LEABHARLANN CHOLÁISTE NA TRÍONÓIDE, BAILE ÁTHA CLIATH | TRINITY COLLEGE LIBRARY DUBLIN
Ollscoil Átha Cliath | The University of Dublin

Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Regulation of Expression of Transcription Factors Nuclear Factor-kappa B and Early Growth Response-1 by *Helicobacter pylori*

A Thesis submitted for the degree of

Doctor of Philosophy (Ph. D.)

By

Mohamed Mahmoud Mohamed Abdel-Latif
BSc. (Pharm.), MSc. (Pharm.)

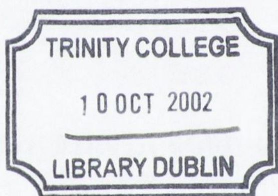
at

University of Dublin, Trinity College
2002

Supervisors

Professor Dermot Kelleher
Department of Clinical Medicine,
Trinity Centre for Health sciences,
St. James's Hospital,
Trinity College
University of Dublin

Professor Kamal Sabra
Department of Clinical
Pharmacology and Therapeutics,
St. James's Hospital,
Trinity College
University of Dublin


TRINITY COLLEGE
10 OCT 2002
LIBRARY DUBLIN
Thesis
6920

DECLARATION

I hereby declare that this thesis has not previously been submitted for a degree at this or any other University and that all work described in this thesis is entirely my own work. I hereby give my permission that this thesis can be borrowed from the library of Trinity College Dublin for the purpose of study.

Mohamed Abdel-Latif

Mohamed M. M. Abdel-Latif

SUMMARY

The Gram-negative bacterium *Helicobacter pylori* colonizes the human gastric epithelium and is a causative agent of peptic ulcer disease and gastric adenocarcinoma. Although *H. pylori* colonization is associated with gastric inflammation, the pathogenic mechanisms remain poorly understood. *H. pylori* initiates an inflammatory and immune reaction within the gastric mucosa which subsequently leads to tissue injury. The induction of the host immune response elicits the production of many pro-inflammatory proteins such as cytokines, chemokines and adhesion molecules that are regulated at the transcriptional level. The results of this study provide a further understanding of the possible pathogenic mechanisms of *H. pylori* infection through initiation of the expression of the transcription factors nuclear factor-kappa B (NF-κB) and the early growth response factor-1 (Egr-1).

Firstly, the exposure of the gastric epithelial cells AGS to *H. pylori* induced NF-κB expression, and this induction requires the direct contact between the gastric epithelial cells and live *H. pylori*, as heat-killed *H. pylori* and *H. pylori* lipopolysaccharide were unable to activate this transcription factor. Interestingly, *in vitro* infection of AGS cells with *H. pylori* strains expressing $CagA^{+}$ and $VacA^{+}$ resulted in NF-κB activation whereas $CagA^{-}$ and $VacA^{-}$ strains do not induce NF-κB DNA-binding. The genotypic and phenotypic differences in clinically acquired *H. pylori* infections may explain in part the variation seen in disease severity. Furthermore, incubation of AGS cells in media of low pH induced the activation of NF-κB and a synergistic effect was seen when the cells were exposed to the combined effect of low pH and *H. pylori* compared to either *H. pylori* or the acidic environment alone.

Secondly, the activation of NF-κB by *H. pylori* was blocked by pretreatment of AGS cells with the antioxidants vitamin C, N-acetylcysteine and thioredoxin. Moreover, these antioxidants inhibited the induction of NF-κB in response to low pH and cytokines like IL-1β and TNF-α and the mitogen phorbol 12-myristate 13-acetate (PMA). These observations indicate a role for reactive oxygen intermediates in *H. pylori*-induced pathogenesis.

Thirdly, *H. pylori* induces the expression of the early growth response-1 (Egr-1)

transcription factor in a dose- and time-dependent manner. Egr-1 is known to be involved in inflammatory and immune responses. Similarly, low pH, cytokines (IL-1β and TNF-α) and the mitogen PMA induced Egr-1 in AGS cells to a level comparable to that seen with *H. pylori*. Importantly, immunohistochemical staining of antral gastric biopsies of patients infected with *H. pylori* indicated increased levels of activated Egr-1 in *H. pylori* infected patients compared to normal individuals. The DNA-binding activity of Egr-1 is regulated by redox, because the activation of Egr-1 expression by *H. pylori* and other antigens was inhibited by antioxidant treatment. This study demonstrates for the first time the induction of Egr-1 expression in gastric epithelial cells in response to *H. pylori* infection. These findings indicate that Egr-1 activation by *H. pylori* may have a key role in the pathogenesis of gastric inflammation.
Finally, the functional significance of Egr-1 expression in regulating the expression of genes involved in inflammation in response to *H. pylori* infection was examined using transient transfection with promoter reporter gene constructs for CD44 and ICAM-1. These studies provided additional support for the role of Egr-1 in regulating the expression of CD44 and ICAM-1, as deletion mutations of the Egr-1 binding site from the CD44 and ICAM-1 CAT reporter constructs significantly abrogated transcriptional activation of these genes. Furthermore, expression of the CD95L (APO-1/Fas) ligand in AGS cells is dependent on the presence of Egr-1 binding sites, whereas using deletion Egr-1 promoter reporter constructs of CD95L abolished the full function of CD95L promoter. Also antisense oligonucleotide directed against Egr-1 blocks *H. pylori*-induced Egr-1 and CD44 expression, whereas the respective control vector had no significant effect on Egr-1 protein levels. These results further indicate a direct role for Egr-1 in the regulation of the expression of genes involved in *H. pylori*-induced inflammation such as CD44.

In conclusion, *H. pylori* activates both NF-κB and Egr-1 in gastric epithelial cells *in vivo* and *in vitro*. The induction of these transcription factors by *H. pylori* and their pathological significance is considered.

Nothing lies beyond the reach of prayer except that which lies outside the will of Almighty Allah.

"They are ill discoveries who think that there is no land when they see nothing but water".

Francis Bacon (1561-1626)

There is always something fascinating about science. Some of the nature's guarded secrets were unrevealed through scientific investigation. Late in the 20th century, scientific curiosity led to the discovery of the human gastric pathogen, *Helicobacter pylori*.

DEDICATION

To my parents

TABLE OF CONTENTS

Table of Contents Page

PREFACE
Title I
Declaration II
Summary III
Dedication VI
Table of Contents VII
Acknowledgements XII
Abbreviations XIV

CHAPTER I- GENERAL INTRODUCTION
1.1 *HELICOBACTER PYLORI* 1
1.1.1 Historic background 1
1.1.2 Nomenclature 2
1.1.3 Microbiological features 2
1.1.4 Colonization of gastric epithelium by *H. pylori* 3
1.1.5 Epidemiology of *H. pylori* infection 4
1.1.6 Mode of transmission 4

1.2 *HELICOBACTER PYLORI* AND GASTRODUODENAL DISEASES 5
1.2.1 *H. pylori* and gastritis 6
1.2.2 *H. pylori* and peptic ulcer disease 6
1.2.3 *H. pylori* and non-ulcer dyspepsia (NUD) 8
1.2.4 *H. pylori* and gastric cancer 9

1.3 PATHOGENIC MECHANISMS OF *H. PYLORI* INFECTION 10
1.3.1 Motility 10
1.3.2 Bacterial enzymatic activity 10
1.3.3 Adhesins 12
1.3.4 *Helicobacter pylori* lipopolysaccharides 12
1.3.5 Heat shock proteins 13
1.3.6 Cytotoxin-associated gene A (*cagA*) and the vaculoating cytotoxin A (*vacA*) 13
1.3.6.1 The *cag* pathogenicity island of *Helicobacter pylori* 14
1.3.6.2 Cytotoxin production and disease outcome 15

1.4 HOST IMMUNE RESPONSE TO *H. PYLORI* 16
1.4.1 Humoral immune response to *H. pylori* 16
1.4.2 Cellular immune response to *H. pylori* 17

1.5 EFFECT OF *H. PYLORI* ON GASTRIC PATHOPHYSIOLOGY 20

1.6 EPITHELIAL RESPONSE TO *H. PYLORI* INFECTION 20
1.6.1 Gastric epithelial cell proliferation 20
1.6.2 Apoptosis 21
1.6.3 Generation of reactive oxygen species by *H. pylori* 22
1.6.4 Cell adhesion molecule expression due to *H. pylori* infection 23

1.7 TRANSCRIPTION FACTORS 24
1.7.1 Nuclear factor-kappa B (NF-κB) 25
1.7.1.1 Members of NF-κB/Rel and Ikappa B proteins 25
1.7.1.2 Conditions that activate NF-κB 28

1.7.1.3 Ikappa B phosphorylation and degradation 28
1.7.1.4 Genes regulated by NF-κB 31
1.7.1.5 *Helicobacter pylori* and NF-κB activation 31

1.7.2 Role of NF-κB in Inflammation and Immunity 33
1.7.2.1 Role of NF-κB in regulating cell proliferation 33
1.7.2.2 Role of NF-κB in regulating apoptosis 34
1.7.2.3 Role of NF-κB in regulating cytokine production 34
1.7.2.4 NF-κB and oxidative stress 35

1.7.3 Inhibition of NF-κB Activation: As a Therapeutic Target 36
1.7.3.1 Phosphothioate and antisense oligonucleotides to NF-κB/Rel 37
1.7.3.2 Proteasome inhibitors 37
1.7.3.3 Glucocorticoids 37
1.7.3.4 Antioxidants 39

1.7.4 Immediate-Early Growth (Egr) Transcription Factors 40
1.7.4.1 The Early Growth Response-1 (Egr-1) Transcription Factor 40
1.7.4.1.1 Egr-1 discovery and characterization 40
1.7.4.1.2 Conditions that activate Egr-1 41
1.7.4.1.3 Genes regulated by Egr-1 (Target genes) 42
1.7.4.1.4 Transcriptional corepressors and coactivators of Egr-1 42
1.7.4.1.5 Mechanisms of Egr-1 activation 45
1.7.4.1.6 Egr-1 activation and oxidative stress 45

1.7.4.2 Role of Egr-1 in Inflammation and Immunity 46
1.7.4.2.1 Role of Egr-1 in regulating cell growth and differentiation 46
1.7.4.2.2 Role of Egr-1 in regulating cell adhesion molecule expression 47
1.7.4.2.3 Role of Egr-1 in regulating of apoptosis 48

1.8 SUMMARY 49

CHAPTER II- MATERIALS AND METHODS
2.1 Chemicals 51
2.2 Carriers 51
2.3 Oligonucleotides and Antibodies 52
2.4 Cell Culture 52
2.4.1 Source of cell lines 52
2.4.2 Cell culture reagents 52
2.4.3 Maintenance of cell cultures 53
2.4.3.1 Adherent cells 53
2.4.3.2 Non-adherent cells 53
2.4.4 Freezing and reconstitution of cells from liquid nitrogen 54
2.4.5 Cell counting and viability 54
2.5 *Helicobacter pylori* and coculture of gastric epithelial cells 54
2.5.1 Bacterial strains 54
2.5.2 Growth conditions of *H. pylori* strains 55
2.5.3 Coculture of gastric epithelial cells with *H. pylori* and other stimuli 55
2.6 Preparation of Blood Samples 55
2.6.1 Isolation of peripheral blood mononuclear cells (PBMCs) 55
2.6.2 Preparation of cytokine rich supernatants from PBMC 56
2.7 Preparation of Cell Protein 56
2.7.1 Preparation of whole cell extracts 56
2.7.2 Protein estimation 57
2.8 Polyacrylamide Gel Electrophoresis (PAGE) 57

2.8.1 Preparation of protein samples and molecular weight markers 57
2.7.2 Polyacrylamide gel electrophoresis (PAGE) 57
2.8.3 Immunoblot detection 58
2.8.4 Enhanced chemiluminescence detection (ECL) 58
2.9 Immunohistochemical Staining of Gastric Biopsies 59
2.10 Electrophoretic Mobility Shift Assay (EMSA) 60
2.10.1 Cell fractionation and nuclear extract preparation 60
2.10.2 Labelling of consensus oligonucleotides for EMSA 61
2.10.3 Preparation of gels for EMSA 62
2.10.4 Preparation of DNA-protein binding reaction 62
2.10.5 Electrophoresis of DNA-protein complexes 63
2.10.6 Supershift and competition assays 63
2.11 Transient Transfection and Evaluation Assays 64
2.11.1 DNA constructs 64
2.11.2 Transformation of bacterial cells 64
2.11.3 Preparation and purification of plasmid DNA from *E. coli* DH5α 64
2.11.4 Transient tansfection 65
2.11.5 Chloramphenicol acetyltransferase (CAT) assay 66
2.11.6 Luciferase reporter gene assay 68
2.12 Flow Cytomerty Analysis 68
2.13 Cell Proliferation Studies 69
2.14 Data Analysis 69

CHAPTER III- EFFECTS OF *HELICOBACTER PYLORI* AND LOW pH ON NF-κB EXPRESSION IN GASTRIC EPITHELIAL CELLS
3.1 INTRODUCTION 70
3.1.1 *H. pylori* and gastric acid secretion 70
3.1.2 Low pH and transcriptional regulation 71

3.2 OBJECTIVES 72

3.3 RESULTS 72
3.3.1 Exposure of gastric epithelial cells to low pH activates NF-κB DNA-binding 72
3.3.2 Supershift and competition assays 73
3.3.3 Effect of low pH on IκB-α protein level 73
3.3.4 *H. pylori* induces NF-κB DNA-binding activity in gastric epithelial cells 74
3.3.5 Effect of different *H. pylori* isolates on NF-κB DNA-binding activity 74
3.3.6 Specificity of *H. pylori*-induced NF-κB DNA-complex 74
3.3.7 Effect of LPS, heat-killed and crude protein of *H. pylori* on NF-κB activation 75
3.3.8 Effect of *H. pylori* on IκB-α protein level 75
3.3.9 Effect of pH combined with *H. pylori* on NF-κB DNA-binding activity 75
3.3.10 Time course of NF-κB activation (combined effects of pH and *H. pylori*) 76
3.3.11 Specificity of NF-κB-DNA-complex induced by *H. pylori* at pH 7.0 76
3.3.12 Expression of NF-κB in patients with *H. pylori* infection 77

3.4 DISCUSSION 77

CHAPTER IV- EFFECTS OF ANTIOXIDANTS ON NF-κB ACTIVATION IN RESPONSE TO *H. PYLORI* AND OTHER STIMULI IN GASTRIC EPITHELIAL CELLS
4.1 INTRODUCTION 83

4.2 OBJECTIVES 85

4.3 RESULTS 85

4.3.1 Effect of antioxidants on NF-κB activation by *H. pylori* 85
4.3.2 Effect of antioxidants on NF-κB activation by low pH 86
4.3.3 Effect of antioxidants on NF-κB activation by low pH and *H. pylori* 86
4.3.4 Effect of antioxidants on NF-κB activation by cytokines and mitogen 86
4.3.5 Effect of thioredoxin on NF-κB activation by cytokine rich supernatants 87
4.3.6 Effect of hydrogen peroxide on NF-κB DNA-binding activity 87
4.3.7 Effect of antioxidants on IκB-α protein level 87
4.3.8 Effect of antioxidants on NF-κB p65 translocation 88
4.3.9 Effect of Trx on the expression of the cell adhesion molecules 88
4.3.10 Effect of Trx on cell proliferation 88

4.4 DISCUSSION 89

CHAPTER V- *H. PYLORI* INDUCES THE EXPRESSION OF THE EARLY GROWTH RESPONSE-1 (Egr-1) PROTEIN IN GASTRIC EPITHELIAL CELLS

5.1 **INTRODUCTION** 94

5.2 OBJECTIVES 94

5.3 RESULTS 95
5.3.1 *H. pylori* induces the expression of Egr-1 95
5.3.2 Effect of *H. pylori* clinical isolates on Egr-1 expression 95
5.3.3 Effect of other bacteria on Egr-1 expression 96
5.3.4 *H. pylori* induces Egr-1 expression in other gastric and non gastric cells 96
5.3.5 Effect of *H. pylori* on Egr-1 DNA-binding activity 96
5.3.6 Signalling pathways involved in Egr-1 induction by *H. pylori* 96
5.3.7 Effect of pH reduction on Egr-1 expression 97
5.3.8 Effect of low pH combined with *H. pylori* on Egr-1 expression 97
5.3.9 Effect of the cytokines Il-1β and TNF-α on Egr-1 expression 98
5.3.10 Effect of the phorbol ester PMA on Egr-1 expression 98
5.3.11 Signalling pathways involved in cytokine-induced Egr-1 expression 99
5.3.12 Signalling pathways involved in PMA- and pH-induced Egr-1 expression 99
5.3.13 Effect of oxidants and antioxidants on Egr-1 expression 100
5.3.14 Expression of Egr-1 in patients infected with *H. pylori* 100

5.4 DISCUSSION 101

CHAPTER VI- BIOLOGICAL ROLE OF EGR-1 TRANSCRIPTION FACTOR IN REGULATION OF CELL ADHESION MOLECULES AND APOPTOSIS

6.1 INTRODUCTION 105

6.2 OBJECTIVES 106

6.3 RESULTS 107
6.3.1 *H. pylori* up-regulates the CAT activity of the CD44 promoter construct 107
6.3.2 Involvement of Egr-1 in the regulation of *H. pylori*-induced CD44 expression 107
6.3.3 Effect of *H. pylori* strains on the CD44 promoter promoter 108
6.3.4 *H. pylori* up-regulates the CAT activity of ICAM-1 promoter construct 108
6.3.5 Regulation of ICAM-1 activation by Egr-1 108
6.3.6 Involvement of Egr-1 binding sites in the regulation of CD95L activation 109
6.3.7 Effect of the antisense Egr-1 oligonucleotide on *H. pylori*-induced Egr-1 expression 109
6.3.8 Effect of the antisense Egr-1 on *H. pylori*-induced CD44 expression 110

6.4 **DISCUSSION** 110

CHAPTER VII- GENERAL DISCUSSION
7.1 *Helicobacter pylori*: A prominent factor in peptic ulceration pathogenesis 113
7.2 *Helicobacter pylori* and transcriptional regulation 114
7.3 Effect of *H. pylori* and Low pH on transcriptional regulation 117
7.4 *Helicobacter pylori* and vitamin C levels 118
7.5 Future prospects 119

REFERENCES 120

APPENDICES
Appendix A: Reagents and Buffers
Appendix B: Publications

ACKNOWLEDGMENTS

IN THE NAME OF ALLAH, THE MOST GRACIOUS, THE MOST MERCIFUL
Many thanks go first and foremost to the glory of Almighty Allah, for making my life what it is and providing me with the energy and patience throughout this thesis.

I wish to express my gratitute to my supervisors, Professor Dermot Kelleher (Department of Clinical Medicine, Trinity College, Dublin) and Professor Kamal Sabra (Department of Clinical Pharmacology and Therapeutics, Trinity College, Dublin). Professor Kelleher introduced me to this field of research on the human gastric pathogen *Helicobacter pylori*. I would like to thank him for his friendship, kindness, boundless enthusiasm, advice and guidance during this work.

Many thanks are also due to Prof. Kamal Sabra for his understanding, encouragement and support during this thesis and also his willingness to offer assistance during this work and during my stay in Ireland. I am extremely grateful to him.

I would like to thank Dr. Henry Windle (my other supervisor) for teaching me the finer things during this work and for his patient guidance and priceless friendship. Most especially, I am deeply grateful to him for giving his time and tremendous efforts in finally bring this thesis to light.

There are many individuals who deserve special thanks for their advice and help throughout this thesis. I would like to express my gratitude to the members of Sir Patrick Dun Immunology Research (SPD) including the past members (Barbara, Yeng and Rachael).

Particularly, to Dr. Anne Murphy (for being a mother before a friend) for her friendship, help and encouragement during this work.

To Dr. Déirdre Ní Eidhin for her friendship and cooperation and for kindly providing cultures of *Helicobacter pylori.*

To Dr. Yuri Volkov for his friendship, advice and many acts of kindness, particularly his useful instructions in the use of the Adobe Photoshop Application.

To Dr. Ana Terres for her endless help and friendship and reading the initial draft of this thesis. I thank her for her endurance and diligence.

To Dr. Sinèad and Jackie for their friendship, help, humane nature and overwhelming support over the years.

Many thanks are also due to my colleagues, Áine Fanning, Áine Fox, Basma, Ali Raoof, Eileen, Claire, Dr. Shah and Shane for their support and I wish them all the best of luck with their work.

Most importantly, another person who deserves a special mention is Dr. Aideen Long for her friendship, valuable assistance and help throughout this work.

A special mentions to Dr. Ross McManus for his friendship and boundless support and particularly his interests in the Pharaoh civilization.

I am indebted to Dr. Katherine Fitzgerald, Department of Biochemistry and Biotechnology Institute, Trinity College, University of Dublin, who offered her help on several occasions during this work.

I greatly appreciate the advice and comments of Dermot O'Toole during my first days and certainly his interests in football. Thanks so much.

I acknowledge the receipt of a postgraduate student scholarship and the financial support from the Egyptian Government (Arab Republic of Egypt). Many thanks are also due to my colleagues in Faculty of Pharmacy, Assiut University, Egypt.

Finally and most importantly, many thanks to my family, my parents, my brothers (Ahmed and Ali), and my sisters (Zinab and Nadia) for their love, support and encouragement throughout my life.

Most of all, many thanks to my wife NERMIN for her support, patience and endless encouragement.

ABBREVIATIONS

AO	acridine orange
Ank	ankyrin
AP-1	activator protein-1
APS	ammonium persulphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
BCR	B cell antigen receptor
$CaCl_2$	calcium chloride
CagA	cytotoxin-associated gene A
cag PAI	*cag* pathogenicity island
CAM	cell adhesion molecule
CAT	chloramphenicol acetyltransferase
CD	cluster of differentiation
CD44 v	variant form of CD44
CD44 v6	variant 6 form of CD44
CD44 v9	variant 9 form of CD44
CD95L	(APO-1/Fas) ligand
DAB	diaminobenzidine tetrachloride
DDI	DNA damage-inducible genes
DMSO	dimethlysulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EB	ethidium bromide
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
Egr-1	early growth response-1
ELAM	endothelial leukocyte adhesion molecule
EGTA	ethyleneglycol bis-(aminoethlether)tetra-acetic acid
EMSA	electrophoretic mobility shift assay
ERK1/2	extracellular signal-regulated kinase 1/2
FCS	foetal calf serum
FITC	fluorescine isothiocynate
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte monocyte-colony stimulating factor
GR	gluocorticoid receptor
GSH	glutathione
HBSS	Hank's Balanced Salts Solution
HCl	hydrochloric acid
HIV	human immunodeficiency virus
Hp	*H. pylori*
H_2O_2	hydrogen peroxide
HRP	horse raddish peroxidase

Hsp	heat shock protein
ICAM-1	intercellular adhesion molecule-1
IE (IEGs)	immediate-early genes
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IκB-α	Ikappa B-alpha
IKK	Ikappa B kinase complex
JNK	c-Jun NH_2-terminal kinase
IL-1β	interleukin-1 beta
IMPDH	inosine-5′-monophosphate dehydrogenase type II
iNOS	inducible nitric oxide synthetase
LB	L-broth medium
kb	Kilobase pairs
kDa	kilodaltons
LFA	leukocyte function antigen
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MAPKKK	mitogen-activated protein kinase kinase kinase
MAb	monoclonal antibody
$MgCl_2$	magnesium chloride
MHC	major histocompatibilty complex
MOI	multiplicity of infection
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS)
NAC	N-acetylcysteine
NaCl	sodium chloride
NaOH	sodium hydroxide
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa B
NGFI-A	nerve growth factor-induced-A
NGFI-B	nerve growth factor-induced-B
NIK	NF-κB-inducing kinase
NP40	nonidet P40
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
O^-_2	superoxide anion
OD	optical density
OH^-	hydroxyl radical
PAF	platelet-activating factor
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDTC	pyrrolidine dithiocarbamate
PFA	paraformaldehyde

PHA	phytohaemaglutinin
PKC	protein kinase C
PMA	phorbol-12-myristate-13 acetate
PMS	phenazine methosulfate
PMSF	phenyl methyl sulphonyl fluoride
PVDF	polyvinylidene difluoride
PAGE	polyacrylamide gel electrophoresis
ROS	reactive oxygen species
RNA	ribonucleic acid
SD	standard deviation
SDS	sodium dodecyl sulphate
Ser	serine
SOD	superoxide dismutase
SRE	serum response elements
SRF	serum response factor
TAE	Tris-acetateEDTA buffer
TBE	Tris-borate EDTA buffer
TBS	Tris-borate saline buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethyl ethylene-diamine
TG	thasipargin
TGF	transforming growth factor
thr	threonine
TLC	thin layer chromatography
TNBS	trinitrobenzenesulfonic acid
TNF-α	tumour necrosis factor-alpha
TRAF	TNF receptor associated factor
TRADD	TNF receptor associated death domaine
Tris	Tris (hydroxymethyl) aminomethane
Trx	Thioredoxin
UV	Ultraviolet
WHO	World Health Organization
VacA	vaculoating cytotoxin A
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4

CHAPTER I

GENERAL INTRODUCTION

CHAPTER I

General Introduction

1.1 *HELICOBACTER PYLORI*

It used to be thought for many years that the stomach was sterile or nearly so, and any possible relationship of microbes to gastric ulceration was not considered. Therefore, the dictum "no acid-no ulcer" has had a major impact on both the way in which the pathogenesis of peptic ulcer disease has been viewed and the way in which this disease has been treated. The isolation of the spiral bacterium, *Helicobacter pylori* in 1984 by Marshall and Warren, was a remarkable revolution in the medical field. Indeed, the discovery of *H. pylori* has changed our understanding about many aspects of gastrointestinal diseases with regard to treatment and gastric physiology. Numerous studies worldwide in several areas of research such as epidemiology, pathogenesis, diagnosis, and treatment of *H. pylori*, have been conducted to clarify the role of the bacterium in the development of peptic ulcer disease. Despite the significant progress that has been made in recent years in these areas, the exact mechanism for the development of inflammation, gastric ulcer and cancer remains unclear. This thesis investigates the effect of *H. pylori* on the regulation of transcription factors during the inflammatory process. This work sheds a new light on the pathogenesis of *H. pylori* infection that might provide a basis for future prevention of gastroduodenal diseases.

1.1.1 Historic Background

The presence of spiral organisms in the stomachs of mammals were observed by Bottcher (1874), followed by similar observations in animals (Bizzozero, 1893). In 1906, spiral and curved bacteria were first seen in the human stomach by Kreinitz. Luck and Seth (1924) reported that the human stomach contains abundant urease activity, and it was subsequently shown that this urease activity disappeared during administration of antibiotics (Langenberg *et al.*, 1984), indicating that the enzyme was of bacterial origin. Doenges (1938) in a histological study of 242 autopsy stomachs found that 43% of human stomachs contained the spirochaetes. In 1975, Steer reported the presence of Gram-negative bacteria on the gastric mucosa with gastritis. However,

the failure to culture the organism resulted in the finding being ignored until 1983, when Marshall and Warren in Perth, Australia, reported finding "small curved and shaped bacilli" in gastric biopsy specimens. It was a combination of scientific curiosity and luck that led to the isolation of these bacteria. Subsequently, the bacterium was isolated in culture from gastric biopsies (Marshall and Warren, 1984). Following this discovery, several reports have confirmed that patients with gastritis or ulcers were infected with the spiral bacteria more than healthy controls (Rollason *et al.*, 1984; Graham, 1989). The successful culture of *H. pylori* and the immense quantity of research that followed this event have resulted in an explosion of new information.

1.1.2 Nomenclature

The spiral microaerophilic bacteria resembled *Campylobacters* by light microscopy and in the guanine plus cytocine contents. Therefore, Marshall and Warren (1984) thought that the new bacterium was a *Campylobacter* found in the pylorus region of the stomach and called it *Campylobacter pyloridis*. The name of the bacterium was changed to *Campylobacter pylori* when linguists pointed out that *pyloridis* was grammatically incorrect and *Campylobacter* became *Helicobacter* (as a new genus), when it was discovered that its 16 subunit ribosomal RNA did not have the characteristic sequences found in *Campylobacter* (Goodwin *et al.*, 1989). The new name *Helicobacter pylori* refers to the helical appearance of this organism *in vivo*, as well as the most common isolation place, the pylorus of the stomach. The genus has expanded rapidly and now includes more than 19 species **[Table 1.1]**, most of which are of non-human origin and are of uncertain pathogenic significance (Owen, 1995).

1.1.3 Microbiological Features

Helicobacter pylori is a small curved S-shaped or slightly spiral Gram-negative rod, 2.5-3.5 μm in length and 0.5-1 μm in width. It has a smooth coat and 1-6 sheathed unipolar flagellae with a terminal bulb **[Fig. 1.1]** (Jones and Curry, 1992). *H. pylori* is a microaerophile, it grows in an atmosphere of 5% O_2 and 5-10% CO_2 on chocolate or blood agar plates at 37°C. *H. pylori* grows poorly at 30°C and 42°C (Mégraud *et al.*,

Members of the genus *Helicobacter* and their host reservoir

Species name	Host reservoir
H. pylori	Man, rhesus monkey, pig, baboon
H. mustlae	Ferret
H. felis	Cat, dog
H. nemestrinae	Pigtailed macaque
H. muridarum	Rat, mouse
H. cinaedi	Human
H. fennelliae	Human
H. acinonyx	Cheetah
H. canis	Human, dog
H. hepaticus	Mouse
H. pullorum	Human, chicken
H. pametensis	Wild birds, pig
H. bilis	Inbred mice

Table 1.1. Species of the genus *Helicobacter* and their host reservoir; most of which are of non-human origin and are of uncertain pathogenic significance. (Reproduced from Owen, 1995).

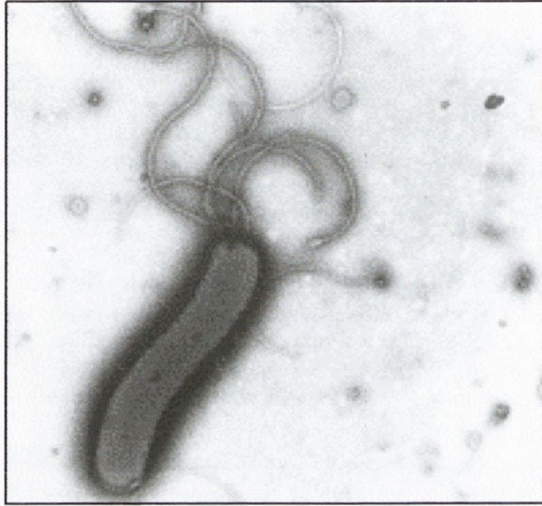


Fig. 1.1. Negatively stained preparation of cultured human isolate of *Helicobacter pylori* showing sheated flagellar filaments. (Magnification X 14^{200}) (Reproduced from Jones and Curry, 1992).

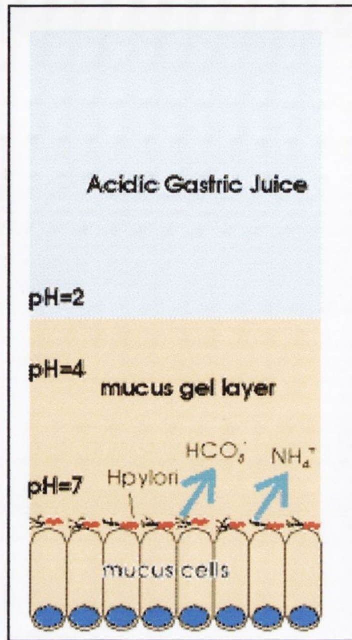
1987). It grows best at neutral pH and becomes non-viable in broth medium at pH 3. *H. pylori* grows, depending on the culture medium, over a wide range of pH (5.5-8.5) with good growth between pH 6.9 and 8.0. Colonies of *H. pylori* from primary culture can be identified on the basis of their morphology, oxidase, catalase, and urease production (Mégraud *et al.*, 1987).

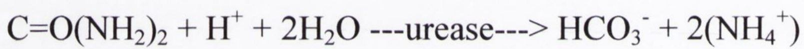
The stomach is protected from its own gastric juice by a thick layer of mucus that covers the stomach lining. However, *H. pylori* is found within and beneath the mucus layer on the gastric epithelium **[Fig. 1.2]**. The organism is protected from gastric acid by the overlying mucus and the secretion of bicarbonate from surface cells. The survival of *H. pylori* can occur between a periplasmic pH of 4.0 to 8.0, but growth and protein synthesis can only occur between a periplasmic pH of 6.0 to 8.0 (Scott, 1998).

1.1.4 Colonization of Gastric Epithelium by *H. pylori*

H. pylori colonizes gastric type mucosa and is found primarily in the antrum of the stomach. It is also seen in the duodenum within areas of gastric metaplasia and in the fundus (Wyatt *et al.*, 1987). Colonization of the gastric mucosa by *H. pylori* is associated with histologic evidence of gastritis **[Fig. 1.3]** (Steer, 1992). *H. pylori* is able to colonize the harsh environment of the human stomach as it avails of a number of mechanisms that facilitate the initial invasion and its persistence. Firstly, its spiral shape and flagellar motility allows the bacteria to penetrate the mucus layer and survive in the semipermeable mucus gel (Hazell *et al.*, 1986). Secondly, the powerful urease enzyme produced by *H. pylori*, creates a basic environment which protect the bacteria from the gastric acid at the colonization stage, and a number of its mucolytic enzymes enhance its penetration into the gastric mucus. Once *H. pylori* reaches the safe environment between the mucus layer and the epithelium, it can adhere to the apical surface of epithelial cells by means of bacterial adhesins.

Formation of adhesion pedestals from the gastric epithelium have been observed in areas of gastric metaplasia in the duodenum, and this is mediated by adhesins on the bacterial surface that bind to specific receptors in the surface of epithelial cells (Lee,





$$C{=}O(NH_2)_2 + H^+ + 2H_2O \text{ ---urease---> } HCO_3^- + 2(NH_4^+)$$

Fig. 1.2. The stomach is protected from its own gastric juice by a thick layer of mucus. The above diagram shows *Helicobacter pylori* inside the mucus lining. *H. pylori* possesses urease enzyme which converts urea, which is abundant in the stomach (from saliva and gastric juices), into bicarbonate and ammonia. This creates a cloud of acid neutralizing chemicals around the bacteria, protecting it from the acid in the stomach. The reaction of urea hydrolysis (urea is broken down to ammonia and carbon dioxide, which in the presence of H^+ converts into HCO_3^-) is shown (Reproduced from the *Helicobacter* foundation web site, http:/www.helico.com).

