected to be

the eight most common cause of death of any type in the world by 2063 (Murray and Lopez, 1997a). Even though the prevalence of infection is decreasing in some developed countries, this still leaves a very large number of people infected. Taking into consideration only children under 15 years of age, it is estimated that over 13 million are infected with *H. pylori* in the USA, 316,000 in Australia and 675, 000 in the UK (H. Mitchell personal communication 2000). This equates to over 16, 000 cases of gastric cancer per annum in the USA, almost 6000 in the UK and 1500 in Australia. In Japan, there are over 70,000 cases of gastric cancer a year. The figures are obviously much higher again in developing countries with China presenting 400,000 new cases of gastric cancer every year (H. Mitchell personal communication 2000). The incidence of antibiotic resistant strains of *H. pylori* is on the rise every year in the UK (Fig 1.9) and in the USA (Fig 1.10). There is clearly a great need for an effective and cost efficient therapy to reduce the impact of *H. pylori* infection on world health.

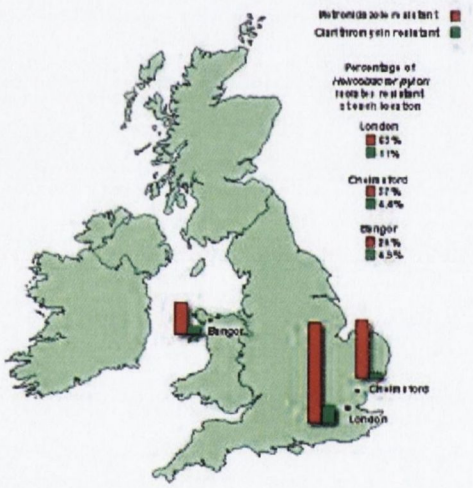


Figure 1.9 – Increasing occurrence of antibiotic resistant strains of *H. pylori* in the UK

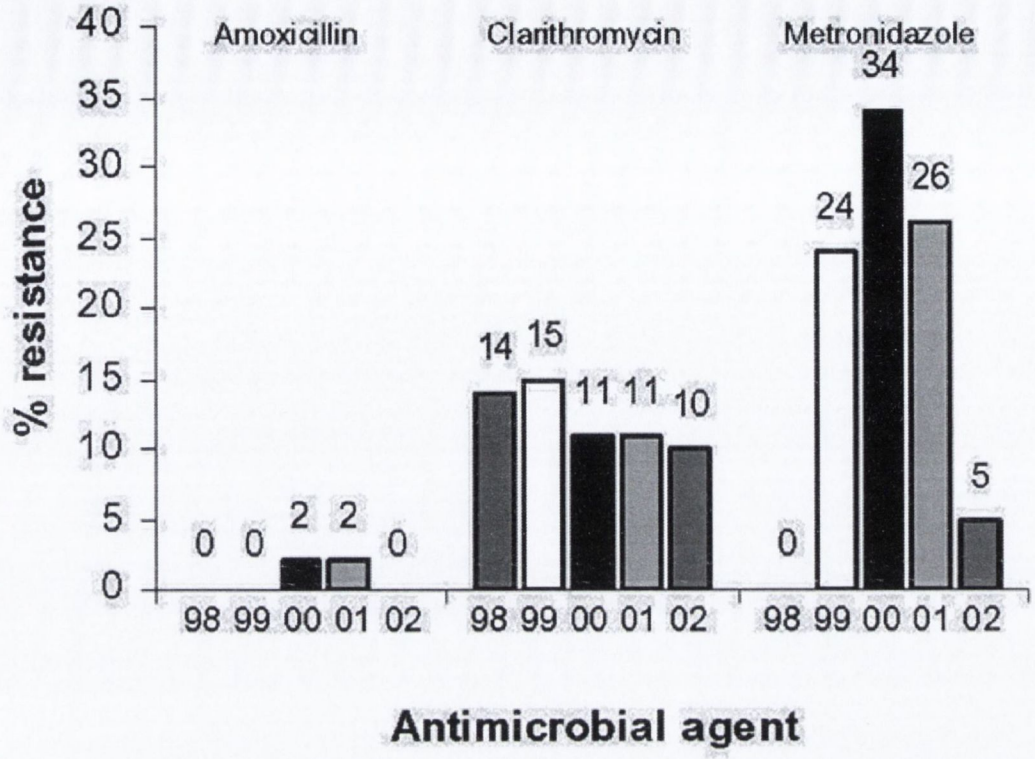


Figure 1.10 – Increasing occurrence of multi-drug resistant strains of *H. pylori* in the USA

1.10 Epithelial Cell Lines as Models of *H. pylori* Infection

For *in vitro* studies of *H. pylori* infection two types of cell culture systems can be used: primary cultures of epithelial cells and cancer cell lines. The characteristics of these cell models are summarised in Table 1.4. Primary cultures are derived from normal mucosal tissue, whereas established tumour-cell lines stem from malignant cells. The major advantage of primary cultures is the extensive preservation of some specific epithelial cell functions, and their close homology to the *in vivo* situation (Anthony P. Moran, 1997). The establishment of primary cultures, however, is laborious and the life span of the cells is very short. Cancer cell lines are readily available commercially and are easy to handle and provide a valuable tool for large-scale experiments. The disadvantage of using these cell lines is the cell transformation involved which may cause loss of specific cell functions and expression of errant cell characteristics (Wagner et al., 1994).

Cancer cell lines can be maintained in culture either as a suspension culture or as an adherent tissue culture. In suspension cultures, cell polarisation is lost. Loss of cell attachment to a matrix may induce apoptosis in non-transformed cells resulting in a

short life span of such cells. Suspension cultures are appropriate for *in vitro* studies where fluorescence-activated cell sorter (FACS) analysis is employed. Tissue cultures are more frequently used as the physiological polarities of the cells are maintained. Certain cell lines form tight monolayers which allow the study of adhesion and invasion of bacteria and allow for the measurement of electrophysiological and transport performance (Corthesy-Theulaz et al., 1996b).

Primary Cultures	***Cancer Cell Lines***
Derived from normal mucosal tissue	Transformed epithelial cells
Short life span	Immortal
Cell functions largely preserved	Loss of some cell function
No unusual features acquired	Gain of unusual features
Influence of donor factors unknown	Large heterogeneity between different cell lines
Laborious establishment	Commercially available, easy to handle

Table 1.4 – Comparison of the characteristics of primary cultures and cell lines as *in vitro* models of infection (Birchmeier, C et al., 1995)

Epithelial cells in the human gastric mucosa are tightly joined to each other, forming a continuous barrier that selectively restricts the movement of substances between compartments (Terres et al., 1998a). These epithelial cells are elongated and column-shaped. The nuclei are elongated and are usually located near the base of the cells (Fig 1.11). Goblet cells (unicellular glands) are found between the columnar epithelial cells of the duodenum. They secrete mucus or slime, a lubricating substance, which keeps the surface smooth.

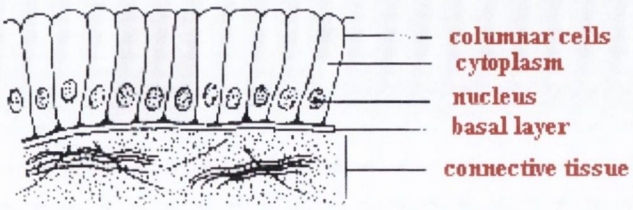


Figure 1.11 – Simple columnar epithelium

Disruption or damage to the epithelial architecture can lead to a deleterious alteration in barrier function. As a result of this, the epithelium requires the paracellular space to be sealed to prevent the passive diffusion of macromolecules (Lewis et al., 1995). Tight junctions, located at the apical poles of epithelial cells, restrict the movement of molecules and are responsible for increasing or decreasing permeability in the intestine (Madara and Pappenheimer, 1987). Tight junctions (Fig 1.12) are mainly composed of the transmembrane protein E-cadherin, which associates with intracellular proteins called catenins (Anderson and Van Itallie, 1995).

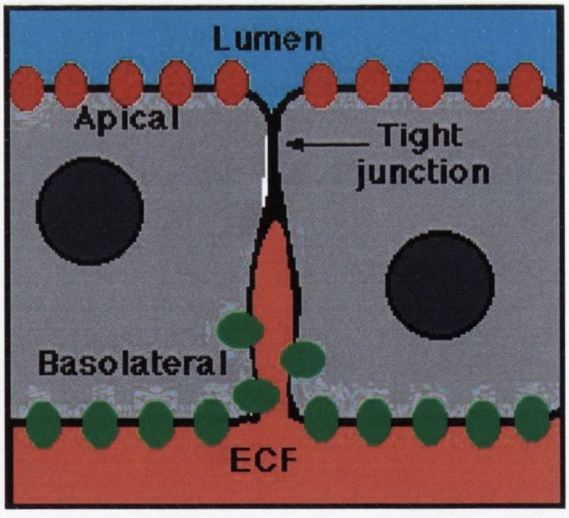


Figure 1.12 – Diagram of a tight junction, sealing the paracellular space between adjoining epithelial cells

The human gastric epithelial-cell lines that are traditionally used as *in vitro* models of *H. pylori* infection are highly permeable, as they do no display a tight phenotype *in vitro* (Terres et al., 1998a). For the majority of the *in vitro* work carried out in this study, the epithelial cell line T84, originally derived from a colon adenocarcinoma, was

used. It represents a well-established tight epithelium with excellent retention of functional phenotype in cultures in which polarity is maintained (Madara et al., 1987). T84 cells form confluent monolayers with functional tight junctions and transepithelial electrical resistance (TER) can monitor barrier function (Taylor et al., 1997). Epithelial cell lines are artificial systems which are very useful for elucidation of specific *H. pylori* factors, however, they can never replace *in vivo* models.

1.11 Introduction to Outer Membrane Vesicles (OMV)

The majority of Gram-negative bacteria naturally produce extracellular outer membrane vesicles (OMV). These spherical bilayered vesicles are liberated from the outer membrane and range in size from 50 to 250 nm in diameter. Similar to the composition of the outer membrane, OMV contain LPS, OMP and phospholipids. They are also known to contain periplasmic proteins (Mashburn-Warren and Whiteley, 2006). The extracellular secretion of products is the major mechanism by which Gram-negative bacteria communicate with and intoxicate host cells. OMV released from the envelope of growing bacteria serve as secretory vehicles for proteins and lipids and for the transport of active virulence factors to host cells (Kuehn, 2005).

Vesiculation is a ubiquitous process for Gram-negative bacteria grown in a variety of conditions (Beveridge, 1999). OMV are thought to form when the outer membrane bulges and pinches off, encapsulating soluble periplasmic cargo (Fig 1.13).

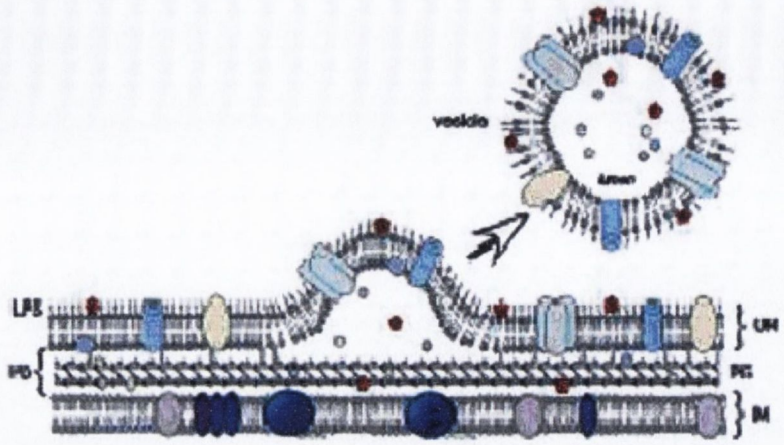


Figure 1.13 – Model of vesicle biogenesis. OMV are known to contain LPS, outer membrane proteins and periplasmic (luminal) proteins. Reproduced from Kuehn and Kesty, Genes and Development, 2005)

In general, pathogenic bacteria produce more vesicles than their nonpathogenic counterparts (Lai et al., 1985; Wai et al., 1995). This is consistent with the theory that pathogenic bacteria have usurped the basic process of vesicle production to disseminate virulence factors and improve survival in the host (Kuehn, 2005). OMV of Gram-negative pathogenic organisms are thought to have the following functions:

(i) toxin delivery to host cells
(ii) transfer of antibiotic resistance determinants
(iii) DNA transfer
(iv) Delivery of antimicrobials
(v) Inter species communication
(vi) Inter kingdom communication
(vii) Modulate host responses

In common with other gram-negative bacteria, studies have demonstrated the ability of *H. pylori* to produce OMV (Keenan et al., 1997) (Fig 1.14).

Figure 1.14 – OMV being shed from the surface of *H. pylori*. (Electron micrograph reproduced from Keenan et al., 2002)

Vesiculating *H. pylori* and vesicle binding to gastric cells have been detected in gastric biopsies and with tissue culture cells (Fiocca et al., 1999; Keenan et al., 2000b). Vesicles containing VacA observed in *H. pylori*-colonized human gastric epithelium biopsies were similar in appearance and composition to *H. pylori* vesicles made *in vitro* (Fiocca et al., 1999; Keenan et al., 2000b). The presence of porins and VacA cytotoxin in these vesicles strongly suggests an offensive role for these structures in the pathogenesis of *H. pylori* infection (Keenan et al., 2000).

Ismail et al., 2003 demonstrated that co-incubating *H. pylori* OMV with AGS cells induced a significant increase in the expression of IL-8, a potent neutrophil activating chemokine (Ismail et al., 2003). It was also reported that *H. pylori* OMV are capable of bearing serologically recognizable Lewis antigens on LPS molecules which may contribute to the chronic immune stimulation of the host. The ability of these vesicles to absorb anti-Lewis autoantibodies indicates that they may, in part, play a role in putative autoimmune aspects of *H. pylori* pathogenesis (Hynes et al., 2005). It was also seen that *H. pylori* OMV, induce a protective response when administered intragastrically to mice in the presence of cholera holotoxin. Immunoblotting later identified this immunodominant protein as lipoprotein 20 (Lpp20). Hybridoma backpacks secreting an IgG1 subclass monoclonal antibody to Lpp20 were generated in *H. pylori* infected mice

and were found to significantly reduce bacterial numbers, providing evidence that this surface-exposed antigen is a true vaccine candidate (Keenan et al., 2000). Furthermore, *H. pylori* OMV are able to induce apoptosis not mediated by mitochondrial pathway in AGS epithelial cells, as demonstrated by the lack of cytochrome c release with an activation of caspase 8 and 3. Apoptosis induced by OMV does not require a classic VacA+ phenotype, as a negative strain can also induce cell death (Ayala et al., 2006).
Due to their small size, adhesive properties and ability to carry virulence factors into host cells, OMV are likely to play a significant role in disseminating virulence factors for Gram-negative bacteria. They can also assist in carving out a niche in the environment by modulating interactions between neighbouring bacteria and between bacteria and the immune system (Kuehn, 2005).

1.12 Host Immune Response to Infection

Host defence against invading microbes is elicited by the immune system, which consists of two branches: innate immunity and acquired immunity. Both components recognize invading microbes as non-self which initiates immune responses to destroy them (Takeda and Akira, 2005) (Fig 1.15). To date, the majority of research has been confined to acquired immunity in response to microbial invasion. In acquired immunity, B and T lymphocytes utilize antigen receptors such as immunoglobulins and T-cell receptors to recognize non-self.

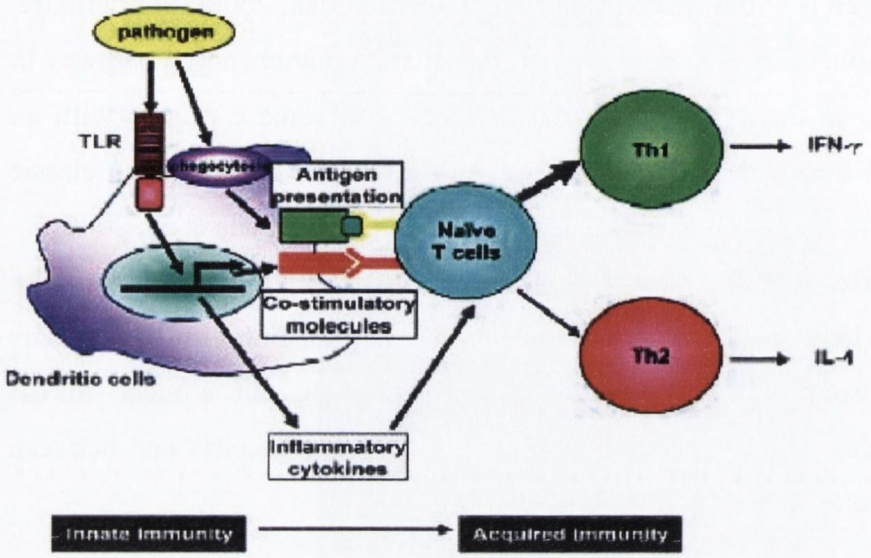


Figure 1.15 – Innate and acquired immunity. Innate immune cells, such as dendritic cells and macrophages, engulf pathogens by phagocytosis and present pathogen derived peptide antigens to naïve T cells. The induction of inflammatory cytokines and co-stimulatory molecules instruct development of antigen-specific acquired immunity. (Reproduced from Takeda and Akira, International Immunology, Vol 17, 2005)

1.12.1 Acquired Immunity

The inflammatory infiltrate into gastric mucosa in response to *H. pylori* infection is comprised of both polymorphonuclear leucocytes (polymorphs) and mononuclear cells. Intra-epithelial lymphocytes and infiltrating neutrophils have been detected on the epithelium and the gastric pits (Robert and Weinstein, 1993). At the acute stage of infection, an increased number of neutrophils are observed in the lamina propria(Anthony P. Moran, 1997). At the chronic stage, there is an increase in T-cells, plasma cells and eosinophils in the lamina propria (Chen et al., 1986). Cell mediated immune responses in the gastrointestinal epithelium and lamina propria are highly specific. *H. pylori* adherence to the epithelium may activate an immune response to release a range of cytokines including: tumour necrosis factor-α (TNF-α), interferon-gamma (IFN- γ) (Karttunen et al., 1990; Karttunen, 1991), interleukin 1 (IL-1) (Mai et al., 1991), IL-4 (Fan et al., 1994), IL-6 (Crabtree et al., 1991) and IL-8 (Crabtree et al., 1993). These cytokines recruit and activate T-cells, neutrophils and other inflammatory

cells. RANTES (regulated on activation normal T cell expressed and secreted) is a CC chemokine produced by epithelial cells, CD8+ T cells, fibroblasts and platelets in response to *H. pylori* infection (Kudo et al., 2005). *H. pylori* induces RANTES through activation of NF-κB (Mori et al., 2003). Microbial antigen expressed on the epithelial cell surface, in association with the major histocompatibility complex (MHC) molecules, may be recognised by T-helper (Th-) cells and cytotoxic T-cells, which in turn produce more cytokines. B-cells are stimulated by cytokines to differentiate into specific anti-body producing cells (Ernst et al., 1994).

A large proportion of immunoglobulin (Ig) secreting B-cells infiltrate the human gastric mucosa and produce a local antibody response, which is primarily of the IgA and IgG classes in *H. pylori* positive patients (Rathbone et al., 1986). The IgG response appears to involve the four IgG subclasses but the major response is restricted to the IgG1 and IgG2 subclasses (Bontkes et al., 1992). The vast majority of individuals infected with *H. pylori* elicit a measurable systemic antibody response, which may reflect the specificity of the antibodies produced in the human mucosa (Stacey et al., 1990).

T-lymphocyte responses to *H. pylori* infection have been determined by studying the proliferative response of peripheral blood mononuclear cells (PBMC) to *H. pylori* antigens and cytokine production by T-cells in culture (Karttunen et al., 1990; Karttunen, 1991). It has been shown that there is reduced peripheral blood lymphocyte proliferation and IFN-γ production to *H. pylori* in *H. pylori* positive individuals. This is consistent with a reduced Th-1 cell response. Cellular immune responses in the gastric mucosa are potentially more important, as it is the site of infection. The gastric T-cell proliferative response to *H. pylori* antigens is also decreased in *H. pylori* positive individuals compared with negative controls. IFN-γ production is decreased by gastric lymphocytes in culture after stimulation with *H. pylori* antigens (Fan et al., 1994). An increase in production of the Th-2 type cytokine, IL-4, in gastric lymphocytes in response to *H. pylori* antigens confirms that this effects extends to the mucosa and suggests that this response might contribute to disease progression (Anthony P. Moran, 1997).

1.12.2 Innate Immunity

The innate immune response to microbial pathogens, in comparison to the acquired immune response, has not been well characterized until recently. At the end of the 20th

century, Toll was shown to be an essential receptor responsible for the recognition of non-self by cells of the innate immune system (Takeda and Akira, 2005). A mammalian homolog of the Toll receptor (now termed TLR4) was shown to induce expression of genes involved in inflammatory responses (Medzhitov et al., 1997). In recent years there has been rapid progress in the understanding that the innate immune system possesses a skilful system that senses invasion of microbial pathogens by Toll-like receptors (TLRs).

Mammalian TLRs comprise a large family of at least 11 members and in some cases the bacterial ligand has also been identified (Table 1.5).

Receptor	Ligand	Origin of Ligand
TLR1	Triacyl lipopeptides	Bacteria and Mycobacteria
	Soluble factors	*Neisseria meningitidis*
TLR2	Lipoproteins	Various pathogens
	Peptidoglycan	Gram positive bacteria
	Lipotechoic acid	Gram positive bacteria
	Lipoarabinomannan	Mycobacteria
	Phenol soluble modulin	*Staphylococcus epidermidis*
	Glycophosphitolphospholipids	*Tyrpanosoma cruzi*
	Glycolipids	*Treponoma mattophillim*
	Porins	*Neisseria*
	Atypical lipopolysaccharide	*Leptospira interogans*
	Atypical lipopolysaccharide	*Porphyromonas gingivalis*
	Zymosan	Fungi
	Heat shock protein 70	Host
TLR3	Double stranded RNA	Viruses
TLR4	Lipopolysaccharide	Gram negative bacteria
	Taxol	Plants
	Fusion protein	Respiratory synctial virus
	Envelope protein	Mouse mammary tumour virus
	Heat shock protein 60	*Chlamydia pneumoniae*
	Heat shock protein 70	Host
	TypeIII repeat extra domain A of fibronectin	Host
	Oligosaccharides of hyaluronic acid	Host
	Polysaccharide fragments of heparan sulphate	Host
	Florinogen	Host
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides	Mycoplasma
	Lipoteichoic acid	Gram positive bacteria
	Zymosan	Fungi
TLR7	Imidazoquinone	Synthetic compounds
	Loxoribine	Synthetic compounds
	Broprimine	Synthetic compounds
	Single stranded RNA	Viruses
TLR8	Imidazoquinone	Synthetic compounds
	Single stranded RNA	Viruses
TLR9	CpG-containing DNA	Bacteria and viruses
TLR10	N.D.	N.D.
TLR11	N.D.	Uropathogenic bacteria

Table 1.5 – Toll-like receptors identified to date and their known ligands

TLR2 recognizes peptidoglycan (Takeuchi et al., 1999) and bacterial lipoproteins (Underhill et al., 1999b), TLR4 recognizes LPS from most Gram-negative bacteria (Poltorak et al., 1998). TLR5 reacts with flagellin (Hayashi et al., 2001) and TLR9 is a receptor for bacterial DNA (Hemmi et al., 2000). Subsequently it has been established that individual TLRs play important roles in recognising microbial components derived for different pathogens (Fig 1.16).

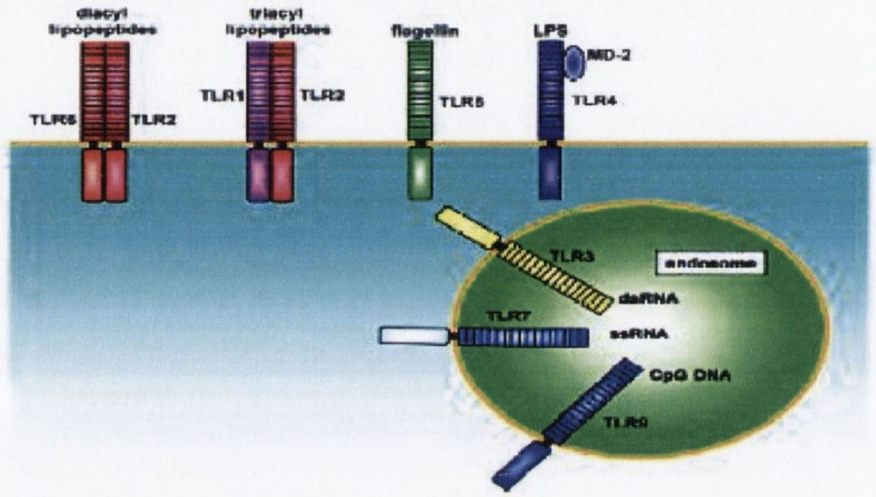


Figure 1.16 – TLRs and their ligands. TLR2 is essential in the recognition of microbial lipoproteins. TLR4 is the receptor for LPS. TLR5 recognizes flagellin. Thus, the members of the TLR family recognize specific microbial components. (Reproduced from Takeda and Akira, International Immunology, Vol 17, 2005)

1.12.3 TLR Signalling to *H. pylori*

In an *in vivo H. pylori* infection, epithelial cells are the first cells to induce innate immune signalling pathways. These cells can express TLR2 and TLR5 among other TLRs (Maeda et al., 2000). Previous work by Torok et al has demonstrated that *H. pylori* lipopolysaccharide (LPS) and flagellin are TLR2 and TLR5 agonists and that expression of these TLRs results in enhanced NF-κB activation upon *in vitro* infection of epithelial cells with *H. pylori*. In addition, IL-8 mRNA levels were found to be elevated in TLR2-expressing epithelial cells upon *H. pylori* infection (Smith et al., 2003). It has been proposed that in addition to activating NF-κB, TLRs may be important for increased IL-8 secretion in *H. pylori* infected cells (Torok et al., 2005). It was reported that *H. pylori* neutrophil activating protein (HP-NAP) was a TLR2 agonist

able to stimulate either neutrophils or monocytes to increase their expression of IL-12, a key cytokine for the differentiation of naïve Th cells into the Th1 phenotype (Amedei et al., 2006). More recently, *H. pylori* heat-shock protein 60 (HSP60) was found to induce IL-8 secretion via TLR2 and mitogen-activated protein (MAP) kinase pathways in human monocytes (Zhao et al., 2007).

1.12.4 TLR Independent Recognition of *H. pylori*

TLRs are membrane bound molecules that recognize microbial components on the surface or within extracellular compartments of cells. Intracellular recognition of bacteria appears to involve a TLR-independent system (Takeda and Akira, 2005). Recent evidence indicates that the nucleotide-binding oligomerization domain (NOD) family of proteins plays an important role in the recognition of intracellular bacteria (Fig1.17) Two members of this family, Nod1 (CARD4) and Nod2 (CARD15), respond to different motifs in peptidoglycan, a component of bacterial cell walls (Chamaillard et al., 2003; Girardin et al., 2003). The reported function of Nod1 is as an important intracellular sensor of gram-negative bacteria in epithelial cells (Philpott and Girardin, 2004).

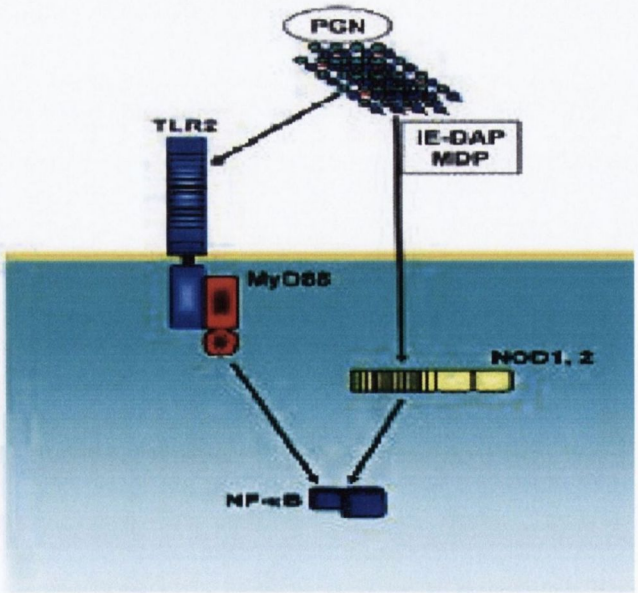


Figure 1.17 – TLR-dependent and independent recognition of microbial components. TLR2 has previously been shown to mediate peptidoglycan (PGN) recognition. However, NOD1 and NOD2 have recently been shown to recognize

motifs found in the layer of PGN. It is possible that TLR2 recognizes lipoprotein contamination in the PGN layer. (Reproduced from Takeda and Akira, International Immunology, Vol 17, 2005)

It has been shown that noninvasive *H. pylori* was recognized by epithelial cells via NOD1. NOD1 detection of *H. pylori* depended on the delivery of peptidoglycan to host cells via a bacterial type IV secretion system, encoded by the *H. pylori* cag pathogenicity island (Viala et al., 2004). It seems likely that Nod1 is a cytosolic host defence molecule that can activate a proinflammatory signaling cascade in response to Gram-negative bacteria which can cause severe mucosal inflammation in animal hosts.

1.13 Rationale and Objectives of the Study

Secreted proteins (the secretome) of *H. pylori* may be of special importance in further examining the effects of *H. pylori* on infected individuals, as these proteins come into direct contact with host cells and tissue. To date, the secreted proteins in the form of *H. pylori* OMV have not been fully characterized. OMV are known to act as vehicles for the delivery of virulence factors from the organism directly to the gastric mucosa. This study proposed to examine the actions of *H. pylori* OMV, independent of the intact organism, using a range of techniques and models. Furthermore, the acute response of duodenal epithelial cells upon *ex vivo* stimulation with *H. pylori* OMV was to be determined.

The objectives of this study were four-fold. The first aims were to proteomically and functionally characterize *H. pylori* outer membrane vesicles by SDS-PAGE and Mass Spectrometry. Secondly, an analysis of the effects on T84 epithelial cell function *in vitro,* following co-incubation with OMV, was to be carried out. This would include internalization of OMV within epithelial cells, cytokine expression and changes at the protein level of expression. Following this, the effect of OMV on the innate immunity of the host was examined with cell lines stably transfected with Toll-like receptors. Finally, the acute response of the duodenum to infection with *H. pylori* OMV using an *ex vivo* model of duodenal explants would be determined.

CHAPTER II

MATERIALS AND METHODS

2.1 REAGENTS

Bovine serum albumin (BSA), ammonium persulphate (APS), phorbol 12-myristate 13-acetate (PMA), β-mercpatoethanol, dimethylsulphoxide (DMSO), ampicillin, chloramphenicol, N,N,N',N'-tetramethlyenediamine (TEMED), Tris (hydroxymethyl) aminomethane, polyoxyethylenesorbitan monolaurate (Tween 20®), 30% H_2O_2, p-nitrophenyl phosphate tablets, nickel chloride, N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), urea, sodium thioglycolate, imidazole, arabinose, Luria-Bertani medium, agar (Select) and wide molecular weight range markers were from Sigma (Poole, Dorset, U.K. and St. Louis, MO, U.S.A.). Lymphoprep™ was from Nycomed Pharma AS, Norway. Enhanced chemiluminescent (ECL) detection kit, the Plus One Silver Stain kit, the IPGphor IEF pH 3 – 10 strips and the protein G sepharose were from Amersham International (Aylesbury, U.K.). Complete® EDTA-free protease inhibitor cocktail and the In Situ Cell Death Detection Kit, TMR red were from Roche Diagnostics (East Sussex, U.K.). Ethanol, methanol, acetone, butanol, glycerol and acetic acid were from BDH Ltd. (Poole, U.K.). Electran 2.6 premix acrylamide: N,N'-methylenebisacrylamide (29.2:0.8) was from BDH, Poole, U.K. All buffer reagents for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were prepared in deionised water (Elga Prima Purelab Ultra). Electrophoresis grade agarose was from GIBCO BRL (Life Technologies, Paisley, Scotland). Polymyxin B BODIPY® FL conjugate, EthD-1 ethidium homodimer-1, Sypro Red protein gel stain and CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) were from Molecular Probes (Eugene, OR, U.S.A.). 1,2-phenylenediamine dihydrochloride (OPD, 2 HCl) tablets were from DakoCytomation (Copenhagen, Denmark). Streptavidin-HRP was from Kirkegaard and Perry Laboratories (Gaithersburg, MD, U.S.A.). The human CXCL8/IL-8 detection ELISA kit, the human IL-12p70 detection ELISA kit and the human CCL5/RANTES detection ELISA kit were from R and D Systems, Minneapolis, Mn). TMB substrate kit for ELISA was from Pierce Laboratories (Rockford, Il). RNA nucleopsin kit was from Machery-Nagel (Duren, Germany). The RETROscript RT-PCR kit was from Ambion (TX, USA). Other reagents were analytical or general preparative grade and were from Sigma, Merck Biosciences or BDH. All cell culture disposables were provided by Nunclon (Roskilde, Denamrk) unless otherwise stated.

2.2 ANTIBODIES

Rabbit anti- *H. pylori* VacA protein toxin, rabbit anti – *H. pylori* CagA antigen were from Austral Biologicals, San Ramon, CA. Mouse anti – HpaA specific Mab (HP30-1:1:6) was a kind gift from Dr Ann-Mari Svennerholm Goteborg, Sweden. The 4G10 anti-phosphotyrosine monoclonal antibody was from Upstate Biotechnology (Lake Placid, NY). The PY 2000 anti-phosphotyrosine monoclonal antibody was from Affiniti Research Products (Exeter, UK). Rabbit anti ZO-1 and rabbit anti-occludin were from Zymed Laboratories (San Francisco, Ca).

ALEXA Fluor ® 488 goat anti-rabbit IgG (H + L), ALEXA Fluor ® 488 goat anti – mouse IgG (H + L), ALEXA Fluor ® 568 goat anti – rabbit IgG (H + L) and ALEXA Fluor ® 546 phalloidin were from Molecular Probes (Eugene, Oregon, USA).

Rabbit polyclonal to creatine kinase B (CKB), rabbit polyclonal to actin, rabbit polyclonal to Galectin-3, rabbit polyclonal to thioredoxin(TRX), rabbit polyclonal to protein disulphide isomerase (PDI) and goat polyclonal to triosephosphate isomerase (TPI) were from Abcam (Cambridge, UK).

Rab5 and Rab 7 were from Santa Cruz Biotechnology (Ca, USA).

The rabbit anti – OipA specific Mab was a kind gift from Dr Yoshio Yamaoko Houston, Tx. The rabbit anti NapA antisera (See Section 2.7) was a kind gift from Dr Veronica Athie Morales (Dept of Biochemistry, Trinity College Dublin).

Rabbit anti- Helicobacter, Rabbit anti-mouse HRP, goat anti-mouse HRP, swine anti-rabbit IgG HRP and rabbit anti-human IgG HRP conjugates were from DakoCytomation (Copenhagen, Denmark). The rabbit anti-goat IgG HRP was from Chemicon

2.3H. PYLORICULTURE

2.3.1 *H. pylori* Strain and Growth Conditions

*H. pylori*strain 11637 was incubated on Columbia blood agar plates (Figure2.1). The bacterial cells were cultivated overnight and microaerophilic conditions were achieved using the MART anoxomat system. For liquid culture, the bacteria were inoculated into Brucella broth base (Difco, Detroit, Mich.) supplemented with 1 % ß-cyclodextran Sigma (Poole, Dorset, U.K) for 72 h at 37°C under microaerophilic conditions with constant rotation (120 rpm) to an OD_{600} of approximately 1.

2.3.2 *H. pylori* Extract (HPE) Preparation

H. pylori grown on Columbia blood agar plates for 3 days were harvested into ice-cold tissue culture medium (see below) without fetal calf serum, unless otherwise indicated (1ml/plate). Suspended bacteria were adjusted to an optical density at 600nm of 2.9, placed on ice for 20 min, and then centrifuged (15,000 x g; 15 min; 4°C). The supernatant (HPE) was analysed on 12 % SDS-PAGE gels and stored at -20°C

2.3.3 *H. pylori* Outer Membrane Vesicle (OMV) Preparation

At 72 h, bacteria were removed from liquid broth by centrifugation in an Eppendorf centrifuge 5417C rotor F45-30-11 (10,000 x g, 15 min, 4°C), and the supernatants were ultracentrifuged in a Sorvall Ultracentrifuge (100,000 x g, 2 h, 4°C) to recover OMV as described previously. After two washes with phosphate –buffered saline (PBS, pH 7.4), aliquots of OMV preparation were overlaid onto carbon-colloidal-coated mesh grids, negatively stained with 1% aqueous uranyl acetate and examined by electron microscopy to confirm the absence of whole cells and flagella. The amount of protein in the OMV preparations was determined using the Bradford method with bovine serum albumin as the protein standard. OMVs were stored at -20°C until use.

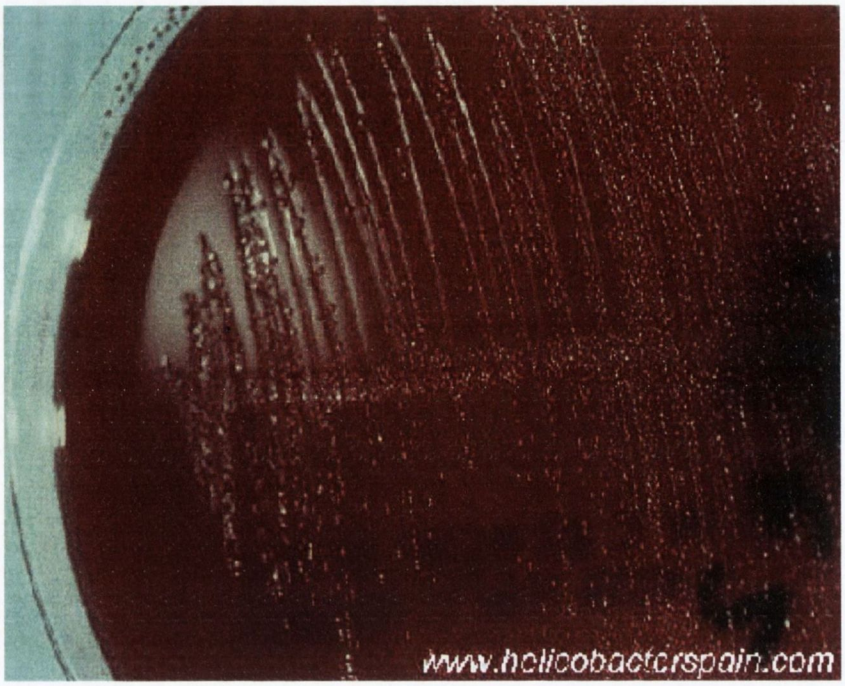


Fig. 2.1 *H. pylori* colonies following anaerobic culture on Columbia blood agar plate. Typical colonies of *H. pylori* after 48 h anaerobic culture on a Columbia blood agar plate. Colonies are 2-3 mm in diameter, irregular, raised, opaque and grey/white after 48 h culture

2.4CELL CULTURE

2.4.1 Source of Cell Lines and Cell Culture Reagents

The epithelial cell lines T84 and AGS, obtained from the American Type Culture Collection (Manassas, Va) were used in this study. RPMI-1640 medium, foetal calf serum (FCS) and Hank's balanced salt solution (HBSS) were obtained from GIBCO BRL (Life Technologies, Paisley, Scotland). Dulbeccos Modified Eagles Medium (MEM), Nutrient F-12 medium and Penicillin/streptomycin/L-glutamine prepared aliquots were purchased from Sigma (Poole, Dorset, U.K.).

The human embryonic kidney cells, HEK 293, HEK TLR2 and HEK TLR4 were a kind gift from Dr Luke O' Neill, Dept of Biochemistry, Trinity College Dublin. MEM Alpha medium was obtained from GIBCO BRL (Life Technologies, Paisley, Scotland).