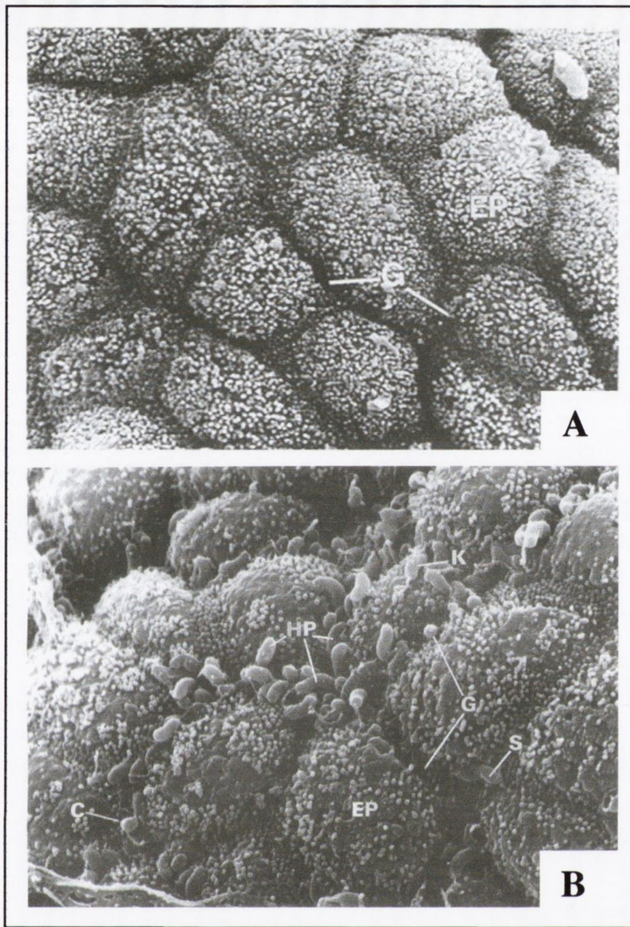


Fig. 1.3. (A) The gastric epithelium surface in a normal stomach showing individual epithelial cells (EP) covered with microvilli. The grooves (G) between the surface of individual cells is clearly seen (SEM X 5936). **(B)** The epithelial surface of gastric mucus-secreting cells from the prepyloric area of a patient with chronic duodenal ulceration. The number of surface microvilli is decreased. The *H. pylori* (HP) are numerous and present in the grooves (G) between individuals epithelial cells (EP). Coccal (C), kidney-shaped (K) and spiral (S) forms of *H. pylori* are seen. (SEM X 3907) (Reproduced from Steer, 1992).

1996). Moreover, adhesion of *H. pylori* to specific blood group (blood group O) may also explain the disease outcome in people with particular blood groups (Boren *et al.*, 1993). All these adaptation mechanisms of survival highlight the importance of *H. pylori* as a human pathogen which has been able to colonize the gastric enviroment, unaccessible to other microbes of the gastrointestinal tract.

1.1.5 Epidemiology of *H. pylori* Infection

Since the original isolation of *H. pylori*, the bacteria have been isolated worldwide, and over one half of the world is infected with this organism (Pisani *et al.*, 1997). The prevalence of *H. pylori* in healthy volunteers varies depending upon age, ethnicity, and country of origin. In some populations, a disproportionately high prevalence of infection is observed in those over 40, which seems to reflect a birth cohort effect (Podolsky *et al.*, 1989). In Western societies, *H. pylori* infection is uncommon in childhood and the prevalence of *H. pylori* seems to increase with advancing age and infecting 50-60% of the population by the age of 60 years (Taylor and Blaser, 1991).

The pattern of *H. pylori* infection in non-Western countries is quite different from that seen in the Western countries, as most *H. pylori* infection occurs earlier in life so that more than 80% of the adult population is infected (Mégraud *et al.*, 1989). The marked difference in infection rates between children in different locations and different birth cohorts may be related to living standards. Situations of economic deprivation and habitational crowding enhance the spread of *H. pylori* during childhood (Mendall *et al.*, 1992). For example, higher infection rates have been found in groups of low socioeconomic status, in institutions, and within families with *H. pylori*-associated ulcer diseases (Taylor and Blaser, 1991). Other demographic factors such as racial/ethnic variations and sex differences have also been linked to infection.

1.1.6 Mode of Transmission

The mode of transmission of *H. pylori* infection has not yet been defined. There is no known reservoir in the environment for this bacterium and man is thought to be the natural host for *H. pylori* (Pisani *et al.*, 1997). Infection with *H. pylori* in domestic cats

also shares many features of long-term *H. pylori* infection in humans, including the development of preneoplastic processes (Esteves *et al.*, 2000). The organism is transmitted from person-to-person via oral-oral or fecal-oral transmission. A study of children and their families provides strong evidence supporting person-to-person transmission of this organism (Drumm *et al.*, 1990). Further support for oral-oral transmission has come from the detection of *H. pylori* DNA in the saliva and dental plaque of infected subjects (Lambert *et al.*, 1993). *H. pylori* has been also cultured from the diarrheal stools of infected children (Thomas *et al.*, 1992).

The geographical, epidemiological, and social patterns of infection are consistent with oral-oral or fecal-oral transmission. For example, a high prevalence of *H. pylori* infection in institutionalized mentally handicapped patients has been observed compared with a control population (Lambert *et al.*, 1990). In addition, strain identification using DNA digest patterns has shown the same strain infecting different members of the same family (Rauws *et al.*, 1989). On the other hand, a common water supply appeared to be the source of infection in Peru (Klein *et al.*, 1991), as well as the consumption of uncooked vegetables and uncooked shellfish in Chile (Hopkins *et al.*, 1993). Interestingly, Mitchell *et al.* (1989) reported that endoscopists have a higher than expected prevalence of infection presumably by handling endoscopes contaminated by patients with *H. pylori*.

1.2 *HELICOBACTER PYLORI* AND GASTROINTESTINAL DISEASES

H. pylori has been identified as the most common bacterial infection of humans worldwide. *H. pylori* lives in the stomach and duodenum and is strongly associated with a wide range of gastrointestinal pathology. Extensive studies have revealed that over 90% of patients with chronic active gastritis are colonized with *H. pylori* and it is the aetiological agent of 70-80% of gastric ulcers, 95% of duodenal ulcers, and has a causal role in up to 60-70% of gastric cancer. This association raised the question of whether *H. pylori* was a mere commensal of a damaged gastric mucosa or a pathogenic bacterium. The causative relation between *H. pylori* and these disorders

has now been well established and the outcome of the disease depends on many factors, including bacterial genotype, host physiology, and dietary habits.

1.2.1 *H. pylori* and Gastritis

The term gastritis may be defined as a heterogeneous group of gastric mucosal disorders which are usually associated with an acute, chronic or mixed inflammatory response. Histologic examinations of areas of gastritis reveal features of both acute and chronic inflammatory change in individual patients. Two distinct forms of gastritis, based on the regional distribution of pathologic changes in the stomach, called corpus-predominant (type A) and antral-predominant (type B) have been defined (Strickland and Mackay, 1973).

The close association between gastric mucosal colonization with *H. pylori* and histologic gastritis was evident in the initial study by Marshall and Warren (1984). Aetiologically, most non-specific histologic gastritis appears to be caused by *H. pylori*. About 100% of patients infected with *H. pylori* have gastritis (Dooley *et al.*, 1989), while 90% of patients with gastritis have *H. pylori* infection (Sobala *et al.*, 1992). A causal relationship has been demonstrated by experimental exposure of human volunteers (Marshall *et al.*, 1985) and animal inoculation studies (Engstrand *et al.*, 1990). Further evidence for a causative role was shown when eradication of *H. pylori* by antimicrobial drug therapy led to significant healing of histologic gastritis (Rauws *et al.*, 1988).

1.2.2 *H. pylori* and Peptic Ulcer Disease

Peptic ulcer disease is a chronic inflammatory condition characterized by breaches in the mucosa of the stomach or duodenum caused by acid damage in areas weakened by inflammation. Peptic ulceration results from a loss of balance between the mucosal protective mechanisms and the aggressive factors of acid and pepsin production, injury, infection, and other agents like non-steroidal anti-inflammatory drugs (NSAIDs) (Soll *et al.*, 1991).

In the early days, treatment of peptic ulcer disease focused on hospitalization with bed rest and prescription of special bland foods. Later, peptic ulcer was believed to be caused by the injurious effect of gastric acid; hence, antacids became the standard of therapy. Other drugs, such as histamine receptor antagonists (H_2RA) and proton pump inhibitors (H^+, K^+-ATPase); bismuth compounds and prostaglandins have also proved to be effective therapy for peptic ulcer disease. Despite these regimens, the problem of high recurrence rate of peptic ulcer remained even after treatment until 1984, when Marshall and Warren reported an association between the presence of spiral organisms on the gastric mucosa and antral gastritis. Subsequent studies have confirmed this association as well as a strong association between *H. pylori*-associated gastritis and duodenal ulcer disease (Graham, 1989).

The causal role of *H. pylori* in the aetiology of gastric ulcer disease is not as clear as the case with duodenal ulcer: One factor which complicates research, is that gastric ulcers have another important cause, namely NSAIDs (Soll *et al.*, 1991). The strongest evidence for the pathogenic role of *H. pylori* in peptic ulcer disease is the marked decrease in recurrence rate of ulcers following the eradication of *H. pylori* infection (Graham *et al.*, 1992; Karita *et al.*, 1994). It seems likely that most of the gastric ulcers that occur in uninfected patients are due to NSAIDs therapy. Graham and colleagues (1991) found that their gastric ulcer patients who were not infected with *H. pylori* had NSAIDs or their metabolites detectable in their blood and urine.

The association between *H. pylori* and duodenal ulcer disease is now well accepted, with more than 90% of duodenal ulcer patients being infected (Graham, 1989; Mégraud and Lamouliatte, 1992). The pathogenic role of *H. pylori* in duodenal ulcer was evident from the numerous treatment studies which demonstrated that duodenal ulcer relapses did not occur in patients in whom *H. pylori* was eradicated (O'Morain *et al.*, 1996; Patchett *et al.*, 1992; Graham *et al.*, 1992; Dooley, 1991; Rauws and Tytgat, 1990). The mechanism(s) by which *H. pylori* infection predisposes to a patient duodenal ulcer remains unclear, although it is speculated that duodenal ulcers occur in areas of gastric

metaplasia infected with *H. pylori*, which subsequently gives rise to duodenal inflammation and ulceration.

The acquisition of *H. pylori* increases with age, whereas the incidence of duodenal ulcer decreases with advancing age, but infection with *H. pylori* makes the duodenum mucosa more vulnerable to other factors, such as gastric acid and diminished duodenal bicarbonate secretion (Lambert *et al.*, 1995). Other factors such as genetic predisposition, environmental factors, blood group O, cigarette smoking and infection are important in the pathogenesis of duodenal ulcer. Thus, the role of *H. pylori* in duodenal ulcer disease seems real, even if the mechanism is not well known. Further work is needed to determine why some individuals with *H. pylori* develop peptic ulcers, but most do not.

1.2.3 *H. pylori* and Non-Ulcer Dyspepsia (NUD)

Non-ulcer dyspepsia refers to chronic, recurrent upper gastrointestinal symptoms of at least four weeks duration in the absence of gastrointestinal or systemic disease. Because of the heterogeneous aetiology of NUD, the role of *H. pylori* is still unclear and also the relationship between gastritis and dyspepsia is also unclear (Lambert, 1993). There is convincing evidence that *H. pylori* is the major cause of chronic non-specific gastritis. A number of studies suggest that the prevalence of *H. pylori*-associated gastritis in non-ulcer dyspepsia ranges between 30% and 70% (Marshall and Warren, 1984; Ormand *et al.*, 1991; Talley, 1990). In treatment studies, some improvement of symptoms after eradication of *H. pylori* has been found. O'Morain and Gilvarry (1993) have shown that *H. pylori* eradication led to a prolonged improvement of symptoms in patients with non-ulcer dyspepsia at one year follow-up. Although the link between *H. pylori* infection and NUD is controversial, it appears that treatment of *H. pylori* infection in those patients results in a significant long-term reduction in symptoms of non-ulcer dyspepsia (Gilvarry *et al.*, 1997).

1.2.4 *H. pylori* and Gastric Cancer

Gastric carcinoma is one of the most common frequently diagnosed cancers world-wide (Parkin *et al.*, 1988), and is the cause of more than 900,000 deaths annually (Pisani *et al.*, 1997). Gastric adenocarcinoma is considered the second leading cause of death from cancer worldwide behind lung cancer (Parkin *et al.*, 1993). Gastric cancer occurs much more frequently in developing countries than in developed countries, which strongly correlated with overcrowding and low socio-economic status. Environmental factors such as high salt intake, nitrite-rich food, low vitamin C, smoking and alcohol intake have all been associated with gastric carcinoma (Correa, 1991). The discovery of *H. pylori* has stimulated research into the relationship between gastric carcinoma and *H. pylori* infection, and now, there is a strong epidemiologic association between both (Parsonnet *et al.*, 1991; Nomura *et al.*, 1991). Moreover, in 1994, the International Research Agency on Cancer (IRAC) added *H. pylori* to its list of known carcinogens, after the EUROGAST study (1993) showed a positive correlation between the prevalence of gastric cancer and *H. pylori* in different countries.

The pathologic mechanisms involved in carcinogenesis associated with *H. pylori* infection are still unclear. It is thought that the carcinogenic effect of *H. pylori* occurs through the development of chronic atrophic gastritis with intestinal metaplasia and reduced acid secretion, increased reactive oxygen intermediate formation with a subsequent increase in N-nitroso compounds (Correa, 1991; O'Connor, 1992). The carcinogenic effect of *H. pylori* infection is modulated by dietary and other environmental factors. Sobala and colleagues (1991) have documented a decrease in gastric vitamin C levels in *H. pylori* infected patients. Vitamin C is an antioxidant and prevents formation of nitrosamines in the stomach and its role related to *H. pylori* will be discussed in detail later on. Inflammatory byproducts or mediators of inflammation could also stimulate cell proliferation and contribute to the risk of DNA damage (Parsonnet *et al.*, 1991). Therefore, the eradication of *H. pylori* might reduce the incidence of gastric adenocarcinoma and prevent hundreds of thousands of gastric cancers.

1.3 PATHOGENIC MECHANISMS OF *H. PYLORI* INFECTION

Although the stomach represents a hostile environment with a low intragastric pH, proteolytic enzymes, and a viscous mucus layer, *H. pylori* has succeeded by a number of adaptations to colonize the gastric mucosa. The colonization of the gastric epithelium by *H. pylori* occurs through production of a number of virulence factors which have been suggested to permit the bacteria to evade the bactericidal properties of gastric acid, damage epithelial cells and induce an inflammatory reaction **[Fig. 1.4]** (Wallace, 1991). Moreover, *H. pylori* can survive in the stomach despite a powerful host immune response, which the organism is capable of evading, possibly by down-regulation of the immune system. The virulence factors, which will be highlighted, include motility, production of certain enzymes, adhesins, lipopolysaccharide, heat shock proteins, vacuolating cytotoxin, and chemotactic factors.

1.3.1 Motility

Motility is considered important for gut pathogens. It has been demonstrated *in vitro* and in animal experiments that *H. pylori* is able to traverse mucus layers and induce local inflammation (Hazell *et al.*, 1986; Eaton *et al.*, 1989) by means of its flagella which allow motility in the gastric juice and gastric mucus. Motility is facilitated by the spiral shape of the bacterium and by the activity of the flagella, which enable the organism to penetrate the mucus, whereas non-motile strains are unable to colonize the gastric epithelium (Hazell *et al.*, 1986). Studies in gnotobiotic piglets have demonstrated that less motile strains of *H. pylori* are less virulent (Eaton *et al.*, 1989).

1.3.2 Bacterial Enzymatic Activity

H. pylori has a wide range of enzymes such as urease, protease, phospholipase, catalase, superoxide dismutase, and others, which may play an important role in *H. pylori*-associated pathogenesis. The most abundant enzyme produced by *H. pylori* is urease, which is considered to be a highly specific tool for the diagnosis of this bacterial infection (Mégraud, 1996). Urease hydrolyses urea into ammonia and carbon dioxide. The ammonia then reacts with water to form ammonium ions that neutralize the gastric acid (Mégraud *et al.*, 1987; Mobley *et al.*, 1991). Urease appears to be

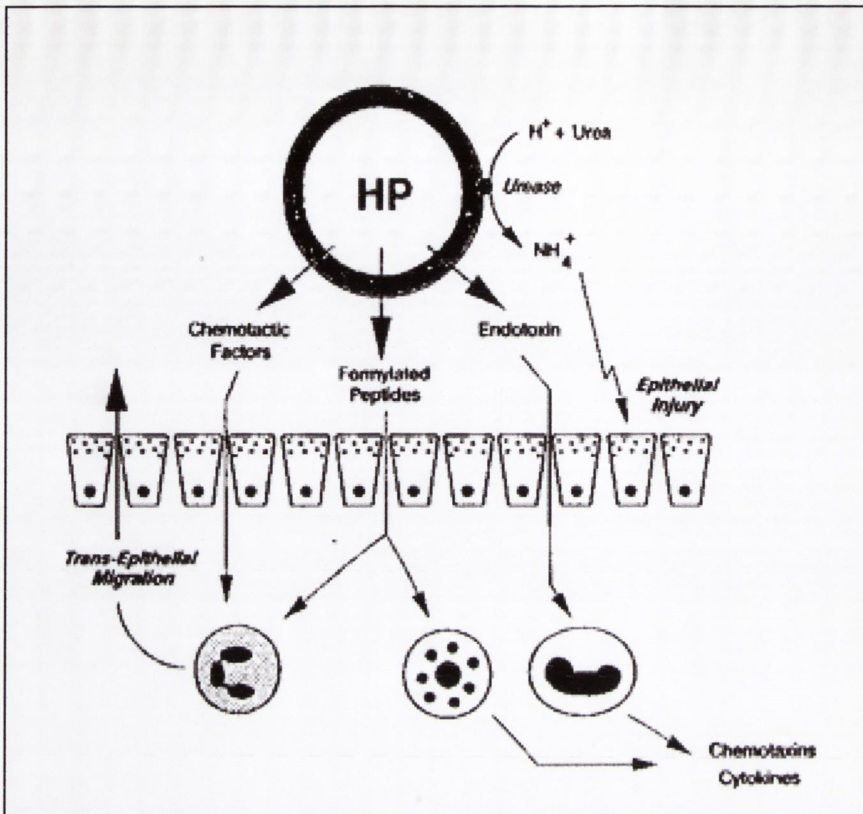


Fig. 1.4. Schematic diagram illustrating some of the possible pathogenic mechanisms through which *Helicobacter pylori* could induce active, chronic gastritis and epithelial injury. The urease activity of this bacterium enables the generation of ammonium, which may have direct cytotoxic actions on the epithelium. Chemotactic factors (possibly platelet-activating factor) and formylated peptides released by the bacterium could contribute to the trans-epithelial migration of neutrophiles. This migration likely damages the junctional compexes between epithelial cells. Formylated peptides can directly activate mast cells within the lamina propria, resulting in the release of pro-inflammatory mediators and cytokines. Endotoxin released from *H. pylori* could activate macrophages to release similar mediators and cytokines (Reproduced from Wallace, 1991).

necessary for protection of *H. pylori* from the bactericidal effect of acidic gastric juice by surrounding the organism with an alkaline micro-environment, and by enhancing bacterial adherence (Marshall *et al.*, 1990). Consistent with this, mutant strains of *H. pylori* lacking urease are unable to withstand low pH and colonize the stomach (Takahashi *et al.*, 1993).

Additionally, a pathogenic role has been proposed for urease. Smoot and colleagues (1990) suggested that urease is cytotoxic and may be responsible for epithelial cell damage associated with *H. pylori*. The pathogenic effect of ammonia to the gastric mucosa has also been documented in several studies. Ammonia in high concentrations induces vacuoles similar to those seen when cells are exposed to the VacA toxin of *H. pylori* (Xu *et al.*, 1990). In rats, the gastric mucosa thins if the animals are fed ammonia in drinking water (Kawano *et al.*, 1991). Finally, urease has been detected within macrophages in the lamina propria suggesting that it may contribute to pathological changes (Mai *et al.*, 1992).

H. pylori possesses the lipolytic enzymes phospholipase A_2 and C. These enzymes digest phospholipids, important components of cell membranes. One by-product is cytotoxic lysolecithin. The activities of phospholipase A_2 and lysolecithin in the basal gastric aspirates of those infected with *H. pylori* are significantly higher than those found in the controls (Langton and Cesareo, 1992). It has been suggested that *H. pylori* disrupts a protective phospholipid-rich layer with hydrophobic properties at the apical surface of mucus cells (Mauch *et al.*, 1993).

H. pylori also produces proteases, which contribute to the disturbance of the gastric mucus layer and allow H_2 back-diffusion towards the gastric mucosa resulting in tissue damage (Slomiany *et al.*, 1987; Sarosiek *et al.*, 1991). Windle and Kelleher (1997) have identified and characterized a zinc-dependent, calcium-stabilized metallopreotease activity from *H. pylori*, which is both expressed on the surface of the bacterium and released in a soluble form. The production of these proteases, lipases and phospholipases may destroy the integrity of the gastric mucus layer and expose it

to further damage from acid and pepsin. Finally, *H. pylori* superoxide dismutase (SOD) protects it from being killed in neutrophil phagocytic vacuoles. SOD converts superoxide into hydrogen peroxide, while catalase then breaks down the hydrogen peroxide into oxygen and water (Mori, 1997). Catalase is an abundant cytosolic enzyme produced by *H. pylori*, which protects the bacteria against H_2O_2 (Hazell *et al.*, 1991).

1.3.3 Adhesins

H. pylori adheres to the mucosal epithelial cells via adhesion pedestals (Hessey *et al.*, 1990). A number of putative "adhesins" such as fibrillar haemagglutinin (Evans *et al.*, 1988), membrane-associated phospholipids such as phosphatidyl ethanolamine (Lingood *et al.*, 1993) and N-acetylnuraminyllactose which binds to sialic acid (Huang et al., 1992) have been identified. Genome analysis identified more than 50 proteins and lipoproteins that may function as adhesins (Tomb *et al.*, 1997). Lewis antigens in *H. pylori* lipopolysaccharide (LPS) may mediate attachment to Lewis receptors and have a role in autoimmune gastritis induction, via formation of antibodies to Lewis antigens (Boren *et al.*, 1993). Corthésy-Teulas *et al.* (1996) have examined the role of pH on adhesion to human polarized epithelial cells and found that adhesion is enhanced at low pH. It has also been shown that low pH modifies the binding of *H. pylori* to host cells (Huesca *et al.*, 1996). The presence of these multiple adhesin genes may represent one of the mechanisms by which *H. pylori* undergoes antigenic variation.

1.3.4 *H. pylori* Lipopolysaccharides

Lipopolysaccharides (LPS), also known as endotoxins, comprise an important group of bacterial surface carbohydrate components, which have been implicated in a variety of biological interactions between Gram-negative bacteria and their hosts. *H. pylori* LPS has been ascribed low immunogenic, which may account for the inability of *H. pylori* to provoke an effective immune response compared to the LPS of other Gram-negative bacteria (Wilson *et al.*, 1996). The basis for the inertness may be caused by the underphosphorylation and underacylation of the lipid A component of this LPS

(Moran *et al.*, 1997). Also, the low endotoxin potency of *H. pylori* LPS may be due to a slow transfer of *H. pylori* LPS from LPS-binding protein to CD14, a cellular receptor for endotoxin (Kirkland *et al.*, 1997). This slower transfer could account for the lower pro-inflammatory effect of *H. pylori* LPS as compared to *Escherichia coli* or *Salmonella typhimurium* LPS (Semeraro *et al.*, 1996). Whether *H. pylori* LPS may contribute in part to the persistence of infection, remains to be determined. Nevertheless, *H. pylori* LPS plays a central role in the induction of gastritis and apoptosis (Piotrowski *et al.*, 1997).

1.3.5 Heat Shock Proteins

Heat shock proteins (Hsp) are produced by all *H. pylori* strains and they are so-called because their synthesis occurs in response to many environmental stresses such as thermal, chemical or physical stress, aiding bacteria to survive the harsh conditions in the stomach (Kaufmann *et al.*, 1991). However, antibodies against the *H. pylori* heat-shock proteins might act as autoantibodies by cross-reacting with the host's heat-shock proteins in the gastric mucosa (Negrini *et al.*, 1991). Furthermore, a specific B-cell response and antibodies against the *H. pylori* HspB protein are found in infected patients and share cross-reactivity with the human homologue, Hsp60 (Sharma *et al.*, 1997). The gene sequences of both bacterial and human heat shock proteins show a high degree of similarity (Macchia *et al.*, 1993).

1.3.6 Cytotoxin-Associated Gene A (*cagA*) and the Vacuolating Cytotoxin A (*vacA*)

Cytotoxic activity is present in about 50-60% of *H. pylori* isolates and it has been suggested in a number of studies that cytotoxin-producing strains are more often isolated from patients with peptic ulcer disease and gastric carcinoma (Figura *et al.*, 1989; Rudi *et al.*, 1997). Production of cytotoxin and related virulence genes are strongly associated with peptic ulcer disease and may play an important role in ulcer pathogenesis. Cytotoxin production is linked with the presence of the cytotoxin-associated gene (*cagA*) and the vacuolating gene (*vacA*). The *cagA* gene encodes for a 120-128 kDa protein (CagA protein), which acts as a serological marker for the *cag* pathogenicity island of *H. pylori* (Tummuru *et al.*, 1993). The CagA protein is an

immunodominant antigen in infected patients and serum antibodies to this protein are found in duodenal ulcer patients (Xiang *et al.*, 1993). Differences in the size of the protein are due to variability in the number of repeating sequences in the gene. Crabtree *et al.* (1991a) have shown that mucosal IgA antibodies to this protein were present in 100% of ulcer patients. It was also reported that only about 60% of *H. pylori* isolates possess CagA, but nearly all subsets associated with idiopathic peptic ulceration are positive.

A second cytotoxin of 87 kDa, VacA (Cover and Blaser, 1992), is responsible for creating vacuoles in epithelial cells, and these vacuoles can be seen *in vivo* in electron micrographs. These vacuoles can also be produced *in vitro* by incubating cell cultures with *H. pylori* supernatants (Figura *et al.*, 1989; Covacci *et al.*, 1993). The *vacA* gene is present in two different forms *1vacA* and *2vacA*. Strains harbouring *1vacA* produce vacuolating toxin, whilst strains with *2vacA* do not. 2vacA is a truncated version of *1vacA* and lacks the core region (Atherton *et al.*, 1995). *H. pylori* vacuolating toxin binds to a 140 kDa protein in human gastric cell lines, and it appears that VacA binding to a specific surface protein receptor is necessary for cell intoxication (Yahiro *et al.*, 1997). The role of cytotoxin production in the pathogenesis of *H. pylori*-associated diseases remains controversial, as animal studies failed to show any difference in bacterial colonization, epithelial vacuolation, or gastritis upon infection with toxigenic and non-toxigenic strains.

1.3.6.1 The *cag* Pathogenicity Island of *Helicobacter pylori*

The term "*cag* Pathogenicity Island" (*cag* PAI) refers to expression of multiple genes in *H. pylori* strains (Censini *et al.*, 1996; Covacci *et al.*, 1997). The *cag* PAI of *H. pylori* contains more than 40 genes encoding putative proteins associated with virulence factors and risk for gastroduodenal diseases (Censini *et al.*, 1996). It is believed that the *cag* gene products may function to export factors involved in *H. pylori* host-cell interaction, including those required to activate interleukin-8 production by gastric epithelial cells. CagA was initially identified in *H. pylori* strains which also secrete vacuolating cytotoxin. It is known that *vacA* and *cagA* genes are

distinct and lie apart on the *H. pylori* genome and the expression of VacA does not require CagA (Tomb *et al.*, 1997; Xiang *et al.*, 1995).

It has been previously reported that coculture of epithelial cells with *H. pylori* triggers signal transduction and tyrosine phosphorylation of an unidentified 145-kDa host protein (Backert *et al.*, 2000), but further studies by Stein *et al.* (2000) demonstrated that this protein is not derived from the host but rather is the bacterial immunodominant antigen CagA. Furthermore, infection of gastric epithelial cells by *H. pylori* strains such as NCTC11637, NCTC11916, and ATCC43579 induced tyrosine phosphorylation of a 145-kDa protein, and it has been suggested the phosphorylated CagA protein may play a crucial role in promoting the inflammatory responses of gastric mucosa to *H. pylori* infection (Asahi *et al.*, 2000). Recently, Higashi *et al.* (2002) have shown that wild-type *H. pylori* CagA protein formed a physical complex with the the SRC homology 2 domain (SH2)-containing tyrosine phosphatase SHP-2 in a phosphorylation-dependent manner and stimulated the phosphatase activity in gastric epithelial cells.

1.3.6.2 Cytotoxin Production and Disease Outcome

Several lines of evidence suggest a strong association between cytotoxin production and the development of peptic ulcer disease and gastric neoplasia. The most important aspect of these toxins is that they represent one of the major virulence factors and therefore they may be considered as key targets for prophylactic and therapeutic vaccine development against *H. pylori* infection. A high prevalence of gastroduodenal diseases is associated with virulent strains rather than non-virulent strains. It has been reported that strains producing vacuolating cytotoxin (Tox^+ VacA) are commonly isolated from patients with peptic ulcer disease, whilst (Tox^- VacA) strains lack cytotoxin activity (Figura *et al.*, 1989; Blaser, 1994). In addition, purified cytotoxin is able to induce ulceration in the stomach of mice (Telford *et al.*, 1994), and this may explain the linkage between cytotoxin production and peptic ulceration. The majority of Tox^+ VacA strains also produce $CagA^+$ whereas strains that do not produce

cytotoxin (Tox^- VacA) carry the cytotoxin gene but lack the gene coding for *cagA* (*cagA*$^-$) (Tummuru *et al.*, 1993; Covacci *et al.*, 1993; Xiang *et al.*, 1995).

The relationship between *H. pylori* toxin production and severity of disease may be due to indirect host-related inflammatory factors rather than to direct effect by the organism. Sozzi and colleagues (1998) have found that infection with $CagA^+$ *H. pylori* is associated with more severe gastritis and intestinal metaplasia. In contrast, Figura *et al.* (1998) found that both $CagA^+$ and $CagA^-$ colonies, infect most patients with non-ulcer dyspepsia and the prevalence of $CagA^+$ colonies was significantly higher in areas with mucosal atrophy. Parsonnet *et al.* (1997) showed that infection with CagA-positive *H. pylori* was associated with a 5.8-fold increased risk for gastric cancer, and *H. pylori*-infected individuals without antibodies to CagA showed a much lower (2.2-fold) risk for gastric cancer.

1.4 HOST IMMUNE RESPONSE TO *H. PYLORI*

H. pylori infection stimulates a strong mucosal and systemic antibody response and a marked mucosal inflammatory response. The induction of local immune response by this organism has stimulated considerable interest in gastric immunology. *H. pylori* may induce mucosal inflammation directly through the production of virulence factors, or indirectly through a consequence of inflammatory response, neutrophil activation and changes in host physiological responses.

1.4.1 Humoral Immune Response to *H. pylori*

The host reaction to *H. pylori* infection is associated with a specific immunoglobulin IgA and IgG response to the bacterium in the gastroduodenal mucosa (Rathbone *et al.*, 1986; Crabtree *et al.*, 1991a), whilst there is little evidence of a local specific IgM response, except in acute gastritis (Sobala *et al.*, 1991). The IgG subclass response in persons infected with *H. pylori* involve all four subclasses but is predominatly of the IgG_1 and IgG_2 subclasses (Bontkes *et al.*, 1992; Andersen and Gaarslev, 1992). Although local IgM, IgA and IgG production occurs *in vivo*, the humoral response to *H. pylori* is unable to clear the infection in humans leading to chronic inflammatory

changes within the gastric mucosa. This variation in immune host response appears to be dependent on the degree of mucosal damage during *H. pylori* infection in addition to genetic heterogeniety of the pathogen (Shimoyama and Crabtree, 1998).

Measurement of serum anti-*H. pylori* antibody levels is a sensitive indicator of *H. pylori* infection and represents a useful tool for epidemiological studies. It has been shown by using this method that individuals infected with CagA-positive *H. pylori* are at increased risk of gastric cancer (Parsonnet *et al.*, 1997). In addition, antibody production by cultured gastric mucosa has been used to detect an immunological history of *H. pylori* infection. Crabtree *et al.* (1993) have shown that patients with gastric cancer and without current *H. pylori* infection were found to have a positive immunoblot, indicating that they had been infected with *H. pylori* in the past. Birkholz *et al.* (1998) have also shown using gastric juice that the majority of *H. pylori*-specific IgA was not of the secretory type. This may be a consequence of the damaged mucosal epithelium at the site of *H. pylori* infection. The secretory IgA is thought to be a major defence against mucosal pathogens. An alternative possibility to account for the lack of surface antibody deposition is that *H. pylori* can catalytically reduce the intramolecular disulfide bonds present in IgG and IgA, a process potentially mediated by the thioredoxin system of *H. pylori* (Windle *et al.*, 2000). Despite the strong antibody response, failure of the host response to clear infection immunologically may not preclude an effective response to prevent reinfection once the host has become free of *H. pylori*.

1.4.2 Cellular Immune Response to *H. pylori*

Colonization of the gastric mucosa by *H. pylori* is associated with a strong inflammatory reaction which may result from either release of products from *H. pylori* or bacterial adherence, but the exact mechanism is still unclear. The initial response to *H. pylori* infection appears to be associated with the inflammatory response, indirectly stimulated by neutrophil infiltration and the release of inflammatory mediators or directly induced by bacterial factors (Crabtree, 1996). In agreement with this, several reports have shown that *H. pylori* secretes products with chemotactic activity. This

results in the recruitment of inflammatory cells (neutrophils, macrophages and monocytes) to the lamina propria with subsequent release of inflammatory cytokines such as IL-1, IL-6, TNF-α and IL-8 as well as reactive oxygen metabolites (Mai *et al.*, 1992; Mai *et al.*, 1991; Mooney *et al.*, 1991; Crabtree *et al.*, 1991b; Noach *et al.*, 1994).

Biopsies from *H. pylori*-infected patients have increased numbers of neutrophils, macrophages, plasma cells and lymphocytes, and increased levels of cytokines such as IL-1β, IL-6, IL-8, IL-10, TNF-α, INF-γ and platelet activating factor (PAF) (Dunn, 1993; Crabtree *et al.*, 1991b; Fan *et al.*, 1993; Noach *et al.*, 1994). An important factor in the pathogenic process of *H. pylori* infection is the induced secretion of IL-8. The induction of epithelial IL-8 secretion following bacterial infection promotes mucosal neutrophil infiltration and activation (Crabtree *et al.*, 1994). Moreover, $CagA^+$ *H. pylori* strains induce significantly greater IL-8 mRNA and protein in gastric epithelial cells than do $CagA^-$ strains (Crabtree *et al.*, 1995; Sharma *et al.*, 1995). The rapid secretion of IL-8 by gastric epithelial cells demonstrates that the gastric epithelium makes an active contribution to regulation of mucosal cellular responses to the bacterium.

Expression of TNF-α, IL-1β, IL-8 (Sharma *et al.*, 1998, Busam *et al.*, 1992, Kopp and Ghosh, 1995) and adhesion molecules such as ICAM-1 (Mori *et al.*, 2000), is regulated by the transcription factor NF-κB through binding to specific sites in the enhancer/promoter region of their genes. Several genes in the *H. pylori cag* PAI appear to be involved in inducing IL-8 response to *H. pylori* including activation of NF-κB and reactive oxygen intermediates (Shimada and Terano, 1998). Activated NF-κB may be involved in the inflammatory process in *H. pylori*-associated gastritis through the up-regulation of cytokines and adhesion molecule expression. Eradication of *H. pylori* from ulcer patients results in a reduction in both antral IL-8 mRNA expression and surface epithelial lesions, suggesting that cytokines play a role in mucosal damage (Moss *et al.*, 1994).

The cellular immune response to *H. pylori* is characterized by a complex array of effector T cells that produce cytokines that facilitate both cell-mediated and antibody responses. Two classes of effector T cells have been identified according to MHC restriction for antigen recognition, T helper (CD4+) and T suppressor (CD8+) (Di Tommaso *et al.*, 1995). At least two distinct types of T helper (Th) lymphocyte subsets called Th1 and Th2 have been identified with distinct patterns of cytokine secretion. Th1 cells secrete IL-2, IFN-γ and TNF-β, and that mediate cell-mediated immunity, macrophage activation, cytotoxicity and help for B cell production of opsonizing and complement-fixing antibodies. Th2 cells secrete IL-4, IL-5, IL-6 and IL-10, and that induce the production of high levels of antibodies of all isotypes including IgE, tend to inactivate macrophages, recruit and activate eosinophils and mast cells (Del Prete, 1998).

The type of Th cell response associated with *H. pylori* infection is controversial. It has been reported that the type of Th cell response against *H. pylori* may vary according to the antigen involved. A polarized Th1 response may play a role in the genesis of peptic ulcer, whereas a local Th0 response, including IL-4 production, may represent individual host responses which contribute to the prevention of ulcer complications (D'Elios *et al.*, 1997). Karttunen *et al.* (1995) have also shown that *H. pylori* infection is associated with a predominant Th1 response. Bamford *et al.* (1998) have shown that gastric T cells produce IFN-γ, IL-2 and low levels of the Th2 cytokines, IL-4 and IL-5. This provides further evidence that Th1 responses are responsible for the inflammatory response and a deficiency of Th2 response results in inability to eliminate the bacteria. Although *H. pylori* can stimulate the immune system to produce cytokines and antibodies, it possesses an immune suppressive activity and that suppresssion of host defense mechanism may contribute to the pathogenesis of this disease (Knipp *et al.*, 1993).

Furthermore, *H. pylori* induces blood monocytes and polymorphs to release reactive oxygen species *in vitro*, and in the gastric and duodenal mucosa of infected patients (Drake *et al.*, 1998; Nielsen and Anderson, 1992). Host tissues are normally protected

against oxidative damage by antioxidants such as vitamins C and E. *H. pylori* gastritis decreases the amount of gastric ascorbic acid, and these ascorbate levels rise after eradication of *H. pylori* (Sobala *et al.*, 1993). Also, tissue concentrations of α-tocopherol (vitamin E) are decreased in subjects with *H. pylori* infection (Wang *et al.*, 1998). It is important to note that variations in host responses with respect to alterations in gastric physiologic responses may be important in regulating the inflammatory response to the pathogen.

1.5 EFFECT OF *H. PYLORI* ON GASTRIC PATHOPHYSIOLOGY

Infection with *H. pylori* produces alterations in gastroduodenal physiology but the connection between these changes and the development of gastric or duodenal ulcers is still unclear. These alterations include increased gastrin secretion, decreased mucosal somatostatin, and enhanced gastrin-releasing peptide (GRP)-stimulated acid secretion, which are reversed following bacterial eradication (Graham *et al.*, 1993; Calam *et al.*, 1996). Many inflammatory mediators have been shown to affect the gastric mucosa and mucosal blood flow, thereby increasing the susceptibility of the mucosa to damage induced by acid or amplifying the inflammatory response through their chemotactic effects on other leucocytes. Beales *et al.* (1997) have shown that IL-8 and other cytokines stimulate gastrin release from canine G cells, and it is known that IL-8 secretion is increased in *H. pylori* infection. The extent of inflammation in the gastric mucosa may be an important factor in determining the changes in physiology associated with *H. pylori* infection.

1.6 EPITHELIAL RESPONSE TO *H. PYLORI* INFECTION

During infection with *H. pylori*, several changes to the epithelium occur such as increased cell proliferation, transcriptional factor activation, up-regulation of adhesion molecules and cell death.

1.6.1 Gastric Epithelial Cell Proliferation

The balance of inhibitory or stimulatory influences on epithelial growth may mediate disease outcome. An increase in epithelial cell proliferation is considered to be one of

the earliest mucosal changes in the development of gastric cancer and serve as an indicator of gastrointestinal adenocarcinoma (Deschner *et al.*, 1972). Increased proliferation of epithelial cells was found to be one of the mechanistic links between *H. pylori* infection and gastric carcinoma. Studies of biopsy samples obtained from *H. pylori*-infected subjects indicate that gastric epithelial proliferation is increased and reverts to normal after eradication of the infection (Cahill *et al.*, 1995). Fan *et al.* (1996a) demonstrated that both *H. pylori* and *H. pylori*-induced cytokines can stimulate proliferation of gastric epithelial cells. Conversely, most studies of gastric epithelial cell lines demonstrate an inhibitory effect of *H. pylori* on epithelial cell growth compared to tissue samples. This is likely due to factors such as cytokine and growth factors released from other cell types within the gastric mucosa. Increased cell proliferation may be a compensatory response to increased cell death that has been shown to occur through *H. pylori*-induced apoptosis.

1.6.2 Apoptosis

Apoptosis plays an important role in the modulation of cell development and differentiation. Apoptotic bodies have been identified in all regions of the stomach, suggesting that apoptosis is important in the turnover of gastric epithelia. It is thought that increased apoptosis predisposes to the development of cancer through increased cell turnover and enhanced mutations **[Fig. 1.5]** (Zarrilli *et al.*, 2000).

A growing body of evidence indicates an association between *H. pylori* infection and increased apoptosis. Moss and colleagues (1996) have found that the number of apoptotic cells is high in *H. pylori*-infected compared to non-infected tissues. *H. pylori* stimulates a decrease in epithelial cell growth and DNA synthesis in parallel with increased apoptotic cell death (Wagner *et al.*, 1997). Alterations in epithelial cell growth caused by *H. pylori* infection were suggested to be an early event in the apoptotic process, as gastric epithelium from children infected with *H. pylori* showed increased apoptotic and proliferating indices compared with non-infected controls (Jones *et al.*, 1997). It is unclear how *H. pylori* infection increases cell loss, but it appears that many factors are involved in the induction of apoptosis. For example,

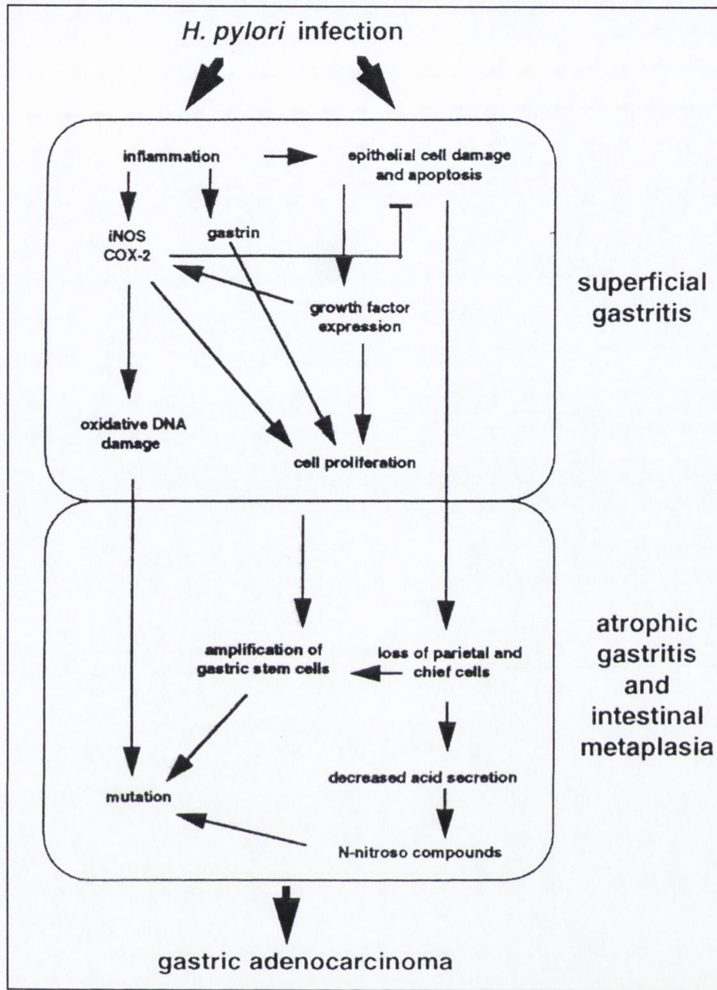


Fig. 1.5. Model for the interaction of environmental factors and *H. pylori* infection in gastric carcinogenesis. *H. pylori* by causing inflammation and cell proliferation, enhances the likelihood of mutation in gastric epithelial cells (Reproduced from Zarrilli *et al.*, 1999).

certain genes encoding for proteins such as p53, Bcl-2 and Fas expression, that are known to regulate cell death were found to be associated with *H. pylori*-induced apoptosis (Houghton *et al.*, 1999; Kodama *et al.*, 1998; Moss *et al.*, 1997).

Apoptosis can be mediated through activation of the CD95 receptor (APO-1/Fas) and ligand system. CD95-mediated apoptosis is considered to be an important mechanism responsible for maintenance of peripheral tolerance and for termination of an immune response. CD95 (APO-1/Fas) is a member of the TNF receptor family (Oehm *et al.*, 1992), and its ligand (CD95L) is a type II transmembrane protein of the TNF family (Suda *et al.*, 1993). This molecule is recognized by the Fas receptor on T cells and after binding of Fas to Fas ligand, cell death by apoptosis occurs. It has been shown that gastric epithelial cells are induced to express Fas and Fas ligand during *H. pylori* infection (Rudi *et al.*, 1998). Thus, the involvement of the CD95 pathway in *H. pylori*-induced gastric epithelial apoptosis may explain the role of CD95 receptor and its ligand system in mediation of epithelial injury caused by *H. pylori*.

1.6.3 Generation of Reactive Oxygen Species by *H. pylori*

Reactive oxygen species (ROS) are generated during the respiratory burst in phagocytic cells through mitochondrial and microsomal electron transport chains and by oxidant enzymes including xanthine oxidase, cyclooxygenase, and lipoxygenase. ROS can be produced endogenously by metabolic processes, cytokines or growth factors and neutrophil or macrophage activation at sites of injury (Janssen *et al.*, 1993). The production of ROS is regulated by several endogenous systems including catalase, superoxide dismutase and glutathione peroxidases. In addition to the catalytic breakdown of ROS, tissues contain several free radical scavengers such as vitamins C and E, in order to protect cells against ROI-induced damage (Scandalios, 1997; Chance *et al.*, 1979; McCord and Fridovich, 1969). High levels of ROIs or depletion of antioxidants have been found in various pathologic conditions such as AIDS, inflammatory diseases and cancer. However, at moderate concentrations, ROIs modulate various cellular functions, cell signalling and gene expression (Schreck *et al.*, 1991).

H. pylori induces directly via bacterial factors or indirectly via IL-8 and other cytokines, neutrophil activation with subsequent ROS production **[Fig. 1.6]** (Crabtree, 1996). Patients with duodenal ulceration have increased plasma concentrations of malandioldehyde, a marker for free oxygen radical reactions and a significant reduction in the concentration of the antioxidant glutathione (Jankowski *et al.*, 1991). Also, gastric and duodenal biopsies have increased levels of ROIs in *H. pylori* infected samples (Davies *et al.*, 1992). The urease produced by *H. pylori* can damage the host by inducing monocytes and polymorphonuclear granulocytes to secrete cytokines and release reactive oxygen radicals (Davies *et al.*, 1994).

In general, *H. pylori* activates neutrophil, monocyte and macrophage infiltrations to produce oxygen free radicals which cause DNA damage to the adjacent cells which subsequently leads to gene modifications that are mutagenic and/or carcinogenic (Baggiolini and Wymann, 1990). Therefore, suppressing the oxidative stress in gastric mucosa could represent a chemopreventive therapy for damage caused by *H. pylori* infection.

1.6.4 Cell Adhesion Molecule Expression due to *H. pylori* Infection

Cell adhesion molecules are multifunctional proteins involved in a number of regulatory processes, including cell growth, differentiation, proliferation, migration, regeneration and interaction between lymphocytes and epithelial cells. The increased expression of adhesion molecules during mucosal inflammation as a result of neutrophil activation and recruitment due to *H. pylori* stimulates the gastric epithelium to participate in the inflammatory process. *H. pylori* induces the expression of several cell adhesion molecules on gastric epithelial cells including the cell surface adhesion molecules CD44, ICAM-1/CD54, lymphocyte function-associated antigen-1 (LFA-1), vascular cell adhesion molecule-1 (VACM-1), human mucosal lymphocyte (antigen)-1 (HML-1/$\alpha^E\beta_7$), very late antigen-4 (VLA-4) and p-selectin (Fan *et al.*, 1995a and b; Hatz *et al.*, 1997; El Kaissouni *et al.*, 1998; Higuchi *et al.*, 1997; Kelleher *et al.*, 1997).

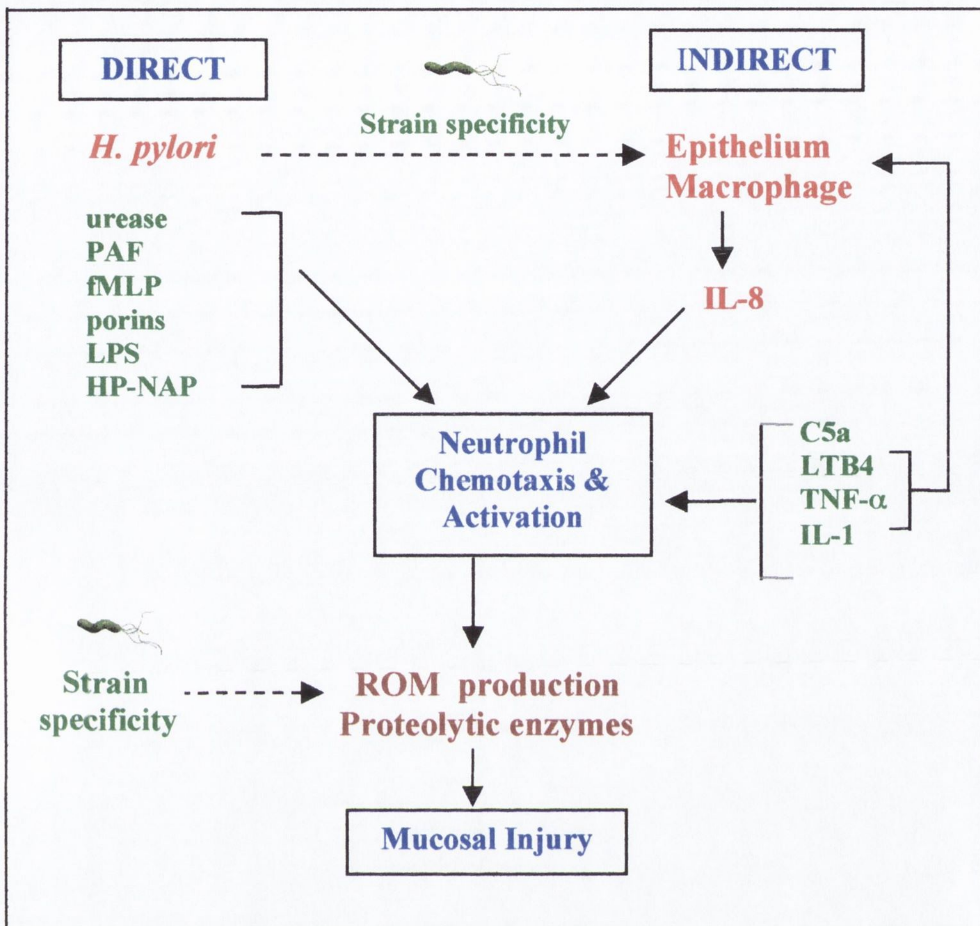


Fig. 1.6. Direct and indirect stimulation of neutrophil chemotaxis and activation in *Helicobacter pylori* infection. PAF; platelet activating factor, fMLP; N-formyl-methonyl-leucyl-phenylalanine, LPS; lipopolysaccharide, HP-NAP; *H. pylori* neutophil activating protein, C5a; complement 5a, LTB4; leukotriene B4, ROM; reactive oxygen metabolite (Reproduced from Crabtree, 1996).

The cell surface adhesion molecule CD44 (Haynes *et al.*, 1989) is expressed on a wide variety of cell types including epithelial cells (Mackay *et al.*, 1994; Fox *et al.*, 1994). CD44 exists in a number of isoforms as a result of alternate splicing of a family of variant exons (termed v1-v10), varying in their molecular weight from 85-90 kDa to 200 kDa (Mackay *et al.*, 1994; Secreaton *et al.*, 1992). CD44 is a multifunctional, ubiquitously expressed glycoprotein that participiates in the recruitment of leukocytes to sites of inflammation (de Grendele *et al.*, 1996). Expression of CD44 is elevated in inflamed tissues (Haynes *et al.*, 1991). CD44 is important in mediating T cell and macrophage adhesion, migration and activation, lymphocyte homing, natural killer cell-mediated cytotoxicity, haemopoesis metastasis, and in the pathogenesis of inflammatory diseases (Haynes *et al.*, 1989; de Grendele *et al.*, 1996; Haynes *et al.*, 1991). Coculture of the gastric epithelial cell line AGS with *H. pylori* or cytokine-rich supernatants from *H. pylori*-activated PBMCs results in up-regulation of CD44 and CD44 v9 expresssion (Fan *et al.*, 1995a and 1996b).

ICAM-1 expression is also up-regulated by *H. pylori* (Fan *et al.*, 1995a; Crowe *et al.*, 1995). ICAM-1, a 90 kDa cell surface glycoprotein, is the prototype member of a family of ligands for the β-2 integrin LFA-1, and is thought to play a critical role in immune system (Doughrety *et al.*, 1988). Yosihida *et al.* (1993) have demonstrated that water extracts of *H. pylori* promote neutrophil-endothelial cell adhesive interactions with up-regulated expression of CD11b/CD18 on neutrophils and initiate a CD11a/CD18- and CD11b/CD18-ICAM-1 dependent PMN adhesion to endothelial cells. Transcriptional activation of the expression of CD44 and ICAM-1 on gastric epithelial cells were examined during in this present study in order to explore the mechanism by which *H. pylori* regulates CD44 and ICAM-1 expression.

1.7 TRANSCRIPTION FACTORS

Epithelial cells and the mucosa form a barrier that impedes the invasion of microorganisms and their products. Once pathogens have detected by epithelial cells, a number of signal transduction pathways are activated within the infected cells and initiate defensive responses by the host. These signalling pathways result in the

activation of NF-κB and/or AP-1, two transcription factors important in driving expression of genes involved in inflammatory response. Direct contact between *H. pylori* and gastric epithelial cells induces NF-κB activation (Keates *et al.*, 1998; Maeda *et al.*, 2000). Moreover, *H. pylori* induces AP-1 DNA-binding and selectively activates the ERK/MAP kinase pathway, results in Elk-1 phosphorylation and increased c-fos transcription (Meyer-ten-Vhen *et al.*, 2000). NF-κB signalling and activity induced by microbial pathogens determine the outcome of the cellular innate immune defense. In the case of *H. pylori* infection, the chronic inflammatory response in the mucosal surface appears to be a prerequiste for the development of gastic adenocarcinoma. Therefore, exploitation of the mechanisms causing activation represents an important field of potenial therapeutic intervention that may be relevant to several inflammatory disease states. In this study, the effect of pH-dependent effect of *H. pylori* on the expresssion of NF-κB and immediate-early growth response-1 (Egr-1) and the signalling pathways that converge on the activation of these transcription factors were studied.

1.7.1 NUCLEAR FACTOR-KAPPA B (NF-κB)

This transcription factor was described as a B cell-specific factor that binds to a specific DNA site (10-base pair motif) in the intronic enhancer of the immunoglobulin κ light chain gene (Sen and Baltimore, 1986). Cloning of this factor revealed a group of proteins, referred to as the family of NF-κB/Rel proteins (Nabel and Verma, 1993; Ghosh *et al.*, 1998).

1.7.1.1 Members of NF-κB/Rel and IkappaB Proteins

NF-κB is the prototype of the Rel protein family of dimeric transcription factors **[Fig. 1.7]** (Perkins, 2000). The Rel protein family has been divided into two groups based on differences in their structure, function, and synthesis. The first group consists of p50 (NF-κB1) and p52 (NF-κB2), which are synthesized as precursor proteins of 105 and 100 kDa, respectively. The second group includes p65 (RelA), Rel (c-Rel), RelB and the Drosophila Rel proteins, dorsal and Dif, which are not synthesized as precursors (Baeuerle and Henkel, 1994; Siebenlist *et al.*, 1994). The subunits of NF-

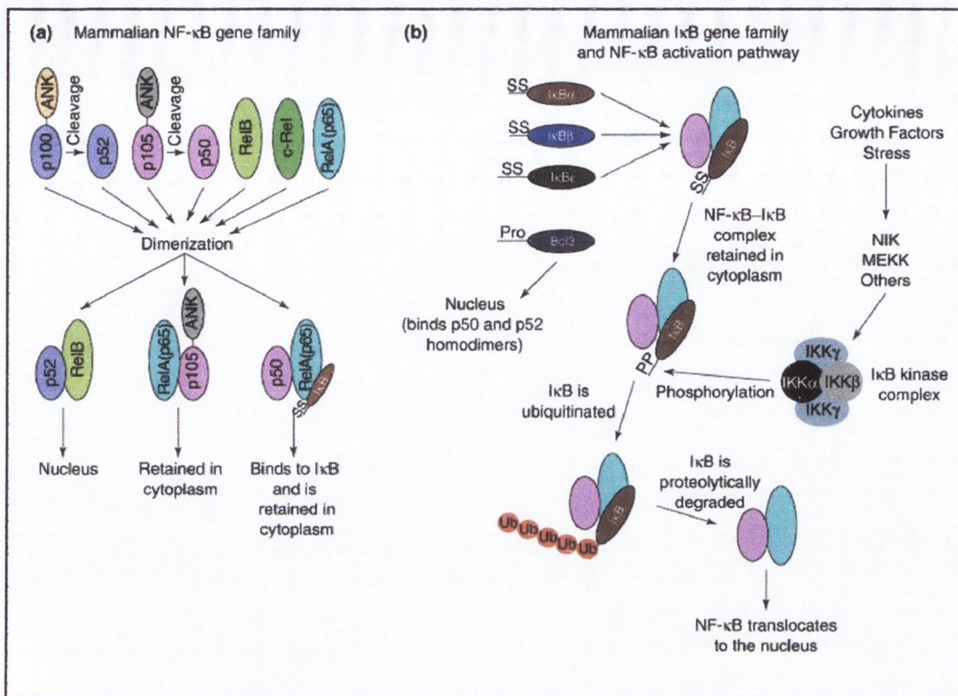


Fig. 1.7. The multigene NF-κB and IκB family and the mechanism of NF-κB activation. **(a)** The five members of the mammalian Rel/NF-κB family. With a few exceptions, all NF-κB subunits homo- and heterodimerize. Most NF-κB complexes then associate with a member of the IκB family, which retains them in the cytoplasm prior to cellular activation. **(b)** The four members of the mammalian IκB family and the mechanism of NF-κB activation. IκB interacts with NF-κB complexes, masking their nuclear localization sequences and retaining them in the cytoplasm. IκB preferentially interacts with c-Rel–RelA complexes, while Bcl3 translocates directly to the nucleus and binds to p50 and p52 homodimers, and functions as a transcriptional activator. Upon cellular stimulation, IκB kinase (IKK) kinases activate the IKK complex, which then phosphorylates two conserved serine residues in the N-terminal domain of IκB. This targets IκB for polyubiquitination and subsequent proteolytic degradation by the proteasome, which then allows NF-κB to translocate to the nucleus and stimulate the expression of its target genes. Abbreviations: PP, phosphorylation sites; Pro, proline-rich transactivation domain present in the N terminus of Bcl3; SS, the two conserved serine residues within the N termini of the IκBs; Ub, ubiquitin (Reproduced from Perkins, 2000).

κB1p50 and NF-κB2p52 and the Rel proteins, RelAp65, RelB and c-Rel, have sequence homology within the N-terminal 300 amino acids, the so-called Rel homology domain, and this region contains the DNA-binding and dimerization domain and the nuclear localization signal (Ghosh *et al.*, 1998; Siebenlist *et al.*, 1994).

Members of both groups of Rel proteins can form homodimers or heterodimers, although NF-κB is classically defined as a p50/RelA heterodimer, the most abundant and biologically active dimer. Other combinations of Rel proteins can function identically to NF-κB, including homodimer of p50, homodimer of p52 and heterodimer of RelAp65/p105. Homodimeric complexes of NF-κB1p50 and NF-κB2p52 have an inhibitory function, whereas Rel protein complexes activate transcription (Ghosh *et al.*, 1998; Siebenlist *et al.*, 1994; Beg and Baldwin, 1993).

NF-κB was initially believed to be lymphoid specific because of its constitutive presence in the nuclei of mature B cells. However, it was subsequently found in the resting state of most cells, where NF-κB is sequestered in the cytoplasm complexed with specific inhibitory proteins, called IkappaB (IκBs) (Baldwin, 1996; Verma *et al.*, 1995; Beg and Baldwin, 1993). Baeuerle and Baltimore (1988a) demonstrated that cytosolic fractions of unstimulated cells contain a form of NF-κB that can be activated *in vitro* upon addition of the detergent desoxycholate (DOC). These detergents were shown to release an inhibitory protein (IκB) from the cytoplasmic NF-κB. IκB reversibly and specifically inactivates NF-κB-DNA binding (Baeuerle and Baltimore, 1988b). IκB family of proteins includes IκB-α, IκB-β, IκB-γ, IκB-ε, and bcl-3. In addition, p105, the precursor of p50, and p100, the precursor of p52, also act as inhibitors of NF-κB (Verma *et al.*, 1995; Inoue *et al.*, 1992; Betts and Nabel, 1996; Ganchi *et al.*, 1992).

IκB-α, a 37 kDa protein, was the first member of IκB family to be identified and the human gene was cloned as MAD-3 (Haskill *et al.*, 1991). MAD-3 is highly homologous to cDNA clones isolated from chicken (Davies *et al.*, 1991), rat (Tewari

et al., 1992), and pig (de Martin *et al.*, 1993), called pp40, RL-TF1, and ECI-6, respectively. IκB-β, an acidic 43 kDa protein, is immunologically unrelated to IκB-α and appears to depend on bound phosphate for its inhibitory activity (Link *et al.*, 1992). The structural features of IκB proteins are complex. IκB proteins contain between five and seven ankyrin (Ank) repeat domains, each about 33 amino acids, which form a unit which interacts with NF-κB/Rel regions (Baeuerle and Baltimore, 1996; Siebenlist *et al.*, 1994; Blank *et al.*, 1992). IκB proteins consist of an N-terminal domain required for proteolytic degradation, a central domain with Ank repeats required for interaction with NF-κB, and a C-terminal domain (PEST domain) which is essential for sequestration of NF-κB in the cytoplasm (Neurath *et al.*, 1998).

The precursor proteins of p50 and p52, so-called p105/IκB-γ and p100/IκB-δ, contain, in addition to the Rel homology domain, Ank repeats and they can function as IκBs (Betts and Nabel, 1996; Naumann and Scheidereit, 1994). Binding of IκBs to NF-κB/Rel blocks the nuclear localization signal of NF-κB/Rel, causing cytoplasmic retention of the ternary complex, NF-κB/Rel-IκB (Ghosh *et al.*, 1998; Finco and Baldwin, 1995; Davies *et al.*, 1991).

Some IκB proteins have been found in the nucleus (Nolan *et al.*, 1993). This suggests that these proteins do not necessarily reside as an anchor in the cytoplasm to fulfill their function. Nuclear uptake of human IκB-α is blocked when RelA is overexpressed which indicates that the subunits mutually control their nuclear uptake in a dynamic fashion (Zabel *et al.*, 1993). The IκB-like protein bcl-3 can function as transcriptional coactivator after association with p52 (Bours *et al.*, 1993), although bcl-3 has been shown to inhibit p50-containing complexes (Nolan *et al.*, 1993; Franzoso *et al.*, 1992). These data indicate that the interaction between IκB and NF-κB/Rel proteins appear to be a central regulatory mechanism on the activity and distribution of NF-κB transcription factors.

1.7.1.2 Conditions that Activate NF-κB

Regulation of NF-κB is part of a cellular response system to many different noxious stimuli **[Table 1.2]**. Signals that activate NF-κB include the cytokines TNF-α, IL-1 and IL-2, LPS, PMA, PHA, ionizing radiation, UV light, H_2O_2, immunoglobulin receptor-cross linking, cross linking of surface CD3 or CD28, free radicals, calcium ionophores, numerous viral and bacterial products, and certain chemical agents (Baldwin, 1996; Verma *et al.*, 1995; Baeuerle and Henkel, 1994; Siebenlist *et al.*, 1994; Beg and Baldwin, 1993). Upon activation, the inhibitory IκBs proteins are phosphorylated and degraded, which facilitate NF-κB/Rel dimers to translocate to the nucleus and bind to target genes and regulate their transcription.

1.7.1.3 IkappaB Phosphorylation and Degradation

Potent NF-κB activators can induce complete degradation of IκBs, particuarly IκB-α, within minutes **[Fig. 1.8]** (Schmid and Alder, 2000). IκB-α is phosphorylated at serine 32 and serine 36 (Traenckner *et al.*, 1995; Brown *et al.*, 1995), followed by ubiquitination on at least two amino-terminal lysines and becomes a target for degradation by the ubiquitin-26S proteasome pathway (Chen *et al.*, 1995; Henkel *et al.*, 1993). NF-κB1p105 and NF-κB2p100 complexes are activated by phosphorylation and cleavage of full-length NF-κB proteins. This results in the N-terminal subunits p50 or p52 complexed to Rel proteins that translocate to the nucleus, whereas the C-termini are degraded (Mercurio *et al.*, 1993).

Recent studies suggest that different kinases are responsible for phosphorylation of IκB-α and so-called IkappaB kinases (IKKs), which target IκB-α to ubiquitin (Régnier *et al.*, 1997). Phosphorylation of IκB-α by IkappaB kinases leads to recognition of the IκB-α molecule by the proteasome complex and subsequent degradation of the IκB-α (Chen *et al.*, 1995). The IkappaB kinases are specific for the N-terminal regulatory serines of IκBs and responsive to a number of potent NF-κB activators, such as IL-1 and TNF-α, which stimulate their activity (Mercurio *et al.*,

Conditions that activate NF-κB transcription factor

Class	Inducing agent
Bacteria	*Shigella flexneri* *Mycobacterium tubercluosis* *Lysteria monocytogenes* *Helicobacter pylori*
Bacterial products	Lipopolysacchride Exotoxin B Toxic shock syndrome toxin 1 Muramyl peptides
Viruses	Human immunodeficiency virus type 1 (HIV-1) Human T-cell leukemia virus type 1 (HTLV-1) Hepatitis B virus (HBV) Herpes simplex virus type 1 (HSV-1) Human herpes virus 6 Newcastle disease virus Sendai virus Epstein-Barr virus (EBV) Adenovirus
Viral products	Double-stranded RNA Tax (from HTLV-1) Hbx (from HBV) MHBs (from HBV) Epstein-Barr nuclear antigen 2; EBNA-2 (from EBV)
Eukaryotic parasite	Theileria parva
Inflammatory cytokines and growth factors	Tumor necrosis factor-alpha (TNF-α) Lymphotoxin (LT) (TNF-β) Interleukin-1 Interleukin-2 Interleukin-17 Interleukin-18 Interferon-gamma (INF-γ) Leukotriene B4 Lymphocyte inhibitory factor Platelet activating factor Nerve growth factor (NGF) Platelet-derived growth factor

Table 1.2 (1). Conditions that activate NF-κB transcription factor. (Reproduced from Baeuerle and Henkel, 1994).