2.4.2 Maintenance of Cell Cultures

T84 cells were grown in a 1:1 ratio of Dulbeccops MEM and Nutrient F-12 medium supplemented with supplemented with L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100 μg/ml) and 10% (v/v) fetal calf serum (Gibco) at 37°C in 5% CO_2. FCS was heat inactivated (56°C, 1 h) to inactivate complement and then aliquoted for storage at -20°C. Supplemented medium was stored at 4°C. Cells were routinely maintained in 75 cm^2 cell culture flasks (Nunclon, Roskilde, Denmark) at 37°C in 5% CO_2 in a humidified incubator. Visual examination was undertaken using phase contrast microscopy. T84 cells were passaged upon reaching 80% confluence. For passage, the cell suspension was aspirated and the cells were washed twice with PBS (1x) containing 1mM EDTA. One to 2 mls of Trypsin/EDTA solution was added to cell culture flasks, ensuring all cells were covered. Cells were incubated at 37°C in 5% CO_2 for 2 – 10 min until cells started to detach from flask. Gentle centrifugation (500 x g, 5 min) was used to pellet the cells, which were subsequently resuspended in complete media for enumeration or seeding.

AGS cells were grown in Nutrient F-12 medium supplemented with supplemented with L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100 μg/ml) and 10% (v/v) fetal calf serum (Gibco) at 37°C in 5% CO_2. AGS cells were maintained as described above.

HEK cell lines were grown in MEM Alpha medium supplemented with supplemented with L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100 μg/ml) and 10% (v/v) fetal calf serum (Gibco) at 37°C in 5% CO_2. The HEK cell lines were maintained by Dr Sinead Smith, Institute of Molecular Medicine, Trinity College Dublin.

2.4.3 Total Cell Lysate Preparation

Cells were harvested by centrifugation and the resulting cell pellet was resuspended in cell lysis buffer (unless otherwise stated this buffer consisted of 1 % v/v NP40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 nM calyculin A, 1 mM PMSF and 10 ug/ml leupeptin (NP40 cell lysis buffer). The cells were lysed on ice for 30 min, with vortexing of the sample every 5 min to promote efficient cell lysis. Detergent-insoluble material, unlysed cells and nuclei were removed by centrifugation for 5 min at 2500 x g. The resulting supernatants, containing total cellular protein, were stored on ice and quantified using the Bradford assay to determine cellular protein content and used for immunoprecipitation experiments or for direct analysis by Western blotting.

2.4.4 Antigen Immunoprecipitation

Immunoprecipitation experiments were carried out using total cell lysates (typically 10 x 10^6 cells / ml lysis buffer). The immunoprecipitation protocol was carried out using protein-G sepharose beads (Pharmacia Biotech), which were washed twice with an excess volume of cell lysis buffer prior to use. Preclearing of any non-specific material from the cell lysates was carried out by adding 100 μl of a 10 % v/v suspension of protein-G sepharose beads to 1 ml of cell lysate. After a two-hour incubation at 4°C with constant end-over-end mixing, the beads were removed by centrifugation at 10,000 rpm for 1 minute. The precleared lysate was divided into two; to one aliquot was added the antigen-specific antibody and to the other aliquot the corresponding control immunoprecipitation antibody was added (the amount of antibody added to the tubes was determined in preliminary experiments and typically ranged from 0.5 μg - 2 μg antibody per reaction per 0.5 ml of lysate. The amount added therefore depended on the affinity of the antibody for its antigen. Lysates (0.5 ml) were incubated with the antibodies on ice for 2 hours with inversion of tubes every 30 min to ensure optimal interaction between antibody-antigen complexes. The antibody-antigen complexes

were collected by adding 100 µl of a 10 % v/v suspension of washed protein-G sepharose beads per tube, for 2 hours or overnight at 4°C with end-over-end mixing. Protein-G sepharose/antibody-antigen complexes were recovered from the cell lysates by centrifugation at 10,000 rpm for 1 min. The pellet was washed three times in ice-cold complete cell lysis buffer. The pellet was then resuspended in 20 µl of 1X SDS-PAGE buffer and boiled for 4 minutes to dissociate the antibody/antigen complex from the protein-G sepharose beads. The protein-G sepharose beads were removed from the sample by centrifugation at 10,000 rpm for 1 min. The supernatants were recovered and immunoprecipitates were analyzed by SDS-PAGE and Western blotting.

2.4.5 Determination of Protein Concentration

The concentration of protein preparations was determined by the method of Bradford (1976) using Bio-Rad Protein Assay Concentrate (Bio-Rad, U.K.). Assay concentrate was freshly diluted 1:5 with distilled water and mixed well prior to each assay. A BSA standard curve was prepared in the appropriate buffer from a 20 mg/ml BSA stock solution [Appendix A] using serial dilutions to yield standards at 20, 10, 5, 2.5, 1.25, 0.625, 0.312, and 0.0625 µg/ml BSA. 5-10 µl of sample or standard was mixed with 1 ml of the diluted BioRad staining solution, vortexed and incubated for 5 min at room temperature. The absorbance of protein solutions were read at 595 nm in triplicate in a Tecan Spectra Fluor Plus spectrophotometer. A specifically designed Excel macro averaged readings, constructed a standard curve and calculated the individual protein concentrations with reference to the standard curve automatically. A commercial BSA solution at a concentration of 2 mg/ml (BSA Protein Standard, Sigma, Poole, U.K.) was used to monitor accuracy and reproducibility of the Bradford method.

2.4.6 Cell Freezing and Revival

A cryopreservation solution was prepared containing 90% FCS and 10% DMSO [Appendix A]. Cells were harvested as for passage, the supernatant decanted and the cells resuspended in ice-cold cryopreservative medium at 2 x 10^6 cells/ml. Cell suspension aliquots (1 ml) were placed into sterile cryovials (Nunclon, Roskilde, Denmark) and the cryovials were then transferred to liquid nitrogen for long-term storage. For cell revival, cryovials removed from storage were quickly thawed in a 37°C water bath. Cells were washed with pre-warmed medium. Gentle centrifugation

(1000 rpm, 3 min) was used to pellet the cells which were subsequently resuspended in 10 ml of RPMI supplemented with 20% FCS and transferred to 25 cm^2 tissue culture flasks and placed at 37°C, 5% CO_2 in a humidified incubator.

2.4.7 Cell Enumeration and Viability

To assess cell yield and viability 10 µl of a 0.4 % trypan blue solution (Sigma) was added to 90 µl of the cell suspension. Cells were counted on a Neubauer haemocytometer under a light microscope. Live cells appear white and non-viable cells appear blue. Enumerations were performed in duplicate. T84 and AGS cells were seeded at a density of 2 x 10^6 cells/ml and used when less than 90% confluent.

2.4.8 Co-culture of Cells with *H. pylori* OMVs and Other Stimuli

(a) T84 cells were adjusted to 1 x 10^6 in complete medium and 1ml added to semi-permeable tissue culture inserts of uncoated polyethylene terephthalate (PET) plastic trace – etched membranes (Falcon, Beckton Dickinson, Oxford, UK) in a 12 well plate. Cells were fed both apically and basally. Cell confluence was measured by phase-contrast microscopy. Measuring transepithelial electrical resistance (TER) using an Endohm apparatus (World Precision Instruments, Sarasota, Fla), monitored the polarization and differentiation of the cells. The resistance monitored at 24 h intervals increased progressively over 10 to 15 days when cells formed high resistance polarized monolayers with stable TER values (~1500 to 2000 Ω/cm^2). At this stage the apical or basolateral cell culture medium was substituted with 10 µg/ml OMV, HPE or media alone as a negative control, incubated for various periods of time, and monitored or processed for 2-dimensional electrophoresis or immunofluorescence staining.

(b) T84 cells were adjusted to 1 x 10^6 in complete medium and 1ml added to 12 well plates. Cells were stimulated in duplicate with OMVs alone (2.5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml, 50 µg/ml), 1 µM PMA as a positive control and media alone as a negative control. Supernatants were collected after 24 h for IL-8 and RANTES detection and stored at -20°C until required. AGS cells were also used for comparative purposes.

(c) T84 cells were adjusted to 1 x 10^6 in complete medium and 100 µl added to wells of 96-well plates. Cells were stimulated in triplicate with OMVs alone (2.5 µg/ml, 5

μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, 50 μg/ml), HPE as a positive control and media alone as a negative control. After 24 h supernatants were removed and the cells were washed, fixed in 4% Paraformaldehyde (PFA) and processed for immunofluoresence staining.

(d) T84 cells were adjusted to 1 x 10^6 in complete medium and 100 μl added to wells in 8 well chamber slides. Cells were stimulated with OMVs alone (2.5 μg/ml , 5 μg/ml, 10 μg/ml), HPE as a positive control and media alone as a negative control. After 24 h supernatants were removed and the cells were washed, fixed in 4 % PFA and processed for immunofluoresence staining. AGS cells were also used for comparative purposes.

(e) AGS cells were adjusted to 1 x 10^6 in complete medium and 1ml added to 12 well plates. Cells were stimulated in duplicate with OMVs alone (2.5 μg/ml , 5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, 50 μg/ml) and media alone as a negative control. Vacuolation of AGS cells was assessed by light microscopy and the neutral red assay.

(f) HEK 293, HEK TLR2 and HEK TLR4 cell lines were adjusted to 1 x 10^6 in complete medium and cells were seeded in 6 well plates, 24 hours before experiment. Cells were stimulated with OMVs, *H. pylori* LPS or *E. coli* LPS (10 μg/ml) for 4, 8 and 24 hours. Cells were then lysed and harvested for RNA extraction (Machery-Nagel).

2.4.9 Neutral Red Assay

After incubation with the OMVs, the medium overlaying AGS cells was removed and replaced with 500 μl of neutral red (0.05 %, v/v) and stained for 4 min. The cells were washed with PBS (x 4) following the removal of the excess stain solution. The neutral red as extracted by the addition of 500 μl of acidified alcohol (70% Ethanol and 0.37% hydrochloric acid). The neutral red uptake was calculated from a neutral red standard curve. The OD was read at 565 nm.

2.4.10 Immunofluorescence Staining

Cells were passaged in the normal manner and quickly spun down at 300 x g for 5 minutes at room temperature and resuspended in sterile tissue culture medium at a concentration of approximately 2 x 10^6 cells per ml. Approximately 150 μl of this cell suspension was placed in each well of an 8 well chamber slide) and the cells were allowed to adhere to the slide for 5 minutes in the cell culture hood. The cells were

fixed with either 4% PFA for 15 min at room temperature or in ice cold methanol for 5 min. The slides were removed and allowed to air-dry for 2 minutes in the cell culture hood to remove any residual methanol. The cells were blocked and permeabilised by the addition of 50 µl of PBS/5% dried skimmed milk/0.1% Tween®/0.05% Triton-X-100 (Blocking buffer – Appendix A) along the centre of the slide. A cover slip was placed on top of the slide and incubated for 30 minutes at room temperature. Unbound serum was removed by incubating the slide vertically in a staining glass jar containing PBS, in which after a few minutes the coverslip detached from the slide. The slide was then washed three times by quickly placing the slide(s) in fresh washes of PBS containing 0.03 % Tween-20 and 0.02 % Triton X-100 (cell staining wash buffer – Appendix A). The primary antibody was typically diluted 1/50 in antibody dilution buffer (Appendix A). The primary antibody solution (50 µl per slide) was added to the slide, a coverslip was added to the slide and the slide was incubated with the antibody for 1 hour at room temperature. The coverslip was removed by a quick wash in staining wash solution and unbound primary antibody was removed by another three quick washes in this solution. The appropriate secondary antibody (typically 1/100 dilution of FITC labelled goat anti-rabbit or TRITC labelled goat anti-mouse in antibody dilution buffer) was added to the slide and incubated for 30 minutes at room temperature in the dark. The coverslip was removed by a quick wash in cell staining wash buffer and unbound secondary antibody was removed by three quick washes in the same solution. The cells were then stained with Hoechst nuclear dye for 2 min at room temperature. The slides were then washed one final time in the cell staining wash buffer. Two drops of fluorescent mounting medium (Dako, Denmark) was placed at opposite ends of the slide, a coverslip was placed on top of the slide and the slide was fastened to the coverslip by gently pressing down on the coverslip. Slides were then immediately analysed or alternatively stored at 4°C in the dark and analysed at a later date. Microscopic observations and photography were performed on a Nikon Eclipse E800 microscope with a Nikon DXM1200 Digital Camera and images were captured using the ACT software program. Confocal images were acquired using confocal laser microscopy (Nikon Eclipse TE 2000-U). Image processing and 3-D cell image analysis was performed using Ultra View LCI and Volocity software (Perkin-Elmer Applied Biosystems, Warrington, UK). Some of the fluorescent images were acquired using the Cellomics™ HCS Kineticscan (Pittsbourg, PA).

Introduction to Cellomics™ HCS Kineticscan Technology

Cellomics is the study of the functional characteristics of genes and proteins in the context of the whole cell and this can be achieved using automated high content analysis technologies. High content analysis is where large quantities of data are obtained from a single experiment, for example, the measurement of multiple pre-selected cellular responses. A kineticscan reader consists of a fluorescence microscope, a plate reader and a flow cytometer. (Fig 2.2). HCS involves a fluorescence-based assay where cells are automatically imaged and analysed using quantitative fluorescence microscopy. Cells are labelled with fluorescent probes whose emissions reflect the cells phenotype, physiological state or cellular distribution of targets. Fluorescent images of the cells are automatically analysed by proprietary image processing algorithms, known as bioapplications.

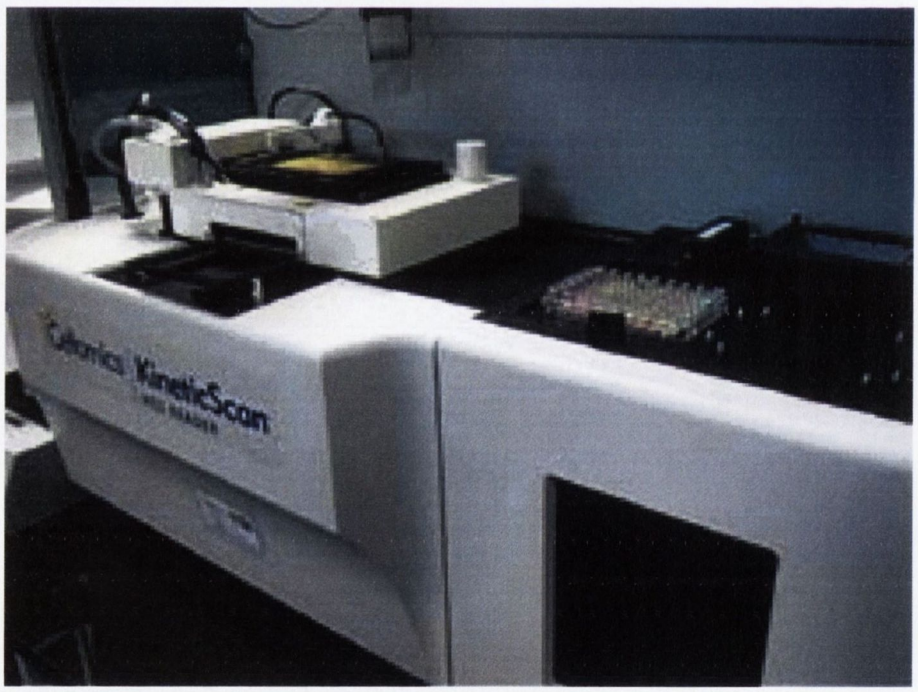


Figure 2.2 - Cellomics™ KineticScan HCS Reader.

2.4.11 Isolation of Polymorphonuclear leukocytes (PMNs)

Normal human PMNs were prepared from the blood of healthy volunteers, using the Lymphoprep™ method. Briefly, blood was obtained from consenting healthy volunteers into tubes containing anticoagulant such as EDTA or heparin. The blood was mixed in a 4:1 proportion with a solution of 6% Dextran in 0.9% NaCl and incubated at 37°C for 45 min. Following this step, the leucocyte rich plasma was removed and was layered onto Lymophoprep (Nycomed, Norway) density gradient and centrifuged at 1500 rpm for 30 min at 4°C. The supernatant was discarded and the neutrophil pellet was washed twice in PBS. Suspending the pellet of cells in H_2O (3ml), followed by the immediate addition of 3.6 % NaCl (1ml) lysed any remaining erythrocytes.

2.4.12 Isolation of Mononuclear Cells from Peripheral Blood (PBMCs)

Briefly, blood was obtained from consenting healthy volunteers into tubes containing anticoagulant such as EDTA or heparin. Blood was mixed with an equal volume of 0.9 % NaCl or PBS. About 30 ml of diluted blood was layered onto Lymophoprep (Nycomed, Norway) density gradient and centrifuged at 1500 rpm for 25 min at RT. Tubes were carefully removed from centrifuge. The mononuclear cell fraction (lymphocytes and monocytes) accumulates as a white band at the interface between the plasma (yellow) and the Lymphoprep layer. The pellet contains the erythrocytes and neutrophils. Most of the platelets are in the yellow plasma. Carefully aspirate the white band of cells, taking as little as possible of the upper layer. The mononuclear cells were dilute in 20 ml PBS and centrifuged at 300g for 10 min. The wash was repeated to remove residual Lymphoprep and platelets. Cells were counted at this point.

2.4.13 Chemotaxis Assay

Cell culture inserts with 5 μm-diameter pore size (Falcon, Becton Dickinson, Paramus, N.J.) were used for chemotaxis of polymorphonuclear leukocytes (PMN). PMNs (10^6) were suspended in HBSS for chemotaxis assays and placed directly in the upper well of the filter insert. PMNs migrating into the lower well containing the chemoattractants were recovered and quantified by counting Hoechst stained PMNs. fM-L-P (1 μM, Sigma) was used as a positive control. Different amounts of OMV (5, 10, 20 μg / ml), were placed in the lower chambers and PMN migration was determined. OMV

neutralised of neutrophil activation with anti-NapA sera (10 µl OMV was incubated with 10 µl of anti-NapA sera for 1 h at 37 °C) or tissue culture media alone as a negative control were also placed directly into the lower chambers. After 110 min at 37°C, the number of PMNs in the lower well was determined by the manner described above.

2.5POLYACRYLAMIDE GEL ELECTROPHORESIS

2.5.1Sample and Molecular Weight Standard Preparation

Following protein estimation, protein was resuspended in 5 µl to 20 µl of 2X reducing sample buffer [Appendix A] as required. Protein samples were boiled at 100°C for 10 min and centrifuged briefly (30 s) to remove any insoluble material. Sigma Broad Range molecular weight standards (5 µg) were run alongside samples.

2.5.2 1D SDS-PAGE

An ATTO system was used for all SDS-PAGE gels (ATTO Corporation, Japan), and protein samples were electrophoresed using a Consort electrophoresis power supply unit. Proteins were separated on reducing gels prepared using a discontinuous buffer system, as described by Laemmli (1970) and adapted by Sambrook and Gething (1989) (Laemmli, 1970; Sambrook and Gething, 1989). Resolving and stacking acrylamide gels were prepared to the required percentage acrylamide in the order indicated in Tables 2.2 and 2.3. APS and TEMED were added last with gentle swirling of the mixture. Electrophoresis was carried out at 150 V, 25 mA per gel for approximately 1.5 h until the dye front had reached just above the gel base. Gels could now be removed from glass plates for staining or for transfer to PVDF membrane by Western blotting.

TABLE 2.1Composition of Resolving Gel for SDS-PAGE

Component	10%	12.5 %
Acryl/Bis 30%	3.3 ml	4.2 ml
Tris 1.5M	2.5 ml	2.5 ml
H_2O	4 ml	3.2 ml
SDS 10 %	100 µl	100 µl
APS 10%	50 µl	50 µl
TEMED	5 µl	5 µl

TABLE 2.2Composition of Stacking Gel for SDS-PAGE

Component	Amount
Acryl/Bis 30%	0.88 ml
Tris 1.0M	1.25 ml
H_2O	4.06 ml
APS 10%	50 µl
TEMED	10 µl

2.5.3 Coomassie Blue Staining

Two different Coomassie blue staining procedures were carried out. The first procedure that was sufficient for staining SDS-PAGE gels that were to be used for mass spectrometry (due to the greater sensitivity of Coomassie G-250 to visualise protein bands). The second procedure was sufficient for staining molecular weight markers and SDS-PAGE gels that were not to be used for mass spectrometry (due to the less sensitive Coomassie R-250).

(a) Colloidal Coomassie G-250 staining (for gels that are to be used for analysis by mass spectrometry)

The SDS-PAGE gel was removed from the glass plates and immersed in fixation solvent buffer (50 % methanol, 2 % of phosphoric acid (85 % stock solution) and 48 % distilled water) overnight at room temperature with continuous agitation. The gel was washed three times in two litres of distilled water. The gel was then immersed in one litre of incubation solvent buffer (34 % methanol, 2 % phosphoric acid (85 % stock) and 17 % w/v ammonium sulphate in distilled water) for 1 hour at room temperature with constant agitation. A total of 0.25 g of Coomassie Blue G-250 powder (BioRad) was dissolved in 20 ml of methanol and this was added to the incubation solvent Buffer (Appendix A). The gel was incubated with this solution for 2-3 days at room temperature with constant agitation (the container was sealed to prevent evaporation). The gel was then washed in approximately two litres distilled water, followed by two washes in 25 % methanol (in distilled water) and the procedure was completed by washing in two litres of distilled water alone. The gel was then placed in a clean clear plastic bag and stored at 4°C prior to analysis of the desired bands by MALDI mass spectrometry.

(b) Coomassie R-250 staining (for gels that are not to be used for mass spec analysis)

The SDS-PAGE gel was removed from the glass plates and immersed in Coomassie R-250 gel staining solution for at least four hours at room temperature with constant agitation. This solution was then poured off (and saved for further use) and the gel was then incubated in Coomassie destain solution until the stained protein bands could be clearly distinguished. The gel was then washed in distilled water and photographed using the SynGene GelDoc capture system.

2.5.4 Silver Staining of Protein or LPS

SDS-PAGE gels that were silver stained were stained by either the Ansorge method or the Amersham Plus One Silver Stain kit unless otherwise stated.

(a) Ansorge Method (for gels not to be used for analysis by mass spec)

The SDS-PAGE gel was removed and fixed in Fixation solution (50% Methanol (Sigma-Aldrich, Germany), 12% Glacial Acetic Acid, 38% deionised water) for 5 min. It was then placed in a solution of 10% ethanol (Merck, Darmstadt, Germany), 5% glacial acetic acid (Sigma-Aldrich, Germany), 85% deionised water for a further 5 min. The gel was then removed and stained in 0.06% potassium permanganate (BDH) for 10 min. Followed by 0.1% potassium carbonate (Sigma) for a further 10 min. The gel would then be washed 3 to 6 times in fresh changes of deionised water. The gel was then placed in the silver solution containing 0.1% silver nitrate (BDH) for 10 min, followed by 2 more washing steps in deionised water. The gel was developed in 2% potassium carbonate, 0.015% formaldehyde (Rectapur, Prolabo) until the desired colour was obtained. To stop the staining the gel was transferred to 1% glacial acetic acid and stored under water, away from light.

(b) Silver Stain for Proteins Plus One Method (for gels that are to be used for analysis by mass spectrometry)

SDS-PAGE gels were stained with the PlusOne™ Silver Staining kit (Amersham Biosciences) as per the manufacturers instructions as follows. Gels were placed in a fixation solution (40% Ethanol, 10% glacial acetic acid) for 30 min, followed by a sensitizing solution (30% ethanol, 0.5% glutardialdehyde (25% w/v), 4% sodium

thiosulphate (5%w/v), 17g sodium acetate) for 30 min. Gels were then washed 3 times with deionised water. For the silver reaction the gels were placed in a silver nitrate solution (2.5% w/v), containing Formaldehyde (37% w/v) which was added immediately prior to staining, for 20 min. The gels were washed again for 2 min and then developed in developer (6.25g sodium carbonate, 0.05ml formaldehyde) for 2 – 5 min. The stopping reaction was carried out with 3.65 g EDTA-Na_2H_2O in 250ml deionised water for 10 min. Gels were stored in water. All of the above solutions were made up to a final volume of 250ml, which was sufficient to stain a 13cm x 13 cm gel. All of the above solutions were made up with deionised water.

2.5.5 Western Blotting

Western blotting was carried out using the semi-dry method for the transfer of electrophoresed proteins to immobilising membranes as described by Towbin *et al.* and was performed using an ATTO semi-dry transfer system (ATTO Medical Supplies, Japan) (Towbin et al., 1979). During SDS-PAGE, polyvinylidene fluoride (PVDF) transfer membrane (0.45 μm, Pall Life Sciences) was briefly saturated with methanol for 10-15 s and then equilibrated in transfer buffer [Appendix A] for approximately 20-30 min prior to semi-dry transfer. Whatmann 3 mm filter paper of the same dimensions was also saturated in transfer buffer prior to semi-dry blot sandwich assembly, which was assembled in the order cathode, filter paper, acrylamide gel, PVDF membrane, filter paper and anode. Electrophoretic transfer was performed at 100 mA per gel for 60 or 90 min as required. Following semi-dry transfer, the PVDF membrane was removed and processed for immunoblotting. The lane containing the molecular weight markers was stained with amido black [Appendix A] and de-stained in water.

2.5.6 Immunodetection and Development

Following transfer, non-specific sites on the membrane were blocked with freshly prepared phosphate buffered saline (PBS)/5% dried skimmed milk/0.1% Tween 20® (blocking buffer) [Appendix A] for 1 h with gentle agitation at room temperature. Membranes were then washed three times with PBS/0.1% Tween 20® (washing buffer) [Appendix A] and incubated with appropriate primary antibodies, diluted as required in blocking buffer, for 2 h at room temperature or overnight at 4°C with gentle shaking in

a sealed plastic bag. Following incubation with primary antibody, membranes were washed several times over 30 min with washing buffer. Membranes were then incubated with the relevant horseradish peroxidase-conjugated secondary antibody, diluted as required in blocking buffer, for 1 h with shaking in a sealed plastic bag. Following incubation with secondary antibody, membranes were finally washed 4 to 5 times with washing buffer over 30 min with the final wash in PBS alone. Membranes were stored in PBS at 4°C if development could not be carried out immediately. Detection of immunoblots was performed using the enhanced chemiluminescence (ECL) method (Amersham, BioSciences). Membranes were incubated for 1 min in a solution of iodophenol (400 μM), luminol (1.25 mM), and hydrogen peroxide (0.1% (v/v)) in 0.1 M Tris-HCl (pH 8.8). The membrane was removed after 1 min and placed between acetate sheets, which were then exposed to Kodak X-OMAT S film for the appropriate time period (range 10 s to 1 min). Exposed films were developed using an automatic developer (CURIX 60, AGFA, Type 9462/100/140, Agfa-Gevaert AG, Munich, Germany).

2.5.7 Stripping of Western Blot Membranes

In some experiments where indicated, PVDF membrane that was exposed to film was stored at 4°C in PBS-T and at a later date the membrane was stripped of its primary and secondary-HRP linked antibodies and reprobed with different antibodies. This procedure was carried out by incubating the PVDF membrane in Western blotting stripping solution (2 % w/v SDS, 62.5 mM Tris-HCl (pH 6.8) and 100 mM β-mercaptoethanol) at 65°C for 30 min. This incubation was carried out in a chemical fume hood due to the dangers associated with β-mercaptoethanol. The membrane was removed from the stripping solution and extensively washed in the fume hood in PBS-T (at least 5 – 10 washes for 5 minutes). Blocking of the membrane, incubation with primary and HRP-labelled secondary antibodies and detection of antigens was carried out as described in Section 2.5.6.

2.5.8 Sample Preparation for SDS-PAGE

a) Acetone Precipitation of Protein Samples

Protein samples were concentrated by acetone precipitation prior to analysis on SDS-PAGE and Western blotting. Total cell lysates or subcellular fractions, containing a predetermined amount of protein, were diluted with at least five volumes of ice-cold acetone. Samples were incubated for at least 20 min at -20°C with inversion of the tubes every five minutes. Precipitated protein was collected by centrifugation at 12,000 x g for 5 min at 4°C. The acetone supernatant was removed and the precipitated protein was allowed to air-dry for 2 min to remove any residual acetone. Protein samples were then ready to be analysed by SDS-PAGE and Western blotting or 2-DE.

b) Trichloroacetic Acid (TCA) Precipitation of Protein Samples

One volume of TCA (Appendix A) (Sigma Poole, Dorset, UK) was added to 4 volumes of a predetermined amount of protein. Samples were incubated for at least 10 min at 4°C. Samples were centrifuged at 14, 000 rpm for 5 min at 4°C. The TCA supernatant was removed and the precipitated protein was then washed in 200 µl acetone. The samples were then centrifuged at 14, 000 rpm for 5 min at 4°C. This was done for a total of two acetone washes. The remaining pellets were then placed on a 95°C heat block for 5-10 min to drive off acetone. Protein samples were then ready to be analysed by SDS-PAGE and Western blotting or 2-DE.

c) Trifluoroethanol (TFE) Precipitation of Protein Samples

Phase partition on OMVs, was performed using the method by Molloy *et al* with some modifications. Briefly, 300–1000 µg of membranes were suspended in 150 µL of 50 mM ammonium bicarbonate. 1000 µL of 2/1 TFE/ CHCl3 mixture was added with strong shaking and themixture was maintained at 0°C for 1 h with periodical vortexing. Centrifugation at 10 000_*g* for 4 min separated the mixture into three phases (Fig. 1). The lower chloroformic and the upper aqueous phases were separated from the insoluble interphase and concentrated by vacuum centrifugation. Two dimensional electrophoresis was carried out as decribed (see below) with the following modifications. The rehydration medium contained 5 M urea, 2 M thiourea, 2% sulfobetaine 3–10, 50 mM DTE, 2% CHAPS, 10 mM Tris-HCl. When TFE was added to the resolubilization medium, it was used at 50% without sulfobetaine. Focusing was performed using a gradient voltage from 300 to 3500 V in 3 h. The voltage was then maintained for 3.5 h and at the end of the period the voltage was increased to 5000 V

for a total of 100 000 Vh. The second dimension was carried out in 12.5% acrylamide SDS gels according to [10] following two equilibration steps (reduction and alkylation). Staining was obtained either by silver nitrate for the analytical or by Coomassie Brilliant Blue R-250 for micropreparative gels.

2.5.9 Two – Dimensional Gel Electrophoresis (2-DE)

Samples to be subjected to isoelectric focusing and second dimension SDS-PAGE were mixed with a sample loading slution (9M urea, 2% CHAPS and a trace of bromphenol blue). IPG buffer (Amersham BioSciences, containing ampholytes, pH range 3 – 10 non-linear, pH 4 – 7 non-linear, pH 6 – 9 non linear) was added to a final concentration of 2% v/v. IPG focusing strips (Amersham BioSciences, 13cm in length pH range 3 – 10, pH 4 – 7, pH 6 – 9) were passively rehydrated overnight at room temperature with 250 µl of sample mix. Isoelectric focusing was on a multiphor II system (Amersham BioSciences), with an initial linear gradient of 0 – 500V over 1 min, then 500 – 3500V linearly over 1h 30min, followed by 3500V for 5h 40 min. Prior to the second dimension, strips were equilibrated in two successive equilibration buffers (Appenice A). The first buffer contained 1% w/v dithiothreitol (DTT) Sigma (Poole, Dorset, UK), while the second contained 2.5% w/v iodoacetamide. Each equilibration was for 15 min with continuous agitation with 10 ml of buffer per IPG gel. Strips were then applied to 12.5% acyrlamide gels and sealed with melted agarose (0.5% w/v agarose in electrophoresis buffer). Electrophoresis was carried out using an Ettan Dalt II (Amersham BioSciences) apparatus, with initial separation at a constant 5W per gel for 30 min followed by 20W per gel until the dye front had migrated approximately 13 cm. Once electrophoresed, gels were transferred to polypropylene containers for staining.

TABLE 2.3 Composition of resolving gel for 2-DE

Component	10%	12%
Acryl/Bis 30%	6.7 ml	8.0 ml
Tris 1.5M	5.0 ml	5.0 ml
H_2O	8.0 ml	6.7 ml
SDS 10 %	200 µl	200 µl
APS 10%	100 µl	100 µl
TEMED	10 µl	10 µl

2.5.10 Scanning and Analysis of Gels

All gel images were obtained using an Agfa Snapscan 1212. Image analysis, in particular to determine differences between gels displaying proteins from treated and untreated cells, was carried out manually initially. Images (exported to TIFF format files) were also subjected to automated anaylsis using Phoretix™ 2D expression software version 2005 (Non-Linear Dynamics, UK).

2.5.11 Peptide Mass Fingerprinting.

Protein samples excised from an SDS-polyacrylamide gel were digested in-gel with trypsin (Promega) using a Investigator Progest digestion robot (Genomic Solutions Ltd., Cambridgeshire, U.K.). Half the sample was desalted and concentrated using a micro C18 column (0.2µl ZipTip, Millipore, Gloucestershire, U.K.) according to the manufacturer's instructions. The peptides were eluted directly from the tip onto the target in 1.5µl alpha-cyano-4-hydroxycinnamic acid (saturated stock prepared in 50:50 acetonitrile: 0.2% TFA, diluted 1/5 in 60:40 acetonitrile: 0.2% TFA). Spectra were obtained on a Micromass TofSpec 2E instrument (Micromass, Manchester, U.K.), equipped with a 337 nm laser and operated in reflectron mode. The data were calibrated using the tryptic peptides of ß-galactosidase (Sigma) and lock mass corrected using a Glu-ibrinopeptide B spike. This was carried out by Dr Catherine Botting in the BMS Unit of Mass Spectrometry and Proteomics in the University of Saint Andrews, Fife, Scotland, UK.

2.5.12 In-House Database Searching.

Monoisotopic peptide masses were selected and searched using BioLynx ProteinProbe (Micromass, Manchester, U.K.) against a FASTA genome database.

2.5.13 On-Line Database Searching.

Monoisotopic peptide masses were selected using BioLynx ProteinProbe (Micromass, Manchester, U.K.) and submitted for peptide mass matching against the NCBI database using the Mascot search engine (Matrix Science).

2.6 RIBONUCLEIC ACID (RNA) EXTRACTION AND ANALYSIS

2.6.1 RNA Extraction from cultured cells

Extraction of RNA was performed using a NucleoSpin® RNA II kit (Machery-Nagel), which contained all of the buffers and filter units described below. Spent media in 6 well plates was removed and cells were gently washed in 1 X PBS. This was then replaced by 350 µl of lysis buffer (RA1) with 3.5 µl of β - mercaptoethanol which immediately resulted in cell lysis and release of nucleic acid into the buffer and increased viscosity. To reduce viscosity the lysate was passed through a 0.9mm needle fitted to a syringe. The lysate was then cleared through Nucleospin® filter unit, placed in a collecting tube, by centrifugation for 1 min at 11, 000 x g. The filter unit was discarded and the lysate mixed with 350 µl of 70% ethanol to adjust the RNA binding conditions. The lysate was then applied to a NucleoSpin® RNA II column, placed in a collecting tube, and centrifuged for 30 s at 8000 x g. The flow-through was discarded. Excess salt was subsequently removed from the column by adding 350 µl of membrane desalting buffer and centrifugation was carried out at 11, 000 x g for 1 min to dry the membrane. 95 µl of DNase reaction mixture was then added to the silica membrane of the column for a 15 min incubation at room temperature. Washing steps were then performed with buffer RA2 for 1 min followed by 2 washes with buffer RA3 for 2 min. RNA was then eluted from the column with 60 µl of RNase free water. The quantity and integrity of RNA extracted was determined using a NanoDrop® ND-1000

Spectrophotometer. The $A_{260/280}$ ratio achieved was usually higher than 1.9, indicating good quality RNA. RNA samples were stored at -80°C until further use.

2.6.2 Reverse Transcription (RT)

The total RNA was transcribed to cDNA using the RETROscript® RT-PCR kit (Ambion TX). Briefly, RNA was diluted (Table 2.4), heated at 70 °C for 3 min and placed on ice.

Table 2.4 RT-PCR Heat Denaturing and Annealing

Component	*Amount*
Total RNA	1 μg
Random Decamers (50μM)	2 μl
H_2O	To a final volume of 12 μl

Then 8 μl of Master Mix (Table 2.5) was added to each sample. The reactions were placed in a thermocycler at 42°C for 2 hours, followed by 92°C for 5 minutes to inactivate the reverse transcriptase. cDNA was stored at –20 °C until required for PCR.

Table 2.5 RT Reaction Master Mix

Component	*Amount*
10X RT Buffer	2 μl
dNTP Mix (2.5mM)	4 μl
RNase Inhibitor (10 units/μl)	0.5 μl
MMLV Reverse Transcriptase (100 units/μl)	0.5 μl
H_2O	1 μl
Total volume	8 μl

2.6.3 Polymerase Chain Reaction (PCR)

PCR was performed using 10 µl of cDNA generated during reverse transcription to confirm expression of TLR mRNA in cell lines. PCR reactions were set up by addition of reagents listed in Table 2.6.

Table 2.6 PCR Reaction Mix

Component	*Amount*
10X RT Buffer (supplied prepared with kit)	5 µl
dNTP Mix (10mM Stock)	1 µl
Fwd Primer (100 pmol/µl)	1 µl
Rev Primer (100 pmol/µl)	1 µl
REDTaq® DNA Polymerase (1 unit/µl)	2.5 µl
cDNA	10 µl
H_2O	29.5 µl
Total volume	50 µl

Primers for TLR2, TLR4 and GAPDH (MWG Biotech) are described in Table 2.7. The amplicon size and annealing temperatures used are also indicated.

Table 2.7 – Primer Sequences for PCR

Target	*Primer Sequences*	*Reference*	*Annealing Temperature*	*Product Size*
TLR2	Forward: 5'-TGATGCTGCCATTCTCATTC-3' Reverse: 5'-CGCAGCTCTCAGATTTACCC-3'	Smith, SM, manuscript in preparation 2007	51°C	157 bp
TLR4	Forward: 5'-ATTCCCCTGAGGCATTTAGG-3' Reverse: 5'-CCCCATCTTCAATTGTCTGG-3'	Smith MF et al., 2003	52°C	100 bp
GAPDH	Forward: 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' Reverse: 5'-CATGTGGGCCATGAGGTCCACCAC-3'	Su B et al., 2003	60°C	983 bp

All PCR reactions were carried out in a thermocycler under the following conditions:

First cycle	95°C, 5 minute	} 1 Cycle
Second cycle	95°C, 30 seconds	
	X°C, 30 seconds	} 30 Cycles
	72°C, 30 seconds	
Final cycle	72°C, 5 minutes	} 1 Cycle

X = Annealing temperature and was usually calculated by subtracting 5°C from the lowest melting temperature (T_m) of a primer pair. Annealing temperatures and primers are listed in Table 2.7. PCR products were stored at -20°C until visualised on 2% agarose gels.

2.6.4 Agarose Gel Electrophoresis

PCR products were separated on 2% agarose gels. Gels were produced by adding 2g of agarose (Melford laboratories, Ipswich,UK) to 100ml of 1X TAE (Appendix A). This mixture was boiled in a microwave until all the agarose was dissolved. The mixture was allowed to cool to about 55°C and 5µl of 10mgml-1 Ethidium Bromide (Sigma Chemical Co, St. Louis, MO, USA) was added. The gel was poured into a large gel tray with inserted combs and allowed to polymerize at room temperature for 30 to 45 minutes. 20µl samples of PCR product were loaded onto the gel. In addition, 6 X loading dye was mixed with a 100 bp ladder (Invitrogen) and added to the gel. The 6X loading dye consists of:

Component	*Amount*
100% Glycerol	3mL
0.5M EDTA, pH8.0	3mL
Bromophenol Blue	3mg
Xylene Cyanol	3mg
Sterile Water	4mL
Total Volume	10mL

The gel was then electrophoresed in a 1X TAE buffer for 45 minutes at 120 volts. Gels were then visualized under UV light and positioning of bands recorded.

2.6.5 Real Time PCR Analysis

Extraction of RNA and reverse transcription was performed as above. The PCR reactions were prepared in 384 well clear reaction plates (Applied Biosystems, Foster City CA) and was performed in the ABI Prism 7900 HT real time thermocyler (Applied Biosystems, Foster City, CA). The reverse transcribed cDNA was diluted in 160 µl of RNase free water and combined with Taqman® Universal PCR master mix and primer/probe (Table 2.8). Fold inductions were calculated using the ΔΔCT method as described in the ABI Prism manual using GAPDH as the denominator and using time point controls for comparison of gene induction (Gibson et al., 1996). Taqman® gene expression assays for IL-8 and GAPDH were from Applied Biosystems (Foster City, CA).