Conditions that activate NF-κB transcription factor

Class	Inducing agent
T cell mitogens	Antigen Lectins (PHA, ConA) Calcium ionophores Anti-CD3 Anti-CD2 Anti-CD28
B cell mitogen	Anti-surface IgM
Fibroblast mitogen	Serum
Protein synthesis inhibitors	Cycloheximide Anisomycin Emetin
Physical stress	UV light Gamma radiation
Oxidative stress	Hydrogen peroxide Butyl peroxide Antimycin A Oxidised lipids
Chemical agents	Okadaic acid Phorbol esters Ceramide Calculin Ouabain Colchicine Nocodazol Sphingomyelinase Dibutyl cAMP Genistein Anthracyclin antibiotics
Others	Prostaglandin E2 (PGE2) Insulin Thrombin

Table 1.2 (2). Conditions that activate NF-κB transcription factor. (Reproduced from Baeuerle and Henkel, 1994)

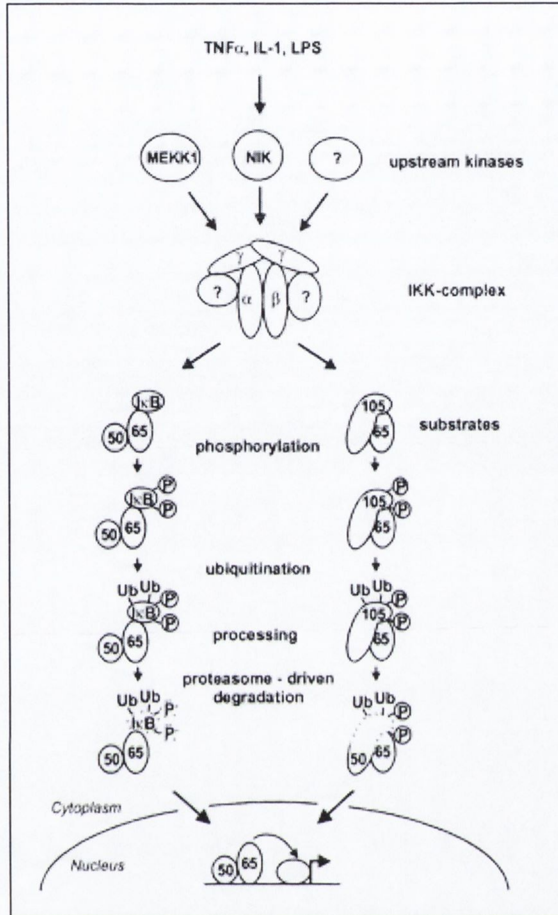


Fig. 1.8. Different signals converge at a multisubunit kinase complex that contains the IκB kinases IKKα and IKKβ (Reprouded from Schmid and Alder, 2000).

1997; Zandi *et al.*, 1997). The extent to which IKK is activated appears to dictate the kinetics of IκB degradation.

The IkappaB kinase complex (IKK) is a 500-900 kDa protein complex, which comprised of three IKK polypeptides. Two of these polypeptides, IKKα (IKK1), and IKKβ (IKK2), are catalytic subunits (Mercurio *et al.*, 1997; Zandi *et al.*, 1997), whereas the third polypeptide, IKKγ (NEMO), is regulatory (Rothwarf *et al.*, 1998). IKKα and IKKβ have very similar structures with protein kinase domains at their N-terminal, a leucine zipper (LZ), and a helix-loop-helix (HLH) motif at their C-terminal region, whereas IKKγ/NEMO is composed of three large α–helix regions, including a LZ, and lacks a catalytic domain (Karin, 1999). IKKα and IKKβ form homodimers and heterodimers in a manner that depends on the integrity of their LZ motifs (Zandi *et al.*, 1997), and both kinases activate NF-κB/Rel when overexpressed. Dominant negative mutations in the kinase domain of either protein can suppress TNF-α or IL-1 induction of NF-κB/Rel. Recent biochemical and gene knockout studies have demonstrated the importance of IKKα for NF-κB activation in morphogentic events, proliferation and differentiation of epidermal keratinocytes (Mercurio *et al.*, 1997, Hu *et al.* 1999). Mice that are lacking the *IKKβ* gene had extensive liver damage from apoptosis and died as embroys. Hepatocyte apoptosis in the knockout mice appears to be induced by TNF-α because crossing the *IKKβ* mice with animals lacking the receptor for TNF-α rescues them from embryonic death ((Li *et al.*, 1999).

IKK activation also requires IKKγ subunit, which interact preferentially with IKKβ, as no IKK or IKKβ activity can be elicited in IKKγ/NEMO-deficient cells (Rothwarf *et al.*, 1998). Both IKKα and IKKβ can be activated by the structurely related upstream kinases MEKK1 and NF-κB-inducing kinase (NIK), most likely through phosphorylation of specific serine residues within the T-loop (activation domain) in the catalytic domain of each IKK (Tojima *et al.*, 2000). NIK is the most potent activator identified to date (Karin and Delhase, 1998) and was suggested to be a direct IKKα kinase (Ling *et al.*, 1998). Moreover, NIK coexpression efficiently stimulates

IKKα–associated IKK activity in IKKβ–expressing cells (Delhase *et al.*, 1999). These results underscore the differences in the regulation of IKKα and IKKβ activities and highlight the specificity of NIK in mediating IKK and NF-κB activation.

Recent evidence suggests that the ubiquitin-proteasome system is involved in the proteolytic processing of IκB-α, as well as of the Rel protein designated p105, which is the precursor of the p50 subunit of NF-κB (Orian *et al.*, 1995). Furthermore, several reports have shown that many kinases phosphorylate IκB-α including protein kinase C (PKC)-ζ, PKA, Raf-1, and dsRNA-dependent kinase (PKR), but only $p90^{RSK}$ appears to phosphorylate IκB-α at serine 32 (Schouten *et al.*, 1997). Despite the progress which has been made in understanding the signalling pathways of NF-κB activation, further studies are still required to clarify the interaction of NF-κB with IκB proteins and the mechanisms by which the IκB kinase complex becomes active in cells.

Certain signalling pathways that lead to activation of NF-κB have been described, and the recent identification of IkappaB kinases is a major step in understanding NF-κB regulation (Neurath *et al.*, 1998). However, identifying the other constituents of the IKK complex will help illuminate the mechanisms involved in the control of NF-κB activity. Activation of NF-κB by double stranded RNA requires the double-stranded RNA-dependent protein kinase (Maran *et al.*, 1994), while IL-1, and TNF activate NF-κB through binding of these cytokines to their specific cell surface receptors **[Fig. 1.9]** (Jobin and Sartor, 2000). For example, TNF binds to its receptor and leads to activation of TNF receptor associated factor (TRAF) protein via a receptor associated adaptive protein, called TNF receptor associated death domaine (TRADD) (Verma *et al.*, 1995; Schmid and Alder, 2000; Hsu *et al.*, 1996). Members of the TNF receptor (TNFR) interact with TRAF proteins, which serve as adaptor protein to recruit NIK, a NF-κB-inducing kinase, and MAPKKK, MEKK1 (Orian *et al.*, 1995). TNF signalling also activates JNK (stress activated protein kinase) and Fas associated protein with a death domaine (FADD) which leads to apoptosis via a caspase-8 initiated cascade (Chinnaiyan *et al.*, 1996). Common steps in all these pathways are suggested by the

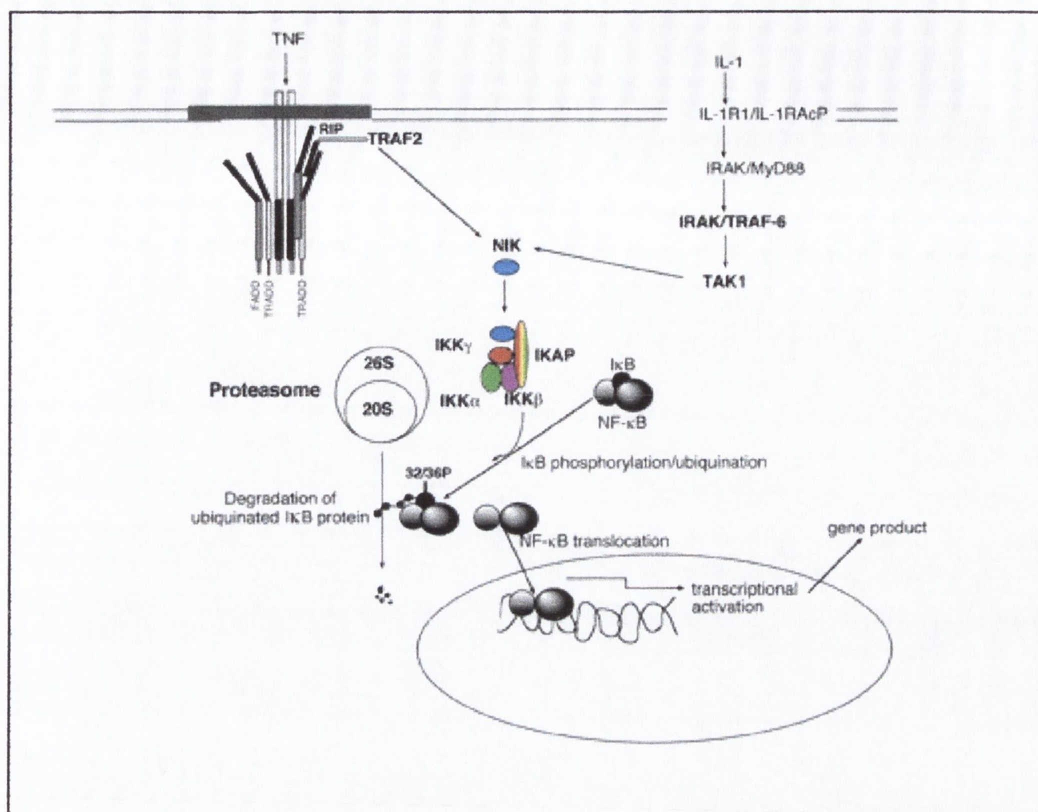


Fig. 1.9. NF-κB signal transduction pathways initiated by IL-1 and TNF. IL-1 or TNF binding to its respective receptor elicits a cascade of transductional signals that converge on NIK. NIK associates with the IKK complex via the action of IKAP, leading to phosphorylation of the IKK and IKK. Activated IKK then phosphorylates IκB, which triggers the ubiquitination/degradation cascade and NF-κB release. Finally, NF-κB translocates into the nucleus and binds to its target DNA sequences. TRAF, TNF receptor-associated factor; TRADD, TNF receptor 1-associated death domain; NIK, NF-κB-inducing kinase; IKK, IκB kinase; IKAP, IKK complex-associated protein; RIP, receptor interacting protein; FADD, Fas-associated death domain; TAK, transforming growth factor-activated kinase; IRAK, IL-1 receptor-associated kinase; IL-1RAcP, IL-1 receptor accessory protein (Reproduced from Jobin and Sartor, 2000).

observation that certain inhibitors like antioxidants and alkylating agents inhibit the phosphorylation and subsequent degradation of IκB-α.

1.7.1.4 Genes Regulated by NF-κB

NF-κB is a key regulator of diverse vital genes required for the immune response, inflammation, differentiation, cell adhesion and apoptosis. More than 150 genes have been identified to be regulated by NF-κB/Rel transcription factors, **[Table 1.3]**. These genes include those encoding cytokines (IL-1, IL-2, IL-6, IL-8, IL-12, INF-γ and TNF-α), cell adhesion molecules (ICAM-1, VACM-1, ELAM-1 and E-selectin), immunoreceptors, acute phase proteins, enzymes, cell surface receptor, chemokines, inducible nitric oxide synthatase (iNOS), and certain transcription factors (Baeuerle and Henkel, 1994; Siebenlist *et al.*, 1994; Schmid and Alder, 2000).

Early gene expression of several viruses including HIV-1, cytomegalovirus and adenovirus are also regulated by NF-κB (Baeuerle and Henkel, 1994). Activation of NF-κB is linked to regulation of programmed cell death, and it has been found to protect against TNF-α-induced apoptosis (Beg and Baltimore, 1996). Knockout mice studies led to the identification of some genes regulated by certain subunits of NF-κB, like IL-2, which is dependent on c-Rel (Köntgen *et al.*, 1995).

1.7.1.5 *Helicobacter pylori* and NF-κB Activation

Non-pathogenic bacterial flora do not elicit an inflammatory response under normal circumstances and are present as commensals in the gastrointestinal tract. However, in the presence of bacterial pathogens, a vigorous immune response is initiated. It has been demonstrated that infection with *H. pylori* activates NF-κB in gastric epithelial cells (Keates *et al.*, 1997; Münzenmaier et al., 1997; Glocker *et al.*, 1998; Maeda *et al.*, 2000). Direct contact between *H. pylori* and gastric epithelial cells activates NF-κB **[Fig. 1.10]** (Kelly and Michetti, 1998). Immunohistochemical studies of biopsies from patients infected with *H. pylori* demonstrated elevated levels of activated NF-κB compared to normal biopsies (Keates *et al.*, 1997; van den Brink *et al.*, 2000). *In vitro*

Target genes which regulated by the transcription factor NF-κB

Class	Target gene
Viruses	Human immunodeficiency virus 1 (HIV-1) Cytomegalovirus (CMV) Adenovirus Simian virus 40 (SV 40)
Immunoreceptors	Immunoglobulin κ light chain T cell receptor β chain T cell receptor α chain MHC class I, MHC class II β_2-microglobulin Invariant chain 1 Tissue factor-1 PAF receptor IL-2Rα chain
Cell adhesions molecules	Endothelial cell adhesion molecule 1 (ELAM-1) Vascular cell adhesion molecule 1 (VACM-1) Intercellular cell adhesion molecule 1 (ICAM-1) E-selectin P-selectin Mucosal vascular addressin cell adhesion molecule-1 (MadCAM-1)
Cytokines and growth factors	Granulocyte/macrophage colony-stimulating factor (GM-CSF) Granylocyte colony-stimulating factor (G-CSF) Macrophage colony-stimulating factor (M-CSF) Melanoma growth stimulating activity (qrox-γ/MGSA) β-Interferon Interleukin-1β Interleukin-2 Interleukin-3 Interleukin-4 Interleukin-6 Interleukin-8 Interleukin-12 Tumor necrosis factor-α (TNF-α) Lymphotoxin (TNF-β) Proenkephalin Macrophage infammatory protein-1 (MIP-1) Macrophage chemotactic protein-1 (MCP-1) Regulated on activation, normal T-cell expressed and secreted (RANTES)

Table 1.3 (1). Target genes whose expression is regulated by the transcription factor NF-κB. (Reproduced from Baeuerle and Henkel, 1994).

Target genes which regulated by the transcription factor NF-κB

Class	Target gene
Acute phase proteins	Angiotensinogen Serum amyloid A precursor Complement factor B Complement factor C4 Urokinase-type plasminogen activator Phospholipase A_2 Cycloxygenase-2 (COX-2) 5-Lipoxygenase (5-LO) 12-lipoxygenase Angiotensinogen
Transcription factors	c-Rel p105 NF-κB1, NF-κB2 IκB-α RelA Interferon regulatory factor 1 (IRF-1) Immediate early response gene (IEX-1) TNF receptor associated factor 1 and 2
Cell cycle	Cyclin D1 P53 C-myc
Antiapoptotic/proapoptotic	Fas, FasL Traf1, Traf2 Mn-SOD BclxL A20 Bfl1/A1 P22/PRG1/IEX-1/DIF-2
Others	Vimentin Perforin Inducible NO-synthase (iNOS) TAP-1 peptide transporter LMP2 proteasome subunit

Table 1.3 (2). Target genes whose expression is regulated by the transcription factor NF-κB. (Reproduced from Baeuerle and Henkel, 1994).

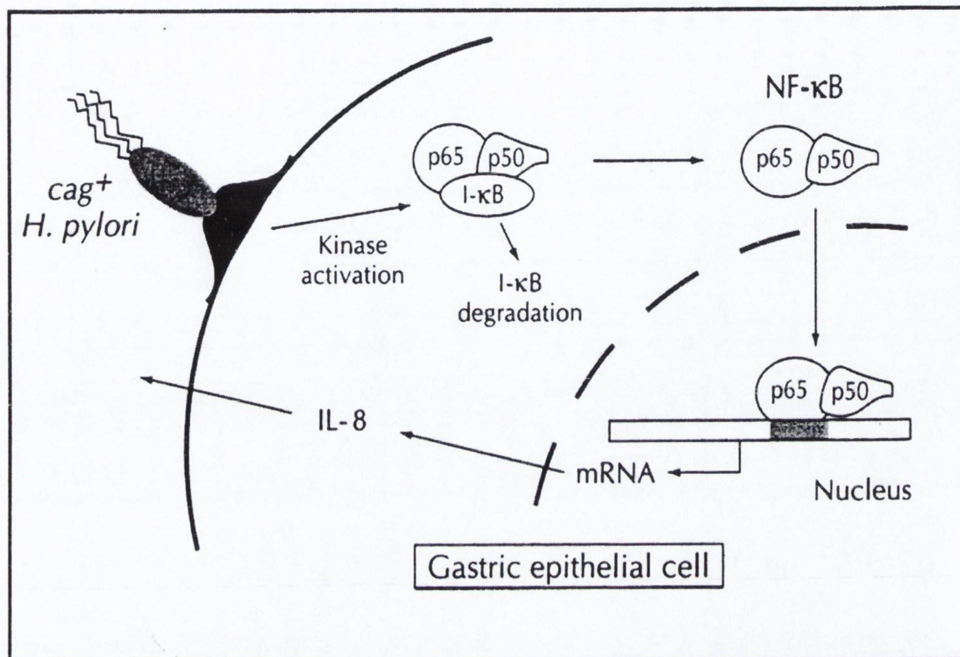


Fig. 1.10. *Helicobacter pylori* activates gastric epithelial cell NF-κB and upregulates interleukin-8 (IL-8) gene expression. Contact between *H. pylori* and the gastric epithelial cell results in the formation of an epithelial cell adhesion pedestal and induces tyrosine phosphorylation of host cell proteins. *H. pylori* infection also results in the phosphorylation and degradation of the inhibitory protein IκB. This releases activated NF-κB dimers which translocate to the nucleus, bind to regulatory sites on the gene promoter, and upregulate interleukin-8 gene expression (Reproduced from Kelly and Michetti, 1998).

infection of AGS and KATO-3 cell lines with *H. pylori* induced NF-κB activation and resulted in nuclear translocation of both p50/p65 heterodimers and p50 homodimers (Keates *et al.*, 1997). Nuclear translocation of NF-κB was accompanied by IL-8 production (Aihara *et al.*, 1997; Sharma *et al.*, 1998).

Unlike other Gram-negative bacteria, *H. pylori* does not use LPS to activate NF-κB, but rather, a secreted bacterial product is required (Münzenmaier *et al.*, 1997). Colonization of gastric epithelial cells with *H. pylori* also results in increased production of the proinflammatory cytokines TNF-α, IL-1, IL-6 and IL-8 (Yamaoka *et al.*, 1997), and increased expression of ICAM-1 (Fan *et al.*, 1995a), all of which are regulated by NF-κB. Activation of NF-κB and up-regulation of epithelial cell IL-8 production require direct contact with live bacteria, while heat-killed *H. pylori* fail to elicit IL-8 production (Aihara *et al.*, 1997; Keates *et al.*, 1997).

Helicobacter pylori strains differ in their ability to induce cytokine production. Several groups have described a link between NF-κB activation and IL-8 secretion (Münzenmaier *et al.*, 1997; Sharma *et al.*, 1998). Consistent with this, $CagA^+$ strains were found to induce higher levels of IL-8, IL-1β, IL-6 and TNF-α and mucosal inflammation than $CagA^-$ strains (Yamaoka *et al.*, 1997). Censini *et al.* (1996) demonstrated that several genes located within the *cag* PAI are required for *H. pylori* to elicit IL-8 secretion. Also, Glocker *et al.* (1998) have shown that NF-κB activation requires six membrane proteins encoded in the *cag* PAI, (CagE, CagG, CagH, CagI, CagL and CagM), since appropriate isogenic mutants of *H. pylori* had no effect on NF-κB activity. Furthermore, AP-1 is also required together with NF-κB for *H. pylori*-induced IL-8 secretion in the gastric epithelial cell line MKN 45 (Aihara *et al.*, 1997). The synergistic effect of pH and *H. pylori* on the exprerssion of NF-κB in gastric cells has not yet been examined and is described in the present study.

1.7.2 ROLE OF NF-κB IN INFLAMMATION AND IMMUNITY

The NF-κB/Rel transcription factors have been the focus of considerable interest over the past few years, because of their broad role in controlling the expression of genes that regulate the inflammatory and immune responses. Here, the importance of NF-κB in regulating cell growth and proliferation, cell apoptosis and cytokine production is summarized.

1.7.2.1 Role of NF-κB in Regulating Cell Proliferation

NF-κB responds to many T and B cell activating signals leading to differentiation and proliferation. In quiescent fibroblasts, NF-κB is transiently activated in the G0-to-G1 transition upon stimulation of fibroblasts with serum (Baldwin *et al.*, 1991). The κB site found in the C-myc upstream promoter conferred serum inducibility on a reporter gene, suggesting the involvement of NF-κB in the immediate-early growth response (Duyao *et al.*, 1990). It has been suggested that inhibition of NF-κB/Rel causes a reduction in serum-induced cyclin D1-associated kinase activity, a key regulator of the G1 transition, and delayed phosphorylation of the retinoblastoma (Hinz *et al.*, 1999). Both levels and kinetics of cyclin D1 expression during G1 phase are controlled by NF-κB/Rel. These results provide a further link between NF-κB/Rel activity and G0/G1-S1 phase transition. The proliferation of the T-cell line HuT78 was found to be stimulated by TNF-α in a manner, which is dependent on NF-κB activation (O'Connell *et al.*, 1995). NF-κB is also involved in the transcriptional activation of several genes encoding growth factors and growth factor receptors. These include the colony-stimulating factors M-CSF, G-CSF, the T cell growth factor IL-2, and the B/T cell growth factor IL-6 (Grilli *et al.*, 1993; Baeuerle, 1991).

Moreover, active forms of NF-κB appear to be involved in sustaining cell proliferation by transcriptionally activating genes for growth factors, their receptors and nuclear growth regulators, as found in T and B cells. As NF-κB is an activator of the β-interferon gene in response to viral infection, it induces a growth arrest in interferon-

induced cells (Lenardo *et al.*, 1989). High levels of NF-κB and related proteins were detected in a metastatic colon cancer cell line (Bours *et al.*, 1994).

1.7.2.2 Role of NF-κB in Regulating Apoptosis

Apoptosis appears to be the main mechanism whereby chemotherapy and radiation induce cell death. The NF-κB/Rel transcription factors are activated by chemotherapy and radiation and this response potently suppresses the apoptotic potential of these stimuli (Beg and Baltimore, 1996; Wang *et al.*, 1996 and 1998). In addition, many cell types become more sensitive to TNF-α-induced apoptosis when transfected with a dominant negative form of IκB-α (Van Antwerp *et al.*, 1996). Furthermore, a role for RelA-containing NF-κB dimers in protecting cells from pro-apoptotic stimuli produced in the embryonic liver has been demonstrated by Beg *et al.* (1995) in mice lacking the RelA subunit led to a massive apoptosis of liver cells.

Adenoviral delivery of a modified form of IκB-α sensitizes chemoresistant tumors to the apoptotic potential of TNF-α and the chemotherapeutic compound Camptothecin (CPT-II), resulting in tumor regression (Wang *et al.*, 1999). Also, radiation-induced cell apoptosis in AT cells was reduced by a dominant negative IκB-α protein (Jung *et al.*, 1995). These data suggest that pro-apoptotic signals can induce NF-κB, which in turn induced the expression of several genes that are anti-apoptotic. Abbadie *et al.* (1993) have also reported that c-Rel induces apoptosis in Hela cells upon expression of the *c-Rel* gene. It is important to mention that RelA can protect from apoptosis, while c-Rel induces apoptosis, and this could be explained by the differences in binding specificity between RelA or c-Rel and the IκB motifs in pro- and anti-apoptotic agents.

1.7.2.3 Role of NF-κB in Regulating Cytokine Production

Cytokines are generally short acting proteins synthesized by several different cell types and convey signals for homeostatic regulation of cellular proliferation, differentiation and other responses (Blackwell and Burke, 1989). Cytokines are

secreted in response to an inflammatory stimulus. Following stimulation, cytokines are produced in a characteristic pattern that is dependent on the stimulus and the cell or tissue type.

NF-κB exerts a wide role on cytokine production by affecting many of the genes involved in its generation, but it is uncertain whether NF-κB works in concert with other transcription factors for regulating the expression of cytokine genes. It has been demonstrated that interaction between NF-κB and NF-IL-6 (Akira and Kishimoto, 1992), NF-ELAM-1 (Whelan *et al.*, 1991), and with other factors on the IL-2 enhancer (Hoyos *et al.*, 1989), is required for the transcriptional activation of IL-6, ELAM-1 and IL-2, respectively.

NF-κB may interact directly with other transcription factors such as glucocorticoid receptors, that alter the ability of NF-κB to bind to DNA (Ray and Prefontaine, 1994). In addition, exposure to TNF-α and IL-1 result in activation of NF-κB and AP-1, which mediate cytokine production (Osborn *et al.*, 1989). Expression of the *β–IFN* gene can be elicited in many cell types and is also regulated by NF-κB (Lenardo *et al.*, 1989). In summary, NF-κB appears to play an important role in the pathogenesis of several inflammatory diseases due to its influence on cytokine production and bacterial infections are known to induce several cytokines through activation of NF-κB.

1.7.2.4 NF-κB and Oxidative Stress

How do unrelated stimuli elicit NF-κB activation? Do all these stimuli use the same signalling pathway in activating NF-κB? Several lines of evidence suggest that increased levels of ROS are responsible for NF-κB activation **[Fig. 1.11]** (Ginn-Pease and Whisler, 1998; Bowie and O'Neill, 2000). Firstly, treatment of certain cells types with H_2O_2 activates NF-κB (Schreck *et al.*, 1991 and 1992a; Meyer *et al.*, 1993) and levels of ROS increase in response to almost all inducers of NF-κB (Schreck *et al.*, 1992b; Meyer *et al.*, 1993; Schmidt *et al.*, 1995). Secondly, increased ROS levels also

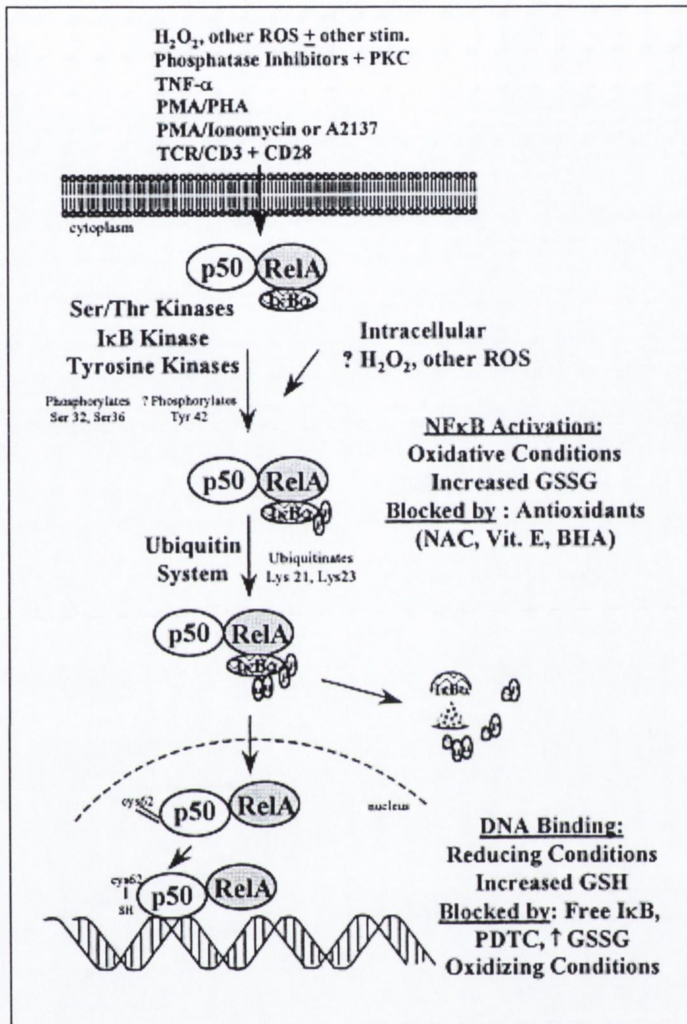


Fig. 1.11. Redox regulation of NF-κB activation: Model depicting the dependencies of IκB-α phosphorylation/degradation on oxidative conditions and of NF-κB binding to DNA recognition sequences on reduction conditions (Reproduced from Ginn-Pease and Whisler, 1998).

occur following a number of cellular injuries including bacterial and viral infections, inflammation, heat shock and ionizing and UV irradiation (Schulze-Osthoff *et al.*, 1995). Thirdly, inhibition or overexpression of certain endogenous oxidant defenses affecting intracellular ROS has been shown to modulate NF-κB activation (Manna *et al.*, 1998; Bonizzi *et al.*, 1999). Schmidt *et al.* (1995) have demonstrated that catalase overexpresssing cell lines have a diminished ability to activate NF-κB. Also, peritoneal macrophages from transgenic mice that overexpressed Cu, Zn-SOD have decreased NF-κB activation in response to PMA as compared with control mice (Mirochnitchenko and Inouye, 1996).However, the activity of IKKβ was significantly elevated in cells exposed to prooxidants but this induction of IKK activity by TNF-α was not affected in cells treated with NAC (Li and Karin, 1999). It appears that the redox control of NF-κB signalling differs in different cell settings with different prooxidants.

Finally, NF-κB activation is inhibited by a wide variety of antioxidants (Schreck *et al.*, 1992b; Meyer *et al.*, 1993; Bowie and O'Neill, 2000). It has been shown that antioxidant treatment prevents the inducible decay of IκB-α in response to various stimuli, suggesting that IκB-α proteolysis is under control of the cell's redox status (Henkel *et al.*, 1993; Beg *et al.*, 1993). Therefore, as redox processes play a critical role in regulating NF-κB activation, a better understanding of the mechanism of redox regulation of NF-κB, particularly *in vivo*, will be beneficial in the design of new treatment strategies for gastrointestinal diseases.

1.7.3 INHIBITION OF NF-κB ACTIVATION: A THERAPEUTIC TARGET?

Because NF-κB is activated by diverse stimuli, inhibition of NF-κB activity has emerged as a potential key event in the control of many disease states (Baeuerle and Baichwal, 1997). A number of compounds that can suppress NF-κB activation including antioxidants, proteasome inhibitors, corticosteroids, salicylates and other immunosuppressants have been identified **[Fig. 1.12]** (Schmid and Alder, 2000).

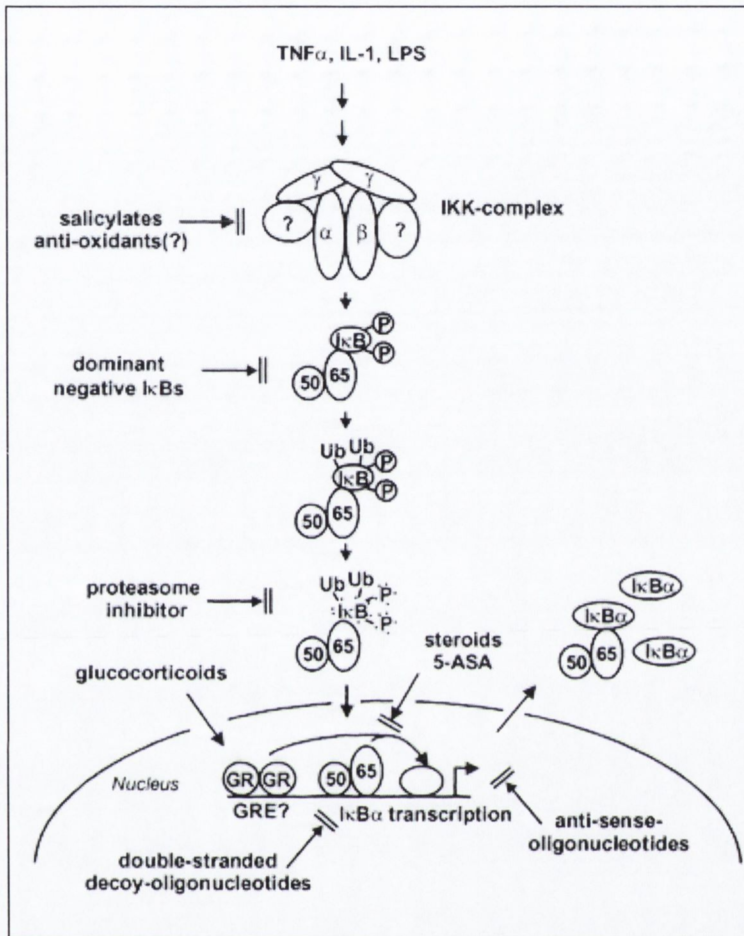


Fig. 1.12. The induction of NF-κB/Rel-mediated transcription factors can be blocked at several levels. While antioxidants and salicylates may block protein kinases, proteasome inhibitors can inhibit proteasome enzymes, and antisense oligonucleotides and double stranded decoy-oligonucleotides can inhibit translation of p65. In addition, corticosteroids lead to blockade of p65 and could deliver genes whose inactivate NF-κB (Reproduced from Schmid and Alder, 2000).

1.7.3.1 Phosphothoiate and Antisense Oligonuclotides to NF-κB/Rel

Double-stranded phosphothioate oligonucleotides that contain κB consensus sequences or antisense oligonuclotides to NF-κB/Rel subunits have been used to block NF-κB/Rel activity (Bielinska *et al.*, 1990). Neurath *et al.* (1996) have shown that local administration of p65 antisense phosphorothioate oligonucleotides abrogates clinical and histological signs of colitis in IL-10 knockout mice and is more effective in treating 2,4,6-trinitrobenezene sulfonic acid (TNBS)-induced colitis than single or daily administration of glucocorticoids.

1.7.3.2 Proteasome Inhibitors

Cellular signals activate a specific IκB kinase to phosphorylate IκB, which in turn serves as the signal for IκB ubiquitination followed by proteolytic degradation by the proteasome. *In vitro* studies have demonstrated that proteasome inhibitors block IκB-α degradation and NF-κB activation, as well as p105 processing both *in vitro* and *in vivo* (Palombella *et al.*, 1994).

Alkylating agents like phenylalanine chloromethylketone (PCK) and its derivative TPCK were the first non-specific proteasome inhibitors discovered that bound to NF-κB, AP-1, CREB and Oct-1 (Palombella *et al.*, 1994; Finco *et al.*, 1994). Although the peptide aldehydes such as N-benzyloxycarbonyl-LL-norvalinan (MG 115) and N-benzyloxycarbonyl-LL-lucinol (MG 132) are potent proteasome inhibitors that specifically block IκB-α degradation after TNF-α stimulation, they had no effect on IκB-α hyperphosphorylation (Palombella *et al.*, 1994; Rock *et al.*, 1994). The most specific proteasome inhibitor described to date is lactacystin, a streptomyces metabolite, which binds irreversibly and specifically to a subunit of the proteasome and acylates the active site NH_2-terminal threonine (Dick *et al.*, 1996).

1.7.3.3 Glucocorticoids

Glucocorticoids, a group of steroid compounds with a broad range of effects on the immune system, have been shown to inhibit the expression of cytokines, adhesion molecules, and enzymes involved in the inflammatory process (Barnes and Adcock,

1993). They exert their effect via binding to the intracellular GR that enters the nucleus, dimerizes and binds to specific DNA targets. Glucocorticoids appear to block NF-κB activation through either *de novo* protein synthesis which involves induction of IκB-α transcription (Auphan *et al.*, 1995), or by direct interaction of the GR with RelA to inhibit DNA binding (Ray and Prefontaine, 1994). In addition, it has been shown that NF-κB/Rel can also repress ligand-dependent activation GR-regulated genes through formation of an inactive protein complex (Ray and Prefontaine, 1994). However, the interaction of the GR with NF-κB/Rel signalling is not specific because NF-κB/Rel subunits can interact with other transcription factors and other nuclear hormone receptors (Gottlicher *et al.*, 1998). Glucocorticoids have numerous undesireable therapeutic side effects that limits their use, therefore, more specific NF-κB inhibitors are required for therapeutic purposes.

Cyclosporin A and Tacrolimus (FK 506) exert their immunosuppressive effects by blocking calcium-dependent NF-κB activation but do not interfere with the PKC pathway, resulting in reduced NF-κB/Rel activation (Schmidt *et al.*, 1990). Salicylates, which are widely used non-steroidal anti-inflammatory drugs, also influence NF-κB/Rel signalling. Salicylates including acetylsalicylic acid (aspirin) and sodium salicylates, have been shown to inhibit NF-κB/Rel activation at high doses (Kopp and Ghosh, 1994). It is thought that the mechanism of NF-κB inhibition by aspirin and sodium salicylate is caused by binding of these drugs to Ikappa B kinase and reduce ATP binding (Yin *et al.*, 1998). However, Alpert and Vilćek (2000) demonstrated that salicylate treatment inhibited the kinase activity of overexpressed IKKα and IKKβ and also decreased p38 kinase activity *in vitro* and this inhibition is not selective and is not likely to be the mechanism by which sodium salicylate exerts its inhibitory effect upon NF-κB activation *in vivo*. Furthermore, sulphasalzine, which is used for treatment of inflammatory bowel disease, inhibits NF-κB activation (Wahl *et al.*, 1998).

1.7.3.4 Antioxidants

The activity of NF-κB is modulated by the redox state of the cell and is activated by a prooxidant state and is therefore potentially inhibited by antioxidants. Many antioxidant agents, including cysteine, metal chelators, dithiocarbamates, quinone derivatives, vitamin E, vitamin C, butylated hydroxylanisole (BHA), α-Lipoic acid, and 2-mercaptoethanol suppress activation of NF-κB in response to diverse stimuli (Bowie and O'Neill, 2000; Schreck *et al.*, 1992a and 1992b; Schmidt *et al.*, 1995; Brennan and O'Neill, 1995). N-acetyl-L-cysteine (NAC) increase glutathione levels in cells and has also been found to inhibit NF-κB induction by H_2O_2, cyclohexamide, dsRNA, IL-1, TNF-α, PMA and LPS in Hela and Jurkat cell lines (Meyer *et al.*, 1993; Schulze-Osthoff *et al.*, 1995). However, Brennan and O'Neill (1995) found that the inhibitory effects of NAC were cell-dependent and failed to inhibit NF-κB activation secondary to IL-1 and TNF-α stimulation in both EL4-NOB and KB cells. Pyrrolidine dithiocarbamate (PDTC), a metal chelator and an antioxidant, has been found to block NF-κB activation by scavenging metal ions required for production of hydroxyl radicals (Schreck *et al.*, 1992b). In addition, PDTC blocks IκB degradation by preventing IκB-α phosphorylation (Henkel *et al.*, 1993).

A number of studies have suggested that thioredoxin is a more potent antioxidant than either glutathione or NAC for NF-κB activation (Schenk *et al.*, 1994; Okamoto *et al.*, 1992; Hayashi *et al.*, 1993). Glutathione peroxidase, an enzyme that catalyzes the conversion of hydrogen peroxide into water, inhibits NF-κB activation by H_2O_2, TNF-α and PMA (Kalebic *et al.*, 1991; Mihm *et al.*, 1991). Finally, vitamin C is a natural powerful antioxidant and is important in the prevention of gastric cancer by scavenging nitrite and preventing the formation of nitroso-compounds, which are carcinogenic. In addition, it is capable of eliminating oxygen radicals, which may cause damage to the gastric epithelium (Mirvish, 1986; Sobala *et al.*, 1989). Recently, it was shown that vitamin C inhibits NF-κB activation in gastric epithelial cells (O'Toole *et al.*, 1997) and also in endothelial cells (Bowie and O'Neill, 2000). The above data suggests that inhibition of NF-κB by antioxidants represents an attractive

strategy for the treatment of inflammatory diseases. In this study, the antioxidants vitamin C, NAC and thioredoxin were tested for their ability to inhibit NF-κB activation by *H. pylori* and other stimuli.

1.7.4 IMMEDIATE-EARLY GROWTH (Egr) TRANSCRIPTION FACTORS

Members of the early growth response (Egr) family of transcription factors are closely related members include Egr-1, Egr-2, Egr-3, Egr-4, NGFI-B and a member of the steroid hormone receptor superfamily. These genes encode nuclear proteins with zinc finger DNA binding domains resembling those of the well-characterized transcription factor, Sp1. In addition, they have several less-well conserved structural features in common. Immediate-early proteins are rapidly induced by diverse extracellular stimuli in many cell types and tissues (Sukhatme, 1990). There is increasing evidence for the involvement of the Egr-1 in the regulation of inflammatory and immune responses (McMahan and Monroe, 1996; Fitzgerald and O'Neill, 1999).

1.7.4.1 The Early Growth Response-1 (Egr-1) Transcription Factor

1.7.4.1.1 Egr-1 Discovery and Characterization

Egr-1 was first identified by Sukhatme and colleagues (1988), as an inducible transcription factor using a differential screening strategy after the addition of serum to quiescent fibroblasts. Egr-1 (Sukhatme *et al.*, 1987) is also known as Krox-24 (Lemaire *et al.*, 1988), nerve growth factor-induced-A; NGFI-A (Milbrandt, 1987), TIS-8 (Lim *et al.*, 1987) and Zif268 (Christy *et al.*, 1988). *Egr-1* encodes an 80-84 kDa inducible protein consisting of 533 amino acids which has a characteristic consensus 9-bp site 5′-GCG (T/G)GG GCG-3′ that is essential for DNA binding (Sukhamte *et al.*, 1988; Lemaire *et al.*, 1988). The protein is highly rich in proline and serine, which constitute one third of its amino acids, and there is a series of three tandem repeats towards the carboxy-terminal end, each of which conforms to the canonical sequence for a zinc finger structure of the Cys_2-His_2 subclass. Each zinc finger domain contains a α-helix and an antiparallel β-sheet held together by a zinc ion and a set of hydrophobic residues **[Fig. 1.13]** (Sukhatme, 1990).

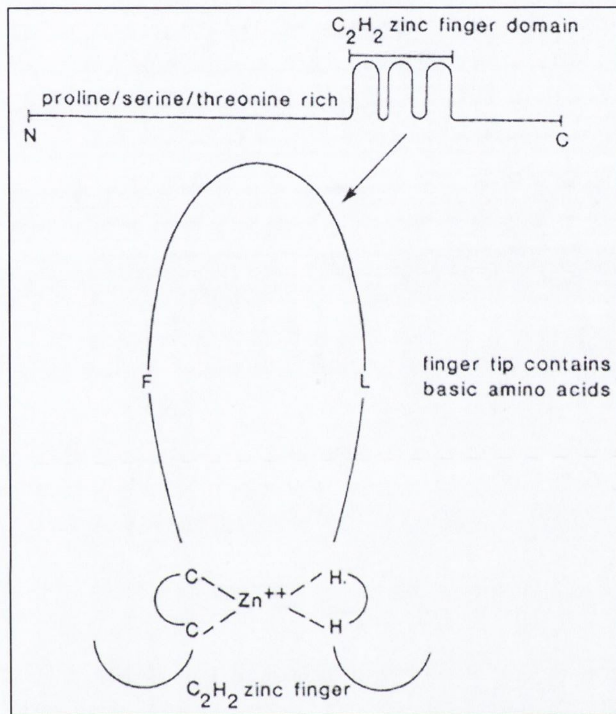


Fig. 1.13. Structure of Early growth response-1 (Egr-1) protein and zinc finger (Reproduced from Sukhatme, 1990).

The binding of Egr-1 to its specific DNA sequence occurs through the interaction of the zinc finger motifs and acts as an activator or repressor of gene expression (Swirnoff and Milbrandt, 1995; Christy and Nathans, 1989). *Egr-1* spans about 3.8 kp and consists of 2 exons and one 700 bp intron. In addition, there are five elements whose sequence is identical to the inner core 10-nucleotide region (CCATATTAGG) of the c-Fos serum response elements, four Sp1 consensus sequences, two AP1 target sequence analogs, and two cAMP response elements (Tsai-Morris *et al.*, 1988).

1.7.4.1.2 Conditions that Activate Egr-1

Egr-1 is rapidly and transiently induced by many extracellular signals including growth factors, cytokines and phorbol esters in fibroblasts, epithelial cells, endothelial cells and lymphocytes (Gashler and Sukhatme, 1995, Sukhatme, 1990). The signals and conditions that can activate Egr-1 transcription factor are summarized in **Table 1.4**. In addition, Egr-1 is also induced by diverse types of DNA-damaging agents such as UV light, ionizing radiation and by environmental stress like shock and chemical stress (Huang and Adamson, 1995; Hallahan *et al.*, 1991; Lim *et al.*, 1998). In mature B-lymphocytes, transient Egr-1 expression is rapidly induced upon stimulation by B cell antigen receptor (BCR) cross linking (Seyfert *et al.*, 1989; McMahan and Monroe, 1995).

Bacterial and viral infection have been shown to induce Egr-1 expression, Coleman *et al.* (1992) have demonstrated that bacterial LPS from *Salmonella minnesota* induces transcriptional activation of Egr-1 and increases Egr-1 protein levels in peritoneal macrophages. Also, infection of human lymphocytes with human T-cell leukemia virus and rat brain with Borna disease virus caused an increased expression of Egr-1 mRNA (Zheng *et al.*, 1993; Fu *et al.*, 1993). Egr-1 was found to be constitutively expressed following infection of human T lymphocytes with the transforming viruses HTLV-I and HTLV-II (Wright *et al.*, 1990). Moreover, the duodenal ulcerogen cysteamine, but not its nonulcerogen and toxic analogue ethanolamine, rapidly increased duodenal mucosal levels of ET-I, which was followed by enhanced

Conditions that activate the transcription factor Egr-1

Class	Inducing agent
Cytokines and growth factors	TNF-α
	IL-1β
	IL-3
	FGF, GM-CSF
	PDGF
	TGF-β
Immunoreceptors	B cell antigen receptor cross linking
	Surface Ig-mediated receptor
DNA-damaging agents	UV light
	Ionizing radiation
	Thapsigargin (TG)
	1-(β-D-arabinofuranosyl) cytosine
	ara-C
Injurious stimuli	Heat shock
	Chemical stress; sodium arsenite
	Anisomycin
	Physical stress
	Hypoxia
	Shear stress
	Mechanical injury
Bacterial products	*Salmonella minnestoa* LPS
Viruses	HTLV-I, HTLV-II
	T-cell leukemia type 1 virus
	Borna disease virus
Oxidative stress	Hydrogen peroxide
	Alkylating agents (MMS)
Chemical agents and drugs	Phorbol esters
	Okadiac acid
	Cysteamine
	Serine/threonine protein phosphatase inhibitor
	Anisomycin

Table 1.4. Conditions that activate the transcription factor Egr-1.

expression of Egr-1 and a decrease in Sp1 in the preulcerogenic stage of duodenal ulceration (Szabo *et al.*, 2000).

1.7.4.1.3 Genes Regulated by Egr-1

Egr-1 binds to a CG-rich consensus DNA sequence: single GC-rich elements (GCEs) with the consensus 5′-TGcG(T/g)(G/A)GG(C/a/t)G(G/T)-3′ (where lowercase letters indicate bases of relatively lower binding affinity), or to a (TCC), motif and overlaping sites consisting of an Sp1 binding site and the GCE consensus or close homolog of these sequences (Swirnoff and Milbrandt, 1995; Christy and Nathans, 1989). The GC-rich consensus target sequence of Egr-1 has been identified in the promoter regions of transcription factors such as jun D and nurr77 and growth factors including platelet-derived growth factor (PDGF-A), insulin-like growth factor-II, basic fibroblast growth factor, epidermal growth factor receptor and TGF-β (Christy and Nathans, 1989; Hu *et al.*, 1994; Silverman et al, 1997; Khachigian *et al.*, 1996; Kim *et al.*, 1989).

Cell cycle regulators such as the retinoblastoma susceptibility gene, Rb (Day *et al.*, 1993), p53 (Nair *et al.*, 1997), cyclin D1 (Yan *et al.*, 1997), and TNF-α (Kramer *et al.*, 1994), and IL-1 (Skerka *et al.*, 1995) are regulated by Egr-1. Egr-1 has also been shown to regulate transcription of CD44 (Maltzman *et al.*, 1996a; Fitzgerald and O'Neill, 1999) and ICAM-1 (Maltzman *et al.*, 1996b). **Table 1.5** shows a list of potential target genes of Egr-1. Initial animal studies with mice having a targeted mutation in Egr-1 suggest that some of these genes are authentic targets for this transcription factor (Yan *et al.*, 1998). Khachigian *et al.* (1996) demonstrated that elevated Egr-1 levels are found at sites of vascular injury and may coordinate the expression of multiple genes involved in the pathogenesis of vascular diseases. However, little is known about the role of Egr-1 expression in the stomach.

1.7.4.1.4 Transcriptional Corepressors and Coactivators of Egr-1

Once bound to DNA, Egr-1 alters gene transcription through mechanisms dependent on both coactivators and repressors. In agreement with this dual function, several

Genes whose expression is regulated by the transcription factor Egr-1

Target gene	Activation/Repression	Cell type
α-Myosin heavy chain	Activation	Muscle
PDGF α-chain	Activation	Kidney
IGF-II	Activation	Kidney
TGF-β1	Activation	Kidney
G α i-2	Activation	Kidney
Thymidine kinase	Activation	Kidney
Phenylethan. N-methytransf.	Activation	PC12
Aldolase C	Activation	Neuronal
Synapsin I	Activation	Neuroblastoma
Neurofilament-light	Activation	Embryon. Carcin.
Adenosine deaminase	Repression	Fibroblast
Nurr77	Activation	Fibroblast
Egr-1	Repression	Fibroblast
Egr-1	Activation	Macrophage
TNF	Activation	T lymphocyte
IL-2	Activation	T lymphocyte
IMPDH	Activation	T lymphocyte
EBV BRLF1 promoter	Activation	B lymphocyte
ICAM-1	Activation	B lymphocyte
CD44	Activation	B lymphocyte
Cu-Zn superoxide dismutase 1	Activation	Hela cells
5-Lipoxygenase (5-LO)	Activation	Drosophila SL2 cells
p53	Activation	Melanoma
CD95L	Activation	T cells
Cyclin D1	Activation	Epithelial cells
Retinoblastoma susceptibility gene;Rb	Activation	Prostate cells

Table 1.5. Genes whose expression is regulated by the transcription factor Egr-1. (Reproduced from McMahon and Monroe, 1996).

activating and one inhibitory domain have been localized within the Egr-1 protein (Gashler *et al.*, 1993; Russo *et al.*, 1993). Transcriptional repressors such as NGFI-A binding proteins 1 and 2 (NAB1 and NAB2) negatively regulate Egr-1 activity. NAB1 has been identified using a yeast two-hybrid system by its ability to bind a 34-amino-acids inhibitory domain of Egr-1, called R1, located 5′ of the zinc finger-binding domain (Russo *et al.*, 1995; Swirnoff *et al.*, 1998). On the other hand, NAB2 functions similarly to NAB1 and has a strong homolog to NAB1 (Svaren *et al.*, 1996), but NAB1 is constitutively expressed in most cell types, whereas NAB2 is rapidly and transiently induced by many of the same stimuli that induce Egr-1. Deletion of the inhibitory domain of Egr-1, R1, results in a marked increase in Egr-1 activity while overexpression of NAB1 decrease Egr-1 transcriptional activity. Egr-1 can function also as a repressor molecule by controlling the expression of downstream target genes. Dinkel *et al.* (1997) have demonstrated that the induction of Egr-1 down-regulates the expression of Fas and CD23, which makes them resistant to Fas-mediated apoptosis in the K46 clones.

The regulatory region of the Egr-1 is relatively complex, containing potential binding sites for many transcription factors. Mutational studies have identified a strong transcriptional activation zone within the amino-terminal region of the protein between amino acids 1 and 281 (Gashler and Sukhatme, 1995). The murine Egr-1 promoter contains five serum response elements (SREs) dispersed over 420 bp upstream of the transcription initiating site. The SRE is bound by a homodimer of the transcription factor serum receptor factor (SRF), which interacts with the neighbouring ETS protein to form what has been termed a ternary complex (Treisman, 1994). Several of the five Egr-1 SREs also contain adjacent ETS motifs capable of supporting the formation of ternary complex (Gashler and Sukhatme, 1995; McMahon and Monroe, 1995). Another regulatory element within the Egr-1 promoter is the CRE (Gashler and Sukhatme, 1995), which is localized to a region 50-75 bp upstream of the transcriptional start site and is occupied by members of the CREB family of transcription factors. Transcriptional coactivators such as CREB-binding protein (CBP) and p300 can interact directly with the activation region of Egr-1 and increase

Egr-1 transactivation (Silverman *et al.*, 1998b). Egr-1 can also interact synergistically with the p65 protein to regulate transcription of the NF-κB1p50 gene, since PMA/PHA-stimulated cells expressing antisense Egr-1 RNA are unable to up-regulate NF-κB1 transcription (Cogswell *et al.*, 1997). Furthermore, Egr-1 can interact synergistically with nuclear factor of activated T cells (NFAT) to augment IL-2 transcription (Decker *et al.*, 1998).

Many promoters contain overlapping Egr-1/Sp1 binding sites, and they can displace one another from many promoters and binding sites (Huang *et al.*, 1997), depending on their affinity for the binding site and Egr-1 phosphorylation state. It is known that Sp1, a zinc finger protein, shares similar consensus binding sites with transcription factor Egr-1 (-GGGCGG-), expressed in all cell types and is required for the expression of several genes (Courey and Tjian, 1992). Also, in the Egr-1 promoter, there are binding sites for the Egr-1 protein itself, which have an autoregulatory feedback loop mechanism (Christy and Nathans, 1989).

The effect of Egr-1 on the enhancement or repression of gene transcription appears to depend on several factors. Firstly, it depends on the ability of Egr-1 to act in concert with multiple-sequence-specific transcription factors. Secondly, it depends on the ability of Egr-1 to interact with transcriptional coactivators and repressors, which may work in an additive, synergistic, or inhibitory fashion to alter Egr-1 activity (Silverman and Collins, 1999). Finally, the activity of Egr-1 is also dependent on the number and relative positions of the Egr-1 binding sites within the Egr-1 gene, which are critical for the expression of target genes. Studies involving the 5-lipooxygenase-gene promoter have shown that the 5 tandem Egr-1 consensus binding sites in the wild-type human 5-LO gene promoter that binds Egr-1 are essential for inducibility (Silverman *et al.*, 1998a). Li-Weber *et al.* (1999) have also demonstrated that all three Egr-1 binding sites in the CD95L promoter are essential for the full function of promoter activity in response to T cell stimulation.

1.7.4.1.5 Mechanisms of Egr-1 Activation

The signalling pathways of Egr-1 activation in response to extracellular signals are poorly understood and many of the molecular mechanisms are speculative **[Fig. 1.14]** (Sukhatme, 1990). Induction of Egr-1 is mediated through different subgroups of mitogen-activated protein kinases (MAPKs), including the extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) (also known as stress-activated kinase protein kinases or SAPKs) and p38 pathways (Silverman and Collins, 1999; Lim *et al.*, 1998). Once Egr-1 is activated, these pathways may lead to an interaction between ternary complex factors (TCF) and serum response factor (SRF) that activates Egr-1 gene transcription by binding to serum response elements (SREs) within its promoter. Treisman *et al.* (1994) have shown that phosphorylation of ETS protein component of the ternary complex by MAP kinase is required for c-Fos induction.

Protein kinases also play a role in the activation of Egr-1 gene expression, which may affect Egr-1 phosphorylation state. Seyfert *et al.* (1990) have shown that protein kinase C inhibitors H7, sangivamycin and staurosporine block anti-receptor antibody-induced expression of Egr-1 in murine B-lymphocytes. Induction of Egr-1 by Il-1β and TNF-α in normal human bone marrow stromal and osteoblastic cells is inhibited by protein kinase inhibitor H7 (Chaudhary *et al.*, 1996). Yamaji *et al.* (1994) also reported that inhibition of tyrosine kinase pathways prevented IE gene activation by acid in renal tubule cells. In agreement with these observations, several other studies have demonstrated that the regulation of Egr-1 gene expression and its DNA binding activity is under the control of protein kinases (Haung and Adamson, 1995; Cao *et al.*, 1993; Huang *et al.*, 1998). It appears that different molecular mechanisms are involved in Egr-1 activation and understanding the mechanism of how these signals elicit the nuclear responses remains to be determined.

1.7.4.1.6 Egr-1 Activation and Oxidative Stress

Oxidative stress and active oxygen species induce various alterations in gene expression by affecting the activities of many signal transduction pathways and

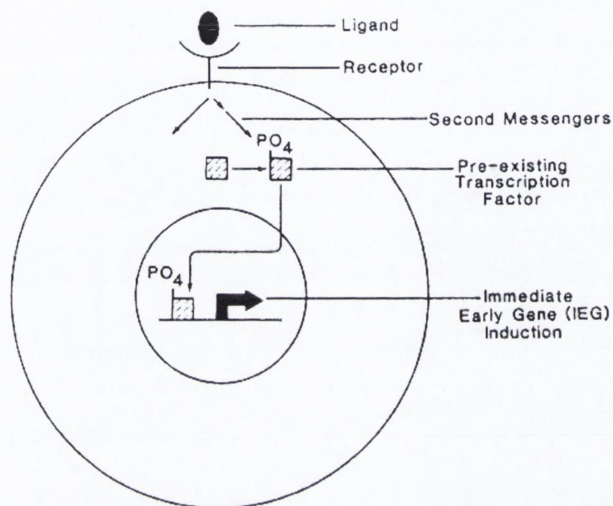


Fig. 1.14. Schematic of signal transduction pathways in a cell. A ligand (growth factor, neurotransmitter, etc.) binds to its cognate receptor, thereby initiating a cascade of second messenger events. One such event includes kinase activation, which phosphorylates preexisting transcription factor rendering it capable of binding to the promoter region of an IEG and activating its transcription (Reproduced from Sukhatme, 1990).

several transcription factors including Egr-1 (Nose and Ohba, 1996; Nose *et al.*, 1991). It appears that the effect of ROI on Egr-1 induction is cell type dependent as several studies have shown that DNA-binding activity of Egr-1 is impaired by treatment of the cells with oxidizing agents (Huang and Adamson, 1993; Esposito *et al.*, 1994; Neguyen *et al.*, 1993). The direct oxidation of Egr-1 results in a loss of binding activity, since zinc finger proteins contain cysteines that are essential for DNA binding (Meyer *et al.*, 1993). However, mild oxidative stress applied to the cells induced early response genes, as the ability of Egr-1 to bind to its specific DNA sequences and to enhance the transcriptional activity of target genes was increased with appropriate concentrations of reactive oxygen (Nose and Ohba, 1996). Furthermore, Egr-1 activity is inhibited by antioxidant treatment (Huang and Adamson, 1995; Huang *et al.*, 1999) and glutathione-depleting agents (Esposito *et al.*, 1994). Ohba *et al.* (1994) demonstrated that hydrogen peroxide acts as a mediator for the TGF-β1-induced transcription of Egr-1 gene; in addition, oxygen radical scavengers such as catalase and NAC inhibited the induction of Egr-1 by TGF-β1. In conclusion, the Egr-1 transcription factor is functionally activated in cells exposed to mild oxidative stress, and this may constitute one of the physiological signalling pathways involved in Egr-1 gene expression.

1.7.4.2 Role of Egr-1 in Inflammation and Immunity

1.7.4.2.1 Role of Egr-1 in Regulating Cell Growth and Differentiation

Egr-1 has been identified as a growth response gene in cultured cells and in response to B cell maturation (Lemaire *et al.*, 1988; Christy *et al.*, 1988; Sukhatme *et al.*, 1987). It is also rapidly induced during differentiation of many cell types such as nerve, bone and myeloid cells during G0-G1 transition (Milbrandt, 1987; Sukhatme *et al.*, 1987). Growth factors are one of several targets of Egr-1; numerous studies have shown that Egr-1 binds to Egr-1 recognition sites within the promoter of PDGF-A chain, PDGF-B chain, bFGF, and FGF-2 (Hu *et al.*, 1994; Silverman *et al.*, 1997; Khachigian *et al.*, 1996). Moreover, both TGF-β1 and TGF-β3 contain Egr-1-binding sites in their promoters, and both mRNA were elevated in response to injury (Kim *et al.*, 1989). Furthermore, antisense oligonucleotides studies have indicated that Egr-1 expression

plays a critical role in macrophage differentiation. This is confirmed by blocking Egr-1 with antisense oligonucleotides that did block the PMA-induced differentiation of HL-60 to macrophages, but had no effect on DMSO-induced granulocytic differentiation (Neguyen *et al.*, 1993). These results provided evidence for the role of Egr-1 in macrophage differentiation. Recently, a binding site for Egr-1 has been identified within the IL-2 promoter, which is an important factor for the proliferation and differentiation of lymphocytes, and Egr-1 binds to this site following stimulation of T cells (Skerka *et al.*, 1995). In addition to the IL-2 gene, the gene encoding inosine-5′-monophosphate dehydrogenase (IMPDH) type II, which is linked to cellular proliferation, is a target for the Egr-1 transcription factor (Zimmermann *et al.*, 1995).

1.7.4.2.2 Role of Egr-1 in Regulating Cell Adhesion Molecule Expression

Cellular adhesion reactions play a crucial role in the induction, expression, and regulation of the immune responses. An early event in inflammation is the recruitment and adhesion of leukocytes to the sites of inflammation. This is mediated by binding of leukocytes to several cell adhesion molecules that are expressed by stimulation. A number of studies have proposed a role for CD44 and ICAM-1 in recruitment of leukocytes to sites of inflammation (de Grendele *et al.*, 1996; Dougherty *et al.*, 1988). It has been previously shown that CD44 and ICAM-1 are expressed at high levels in gastric epithelial cells, endothelial cells and B cells (Fan *et al.*, 1995a; Fitzgerald and O'Neill, 1999; Maltzman *et al.*, 1996a). In addition, *H. pylori* infection up-regulates the expression of CD44 and ICAM-1 on gastric epithelial cells (Fan *et al.*, 1995a). Importantly, the expression of CD44 (Fitzgerald and O'Neill, 1999; Maltzman *et al.*, 1996a) and ICAM-1 (Maltzman *et al.*, 1996b) were found to be controlled by the activity of Egr-1. Using transient transfection studies, deletion mutations of the Egr-1 binding site from the CD44 and ICAM-1 promoters significantly abrogated transcriptional induction of these genes (Maltzman *et al.*, 1996a and 1996b). Therefore, *Egr-1* and its transcription factor product appears to play a critical role in regulating levels of migration and adhesion molecules during the initiation of the immune responses.

1.7.4.2.3 Role of Egr-1 in Regulating Apoptosis

Many genes have been identified as DNA damage-inducible (DDI) genes or as growth arrest and DNA damage genes (GADD) including the Egr-1 transcription factor. The importance of Egr-1 as a DDI gene is highlighted by the fact that a number of DDI genes such as metallothionein, ras, fos, jun B and jun D contain the GC-rich Egr-1 response element in their promoter region (Searle *et al.*, 1984; Rachal *et al.*, 1989). Because Egr-1 is induced very early in the apoptotic process (Muthkkumar *et al.*, 1995; Ahmed *et al.*, 1996), it is expected to mediate the activation of downstream genes that play crucial roles in growth control. Recently, the human Egr-1 gene was found to be localized to human chromosome 5q31.1, a region known to be often deleted from patients suffering from therapy-induced acute myeloid leukemia (Nagarajan *et al.*, 1990).

Functional studies have suggested that Egr-1 functions as tumor suppressor gene and as anti-proliferative protein in a number of tumor cells (Huang *et al.*, 1994; Swirnoff and Milbrandt, 1995), and acts to increase the potency of apoptotic agents (Muthukkumar *et al.*, 1995; Huang *et al.*, 1995). Consistent with this, apoptosis-inducing stimuli such as TG and ionizing radiation, up-regulate Egr-1 expression and blockade of Egr-1 expression with a dominant negative construct or antisense oligmers inhibited Egr-1 function, indicating that Egr-1 is essential for apoptosis induced by TG and ionizing radiation (Ahmed *et al.*, 1996; Muthukkumar *et al.*, 1995). Similarly, studies on mouse fibroblast NIH 3T3 cells also have indicated that Egr-1 may confer resistance to growth inhibition caused by ultraviolet radiation (Huang and Adamson, 1995). The mechanism of the protective function of Egr-1 in these cell types is poorly understood, but it has been postulated that Egr-1 may regulate the expression of genes such as metallothionein and ras, whose promoters contain Egr-1 sites, which have protective function (Searle *et al.*, 1984; Rachal *et al.*, 1989). Li-Weber *et al.* (1999) demonstrated the necessity of the presence of Egr-1 binding sites in regulating the expression of CD95 (APO-1/Fas) ligand in activated T cells, which is a major cause of activation-induced T cell apoptosis. In addition, deletion mutations studies showed

that all three binding sites of Egr-1 (-120, -180 and -680) are required for CD95L promoter activity.

Other Egr-1-dependent genes that may influence cell survival and cell death is the tumor suppressor protein, p53, an essential mediator of cell cycle growth arrest and apoptosis (Lane, 1992). In many tumor cells, p53 is the most commonly mutated tumor suppressor gene, and most of the commonly occurring mutations in p53 are located in the DNA-binding or transactivation domains of the protein (Hollstein *et al.*, 1994). Several immediate-early genes require wild-type p53 for apoptosis. In addition, the apoptotic action of Egr-1 is mediated via wild-type p53 (Nair *et al.*, 1997). These results establish a direct functional link between Egr-1 and p53-mediated cell death and suggest that mutant forms of p53 in tumor cells may provide resistance to the anti-proliferative effects of Egr-1. The mechanisms by which Egr-1 induces these distinct effects are unclear, but Egr-1 binding sites have been identified in the promoter regions of a number of genes regulating cell cycle, cell proliferation and apoptosis, which may illustrate the proapoptotic action of Egr-1.

1.8 SUMMARY

Despite the recent advances that have been made in our understanding of the aetiology and pathogenesis of peptic ulceration, the exact mechanisms of ulcer development and resolution are poorly understood. In addition, the discovery of *H. pylori* by Marshall and Warren (1984), followed by the high volume of work identifying the role of this human pathogen in the pathogenesis of peptic ulceration, has transformed the way physicians cure some of the most prevalent gastrointestinal pathologies. There is clear evidence that *H. pylori* eradication not only heals peptic ulcer, but also prevents ulcer recurrences. It has been appreciated that the gastric mucosal immune response may represent one main pathogenic mechanism in *H. pylori*-associated diseases (Hatz *et al.*, 1992). A somewhat better understanding exists concerning the mechanisms of gene expression that occurs at the transcriptional level and many of proinflammatory cytokines have been shown to be regulated by many transcription factors.

Genetic variations in transcription factors and/or their binding site sequences contribute to several disease states including cancer (Wang *et al.*, 1999; Taniuchi *et al.*, 1997). NF-κB and Egr-1 are induced in various cell types and tissues by many stimuli and function to control initiation of transcription of several genes involved in cell proliferation, differentiation, signal transduction and apoptosis. In order to understand the regulatory mechanisms of *H. pylori* infection in gastroduodenal ulceration, this present study examines the expression of the transcription factors NF-κB and Egr-1 in response to *H. pylori* in gastric epithelial cancer cells under various redox conditions. Identification of specific and efficacious inhibitors of such transcription factors might provide a new approach for the treatment of gastroduodenal diseases.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

Materials and Methods

2.1 CHEMICALS

Recombinant human TNF-α was a gift from Cetus Corporation, Emeryville, CA. Poly(dI-dC) was obtained from Pharmacia (Biosystems, Milton Keynes, UK). [γ-^{32}P]ATP (35 pmol, 3000 Ci/mmol) and D-THREO [dichloroacetyl-1-^{14}C] chloramphenicol (56 mCi/mmol) were from Amersham International (Aylesbury, UK). The Luciferase Assay System was from Promega Inc. (Madison, WI). Bovine albumin, ammonium persulphate, acrylamide:bisacrylamide (29:1), Nonidet P40 (NP40), PMA, leupeptin, 2-mercaptoethanol, acetyl coenzyme A, ascorbic acid (vitamin C), N-acetyl cysteine (NAC), ampicillin, EDTA, IL-1β and PMSF were obtained from Sigma (Poole, Dorset, UK and St. Louis, MO., USA). The recombinant thioredoxin was a gift from Dr. Henry Windle (Department of Clinical Medicine, St. James's Hospital, Trinity College, Dublin 8, Ireland). Agarose was purshased from GIBCOBRL (Life Technologies, Paisley, Scotland). Sodium chloride, magnesium chloride, calcium chloride, ethanol, methanol, Tween-20, acrylamide, bisacrylamide, chloroform, glycerol, acetone, acetic acid, dimethylsulphoxide, TEMED and Tris(hydroxymethyl)aminomethane (Tris) were obtained from BDH Ltd (Poole, UK). Ethylether was obtained from Lennox Laboralorties LtD., Dublin, Ireland. Ethanol was obtained from Merck KgaA, Darmstadt, Germany. The PKC inhibitors calphostein C, rottlerin and Gö6976; the phosphatidylinositol 3-kinase inhibitor worthmannin; the tyrosine inhibitor herbimycin A and the p38 MAP kinase inhibitor SB203580 and the MEK MAP kinase inhibitor PD98059 were purchased from CALBIOCHEM, Novabiochem Corp., La Jolla, CA.