Table 2.8 Real Time PCR Reaction Mix

Component	*Amount*
cDNA rxn	4.5µl
Taqman® Universal PCR Master Mix (2X)	5µl
Taqman® gene expression assay (20X)	0.5µl
Total volume	10µl

2.7 ENZYME LINKED IMMUNOSORBENT ASSAYS

2.7.1 Detection of Secreted IL-8

The human IL-8 ELISA was performed in a flat-bottomed 96-well plate and was carried out according to the manufacturers (RnD Systems) instructions as follows: The capture antibody was diluted to working concentration of 4 µg/ml in PBS. Each well was coated with 100 µl of this solution, the plate was sealed and incubated overnight at room temperature. The top left hand well (A1) was left un-coated throughout the whole

experiment and was used to determine any optical imperfections in the plate due to the background absorbance of the plastic. The absorbance of this reading was subtracted from all other absorbances obtained at the end of the experiment). The following day, unbound capture antibody was aspirated and each well was completely filled with wash buffer and was washed thoroughly for a total of three times. After the last wash, any remaining buffer was removed by inverting the plate and blotting it against clean paper towel. The plate was then blocked with 300 µl of blocking buffer and incubated for one hour at room temperature. The blocking buffer was aspirated and the plate was washed for a total of three times. Recombinant IL-8 standards of known concentration and cell culture supernatants of unknown concentration were prepared in triplicate. A seven point standard curve of recombinant IL-8 standard was prepared by making 2-fold serial dilutions in reagent diluent with a top standard of 1000 pg/ml used (0 – 1000 pg/ml IL-8). 100 µl of this solution was added to the wells in triplicate. Samples of unknown IL-8 concentration were either left undiluted or diluted 1:4 with reagent diluent and 100 µl of this solution was also added to the wells in triplicate. The plate was incubated for two hours at room temperature. Unbound standard or sample was aspirated from the plate and the plate was washed three times with wash buffer. The detection antibody was diluted to working concentration (100 ng/ml) with reagent diluent and each well was coated with 100 µl of this solution and the plate was incubated for two hours. Unbound detection antibody was aspirated and the plate was washed three times with wash buffer solution. A working stock of streptavidin-HRP was prepared (1/200 dilution in reagent diluent) and 100 µl of this solution was added to each well of the plate. The plate was incubated for 20 minutes in the dark. After aspiration of unbound streptavidin-HRP and three washes of the plate, 100 µl of substrate solution (TMB solution, Pierce, MSC, brought to room temperature) was added to each well and incubated in the dark for 20 minutes. 100 µl of stop solution was added to each well of the plate and the absorbance values of each well were immediately read on a 96-well plate reader at 450 nM wavelength (Wallac Victor2 microplate reader). A standard curve of recombinant IL-8 was determined, plotting the absorbance (OD) at 450 nm versus concentration of IL-8 (pg/ml). The equation of the line of the standard curve was determined using a Microsoft excel software program. The concentration of IL-8 in the unknown samples was determined by using the equation of the line from the standard curve.

2.7.2 Detection of Secreted IL-12

The human IL-12p70 ELISA was performed in a flat-bottomed 96-well plate and was carried out according to the manufacturers (RnD Systems) instructions as detailed for the IL-8 ELISA.

2.7.3 Detection of RANTES

The human chemokine RANTES ELISA was performed in a flat-bottomed 96-well plate and was carried out according to the manufacturers (RnD Systems) instructions as detailed for the IL-8 ELISA.

2.7.4 *H. pylori* ELISA

96-well Maxisorp plates were coated overnight in a moist chamber at 4°C with 100 µl of T84 supernatants in 0.05 M carbonate buffer (pH 9.6) [Appendix A]. Plates were washed three times with washing buffer [Appendix A] and blocked for 1 h with blocking buffer [Appendix A]. Plates were washed three times with washing buffer and Rabbit anti- *H. pylori* antibody, diluted as required, was added and incubated for 1 h in a moist chamber at 37°C. Following five further washes, bound antibody was detected with goat anti-rabbit IgG alkaline phosphatase conjugate as required, followed by p-nitrophenyl phosphate substrate [Appendix A]. Reactions were allowed to develop for 30 min and stopped with 50 µl of 3 M NaOH [Appendix A]. Optical densities were read immediately at 405 nm on a Wallac Victor2 microplate reader.

2.7.5 Cytokine and Growth Factors Array

The evidence investigator™ (Randox Laboartories, Antrim, UK) quantitatively tests for IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFNγ, EGF, MCP-1 and TNFα simultaneously in a single serum/supernatant sample. Assay was carried out as per manufacturers instructions.

Introduction to RANDOX evidence investigator ™ Technology

The evidence investigator™ Biochip Array technology (Fig 2.3) is used to perform simultaneous quantitative detection of multiple analytes from a single sample. The core technology is the Randox Biochip, a solid-state device containing an array of discrete test regions containing an array of discrete test regions containing immobilised antibodies specific to different cytokines and growth factors. A sandwich chemiluminescent immunoassay is employed for the cytokine array. Increased levels of cytokine in a specimen will lead to increased binding of antibody labelled with HRP and thus an increase in chemiluminescence being emitted. The light signal generated from each of the test regions on the biochip is detected using digital imaging technology and compared to that from a stored calibration curve. The concentration of anaylte present in the sample is calculated from the calibration curve.

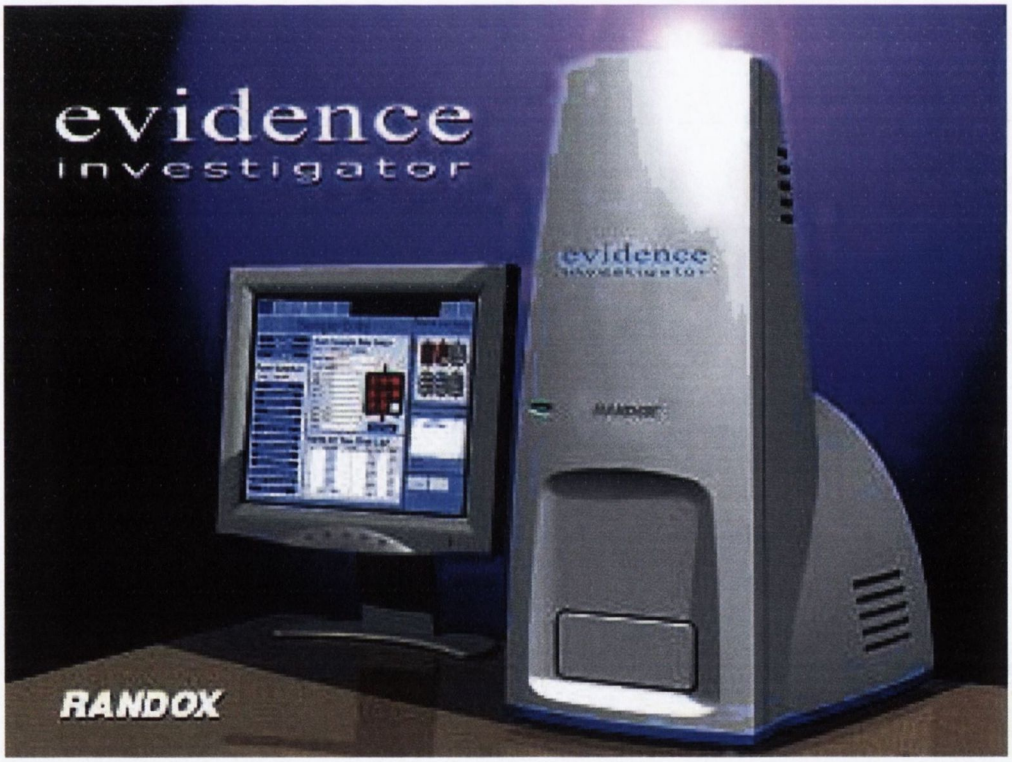


Figure 2.3 – RANDOX evidence investigator™

2.8 CLONING AND PURIFICATION OF NAPA FOR ANTIBODY PRODUCTION

2.8.1 NapA Cloning and Purification

Full length NapA (HP243) was amplified from *H. pylori* reference strain (NCTC 11638) genomic DNA using oligonucleotide primers specific for the 5' and 3' termini of the napA gene. Primers were designed to incorporate flanking *NdeI* and *BamHI* restriction endonuclease sites. Forward primer: 5'-GAAGGACTTCATATGAAGACATTTG-3', reverse primer: 5'-CGTGAATGGATCCTCATGCTGACTTCT-3'. The amplimer was digested with the appropriate enzymes and ligated into the expression vector pET16b (Novagen), which introduces a His-Tag. Following transformation into *E. coli* DH5ß recombinant plasmids were purified and sequenced using universal sequencing primers to verify their identity. Purified wt plasmids were used to transform *E. coli* BL21pLysS. Expression of His-tagged NapA was induced with IPTG (isopropyl-ß-D-thiogalactopyranoside). Appropriate molecular weight and expression were verified by Coomasie staining of a 15% SDS-PAGE gel. The recombinant protein was purified from bacterial lysates using a nickel (Ni^{2+}) beads column following the pET system manufacturer's instructions (Novagen). This work was carried out by Dr. Veronica Athie Morales.

2.8.2 Antibody Production

Antibodies were raised by inoculating naïve rabbits at days 1, 15 and 29 with 800 µg of recombinant His-tagged NapA emulsified (1:1) with either complete (initial inoculation) or incomplete (1^{st} and 2^{nd} boost) Freund's adjuvant. Bleed test and bleed out samples were respectively obtained at days 22, 30 and 70. Antibody titers and specificity against wt NapA were analysed by indirect ELISA using pre-bleed sera as negative control. This work was carried out by Dr. Veronica Athie Morales.

2.9 CLINICAL SAMPLES

2.9.1 Collection of Duodenal Biopsy Samples

Clinical samples were obtained from volunteers attending for endoscopies at St James's Hospital Endoscopy Unit, Dublin 8, Ireland. Patients who had taken nonsteroidal anti-inflammatory drugs, proton pump inhibitors or antibiotics during the preceding 3 months were excluded from the study. Only volunteers who were Clo test negative for *H. pylori* were suitable to partake in this study. Endoscopies were performed under local anesthesia and two biopsy samples were obtained from adjacent areas of the gastric antrum. Samples were transferred to ice-cold L-15 media (Appendix A) immediately after removal for transport to the laboratory where they were immediately rinsed with fresh media at 4°C.

2.9.2 Detection of Viable and Non-Viable Cells in Duodenal Explants

Metabolic studies requiring short studies (6 – 24 hours) explant culture at 37°C were inititated at this time as previously described (Poole et al., 1996). Explants were placed in fresh media supplemented with 25 μM of the viable cell probe CMFDA (Molecular Probes, Eugene, Oregon) and 25 μM of the dead cell probe ethidium homodimer (EthD-1) (Molecular Probes, Eugene, Oregon). The tissue was incubated in the dark at 37°C. The explants were then washed with fresh media, fixed with 2% PFA for 15 min and washed thoroughly. For sectioning, each explant was mounted in the antero-posterior orientation and frozen in OCT (TissueTek, USA). Multiple 5 μm sections were then cut from each block in an antero-posterior orientation and dried overnight at room temperature in the dark, onto pre-prepared glass slides coated with poly-L-lysine (Sigma, Poole, Dorset, UK). Representative sections were mounted under coverslips in anti-fade mounting media for immunofluorescence (Dako, Denmark) for qualitative assessment of dye penetration and to determine the viability of the explants post excision.

2.9.3 Culture of Human Duodenal Explants

Explants were cultured as previously described (Olfat et al., 2002). Two explants were applied to each insert cup (Transwell, Costar) with the mucosal side facing up. Auturp medium (Appendix A), supplemented with 2 mg/L Nalidixic acid (Sigma – Aldrich)

and 4 ml/L Skirrows supplement (Oxoid), was added to the wells to allow contact with the bottom of the insert cup. Thus, a capillary contact between the explants and the culture medium from below was established (Figure2.4). The inserts cup's membrane pore size (0.4 μm) prevented *H. pylori* OMVs from passing through the insert to the medium below. The explants were infected with *H. pylori* OMVs after 1h of incubation at 37°C in 5% CO_2.

2.9.4 Inoculation of Human Duodenal Explants

After explants had become accustomed to their ex-vivo environment, 10 μg/ml OMV resuspended in Auturp medium was applied to each insert cup to cover the explants. A set of 2 uninfected explants were included in each plate as a control. After coincubation for 2 h at 37 °C, the OMV suspension was removed and the explants were washed 3 times in fresh culture medium. The culture medium underneath the insert cups was removed and stored at -20 °C for subsequent IL-8 and RANTES quantification analysis.

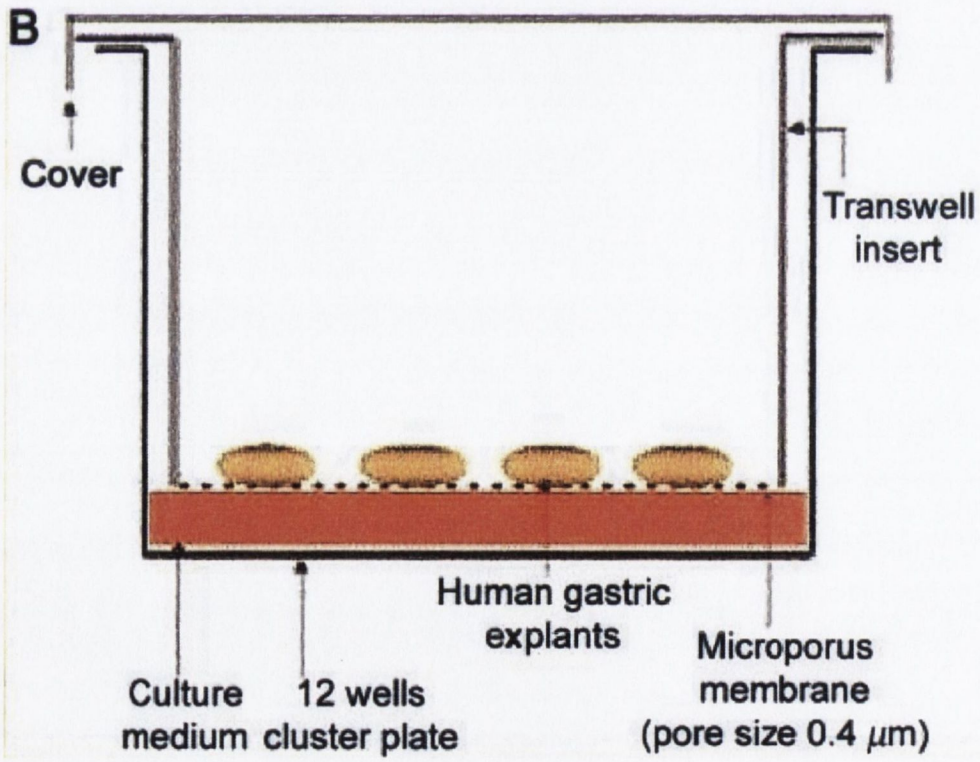


Figure 2.4– Assembly of the insert cup (Transwell, Costar) and culturing of human explants. Reproduced from Olfat et al., JID 2002; 186 (1 August)

CHAPTER III
PROTEOMIC AND FUNCTIONAL CHARACTERISATION OF *H. PYLORI* OUTER MEMBRANE VESICLES

3.1 INTRODUCTION

H. pylori is a gram negative bacterium which colonises the human gastric mucosa and causes acute and chronic gastritis, gastroduodenal ulcers and gastric cancer (Covacci et al., 1999). More than half the worlds population is infected with this organism. *Helicobacter pylori*, like most gram negative pathogens, releases a large, complex group of proteins and lipids into the extracellular milieu via outer membrane vesicles (OMV) which are constitutively shed from the surface of the organism (Keenan et al., 2000a). Studies of vesicles from diverse bacterial origins support a common function. Vesicles are a means by which bacteria interact with other cells in their environment and for pathogens to actively export virulence factors to host cells (Kuehn and Kesty, 2005). By electron microscopy (EM), vesicles appear round with a bilayer membrane, electron dense luminal content, and an average diameter of 50-250 nm, which is strain dependent (Beveridge et al., 1999). Vesiculation is a ubiquitous processs for Gram negative bacteria grown in a variety of environments including liquid culture, solid culture and biofilms (Beveridge, et al., 1999). It has been demonstrated that OMV formation and release is an energy sink (Kesty and Kuehn, 2004), therefore it is unlikely that *H. pylori* would produce OMV without function. Based on events viewed by EM, OMV are thought to form when the outer membrane bulges and pinches off, encapsulating soluble periplasmic proteins (Chatterjee and Das, 1967). *H. pylori*OMV have been shown to contain proteins, lipopolysaccharide (LPS) and lipoproteins (Keenan et al., 1997). Importantly, *H. pylori* OMV have been shown to augment pro-inflammatory cytokine production by gastric cells and modulate cell proliferation. As such biological activity may influence gastric and extra-gastric disease progression in vivo, identification of the protein constituents comprising *H. pylori* OMV represents an important but hitherto undocumented milestone to aid our understanding of alternative strategies available to the pathogen to influence host pathogen interactions. For this study, *H. pylori* OMV were subjected to 1D SDS-PAGE, prior to analysis of the separated components by tandem Mass Spectrometry (MS). This approach circumvented the resolution problems encountered when attempting to separate membrane protein-rich vesicles by standard two-dimensional electrophoresis. Western blotting analyses and functional studies were performed to confirm the presence and activity of selected virulence factors identified in the OMV proteome.

3.2 OBJECTIVES

1. To purify outer membrane vesicles from whole cells.
2. To separate OMV and analyze their proteomic composition.
3. To confirm the presence of identified proteins.
4. To assess the biological activity of the proteins and virulence factors identified within the OMV.

3.3 RESULTS

3.3.1 Isolation of *H. pylori* OMV.

Outer membrane vesicles released by the human pathogen *H. pylori* are of special interest because they come into direct contact with host tissues and may be a vehicle for the delivery of virulence factors to the gastro-duodenal mucosa. To identify the protein composition of OMV, *H. pylori* was cultured in a protein free medium of 2.8 % Brucella broth supplemented with 1 % ß- cyclodextran in order to recover OMV released into the broth supernatant during growth. Growth in this medium compared favourably with the organism's growth in the same medium supplemented with fetal calf serum. Electron microscopic analysis of the material recovered in the broth supernatant revealed the presence of membrane enclosed vesicle – like structures of uniform size, approximately 20 nm in diameter (Fig 3.1a). There was no evidence of contamination of these OMV preparations with whole cells, cellular debris or fragments of flagellae. Figure 3.1 b shows the 1D protein profile of OMV stained with either colloidal Coomassie blue (Lane a) or silver (Lane b). For mass spectrometry analysis, a colloidal Coomassie stained gel (6.5 x 9 cm) similar to that shown in Fig 3.1b was divided into 12 prior to trypsin digestion and analysis described in Methods.

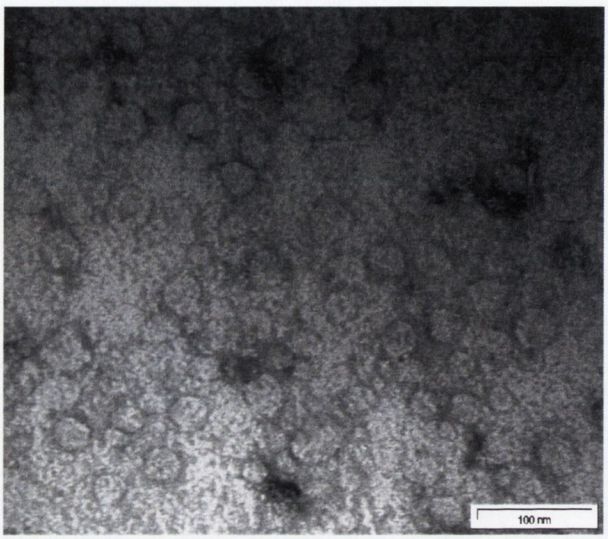


Figure 3.1a

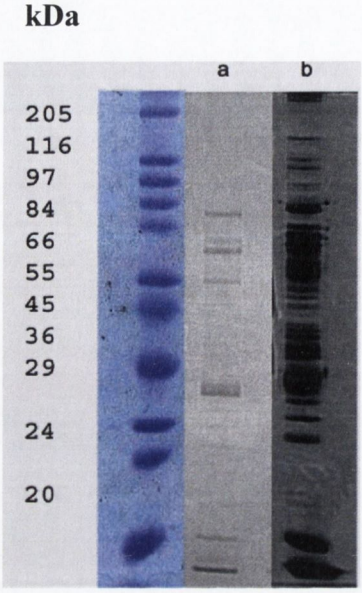


Figure 3.1b

Figure 3.1 (a) Samples of OMV were overlaid onto carbon-colloidal-coated mesh grids, negatively stained with 1% aqueous uranyl acetate and examined by electron microscopy to confirm the absence of whole cells and flagella. (Bar = 100nm). OMV were purified as described in methods (b) - Samples of OMV were electrophoretically separated by SDS-PAGE (12.5 %) and stained with both colloidal Coomassie blue (a) and silver (b). Molecular weight markers are shown on left hand side.

3.3.2 Identification of Proteome of *H. pylori* OMV

In total, 99 *Helicobacter* proteins were unambiguously identified by tandem mass spectrometry using OMV preparations separated by 1D SDS-PAGE (12.5 %). Of these 35 % were outer membrane proteins, thus reflecting the membrane rich nature of the vesicle preparations (Table 3.1). These included iron transporters (frpB/ B1), adhesins (HpaA, HopZ), lipoproteins (lpp20, lipoprotein A), multiple OMPs of unknown function and the proinflammatory protein, OipA.

Of particular interest was the identification of molecules considered to be virulence factors, including: CagA, VacA, Urease, NapA, CagA and OipA (Table 3.2) and one novel adhesin (hopZ).

When the vesicles pinch off from the outer membrane, they encapsulate contents of the periplasm. A number of *H. pylori* proteins have been identified in the periplasm including KatA, HspB and urease (Harris and Hazel, 2003, Phadnis et al., 1996). Additionally, these and other proteins (e.g. ribosomal proteins), not classically regarded as being associated with the outer membrane, were identified within the proteome of the OMV (Table 3.3). This phenomenon of cytoplasmic proteins being present within the OMV of gram-negative organims has been widely reported and the significance of this finding will be considered in the discussion.

Name	MW	pI	Locus	% Sequence Coverage	Mainrole
Putative OMP	102,653	9.368	HP0655	15	Cell Envelope
Iron-regulated OMP (frpB)	97,384	9.281	HP1512	35	Transport and Binding Proteins
Probable iron-regulated OMP frpB1	88,556	8.785	HP0876	16	Transport and Binding Proteins
Probable OMP	78,103	5.839	HP0373	1	Hypothetical
OMP 25	76,345	9.353	HP1156	5	Cell Envelope
OMP 5	75,739	8.615	HP0227	17	Cell Envelope
Probable OMP (flagellar hook)	73,343	7.666	HP0710	10	Hypothetical
OMP 27	69,691	9.535	HP1177	18	Cell Envelope
OMP 17 / SabA	69,419	9.398	HP0725	9	Cell Envelope
Putative OMP	63,653	9.903	HP0839	18	Transport and Binding Proteins
Probable iron-regulated OMP frpB2	62,690	8.589	HP0915	20	Transport and Binding Proteins
OMP	59,416	9.658	HP0486	8	Cell Envelope
OMP 20 / HopC	55,931	9.362	HP0912	10	Porin
Outer Membrane Protein (hefA)	54,608	9.388	HP0605	7	Hypothetical
OMP 7	53,292	9.629 4	HP0252	9	Cell Envelope
OMP 6 / HopA	53,063	9.585 8	HP0229	15	Porin
Probable OMP	52,747	9.922	HP0209	15	Hypothetical
OMP 8	47,555	9.507	HP0254	14	Cell Envelope
OMP 32	42,975	9.811	HP1501	27	Cell Envelope
OMP 22	41,970	9.24	HP0923	8	Cell Envelope
OMP 12	41,695	9.241	HP0477	7	Cell Envelope
Rare lipoprotein A	35,476	9.821 3	HP1571	17	Cell Envelope
OipA	34,186	10.27 1	HP0638	17	Cell Envelope
Cell binding factor 2	34,030	9.964	HP0175	21	Cell Envelope
Putative OMP	31,890	9.863	HP0127	13	Cell Envelope
Putative OMP 14	30,346	9.441	HP0671	18	Cell Envelope
OMP	30,151	9.641	HP1564	22	Cell Envelope
Probable OMP	29,958	9.491	HP0694	17	Hypothetical
OMP 15 / HopE	29,928	9.113	HP0706	41	Porin
Flagellar Sheet Adhesin (HpaA)	28,348	9.441	HP0410	56	Cell Envelope
OMP 31	28,152	9.895	HP1469	20	Cell Envelope
OMP 30	26,762	10.1	HP1395	8	Cell Envelope
OMP 11	20,924	9.395	HP0472	46	Cell Envelope
OMP 18 peptidoglycan associated lipoprotein precursor	19,977	5.896	HP1125	6	Cell Envelope
20 kDa membrane-associated lipoprotein Lpp20	19,108	10.14 8	HP1456	61	Cell Envelope

Table 3.1 Membrane proteins identified in the OMV of *H. pylori*

Name	*MW*	*pI*	*Locus*	*% Sequence Coverage*	*Mainrole*
Vacuolating Cytotoxin A	139,789	9.395	HP0887	22	Cellular processes / Toxin Production
Cag PAI protein (cag26)	132,386	9.425	HP0547	20	Cellular processes / Pathogenicity
OipA	34,186	10.271	HP0638	17	Proinflammatory Protein
Urease	26,539	8.959	HP0073	62	Central Intermediary Metabolism
NapA	16,933	5.83	HP0243	57	Cellular processes

Table 3.2 Virulence factors identified in the OMV of *H. pylori*

Name	MW	pI	Locus	% Sequence Coverage	Mainrole
Iron(III) dicitrate transport protein	94,827	9.785	HP0686	28	Transport and Binding Proteins
NADH2 dehydrogenase (ubiquinone) (EC 1.6.5.3) chain NQO3	94,229	5.117	HP1266	15	Energy Metabolism
Flagellar hook-associated protein 2 (HAP2)	72,806	4.999	HP0752	16	Cellular Processes
Chaperone and heat shock protein C62.5	71,274	5.260	HP0210	14	Protein Fate
90Kda chaperone	71,140		JHP0196	12	Molecular Chaperones
Transketolase A (tktA)	70,584	6.746	HP1088	4	Energy Metabolism
Putative Flagellar Basal-Body Rod Hook Protein	67,122	5.791	HP0908	18	Cellular processes
Chaperone protein dnaK (Heat shock protein 70) (HSP70).-	67,064	4.756	HP0109	11	Protein Fate
GTP-binding protein, fusA-homolog (yihK)	66,676	5.093	HP0480	8	Unknown Function
Quinone-reactive Ni/Fe hydrogenase, large subunit (hydB)	64,396	7.287	HP0632	12	Energy metabolism
CTP Synthase	60,615	7.838	HP0349	32	Purines, pyrimidines, nucleosides and nucleotides
ABC transporter, ATP-binding protein (yheS)	59,931	5.618	HP0853	13	Transport and binding proteins
Catalase	58,628	9.036	HP0875	6	Cellular processes
H. pylori (HspB)	58,227	5.4772	HP0010	14	Protein Fate
Phosphoglycerate dehydrogenase	57,905	7.1779	HP0397	27	Amino Acid Biosynthesis
Lysyl-tRNA Synthetase	57,782	5.4328	HP0182	5	Protein synthesis
Gmp synthetase (guaA)	56,977	7.0891	HP0409	16	Purines, pyrimidines, nucleosides and nucleotides
Flagellin B (FlaB)	53,881	6.327	HP0115	28	Cellular processes
HopZ protein precursor	53,292	9.6294	HP0252	14	Cell Envelope
Flagellin A	53,284	6.322	HP0601	18	Cellular processes
IMP Dehydrogenase (guaB)	51,688	8.215	HP0829	17	Purines, pyrimidines, nucleosides and
ATP-dependent proteinase, ATP-binding chain	51,166	6.6788	JHP1288	21	Molecular Chaperone
Biotin carboxylase	51,001	7.4209	HP0370	8	Fatty acid and phospholipid metabolism
Fumarase (fumC)	50,974	7.287	HP1325	7	Energy Metabolism
Heat Shock Protein	50,190	5.5265	HP0280	6	Protein Fate
TolB	47,797	9.558	HP1126	20	Cellular Processes
Nickel and cobalt resistance protein	47,710	8.776	HP0971	5	Unknown Function
Isocitrate dehydrogenase (icd)	47,531	7.952	HP0027	17	Energy Metabolism
Serine hydroxy methyl transferase (glyA)	45,710	6.992	HP0267	22	Amino Acid Biosynthesis
Chlorohydrolase	45,541	6.196	HP0267	22	Cellular Processes
HopW protein precursor	43,084	9.811	HP1501	17	Cell Envelope
Synthesis of [Fe-S] cluster (nifS)	42,403	6.154	HP0220	15	Biosynthesis of cofactors, prosthetic

					groups, carriers
Protein E (gcpE)	39,278	9.467	HP0625	40	Unknown Function
Iron(III) ABC transporter, periplasmic iron-binding protein	37,552	9.755	HP1561	23	Transport and Binding Proteins
Iron(III) ABC transporter, periplasmic iron-binding protein (ceuE)	37,102	9.677	HP1562	33	Transport and Binding Proteins
Asparaginase (EC 3.5.1.1) II	35,538	9.545	HP0723	20	Energy Metabolism
Rare lipoprotein A	35,476	9.821 3	HP1571	10	Cell Envelope
Pyruvate ferredoxin oxidoreductase (porB)	34,953	8.201	HP1111	26	Energy Metabolism
30S Ribosomal Protein S2	30,687	7.627	HP1554	17	Protein Synthesis
Ribosomal protein L2	30,271	11.00 8	HP1316	31	Protein Synthesis
Enoyl-Acyl Protein Carrier Protein Reductase	29,981	7.802	JHP0181	16	FattyAcidMetabolism
Cell division inhibitor (minD)	29,294	7.131	HP0331	25	Cellular Processes
Cysteine-rich protein; HcpA	28,109	8.406	HP0211	4	Hypothetical Proteins
Ribosomal protein L4	24,023	9.942	HP1318	22	Protein Synthesis
Alkly hydroperoxide reductase (tsaA)	22,235	6.248	HP1563	38	Cellular Processes
Ribosomal protein L3	21,192	10.94 1	HP1319	28	Protein Synthesis
Nonheme iron-containing ferritin (pfr)	19,285	5.486	HP0653	58	Transport and Binding Proteins
Ribosomal protein L10	18,573	9.998	HP1200	26	Protein Synthesis
3-dehydroquinase type II (aroQ)	18,483	4.929	HP1038	21	Amino Acid Biosynthesis
Adhesin-thiol peroxidase (Fragment).-	18,292	8.18	HP0390	28	Unknown Function
NADH-ubiquinone oxidoreductase, NQO6 subunit (NQO6)	17,808	9.763	HP1261	28	Energy Metabolism
Ribosomal protein S5	16,538	10.61 9	HP1302	34	Protein Synthesis
Ribosomal protein L16	16,045	11.14	HP1312	26	Protein synthesis
Ribosomal protein S8	15,184	1.353	HP1305	40	Protein Synthesis
Pyruvate ferredoxin oxidoreductase, delta subunit	15,108	6.647	HP1109	22	Energy Metabolism
Ribosomal protein S9	14,508	11.37 5	HP0083	19	Protein Synthesis
50S Ribosomal protein L20	14,031	11.83 1	HP0126	19	Protein Synthesis
Ribosomal protein S11	14,029	11.05 9	HP1295	12	Protein Synthesis
Ribosomal protein L17	13,364	11.06 9	HP1292	18	Protein Synthesis
Ribosomal protein S10 (rps10)	11,917	9.861	HP1320	23	Protein Synthesis

Table 3. 3 Other proteins identified in the OMV of *H. pylori*

3.3.3 Assessing the Biological Activity of Virulence Factors Identified

Immunoblotting verified the presence of OMV-associated: CagA (120 kDa), VacA (87 kDa), OipA (34 kDa), HpaA (30 kDa) and NapA (15kDa) (Fig. 3.2). To evaluate the functional biological activity of VacA within OMV, the ability of the OMV to induce vacuolation in gastric epithelial cells was evaluated. Additionally, the neutrophil chemoattractant activity of OMV-NapA was tested and the ability of OMV to induce IL-8 secretion in cell lines and duodenal explants was evaluated.

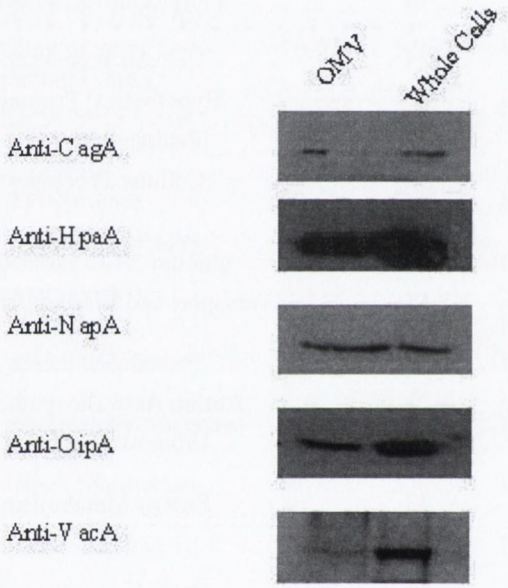
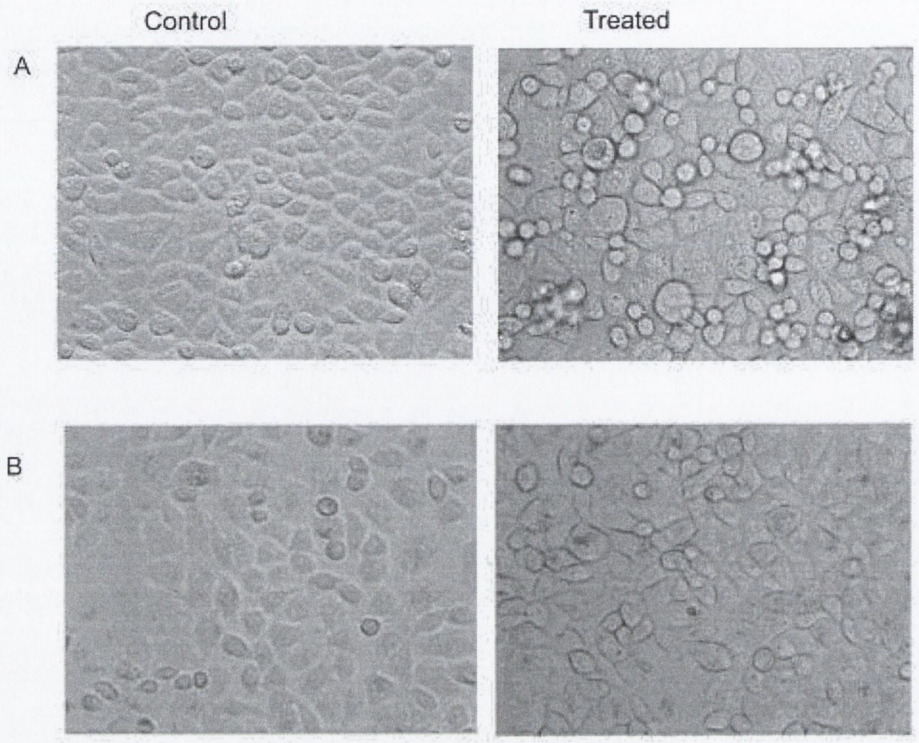


Figure 3.2. Verfification of presence of selected proteins in OMV by immunoblotting. Lane (1) outer membrane vesicles; (2) whole cells. Molecular mass standards (kDa) are indicated (left). Immunoblotting of OMV transferred to PVDF verified the presence of CagA, VacA, OipA, HpaA and NapA proteins in the outer membrane vesicles.

Firstly, exposure of AGS gastric epithelial cells to OMV for 24 h resulted in vacuolation, which was clearly visible by light microscopy (Fig. 3.3a). Localisation of neutral red within the vacuoles induced by the OMV was visible by light microscopy within 4 min after the addition of the dye (Fig. 3.3b). AGS cells stimulated with OMV accumulated significantly more dye than control cells (Fig. 3.3c) indicating the presence of intracellular vacuoles in the treated samples.



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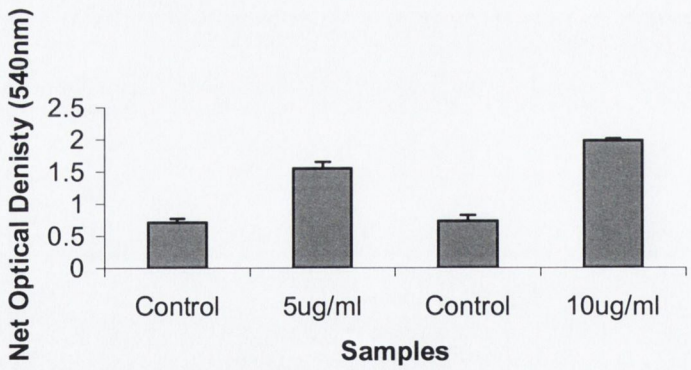


Figure 3.3 – *H. pylori* OMV induce vacuolation in AGS cells and neutral red uptake. AGS cells were grown in 6 well plates and stimulated with 10 μg/ml OMV for 24 hours. Vacuolation was viewed by normal phase contrast microscope(A) and via the neutral red assay(B) and (C) .

Secondly, the ability of OMV to act as chemoattractants for neutrophils due to the presence of OMV-associated NapA (Cooksley et al., 2003) was evaluated. The data shown in Fig. 3.4 clearly demonstrates that OMV were potent chemoattractants for PMN and that this activity was dose dependent. This chemotactic response was completely abolished if the OMV were first treated with anti-NapA polyclonal antiserum indicating that NapA was solely responsible for this effect. Pre-treatment of OMV with NapA pre immune serum had no effect on OMV-induced - PMN chemotaxis.