2.2 CARRIERS

Vitamin C (sodium salt) was dissolved in phosphate buffered saline (PBS), pH 7.4; NAC in DMSO (both used immediately before each experiment) and Trx in 20 mM Tris-HCl, pH 7.4 (stored at -20^{0}C). PMA; NAC; IL-1β; the PKC inhibitors calphostein C, rottlerin and Gö6976; the phosphatidylinositol 3-kinase inhibitor worthmannin; the

tyrosine inhibitor herbimycin A and the MAP kinase inhibitors SB203580 and PD98059 were dissloved in DMSO and stock solutions were kept at -20^{0}C. In the cases of all inhibitors and other agents, appropriate concentrations were made in cell culture medium just prior to use. The final concentration of DMSO was adjusted to 0.1% (v/v) in all experiments.

2.3 OLIGONUCLEOTIDES AND ANTIBODIES

NF-κB consensus oligonucleotide was obtained from Promega (Promega Corp., Madison, WI). Egr-1 consensus oligonucleotide, polyclonal antibody to Egr-1 (588), polyclonal antibodies to I kappa B-alpha (IκB-α) and p65, anti-Egr-1 antibody (sc-110X), anti-p50 (sc-114X), anti-RelAp65 (sc-109X) and anti-c-Rel (sc-70X) for gel supershift assays were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antisense Egr-1 oligonucelotides were a gift from Dr. Timothy McCffary (Department of Biochemistry and Molecular Biology, The George Washington University, Washington, DC, USA).

2.4 CELL TISSUE CULTURE

2.4.1 Source of Cell Lines

The gastric epithelial cell line AGS, derived from human gastric adenocarcinoma was obtained from the European Collection of Animal Cell Cultures, ECACC (Porton Down, Salisbury, UK). The gastric cancer cell line KATO-3 and the colonic cancer cell line HT29 was obtained from the American Type Culture Collection, ATCC (Manassas, VA). HuT 78, a T-cell lymphoma derived from peripheral blood of a patient with a Sezary lymphoma and the colonic cancer cell line T84 were obtained from the European Collection of Animal Cell Cultures, ECACC (Porton Down, Salisbury, UK).

2.4.2 Cell Culture Reagents

RPMI 1640 medium, foetal calf serum, penicillin, streptomycin, L-glutamine, Hank's Balanced salt solution (HBSS) and trypsin were obtained from GIBCOBRL (Life Technologies Renfrewshire, Paisley, Scotland). Dulbecco's MEM-nutrient mix F-12

medium was obtained from Sigma (Poole, Dorset, UK and St. Louis, MO., USA). 'Lymphoprep' density gradient was obtained from Nycomed Pharma AS (Oslo, Norway).

2.4.3 Maintenance of Cell Cultures

Cell lines were grown in complete medium supplemented with 10% foetal calf serum (FCS), 100 Units/ml penicillin, 100 μg/ml streptomycin and 2mM L-glutamine (Harlow and Lane, 1988). The medium was stored at 4^0C and used within two weeks, as glutamine can become enzymatically converted by serum enzymes (Griffiths, 1992). A stock solution of penecillin/streptomycin and L-glutamine was prepared and stored in aliquots at -20^0C [Appendix A] until required. Aliquots of FCS were prepared by incubation at 56^0C in a water bath for 60 min, to heat inactivate complement, and stored at -20^0C. Cells were maintained in culture flasks (Nunclon, Kamstrop, Roskilde, Denmak) at 37^0C in a CO_2 incubator and examined regularly by phase contrast microscopy. Cells were spilt between 2 to 4 days, depending on their seeding concentration, for experimental use.

2.4.3.1 Adherent Cells

Confluent AGS, KATO-3 and HT29 cells grown in RPMI 1640 medium or T84 cells grown in Dulbecco's MEM-nutrient mix F-12 medium were removed from culture flasks by trypsinization with 1x trypsin, after discarding the spent medium containing dead cells and metabolites. Cells were then washed twice with Hanks balanced salts solution (HBSS solution containing 0.02 M HEPES buffer) [Appendix A] at 2000 rpm for 5 min, to remove cell debris and resuspended in complete medium for experimental procedures.

2.4.3.2 Non-Adherent Cells

Confluent HuT 78 cells (20 ml) were placed on 5ml 'lymphoprep' density gradient and centrifuged at 1200 rpm for 25 min. The buffy coat containing the cells was removed, washed twice with HBSS solution at 2000 rpm for 10 min, to remove cell debris and dead cells, and resuspended in complete medium.

2.4.4 Freezing and Resuscitation of Cells from Liquid Nitrogen

Stocks of viable cells were maintained by cryopreservation. Cell lines were frozen in aliquots ($1x10^6$ cells/ml) in filtered FCS containing 10% dimethysulphoxide (DMSO) (Hay, 1992) [Appendix A] in sterile cryostat tubes (Sarstedt, Numebrech, Germany) at -70^0C, and then transferred to liquid nitrogen (Harlow and Lane, 1988). Cells were subclutured the day before freezing; adherent cells were subcultured after trypsinization and washing in complete medium, to inactivate trypsin, and non adherent cells were washed as in section 2.2.3. Frozen cells were resuscitated by rapid defrosting and resuspended in 10 ml complete medium. Cells were then centrifuged at 1500 rpm for 5 min, washed twice with HBSS and then resuspended in complete medium in a tissue culture flask in a CO_2 incubator at 37^0C (5 ml complete medium in 25 ml tissue culture flask).

2.4.5 Cell Counting and Viability

In order to assess cell number and viabilty, Acridine orange (AO) and Ethidium bromide (EB) fluorescence staining (Lee *et al.*, 1975) was used. Cell counting was performed by diluting cells with EB/AO working solution (1/10) [Appendix A] and counted on a Neubauer haemocytometer (Hudson and Hay, 1976). AGS cells were seeded at concentration $5x10^5$ cells/ml and used when ~80% confluent (Fig. 2.1). If cell viability was less than 80%, cells were discarded.

2.5 *H. PYLORI* AND COCULTURE WITH GASTRIC EPITHELIAL CELLS

2.5.1 Bacterial Strains

H. pylori reference strains NCTC 11637 and NCTC 11638 (both $VacA^+$ and $CagA^+$) and isolates 92-701 and 93-1000 (both $VacA^-$ and $CagA^-$) and 92-1099 ($VacA^+$ and $CagA^-$) (the last three strains do not possess the *cagA* gene and do not express the CagA protein, were kindly provided by Prof. Cyril Smyth, Microbiology Dept., Moyne Institute, Trinity College, Dublin, Ireland). *Escherichia coli* (C-600) and *Campylobacter jejuni* (clinical isolates), were used as control bacteria.

Fig. 2.1. The gastric epithelial cells AGS were grown in 6-well plates at a concentration $5x10^5$ cells/ml and photographed at X400 magnification.

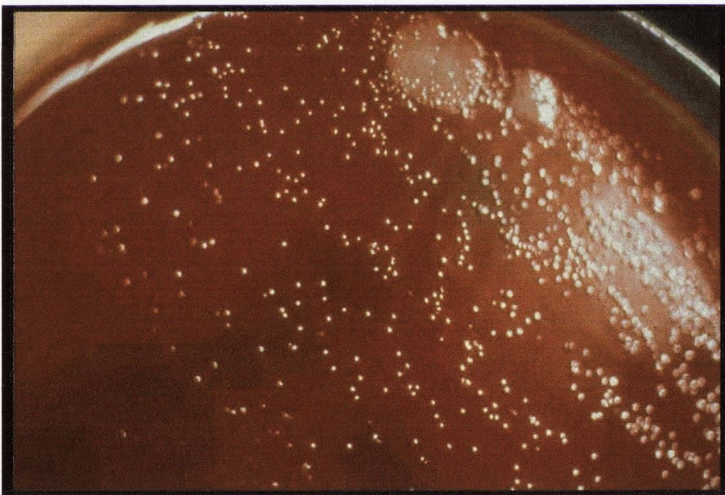


Fig. 2.2. Morphology of 3 days old *Helicobacter pylori* colonies grown on blood agar medium. Colonies are circular, 1-2 mm in diameter, non-hemolytic to blood agar and translucent in appearance (Magnification X400).

2.5.2 Growth Conditions of *H. pylori* Strains

H. pylori strains were grown in a microaerobic humidified atmosphere on either 7% lysed horse blood Columbia agar in an anaerobic jar (Oxoid) using a Gas Generating kit for Campylobacters (Oxoid) or modified brain heart infusion broth medium with constant shaking at 150 rpm (Orbital Incubator S1 50, Stuart Scientific, UK) at 37^0C (Xia *et al.*, 1993). Fig. 2.2 shows colonies of *H. pylori* from primary culture on supplemented blood agar at 37°C, usually take 3 to 4 days to appear and they are circular, convex and translucent in appearance. After 48-72 h, bacteria were harvested in phosphate-buffered saline; PBS (pH 7.4); (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl) or RPMI 1640 medium without antibiotics and resuspended to a concentration of $6x10^8$ colony-forming units/ml using the McFarland standard kit and used immediately.

2.5.3 Coculture of the Gastric Epithelial Cells with *H. pylori* and Other Stimuli

AGS cells were removed from flasks by trypsin/EDTA treatment and seeded at a density of $5x10^5$ cells/ml in 6-well plates. Confluent AGS cells were cultured alone or with *H. pylori* at different doses for different periods of time. In some experiments, AGS cells were treated with interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) or the mitogen phorbol 12-myristate 13-acetate (PMA). AGS cells were also incubated in media of different pH values for the indicated times (as indicated in figure legends). To adjust media to the required pH value, 0.1 M HCl or 0.1 M NaOH was added to the cell culture medium and titrated to the required pH. The pH of the culture medium was measured before and after the incubation to ensure that the pH remained unchanged. For controls, equivalent volumes of sterile deionized water were added. *E. coli* and *Campylobacter jejuni* were used as control bacteria. Each experiment was performed in triplicate and representative results are shown.

2.6 PREPARATION OF BLOOD SAMPLES

2.6.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood samples were obtained by venupuncture from healthy volunteers into 10 ml heparinized vacutaniers (Becton Dickinson UK Ltd., Cowley, Oxford, UK).

PBMCs were separated as described by Boyum (1968), by diluting heparinized blood in half with HBSS solution and layering this solution onto 'lymphoprep' density gradient (20 ml blood/HBSS and 5 ml lymphoprep) and centrifuged at 1200 for 25 min. The buffy coat containing PMBCs was removed, diluted 1/5 in HBSS solution and centrifuged at 2000 rpm for 15 min. The supernatant was removed and PMBCs were washed with HBSS solution by centrifugation. Cells were finally resuspended in RPMI 1640 medium for use.

2.6.2 Preparation of Cytokine Rich Supernatants from PBMC

PBMCs were obtained from healthy donors (sero-negative for *H. pylori*) as described in Section 2.5.1. Supernatants were then prepared by co-culturing PBMCs ($2x10^6$ cells/ml) for 18 h with *H. pylori* (15 μg/ml), PMA (10 ng/ml) plus phytohaemagglutinin (10 μg/ml), *E.coli* (15 μg/ml) or TNF-α (20 ng/ml), respectively, in RPMI 1640. After 18 h stimulation, PBMCs were washed three-times with HBSS and incubated in RPMI 1640 medium for a further 48 h. The medium at the end of this period was collected and cultured with AGS cells (Fan *et al.*, 1996a).

2.7 PREPARATION OF CELL PROTEIN

2.7.1 Preparation of Whole Cell Extracts

Confluent cells in six-well plates (2 ml volume) were treated as indicated in the figure legends where appropriate, and at the end of the stimulation, whole cell extracts were prepared as described by Kelleher and Long (1992). Treatment was terminated by washing cells with ice-cold PBS, pH 7.4 [Appendix A], and cells were collected by scraping and centrifugation , and all the following steps were carried out on ice. Cells were lysed in PBS (0.5 ml) containing 1μl PMSF (0.1 mM), 3 μl leupeptin (2 mg/ml) and 0.5 ml 1% NP40 [Appendix A], vortexed every 5 min for 30 min to deposit cell debris. The supernatants were removed as cell lysates and stored at -20^0C until required.

2.7.2 Protein Estimation

The protein concentration of cell extracts was determined by the dye-binding method of Bradford (Bradford, 1976), in order to standardize the amount of the protein of each lysate for Western blotting [Appendix A]. Bovine serum albumin (BSA) was used as the protein standard.

2.8 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

2.8.1 Preparation of Protein Samples and Molecular Weights Standards

Following protein estimation, test samples were aliquoted to yield 50 μg of sample protein. This volume was diluted 1:5 with ice-cold acetone to concentrate the protein sample, vortexed and allowed to incubate at -20^{0}C for at least 30 min. The tubes were then centrifuged at 15,000 g for 2 min and the supernatant was discarded. Excess acetone was allowed to evaporate at room temperature for 15 min. Samples containing 50 μg were then suspended in 20 μl 1x sample buffer [Appendix A]. Samples and molecular weight standers were vortexed and boiled for 5-10 min and centrifuged to precipitate any insoluble solids.

2.8.2 Polyacrylamide Gel Electrophoresis (PAGE)

Equivalent amounts of proteins in cell extracts were separated on 10% non-reducing polyacrylamide gel prepared by the method of Laemmli, (1970) with some modifications. Both resolving and stacking gels were prepared in the order indicated in **Table 2.1** [See Appendix A for composition of solutions]. The APS and TEMED were added last with gentle swirling of the mixture. Samples containing 50μg and standards (5μl) were loaded into the wells of the gel and the electrodes were connected and the gels were run at 25 mA. At the end of electrophoresis, the proteins on the gel were electrotransferred to PVDF membrane (Gelman Sciences Inc., Ann Arbor, MI, USA) by semi-dry transfer as described by Towbin (1979). Following transfer, the PVDF membrane was removed and processed for immunoblotting. The lane containing the molecular weight marker was stained with Commaasie Blue [Appendix A], followed by destaining with 50% methanol.

Component	Resolving gel (10%)	Stacking gel (10%)
Distilled water	8.23 ml[a]	5.55 ml
Resolving buffer (1.5 M Tis, pH 8.8)	5.0 ml	--
Stacking buffer (1 M Tris, pH 6.8)	--	3.05 ml
Acrylamide/Bisacrylamide	6.66 ml	1.33 ml
10% APS	100 µl	50 µl
TEMED[b]	10 µl	10 µl

10% APS: 0.1g/1ml

a: Volume (20 ml) is sufficient for 2 gels

b: N,N,N′,N′-tetra-methylenediamine

Table 2.1: Composition of gels for PAGE electrophoresis

2.8.3 Immunoblot Detection

Non-specific sites were blocked by incubation of the membranes with a freshly prepared PBS containing 5% non-fat skimmed milk (Blotto-Tween solution) [Appendix A] for 1h with gentle shaking (Orbital Shaker S03, Stuart Scientific, UK) at room temperature. The blots were then washed twice with PBS and incubated with specific primary antibodies (Egr-1, p65 or IκB-α) in a sealed plastic bag in a dilution 1:1000 in Blotto-Tween for 1h with shaking at room temperature followed by washing (x3), 10 min each time, with PBS-Tween. Blots were then incubated with the relevant secondary antibody (Swine anti-rabbit for polyclonal antibodies) horseraddish peroxidase conjugate in a dilution 1:2000 in PBS-Tween for 1h at room temperature with gentle shaking. Blots were washed again (x3), 10 min each time, with PBS-Tween. The immunoblots were developed using enhanced chemiluminescence (ECL).

2.8.4 Enhanced Chemiluminescence (ECL)

Detection of the immunoblots was performed by enhanced chemiluminescence (ECL) method. The blot membranes were incubated for 1 min in a solution of iodophenol (400 µM), luminol (1.25 mM) and hydrogen peroxide (0.01% v/v) in 0.1M Tris-HCl (pH 8.8) [Appendix 1]. The membrane was put between two acetate sheets, excluding air bubbles, and any residual developing buffer solution was wiped out. Blots were exposed to Kodak X-OMAT S film for 10-30 seconds. Exposed films were then

developed using automatic developer (CURIX 60, AGFA, Type 9462/100/140, Agfa-Gevaert AG München, Germany).

2.9 Immunohistochemical Staining of Gastric Biopsies

Immunohistochemistry studies were carried out in order to assess the expression of NF-κB and Egr-1 transcription factors *in situ* in the human stomach in response to colonization by *H. pylori*. Immunohistochemical staining was performed on archival formalin-fixed, paraffin-embedded antral specimens that had been obtained from dyspeptic patients attending the gastroenterology clinic at St. James's Hospital, Dublin, Ireland. Antral specimens represented patients with normal gastric mucosa (n=5), patients with chronic gastritis (*H. pylori*-negative) (n=5) and patients with *H. pylori* infection (n=10). Paraffin sections (4 μm thick) were mounted on slides coated with 3-aminopropyltriethoxy-silane, sections were dewaxed and rehydrated through the xylene and graded alcohols series to distilled water. Endogenous peroxidase activity was quenced by incubating sections in 0.3% H_2O_2 in methanol for 30 min at room temperature. For epitope retrieval, the sections were microwaved in citrate buffer solution at pH 6.0 [Appendix A] on high power for 20 min. Slides were allowed to cool down at room temperature for 15 minutes. Sections were rinsed in running water for 2-3 min and transferred to TBS [Appendix A]. Non specific binding was blocked by covering the slides with 0.1% bovine serum albumin for 10 min at room temperature. Any excess buffer solution was wiped out from the slides and incubated with an appropriate primary antibodies; anti-RelAp65 polyclonal antibody (1:50) or anti-Egr-1 polyclonal antibody (1:50) in PBS overnight at 4^0C. Sections were washed twice in PBS, 5 min each time, and excess buffer solution was decanted and incubated with the secondary peroxidase-conjugated swine anti-rabbit immunoglobulin; Ig (DAKO, 1:50) in PBS for 1 h at room temperature followed by washing slides with PBS. Bound antibody was visualised by the avidin-biotin peroxidase method (Hsu *et al.*, 1981) using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The ABC reagent must be made up 30 min before use and allowed to stand at room temperature. The peroxidase activity was detected by diaminobenzidine tetrachloride (DAB, Sigma, St. Louis, MO). DAB

solution was prepared by dissolving one DAB tablet and one Urea Hydrogen Peroxide tablet in 5 ml of distilled water and the solution was vortexed and used within 1h of its preparation. When DAB was added to the slides, a brown reaction product was formed within 5-10 min. Finally, slides were further washed in running water for 2 min and briefly counterstained with Mayer's hematoxylin, slides were dehydrated in graded of alcohols as before and mounted with permount. Sections were considered as immunopositive when cells showed distinct brown staining. In some experiments, paraffin sections were stained without adding the polyclonal anti-Egr-1 or anti-RelAp65 antibodies or use of appropriate Ig control, the rabbit polyclonal Ig antibody (DAKO, 10 μg/ml) and used as controls.

2.10 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

2.10.1 Cell Fractionation and Nuclear Extract Preparation

Nuclear extracts were prepared as described by Osborn *et al.* (1989). Confluent cells in six-well plates (2-ml volume) were treated as shown in figure legends and treatments were terminated by removal of the medium and washing the cells twice with ice-cold PBS. All subsequent steps were carried out on ice using ice-cold buffers. Cells were harvested by scraping with a cell scraper, and transferred to centrifuge tubes on ice. The cells were pelleted by centrifugation at 1400 rpm for 5 min and washed once in (1 ml) buffer A [Appendix A] and centrifuged at 10,000 rpm for 10 min. The pellet of cells was then resuspended in buffer A (20 μl) containing 0.1% (v/v) Nonidet NP40 for 10 min on ice and lysed cells were centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the nuclear pellet was extracted with (15 μl) buffer C [Appendix A] for 15 min on ice. After incubation, the nuclei were centrifuged at 10,000 rpm for 10 min and the supernatant was diluted with 4 volumes of buffer D [Appendix A]. In some experiments, nuclear extracts were prepared from HuT 78 cells, as these cells contain high levels of constitutive NF-κB, and used as positive controls for EMSA assays. The nuclear extracts were used immediately or stored at -70^{0}C until required.

2.10.2 Labelling of Consensus Oligonucleotides for EMSA

5`-end-labelling of the 22 base pair oligonucleotide containing the NF-κB consensus sequence (5`-AGT TGA GGG GAC TTT CCC AGG C-3`)(3`-TCA ACT CCC CTG AAA GGG TCC G-5`) or the 27 base pair Egr-1 consensus sequence (5`-GGA TCC AGC GGG GGG GAG CGG GGG CGA-3`) was performed by the method of Sambrook *et al.* (1989). The reaction mixture was assembled in a sterile microcentrifuge tube in a final volume of 50 μl with sterile water, **Table 2.2**.

Component	Amount
5`-ends of DNA probe (1-50 pmol)	5.7 μl
T4 polynuclotide kinase (10-20 U)	2.5 μl
10X Kinase buffer	5 μl
[γ-^{32}P]ATP (3000 Ci/mmol, 10mCi/ml, 50pmol)	15 μl
Sterile water	21.8 μl
	Final volume=50μl

Table 2.2: Constituents of labelling reaction of consensus oligoneucleotides

The reaction mixture was incubated in a pre-warmed perspex box at 37^{0}C for 10 min and the reaction was terminated by addition 2 μl of 0.5 M EDTA. To this mixture, 50 μl of phenol:chloroform solution (1 part TE-saturated phenol and 1 part choroform:isoamyl alcohol (24:1 ratio) was added to extract the DNA, and vortexed for 1 min and centrifuged at 13,000 g for 2 min. The upper aqueous layer was transferred to a fresh tube and 2 μl of 5 M NaCl was added. The tube was vortexed and 100 μl of ethanol was added followed by incubation at -70^{0}C for 30 min to allow ethanol precipitation of the DNA. The mixture was centrifuged at 13,000 g for 5 min, the supernatant was carefully removed and the pellet was dried in vacuum dryer. Finally, the pellet was resuspended in 50 μl of TE buffer [Appendix A]. To calculate the activity of the labelled oligonucleotide, 1 μl of this solution was counted in 5 ml

Ecosint and counted using using a Wallac 1409 DSA Liquid Scintillation Counter. 10,000 cpm of labelled oligonucleotide was used per reaction for gel shift assays.

2.10.3 Preparation of Gels for EMSA

Proteins in nuclear extracts were separated on 5% polyacrylamide non-reducing gels using the ATTO gel system (ATTO, Japan). The glass plates were washed and cleaned throughly to remove any residue which may interfere with running of the gel and allowed to air dry. The composition of the gel is indicated in **Table 2.3** [See Appendix A for composition of solutions]. Both DTT and TEMED were added last to the gel with gentle swirling of the gel solution. The gel mixture was poured gently into a 1 mm thick gel mould, and allowed to polymerize for 30 min at room temperature.

Component	Amount
Distilled water	19.4 ml
Acrylamide Mix (40%)[a]	3.125 ml
10X TBE buffer	2.5 ml
Ammonium persulphate	0.05 g
TEMED[b]	15 μl
DTT[c]	5 μl

a: Accugel (29:1) b: N,N,N′,N′-tetra-methylenediamine
c: Dithiothreitol

Table 2.3: Formulation of gels for EMSA

2.10.4 Preparation of DNA-Protein Binding Reaction

Binding reaction was prepared for detection of NF-κB or Egr-1 activity by electrophoresis as described by Sen and Baltimore (1986). Nuclear extracts (4 μg of protein) were incubated with 10000 cpm of the ^{32}P-labeled NF-κB or Egr-1 consensus oligonucleotides (Section 2.9.2). The assay was performed in 20 μl binding reaction in the presence of binding buffer [Appendix A] and 2 μg of poly(dI-dC) as non specific competitor, **Table 2.4**. The reaction mixture was then incubated for 30 min at room

temperature. The binding reaction was terminated by addition of one tenth volume of gel loading dye [Appendix A] prior to loading the samples onto the gel for electrophoresis.

Component	Amount
Nuclear extract	2-4 μg
10X binding reaction buffer	1 μl
[γ-^{32}P]ATP labelled DNA probe	1 μl
Poly (dI-dC)	2 μl
Sterile water up to a final volume of	20 μl

Table 2.4: DNA-protein binding reaction

2.10.5 Electrophoresis of DNA-Protein Complexes

The DNA-protein complexes were separated on 5% polyacrylamide gels that had been pre-run in 0.5X TBE buffer [Appendix A] for 30 min at 80 V. The gels were run in 0.5X TBE buffer at 150 V for 2-3 h or until the bromophenol blue dye was three-quarters of the way down the gel. After electrophoresis was performed, the power supply (Consort EUSS microcomputer electrophoresis power supply) was disconnected and the gels were removed from the plates and transferred to a piece of filter paper and wrapped with cling film. The gels were then dried on an automatic dryer and exposed to Kodak X-OMAT S film for 24-48 h at -70^{0}C with intensifying screens. The autoradiograph films were developed using CURIX 60, AGFA, Type 9462/100/140 (Agfa-Gevaert AG München, Germany).

2.10.6 Supershift and Competition Assays

Competition assays or supershift assays were performed in order to identify the specificity of DNA-protein complexes. For competition assays, 100-fold molar excess of unlabeled oligonulcelotide (NF-κB or Egr-1) was added to the nuclear extract (4 μg protein) 30 min prior to addition of the labelled probe at room temperature. In

supershift assays, 0.5 μl of rabbit antisera to specfic NF-κB/Rel subunits (anti-p50, anti-RelAp65, and anti-c-Rel), or anti-Egr-1 antibodies was incubated with nuclear extracts for 30 min at room temperature prior to the binding reaction.

2.11 TRANSIENT TRANSFECTION AND EVALUATION ASSAYS

2.11.1 DNA Constructs

The chloramphenicol acetyltransferase (CAT) promoter plasmid pRb containing 1.7 kb of the CD44 upstream regulatory region was a generous gift from Dr. Emma Shtivelman (University of California, San Francisco, CA). Dr. John Monroe (Department of Pathology and Laboratory Medicine, University of Pennsylvania, PA) kindly provided pCAT reporter plasmid containing 1.1 kb of the murine ICAM-1 upstream regulatory region and constructs pBLCD44, pBLmCD44, pBLICAM-1 and pBLmICAM-1. The luciferase reporter plasmid containing the human CD95L promoter spanning -860 to +100 and the mutant CD95L promoter carrying point mutations at the -680, -180 and -120 Egr-1 sites were from Dr. Min Li-Weber, Tumor Immunology Program, German Cancer Research Center (DKFZ), Heidelberg, Germany

2.11.2 Transformation of Bacterial Cells

Competent *E. coli* DH5α cells were transformed with plasmid DNA (0.1-1 μg) by incubating 100 μl competent cells with 1 μg plasmid DNA on ice for 30 min, followed by heat shocking the cells for 1-2 min at 42^0C. Cells were cooled on ice for 1-2 minutes before being transferred to 1 ml warm broth (LB) medium [Appendix A]. Cells were allowed to recover for 2 h with gentle shaking at 37^0C. Transformed cells were then plated onto L-agar containing ampicillin and left for 12-24 h at 37^0C. A single colony purification was then performed by taking a single colony and growing this overnight at 37^0C.

2.11.3 Preparation and Purification of Plasmid DNA from *E. coli* DH5α

LB broth (10 ml) was inoculated with pure colonies, and cells were grown for 16-18 h at 37^0C under ampicillin antibiotic selection. Stock solutions were made by adding

930 μl aliquots of growing culture into sterile cryotubes containing 70 μl DMSO and stored at -70^{0}C. Plasmid DNA was isolated from *E. coli* DH5α, as described previously (Feliciello *et al.*, 1993). Briefly, cells were harvested by centrifugation at 2,800 rpm for 20 min at room temperature. The pellet was resuspended in 1ml ice-cold STE [Appendix A], and cells were harvested by spinning at 8,000 g for 1 min. The pellet was resuspnded in 250 μl of ice-cold solution I [Appendix A] followed by adding 500 μl solution II [Appendix A] and mixed gently and incubated for 3-5 min on ice. To this solution, add 750 μl ice-cold first precipitation solution [Appendix A] was added. After 10 min incubation, samples were centrifuged at 12,000 g for 5 min and the supernatant containing plasmid DNA was carefully transferred to clean tubes. The supernatant was mixed with 700 μl isopropanol to precipitate DNA and tubes were centrifuged immediately at 12000 g for 5 min. The pellet was resupended in 250 μl TE buffer [Appendix A] containing 10 μg /ml RNase and incubated for 15 min at room temperature, followed by adding 300μl final precipitation solution [Appendix A] with mixing and incubated for another 10 min at room temperature. Tubes were then centrifuged at 12,000 g for 5 min and the supernatant carefully removed and DNA pellets were washed in 70% ethanol, air-dried for 15 min, redissolved in TE buffer (25 μl/tube) and DNA solutions were stored at -20^{0}C. The plasmid concentration was determined using horizontal agarose gel (1%) electrophoresis, and the concentration of the plasmid was estimated from this gel.

2.11.4 Transient Transfection

The gastric epithelial cells AGS were seeded in 24-well plates at a density of $5x10^5$ cells/ml medium in 250 μl volume until grown to ~ 80% confluence, the day before transfection. The cells were transfected with 1μg of plasmid constructs carrying the CAT reporter gene (pCD44, pBLCD44, pBLmCD44, pICAM-1, pBLICAM-1 or pBLmICAM-1) or 1 μg of plasmid carrying the luciferase reporter gene under control of the wild-type of CD95L or the mutant CD95L by GenePORTER transfection reagent (Gene therapy Systems Inc., San Diego, CA) according to the manufacturer's instructions. The DNA (1 μg) was diluted with serum-free medium using half of the

transfection volume (250 μl), and the GenePORTER reagent (5 μl) was diluted with serum-free medium using the other half of the transfection volume. The diluted DNA was added to the diluted GenePORTER reagent and mixed rapidly and incubated at room temperature for 10-45 min. The culture medium was aspirated from the cells and the DNA-GenePORTER mixture was added to the cells and incubated at 37°C for 3-5 h. Post transfection, one volume of culture medium containing 10% FCS (250 μl) was added and the cells were incubated overnight under 5-10% CO_2 at 37°C. The transfected cells were allowed to recover overnight and then incubated for 24 h in the absence or presence of *H. pylori* cells. Following treatment, culture medium was aspirated and cells were lysed with 100 μl of reporter lysis 5X buffer (Promega) and used immediately or stored at -70°C untill required. Expression assays were performed on cell lysates for CAT or Luciferase assay.

To measure transfection efficiency, 1 μg of pSV-β-galactosidase control vector (Promega) was simultaneously transfected into the cells and β-galactosidase activity was determined by a colourimetric assay (Promega). The standard assay is performed by adding a dilute sample to an equal volume of assay 2X buffer, which contains the substrate ONPG (o-nitrophenyl-b-D-galactopyranoside). Samples are incubated for at least 30 min, during which time the β-Galactosidase hydrolyzes the colorless substrate to o-nitrophenyl, which is yellow. The absorbance at 420 nm was measured by spectrophotometry. All transfection assays were carried out in triplicate in three independent experiments with two different plasmid preparations.

2.11.5 Chloramphenicol Acetyltransferase (CAT) Assay

At the end of stimulation, AGS cells were washed twice with PBS, scraped and resuspended in 1 ml PBS. Cells were centrifuged at 12,000 for 5 min at 4^0C and the pellets were resuspended in 100 μl of 0.25 M Tris-HCl (pH 8.0). Cells were lysed by four repeated freeze-thaw cycles in liquid nitrogen and at 37^0C. The cells were then centrifuged again at 12,000 for 5 min at 4^0C. The supernatant was removed and assayed for protein by the method of Bradford (1976). The CAT activity was determined as described by Fitzgerald and O'Neill (1999). Briefly, equal amounts of

protein (60 μg) from different cell extracts were incubated with 1 mM acetyl coenzyme A and 0.3 μCi D-THREO [dichloroacetyl-1-^{14}C] chloramphenicol (56 mCi/mmol) in a final volume of 91.5 μl overnight at 37^{0}C, as described in **Table 2.5**.

Component	Amount
Cell extract protein	60 μg
Acetyl CoA (Stock 100 mM)	9 μl
[^{14}C]Chloramphenicol (0.3 μCi)	3 μl
0.25 M Tris-HCl, pH 8.0	79.5 μl

Table 2.5: Preparation of CAT assay reaction

The reaction was terminated by addition of 350 μl ethylacetate and samples vortexed for 30 s. Samples were then centrifuged at 12,000 x g for 1 min and the upper phase (300 μl) was removed into a fresh tube and dried under vacuum. The pellet was resuspended in 12 μl ethylacetate, vortexed and spotted on silica-thin layer chromatograph (TLC) plate (0.2 mm thickness), allowing the solvent to dry between each application. The TLC plate was run for 30 min in TLC chamber that had been equilibrated with chloroform:methanol (19:1 v/v) for 30 min. After running the TLC plate, the plate was dried, autoradiographed to locate the acetylated and non-acetylated species of [^{14}C] chloramphenicol. The plates were then cut, mixed with 5 ml Ecosint and counted using using a Wallac 1409 DSA Liquid Scintillation Counter. The CAT activity was expressed as % acetylation by dividing cpm from acetylated chloramphenicol (top spots) by cpm from non-acetylated chloramphenicol plus acetylated chloramphenicol (bottom spots + top spots). The data results were expressed as means ± SD.

2.11.6 Luciferase Reporter Gene Assay

Following stimulation, cells were washed twice with PBS, after discarding the medium. Cells were then lysed in 120 μl of 1x cell lysis buffer (Promega) at room temperature for 15 min and whole cell lysates were collected by scraping and centrifugation at 12,000 g for 5 min at 4^0C. The Luciferase reporter gene activity was measured by incubating 20 μl of the cell lysates with the Luciferase Assay Reagent (Promega) according to the manufacturer's instructions. The light emission was measured immediately (45 s) after the addition of the Luciferase Assay Reagent on a luminometer (Mediators PhL, version 1.6, Diagnostic Systems). Luminescence was corrected for protein according to the dye-binding assay (Bradford, 1976). The Luciferase activity of each sample was normalized for β-galactosidase activity and the results were presented as a Luciferase Relative Activity.

2.12 FLOW CYTOMETRY ANALYSIS

AGS cells were grown to confluence in 6-well plates and then the cells were incubated with thioredoxin for 24 h at 37^0C. After pretreatment with thioredoxin, cells were stimulated with either *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for 24 h at 37^0C. The cells were washed with PBS and prepared for flow cytometry analysis. All labelling steps were performed at room temperature. Approximately $1x10^5$ cells were aliquoted to each Facs tube in a final volume of 200 μl. Cells were incubated with 5 μl of the following primary antibodies, CD44 (L3D.1) and ICAM-1 (CD54) for 15 min. Tubes were washed twice with PBS/BSA/Azide [Appendix A] by centrifuged at 200 g for 2 min. 100 μl fluorescein isothiocynate (FITC)-conjugated rabbit F(ab)2' fragment of rabbit anti-mouse IgG (Dakopotts, Glostrup, Denmark) (1:50 in PBS), was added to each tube, vortexed, incubated in the dark for 10 min and washed twice with PBS/BSA/Azide. At this point the cells were fixed with 0.5% p-formaldehyde (0.5 ml PFA) [Appendix A]. Samples were stored at 4^0C for up to 7 days before acquisition a Becton Dickinson FACScan flow cytometer. Cells were analysed using Lysys software.

2.13 CELL PROLIFERATION STUDIES

AGS cells ($1x10^5$ cells/ml) were cultured in 96-well plates in triplicate overnight at 37^0C. AGS cells were then incubated alone or in the presence of various doses of thioredoxin for 24-72 h. In some experiments, serum-starved AGS cells, 0.5% serum or 10% serum were used. To test the effect of thioredoxin on cell proliferation in response to *H. pylori* and TNF-α, AGS cells were incubated with 10 μg/ml thioredoxin for 24 h at 37^0C and then stimulated with either *H. pylori* strain 11638 ($6x10^8$ cfu/ml), TNF-α (20 ng/ml) or PMA (10 ng/ml) for an additional 24 h. To the cultured cells, 20 μl of freshly prepared PMS (phenazine methosulphate)/MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium, inner salt; MTS) solution was added to each well and the plates were incubated for 4 h at 37^0C. The absorbance of these wells was read at 490 nm using an enzyme-linked immunosorbant assay (ELISA) plate reader (Labsystems Multiskanplus, type 314, Finland). Under the experimental conditions described and in the range of thioredoxin concentrations used, cell viability was greater than 90% as assessed by microscope using AO/EB fluorescence staining.

2.14 DATA ANALYSIS

Experiments were conducted a minimum of three separate times each and data were analyzed after calculation of the mean and standard deviation for each experiment, using Students T test and results are expressed as mean ± SD.

CHAPTER III

EFFECTS OF *HELICOBACTER PYLORI* AND LOW pH ON NF-κB EXPRESSION IN GASTRIC EPITHELIAL CELLS

CHAPTER III

EFFECTS OF *HELICOBACTER PYLORI* AND LOW pH ON NF-κB EXPRESSION IN GASTRIC EPITHELIAL CELLS

CHAPTER III

3.1 INTRODUCTION

3.1.1 *H. pylori* and Gastric Acid Secretion

Helicobacter pylori colonizes the human gastric epithelium and is associated with the development of peptic ulcer disease and gastric carcinoma (Blaser, 1992; Moss and Calam, 1992). Infection with this gastric pathogen is characterised by neutrophil infiltration into the gastric mucosa (Dixon, 1991; Crabtree, 1996) and strong cellular response which may be important in gastric epithelial damage and ulceration (Weiss, 1989). This cellular response appears to be dependent on the host-bacteria interaction and bacterial cytotoxicity.

The role of gastric acid in the pathogenesis of peptic ulcer disease is clearly evident. *H. pylori* infection causes inflammation of the gastric and duodenal mucosa, which results in a disturbance of the regulation of gastric acid and pepsin secretion. *H. pylori* infection and gastritis in the corpus suppress acid secretion and increase gastric juice pH, resulting in hypergastrinemia. Eradication of *H. pylori* normalizes acid secretion and serum gastrin levels (Furuta *et al.*, 1998). The acid suppression by *H. pylori* appears to occur for several weeks after first infection (Graham *et al.*, 1988), which could be due to acid-suppressing factors from *H. pylori* (Cave and Vargas, 1989; Beil *et al.*, 1994). Several substances, including the cytokines IL-1β and TNF-α, induced by *H. pylori*, have been reported to inhibit acid secretion. (El-Omar, 2001; Noach *et al.*, 1994, Kobayashi *et al.*, 1996).

Duodenal ulcer patients differ from asymptomatic individuals infected with *H. pylori* by their increased acid secretion (Blair *et al.*, 1987), causing gastric metaplasia of the duodenum which in turn is then colonized by *H. pylori*. Maximal acid output is increased in *H. pylori* infected patients with duodenal ulcers and conditions of low pH appear to be important for causing mucosal damage (Calam *et al.*, 1997; Gillen *et al.*, 1998). Furthermore, *H. pylori* infection is also associated with elevated acid secretion during stimulation with acid-peptone and gastrin-releasing peptide (GRP) (El-Omar *et al.*, 1993). Eradication of *H. pylori* decreases basal acid secretion by about 60% in

duodenal ulcer patients (Moss and Calam, 1993). This increase in acid secretion by *H. pylori* in duodenal ulcer patients is thought to occur through decreasing somatostatin release and increasing gastrin release, leading to mucosal ulceration (Calam, 1995; Levi *et al.*, 1989). Further studies have shown that eradication of *H. pylori* normalized acid secretion via restoration of somatostatin secretion and reduction in the serum gastrin level (Graham *et al.*, 1990; El-Omar *et al.*, 1993).

3.1.2 Low pH and Transcriptional Regulation

Increasing evidence has indicated that an acidic environment contributes to the regulation of expression of gene transcription during the inflammatory process. The decrease of pH in inflammatory lesions is due to increased metabolic acid generation during cell activation (Wright *et al.*, 1986). Zabel *et al.* (1991) demonstrated that NF-κB could form a complex with DNA over a broad pH range. Reduced pH was found to potentiate the synthesis of TNF-α by macrophages via increased NF-κB binding activity in the nucleus (Bellocq *et al.*, 1998). Yamaji *et al.* (1994) reported that exposure of epithelial cells from mouse proximal tubules to an acid enviroment led to transcriptional activation of immediate early genes. These findings indicate the importance of an acidic environment in the regulation of several genes which might play a role the pathogenesis of many inflammatory disease states.

NF-κB participates in the regulation of the transcription of multiple cellular genes including genes encoding for cytokines, cell adhesion and acute phase proteins (Baeuerle and Henkel, 1994). *H. pylori* infection of gastric epithelial cell lines has been shown to induce NF-κB (Keates *et al.*, 1997; Münzenmaier *et al.*, 1997, Maeda *et al.*, 2000). The effect of low pH alone, or combined with *H. pylori,* on NF-κB and IκB-α protein expressions has not been addressed. In this chapter, the effect of these factors on NF-κB transcription was examined in the gastric epithelial cancer cells AGS.

3.2 OBJECTIVES

The aims of this chapter were to examine:

1. The effect of incubation of gastric epithelial cells in media of different pH values on NF-κB DNA-binding activity.
2. The effect of *H. pylori* on NF-κB DNA-binding activity.
3. The combined effect of low pH and *H. pylori* on NF-κB DNA-binding activity.
4. The effect of low pH and *H. pylori* on IκB-α protein level.
5. Immunohistochemical staining of NF-κB expression in gastric biopsies from patients infected with *H. pylori*.

3.3 RESULTS

3.3.1 Exposure of gastric epithelial cells to low pH activates NF-κB DNA-binding

Nuclear extracts from AGS cells in the resting state revealed no activation of NF-κB and minimal levels of active NF-κB were detected only at 24 h of incubation (Fig. 3.1). The exposure of AGS cells to low pH for 1 h resulted in the induction of NF-κB DNA-binding activity, and this DNA-binding activity was increased as the pH was reduced from pH 7.4 (pH of the culture medium) to pH 6.6 by the addition of 0.1 M HCl (Fig. 3.2A). It is evident that NF-κB activation is stronger at pH 7.0 (lane 5) and pH 6.8 (lane 6) than that seen at pH 7.4 (lane1) in resting AGS cells. Cell viability and pH value were regularly regularly before and after incubation of AGS at different pH values. Cell counts were unchanged at the pH range from pH 7.4 (pH of the culture medium) to pH 6.4 at the end of each incubation as determined by EB/AO staining.

Incubation of AGS cells at a pH value less than pH 6.4 resulted in decreased NF-κB DNA-binding activity. This may be due to the toxic effect of low pH, as demonstrated by compromised cell viability as judged microscopically by staining the cells with EB/AO. On the other hand, exposure of AGS cells to media of high pH values had no effect on the DNA-binding activity of NF-κB (Fig. 3.2B).

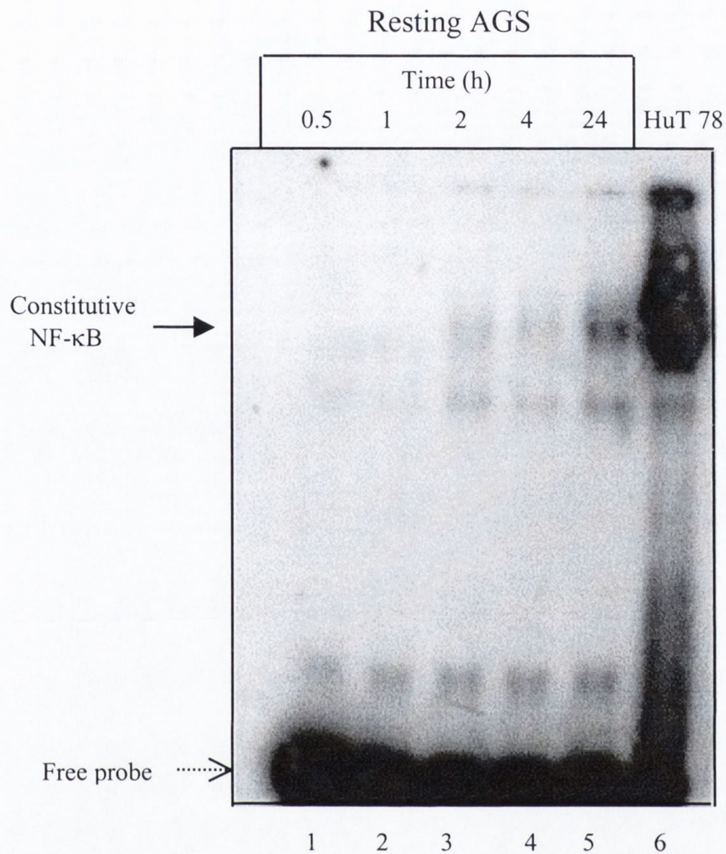


Fig. 3.1. Electrophoretic mobility shift assay (EMSA) for analysis of NF-κB DNA-binding in AGS cells. AGS cells were incubated at pH 7.4 for various periods of time prior to subjecting nuclear extracts to EMSA. HuT 78 cells, a T-cell lymphoma derived from peripheral blood of a patient with a Sezary lymphoma, known to express high levels of constitutive NF-κB served as a positive control (lane 6). The solid arrow indicates the position of the induced NF-κB-DNA binding complex and the dashed arrow indicates the position of free probe.

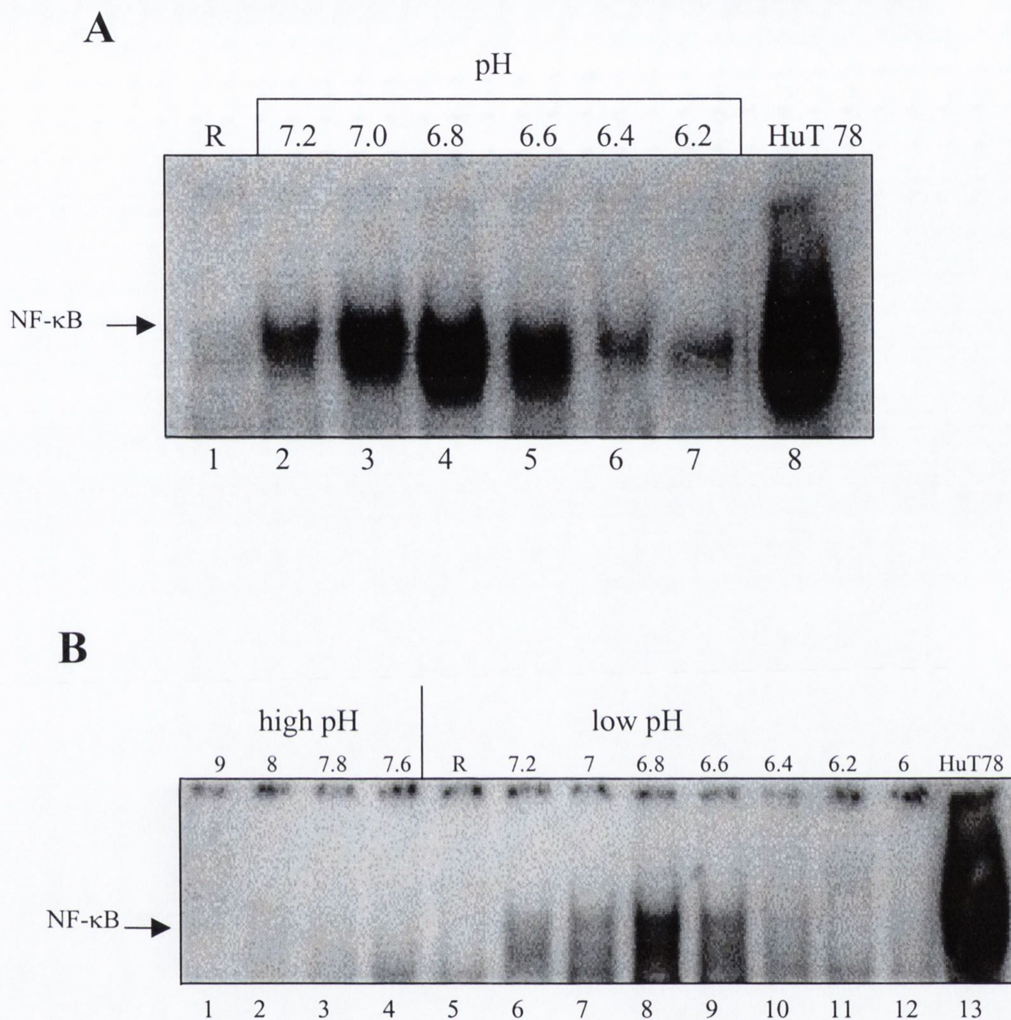


Fig. 3.2. Effect of pH on NF-κB DNA-binding in AGS cells. **(A)** AGS cells were incubated in media of different pH values ranging from pH 7.4 (pH of the culture medium, lane 1) to pH 6.2 (lane 7). HuT 78 cells were used as a positive control (lane 8). The pH of the culture medium was adjusted by adding 0.1 M HCl. **(B)** AGS cells were incubated in media of different pH values. AGS cells were exposed to media of different pH values for 1 h and nuclear extracts were prepared and assayed for NF-κB DNA-binding by EMSA. Each experiment was repeated three times and representative gels are shown.

3.3.4 Supershift and competition assays

The specificity of NF-κB-DNA complexes was confirmed by competition assays. A 100-fold molar excess of unlabelled NF-κB oligonucleotide was added to the EMSA-binding reaction containing cell extracts prepared from AGS cells incubated at pH 7.0 as described under the Methods section. The addition of unlabelled NF-κB oligonucleotide completely abolished NF-κB-DNA complex formation (Fig. 3.3).

Supershift studies were then performed to characterize the nature of the NF-κB-DNA complex using antibodies against various NF-κB subunits (p50, RelA/P65 and c-Rel). Antibodies to p50 and RelA resulted in a supershift of the NF-κB-DNA complex which demonstrated the presence of both p50 and RelA components in the NF-κB heterodimer complex (Fig. 3.3). Anti-c-Rel antibody had no effect on the formation and migration of the NF-κB-DNA complex. These findings indicate that p50/RelA of the NF-κB-DNA complex is active in gastric epithelial cells at pH 7.0.

3.3.5 Effect of pH on IκB-α protein level

To investigate the effect of pH on IκB-α protein level, AGS cells were incubated in media of different pH, ranging from pH 7.4 to pH 6.6 for 1 h. Total cell extracts were prepared and subjected to immunoblotting with antiserum against IκB-α. In cell extracts from unstimulated AGS cells, a single 37 kDa band was detected on Western blots (Fig. 3.4A). The results also show that as the pH was decreased from 7.4 (lane 1) to 6.6 (lane 5), the IκB-α levels decreased. This event is coincident with the appearance of activated NF-κB, as demonstrated in gel retardation assay, upon exposure to the same conditions (Fig. 3.2).

Interestingly, as the pH decreased beyond pH 6.6, there is an increase in IκB-α levels (pH 6.4, lane 6). This increase in IκB-α was associated with reduced NF-κB-DNA complex formation. Fig. 3.4B shows a time-course of IκB-α degradation at pH 7.0, indicating that IκB-α degradation was evident by 30 min (lane 6). These results suggest that a small decrease in pH may be considered a significant factor in

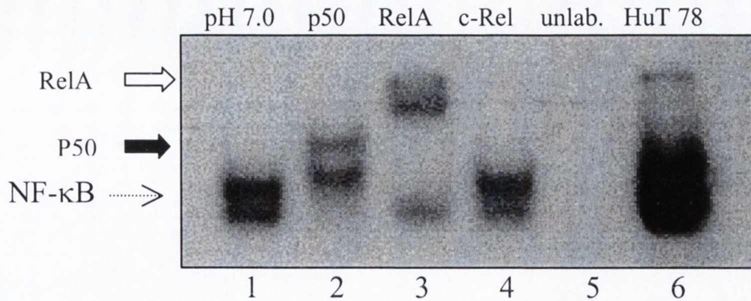


Fig. 3.3. Supershift and competition assays. Nuclear extracts from AGS cells incubated at pH 7.0 were analyzed for NF-κB DNA-binding by EMSA. The binding reaction was carried out after a 30 min incubation with or without 0.5 μl of rabbit anti-NF-κB subunits, p50 (lane 2), RelA (lane 3) and c-Rel (lane 4), or with an excess (100-fold) of unlabelled NF-κB oligonucleotide (lane 5). HuT 78 cells serve as a positive control (lane 6). The solid arrow indicates the location of the p50 supershifted complex, the open arrow indicates the location of the RelA supershifted complex and the dashed arrow indicates the position of the induced NF-κB-DNA binding complex.

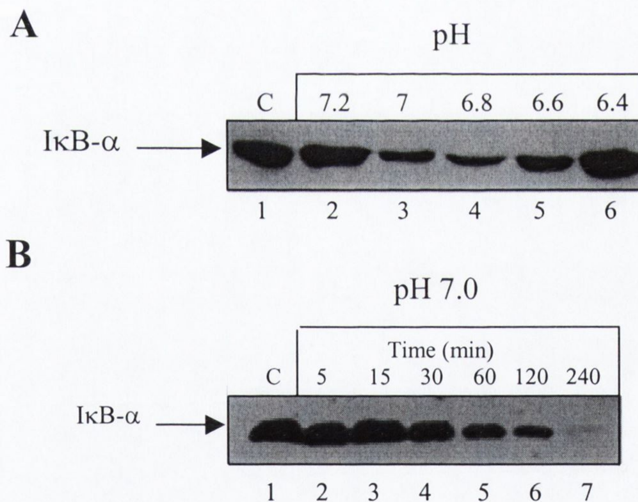


Fig. 3.4. The effect of pH on IκB-α protein expression. **(A)** AGS cells were incubated at different pH values ranging between pH 7.4 (pH of the culture medium, lane 1) to pH 6.4 (lane 6) for 1 h. Proteins in total cell extracts were separated by 10% polyacrylamide gel electrophoresis and transferred onto PVDF membrane followed by Western blot analysis using anti-IκB-α antibody. The arrow indicates the position of 37 kDa IκB-α band on Western blots. **(B)** Time course of IκB-α degradation in AGS cells incubated at pH 7.0. AGS cells were exposed to pH 7.0 for different periods of time (lanes 2-7). Unsitmulated AGS cells are shown in lane 1. Each experiment was performed three times and one blot is shown.

regulating the activity of NF-κB DNA-binding activity, as indicated by EMSA and Western blotting data.

3.3.6 *H. pylori* Induces NF-κB DNA-binding activity in Gastric Epithelial Cells

Previous studies have shown that *H. pylori* activates NF-κB in gastric epithelial cells *in vitro* and *in vivo* (Keates *et al.*, 1997; Maeda *et al.*, 2000; van de Brink *et al.*, 2000). Time course experiments indicated that the NF-κB DNA-binding activity was evident as early as 30 min (lane 2) and up to 24 h stimulation (lane 6) of AGS cells with *H. pylori* strain 11638 at a multiplicity of infection (MOI) of 100:1 (Fig. 3.5A). Coculture of AGS cells with *H. pylori* results in the activation of NF-κB DNA-binding activity in a dose-dependent manner (Fig. 3.5B).

3.3.7 Effect of *H. pylori* isolates on NF-κB DNA-binding activity

Different *H. pylori* strains vary in their ability to induce cytokine production and gastric mucosal inflammation. Therefore, the ability of different *H. pylori* isolates to induce NF-κB activation was investigated (Fig. 3.6A). AGS cells were incubated with either NCTC 11367 ($CagA^+$ and $VacA^+$) or isolates 92-1099 ($CagA^-$ and $VacA^+$), 93-1000 ($CagA^-$ and $VacA^-$) or 92-701 ($CagA^-$ and $VacA^-$) for 2 h and nuclear extracts were prepared and assayed by EMSA. $CagA^+$ and $VacA^+$ *H. pylori* (lane 2) induced strong NF-κB activation, while *H. pylori* isolates 92-1099 (lane 3), 93-1000 (lane 4) or 92-701 (lane 5) failed to activate NF-κB in AGS cells (Fig. 3.6A). These results indicate that *H. pylori* strains bearing the *cag* PAI are associated with NF-κB activation compared to $CagA^-$ *H. pylori* strains.

3.3.8 Specificity of *H. pylori*-induced NF-κB DNA-complex

Competition and supershift assays revealed the specificity of the *H. pylori*-induced NF-κB-DNA complex (Fig. 3.6B). Antibodies directed against NF-κB subunits recognized both p50 and p65/RelA of the *H. pylori*-induced NF-κB heterodimer complex.

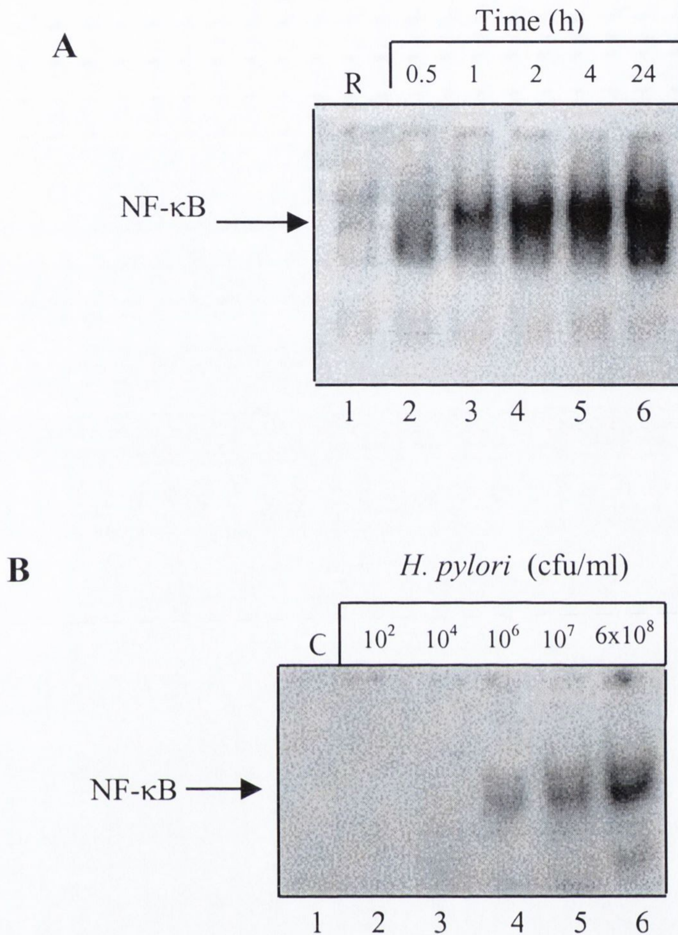
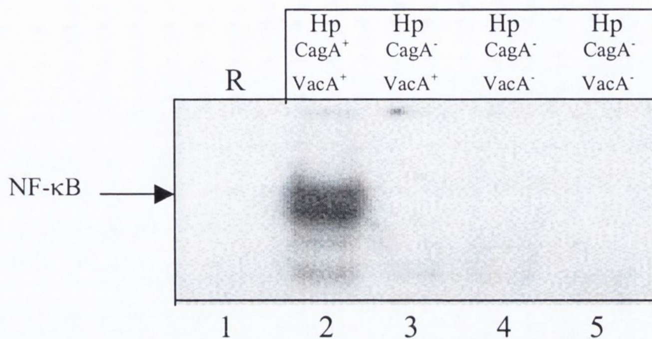


Fig. 3.5. Electrophoretic mobility shift assay analyses of *H. pylori*-induced NF-κB DNA-binding in AGS cells. **(A)** Time course of NF-κB DNA-binding in AGS cells cocultured with freshly harvested suspension of *H. pylori* strain 11638 ($6x10^8$ cfu/ml) at a multiplicity of infection (MOI) 100:1. Lane 1, nuclear extract from unstimulated AGS cells. Lanes 2 to 6, nuclear extracts from AGS cells cocultured with *H. pylori* for 30 min to 24 h. **(B)** Effect of *H. pylori* doses on NF-κB DNA-binding. AGS cells were cocultured for 2 h with different doses of *H. pylori* ranging from 10^2 to $6x10^8$ cfu/ml (lanes 2-6). Each experiment was performed three times and a representative gel is shown.

A



B

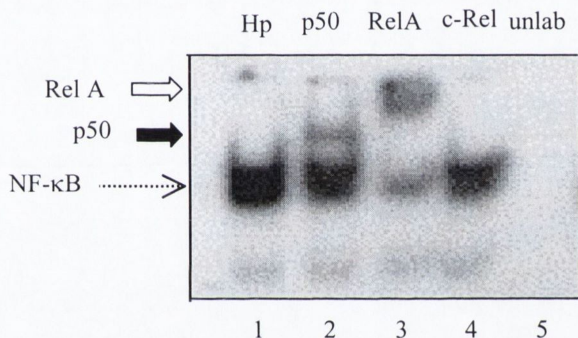


Fig. 3.6. (A) Effect of different *H. pylori* strains on NF-κB DNA-binding in AGS cells. AGS cells were cocultured with *H. pylori* strains, NCTC 11637 (VacA$^+$ and CagA$^+$, lane 2), 92-1099 (VacA$^+$ and CagA$^-$, lane 3), and VacA$^-$ and CagA$^-$ stains; 92-701 (lane 4) and 93-1000 (lane 5) for 2 h. Nuclear extracts were prepared and analyzed for NF-κB DNA-binding by EMSA. **(B)** Supershift analysis was performed on nuclear extracts from AGS cells stimulated with *H. pylori* using 0.5 μl of rabbit antisera against p50 (lane 2), RelA (lane 3) and c-Rel (lane 4). A competition assay for NF-κB was also performed using an excess (100-fold) of unlabelled NF-κB oligonucleotide (lane 5). The solid arrow indicates the location of the p50 supershifted complex, the open arrow indicates the location of the RelA supershifted complex and the dashed arrow indicates the position of the induced NF-κB-DNA binding complex. A representative gel of three independent experiments with similar results is shown.

3.3.9 Effect of LPS, heat-killed and crude protein of *H. pylori* on NF-κB activation

A direct contact between live *H. pylori* and AGS cells appears to be necessary for NF-κB activation (Fig. 3.7). Coculture of AGS cells with *H. pylori* LPS (15 μg/ml, lane 2), heat-killed *H. pylori* (used at the same concentration as with live *H. pylori* cells; $6x10^8$ cfu/ml, lane 3) or *H. pylori* sonicate (15 μg/ml, lane 4) failed to induce NF-κB DNA-binding, while live *H. pylori* cells, strain 11638 (lane 5) resulted in a strong induction of NF-κB.

3.3.10 Effect of *H. pylori* on IκB-α protein level

The level of IκB-α in AGS cells after stimulation with *H. pylori* was determined over the same time course used to monitor NF-κB DNA-binding activity. Immunoblotting with anti-IκB-α antibody demonstrated clearly that exposure of AGS cells to *H. pylori* results in accelerated degradation of IκB-α (Fig. 3.8). IκB-α degradation was observed as early as 15 min (lane 3) with maximal IκB-α degradation observed at 2-4 h (lanes 6+7) postinitiation of stimulation. The degradation of IκB-α and activation of NF-κB were coincident events which occurred following exposure of AGS cells to *H. pylori*.

3.3.11 Effect of pH combined with *H. pylori* on NF-κB DNA-binding activity

To investigate the combined effect of pH and *H. pylori* on NF-κB DNA-binding activity, AGS cells were incubated in media of different pH (7.4 -6.6) in the absence and presence of *H. pylori* strain 11638. As the pH decreased from pH 7.4 to pH 6.6, the NF-κB DNA-binding activity was enhanced (Fig. 3.9A). The induction of NF-κB DNA-binding activity was stronger when AGS cells were exposed to conditions of low pH in the presence of *H. pylori* compared to low pH alone. The combined effect of *H. pylori* to low pH appears to be synergistic which is clearly demonstrated in chapter IV in Fig. 4.6A and B. The synergistic effect seen due to the combined effect of low pH and *H. pylori* may be a key factor in mucosal inflammation during *H. pylori* infection.

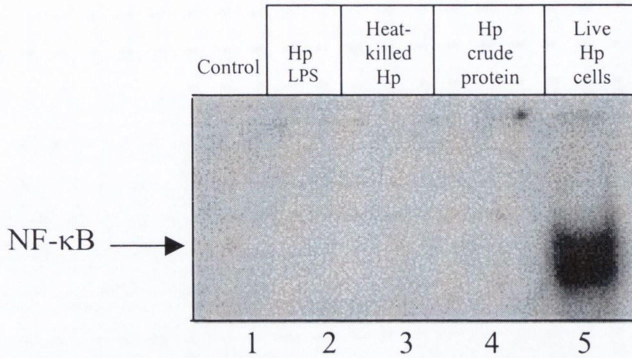


Fig. 3.7. Live *H. pylori* cells induce NF-κB DNA-binding in AGS cells. AGS cells were cocultured with *H. pylori* LPS (15 μg/ml; lane 2), heat-killed *H. pylori* (lane 3), *H. pylori* sonicated crude protein (15 μg/ml; lane 4) or freshly harvested suspension of *H. pylori* strain 11638 ($6x10^8$ cfu/ml; lane 5). Unstimulated AGS cells are shown in lane 1.

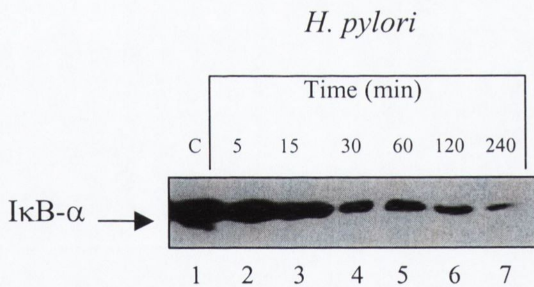
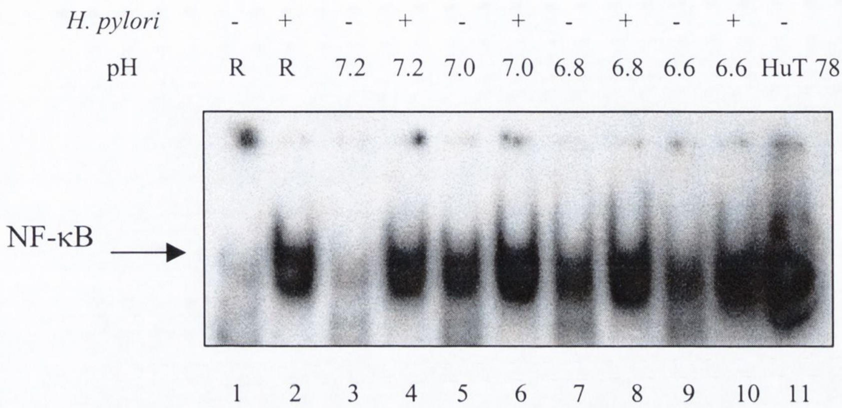


Fig. 3.8. Effect of *H. pylori* on IκB-α protein level. AGS cells were stimulated with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for the indicated times (lanes 2-7). Cell extracts were analyzed for IκB-α expression by Western blotting using antiserum against IκB-α. Lane 1, unstimulated AGS cells. A representative result of three different experiments with similar results is shown.