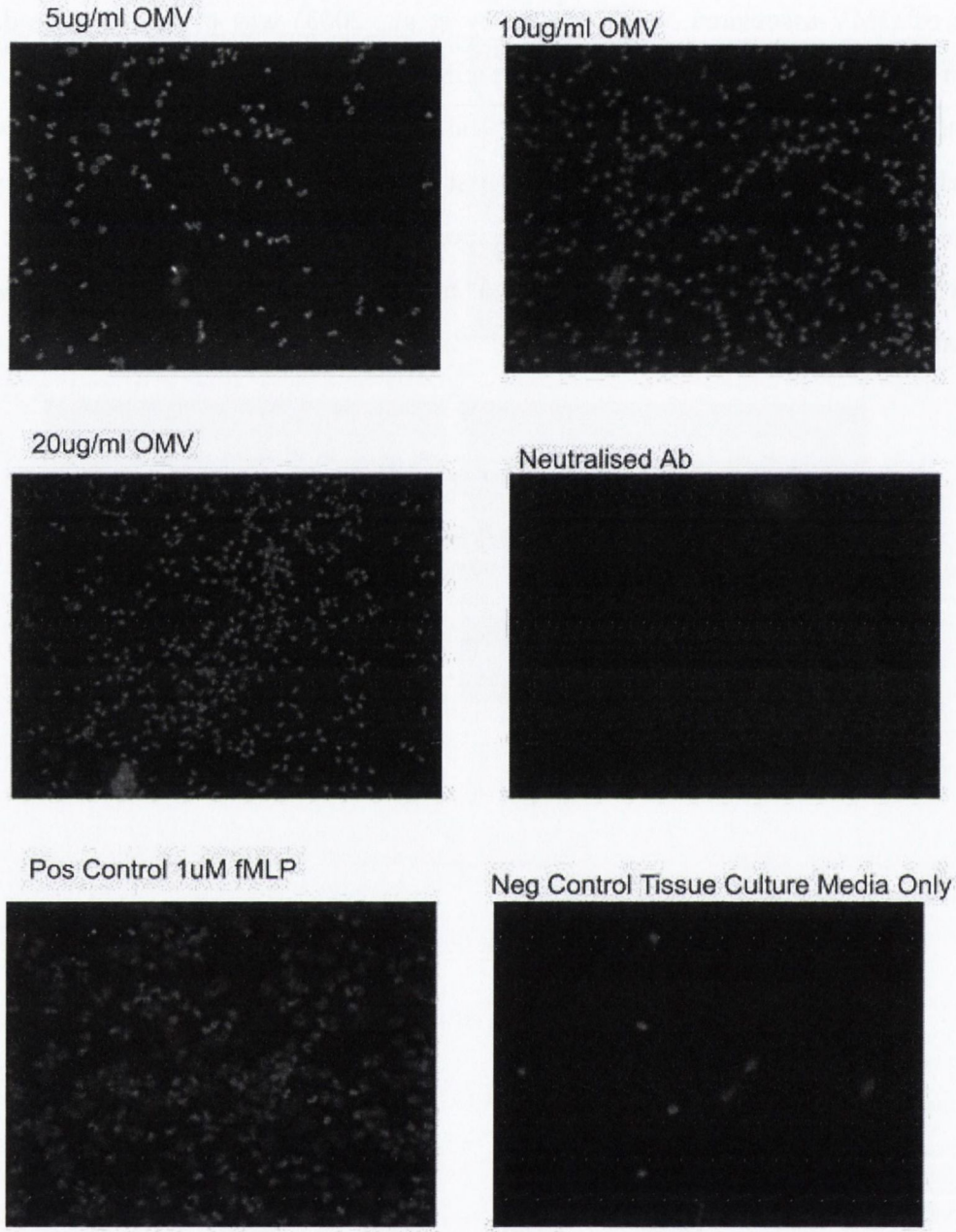
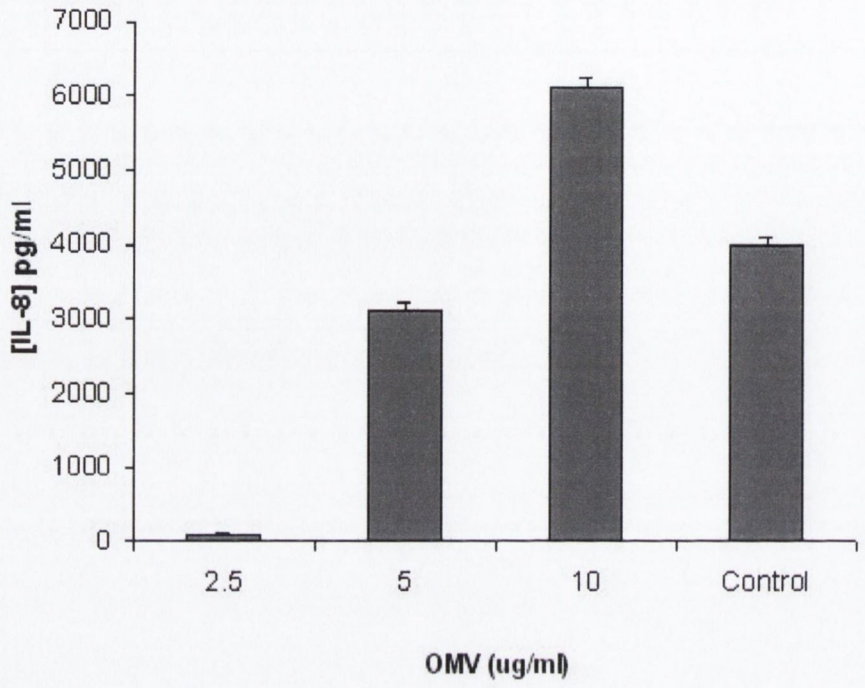
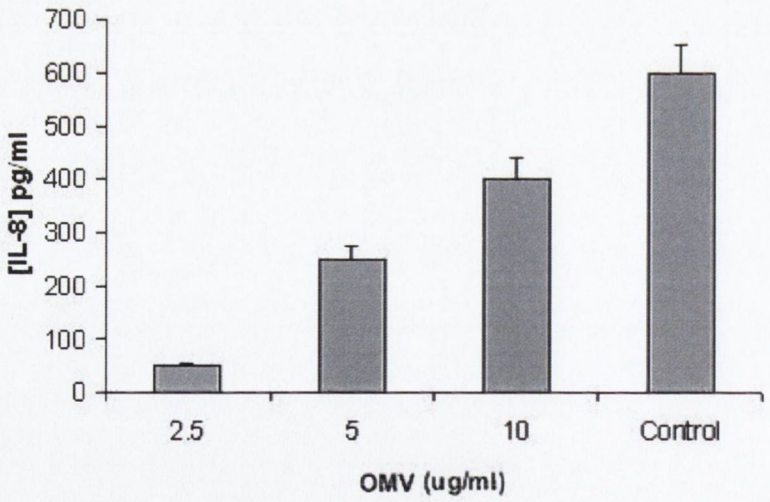


Figure 3. 4 - OMV induce chemotaxis of human neutrophils through NapA. Cell culture inserts with 5 μM diameter pore size were used to monitor the chemotaxis of PMNs. PMNs (10^6) were suspended in HBSS for chemotaxis assays and placed directly in the upper well of the filter insert. PMNs migrating into the lower well containing the varying concentrations of OMV were recovered and quantified by counting Hoechst stained PMNs. fM-L-P (1μM) was used as a positive control.

OMV also induced a dose dependent secretion of the proinflammatory cytokine IL-8 by both T84 (Fig, 3.5a) and AGS cells (Fig. 3.5b). Interestingly, OMV induced approximately 12-15 fold more IL-8 secretion in colonic epithelial cells compared to gastric epithelial cells. Attempts were made to abrogate OMV-stimulated chemokine secretion by blocking OMV-associated OipA with specific antiserum, as OipA reportedly induces IL-8 secretion (Yamaoka et al., 2002). However, no attenuation of IL-8 production was observed. It is likely that other OMV-associated factors (e.g. LPS, CagA, Hsp60, NapA, urease) also modulate IL-8 production. Additional attempts were made to block chemokine expression by pretreating the OMV with both polymyxin B and anti-NapA antiserum, however neither treatment (alone or combined) had any effect on IL-8 expression.



A



B

Figure3. 5 – *H. pylori* OMV induce IL-8 production in (a)T84 and (b)AGS cells. Cells were grown in 6 well plates and stimulated with 10 μg/ml OMV. Supernatants were collected at various time points for ELISA protein assay. OMV induce IL-8 at the protein level in both cell lines. The control used was 1 μm PMA.

3.4 DISCUSSION

The first step towards elucidating the potential role that OMV have in pathogenesis was development of a reliable method of production, purification and initial characterization of these vesicles. This chapter describes the development of a reliable method of producing OMV to be used for *in vitro* and *in vivo* studies, in order to gain an insight into the functions of these antigens. This chapter reports the identity of the proteomic composition of *H. pylori* OMV and the functional relevance of some recognised virulence factors. Providing a protective environment for the transmission of virulence factors from bacteria into the host target cells both locally and away from the site of colonisation, OMV represent a potentially important vehicle for delivering antigens to either epithelial or non-epithelial gastric cells where they can exert their pathogenic potential (Ricci et al., 2005).

The proteomic analysis of *H. pylori* OMV presented in this paper revealed the presence of several OMV-associated virulence factors including NapA, CagA, VacA, and OipA. Importantly, functional assays demonstrated that these OMV are potent neutrophil attractants (NapA mediated) also capable of inducing vacuolation in gastric epithelial cells. As shown by others (Ismail et al., 2003), OMV induced IL-8 secretion in gastric cells however we extend these observations and demonstrate that OMV provoke 12-15-fold more IL-8 secretion in non-gastric epithelial cells (T84 colonic cells).

OMV are generally considered as vehicles that transport and deliver virulence factors and immunogens to either epithelial cells or non-epithelial cells at their site of production. In general, pathogenic bacteria produce more vesicles than their non-pathogenic counterparts, suggesting an important functional role (Wai et al., 1995). The importance of *H. pylori* OMV as an emerging virulence factor of the pathogen is underscored by studies demonstrating constitutive vesiculation of *H. pylori* ex vivo in gastric biopsies in addition to vesicle adherence to gastric cells in vitro (Fiocca et al., 1999, Keenan et al., 2000). Constitutive shedding of OMV *in vitro* thus mimics the *in vivo* situation. The identification of OMV-associated adhesins (HpaA, SabA) in this present study also provides evidence for a mechanism whereby the vesicles could adhere to host tissue. Presumably, such a close association would ultimately facilitate entry of the OMV into the host cell either by fusion with the host cell membrane or internalization via endocytosis. Our finding that OMV are internalized by colonic epithelial cells (See Chapter 4) also demonstrates that *H. pylori* OMV can efficiently

deliver virulence factors such as CagA directly to host cells in the absence of a type IV secretion system.

Of the 99 OMV-associated proteins identified in this study, 35 % were classified as outer membrane proteins and the remainder were cytoplasmic or periplasmic proteins. Other groups have identified cytoplasmic proteins as components of OMV preparations (Uli et al., 2006, Vaughan et al., 2006, Ferrari et al., 2006, Vipond et al., 2006, Williams et al., 2006, Balsolobre et al.,2006) obtained from various gram negative pathogens. The presence of ribosomal proteins, heat shock proteins (HSP), chaperones and elongation factors (EF) in OMV preparations appears to be a feature common to all proteomic studies of OMV. Typically such molecules are found in the cytoplasm whereas others (e.g. KatA) have dual cytoplasmic and periplasmic distribution (Harris and Hazell, 2003). A cotranslational interaction between ribosomal proteins and others involved in translation (e.g. EF, chaperones, HSP) has been proposed to account for the co-localisation and association of such cytoplasmic constituents within OMV (Herskovits et al., 2002). Similarly, other proteomic studies have identified the presence of cytoplasmic proteins associated with membraneous material in *H. pylori* (Backert et al.,2005), including DnaK (HSP70), CagA, VacA and flagelin A, all of which were identified within the proteome of the OMV. Autolysis (Phadnis et al., 1996) of *H. pylori* may provide an alternative mechanism for cytosolic constituents to become membrane associated although others (Vanet and Labigne, 1998) argue that specific secretory mechanisms may be involved such as a type III secretion pathway or an ABC transporter system.

In this study, 35 OMP were identified in total. Outer membrane proteins are often associated with the virulence of many pathogens and there has been an increasing interest in the role of *H. pylori* OMP, especially OipA and BabA in gastroduodenal disease (Yamaoka et al., 2005). The functions of 3 of these OMP have been identified. OMP 6, OMP 20 and OMP 15 have previously been designated HopA, HopC and HopE, respectively, and function as porins (Doig et al., 1995). Until recently, VacA, CagA, NapA, urease and OipA were all considered virulence factors from predictions based on *in vitro* studies. Virulence factors are related to the ability of a microbe to induce disease, so true virulence factors must therefore have a disease association, an *in vivo* correlate with disease such as increased mucosal inflammation or both (Lu et al.,

2005b). However, for the purpose of this study all putative virulence factors identified will be considered.

H. pylori infection elicits an inflammatory cell response and the severity of mucosal injury appears to be directly correlated with the extent of neutrophil infiltration (Cover and Blaser, 1992). It has been suggested that activated leukocytes may be responsible for some tissue damage seen in cases of *H. pylori* associated gastritis. Neutrophil activating protein, NapA (Evans et al., 1995), was so named because of its ability to mediate neutrophil adhesion to endothelial cells (Yoshida et al., 1993) and to bind both mucin and neutrophil glycosphingolipids (Namavar et al., 1998). NapA was also shown to bind iron *in vitro* (Tonello et al., 1999) and to protect cells against oxidative stress induced lethality (Cooksley et al., 2003). More recently NapA has been shown to be an immunomodulatory molecule which functions through TLR2 (Amedei et al., 2006).

NapA was identified in the proteome of *H. pylori* OMV and its presence was confirmed by immunoblotting. From the data currently available it seems clear that NapA has a protective role for *H. pylori* but it seems unlikely that it facilitates the pathogenicity of the organism as it was not originally associated with a disease outcome (Lu et al., 2005). However, NapA is known to induce activation of human neutrophils involved in the inflammatory response linked with *H. pylori* associated gastritis. This study shows that increasing amounts of OMV induced increased neutrophil chemotaxis and that antibody mediated neutralization of NapA protein of the OMV, prevented neutrophil migration (Fig 3.4). Thus it appears that the NapA contained within the vesicles is biologically active and that neutrophil migration is dependent on its presence. Consequently, OMV could potentially elicit an inflammatory cell response *in vivo*, independently of intact bacteria, which may be responsible for some of the tissue damage seen in cases of *H. pylori* gastritis. Additionally, OMV induced neutrophil chemotaxis may be responsible for extra gastric manifestations of inflammation and may have implications for duodenal ulceration.

Among several virulence factors produced by *H. pylori*, the vacuolating cytotoxin VacA, plays a potentially pivotal role in pathogenicity (Ricci et al., 2005). VacA is a protein toxin which induces cytoplasmic vacuoles in eukaryotic cells in culture (Ricci et al., 2000). VacA was identified within the proteome of the OMV and its presence

confirmed by immunoblotting. Its biological activity was assessed in vitro by inducing vacuolation in AGS cells, which was confirmed by light microscopy and the neutral red assay (Fig 3.3).

The outer inflammatory protein (OipA), an inflammation related protein, was also identified in the proteome of the OMV. The OipA protein is an important virulence factor associated with enhanced IL-8 secretion, increased inflammation and pronounced neutrophil infiltration *in vivo* as well as *in vitro*, validated by the clinically important presentation of peptic ulcer (Yamaoka et al., 2002; Kudo et al., 2004). However more recent data suggests that its effect on epithelial cells may be limited and that its main function is to induce genes within the CagA PAI. In this study we found that exposing T84 and AGS epithelial cells to OMV induced a dose dependent increase in expression of IL-8 secretion. With a few exceptions, IL-8 production in vitro requires the attachment of viable *H. pylori* to the epithelial cells (Kudo et al., 2004).

Due to their small size, adhesive properties and ability to deliver toxins into host cells of the gastric mucosa, OMV are likely to play an important role in the pathogenesis of gram negative infection (Kuehn and Kesty, 2005). In summary, this study provides substantial support for the emerging hypothesis that OMV from pathogenic organisms are indeed potential virulence factors (Kuehn and Kesty, 2005). A combination of the pro-inflammatory chemokine and the potent neutrophil attractant activity of *H. pylori* OMV equip the organism with the means to extend its pathogenic reach beyond the gastric mucosa and deliver toxins and other factors directly into host tissue. *H. pylori* OMV also harbour at least 3 well defined immunomodulatory molecules, OMP16 (Rathinavelu et al., 2005) and cell binding factor 2 (Pathak et al., 2006)), in addition to the well characterized NapA (Amedei et al., 2006)). Secreted components of *H. pylori* are known to mediate multiple pathogen host interactions during infection (Bumann et al., 2001). Much work has been done on characterising and identifying the proteins present in the *H. pylori* secretome (Bumann et al., 2002) and of the sarcosine insoluble OMPs of strain 26695 (Baik et al., 2004), and this is the first complete identification of the proteome of *H. pylori* OMV.

CHAPTER IV
THE EFFECTS OF *H. PYLORI* OUTER MEMBRANE VESICLES ON COLONIC EPITHELIAL CELLS: AN *IN VITRO* MODEL

4.1 INTRODUCTION

The gastrointestinal epithelium represents the first cellular barrier against intraluminal insults. In the human stomach, the interface between the epithelial cells and the gastric mucus represents the ecological niche for *H. pylori* (Terres et al., 2003). The direct effects in epithelial cells upon stimulation with *H. pylori* have been widely studied in vitro. Effects such as loss of epithelial barrier function (Papini et al., 1998; Terres et al., 1998a), cytoskeletal rearrangement (Ashorn et al., 2000), apoptosis (Fan et al., 2000), and induction of nuclear factor kappa B, interleukin 8 and cyclooxygenase – 2 have been reported. *H. pylori* OMV also induce apoptosis in gastric (AGS) epithelial cells (Ayala et al., 2006) and induce proliferation and IL-8 secretion (Ismail et al., 2003). The same authors report a decrease in cell viability at higher OMV doses (Ismail et al., 2003). OMV bear serologically recognisable Lewis antigens on lipopolysaccahride molecules and it is also thought that this may contribute to the chronic immune stimulation of the host (Hynes et al., 2005).

This present study found that OMV are capable of inducing IL-8 expression in both AGS and T84 cell lines, that they have a vacuolating effect on AGS cells and that they behave as chemoattractants for neutrophils. In this chapter T84 cells were grown in confluent monolayers, on semi permeable inserts, and apically stimulated with OMV in an *in vitro* model of infection. As an aim of this project was to look at the initial effects of OMV stimulation on duodenal biopsies in an *ex vivo* model of ulcerogenesis, this seemed like an excellent method through which this technique could be validated. As there are no duodenal human epithelial cell lines commercially available, the T84 cell line was used. The human intestinal epithelial cell line T84 differentiates in vitro into polarised monolayers with functional tight junctions which maintain an ion-transporting phenotype (Madara et al., 1987). This represents a good *in vitro* model for the study of the physiological response to the gastrointestinal pathogen *H. pylori* and its released components, OMV.

For this chapter a novel assay approach to examining the effects of OMV on tight junctions using the Cellomics HCS Kineticscan Reader was developed.

4.2 OBJECTIVES

1. To determine whether OMV can become internalised in epithelial cells
2. To determine whether a transfer of OMV from the apical to the basolateral pole of the epithelium takes place
3. To examine the effects of OMV on tight junctions and barrier function of a model intestinal epithelium
4. To determine whether OMV can induce dome formation in T84 cell monolayers
5. To examine the effects of OMV on T84 cells protein expression profiles by 2-DE analysis. Identify differentially regulated proteins using mass spectrometry.
6. To examine the inflammatory cytokine response in epithelial cells in response to OMV stimulation.

4.3 RESULTS

4.3.1 Internalisation of OMV in Epithelial Cells

Although *H. pylori* is not considered an invasive pathogen, numerous clinical and experimental observations support the notion that *H. pylori* is able to invade epithelial cells in the gastric mucosa (Amieva et al., 2003). OMV are natural vehicles for directed intercellular transport of bacterial virulence factors into host cells and tissues (Kuehn and Kesty, 2005). Vesicle surface factors can mediate adhesion to eukaryotic cells as well as subsequent internalisation. Based on their size, adhesive and proteolytic properties, OMV can interact with cells that are not readily accessible by the infecting bacteria (Kuehn and Kesty, 2005). To date, the internalisation of OMV in epithelial cells has been assessed by immunogold labelling (Ricci et al., 2002). Using the OMV proteomic data previously discussed (Chapter 3) and a panel of antibodies specific for proteins known to be associated with OMV, immunostaining techniques were used to monitor internalised OMV within epithelial cells by confocal microscopy. T84 cells were seeded onto 8 well Permanox slides and incubated with OMV (10μg/ml) for 1 h or tissue culture medium alone as a control. Immunostaining methods were as previously described (Chapter 2). Figure 4.1a shows OMV internalised within cells (OMV green). This was not due to non-specific binding of the secondary antibody (Fig 4.1b). To assess whether internalised OMV had become associated with endosomal vesicles, double staining for internalised OMV and the early endosome marker Rab5 was carried

out. There was no evidence of co-localisation between internalised OMV and endosomal vesicles (Fig 4.1c).

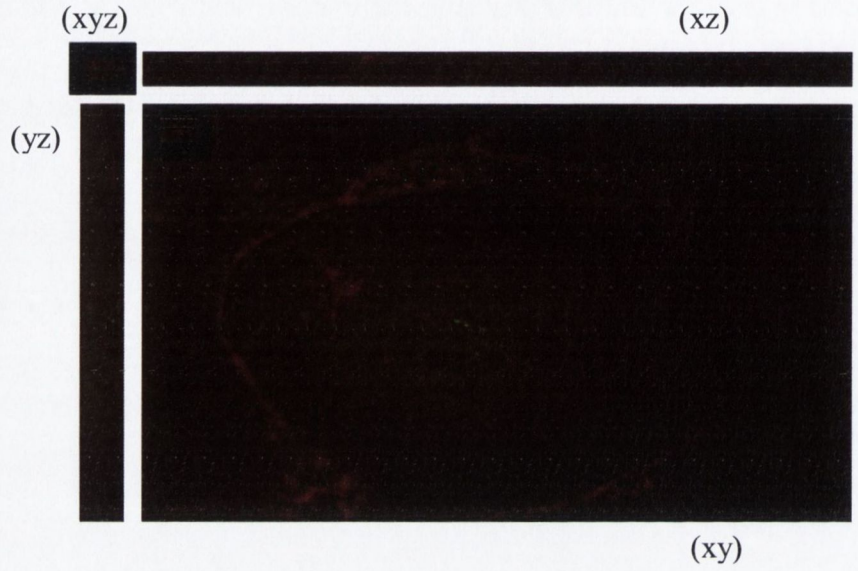


Figure 4.1a – *H. pylori* OMV internalized within epithelial cells, visualized by Confocal microscopy. In all images, XYZ shows the three-dimensional image, XY represents the normal confocal image, YZ shows the image cut vertically and XZ shows the image cut horizontally. T84 cells were seeded in an 8-well chamber slide and incubated with OMV (10 μg/ml). To assess whether OMV had become internalised within epithelial cells, anti-HpaA was used to stain OMV (green) and ALEX phalloidan 546 was used to stain for the actin cytoskeleton (red). Images were acquired on a Nikon Eclipse confocal microscope. OMV were internalized within the epithelial cells.

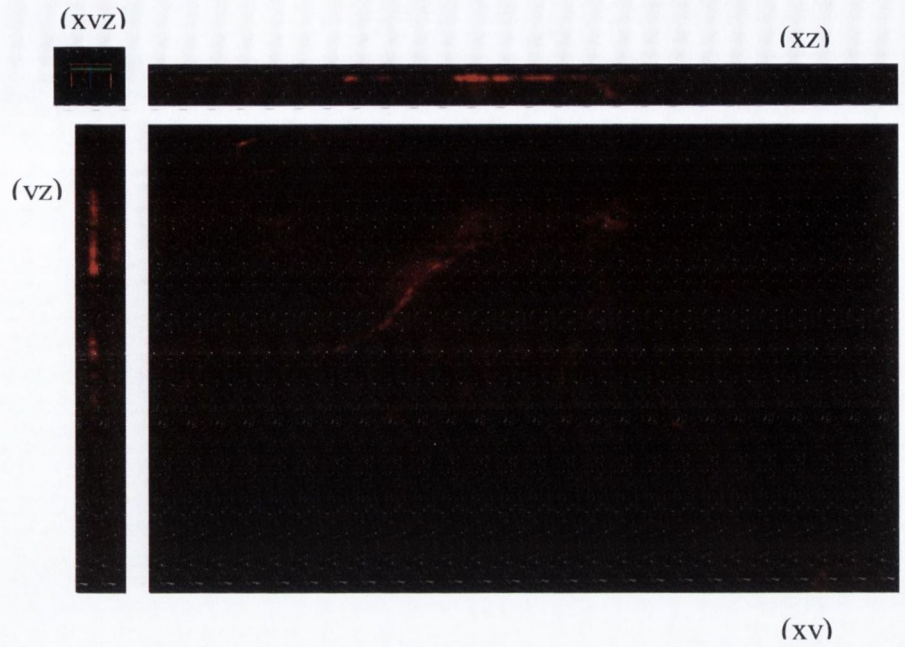


Figure 4.1b – Control image showing no non-specific binding. In all images, XYZ shows the three-dimensional image, XY represents the normal confocal image, YZ shows the image cut vertically and XZ shows the image cut horizontally. T84 cells were seeded in an 8-well chamber slide and incubated with tissue culture medium alone. To assess whether non-specific binding had occurred, immunostaining was carried out as previously described. Images were acquired on a Nikon Eclipse confocal microscope.

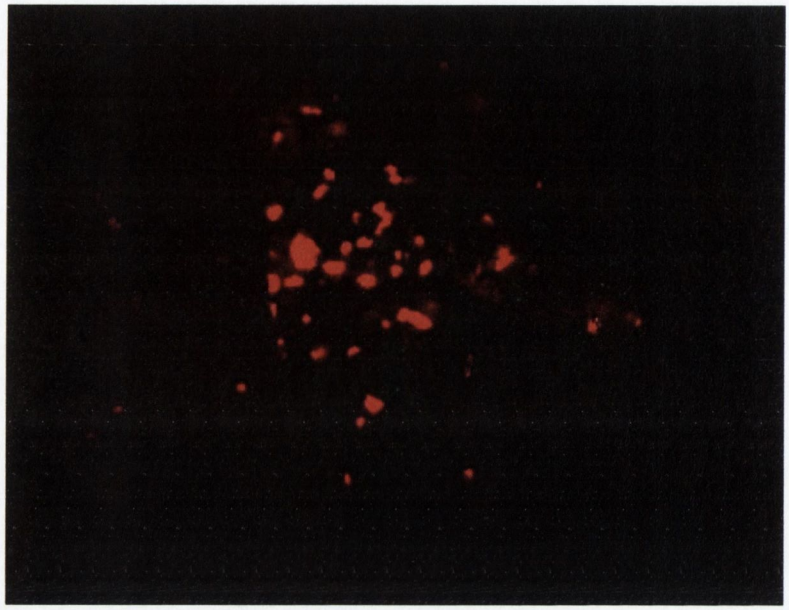


Figure 4.2 – *H. pylori* OMV are not associated with intercellular endosomes. T84 cells were seeded in an 8-well chamber slide and incubated with OMV. To assess whether internalised OMV had become associated with endosomal vesicles, double staining took place for internalised OMV (anti HpaA - green) and the early endosomal marker Rab5 (red). There was no evidence of co-localisation between internalised OMV and endosomal vehicles

4.3.2 Apico-Basal Transfer of OMV in Epithelial Cells

Bacterial LPS at the apical surface of T84 cells has been shown to be transferred in small amounts from the apical to the basal surface of the epithelium (Beatty and Sansonetti, 1997; Beatty et al., 1999). To determine if this was the case with OMV in the *in vitro* model, mature monolayers of T84 cells growing in semi-permeable inserts were apically incubated with OMV (10μg/ml). Using an antibody against *H. pylori*(Dako) the presence of OMV in the basal compartment at different time points was evaluated. As shown in Fig 4.3, the antibody recognised OMV. Attempts were made to detect OMV in the basal compartment using this assay. However, the antiserum cross-reacted with a component(s) of the basolateral medium to a very significant extent and this reactivity masked, and thus prevented, the detection of OMV in the basal compartment using this method.

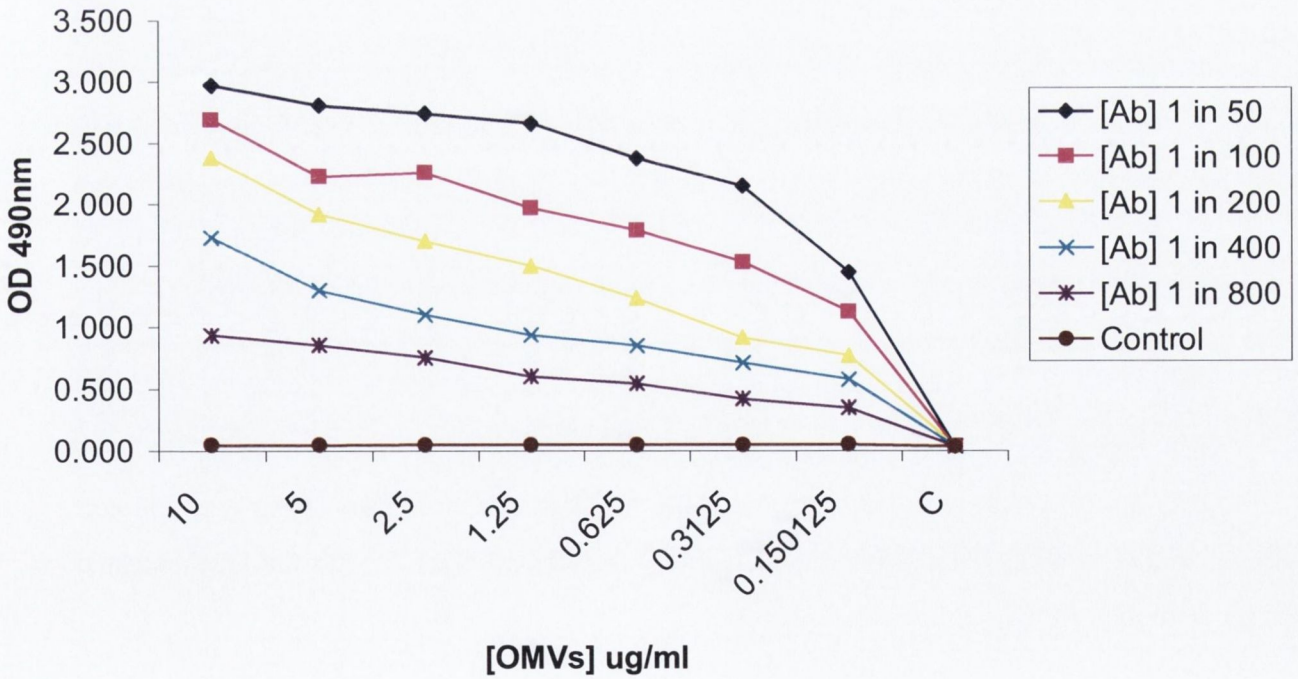


Figure 4.3 – DAKO *H. pylori* antibody recognizes OMV

Decreasing amounts of OMV were coated on a 96 well plate overnight and OMV were detected using a commercial (DAKO) anti-*H. pylori* antiserum (1/50 – 1 / 800) as the primary antibody.

4.3.3 Effects of OMV on Tight Junctions and Barrier Function in Epithelial Cells

The epithelium is the interface between the lumen and the underlying cells. The portion of the epithelial cell exposed to the lumen is referred to as its apical surface and the remainder of the cell surface is known as the basolateral surface. Tight junctions seal adjacent epithelial cells in a narrow band just beneath their apical surface and prevent the passage of molecules and ions through the intracellular space. They also block the movement of integral membrane proteins between the apical and basolateral compartments of the cell. T84 cells are considered to be a model tight epithelium as they have functional tight junctions. *H. pylori* colonizes the gastric mucosa, possibly by affecting epithelial – cell function (Terres et al., 1998). To investigate the effect of *H. pylori* OMV on epithelial-barrier function, the transepithelial electrical resistance (TER) of confluent cell monolayers grown on filters after exposure to OMV in the apical bathing medium for 1 h using HPE as a positive control was measured. OMV (10μg/ml) had no effect on TER in comparison with the drop in TER caused by HPE (Fig 4.4a). To determine if a change in TER was associated with a change at the molecular level of the tight junctions, the expression of the tight junctional protein ZO-1 was analysed. This was done by immunostaining the monolayers with a ZO-1 antibody (Zymed) and the appropriate secondary. In the control monolayers, ZO-1 was expressed exclusively at the tight junctions (Fig 4.4b). There was no evidence of redistribution of ZO-1 in monolayers treated with OMV (Fig 4.4c). In HPE treated monolayers there was a complete disruption of tight junctions and a redistribution of ZO-1 throughout the cell (Fig 4.4d). For a more complete analysis of the monolayers treated with OMV, images of the immunostained cells were acquired using the Cellomics Kineticscan HCS reader (Fig 2.2) and preliminary analyses was then conducted using propreitary image processing algorithms. This was achieved by first identifying and segmenting the cells into discrete objects to be measured. The object was identified by using a whole cell label (Fig 4.5). There was no evidence of a redistribution of ZO-1in the OMV treated monolayers using this technology.

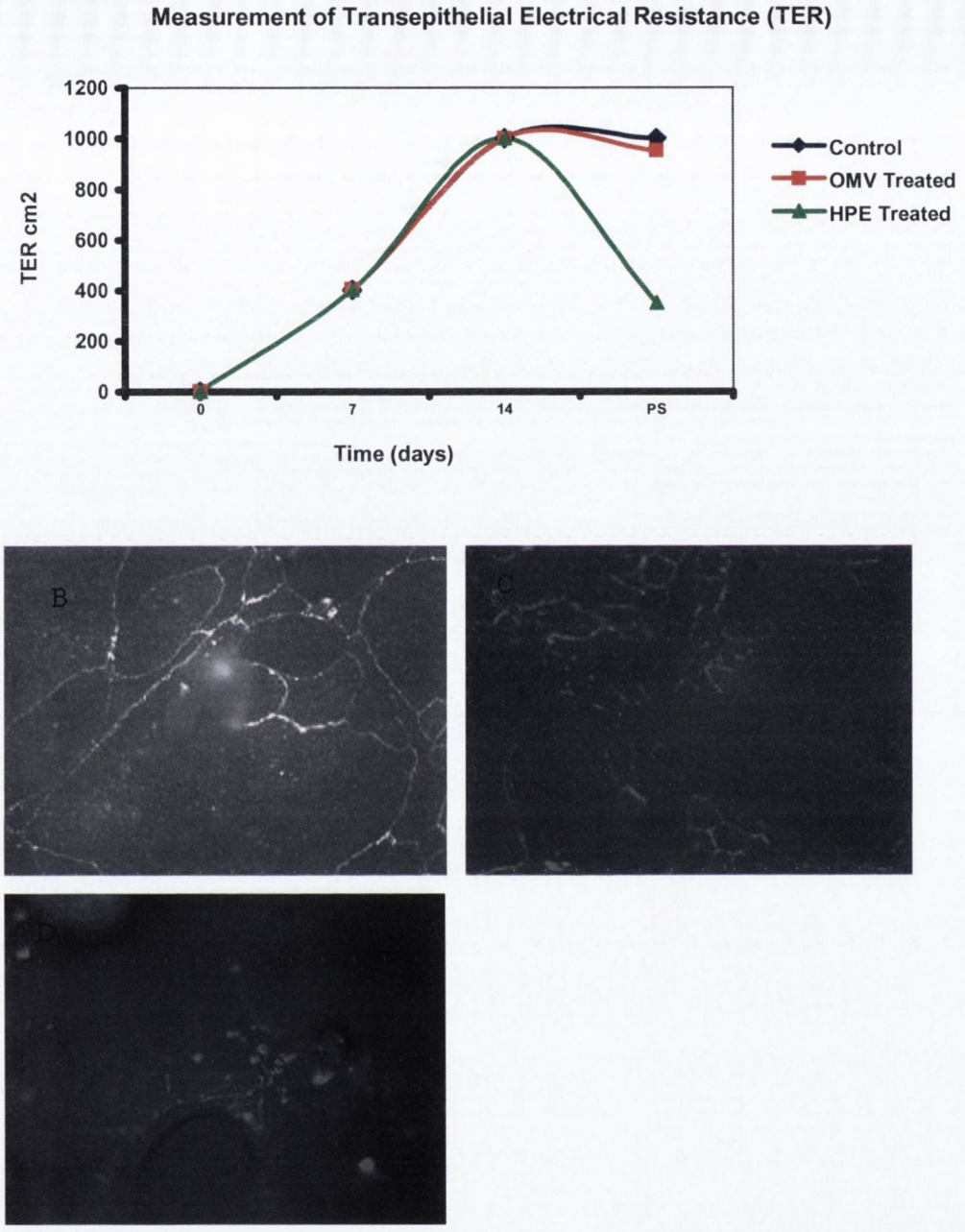


Figure 4.4 – Measurement of TER and staining of T84 monolayers. T84 cells were seeded onto semi-permeable inserts. TER was monitored until cells were fully differentiated and polarized. Cells were then stimulated with either OMV (10 μg/ml) or HPE (500 μg/ml) as indicated and then TER was measured once post stimulation (PS). (B) – control, (C) OMV treated, (D) HPE treated. T84 cells were seeded onto semi-permeable inserts. TER was monitored until cells were fully differentiated and polarized. Cells were then left untreated (B) or stimulated with OMV (10 μg/ml) (C) or HPE (500 μg/ml) (D) and stained with anti-ZO-1.

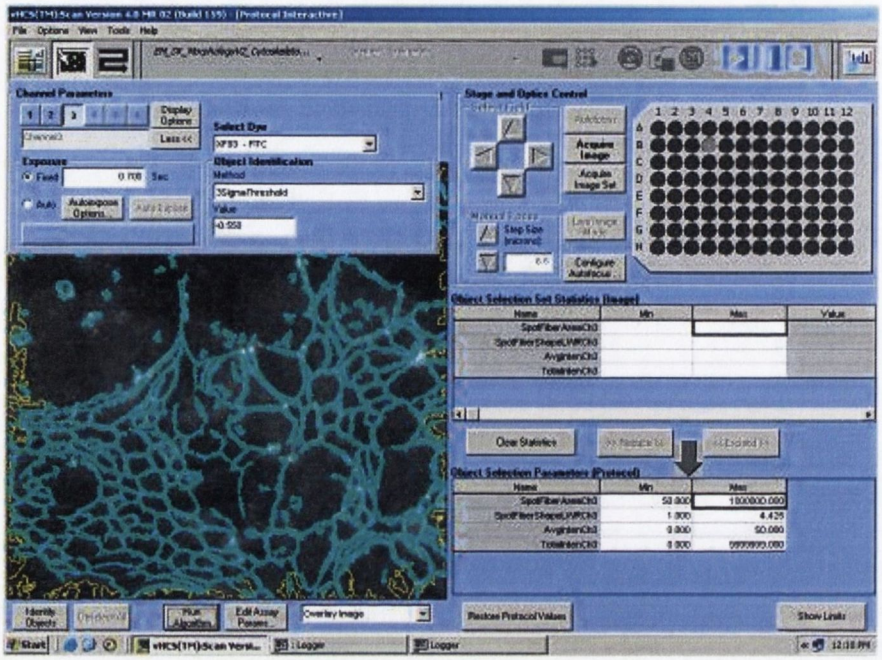


Figure 4.5 – Tight junctions stained with a fluoescent cell label and analysed by Cellomics™ HCS Kineticscan Reader for changes at the molecular level. Representative screen shot from the Cellomics™ machine. T84 cells were seeded onto semi-permeable inserts. TER was monitored until cells were fully differentiated and polarized. Cells were then stimulated with OMV and stained with anti-ZO-1 to examine the effects on tight junctions using the novel assay approach of Cellomics™ HCS Kineticscan.

4.3.4 OMV Do Not Induce Dome Formation

Domes are localized blister like areas of fluid accumulation between the cultured monolayers and the impermeable substrate (Rotoli et al., 2002) which have been considered as good indicators of fluid transport, weak attachment to the substrate and a sign of epithelial differentitation (Terres et al., 2003). Their presence is said to demonstrate the maintenance of a differentiated polarized phenotype and functioning tight junctions (Terres et al., 2003). In this study, *H. pylori* extract (HPE) was used as a positive control for disruption of epithelial-barrier function. HPE is cell free and contains proteins and lipopolysaccharide (LPS). This HPE is known to induce the formation of fluid filled semi-spheric domes (Terres et al., 2003) and to disrupt epithelial barrier function in T84 monolayers (Terres et al., 1998). HPE was used as a positive control by which to assess the effects, if any, of OMV on tight junctions and their ability to induce dome formation in T84 monolayers. To determine the ability of OMV to induce dome formation on polarized epithelia, T84 cells were grown to confluency on semi permeable inserts. The apical bathing medium was substituted with OMV (10μg/ml), HPE as a positive control or tissue culture medium alone and the monolayers were examined in situ under a phase contrast microscope. After 1 h of incubation at 37°C, monolayers treated with OMV showed no signs of dome formation. Numerous circular structures appeared evenly spread across the monolayers treated with HPE indicating dome formation. There was also a concomitant decrease in the TER of the monolayers treated with HPE after 1 h. There was no such decrease in the control monolayers or the monolayers treated with OMV. The monolayers were cocultured with their various stimuli for 24 h but OMV were unable to induce dome formation (Fig 4.6). HPE associated dome formation is not associated with cell death (Terres et al., 2003) and at 24 h the epithelium exhibited signs of complete reattachment and recovery of normal TER levels (Fig 4.7).

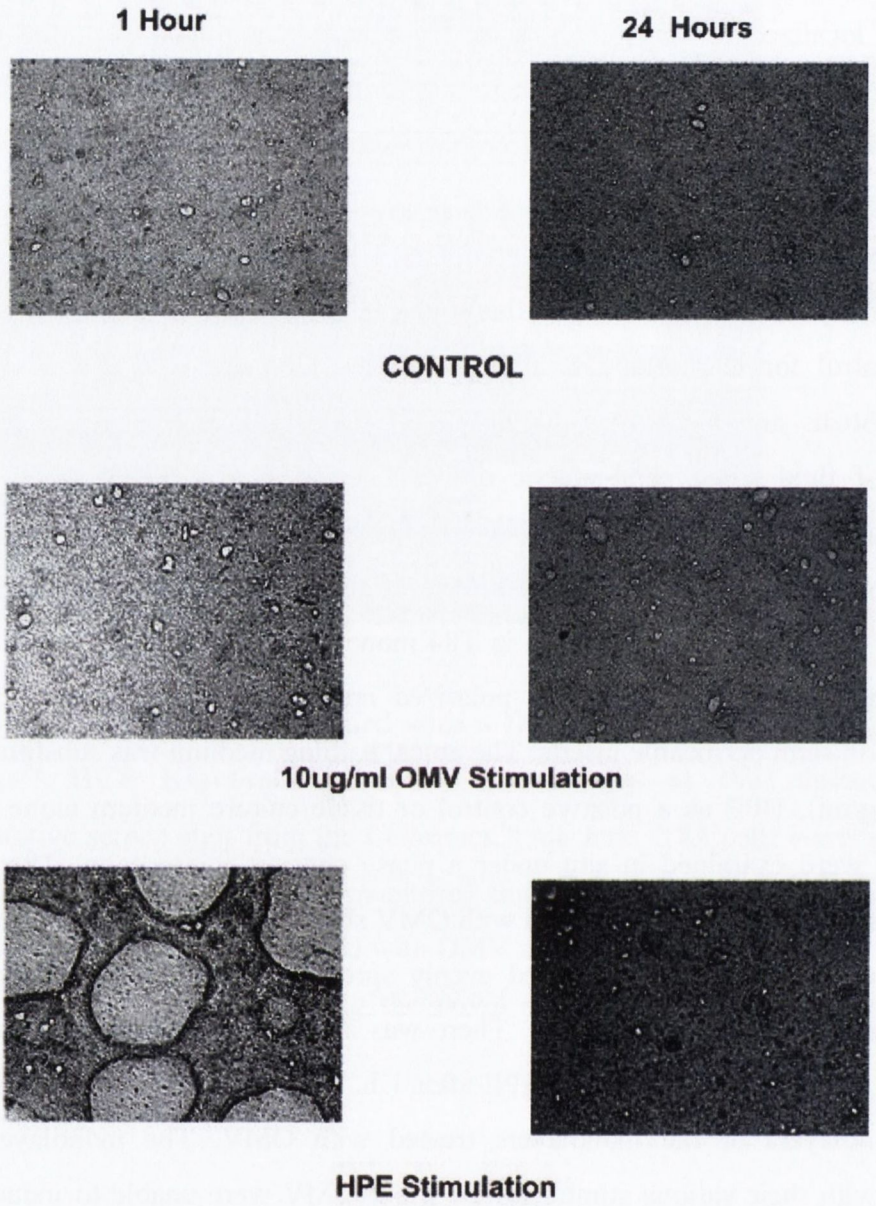


Figure 4.6 – *H. pylori* OMV do not induce dome formation in T84 monolayers, in comparison with HPE. In all panels, images on the left were taken after 1 hour and images on the right after 24 hours stimulation. T84 cells were seeded onto semi-permeable inserts. TER was monitored until cells were fully differentiated and polarized. Cells were then stimulated with various stimuli and monitored by phase contrast microscopy for dome formation. OMV (10 μg/ml) did not induce dome formation in comparison to HPE (500 μg/ml), which induced dome formation within 1 hour of stimulation.

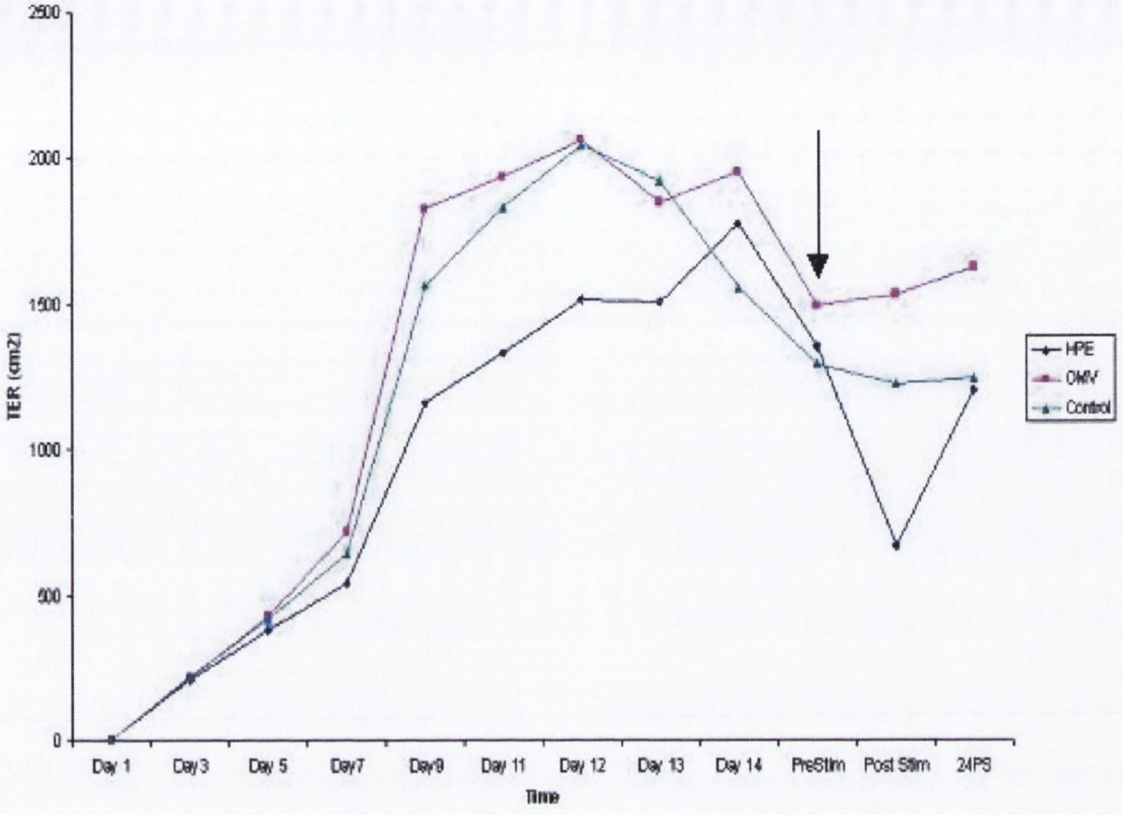


Figure 4.7 – *H. pylori* OMV have no significant effect on TER of T84 monolayers after 24 hours stimulation. In comparison, HPE induces a dramatic decrease in TER after 1 hour but TER values are restored to normal levels after 24 hours. Arrow indicates point at which apical stimulation occurred. T84 cells were seeded onto semi-permeable inserts. TER was monitored until cells were fully differentiated and polarized. Cells were then incubated with HPE (500µg/ml), OMV (10 µg/ml) or left untreated and TER was monitored for 24 h.

4.3.5 Protein Profiling of Epithelial Cells upon Stimulation with OMV

The host cell response to infection by *H. pylori* in a number of models of pathogenesis has been characterised at the level of global approaches such as transcriptomics and proteomics (Backert et al., 2005a). In many of these investigations it is normal to find the upregulation of genes involved in the inflammatory response but this observation can be seen in epithelial cells aswell. The early protein expression 2-DE profiles of colonic epithelial cells exposed to *H. pylori* OMV were examined by 2-DE and LC-MS/MS. To examine the proteomic response of epithelial cells to OMV stimulation, T84 cells were grown to confluency on semi permeable inserts and stimulated with OMV (10 μg/ml) at 37°C for 24 h. After which the cells were washed with PBS and harvested by scraping into 8M urea lysis buffer. For the resolution of proteins, a small 2-DE gel system (13 x 13 cm) was used. Figure 4.5a shows a typical 2-DE protein pattern of control T84 cells. Using the Phoretix Expression (Non-Linear, Horsham, UK) software, this was compared with the protein profile of T84 cells stimulated with *H. pylori* OMV. The rectangles in Fig 4.8a and 4.5b highlight areas of the 2-DE gels where major differences were observed in comparing the control gel to that of the stimulated cells.

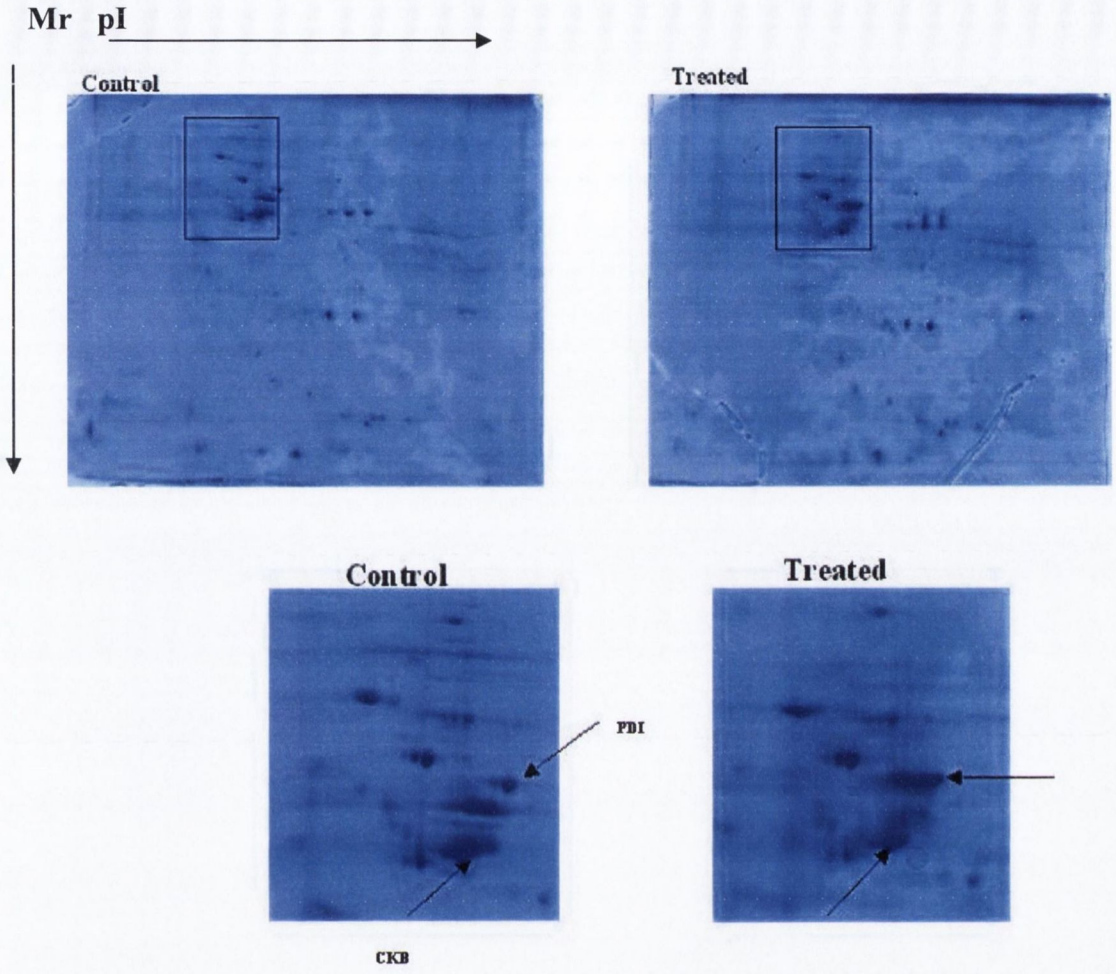


Figure 4.8a – 2-DE gels of control and treated T84 monolayers. Rectangles in each whole gel are enlarged below. Arrows indicate differences in expression noted between gels. T84 cells were seeded onto semi-permeable inserts. TER was monitored until cells were fully differentiated and polarized. Cells were then stimulated with OMV (10 μg/ml) for 24 hours. Cells were then washed, lysed and prepared for 2-DE. Samples were run on 12.5 % SDS-PAGE and stained with Colloidal Coomassie blue.

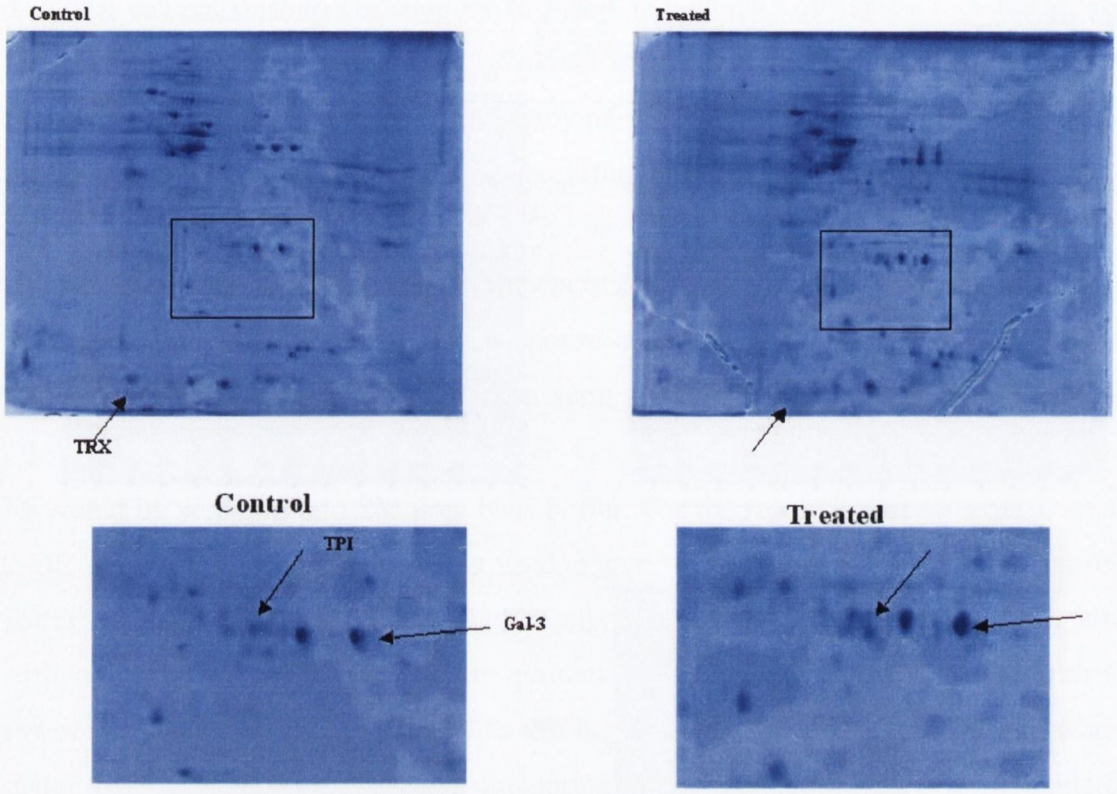


Figure 4.8b - 2-DE gels of control and treated T84 monolayers. Rectangles in each whole gel are enlarged below. Arrows indicate differences in expression between gels. T84 cells were seeded onto semi-permeable inserts. TER was monitored until cells were fully differentiated and polarized. Cells were then stimulated with OMV (10 μg/ml) for 24 hours. Cells were then washed, lysed and prepared for 2-DE. Samples were run on 12.5 % SDS-PAGE and stained with Colloidal Coomassie blue.

Altogether, 5 host cell protein spots were differentially regulated from treated samples as compared to control samples (Table 1). Proteins are referred to as 'differentially regulated' when the difference in spot abundance is equal to or larger than twofold. Three host proteins that were upregulated by twofold or more (protein disulphide isomerase PDI, triosephosphate isomerase TPI and Galectin –3 carbohydrate recognition domain) and 2 proteins that were down regulated by twofold or more (thioredoxin and creatine kinase B CKB) in response to stimulation by *H. pylori* OMV were identified. The results of 5 independent experiments suggest that stimulation of mature T84 monolayers by *H. pylori* OMV led to a small change in the protein profile. Interestingly, 4 of these proteins were previously reported as differentially regulated by *H. pylori* infection in gastric epithelial cells.

Name	***Mr***	***Location***	***Function***
Protein Disulphide Isomerase (PDI)	57, 119	Endoplasmic Reticulum	Isomerase Enzyme Protein Metabolism
Creatine Kinase B (CKB)	42,000	Cytoplasm	Promote Cell Differentiation
Triosephosphate Isomerase (TPI)	26,671	Cytoplasm	Isomerase Enzyme Energy Metabolism
Galectin-3 (Gal-3)	26,190	Cytoplasm	Sugar binding protein Transcription Regulator Activity
Thioredoxin	11,694	Cytoplasm	Oxidoreductase Enzyme Energy Metabolism

Table 4.1 – Five host proteins differentially regulated by stimulation with *H. pylori* OMV

Antibodies to the specific proteins that were differentially regulated by stimulation with the OMV were purchased (AbCam) to validate the proteomic data derived from 2-DE. T84 cell lysates were electrophoretically separated by 1D SDS-PAGE (6.5 x 9 cm), transferred to PVDF membrane, blocked with 5 % Marvel and probed for the specific proteins. All 5 of the antibodies recognised proteins in both cell lysates (Fig 4.5c) but

the expression of the proteins did not differ between the control and treated lysates. This may be due to the fact that the 2-DE approach is more sensitive in detecting subtle changes in expression.

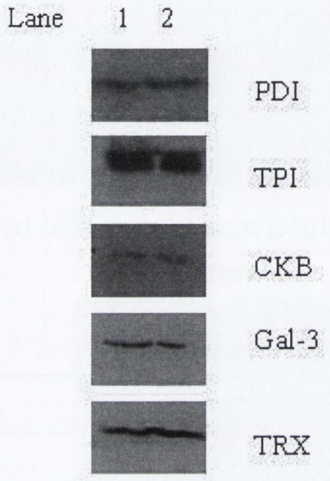


Figure 4.9 – Immunoblotting for proteins, seen to be differentially regulated inT84 cell monolayers in response to *H. pylori* OMV stimulation. Lane 1 – T84 Lysate (Control), lane 2 – T84 Lysate (Treated). T84 cell and biopsy lysates were electrophoretically separated on a 1D SDS-PAGE gel (6.5 x 9 cm), transferred to PVDF membrane, blocked with 5 % Marvel and probed for the specific proteins. PDI and TPI were recognised by cell lysates but no difference in expression was noted between control and treated lysates.

4.3.6 Induction of Cytokine Expression in T84 Epithelial Cells by OMV

Epithelial cells respond to infection with *H. pylori* by activating numerous signal transduction cascades (Smith et al., 2003). To date, the responses that have been best characterised are those which lead to expression and activation of c-fos and c-jun via mitogen activated protein kinase pathways (Meyer-ter-Vehn et al., 2000) and NF-κB (Keates et al., 1997). Activation of these pathways results in the production of IL-8 by epithelial cells. OMV also induce IL-8 expression in AGS cells (Ismail et al., 2003) and data from this present study demonstrate that they also induce IL-8 expression in non-polarised T84 epithelial cells. It was decided to examine whether OMV could induce RANTES protein expression in fully differentitiated mature T84 monolayers. Cytokines in both the apical and basolateral supernatants were measured by ELISA. The Randox Evidence Investigator™ was also used to look at the expression of a range of cytokines and growth factors possibly induced by OMV.

RANTES (regulated on activation normal T cell expressed and secreted) is a CC chemokine, which plays an important role in the inflammatory response associated with *H. pylori* infection. It is produced by epithelial cells, CD8+ T cells, fibroblasts and platelets and mediates the trafficking and homing of classical lymphoid cells such as T cells and monocytes (Kudo et al., 2005). Increased RANTES production is a feature of *H. pylori* induced gastric inflammation (Shimoyama et al., 1998; Yamaoka et al., 1998; Kikuchi et al., 2000; Park et al., 2001; Cottet et al., 2002). OMV (10 μg/ml) induced IL-8 secretion by differentitated T84 cells and this was recovered in both the apical and basal compartments (Fig. 4.10). The results are strikingly similar for both sets of samples but more IL-8 is secreted apically over time.

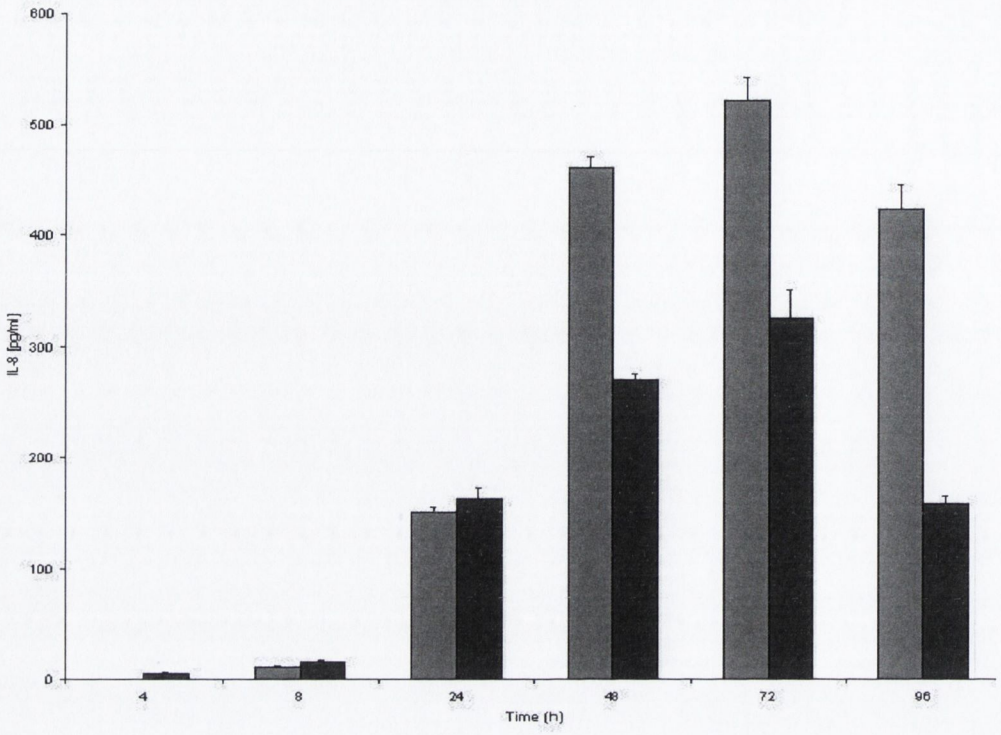


Figure 4.10 – *H. pylori* OMV induce IL-8 expression in polarized T84 cells both apically and basally

T84 cells were seeded onto semi-permeable inserts. TER was monitored until cells were fully differentiated and polarized. Cells were then stimulated with OMV (10 μg/ml). Supernatants were collected apically and basally and an ELISA protein assay for IL-8 was performed. OMV induce IL-8 both apically and basally in T84 cells. More IL-8 is induced apically than basally. (Apical – grey bars, Basal – black bars).

Figure 4.11 shows RANTES expression in the apical and the basal supernatants, of polarised T84 cells. There appears to be a higher expression of RANTES in the apical portion of the cell. However, the induction of RANTES is equally high in the control as well as the treated cells as if the cells constitutively expressed RANTES.

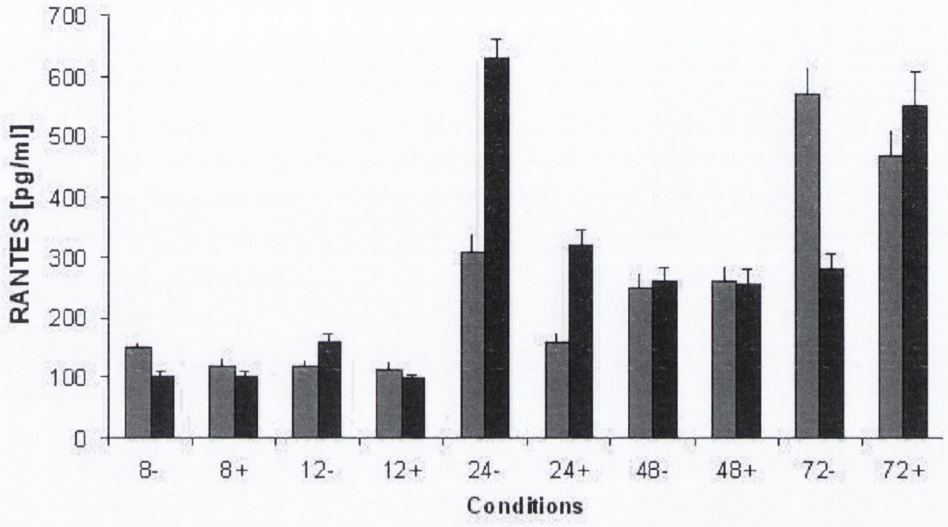


Figure 4.11- RANTES expression in polarized T84 cells in response to *H. pylori* OMV. T84 cells were seeded onto semi-permeable inserts. TER was monitored until cells were fully differentiated and polarized. Cells were then stimulated with 10 μg/ml OMV. Supernatants were collected apically and basally and an ELISA protein assay for RANTES was performed. OMV induce a small amount of RANTES both apically and basally in T84 cells. More RANTES is induced apically than basally, but there is no significant difference in RANTES production between control and treated cells. (Apical – grey bars, Basal- black bars, - Neg controls, + Treated samples).

Figure 4.12 shows that when a broad range of cytokines were screened by the Randox Evidence Investigator™, only IL-8 was abundantly expressed by the cells, at levels comparable to those seen by ELISA (3000pg/ml at 24 h). IL-4 and VEGF were also detected but at low levels, approximately 30pg/ml and 70pg/ml respectively. There was no evidence of induction of IL-2, IL-6, IL-10, IFNG, TNFA, IL-1A, IL-1B, MCP-1 or VEGF.

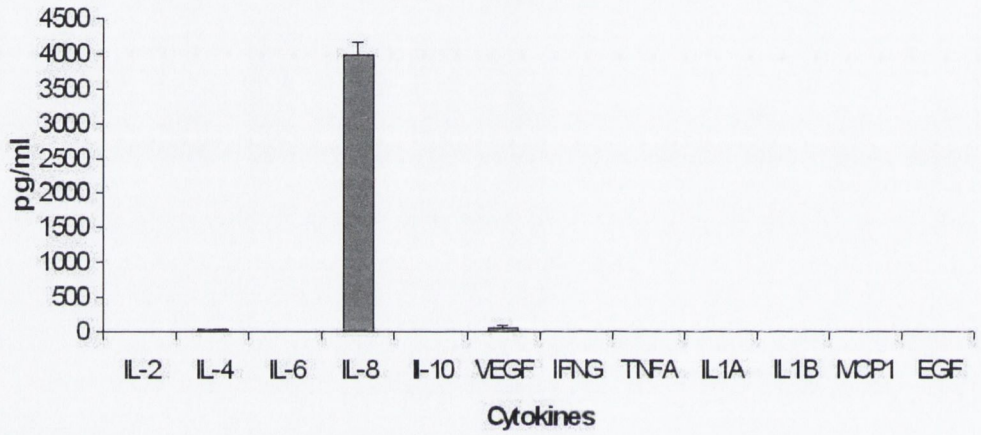


Figure 4.12 – T84 cytokine response to stimulation with *H. pylori* OMV. This assay was carried out on the Randox evidence investigator Biochip. T84 cells were seeded onto semi-permeable inserts. TER was monitored until cells were fully differentiated and polarized. Cells were then stimulated with 10 μg/ml OMV. Supernatants were collected and assayed for cytokine expression using the RANDOX evidence investigator. IL-8 was the major cytokine induced. Small amounts of IL-4 and VEGF were produced.

4.4 DISCUSSION

This chapter details the initial effects of OMV on epithelial cells, an *in vitro* model of infection. There is a large body of data available on the effects of *H. pylori*on epithelial cells but little has been reported on the effects of the OMV on these cells. As it was ultimately proposed to examine the effects of OMV on human duodenal explants in an *ex vivo* model of ulcerogenesis, the *in vitro* model of infection was essential for optimising the techniques to be used. The colonic epithelial cell line T84 was chosen as it becomes polarised and differentitated when grown on a semi permeable substrate, has functional tight junctions and represents a model 'tight' epithelium (Terres et al., 1998). In this study the effect of OMV on epithelial cell function was examined. The internalization of OMV in cell lines was the first objective because in cell lines and biopsies, less than 10 % of *H. pylori* are internalised (Birkness et al., 1996; Amieva et al., 2002). However, in biopsies the organism never reaches the lamina propria, and only *H. pylori* OMV seem to transverse the gastric epithelial layer (Fiocca et al., 1999). Once internalized, OMV can deliver bacterial DNA and virulence factors such as enterotoxins and enzymes into the host cell (Yoshimura et al., 1993). The proteomic data on *H. pylori* OMV, identified the HpaA protein as a component of OMV to which specific antiserum was available. The anti-HpaA was fluorescently labelled to enable immunostaining of internalized OMV using confocal laser microscopy.

Epithelial cell lines incubated with broth culture filtrate (BCF) from VacA producing strains of *H. pylori* or with purified VacA show energy-dependent uptake of the toxin. Its selective internalization into endosomes and related vacuoles (Garner and Cover, 1996; Ricci et al., 1997) is likely to occur by a process of receptor mediated endocytosis (Ricci et al., 2000). However, when double immuno-staining for OMV and the early endosomal structure Rab-5 was carried out, no co-localisation between the OMV and endosomes was evident. Although gastric absorption of antigenic material by an active process compatible with endocytosis has been reported (Curtis and Gall, 1992), little data is available on gastric luminal endocytosis (Ricci et al., 2002). However this internalization of OMV occurs within epithelial cells, it is an ideal method by which to deliver their virulent cargo directly to the host mucosa.

The internalisation of OMV may or may not be accompanied by translocation of the OMV from the apical to the basolateral compartment. Due to technical difficulties with this assay the detection of OMV was prevented in the basolateral compartment due to cross-reactivity between the antiserum and the basolateral medium. This vectorial

traficking across polarized intestinal epithelial cells has been described for *H. pylori* LPS (Terres et al. Unpublished observations) and for *Shigella* LPS (Beatty et al., 1999). Tight junctions selectively regulate the passage of molecules and ions via the paracellular pathway (Cereijido et al., 1988), and also restrict the lateral movement of molecules in the cell membrane (van Meer et al., 1985)(Dragston et al., 1981). It has been shown that bacterial extracts from *H. pylori* can alter the paracellular barrier function of the T84 cell line (Terres et al., 1998). In this study the stimulation of T84 monolayers with *H. pylori* OMV had no significant effect on transepithelial electrical resistance (TER), did not induce changes in the expression of the tight junction protein ZO-1 and did not induce dome formation up to 24 hours. The HPE used as a positive control for these experiments was capable of inducing dramatic decreases in TER, a displacement of ZO-1 from cell-cell junctions and of inducing dome formation within 30 min of stimulation. It is likely that the amount of OMV used for these experiments, (10 μg/ml), was too low, in comparison to the concentration of the HPE, to elicit these responses from the T84 monolayers. A dose response was carried out ranging from 10 – 50 μg/ml OMV but no dome formation occurred. The HPE is known to contain LPS and lipoproteins but no further characterisation of its components has taken place. Its protein concentration of 0.5 mg/ml is a great deal more concentrated than that of the OMV and it is likely that this is not comparable to that seen during a normal human *H. pylori* infection. It seems clear from these results that *H. pylori* OMV have no effect on the barrier function of T84 or adherence to the extracellular matrix.

H. pylori elicits a range of host responses during infection including the activation of various signal transduction pathways, change in cell morphology, actin cytoskeletal rearrangements, apoptosis and cytokine induction (Backert et al., 2005a). In this study 2-DE and LC-MS/MS were used to characterize the changes in the protein profile during *H. pylori* stimulation of T84 epithelial cells. Using 2-DE, approximately 200 protein spots were resolved per gel, among which 10 showed differential intensities between treated and untreated samples. Of these 10 spots, 3 upregulated and 3 downregulated host proteins on CCB stained gels were detected, whose abundance differed significantly (twofold or more) between T84 control cells and T84 cells stimulated with *H. pylori* OMV. Interestingly, 4 of these proteins have been previously reported to be differentially regulated by *H. pylori* infection in gastric epithelial cells. Expression of the protein Galectin – 3 is upregulated by gastric epithelial cells

following adhesion of *H. pylori*, suggesting that in addition to colonisation this protein also plays an important role in the host response to infection (Fowler et al., 2006). In 2004, He et al., used a proteomic approach to globally analyse the protein profiles of paired surgical specimens of primary gastric adenocarcinoma and nontumour mucosa aimed at identifying specific disease associated proteins as potential clinical biomarkers *in H. pylori* infection (He et al., 2004). They reported the up-regulation of the glycoloytic enzyme TPI, which is involved in the triose stage of the glycolytic pathway. Significant over expression of TPI has also been found in lung adenocarcinoma (Chen et al., 2002) and in squamous cell carcinoma of the bladder (Montgomerie et al., 1997). They also reported the up regulation of PDI, which is a multifunctional chaperone in the endoplastic reticulum (Noiva, 1999) which is often upregulated under stress conditions. They observed the down regulation of CK-B, which has been long related to malignant tumors of the gastrointestinal tract (Koven et al., 1983; Hirata et al., 1989). The down regulation of this enzyme may reflect the poor differentiation state at the late stages of gastric cancer. This approach appeared to produce new and interesting data, although these results were not validated by Western blotting. However this may be simply due to a lack of sensitivity inherent in 1D SDS-PAGE in comparison to the 2-DE approach.

Epithelial cells are the first line of defence to bacterial infection and actively participate in host innate immunity (Ding et al., 2005). The enhanced mucosal inflammation has been thought to play an important part in *H. pylori* induced pathogenesis (Ding et al., 2005). Infection with the organism induces multiple cellular signalling pathways in gastric epithelial cells, causing increased inflammatory cytokine production including IL-8, IL-1, IL-6 and RANTES. In addition to specific virulence factors, vesicles contain compounds that are recognized by eukaryotic cells in the innate and acquired immune response pathways (Kuehn and Kesty, 2005). An overstimulated inflammatory response to a pathogenic organism is destructive for the host. Outer membrane proteins, LPS and lipoproteins in OMV are biologically active molecules that can activate immune cells via Toll-like receptors (TLRs) (Galdiero et al., 1999; Akira et al., 2001) but this will be discussed in greater detail in the next chapter. In this study the ability of OMV to induce IL-8 and RANTES production from polarised T84 epithelial cells was examined and their ability to induce a range of cytokines and growth factors using the Evidence Investigator™. It was found that coincubation of *H. pylori* OMV and fully differentiated T84 cells induced a significant increase in IL-8 expression, apically and basally. The

results for the induction of RANTES expression by *H. pylori* OMV were inconclusive. Although RANTES expression appeared to be higher in the apical portion of the cells, no conclusions can be drawn from this as RANTES appeared to be constitutively expressed by the cells at very high levels. The results from the Evidence Investigator™ were satisfactory insofar as IL-8 was by far the most highly expressed cytokine. OMV were shown to induce VEGF and IL-4. VEGF overexpression is accompanied by an increase in gastric neo-angiogenesis (Tucillo et al., 2005). which could make this finding highly significant. It has also been reported that impaired mucosal immunity, particularly involving the secretion of IL-4, may contribute to *H. pylori* eradication failure (Borody et al., 2002). However, the levels of IL-4 secreted in this study (30 pg/ml) do not appear to be physiologically relevant as Serrano (2007) reported similar levels secreted in patients diagnosed as *H. pylori* negative.

In summary, in this study it was found that OMV, which are constantly shed from the surface of *H. pylori* in vivo, have a measurable effect on the mature monolayers of T84 cells. Internalisation of OMV was observed after 1 – 2 hours, changes at the protein level of the host cells after 24 hours and epithelial cell IL-8 production. However, any effect on barrier function or dome formation in mature monolayers of T84 cells after 24 hours were not observed. These findings support the theory that OMV play a role in promoting host responses independently of the intact organism.

CHAPTER V
THE ROLE OF TOLL – LIKE RECEPTORS IN THE RESPONSE OF EPITHELIAL CELLS TO *H. PYLORI* OUTER MEMBRANE VESICLES

5.1 INTRODUCTION

Cells belonging to the innate immune system sense and respond to microbial products via the Toll – like receptor family (Smith et al., 2003). Toll – like receptors (TLR) are an evolutionarily conserved family of cell surface molecules and trans membrane proteins that are involved in innate immune recognition of pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2000). PAMPS are generally unique, chemically diverse products released by microorganisms and examples include lipopolysaccharides (LPS), peptidoglycan, lipoproteins, bacterial DNA, bacterial flagella, double-stranded RNA and yeast zymosan (Smith et al., 2003; Ferrero, 2005). The extracellular regions of TLRs are diverse but contain variable numbers of leucine-rich repeat regions and conserved cysteine domains that are thought to contribute to structure and function (Mandell et al., 2004). So far, a total of 11 TLRs have been identified in mammalian cells (Takeda et al., 2003; Zhang et al., 2004). When these receptors interact with diverse microbial products and PAMPs, they transduce cellular signals that are responsible for the activation of genes that control host innate and adaptive immune responses, including proinflammatory cytokine expression (Ding et al., 2005). To date, TLR2 and TLR4 are the two best characterised of the Toll family. TLR2 recognizes peptidoglycan, bacterial lipoproteins and some LPS (Takeuchi et al., 1999; Underhill et al., 1999a). However its ability to recognize peptidoglycan has recently been refuted (Travassos et al., 2004). TLR4 recognizes LPS from gram - negative bacteria (Takeuchi et al., 1999).

With respect to *H. pylori*, the intestinal epithelial cell layer is thought to represent the first line of defence and the trigger for host innate and inflammatory responses (Cario et al., 2002). It has been demonstrated that intestinal epithelial cells constitutively express several TLRs in vitro and in vivo (Cario et al., 2000). It has been widely reported that epithelial cells respond to *H. pylori* infection at least in part via TLR2 and TLR5 (Smith et al., 2003). It has also been suggested that distinct TLRs are responsible for the recognition of *Helicobacter* LPS versus intact *Helicobacter* bacteria. In 2004, Mandell et al, showed that the cytokine-inducing activity of *Helicobacter* LPS was mediated by TLR4 and that the cytokine response to whole *H. pylori* was mediated via TLR2. It was also reported that TLR2 mediates changes in the gene expression profile of epithelial cells during *H. pylori* infection (Ding et al., 2005) and that the *H. pylori* neutrophil

activating protein (HP-NAP) is a TLR2 agonist capable of inducing the expression of IL-12 and IL-23 by neutrophils and monocytes (Amedei et al., 2006).

It has not been reported to date as to which TLRs are involved in epithelial cell recognition of OMV. Outer membrane proteins (OMPs) present in all vesicles are all biologically active molecules that can activate immune cells via TLRs and induce neutrophil migration (Galdiero et al., 1999; Akira et al., 2001). Vesicles may enable bacteria to escape immune detection during colonization (Kuehn and Kesty, 2005). In this study, the role of TLRs in the recognition of *H. pylori* OMV by epithelial cells was investigated. The ability of *H. pylori* OMV to engage different TLRs was assessed by measuring IL-8 induction in Human Embryonic Kidney 293 (HEK 293) cells, stably transfected with either TLR2 or TLR4. The ability of OMV to stimulate IL-8 from the gastric cell line AGS and the intestinal cell line T84 was also tested. The work in this chapter was carried out in conjunction with Dr Sinead Smith.

5.2 OBJECTIVES

1. Determine the TLR expression profile in epithelial cell lines used.
2. Monitor IL-8 expression in the TLR-mediated response to *H. pylori* OMV.
3. Investigate the role of LPS and NapA in the cellular response to OMV

5.3 RESULTS

5.3.1 Toll-like Receptor mRNA Expression in AGS, T84 and HEK Epithelial Cell Lines

In order to determine which TLRs are expressed on the epithelial cell lines and thus may contribute in the response to *H. pylori* OMV, the TLR2 and TLR4 mRNA expression profile was examined using reverse transcription PCR (RT-PCR). To this end, total RNA was isolated from each cell line, and reverse transcription was carried out to generate cDNA. The resulting cDNA was used as a template in a PCR assay for TLR2, TLR4 or GAPDH. As expected, HEK TLR2 cells express mRNA for TLR2 (Fig 5.1), HEK TLR4 cells express mRNA for TLR4 and HEK 293 expressed mRNA for neither. Figure 5.2 shows that polarized T84 cells express mRNA for TLR2 and TLR4 and Fig 5.3 shows AGS express mRNA for TLR4 but not TLR2.

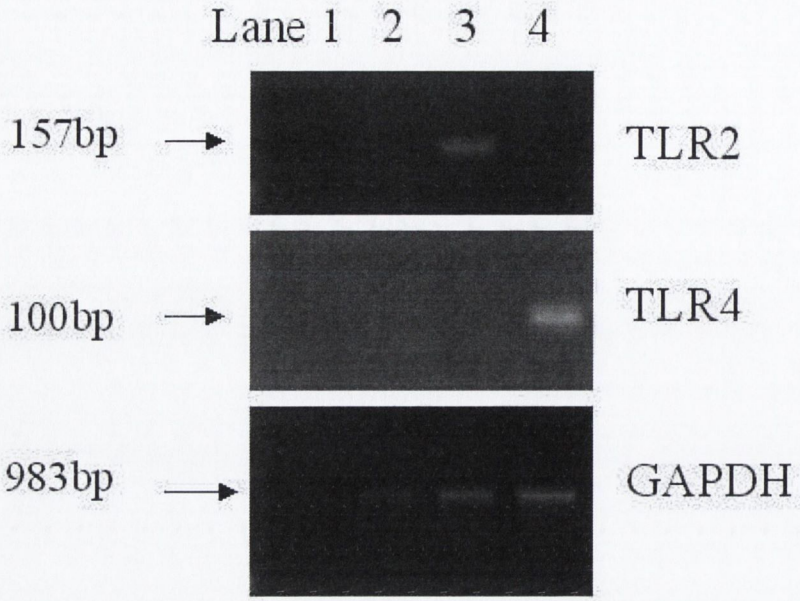


Figure 5.1 – Toll-like receptor (TLR) expression profile in HEK cell lines. In order to confirm the TLR expression profile of the HEK cell lines used, total RNA was isolated from HEK293, HEK TLR2 and HEK TLR4 cells. Reverse transcription was carried out to generate cDNA. The resulting cDNA was used as template in a PCR assay for TLR2, TLR4 or GAPDH (control). PCR products were analysed by agarose gel electrophoresis on a 2% agarose-1X TAE gel. A minus-template PCR was set up to verify that none of the PCR reagents were contaminated (Lane 1). As expected, HEK293 cells were did not express TLR2 or TLR4 (Lane 2). HEK TLR2 cells expressed TLR2 mRNA (Lane 3) and HEK TLR4 cells expressed TLR4 mRNA (Lane 4).

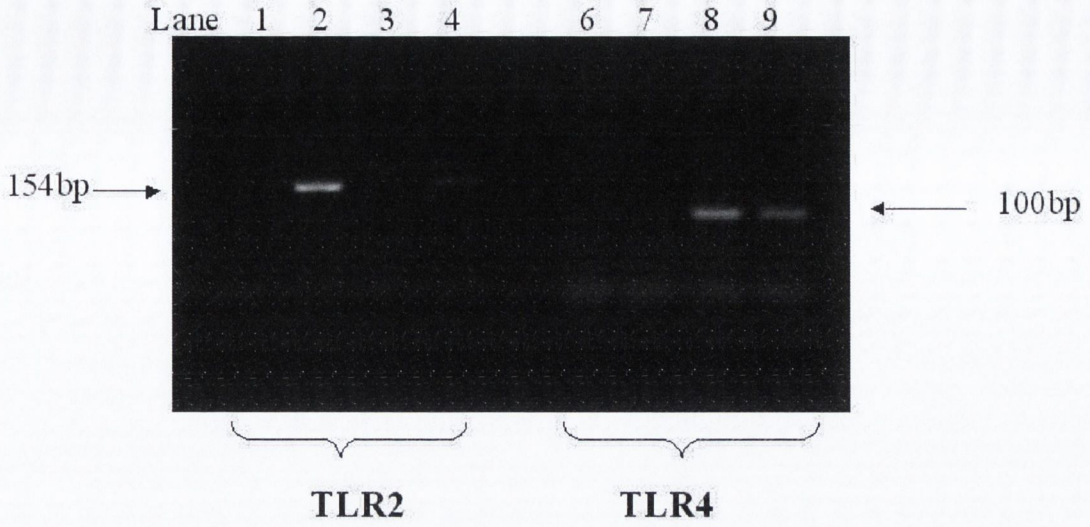


Figure 5.2 – Expression of Toll-like receptor mRNA in T84 cell lines In order to confirm the TLR expression profile of the T84 cell line used, total RNA was isolated from HEK293, HEK TLR4, HEK TLR2 and T84 cells. Reverse transcription was carried out to generate cDNA. The resulting cDNA was used as template in a PCR assay for TLR2 orTLR4. PCR products were analysed by agarose gel electrophoresis on a 2% agarose-1X TAE gel. A minus-template PCR was set up to verify that none of the PCR reagents were contaminated (Lane 1). HEK TLR2 cells expressed TLR2 mRNA (Lane 2), HEK TLR4 cells expressed TLR4 mRNA (Lane 8), T84 cells weakly expressed TLR2 (lane 4) and TLR4 (Lane 9). Lanes 6 –9 had the same lay out.

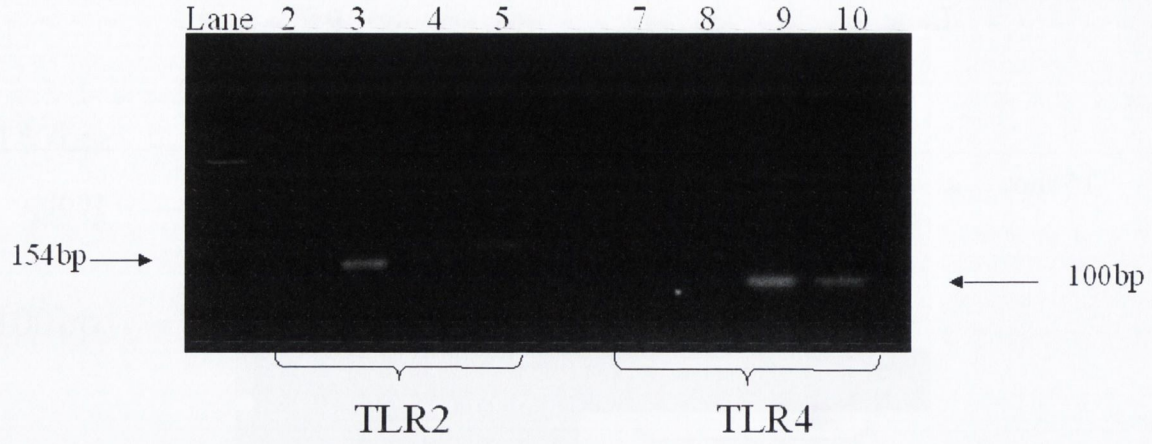


Figure 5.3 – Expression of Toll-like receptors mRNA in AGS cells. In order to confirm the TLR expression profile of the AGS cell line used, total RNA was isolated from HEK293, HEKTLR4, HEK TLR2 and AGS cells. Reverse transcription was carried out to generate cDNA. The resulting cDNA was used as template in a PCR assay for TLR2 or TLR4. PCR products were analysed by agarose gel electrophoresis on a 2% agarose-1X TAE gel. A 100 kb ladder was run on the gel (Lane 1). HEK 293 cells expressed neither TLR2 nor TLR4 (Lanes 2 and 7). HEK TLR2 cells expressed TLR2 mRNA (Lane 3), HEK TLR4 cells expressed TLR4 mRNA (Lane 9), AGS cells expressed TLR4 (Lane 10) but not TLR2 (Lane 5).

5.3.2 *H. pylori* OMV Induce IL-8 Expression in HEK Cell Lines

To determine which, if any, TLRs may play a role in the response of epithelial cells to *H. pylori* OMV, IL-8 induction in HEK cells transfected with TLR2, TLR4 or neither was evaluated. It is widely accepted that *E.coli* LPS is a TLR4 ligand and it has been previously reported in the literature that *E. coli* LPS induced IL-8 expression via TLR4 (Takeuchi et al., 1999). During an initial control experiment, HEK293, HEK-TLR2 and HEK-TLR4 cells were treated with 10 μg/ml *E. coli* LPS (Alexis) and total RNA was isolated at intervals over a 48 hour period. Reverse transcription was carried out to generate cDNA, which was then used as template in a real-time PCR-based IL-8 assay. As expected, it was observed that IL-8 mRNA was induced in HEK-TLR4 cells but not HEK293 or HEK-TLR2 cells (Figure 5.4a). In contrast with *E. coli* LPS, previous work in our laboratory has demonstrated that TLR2 is involved in the recognition of *H. pylori* LPS (Dr. Sinead Smith et al., manuscript in preparation; Figure 5.4b). This observation is in keeping with the findings of Smith et al., 2003.

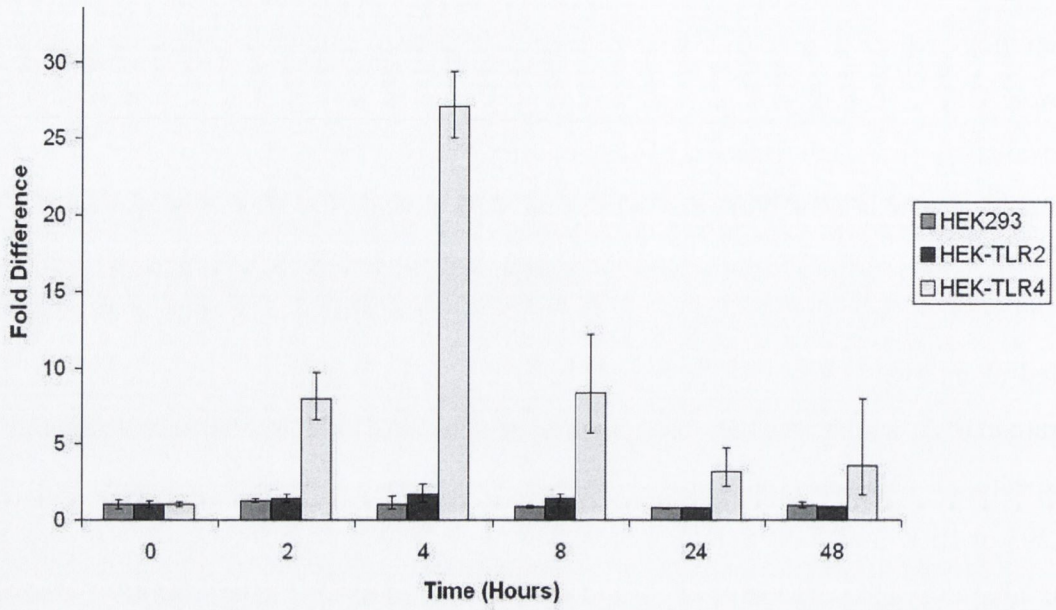


Figure 5.4a – *E. coli* LPS induced IL-8 mRNA in HEK-TLR4 cells. *E. coli* LPS is a well known TLR4 agonist. During a control experiment, HEK293, HEK-TLR2 and HEK-TLR4 cells were treated with 10 μg/ml of *E. coli* LPS and total RNA was isolated at intervals of a 48-hour period. Reverse transcription was carried out, followed by a real-time PCR-based IL-8 assay. As expected, it was observed that IL-8 mRNA expression was induced in HEK-TLR4 cells only (adapted from Smith, S. et al., manuscript in preparation).

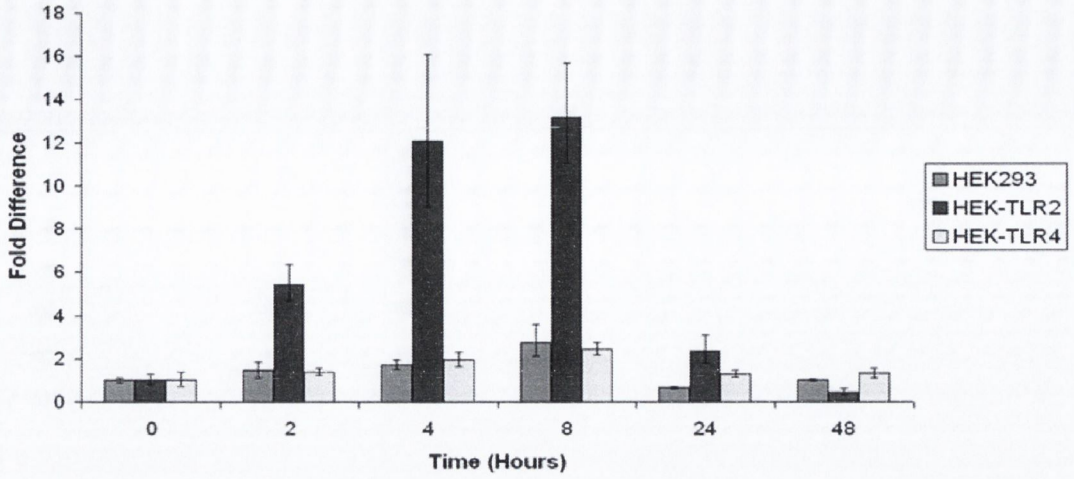


Figure 5.4b – *H. pylori* LPS induced IL-8 mRNA expression in HEK-TLR2 cells. The ability of *H. pylori* LPS to stimulate IL-8 mRNA expression was tested. HEK293, HEK-TLR2 and HEK-TLR4 cells were treated with 10 μg/ml of *H. pylori* LPS (NCTC 11637). Total RNA was isolated over a 48 hour time-course. Reverse transcription was carried out, followed by a real-time PCR-based IL-8 assay. It was observed that IL-8 mRNA expression was induced in HEK-TLR2 cells only, suggesting a role for TLR2 in the recognition of *H. pylori* LPS (Smith, S. et al., manuscript in preparation).

In order to investigate whether TLR2 and/or TLR4 play a role in the response of epithelial cells to *H. pylori* OMVs, the HEK cell lines were treated with 10 μg/ml OMVs and IL-8 mRNA expression was monitored over time. Treatment of HEK293 cells with OMVs lead to increased expression of IL-8 mRNA, with a maximum induction of 25-fold following 4 hours stimulation (Fig 5.5). This suggests that there are components present in the OMVs, capable of inducing IL-8 expression independent of TLR2 or TLR4. However, expression of TLR2 in the HEK cells was found to greatly enhance IL-8 induction in response to *H. pylori* OMVs, with a 630 fold induction following 4 hours of treatment (Fig 5.5). OMVs were also shown to drive IL-8 mRNA expression in HEK-TLR4 cells. The level of induction in HEK-TLR4 cells was greater than that of HEK293 cells, with a peak induction of 88 fold following 8 hours stimulation, but significantly lower than the level of IL-8 induction by OMVs in HEK-TLR2 cells.

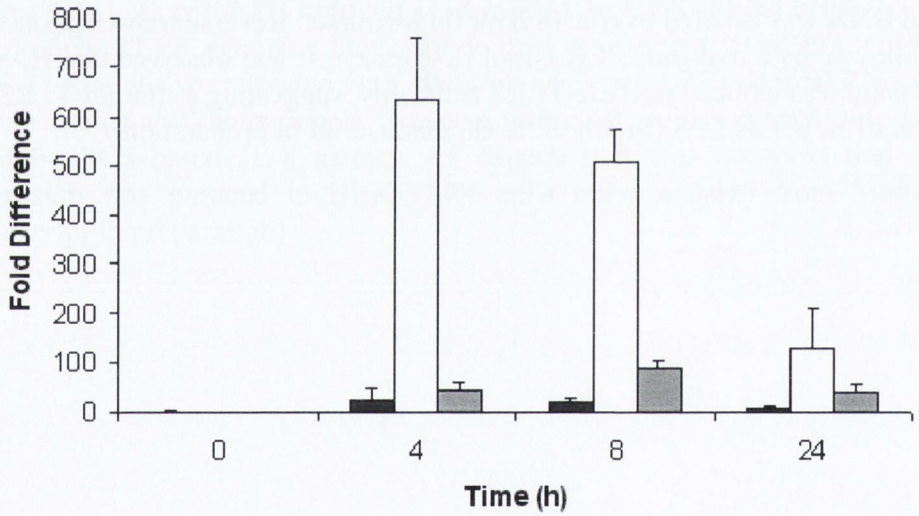


Figure 5.5 – *H. pylori* OMV induce mRNA IL-8 in HEK cell lines
The ability of *H. pylori* OMV to stimulate IL-8 mRNA expression was tested. HEK293 (black bars), HEK-TLR2 (white bars) and HEK-TLR4 (grey bars) cells were treated with 10 μg/ml of *H. pylori* OMV (NCTC 11637). Total RNA was isolated over a 48 hour time-course. Reverse transcription was carried out, followed by a real-time PCR-based IL-8 assay. It was observed that IL-8 mRNA expression was induced in all HEK cell lines but the effect was higher in HEK-TLR2 cells only, suggesting a role for TLR2 in the recognition of *H. pylori* OMV. (Black bars – Hek 293, White bars – TLR2 and Grey bars – TLR4)

In order to monitor IL-8 expression at the protein level in the HEK cell lines treated with *H. pylori* OMVs, supernatants were collected from the above experiment and assayed for IL-8 expression by ELISA. OMVs were found to stimulate IL-8 expression in all of the cell lines tested, but to the greatest level in HEK-TLR2 cells (Figure 5.6). It was observed that by 24 hours post stimulation, HEK TLR4 cells were producing approximately 70% as much IL-8 as HEK TLR2 cells. This was not the case at the mRNA level of expression.

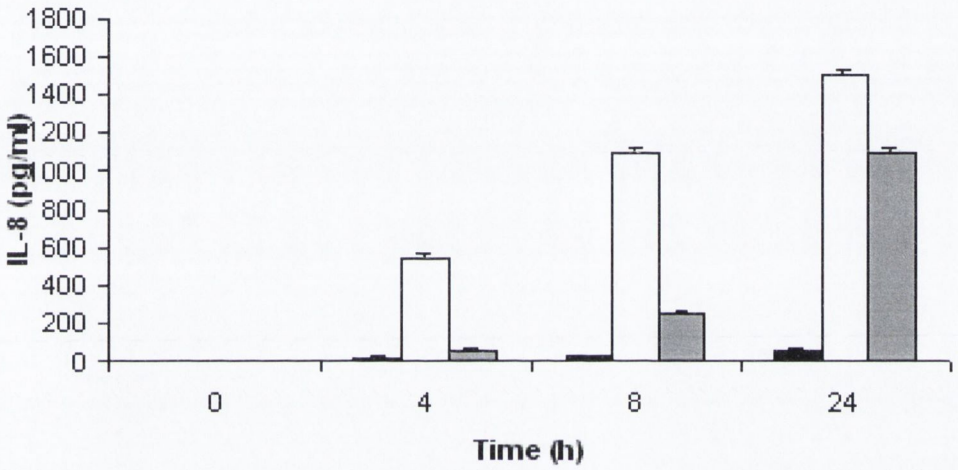


Figure 5.6 – *H. pylori* OMV induces protein IL-8 expression in HEK cell lines
HEK cells were grown in 6 well plates and stimulated with 10 μg/ml OMV. Supernatants were collected at various time points for ELISA protein assay. OMV induce IL-8 at the protein level in all HEK cell lines. Black bars – 293, White bars – TLR2 and Grey bars – TLR4

5.3.3 *H. pylori* OMV, *H. pylori* LPS and *E. coli* LPS Induce IL-8 Expression in Polarized Intestinal Epithelium (T84)

TLR2 and TLR4 are both expressed in polarized monolayers of T84 cells (Fig 5.2). As TLR2 and TLR4 are differentially expressed at the apical pole of differentiated T84 cells (Cario et al., 2002) the cells were first grown on semi-permeable inserts for 17 days and the TER monitored daily (Fig 5.7). When the cells were fully polarized and differentitated (TER > 1500 Ωcm^2), they were co-incubated with various stimuli (10 μg/ml) and the TER readings were taken for up to 4 days post stimulation. None of the stimuli induced a dramatic decrease in TER post stimulation (Fig 5.7).

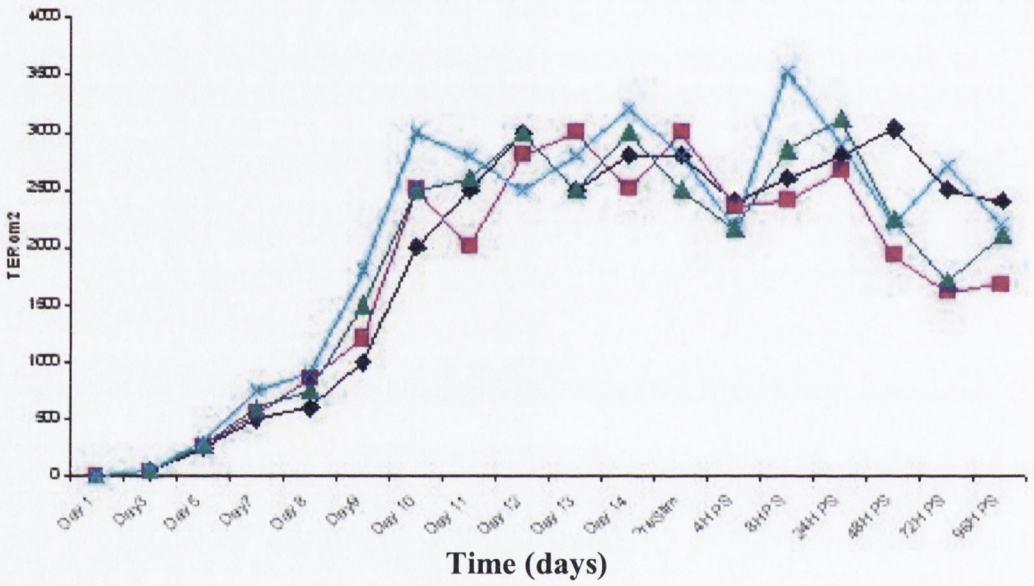


Figure 5.7 – Transepithelial electrical resistance (TER) of the T84 monolayers did not significantly decrease in response to stimuli.
T84 cells were seeded onto semi permeable inserts and TER was measured daily. Cells were stimulated on Day 14. TER was measured for the next 4 days. No significant decrease in TER was observed. (Control – Black, Pink – 10μg/ml OMV, Green – 10μg/ml *E. coli* LPS and Blue – 10μg/ml *H. pylori* LPS)

The cells were harvested over a range of time points for quantitative RT real-time PCR. Figure 5.8 shows the amount of IL-8 induced by the various stimuli. All of the stimuli exerted a similar effect on the polarized T84 cells but at 72 hours post stimulation, *H. pylori* LPS, induces the most mRNA IL-8 expression with a 3-fold change in comparison to non-stimulated control cells. In comparison with the amount of IL-8 induced in HEK TLR2 cells (Fig 5.5), the OMV induced a lot less IL-8 in the polarized T84 cells. This may be due to a lower expression of TLR2 in the polarized T84 cells.

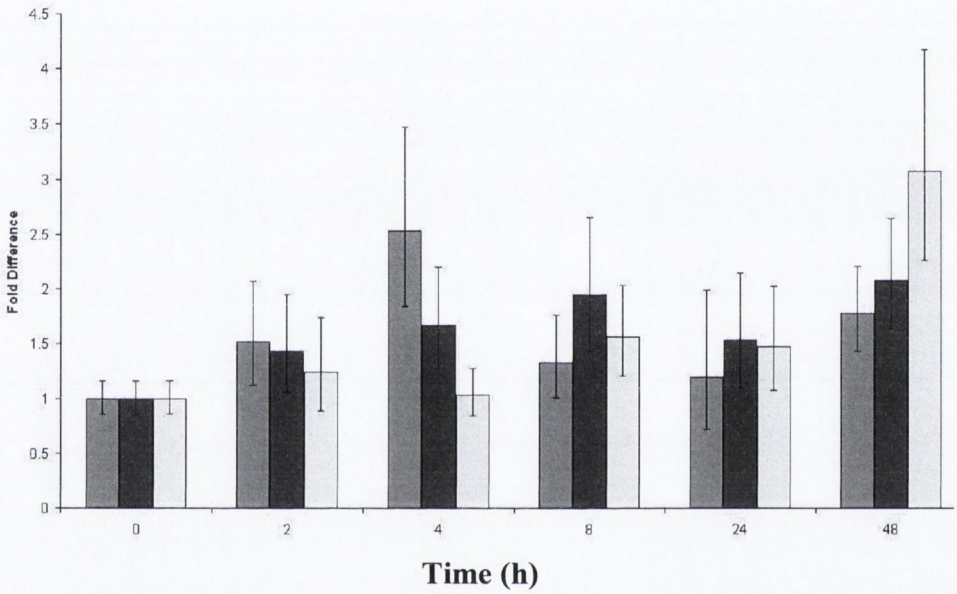


Figure 5.8 – mRNA IL-8 induction in polarized T84 monolayers in response to various stimuli.
The ability of various stimuli to stimulate IL-8 mRNA expression was tested. T84 cells were seeded onto semi-permeable inserts. When the cells were fully polarized and differentiated they were stimulated with various stimuli. T84 cells were treated with 10 μg/ml of *H. pylori* LPS (NCTC 11637), *E. coli* LPS and OMV. Total RNA was isolated over a 48 hour time-course. Reverse transcription was carried out, followed by a real-time PCR-based IL-8 assay. It was observed that IL-8 mRNA expression was induced in T84 cells by all three stimuli. (OMV – grey bars, *E. coli* LPS – black bars, *H. pylori* LPS – white bars)

Again to determine if the message seen at the RNA level was translated to the protein level, supernatants from a similar experiment were assayed to examine protein IL-8 expression in the polarized cells in response to various stimuli. Figure 5.9 shows the protein IL-8 produced by the cells. At the protein level, *H. pylori* OMV induced the highest amount of IL-8, which increased steadily over time. *E. coli* and *H. pylori* LPS elicited a very similar induction of IL-8. Again, this is slightly at variance to the results seen by the quantitative RT real-time PCR.

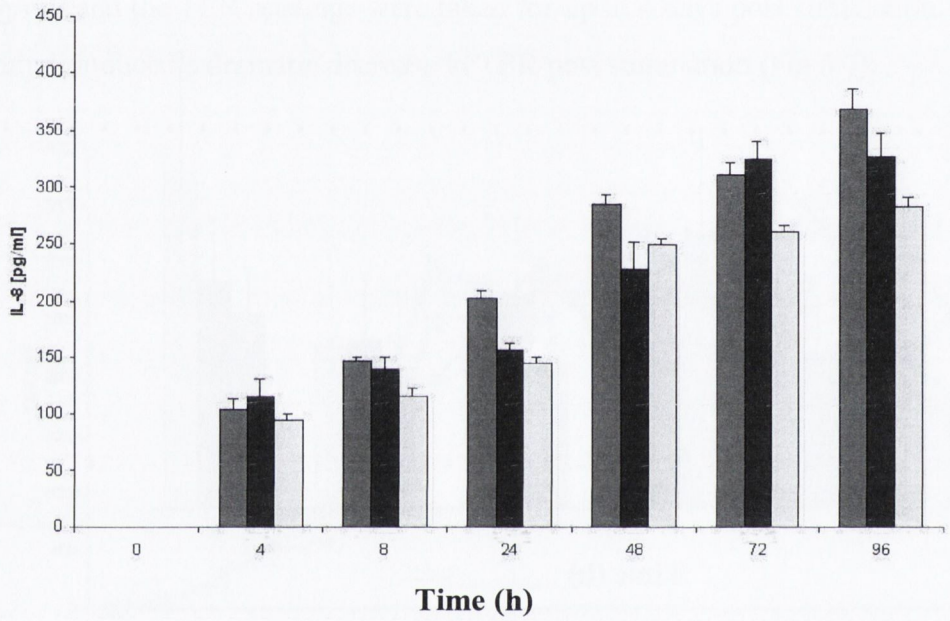


Figure 5.9 – Protein IL-8 induction in polarized T84 monolayers in response to various stimuli.
T84 cells were seeded onto semi-permeable inserts. When the cells were fully polarized and differentiated they were stimulated with various stimuli. Supernatants were collected at various time points for ELISA protein assay. IL-8 induction was seen in response to all stimuli and it increased with time. (10μg/ml OMV – grey bars, 10μg/ml *E. coli* LPS – black bars, 10μg/ml *H. pylori* LPS – white bars)

5.3.4 *H. pylori* OMV Induce IL-8 Expression in Gastric Epithelial Cells (AGS)

TLR4 is expressed in AGS cells (Fig 5.3). AGS cells were grown to confluency and stimulated with OMV (10 μg/ml). The cells were harvested at various time points for quantitative RT real-time PCR. Figure 5.10 shows that OMV were capable of inducing mRNA IL-8 expression in AGS cells. IL-8 levels increased over time and were maximal at 8 hours. The levels of IL-8 induced are very similar to those induced in the polarized T84 cells (Fig 5.8) but are again, far lower to those achieved in the HEK TLR2 cells (Fig 5.5). This is possibly due to the absence of an endogenously expressed TLR2.

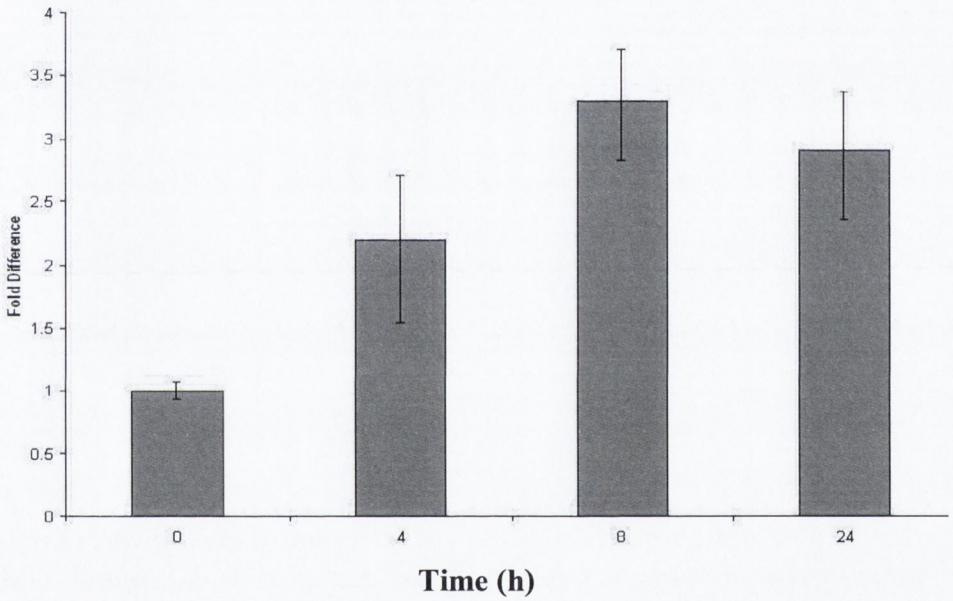


Figure 5.10 – *H. pylori* OMV induce mRNA IL-8 expression in AGS cells
The ability of *H. pylori* OMV to stimulate IL-8 mRNA expression in AGS cells was tested. AGS cells were treated with 10 μg/ml of *H. pylori* OMV (NCTC 11637). Total RNA was isolated over a 48 hour time-course. Reverse transcription was carried out, followed by a real-time PCR-based IL-8 assay. It was observed that OMV induce IL-8 in AGS cells.

Supernatants from the above experiment were assayed for protein IL-8 expression. Figure 5.11 shows that *H. pylori* OMV only induced an increased level of IL-8 in AGS cells, in comparison to control untreated cells, at 24 hours post stimulation. Prior to this, the levels induced were much lower in comparison to those of the polarized T84 cells (Fig 5.9).

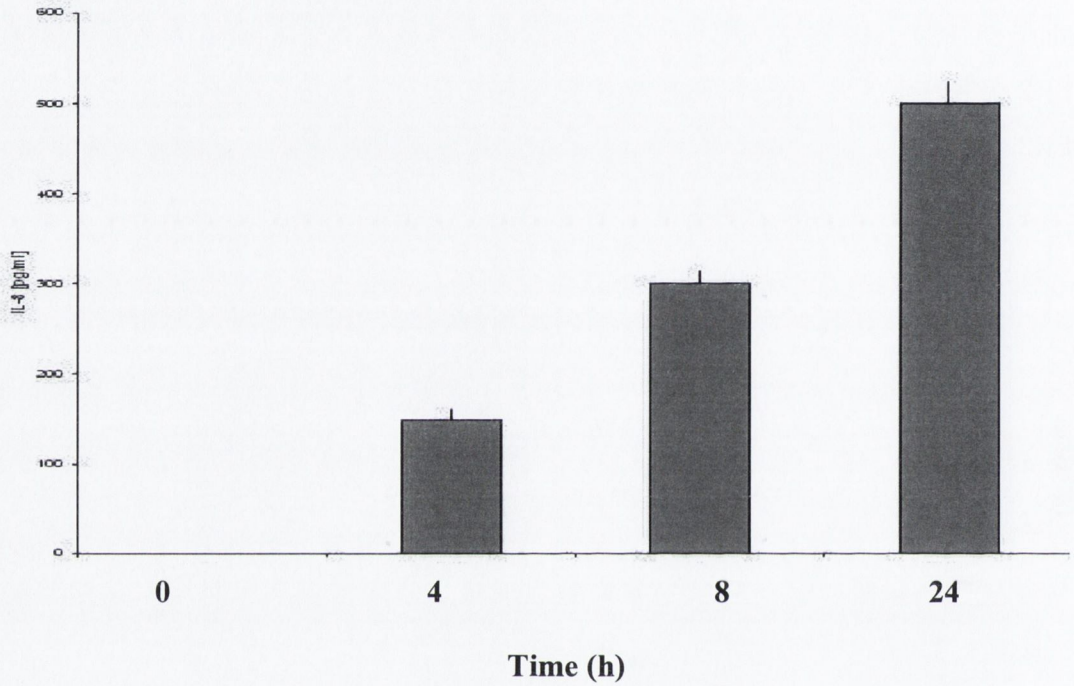


Figure 5.11 – Protein IL-8 expression in AGS cells in response to *H. pylori* OMV
AGS cells were grown in 6 well plates and stimulated with 10 μg/ml OMV. Supernatants were collected at various time points for ELISA protein assay. OMV induce IL-8 at the protein level in AGS cells, which increases with time.

5.3.5 Pre-incubating *H. pylori* OMV with Polymyxin B / NapA anti-serum Fails to Inhibit Induction of IL-8 Expression in HEK TLR2 Cells

H. pylori OMV induced the highest levels of mRNA IL-8 expression in HEK TLR2 cells with a 630-fold change in comparison to nonstimulated control cells. Using the proteomic data of the OMV being used in these experiments, experiments were conducted in an attempt to inhibit the IL-8 expression being induced in this cell line in response to *H. pylori* OMV. The proteomic analysis of *H. pylori* OMV (Tables 3.1, 3.2, 3.3) demonstrated that OMV contain LPS and the neutrophil activating protein, HP-NAP. Although there has been some controversy in this area, it has been reported that *H. pylori* LPS is a TLR2 agonist (Smith et al., 2003) and this was verified by our own results (Fig 5.5b). HP-NAP was found to be a TLR2 agonist able to stimulate neutrophils and monocytes to increase expression of IL-12, thus promoting Th1 immune responses (Trinchieri, 2003; Amedei et al., 2006).

Firstly, inhibition of IL-8 induction with Polymyxin B (PX) was attempted. PX acts specifically on gram-negative bacteria by electrostatic and hydrophobic interactions with ionic cell envelope components like phospholipids and LPS (Schindler and Teuber, 1975). PX binds to the lipid moiety of LPS and is a well-known inhibitor of the activation properties of LPS.

Before attempting to coincubate PX with OMV, preliminary experiments were carried out where HEK TLR2 cells were stimulated with *H. pylori* LPS (Fig 5.12a) and HEK TLR4 cells were stimulated with *E. coli* LPS (Fig 5.12b) with increasing concentrations of PX in both. This was done to determine the appropriate concentration of PX to use in order to inhibit IL-8 induction. The results were very encouraging in both experiments, although inhibition was higher in the HEK TLR4 cells stimulated with *E. coli* LPS and PX.

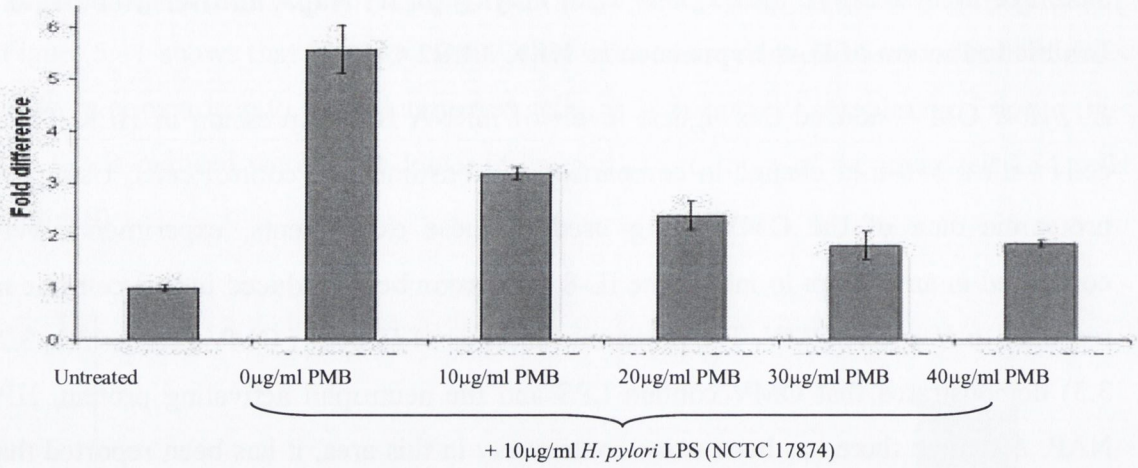


Figure 5.12a - Polymyxin B inhibits induction of IL-8 in HEK TLR2 cells in response to *H. pylori* LPS

The ability of Polymyxin B to inhibit IL-8 induction was tested. HEK-TLR2 cells were treated with 10 μg/ml of *H. pylori* LPS (NCTC 11637) and increasing concentrations of PX. Total RNA was isolated at 8 hours post-treatment. Reverse transcription was carried out, followed by a real-time PCR-based IL-8 assay. It was observed that PX inhibits IL-8 induction in HEK TLR2 cells in response to *H. pylori* LPS. (Adapted from Smith, S et al., manuscript in preparation).

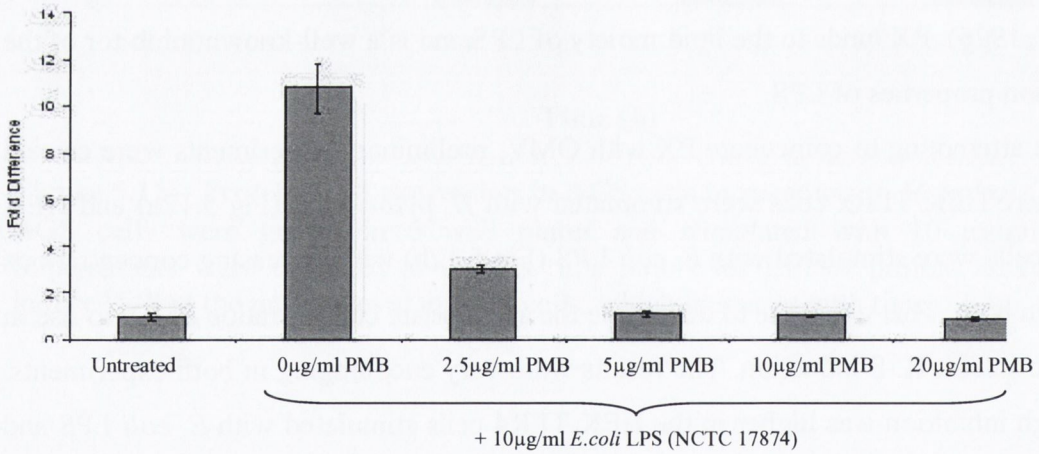


Figure 5.12b – Polymyxin B inhibits induction of IL-8 in HEK TLR4 cells in response to *E. coli* LPS

The ability of Polymyxin B to inhibit IL-8 induction was tested. HEK-TLR4 cells were treated with 10 μg/ml of *E.coli* LPS and increasing concentrations of PX (0, 2.5, 5, 10 and 20 μg/ml PMB). Total RNA was isolated 8 hours post stimulation. Reverse transcription was carried out, followed by a real-time PCR-based IL-8 assay. It was observed that PX inhibits IL-8 induction in HEK TLR4 cells in response to *H. pylori* LPS. (Adapted from Smith, S et al., manuscript in preparation).

HEK TLR2 cells were stimulated with OMV alone and with OMV (10 μg/ml) that had been pre-treated with PX (30 μg/ml) for 1 hour at 37 °C. Cells were harvested at 8 hours post stimulation and quantitative RT-PCR was performed. Figure 5.13 shows that PX was unable to inhibit IL-8 induction by *H. pylori* OMV in HEK TLR2 cells. This could be due to many factors but it seems likely that: i) LPS does not solely modulate IL-8 production, ii) PX can not completely inhibit the action of the LPS component.

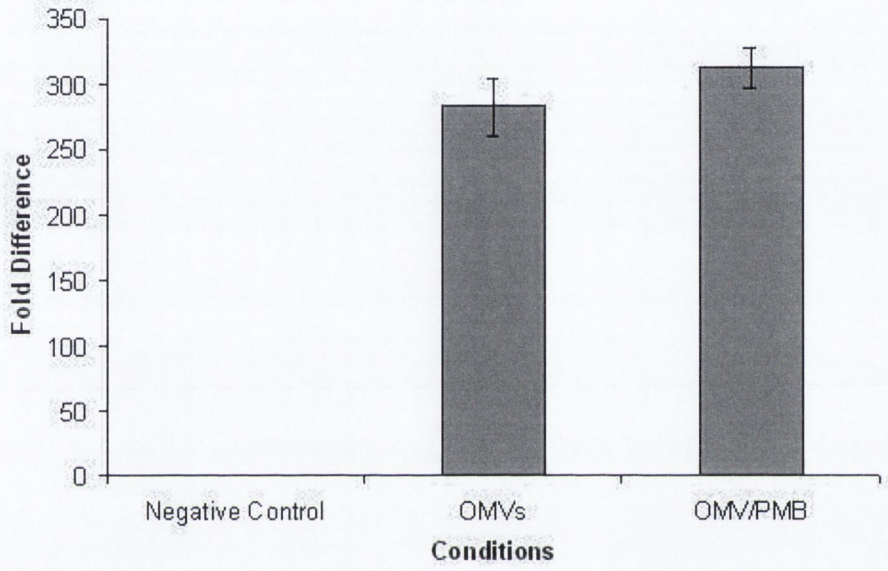


Figure 5.13 – Polymyxin B fails to inhibit IL-8 induction in HEK TLR2 cells stimulated with *H. pylori* OMV

HEKTLR2 cells were grown in 6 well plates and stimulated with 10 μg/ml OMV and 30 μg/ml PMB. Cells were harvested at 8 hours post stimulation for quantitative RT-PCR. PMB does not inhibit IL-8 induction in HEK TLR2 cells in response to OMV.

Secondly, inhibiting IL-8 induction by *H. pylori* OMV with NapA antiserum was attempted. This present study has found that OMV were potent chemoattractants for PMNs and that this function was dose dependent. This chemotactic response was completely abolished if the OMV were first treated with anti-NapA polyclonal antiserum (Fig 3.4) indicating that NapA was solely responsible for this effect. OMV were pretreated with increasing concentrations of NapA antiserum for 1 hour at 37 °C. Figure 5.14 indicates that NapA antiserum was unable to inhibit IL-8 induction by *H. pylori* OMV in HEK TLR2 cells.

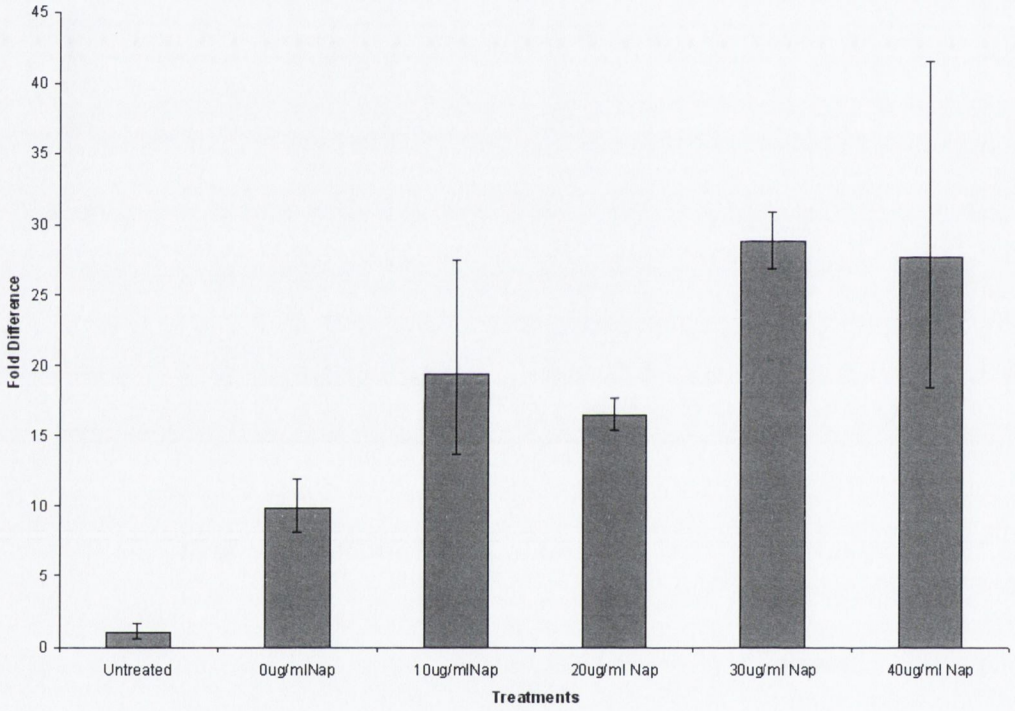


Figure 5.14 – NapA antiserum fails to inhibit IL-8 induction in HEK TLR2 stimulated with *H. pylori* OMV

HEKTLR2 cells were grown in 6 well plates and stimulated with 10 μg/ml OMV and increasing concentrations of NapA antiserum. Cells were harvested at 8 hours post stimulation for quantitative RT-PCR. NapA does not inhibit IL-8 induction in HEK TLR2 cells in response to OMV.

Finally, inhibiting IL-8 induction by *H. pylori* OMV using a combination of PX and NapA antiserum was attempted. Briefly, HEK TLR2 cells were stimulated with OMV (10 μg/ml), OMV pre-treated with PX (30 μg/ml), OMV pre-treated with NapA antiserum (30 μg/ml), OMV pre-treated with a combination of PX (30 μg/ml) and NapA antiserum (30 μg/ml) or tissue culture media alone. Cells were harvested and quantitative RT-PCR was performed on all samples. The results were unsatisfactory (Figure 5.15). HEK TLR2 cells stimulated with OMV pre-treated with PX show a small inhibition of IL-8 induction, in comparison with cells treated with OMV alone. OMV pre-treated with NapA antiserum show no inhibition of IL-8 induction. HEK TLR2 cells stimulated with a combination of PX (30 μg/ml) and NapA antiserum (30 μg/ml) again show no overall inhibition of IL-8 induction, in comparison with cells treated with OMV alone.

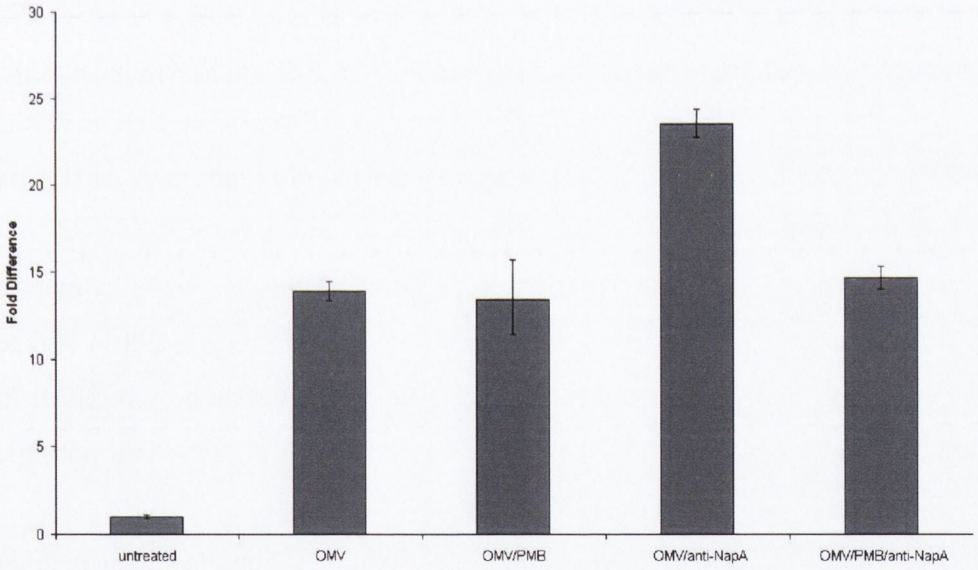


Figure 5.15 – *H. pylori* OMV pre-treated with combination of Polymyxin B and NapA antiserum appears to inhibit IL-8 induction by 50 % in HEK TLR2 cells
HEKTLR2 cells were grown in 6 well plates and stimulated with 10 μg/ml OMV and 30 μg/ml PMB, 30 μg/ml NapA or a combination of the two. Cells were harvested for quantitative RT-PCR at 8 hours post stimulation. In this experiment PMB does not inhibit IL-8 induction in HEK TLR2 cells in response to OMV. NapA alone does not inhibit IL-8 induction in HEK TLR2 cells in response to OMV. A combination of the two treatments appeared to not inhibit IL-8 induction either

5.4 DISCUSSION

In this study the role of Toll-like receptors in the response of epithelial cells to *H. pylori* OMV has been determined by examining cytokine responses at the mRNA and protein level of expression. TLRs are transmembrane proteins that function as recognition receptors for the detection and response to microbial ligands (Immler and Hoffman, 2001). Previous studies have provided evidence that TLR2 and TLR4 are important contributing factors to the inflammatory response induced by *H. pylori* (Smith et al., 2003; Mandell et al., 2004). The data presented here demonstrates that infection of epithelial cells *in vitro* with *H. pylori* OMV induces IL-8 cytokine responses primarily via TLR2 and via TLR4 to a much lesser degree. It has been previously reported that TLR2 is a critical receptor for the recognition of intact *H. pylori* bacteria (Mandell et al., 2004) so therefore it is surprising that this study demonstrates that TLR2 enhances epithelial cell responses to *H. pylori* OMV. This was seen in cell lines stably transfected with TLR2 and TLR4. The IL-8 expression of HEK TLR2 cells in response to OMV stimulation was approximately 10 fold higher than that of HEK TLR4 and HEK 293 and approximately 100-fold higher than that of polarized T84 cells and AGS cells at the mRNA level of expression. Although the protein levels of expression were still the highest in HEK TLR2 cells, HEK TLR4 cells expressed a much higher level of IL-8 in comparison with the mRNA data.

Recently, a secreted *H. pylori* antigen containing peptidyl prolyl cis-, trans-isomerase (HP0175) activity has been shown to interact with TLR4 (Basak et al., 2005) and to induce IL-6 in a TLR4 dependent manner (Pathak et al., 2006). The protein HP0175 was identified within the proteome of *H. pylori* OMV (Table 3.1). This is a possible reason for TLR4 mediated induction of IL-8 by OMV.

A small amount of OMV-induced IL-8 secretion occurs in a TLR-independent manner in untransfected HEK239 cells. It is likely that this component of IL-8 secretion is mediated by NOD proteins, the recently described intracellular pathogen recognition receptors (Ferrero, 2005), known to be expressed in this cell line (Viala et al., 2004). Like TLRs, NODs have been shown to act as sensors for bacteria. It is possible that the OMV contain something that is recognised by a NOD. HEK 293 cells have been shown to express NOD proteins and several *H. pylori* strains were found to induce NF-kB activation in HEK293 cells (Viala et al., 2004). A possible candidate could be peptidoglycan or peptidoglycan associated proteins such as OMP18, a component of the OMV proteome.

Furthermore, this data using highly purified LPS from *H. pylori* 17874 indicated this LPS was a TLR2 agonist (S. Smith et al., 2006, unpublished observation). This is consistent with the findings of Smith, MF et al but in contrast to Kawahara et al and Mandell et al who reported that *H. pylori* LPS is a TLR4 ligand. One possible explanation for the difference in the results is that the concentration of *H. pylori* LPS used for cytokine stimulation in this study is much higher than the dosages at which Mandell et al detect TLR4 agonist activity in their *H. pylori* LPS.

Although OMV potently induced IL-8 secretion in a TLR-2 and TLR-4 dependent manner it remains unclear which component(s) of the OMV is responsible for this effect. Neutralisation of NapA and LPS with specific antiserum and Polymyxin B, respectively, failed to abrogate chemokine release, even though both of these are known inducers of epithelial cell IL-8 secretion. Similarly, blocking OipA (Yamaoka et al., 2002, Kudo et al., 2004) with specific anti-serum did not diminish IL-8 secretion. However, our proteomic analysis of *H. pylori* OMV revealed the presence of other OMV components known to promote IL-8 secretion including Hsp60 (Takenaka et al., 2004, Zhao et al., 2007), CagA (Kim et al., 2006), and urease (Beswick et al, 2006), thus it is likely that any or all of these could contribute efficiently to chemokine secretion in the absence of functional NapA, OipA, or LPS. It has been reported, however, that CagA has been linked to IL-8 secretion from epithelial cells (Graham and Yamaoka, 2000). In 2004, Mandell et al, suggested that cagA induced IL-8 secretion from transfected HEK cells is a TLR2 dependent process, since HEK cells lacking TLR2 failed to secrete IL-8 when stimulated with CagA+ or CagA mutant *H. pylori* bacteria. If this is the case, then 100 % inhibition of IL-8 induction by OMV will be virtually impossible due to the presence of the CagA 26 protein within the proteome of the OMV. Further work in this area may yet determine what combination of factors within the OMV is capable of driving the inflammatory cytokine response.

Evaluation of the role of Toll-like receptors (TLR) in the response of epithelial cells to *H. pylori* OMV suggested that there may be qualitative differences between the ability of whole live *H. pylori* and OMV to elicit pro-inflammatory responses in epithelial cells. Others (Smith et al., 2003, Mandell et al., 2004) have demonstrated that live *H. pylori* provoke gastric cellular activation via TLR-2 and TLR-5, with TLR-4-mediated responses making little or no contribution. Thus it is interesting to note that OMV induce TLR-4-mediated IL-8 transcript in epithelial cells. However, the gastric cell line

AGS (TLR-4 positive and TLR-2 negative) lacks the co-receptor CD14 and is non-responsive to *H. pylori* LPS. Thus, the TLR-4 signalling is unlikely to be mediated by OMV-associated LPS. Although the levels of IL-8 transcript produced via the TLR-4 route are modest when compared with the levels seen in TLR-2 mediated OMV stimulation, the IL-8 protein expression levels are high and comparable in both cases. Signalling via TLR-2 and 4 results in distinct patters of pro-inflammatory gene transcription, with TLR-4 agonists yielding a more inflammatory phenotype than TLR-2 agonists (Toshakou et al., 2002). Additional qualitative differences in epithelial cell responses to *H. pylori* and OMV include release of MCP-1 and IL-6 from gastric cells (Mori et al., 2001, Lu et al., 2007) by *H. pylori* but not by OMV. These data suggest that OMV have the potential to induce qualitatively different signalling responses compared to whole bacteria. Further, OMV can readily enter epithelial cells, a rare occurrence with live *H. pylori*, and potentially interact with host signalling pathways. Such events could result in synergistic innate immune signalling between cell surface TLRs and cytoplasmic pathogen recognition receptors such as Nod-1.

In conclusion, the current study examined the role of TLRs in epithelial cells cytokine responses to *H. pylori* OMV. Evidence that TLR2 is involved in *H. pylori* OMV induced IL-8 expression by epithelial cells, has been presented. Although complete inhibition of IL-8 induction by pre-treating OMV with a combination of Polymyxin B and NapA antiserum proved unsuccessful, it is clear that the HP-NAP and the LPS component of the OMV are two very active TLR2 agonists.

CHAPTER VI
IMMUNE AND EXPLANT TISSUE RESPONSES TO *H. PYLORI* OUTER MEMBRANE VESICLES

6.1 INTRODUCTION

The development of duodenal ulceration in humans is highly associated with gastric colonisation by *H. pylori*(Dixon, 1991; Dixon, 1996; Dixon et al., 1996; Ekstrom et al., 2001). However, the acute response of the duodenum upon infection is poorly understood. This is probably due to the fact that patients only relate symptoms after the duodenal ulcer has already formed and secondly because animal models of *H. pylori* infection are frequently associated with gastritis rather than duodenal ulceration. The use of animal models is also difficult due to the high tropism of *H. pylori* for human vs animal epithelia. Finally, human duodenal cell lines are not commercially available, therefore the use of colonic epithelial cell lines such as T84 or CACO-2 represents a possible alternative. However, colonic epithelial cell lines are specialised in electrolyte and water absorption, while duodenal cells are considered to be a more "leaky" epithelia. To overcome these problems, an *in vitro* explant-culture system (IVEC) of duodenal biopsies from healthy donors was developed to study the effect of the epithelial interaction with OMV.

In preliminary experiments it has been demonstrated that the OMV of *H. pylori*, contain many biologically active virulence factors. Using a range of epithelial cell lines, it has been shown that OMV have the potential to become internalized within epithelial cells, to induce IL-8 cytokine expression via TLR2 and to elicit changes at the protein level. In this study, the interaction between *H. pylori* OMV and the duodenal epithelium using a human duodenal explant system was examined. The IVEC system was originally established successfully with pig explants (Meyer-Rosberg and Berglindh, 1996). In 2002, Olfat et al. used a similar system with gastric biopsies for the study of *H. pylori* infection (Olfat et al., 2002). For this work, viable human duodenal biopsies from patients with normal duodenum were cultured and exposed *ex vivo* to bacterial soluble components. In this chapter, other clinical aspects of the effects of *H. pylori* OMV were examined. These include determining if sera from *H. pylori* infected individuals contain more antibodies to OMV proteins than sera from *H. pylori* negative individuals. Recently, Amedei et al., 2006 reported that the neutrophil activating protein of *H. pylori* (NapA) promotes Th1 immune responses. As *H. pylori* OMV are known to contain NapA (Table 3.2, Chapter 3), the ability of OMV to act as immunomodulators by inducing expression of IL-12 by neutrophils and monocytes was also examined.

6.2 OBJECTIVES

1. To find biopsy donors for the study and to determine their suitability by confirming lack of *H. pylori* infection.
2. To establish explant cultures of duodenal biopsies from normal human donors and determine their viability
3. To establish an *in vitro* explant-culture system (IVEC) system and stimulate biopsies with OMV
4. To examine cytokine expression in treated biopsies, compared to control untreated biopsies, particularly: IL-8, IL-12 and RANTES.
5. To assess OMV antigenic potential by analysing the reactivity of human sera from infected patients when compared to uninfected individuals.
6. To determine if OMV can act as immunomodulators by stimulating human neutrophils and PBMCs to increase IL-12 expression.

6.3 RESULTS

6.3.1 Biopsy Collection and Patient Criteria

Duodenal biopsies were collected at the Endoscopy Unit at St. James Hospital, Dublin 8, in collaboration with Professor Dermot Kelleher's team of gastrointestinal registrars. Biopsies were collected from healthy volunteers who were fully informed about the study and had given their written consent of participation prior to the endoscopy procedure. Ethical approval was obtained prior to the commencement of the project from XXX. All participants were Clo test negative and culture negative (were not infected with *H. pylori*) at the time of biopsy collection. Biopsies were transported in ice-cold L15 medium (Gibco), supplemented with Pen/Strep from the Endoscopy unit to the laboratory.

6.3.2 Assessment of Viability of *Ex Vivo* Cultured Tissue

The reliability of data derived from this study was dependent on sample viability. Where tissue samples are obtained at biopsy, there is usually little doubt expressed as to the viability of the preparation although this is seldom assessed (Poole et al., 1996). Hence, a novel approach to determine the viability of the duodenal explants after 24

hours of *ex vivo* culture by using fixable non-toxic fluoroprobes was developed. An aim of this work was to examine the acute response of the duodenum upon infection by *H. pylori*, so it became necessary to devise this protocol, which would confirm that the biopsies were still viable. It would then be possible to confirm that changes at the protein level or differences in cytokine expression between treated and untreated biopsies were due to stimulation with *H. pylori* OMV and not due to the tissue having become necrotic.

Detection of viable and non-viable cells in biopsies was carried out using the fixable fluoroprobes 5-Chloromethylfluorescein diacetate (CMFDA) and ethidium homodimer-1. CMFDA is a glutathione-reactive dye, which freely diffuses into the cytoplasm of viable cells. CMFDA can only be loaded into living cells where the plasma membrane is intact and intracellular processes maintained (Poole et al., 1993). In contrast, ethidium homodimer-1 labels the nuclei of non-viable cells in which plasma membrane integrity and cellular metabolism has been compromised (Poole et al., 1993). Ethidium homodimer-1 has very low membrane permeability and is thus excluded from viable cells. CMFDA appears green and Ethidium homodimer-1 appears red when excited at a wavelength of 488nm, hence green indicating viable tissue and red indicating non-viable cells.

Biopsies were placed in fresh Autrup medium supplemented with 25 μM CMFDA and 25 μM ethidium homodimer-1 at 37 °C for 24 hours to achieve maximal dye penetration. After stimulating them over various timepoints, it was determined that 24 hours represented the time point for maximal dye penetration and minimal loss of viability. Biopsies were then washed, immediately embedded in OCT and snap frozen in liquid nitrogen cooled isopentane. The samples were then sectioned on a -25 °C cryostat. All cryosections were examined on a Leitz light microscope. Epi-illumination with a 50W mercury vapour lamp using a conventional (488 nm) fluorescein filter block simultaneously excites CMFDA and ethidium homodimer-1. Figure 6.1 shows an excised duodenal biopsy segment after 24 hours. The entire epithelial cell layer remains viable (green) and dead cell nuclei (red) are predominantly localised at the cut margins of the biopsy. The lamina propria remains un-stained due to the lack of dye penetration. To allow for dye penetration to this area of the biopsy, longer incubation times were required, which resulted in an exponential increase in cell death. Hence, since

incubation for 24 hours guaranteed viability of the epithelial cell layer, this time point was used for further experimentation.

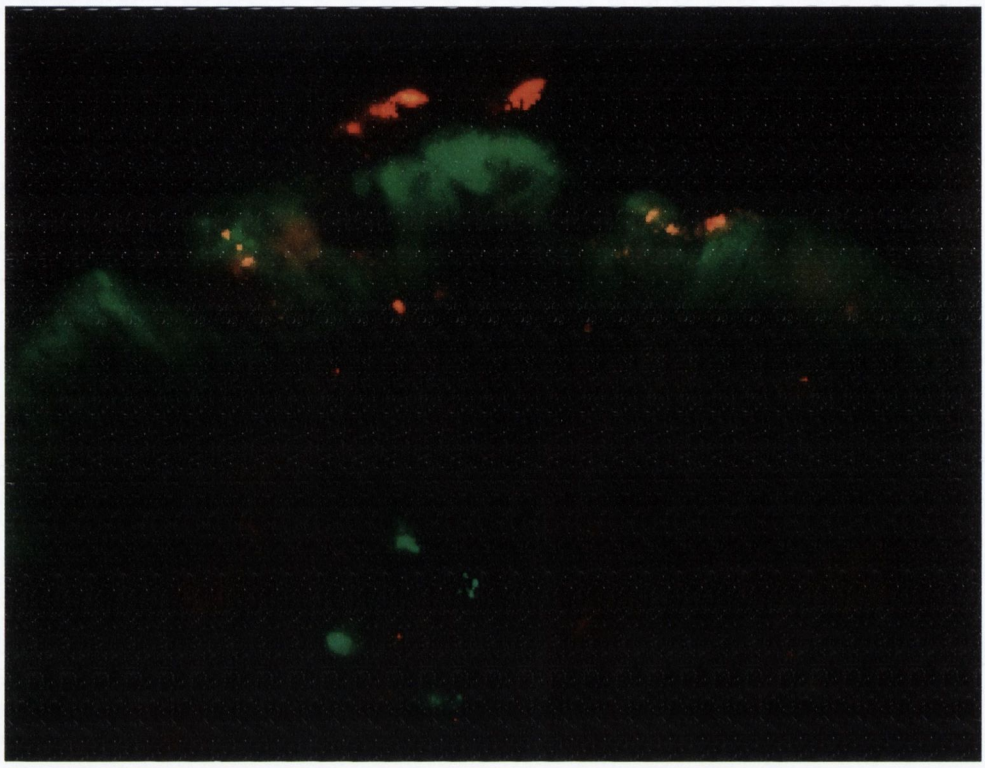


Figure 6.1 – Cryosection of duodenal explant stained with the fixable fluoroprobes CMFDA and ethidium homodimer-1

Biopsies were placed in fresh Autrup medium supplemented with 25 μM CMFDA and 25 μM ethidium homodimer-1 at 37 °C for 24 hours to achieve maximal dye penetration. Biopsies were then washed, immediately embedded in OCT and snap frozen in liquid nitrogen cooled isopentane. The samples were then sectioned on a -25 °C cryostat. All cryosections were examined on a Leitz light microscope (magnification – 100x). Green tissue indicates living tissue. Red tissue indicates dead cells.

6.3.3 Establishing an IVEC System for Biopsy Stimulation with OMV

Once the viability of the biopsies had been established for up to 24 hours post excision, the biopsies could then be stimulated with OMV. Upon removal to the laboratory, biopsies were immediately processed as described by Olfat et al., (2002). Basically, biopsies were transferred to an insert cup with a permeable membrane of 0.45 μm pore size with the mucosal side facing up. Auturp medium (see Material and Methods Chapter II) supplemented with 2 mg/L nalidixic acid and 4 ml/L DENT supplement, was added to the wells to allow contact with the bottom of the insert cup. Thus a capillary contact between the biopsies and the culture medium from below was established (Fig 2.4 Chapter II). Under these conditions biopsies can maintain a viable epithelium up to 7 days in culture (Autrup et al., 1978). For this study, biopsies were cultured *ex vivo* for a maximum of 24 hours.

6.3.4 Induction of Cytokine Expression in Duodenal Explants by *H. pylori* OMV

In the human gastric mucosa, *H. pylori* has the opportunity to access its natural binding receptors, such as the fucosylated blood group antigens (Boren et al., 1993). These allow the bacteria to adhere to gastric epithelial cells and trigger host inflammatory responses (Guruge et al., 1998). Clinical studies have demonstrated increased IL-8 concentration in gastric biopsy specimens obtained from *H. pylori* infected individuals (Rieder et al., 2001). For this study, one biopsy was used as a control and a second biopsy from the same donor was stimulated with 10 μg/ml OMV. After coincubation for 1 h at 37 °C, the OMV suspension was removed and the explants were washed with Autrup medium. The culture medium underneath the insert cup was replaced with fresh medium after each time point, and stored at –20 °C for subsequent IL-8 quantification. Increased IL-8 production was demonstrated in response to *H. pylori* OMV (Fig 6.2). A significant difference in IL-8 production between control and treated biopsies was seen as early as 12h post stimulation. Results are a mean representation of 4 individual experiments with biopsies from 4 separate donors.

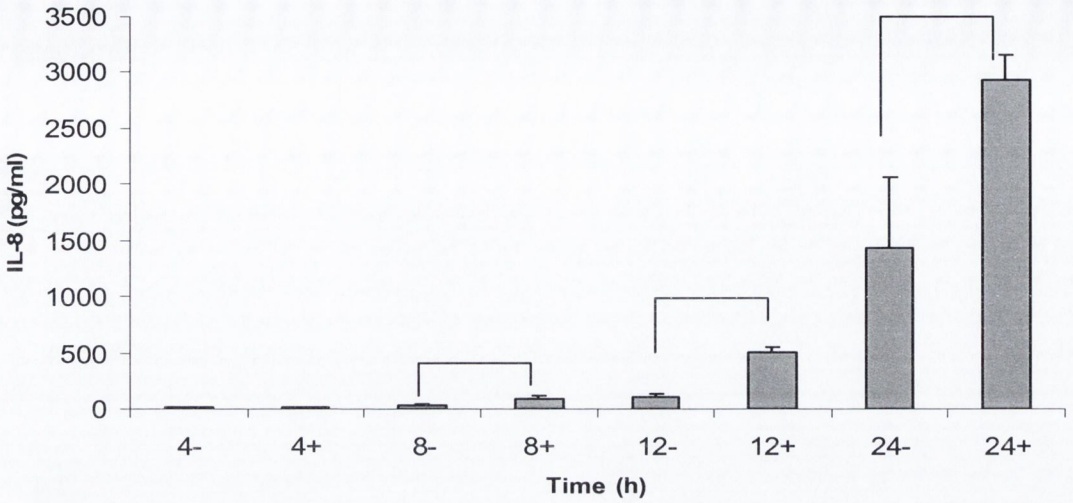


Figure 6.2 – IL-8 expression in duodenal explants stimulated with *H. pylori* OMV, from 4 to 24 hours.

Biopsies were stimulated with 10 μg/ml OMV. After coincubation for 1 h at 37 °C, the OMV suspension was removed and the explants were washed with Autrup medium. The culture medium underneath the insert cup was replaced with fresh medium after each time point, and stored at –20 °C for subsequent IL-8 quantification analyses. Increase of IL-8 production was demonstrated in response to *H. pylori* OMV. (+ OMV treated, - not OMV treated).

Increased RANTES expression is a feature of *H. pylori* induced gastric inflammation (Shimoyama et al., 1998; Yamaoka et al., 1998; Kikuchi et al., 2000; Park et al., 2001; Cottet et al., 2002). As it is produced by epithelial cells, CD8+ T cells, fibroblasts and platelets, the culture medium was assayed for RANTES production. No significant change in RANTES production was demonstrated in response to stimulating duodenal biopsies with *H. pylori* OMV in comparison with uninfected control biopsies (Fig 6.3).

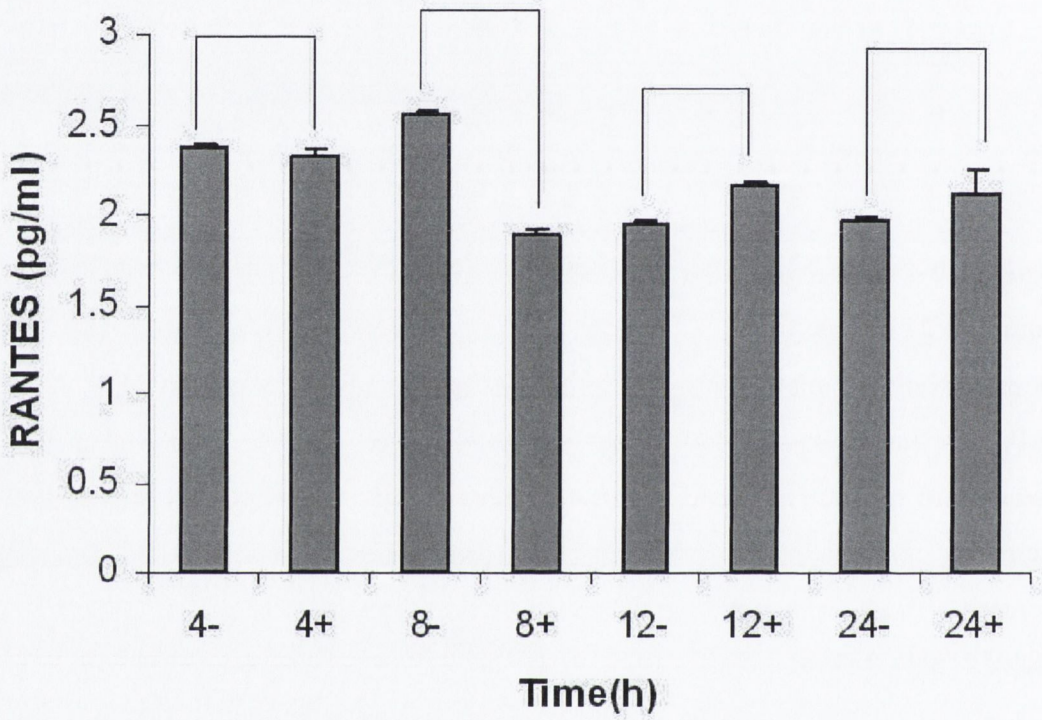


Figure 6.3 – RANTES expression in duodenal explants stimulated with *H. pylori* OMV, from 4 to 24 hours.

After coincubation for 1 h at 37 °C, the OMV suspension was removed and the explants were washed with Autrup medium. The culture medium underneath the insert cup was replaced with fresh medium after each time point, and stored at –20 °C for subsequent RANTES quantification analyses. RANTES production was not demonstrated by duodenal explants in response to *H. pylori* OMV.

6.3.5 Immunoblotting of *H. pylori* OMV Probed with Sera from *H. pylori* Infected and Uninfected Individuals

A biobank of sera from *H. pylori* infected and un-infected individuals was available. Recognition of OMV by sera from both positive and negative subjects was assessed by Western blotting. Figure 6.4 shows that sera from the infected patients recognize more of the OMV proteins than sera from the uninfected patients. Some of the negative controls recognize OMV proteins, but this could be due to a false negative or a patient who formerly tested positive for *H. pylori* but still has antibodies to the organism in their blood.

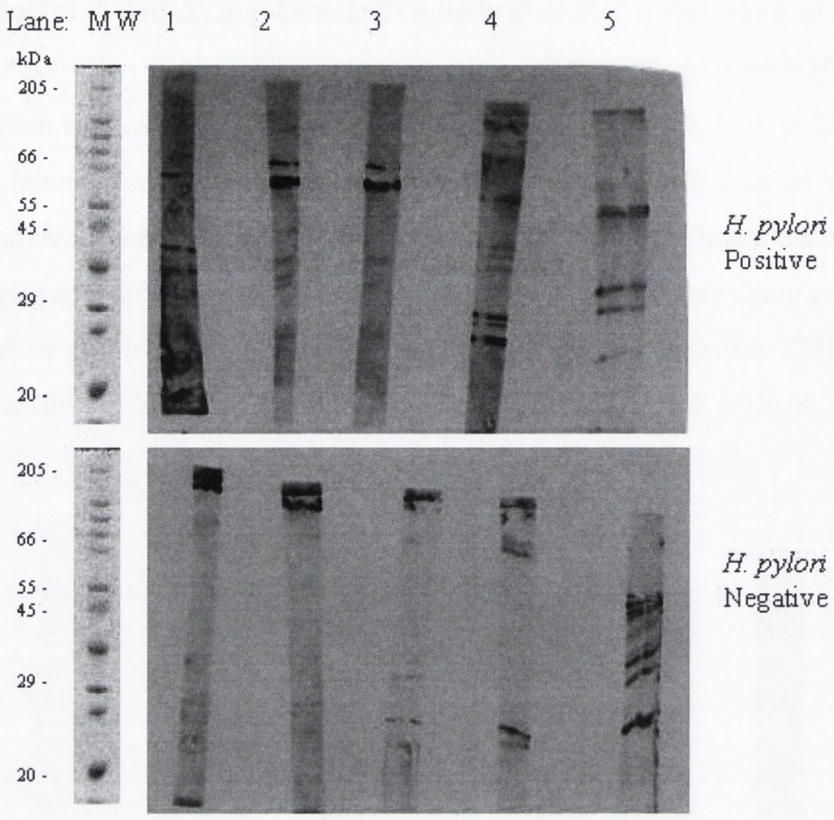


Figure 6.4 – Immunoblotting with human sera for recognition response to *H. pylori* OMV. Lane 1-5 represent different sera samples (5 positive, 5 negative).

Samples of OMV were electrophoretically separated on a 1D SDS-PAGE gel (6.5 x 9 cm), transferred to PVDF membrane and blocked with 5 % Marvel. The membrane was then cut into strips and each individual strip was then probed with one of the collected sera. The purpose of this was to determine if sera from *H. pylori* infected individuals could recognise OMV proteins, in comparison with sera from the uninfected control group. Sera from the infected patients recognize more of the OMV proteins than sera from the uninfected patients. Some of the negative controls recognize OMV proteins, indicated by one or two bands lighting up on the blots, but this could be due to a false negative or a patient who formerly tested positive for *H. pylori* but still has antibodies to the organism in their blood.

6.3.6 *H. pylori* OMV do not Stimulate IL-12 Production in Neutrophils or PBMCs

Amedei et al. (2006) showed that the *H. pylori* (NapA) was a TLR2 agonist capable of stimulating neutrophils to increase IL-12 expression, which acts as a key cytokine for the differentiation of naïve Th cells into the TH1 phenotype. They suggested that NapA could be used as a new tool for promoting Th1 immune responses (Amedei et al., 2006). Based on this, neutrophils and PBMCs were cultured in the presence of OMV (10μg/ml) or PBS as a control. A commercial ELISA was used to quantify the amount of IL-12p70 present in culture supernatants. Figure 6.5 shows there was no increase in IL-12 protein accumulation in neutrophil culture supernatants over time or a significant difference between baseline levels of IL-12 in control cells in comparison to cells cocultured with OMV. The same was true for experiments with the PBMCs (Fig 6.6).

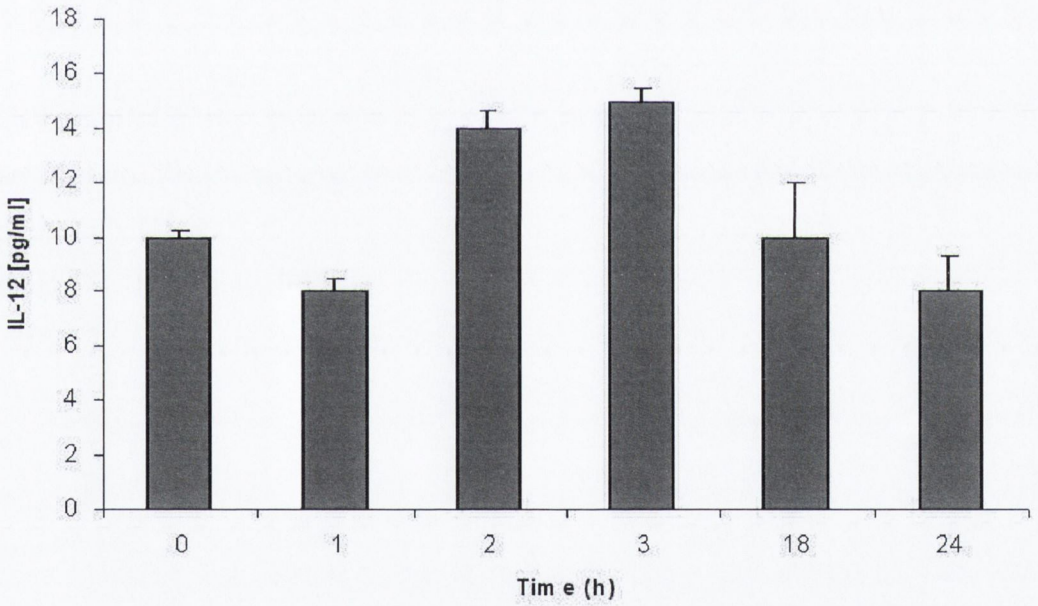


Figure 6.5 – IL-12 expression in human neutrophils in response to stimulation with *H. pylori* OMV. Neutrophils were cultured in the presence of 10μg/ml OMV or PBS as a control. A commercial ELISA quantified the amount of IL-12p70 present in culture supernatants. There was no increase of IL-12 protein accumulation in neutrophil culture supernatants over time or a significant difference between baseline levels of IL-12 in control cells in comparison to cells co-cultured with OMV.

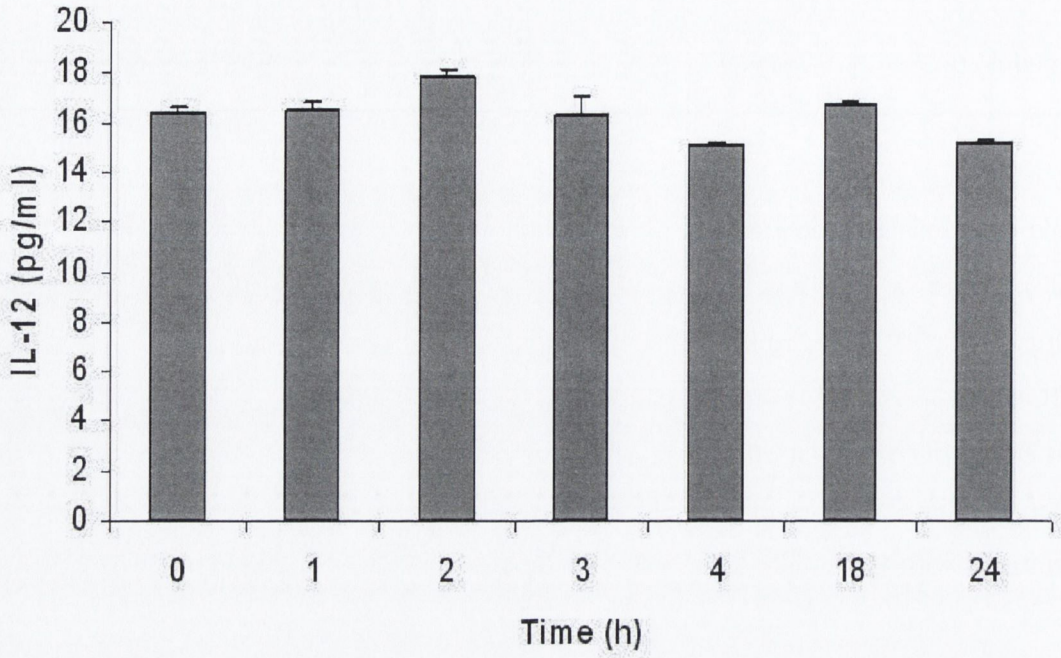


Figure 6.6 – IL-12 expression in human PBMCs in response to stimulation with *H. pylori* OMV. PBMCs were cultured in the presence of 10μg/ml OMV or PBS as a control. A commercial ELISA quantified the amount of IL-12p70 present in culture supernatants. There was no increase of IL-12 protein accumulation in PBMC culture supernatants over time or a significant difference between baseline levels of IL-12 in control cells in comparison to cells co-cultured with OMV.

6.4 DISCUSSION

In this study the effects of *H. pylori* OMV on duodenal explants were examined. As the first barrier against intraluminal insults, it is widely believed that the duodenal epithelium must have a relevant role in the sequence of events that lead eventually to ulceration. Epithelial responses in the proximal duodenum (most common place of ulceration) may occur against potentially secreted or released bacterial elements, like OMV, without the need for direct bacterial-epithelial contact (Olfat et al., 2002). This study has shown that duodenal explants can be loaded ex vivo with fluoroprobes, which simultaneously reveal viable and non-viable cells within an unstained extracellular matrix. In the experiment described, the red fluorescent signal produced by ethidium homodimer-1 clearly marked the nucleus, but not the cytoplasm, of non-viable cells, and was contrasted against the cytoplasmic distribution of the fluorescent green CMFDA in viable cells (Poole et al., 1996). This optimized protocol confirmed viability of the biopsies for up to 24 hours post excision.

As the natural niche for *H. pylori* is the acidic environment of the human stomach, developing an *in vitro* model to mimic these conditions has long been of interest (Olfat et al., 2002). Cell culture and animal models, although beneficial, do not mimic *in vivo* infection conditions exactly due to the specific tropism that *H. pylori* exhibits for the human gastric mucosa. The IVEC model used in this study represents an alternative and novel model for the study of *H. pylori* infection. Using this model, it was observed that IL-8 was maximally secreted between 12 – 24 h (Fig 6.4). In the IVEC model, background production of IL-8 was consistently detected in explants not treated with *H. pylori* OMV. However the levels of IL-8 produced in the biopsies stimulated with *H. pylori* OMV were significantly higher at only 12 hours post stimulation. The data clearly show that *H. pylori* OMV can elicit an inflammatory response in duodenal biopsy specimens, and that this response is not restricted to adherent *H. pylori* (Olfat et al., 2002). Little is known about the early duodenal inflammatory response to colonization of the stomach by *H. pylori*. These data show that an inflammatory response has a rapid onset ($\leq$12 hours). This data also demonstrates again, that *H. pylori* OMV can indeed act as virulence factors that equip *H. pylori* with a potent means of extending its pathogenic reach beyond that of the human stomach.

The lack of any RANTES production in the duodenal explants in response to stimulation with *H. pylori* OMV was not surprising. This is likely due to the fact that duodenal biopsy specimens contain many nonepithelial cells, including macrophages,

and RANTES induction patterns in these cells might be different from those in epithelial cells (Kudo et al., 2005).

Towards the end of this study, other outcomes of infection with *H. pylori* OMV were examined, in particular the immune response. The clinical course of infection with *H. pylori* is highly variable and is influenced by both microbial and host factors (Suerbaum and Michetti, 2002). Using human sera obtained from *H. pylori* infected and un-infected individuals, it was determined that sera from *H. pylori* infected individuals recognized more OMV proteins, in comparison with sera from uninfected individuals. This seems to strengthen the case that OMV are indeed immunogenic, capable of eliciting an immune response in the host. However, OMV are not capable of stimulating IL-12 production by neutrophils and PBMCs. This suggests that OMV alone are not potent enough to elicit the cells of the innate immune system to produce IL-12 and would be unable to promote the differentiation of naïve Th cells into the Th1 phenotype (Amedei et al., 2006).

In conclusion, although many cell lineages and animal models have been used to study *H. pylori* infection, alternative models of infection are currently being sought. The possibility of culturing human gastric mucosa in vitro is an interesting alternative to the methods and models currently available. The IVEC technique is a novel method by which to study the interactions of duodenal explants with *H. pylori* OMV and may become a useful model for studies of bacteria-host interactions. The data in this study has shown that although OMV can not act as immunomodulators of the host response to infection, they can induce a rapid inflammatory cytokine response in the explant tissue. The early duodenal response to *H. pylori* infection and stimulation by OMV warrants further study.

CHAPTER VII

GENERAL DISCUSSION

7.1 Background

The aim of this study was to determine the pathogenic potential of *H. pylori* OMV. To that end, *H. pylori* OMV were characterized both proteomically and functionally to gain a better insight to their actions *in vivo*. The effects of OMV in an *in vitro* model were examined by determining their effects on epithelial cell function. This led to determining the effects of OMV on components of the innate immune system, mainly Toll-like receptors. Finally, the study examined the effects of OMV on human duodenal explants in an *ex vivo* model of infection.

The discovery of *H. pylori* and its role in gastroduodenal diseases has been one of the most significant discoveries in the area of gastroenterology. *H. pylori* is now accepted as the cause of chronic atrophic (type B) gastritis and is associated with the development of duodenal ulcers in over 90 % of sufferers (Coghlan et al., 1987; Kuipers et al., 1995). Infection with this organism is also associated with an increased risk of gastric adenocarcinoma and is an important co-factor in the develoment of gastric cancer (Forman et al., 1991; Nomura et al., 1991; Forman et al., 1994; Forman, 1995; Webb and Forman, 1995). Eradication of *H. pylori* from infected individuals leads to the rapid disappearance of clinical symptoms, the rapid termination of neutrophil migration into the gastric mucosa and a reduction of chronic inflammation and ulceration (Moran, 1997).

The ability of gram negative bacteria to naturally produce extracellular outer membrane vesicles (OMV) is well established (Chatterjee and Das, 1967; Kadurugamuwa and Beveridge, 1995; Beveridge and Kadurugamuwa, 1996; Kadurugamuwa and Beveridge, 1996; Beveridge, 1999; Kadurugamuwa and Beveridge, 1999) but the function of this ubiquitous process is still being elucidated. In general, pathogenic bacteria produce more vesicles than their nonpathogenic counterparts (Lai et al., 1985; Wai et al., 1995). This is consistent with the theory that pathogenic bacteria have usurped the basic process of vesicle production to disseminate virulence factors and improve survival in the host (Kuehn and Kesty, 2005).

Vesiculating *H. pylori* and vesicles binding to gastric cells have been detected in gastric biopsies and tissue culture cells (Fiocca et al., 1999; Keenan et al., 2000a).

The presence of porins and VacA cytotoxin in these vesicles strongly suggests an offensive role for these structures in the pathogenesis of *H. pylori* infection (Keenan et al., 2000). Ismail et al., (2003) demonstrated that coincubating *H. pylori* OMV with AGS cells induced a significant increase in the expression of IL-8, a potent neutrophil

activating chemokine. It was also reported that *H. pylori* OMV are capable of bearing serologically recognizable Lewis antigens on LPS molecules which may contribute to the chronic immune stimulation of the host (Hynes et al., 2005). Furthermore, *H. pylori* OMV are able to induce apoptosis in AGS epithelial cells (Ayala et al., 2006). Due to their small size, adhesive properties and ability to carry virulence factors into host cells, OMV are likely to play a significant role in disseminating virulence factors for Gram-negative bacteria. They can also assist in carving out a niche in the environment by modulating interactions between neighbouring bacteria and between bacteria and the immune system (Kuehn and Kesty, 2005).

OMV are possibly involved in spreading the pathogenesis of *H. pylori* far beyond the reaches of the human stomach. Therefore, this study was concerned with further characterizing *H. pylori* OMV and investigating their role as pathogenic agents of the organism using a wide variety of techniques and tools. The schematic figure provides a summary of the work that was undertaken in this study.

SUMMARY OF WORK

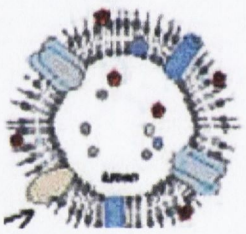
Proteomic Data
OMV known to contain:
VacA
NapA
OipA
CagA
OMPs

Biological Activity
Known to cause:
Vacuolation in gastric epithelial cells
PMN migration
IL-8 secretion

TLRs are members of the pattern recognition receptors and play a central role in the initiation of innate cellular immune responses

Toll-like Receptors
IL-8 expression known to be mediated by TLR2 and TLR4

The cytokine-inducing activity of whole *Helicobacter pylori* is known to be mediated by TLR4 and TLR2.



OMV capable of delivering virulence factors directly to the host tissues

***In vitro* Cell Based Model of Infection**
OMV known to become readily internalized
Cause vacuolation
Induce changes at the protein level of expression
Have profound effects on cytokine expression (e.g. IL-8 and VEGF)

Proteins known to be involved in tumourgenesis (e.g. **TPI, PDI, CKB**)

VEGF is overexpressed in cases *H. pylori* infection and in gastric cancer. VEGF is the most well characterized angiogenic factoran plays a pivotal role in tumor-associated microvascular angiogenesis.

***Ex vivo* Model of Infection**
OMV induce IL-8 secretion in duodenal biopsies as early as 12 hours post stimulation. OMV can indeed act as virulence factors that equip *H. pylori* with a potent means of extending its pathogenic reach beyond that of the human stomach

IL-8 expression is a known mediator in the inflammatory response and causes gastric damage

Immunogenicity of OMV
Sera from *H. pylori* infected individuals recognized OMV proteins, in comparison with sera from uninfected individuals. OMV are capable of eliciting an immune response

7.2 Proteomic and Functional Characterization of *H. pylori* OMV

The first step towards elucidating the potential role that OMV have in pathogenesis was the development of a reliable method of production, purification and initial characterization of these vesicles. This study reports the identity of the proteomic composition of *H. pylori* OMV and the functional relevance of some recognised virulence factors. OMV provide a protective environment for the transmission of virulence factors from bacteria into the host target cells both locally and away from the site of colonisation. They represent a potentially important vehicle for delivering antigens to either epithelial or non-epithelial gastric cells where they can exert their pathogenic potential (Ricci et al., 2005). In summary, this part of the study provides substantial support for the emerging hypothesis that OMV from *H. pylori* are indeed potential virulence factors that equip the organism with a potent means of extending its pathogenic reach beyond the gastric mucosa. To the best of my knowledge this is the first extensive proteomic characterization of *H. pylori* OMV. It also provides evidence that the virulence factors identified within the vesicles are biologically active, capable of inducing cytokine secretion and neutrophil migration independent of the intact organism.

7.3 Effects of OMV on Colonic Epithelial Cells: an *in vitro* model

Part of this study involved monitoring the effects of OMV on epithelial cells, in an *in vitro* model of infection. There are almost no data available on the effects of *H. pylori* OMV on epithelial cells. The colonic epithelial cell line T84 was used for this purpose, since these cells become polarised and differentiated when grown on a semi-permeable substrate, have functional tight junctions and represent a model 'tight' epithelium. This cell line was also used in previous studies and is a good *in vitro* model for the study of the physiological response to *H. pylori* (Terres et al., 1998a; Terres et al., 1998b; Terres et al., 2003). OMV were observed to be internalized within the cells by confocal laser microscopy. As no co-localisation between the OMV and endosomal structures was seen, this process of internalization appears to be independent of receptor mediated enodocytosis. This internalization of OMV within epithelial cells is an ideal method by which to deliver their virulent cargo directly to the host mucosa.

H. pylori elicits a range of host responses during infection including the activation of various signal transduction pathways, change in cell morphology, actin cytoskeletal rearrangements, apoptosis and cytokine induction (Backert et al., 2005a). Additionally,

work in this laboratory has shown that bacterial extracts from *H. pylori* (HPE) can alter the paracellular barrier function of the T84 cell line (Terres et al., 1998a). In this study the stimulation of T84 monolayers with *H. pylori* OMV had no significant effect on transepithelial electrical resistance (TER), did not induce changes in the expression of the tight junctional protein ZO-1 and did not induce dome formation. The HPE used as a positive control for these experiments was capable of inducing dramatic decreases in TER, a displacement of ZO-1 from cell-cell junctions and of inducing dome formation within 30 min of stimulation.

Furthermore, in this present study, 6 proteins were shown to be differentially expressed in T84 monolayers treated with OMV vs untreated samples when 2D electrophoresis was used for the analysis. Of these 10 proteins, 3 upregulated, (protein disulphide isomerase PDI, triosephosphate isomerase TPI and Galectin –3 carbohydrate recognition domain) and 2 downregulated host proteins (thioredoxin and creatine kinase B CKB) were characterized by mass spectrometry. It was found that 4 of these proteins had been previously reported to be differentially regulated by *H. pylori* infection in gastric epithelial cells. However, Western blotting with specific antibodies to the proteins failed to show a difference in expression between the control and treated lysates. This may be due to the fact that the 2-DE approach is more sensitive in detecting subtle changes in expression.

It was also seen that coincubation of *H. pylori* OMV and T84 cells induced a significant increase in IL-8 expression, apically and basally. It is hypothesized that cytokine release (especially IL-8) due to *H. pylori* infection and the subsequent influx of inflammatory cells causes a massive release of reactive oxygen species during the inflammatory reaction (Berg et al., 2006). To date, only AGS cells have been demonstrated to produce an increased amount of IL-8 when cocultured with *H. pylori* OMV (Ismail et al., 2003). The data presented in this thesis demonstrate that differentiated T84 monolayers behave similarly and secrete IL-8 both apically and basally. *H. pylori* OMV also stimulated VEGF and IL-4 secretion. As VEGF is an important modulator of gastric mucosal repair and is overexpressed in gastric cancer this is an interesting observation. VEGF is the most well characterized angiogenic factor (Peek and Blaser, 2002), is a potent stimulator of neo-angiogenesis in response to mucosal injury in the GI tract (Jones et al., 1994) and plays a major role in tumour-associated microvascular angiogenesis (Conolly et al., 1989). To date there is no data

available on the ability of OMV to induce VEGF expression. It has been reported that *H. pylori* broth culture filtrate (BCF) up-regulates VEGF expression in gastric epithelial cells and this effect is specifically related to VacA toxin and seems to depend on the activation of an EGFR, MAP kinase and COX-2 mediated pathway (Caputo et al., 2003). The MKN-28 cells expressed VEGF at levels comparable to those of the T84 cells co-incubated with OMV (approximately 75 pg/ml). This could potentially be a very interesting observation if OMV are capable of inducing similar concentrations of VEGF as whole bacteria.

OMV were also shown to induce IL-4 secretion in T84 monolayers (approximately 30pg/ml). IL-4 has many biological roles, including the stimulation of activated B-cell and T-cell proliferation, and the differentiation of CD4+ T cells into Th2 cells. It is a key regulator in humoral and adaptive immunity. IL-4 induces B-cell class switching to IgE, and up-regulates MHC class II production. There is no data currently available on the effects of *H. pylori* OMV on IL-4 expression in epithelial cells. However, Mirlashari et al., 2007 studied the ability of OMV from *Neisseria meningitidis* to induce secretion of pro-inflammatory cytokines, including IL-4, in a human whole blood model. OMV did not induce release of IL-4 in doses from 0.001–10 μg/ml. It has also been reported that IL-4 showed no significant increase in patients with chronic gastritis or duodenal ulcer in comparison to patients diagnosed as *H. pylori* negative (Serrano et al., 2007). The control levels of IL-4 are comparable to those seen in this study. This suggests that the *H. pylori* OMV induced IL-4 is not physiologically relevant.

In conclusion, this part of the study found that OMV, which are constantly shed from the surface of *H. pylori in vivo,* have a measurable effect on the mature monolayers of T84 cells. These findings support the theory that OMV play a role in promoting host responses in *H. pylori* associated disease.

7.4 The Role of Toll-like Receptors (TLRs) in the Epithelial Cell Response to OMV Stimulation

Innate immunity is considered to be important for the elimination of invading pathogens. Mammalian TLRs are members of the pattern-recognition receptor (PRR) family and play a central role in the inniation of innate cellular immune response to microbes. As a significant part of the work in this study involved examining the effects of OMV on epithelial cells, it was decided to determine which if any TLRs mediated epithelial cell response to OMV stimulation. It has not been reported to date as to which TLRs are involved in the epithelial cell recognition of *H. pylori* OMV. OMP present in

vesicles are all biologically active molecules that can activate immune cells via TLRs and induce neutrophil migration (Galdiero et al., 1999, Akira et al., 2001). This study has demonstrated that co-incubation of epithelial cells with *H. pylori* OMV induced IL-8 cytokine responses via TLR2 and via TLR4 to a much lesser degree at the message level and very comparable amounts of IL-8 at the protein level. Due to the clinical significance of *H. pylori* infection, the interaction between TLR and *H. pylori* is one of the most extensively studied. As the first step in *H. pylori* infection is the adherence to gastric epithelial cells by the bacterium, it is logical to postulate that TLRs would play a significant role in detection of the bacterium, as well as the subsequent cellular and inflammatory response (Harris et al., 2006). These findings further elucidate the effects that *H. pylori* and its secreted components have on the innate immune system and could provide a potential strategy to modulate OMV function by interfering with innate signalling.

7.5 Immune and Duodenal Explant Responses to *H. pylori* OMV

In the final phase of this study the effects of *H. pylori* OMV on duodenal explants were examined. An ex vivo model of ulcerogenesis, using duodenal explants would provide the most realistic model of the actions of OMV during *H. pylori* infection. The IVEC model used in this study represents an alternative and novel model for the study of *H. pylori* infection. Using this model, it was observed that OMV induced IL-8 secretion 12 – 24 h after exposure to OMV. Despite the high levels of background production of IL-8 in untreated explants, the levels of IL-8 produced in the biopsies stimulated with *H. pylori* OMV were significantly higher at only 12 hours post stimulation. This study has clearly shown that *H. pylori* OMV can elicit an inflammatory response in duodenal biopsy specimens, and that this response is not restricted to adherent *H. pylori* (Olfat et al., 2002). These data show that an inflammatory response has a rapid onset ($\leq$8 hours) and that *H. pylori* OMV can indeed act as virulence factors that equip *H. pylori* with a potent means of extending its pathogenic reach beyond that of the human stomach.

In conclusion, the possibility of culturing human gastric mucosa *in vitro* is an interesting alternative to the methods and models currently available. The IVEC technique is a novel method by which to study the interactions of duodenal explants with *H. pylori* OMV and may become a useful model for studies of bacteria-host interactions.

7.6 Future Work

The present study contains many interesting results and highlights the future lines of investigation that may be followed. Secreted components of *H. pylori* are known to mediate multiple pathogen host interactions during infection (Bumann et al., 2001) and in this context it is reasonable to consider OMV as a complex group of secreted components, which exert more profound and possibly synergistic effects on host tissue. This study represents the first extensive identification of the proteome of *H. pylori* OMV. Functional studies can now be carried out on proteins with potential pathogenic functions identified within the proteome of the OMV to determine their biological activity and possibly assess their usefulness as therapeutic targets and/or vaccine candidates.

The need for a vaccine against *H. pylori* makes the characterization of all the antigenic components of OMV an important future study. *H. pylori* strains resistant to antibiotics commonly used as treatment are emerging (Koletzko et al., 2006). Therefore, future priorities for vaccine development against *H. pylori* infection possibly should include a combination of strong protective antigens, similar to those found within the OMV (e.g. NapA). Other candidate vaccine antigens for a subunit preparation should likely contain virulence factors known to be present in OMV also. As OMV contain virulence factors that operate independedntly of the intact organism, it would be important that they be represented in a future subunit vaccine in the hope that the proinflammatory activity of the OMV could be neutralized.

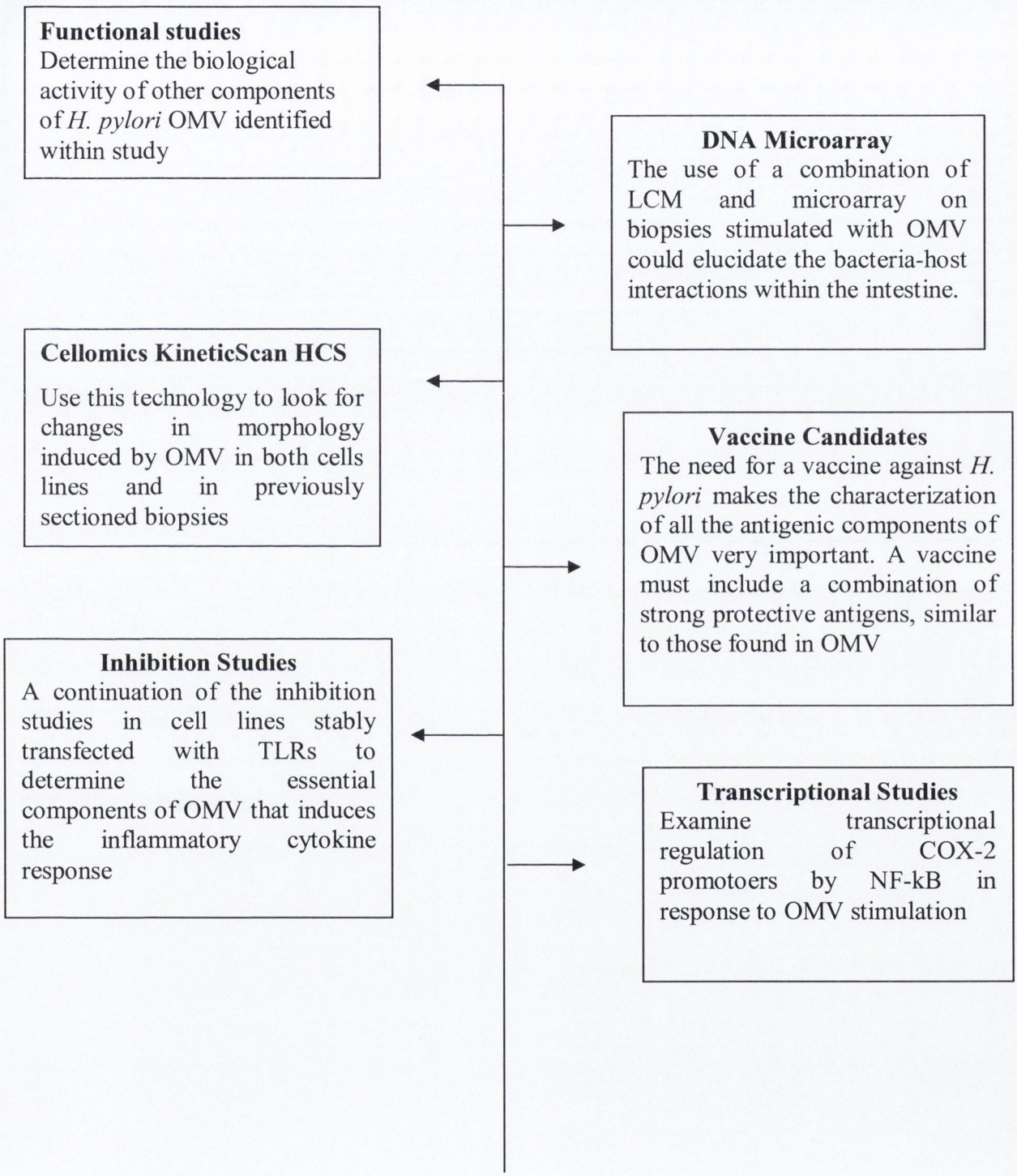
The findings of the *in vitro* model of this study support the theory that OMV play a role in promoting host responses to support persistence of *H. pylori* in the human gastric mucosa. Further work in this area, may yet determine what combination of factors within the OMV is capable of driving the inflammatory cytokine response. An array of new treatment options for *H. pylori* infection could involve tissue-specific suppression of TLR signalling- pathways by either chemical means and introduction of natural TLR suppressors and antagonists. Further exploration in this area of negative regulatory mechanisms may be fruitful for the development of more effective treatments for *H. pylori* infection.

The use of High Content Screening assays (Cellomics) was a novel approach used to assess the effects of OMV on tight junctions in the T84 model of infection. The KineticScan could be very advantageous in determining changes in cell morphology in response to OMV stimulation. *H. pylori* is known to elicit effects on cell morphology

such as actin cytoskeletal rearrangement (Su et al., 2003). Although no effects on tight junctions in response to co-incubation of T84 cells with OMV were seen in this present study, perhaps a longer time course and a dose response could be used to look at the effects, if any, on the actin cytoskeleton.Other avenues of investigation that can be carried out using the Cellomics HitKits® HCS reagent kits might involve examining transcriptional regulation of COX-2 promotoers by NF-kB, looking at signalling events, activation of transcription factors and cell mobility in response to OMV stimulation.

This study has provided some information on the early duodenal response to *H. pylori* infection and stimulation by OMV warrants further study. The acute response of the duodenum upon infection is poorly understood. This is probably due to the fact that patients only relate symptoms after the duodenal ulcers have already formed. In future studies, the use of laser capture microdissection (LCM) could specifically capture cells of interest from treated and control biopsy specimens. By this method the exact morphologies of both the captured cells and the surrounding tissue can be preserved. This accurate technique is compatible with subsequent analysis of protein and nucleic acids. Captured cells could then be used for the molecular analysis of the host-microbial relationship within the intestine by using DNA microarrays and also to determine the differential proteomic profiles by using ProteinChip technology. The DNA microarray results can be validated by quantitative real-time PCR of reverse transcribed material from both LCM derived epithelial cell populations and the original biopsy samples. Where respective commercial antibodies are available, a selection of the identified differentially expressed genes will be monitored at the translational level by Western blot anlaysis. The ProteinChip results can be validated by mass spectrometry. This approach could be very informative about the effects of OMV on duodenal explants, independent of the intact organism itself. However, due to the complex nature of the biopsy work it would be appropriate to conduct preliminary experiments involving the coincubation of OMV and T84 or AGS cell lines and carrying out a microarray to determine what is occurring at the gene expression level. Such a strategy will definitively answer the questions regarding the role(s) of OMV in *H. pylori* infection and the host immune response to the organism.

SUMMARY OF FUTURE WORK



7.7 Concluding Remarks

Gastric colonisation with *H. pylori* currently affects at least half the world's population. The prevalence of *H. pylori* infection is higher in developing countries (about 80 %) than in the industrialised world (20 – 50 %), reflecting the influence of socioeconomic factors. *H. pylori* infection is the major cause of gastric adenocarcinoma (Pritchard and Crabtree, 2006). There are currently no predictors for gastric cancer, which remains a major killer and has been projected to be the eight most common cause of death of any type in the world by 2063 (Murray and Lopez, 1997a; Murray and Lopez, 1997b). This study provides substantial support for the emerging hypothesis that OMV from *H. pylori* are indeed potential virulence factors that equip the organism with a potent means of extending its pathogenic reach beyond the gastric mucosa. This study has highlighted the profound affects that OMV can elicit *in vitro* and *ex vivo*, independently of the intact organism. OMV have been shown to induce cytokine release and neutrophil migration. One of the most important findings of this study is that OMV induces IL-8 cytokine responses primarily via TLR2, although TLR4 is also involved to a much lesser degree. The proteomic characterization of the OMV components in this study could also contribute to the ongoing search for a suitable vaccine candidate for *H. pylori* infection. The OMV putatively function in dissemination of virulence factors from the organism to the host and may have a role in immune evasion of the bacterium. It is clear, therefore, that these proteins represent good targets for development of a vaccine against *H. pylori*. In the future they may form part of a composite vaccine with the toxins or with other as yet uncharacterised antigens of *H. pylori*.

APPENDICES

Appendix A

Reagents and Buffers

Cell Culture Media

Complete RPMI-1640 Medium

RPMI 1640

Heat inactivated Foetal Calf Serum (FCS)

Penicillin/streptomycin/L-glutamine Culture Cocktail

(Gives a final concentration of 2 mM L-glutamine,

100 μg/ml penicillin and 100 U/ml streptomycin)

Hanks Balanced Salts Solution

HBSS

N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid,

(HEPES) (1 M)

Dulbeccos Hams F12 Medium

Dulbeccos Modified Eagles Medium

Heat inactivated Foetal Calf Serum (FCS)

Penicillin/streptomycin/L-glutamine Culture Cocktail

(Gives a final concentration of 2 mM L-glutamine,

100 μg/ml penicillin and 100 U/ml streptomycin)

Hams F12 Medium

Heat inactivated Foetal Calf Serum (FCS)

Penicillin/streptomycin/L-glutamine Culture Cocktail

(Gives a final concentration of 2 mM L-glutamine,

100 μg/ml penicillin and 100 U/ml streptomycin)

(An equal 50:50 ratio of these two complete media were used)

L-15 Leibovitz Medium

L-15

Penicillin/streptomycin/L-glutamine Culture Cocktail

(Gives a final concentration of 2 mM L-glutamine,

100 μg/ml penicillin and 100 U/ml streptomycin)

Autrup Medium

CMRL 1066 Media (10X)

Sterile Endotoxin free water

Glucose

Tricine

Hydrocortuisone Hemisuccinate

β-Retinyl acetate

Glutamine

Bovine Serum Albumin

DMSO

Nalidixic Acid

Penicillin/streptomycin/L-glutamine Culture Cocktail

(Gives a final concentration of 2 mM L-glutamine,

100 μg/ml penicillin and 100 U/ml streptomycin)

Cell Culture Cryopreservative Medium

FCS

Dimethylsulfoxide

10X Phosphate Buffered Saline (PBS)

$Na_2HPO_4.2H_2O$ (8 mM)

KH_2PO_4 (1.5 mM)

NaCl (137 mM)

KCl (2.7 mM)

Make up to 1 L final volume. Dilute to 1X PBS

and pH to 7.4

1X Phosphate Buffered Saline and 1mM EDTA

100mM EDTA

PBS

25X Protease Inhibitor Cocktail

One tablet dissolved in 2ml distilled water.

Store 200µl Aliquots in -20°C.

RIPA cell lysis buffer:

1 % v/v NP40 in 10 mM Tris-HCl (pH 7.3) containing 1 % w/v sodium deoxycholate, 0.1 % w/v SDS, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF and 10 μg/ml leupeptin.

Preparation of activated sodium orthovanadate (Na_3VO_4):

A 100 mM solution of sodium orthovanadate was prepared in distilled water. The pH was adjusted to 10.0 to give a yellow colour. The solution was boiled until it turned colourless and allowed to cool to room temperature. The pH was readjusted to 10.0 and the sample was boiled and allowed to cool (this step was repeated a number of times) until the solution remained colourless and the pH stabilised at 10.0. The solution was aliquoted and stored at –20°C.

Preparation of pervanadate:

A 1/88 dilution of 30 % H_2O_2 was prepared in sterile PBS. An equal volume of this solution was added to an equal volume of 100 mM Na_3VO_4. The solution was mixed well and a 1/10 dilution of this solution was added to the cells (which were resuspended at a concentration of 50 x 10^6 cells per ml in a sterile eppendorf) for 10 minutes at 37°C.

H. pylori Culture Media

NOTE: Sterilise all media by autoclaving

Brain Heart Infusion (BHI) Medium

BHI powder

Distilled water

FCS

DENT supplement

Protein Free Brain Heart Infusion (BHI)/1% β-Cyclodextran

β-Cyclodextran

BHI media

Brain Heart Infusion (BHI) Agar

BHI powder

Agar

Distilled water

Columbia Blood Agar *H. pylori*

Columbia agar base

Sterile horse blood

Distilled water

Boil Columbia agar base in the water to dissolve,

cool to 50°C and add horse blood with swirling.

Brucella Broth

Brucella broth

Distilled water

Urease Test

5mM Tris pH 6.8

4M Urea

Phenol red

Urease

Polyacrylamide Gel Electrophoresis (PAGE) Reagents

2X Reducing Sample Buffer

Tris HCl 1.0 M pH 6.8

Glycerol

Sodium dodecyl sulphate (SDS) 10%

β-mercaptoethanol

Bromophenol Blue (0.1 %)

Deionised water

Stacking Gel Buffer (1.0 M Tris HCl pH 6.8)

Tris base

Deionised water

Adjust to pH 6.8 with concentrated HCl and make

up to final volume.

Resolving Gel Buffer (1.5 M Tris HCl pH 8.8)

Tris base

Deionised water

Adjust to pH 8.8 with concentrated HCl and make

up to final volume.

10% Ammonium Persulphate (APS)

APS

Deionised water

Make up fresh before use.

Electran 2.6 Acrylamide/Bisacrylamide Premix

Acrylamide

Bisacrylamide

Deionised water to final volume

Water-Saturated Butanol

Butanol

Deionised water

Mix well and allow settle. Use top layer.

10X SDS-PAGE Running Buffer

Tris base

Glycine

SDS

Dissolve in deionised water to a final volume of 1 L

and dilute 1:10 in deionised water before use.

2-DE Lysis Buffer

Urea

Thiourea

CHAPS

Tris Base

Dissolve in deionised water to a final volume of 10ml. Store in 500µl aliquots

At –20°C

2-DE Equilibration Buffer

Tris HCl pH 8.8

Urea

Glycerol (v/v)

SDS (w/v)

Bromophenol Blue

Dissolve in deionised water to a final volume of 200ml. Store in 10 ml aliquots

At –20°C

2-DE Rehydration Buffer (Sample Loading Buffer)

Urea

CHAPS

Bromophenol Blue

Dissolve in deionised water to a final volume of 10ml. Store in 500µl aliquots

At –20°C.

Coomassie Blue Gel Stain

Coomassie Brilliant Blue R-250

Methanol

Glacial Acetic Acid

Deionised water

Dissolve the Coomassie Blue in the methanol, add the

water and finally add the acetic acid. Filter through a

Whatman # 1 filter and store in a lightproof container.

De-stain Solution

Methanol

Glacial Acetic Acid

Distilled water

Coomassie stain G-250 Fixation Solvent (for mass spectrometry analysis):

Methanol

Phosphoric Acid

Distilled water

Coomassie stain G-250 Incubation Solvent (for mass spec analysis):

Methanol

Phosphoric Acid

17% w/v ammonium sulphate

Distilled water

Western Blotting Reagents

Transfer Buffer

Tris base

Glycine

SDS

Dissolve in 350 ml deionised water, add 100 ml of

methanol and make up to final volume of 500 ml.

Blocking Solution

Skimmed dried milk powder (Marvel)

PBS

Polyoxyethylenesorbitan monolaureate (Tween 20®) 100 µl

Make up fresh before use.

PBS/0.1% Tween Washing Solution

PBS

Tween 20®

Primary and Secondary Antibody Diluent Solution

Skimmed dried milk powder (Marvel)

PBS

Immunoassay Buffers and Reagents

0.1 M Carbonate Buffer

Soln. A: Na_2CO_3 (0.1 M)

Soln. B: NaH CO_3 (0.1 M)

Take 70 ml of soln. A and add soln. B until pH is at 9.6 (approximately 190 ml of soln. B).

0.05 M Carbonate Buffer

Soln. A: Na_2CO_3 (0.05 M)

Soln. B: NaH CO_3 (0.05 M)

Take 70 ml of soln. A and add soln. B until pH is at 9.6 (approximately 190 ml of soln. B).

Immunoassay Blocking Buffer

Skimmed dried milk powder (Marvel)

PBS

PBS, pH 7.0

NaCl

KCl

$Na_2HPO_4.2H_2O$

KH_2PO_4 (anhydrous)

Dissolve in 9.5 L deionised water, adjust pH to 7.0 if necessary, and complete to 10 L. Sterilise by autoclaving.

Assay Diluent (R&D Systems)

PBS, pH 7.2-7.4/0.05% Tween

Delipidised Bovine Serum

PBS/0.05% Tween Washing Solution

PBS

Tween 20

Tris Buffered Saline (TBS) pH 7.6

Tris base

NaCl

Dissolve in 950 ml deionised water, adjust pH to 7.6

if necessary, and complete to 1 L. Sterilise by autoclaving.

TBS, pH 7.6/0.05% Tween

TBS, pH 7.6

Tween 20®

2 N H_2SO_4

H_2SO_4 (36 N)

Deionised water

2.5 N H_2SO_4

H_2SO_4 (36 N)

Deionised water

1 M HCl

Concentrated HCl (12.1 N)

Deionised water

3 M NaOH

NaOH

Dissolve in 90 ml deionised water and complete to 100 ml.

1.2 M NaOH/0.5 M HEPES

NaOH

HEPES

Dissolve in 90 ml deionised water and complete to 100 ml.

Horseradish Peroxidase Substrate

1,2-phenylenediamine dihydrochloride (OPD, 2 HCl)

1 X 2 mg tablet

Dissolve in 5 ml of deionised water and add 5 µl of 30% H_2O_2.

Alkaline Phosphatase Substrate

p-nitrophenyl phosphate

Dissolve in 5 ml of 1 M diethanolamine buffer,

pH 9.8, containing 0.5 mM $MgCl_2$.

Capture antibody:

A 720 µg/ml solution of mouse anti-human IL-8 was prepared, aliquoted and stored at –20°C. This stock solution was diluted to a final concentration of 4 µg/ml in PBS immediately prior to use.

Detection antibody:

An 3.6 µg/ml stock solution of biotinylated goat anti-human IL-8 was prepared in Reagent Diluent and aliquoted and stored at –20°C. This solution was diluted to a working concentration of 20 ng/ml in Reagent Diluent immediately prior to use.

IL-8 standard:

A 100 ng/ml stock solution of recombinant IL-8 was prepared in distilled water and aliquoted and stored at –20°C. A seven point standard curve of this recombinant solution (using 2-fold serial dilutions in Reagent Diluent (0 pg/ml – 1000 pg/ml of recombinant IL-8)) was prepared prior to use.

Immunstaining Buffers and Reagents

Immunostaining blocking buffer:

(a)

Skimmed dried milk powder (Marvel)

PBS

(b)

BSA

PBS

Staining wash solution:

0.05 % v/v Tween-20

0.02 v/v % Triton X-100

PBS

Antibody dilution buffer:

(a)

3 % w/v BSA

PBS

(b)

Skimmed dried milk powder (Marvel)

PBS

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