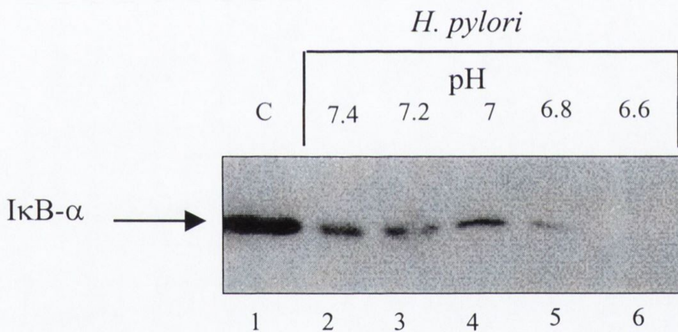


Fig. 3.9. Effects of pH and *H. pylori* on NF-κB DNA-binding in AGS cells. **(A)** NF-κB activation by either low pH or low pH combined with *H. pylori* in AGS cells. AGS cells, in which the pH of the medium was adjusted to the required pH (as indicated above each lane) by addition of 0.1 M HCl, were cocultured for 2 h with *H. pylori* strain 11638 ($6x10^8$ cfu/ml). C, nuclear extract from resting AGS cells at pH 7.4. **(B)** Effect of combined pH and *H. pylori* on IκB-α protein level. AGS cells, in which the pH of the culture medium was adjusted to different pH values as indicated above each lane, were cocultured with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for 2 h (lanes 2-6). Total cell extracts were prepared and analysed for IκB-α in Western blotting. Each experiment was performed three times with similar results and a representative gel is shown.

These findings are consistent with the results observed for IκB-α expression. Exposure of AGS cells to conditions of low pH in the presence of *H. pylori* resulted in a rapid diminution in IκB-α levels (Fig. 3.9B). As the pH of the culture medium was reduced from pH 7.4 to pH 6.6 (lanes 2-6), significant degradation of IκB-α occurs which parallel the increases seen in the activation of NF-κB DNA-binding activity.

3.3.12 Time course of NF-κB activation (combined effects of pH and *H. pylori*)

AGS cells were incubated at pH 7.0 in the absence or presence of *H. pylori* for different periods of time and nuclear extracts were analyzed by EMSA. The induction of NF-κB DNA-binding at pH 7.0 is time-dependent (Fig. 3.10A). A detectable increase in the levels of NF-κB was seen as early as 30 min and continued to increase up to 24 h. However, exposure of AGS cells to *H. pylori* strain 11638 at pH 7.0 induced significantly more NF-κB DNA-binding activity compared to that seen at pH 7.0 alone (Fig. 3.10A).

Western blot data showed that the degradation of IκB-α was enhanced as the time of incubation was increased above 15 min with significant IκB-α degradation evident seen at 30 min-1 h (Fig. 3.10B). This observation indicates that activation of NF-κB DNA-binding under these conditions is also associated with degradation of the inhibitor IκB-α.

3.3.13 Specificity of the NF-κB DNA-complex induced by *H. pylori* at pH 7.0

To identify the composition of the NF-κB DNA-complex induced by the combined effect of pH 7.0 and *H. pylori*, antibodies to RelA, p50 and c-Rel were preincubated with nuclear extracts from AGS cells stimulated *H. pylori* at pH 7.0. Antibodies to RelA and p50 recognized this NF-κB DNA-complex, while c-Rel antibody had no effect on the formation of this complex (Fig. 3.11). Moreover, competition assays with a 100-fold molar excess of unlabelled NF-κB oligonuclotide confirmed the specificity of NF-κB DNA-complex-induced at pH 7.0 in the presence of *H. pylori* (Fig. 3.11).

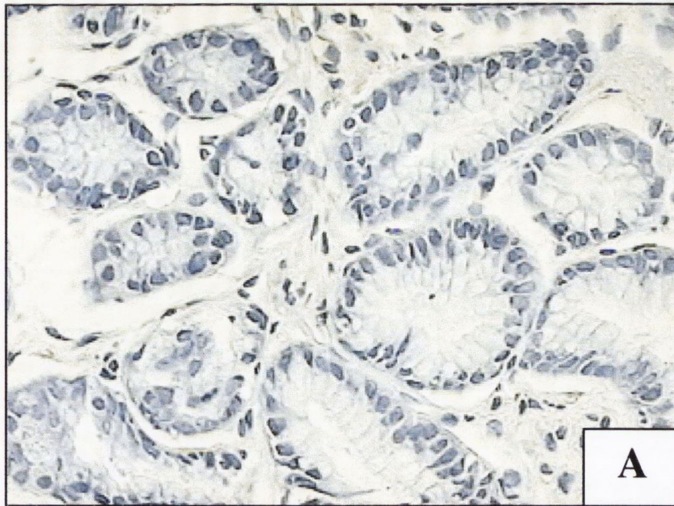
3.3.14 Expression of NF-κB in patients with *H. pylori* infection

The expression of NF-κB in antral gastric mucosa, the main site of colonization, was examined in histologically normal gastric biopsies and biopsies from patients with chronic gastritis and patients infected with *H. pylori* with using immunohistochemical procedures. Staining was performed with an antibody directed against the RelA/p65 subunit of NF-κB. Little or no activated NF-κB was detected in epithelial cells from normal gastric mucosa (Fig. 3.12A). Gastric specimens from *H. pylori*-colonized stomachs showed strong NF-κB expression compared to normal mucosa (Fig. 3.12B).

Immunostaining of antral biopsy specimens from *H. pylori*-negative patients showed little or no NF-κB expression (Fig. 3.13A). *H. pylori*-infected antral samples containing a lymphoid aggregate showed activated NF-κB (Figure 3.13B). Furthermore, antral biopsy specimens from *H. pylori* infected patients stained without the use of RelA/p65 antibody showed no active NF-κB (Fig. 3.13 C). These findings indicate that NF-κB expression is enhanced in the gastric mucosa in response to *H. pylori* colonization.

3.4 DISCUSSION

Accumulating evidence implicates a role for NF-κB in the mucosal inflammation of the stomach (van den Brink *et al.*, 2000) and intestine (Neurath *et al.*, 1996). Nuclear levels of p65 are increased in the lamina propria of patients with Crohn's disease in comparison to patients with ulcerative colitis and controls (Schreiber *et al.*, 1998). Rogler *et al.* (1998) have also demonstrated that NF-κB is activated in biopsy specimens from inflamed mucosa from patients with Crohn's disease, ulcerative colitis and unspecific colitis. The results presented in this study demonstrate an increase in NF-κB DNA-binding activity when the gastric epithelial cells AGS were exposed to conditions of low pH. The activation of NF-κB was markedly increased as the pH was reduced from pH 7.4 to pH 6.6. However, when the pH was reduced beyond pH 6.6, the NF-κB DNA-binding activity decreased and cell viability was compromised. Western blot analyses support the results of mobility shift assays and indicate that NF-



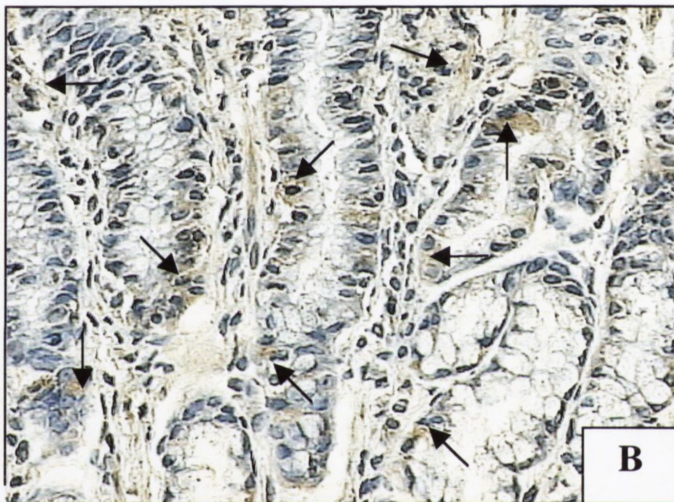
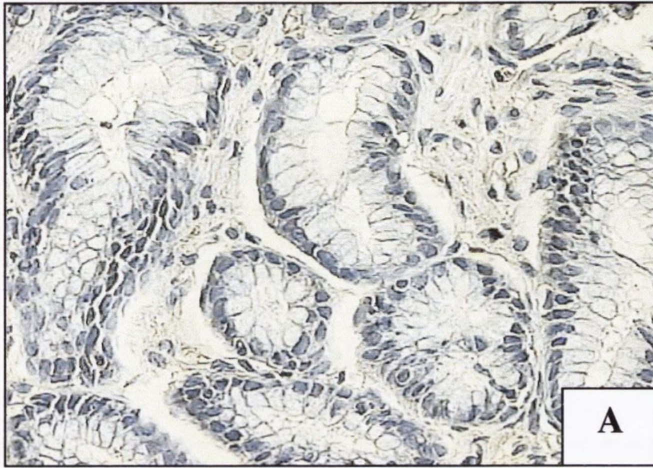
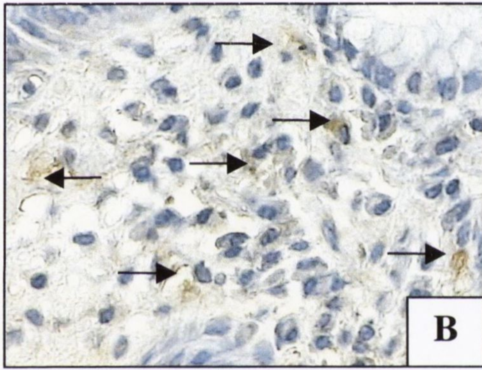


Fig. 3.12. Immunohistochemical detection of activated NF-κB in antral biopsy specimens from *H. pylori*-infected patients. Antral biopsy sections were stained with the NF-κB p65 antibody and peroxidase DAB followed by counterstaining with Mayer's hematoxylin. **(A)** Normal antral biopsy specimens showing no expression of NF-κB p65. NF-κB expression was evident in *H. pylori*-infected samples. **(B)** Antral biopsy specimens from *H. pylori*-infected patients indicate increased NF-κB p65 staining (brown), as indicated by arrows. (Magnifications A and B, X 600).





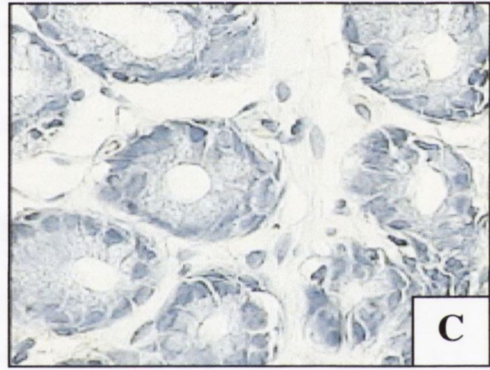


Fig. 3.13. Immunohistochemical staining of antral samples from normal and *H. pylori*-negative patients with chronic gastritis. **(A)** The mucosa of *H. pylori*-negative patients with chronic gastritis shows minimal activation of NF-κB p65 (brown). **(B)** Antral biopsy specimens containing lymphoid aggregates from *H. pylori*-infected patients indicate increased NF-κB p65 activity, as indicated by arrows. **(C)** Antral specimens from the same *H. pylori* positive NF-κB p65 stained subjects (as in Fig. 3.12B) without the addition of the NF-κB p65 antibody showing no staining of cells. (Magnifications A, B and C, X 600).

κB DNA-binding activity is pH-sensitive in gastric cells. The pH-dependent induction of NF-κB DNA-binding activity appears to rely on IκB-α degradation.

These findings point to the importance of low environmental pH as one of the possible contributory factors involved in mucosal inflammation in hyperacidic or duodenal ulcer disease states. Consistent with these observations, it has been shown that exposure of macrophages to the acidic environment of inflammatory lesions leads to increased NF-κB DNA-binding activity and this activity was inhibited by treatment with PDTC or N-acetyl-leucinyl-norleucinol (Bellocq *et al.*, 1998). The importance of the role of gastric acid in the pathogenesis of peptic ulcer disease is clearly demonstrated by the rapid symptomatic improvement and ulcer healing upon treatment with proton pump inhibitors. However, H_2-receptor antagonist treatment results in ulcer recurrence, which may be linked to changes in parietal cell sensitivity to secretogogues (Chiverton and Hunt, 1989).

Although the stomach is protected from the deleterious effect of gastric acid, acid plays a critical role in the pathogenesis of tissue injury due to impairment of the mucosal defence mechanisms. These mechanisms include the surface water-insoluble mucus layer overlaying epithelial cells, mucosal blood flow, rapid cell turnover and surface hydrophobicity and secretion of bicarbonate. Under certain circumstances, aggressive factors such as acid, pepsins and bile acids that present in the lumen as substances necessary for the normal process of digestion, may cause mucosal inflammation. Acid contributes to mucosal injury through direct damage of the gastric epithelium, inhibition of repair of injury or interference with hemostatic mechanisms. A very high acid output, as in patients with Zollinger-Ellison syndrome, is sufficient to cause ulceration without the additional factor of *H. pylori* (Talley, 1990).

The idea that changes in pH could control the transcription of genes was documented in several previous studies. A decrease in the extracellular pH from pH 7.4 to 6.9 caused a marked increase in the expression of mRNA for phosphoenol pyruvate carboxykinase (Kaiser and Curthoys, 1991). Yamaji *et al.* (1994) have also shown that

the exposure of renal epithelial cells to an acidic environment led to transcriptional activation of immediate early genes such as c-Fos and c-Jun. Recent studies by van den Brink *et al.* (2000) have demonstrated that the uninflamed antrum of the stomach has high NF-κB expression and activity. This suggests the possibility of a direct link between acidic environment in the stomach and NF-κB activation in the absence of *H. pylori* infection. However, the presence of *H. pylori* infection greatly increased the number of cells containing active NF-κB.

In this study, *H. pylori* induces NF-κB *in vitro* and *in vivo*. The expression of NF-κB by *H. pylori* in AGS cells occurs in a dose- and time-dependent manner. Immunohistochemical staining showed increased expression of NF-κB p65 in the gastric mucosa of patients with *H. pylori* infection compared to normal gastric mucosa. These findings are consistent with data from gastric biopsy specimens showing increased NF-κB p65 in *H. pylori*-infected patients (Keates *et al.*, 1997; van den Brink *et al.*, 2000). Additionally, the results showed that $CagA^+$ *H. pylori* strains were able to induce a strong NF-κB activation compared to $CagA^-$ strains. There is considerable data showing that infection with $CagA^+$ strains is associated with increased risk of peptic ulceration than with $CagA^-$ strains. Sharma *et al.* (1998) demonstrated that $CagA^+$ strains, compared with $CagA^-$ strains, induce enhanced nuclear localization of a Rel-containing NF-κB binding complex.

It has been demonstrated that *H. pylori* triggers, in a Cag-dependent manner, cellular events such as the reorganization of the actin cytoskeleton and pedestal formation (Segal *et al.*, 1996). Further studies have been shown that infection of gastric epithelial cells by *H. pylori* induced tyrosine phosphorylation and dephosphorylation of several 125-135 kDa and 75-80 kDa proteins (Backert *et al.*, 2000; Stein *et al.*, 2000). Therefore, the phosphorylated CagA protein may play a crucial role in promoting the inflammatory responses of gastric mucosa to *H. pylori* infection. Several *cag* PAI gene products but not CagA were shown to be essential for NF-κB activation by Glocker *et al.* (1998). Asahi *et al.* (2000) demonstrated that infection of gastric epithelial cells by *H. pylori* induces tyrosine phosphorylation of CagA protein, indicating that the

tyrosine phosphorylated protein was not a host cellular protein but rather derived from *H. pylori*. Recently, it has been shown that CagA interacts directly with a tyrosine phosphatase in a phosphorylation-dependent manner (Higashi *et al.*, 2002). The variations among *H. pylori* strains in their capability to activate transcrpition factors such as NF-κB and other transcription factors might explain why infection with *H. pylori* progress to peptic ulceration in some groups of individuals and others develop only superficial gastritis. Furthermore, the activation of NF-κB requires live *H. pylori* since *H. pylori* LPS, heat-killed *H. pylori* and crude *H. pylori* protein extracts failed to induce NF-κB in AGS cells.

There is an association between *H. pylori*-induced gastritis, hyperacidity and the development of duodenitis and duodenal ulcer disease (Tytgat and Rauws, 1990). Infection with *H. pylori* results in paradoxical changes in gastric acid secretion. In duodenal ulcer patients, *H. pylori* elevates gastric acid secretion, so that more acid enters to the duodenum causing mucosal injury (Calam *et al.*, 1997; Gillen *et al.*, 1998). On the other hand, *H. pylori* also decreases acid secretion through production of acid-inhibitory substances (Beil *et al.*, 1994; Jablonowski *et al.*, 1994) and increases the secretion of cytokines like IL-1β and TNF-α (El-Omar, 2001; Noach *et al.*, 1994). Logan *et al.* (1995) reported that a decrease in gastric acid secretion enables *H. pylori* to survive in the corpus. Mucosal damage in the corpus becomes more severe and results in further decrease in acid secretion (Hunt, 1992). Eradicating the infection restored gastrin to control levels and led to a marked improvement in gastric inflammation (Rademaker and Hunt, 1991).

The results presented in this study also demonstrate an increase in NF-κB DNA-binding activity by low pH combined with *H. pylori* compared to either *H. pylori* or low pH alone. The induction of NF-κB in response to low pH combined with *H. pylori* is associated with a reduction in IκB-α protein levels, thereby, allowing the translocation of NF-κB from the cytoplasm to the nucleus (Baeuerle and Baltimore, 1989; Beg *et al.*, 1993). Changes in pH at the epithelial cell level are very small. Since *H. pylori* colonizes beneath the mucus layer, where the pH in the vicinity of mucosal

cells is high, the ammonia generated by hydrolysis of urea protects the organism by buffering gastric acid (Mégraud *et al.*, 1987). After eradication the acid suppression by *H. pylori* infection was removed, ammonia concentration in gastric juice decreased and gastric juice pH and serum gastrin level decreased significantly to the same level as those in controls (Furuta *et al.*, 1998).

A number of mechanisms propose to explain how *H. pylori* affects gastric acid secretion (Mohamed and Hunt, 1994). Firstly, *H. pylori* may attach to the parietal cell and/or other cells involved in the control of gastric acid secretion and alter function directly. Secondly, *H. pylori* may secrete compounds that act directly on the parietal cell and/or other cells involved in the control of gastric acid secretion. Finally, this increase in gastric acid secretion could be due to gastritis. *In vitro* studies have shown that IL-1, IL-2, TNF-α and IFN-γ can increase gastrin release (Hazell *et al.*, 1986) and all of these cytokines are increased during infection with *H. pylori* (Yamaoka *et al.*, 1997; Crabtree *et al.*, 1991b, Fan *et al.*, 1993). The transcription of these cytokines is regulated by NF-κB.

Cytokines contribute to mucosal damage directly or indirectly by mediating inflammatory response to *H. pylori*. The adherence of *H. pylori* to the gastric epithelium initiates an immune response or stimulates mononuclear cells to release cytokines which recruit and activate T-lymphocytes, neutrophils and other inflammatory cells. The gastric mucosal levels of the proinflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α are increased in *H. pylori*-infected subjects. In particular, IL-1β is an important proinflammatory cytokine with profound effects on gastric physiology and is involved in *H. pylori*-induced gastritis (El-Omar, 2001). IL-1β can also induce expression of IL-8 (Noach *et al.*, 1994) and stimulate gastrin release from cultured rat antral G cells (Weigert *et al.*, 1996). Beales and Calam (1998) have shown that IL-1β and TNF-α inhibit acid secretion in cultured rabbit parietal cells. Polymorphisms in the IL-1β gene that correlate with higher levels of this cytokine have been found to increase the risks of hypochorhydria and gastric atrophy in response to *H. pylori* infection and to increase the risks of gastric cancer (El-Omar *et*

al., 2000). Thus, variation in host genetic factors that affect IL-1β may determine why certain individuals infected with *H. pylori* develop gastric cancer while others do not.

In summary, an acidic pH clearly has a role in activating NF-κB DNA-binding and this effect is enhanced in the presence of *H. pylori*. Acidic pH may influence the transcription of many genes during inflammation. Therefore, inhibition of gastric acid might protect patients with peptic ulcer disease against further epithelial injury and mucosal inflammation.

CHAPTER IV

EFFECTS OF ANTIOXIDANTS ON NF-κB ACTIVATION BY *HELICOBACTER PYLORI* AND OTHER STIMULI IN GASTRIC EPITHELIAL CELLS

CHAPTER IV

4.1 INTRODUCTION

H. pylori infection produces oxygen free radicals secondary to the accumulation of activated neutrophils in the inflammatory infiltrate of *H. pylori*-associated gastritis (Nielsen and Anderson, 1992; Davies *et al.*, 1994). In addition, the urease produced by *H. pylori* can damage the host by inducing monocytes and polymorphonuclear granulocytes to secrete cytokines and release reactive oxygen radicals (Mai *et al.*, 1991; Nielsen and Anderson, 1992). Furthermore, gastric and duodenal tissue biopsies have increase levels of ROIs in *H. pylori*-infected samples (Davies *et al.*, 1992). It was also shown that patients with duodenal ulceration have an increased plasma concentration of malondialdehyde, a marker for free oxygen radical and a significant reduction in the concentration of the antioxidant glutathionine (Jankowski *et al.*, 1991).

The redox regulation of NF-κB has received increased interest because this protein controls the inducible expression of a wide range of genes involved in inflammatory and immune responses (Baeuerle, 1991). Reactive oxygen intermediates (ROIs) serve as a second messenger in NF-κB activation in various systems (Schreck *et al.*, 1991; Suzuki *et al.*, 1994; Mihm *et al.*, 1991; Staal *et al.*, 1990; Bowie and O'Neill, 2000). In this study, the effects of the antioxidants; vitamin C, NAC and thioredoxin on NF-κB activation by *H. pylori* and other stimuli were examined.

Vitamin C is one of the most labile nutrients in the diet that is present in citrus and soft fruits and the growing points of vegetables. Ascorbic acid is normally secreted by the gastric mucosa and its concentrations are higher in gastric juice than in plasma. The amount of vitamin C required by human adults is 30-60 mg/day. Vitamin C is a powerful natural antioxidant that is capable of eliminating oxygen free radicals which may cause gastric epithelial damage (Mirvish, 1986). Ascorbic acid also diminishes the generation of harmful substances such as N-nitroso compounds and protects against gastric cancer (Mirvish, 1994). In addition, a low intake of ascorbic acid increases the risk of gastric cancer (Hansson *et al.*, 1994). The link between *H. pylori*

and ascorbic acid levels has been documented by several investigators. *H. pylori* infection decreases the amount of ascorbic acid in gastric juice which subsequently increases susceptibility to the risk of free oxygen radicals (O'Conner *et al.*, 1989; Sobala *et al.*, 1989). Decreased ascorbate levels were returned to normal levels after eradication of *H. pylori* (Sobala *et al.*, 1993).

N-acetyl-L-cysteine (NAC) is a thiazolidine derivative which has been employed as a glutathione precursor for cysteine supply for GSH biosynthesis and it also acts as an antioxidant. Several studies have shown that high concentrations of NAC (20-30 mM) inhibit NF-κB activation (Schreck *et al.*, 1992b; Mihm et al., 1991; Staal *et al.*, 1990)

The role of thioredoxin (Trx) in signal transduction and gene expression has emerged as an important biomedical research area. Thioredoxin has been isolated and characterized from a wide variety of prokaryotic and eukaryotic cells (Holmgren, 1985). It is also secreted and rapidly taken up by various cell types (Ericson *et al.*, 1992). Thioredoxin, 13 kDa, is a small ubiquitous protein with redox-active half-cysteine residues in an exposed active center [-Trp-Cys-Gly-Pro-Cys-] (Holmgren, 1985). Thioredoxin participates in redox reactions through reversible oxidation of its active center dithiol to a disulphide and it catalyzes dithiol-disulphide exchange reactions (Holmgren, 1985). It serves as a hydrogen donor for ribonuclotide reductase, which is essential for DNA synthesis, and for enzymes reducing sulphate or methionine sulfoxide (Reichard, 1993). Thioredoxin can regulate different systems via thiol redox control. The redox regulation of these systems involves changes in activity of an enzyme, a receptor, or a transcription factor via dithiol/disulphide interchange reactions (Holmgren, 1981; Holmgren, 1985; Gleason and Holmgren, 1988). It has been reported that Trx regulates redox-sensitive molecules such as NF-κB, AP-1 and the glucocorticoid receptor (Okamoto *et al.*, 1992; Abate *et al.*, 1990; Grippo *et al.*, 1983).

The transcription factor NF-κB and related proteins share a characteristic sequence motif with a cysteine and three arginine residues in the DNA binding region (Kumar *et*

al., 1992; Kieran *et al.*, 1990). The cysteine residues of cytoplasmic and nuclear proteins are in the reduced state. The NF-κB activating signal cascade requires disulphide formation at a critical cysteine residue of one of the regulatory proteins in the cascade. The reduced form of Trx [Trx-$(SH)_2$] is a powerful protein disulphide oxidoreductase inside the cell (Holmgren, 1985; Holmgren, 1988), and acts as a potent reductant for NF-κB. Several studies have reported that the activity of NF-κB can be modulated by human and bacterial Trx (Weichsel *et al.*, 1996; Matthews *et al.*, 1992). The induction of NF-κB activation and nuclear translocation is strongly inhibited by Trx (Schenk *et al.*, 1994; Meyer *et al.*, 1993).

4.2 OBJECTIVES

The aims of this study were to investigate:

1. The role of vitamin C and NAC in the regulation of pH-dependent NF-κB activation in gastric epithelial cells.
2. The role of vitamin C and NAC in the regulation of NF-κB activation by *H. pylori.*
3. The effect of hydrogen peroxide on NF-κB activation.
4. The effect of *H. pylori* Trx in the regulation of NF-κB activation by *H. pylori* and other stimuli.
5. The effect of *H. pylori* Trx on cell proliferation of AGS cells.
6. The effect of *H. pylori* Trx on *H. pylori*-induced CD44 expression on AGS cells.

4.3 RESULTS

4.3.1 Effect of antioxidants on NF-κB activation by *H. pylori*

AGS cells were preincubated with different amounts of vitamin C, NAC or *H. pylori* Trx for different periods of time, the medium was refreshed, and then cells were further incubated with *H. pylori* strain 11638 for 2 h. Cell viability was unchanged by any of the tested concentration of the agents used over the time course studied as determined by EB/AO staining. At the end of the incubation, nuclear cell extracts were prepared and analyzed by EMSA. Coculture of AGS cells with *H. pylori* caused a strong induction of NF-κB and this induction was inhibited when the cells were

preincubated with vitamin C (30 mM), NAC (20 mM) or Trx (10 μg/ml). The inhibition of NF-κB by these antioxidants is dose- and time-dependent manner. The inhibitory effect of the antioxidants NAC (Fig. 4.1A), vitamin C (Fig. 4.1B), respectively, and Trx (Fig. 4.2) was observed as early as 30 min with maximal inhibition seen at 1-2 h. Treatment of AGS cells with the vehicle alone DMSO (0.1% (v/v) or 20 mM Tris-HCl, pH 7.4 had no effect on NF-κB DNA-binding activity.

Dose-response experiments showed that maximal inhibition of NF-κB activation was detected using 10 mM vitamin C and 20 mM NAC (Fig. 4.3A and B), respectively, and 5-10 μg/ml Trx (Fig. 4.4). The inhibition of *H. pylori*-induced NF-κB binding activity by the tested antioxidants indicates a role for ROI in NF-κB activation in AGS cells.

4.3.2 Effect of antioxidants on NF-κB activation by low pH

AGS cells were preincubated with vitamin C (30 mM) and NAC (20 mM) for 1 h, the culture medium was refreshed, and then cells were incubated in media of different pH values ranging from pH 7.4 to pH 6.6. Cell counts were routinely checked at the end of the incubation by EB/AO staining. No change in cell numbers or pH of the cell culture medium in the presence or absence of vitamin C or NAC was noticed over the time course studied. Pretreatment of AGS cells with vitamin C (30 mM) and NAC (20 mM) blocked low pH-induced-NF-κB DNA-binding activity (Fig. 4.5A and B).

4.3.3 Effect of antioxidants on NF-κB activation by low pH and *H. pylori*

Similarly, preincubation of AGS cells with vitamin C (30 mM), NAC (20 mM) or Trx (10 μg/ml) for 1 h completely abrogated the NF-κB activation seen in response to *H. pylori* strain 11638 under acidic conditions (Fig. 4.6A and B).

4.3.4 Effect of antioxidants on NF-κB activation by cytokines and mitogen

To further explore the effect of antioxidants on NF-κB activation, AGS cells were preincubated with vitamin C (30 mM), NAC (20 mM) and Trx (10 μg/ml) followed by

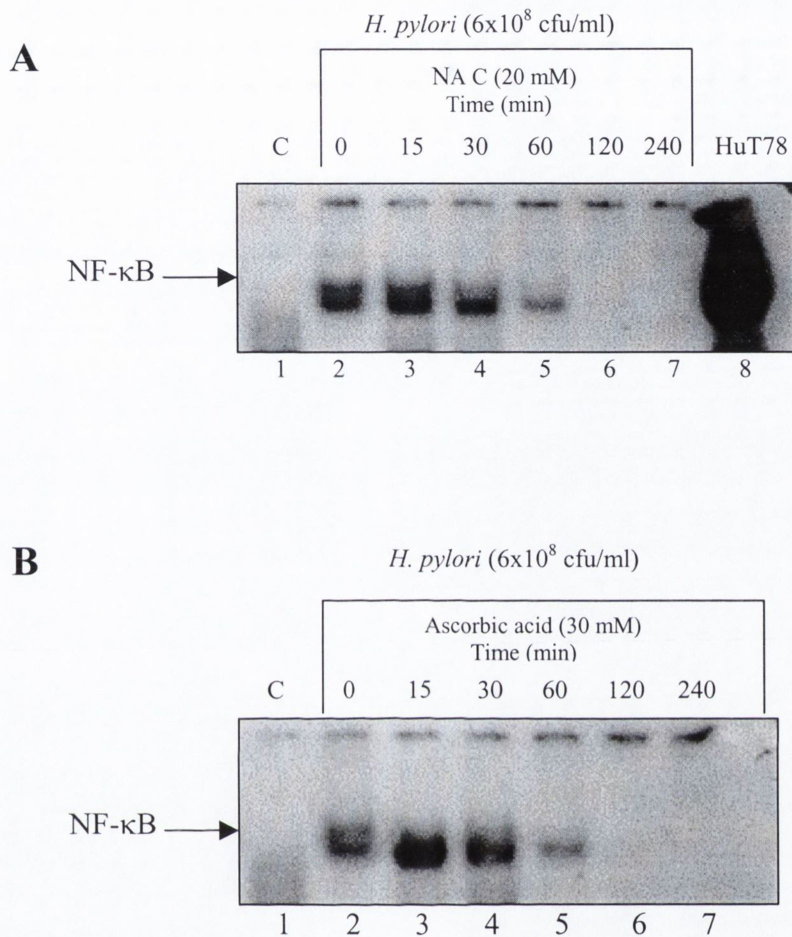


Fig. 4.1. Electrophoretic mobility shift assay anaylsis of NF-κB DNA-binding activity. Time course of NF-κB inhibition by NAC (20 mM) **(A)** and ascorbic acid (30 mM) **(B)** secondary to treatment of AGS with *H. pylori* strain 11638 ($6x10^8$ cfu/ml). Nuclear extracts were prepared and analyzed for NF-κB DNA-binding activity by EMSA. Lanes 3-6, time periods of preincubation with either NAC or ascorbic acid for 15, 30, 60, 120 and 240 min before stimulation with *H. pylori*. Control AGS cells are shown in lane 1 and *H. pylori*-induced NF-κB in AGS cells in lane 2. HuT 78 cells were used as a positive control (lane 8) in panel A. Each experiment was performed three times with similar results and representative gels are shown.

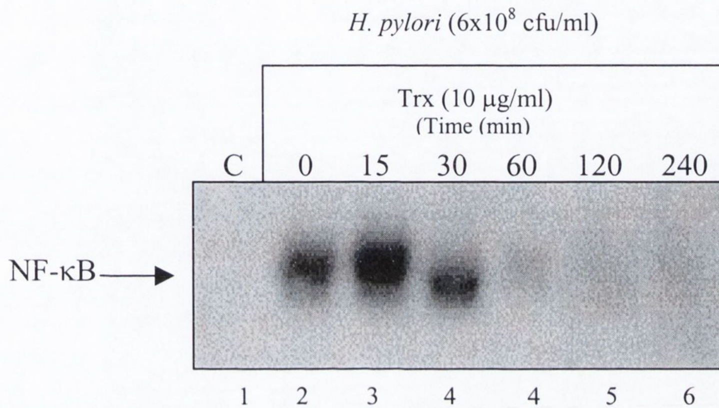
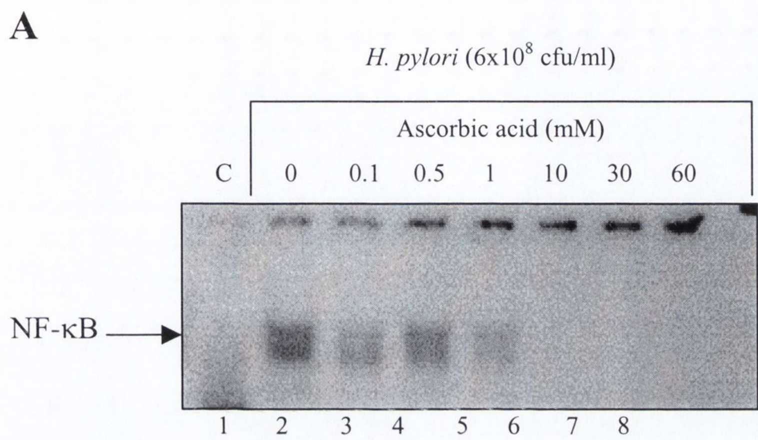


Fig. 4.2. Time course of NF-κB inhibition by *H. pylori* Trx treatment. AGS cells were pretreated with Trx (10 μg/ml) for different periods of time (as indicated above each lane) and then stimulated with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for 2 h and cell extracts were prepared and analyzed for NF-κB DNA-binding activity by EMSA. Lanes 3-6, time periods of preincubation with Trx for 15, 30, 60, 120 and 240 min prior to stimulation. Control AGS cells are shown in lane 1 and *H. pylori*-induced NF-κB in AGS cells in lane 2. A representative result of three independent experiments with similar results is shown.

A



B

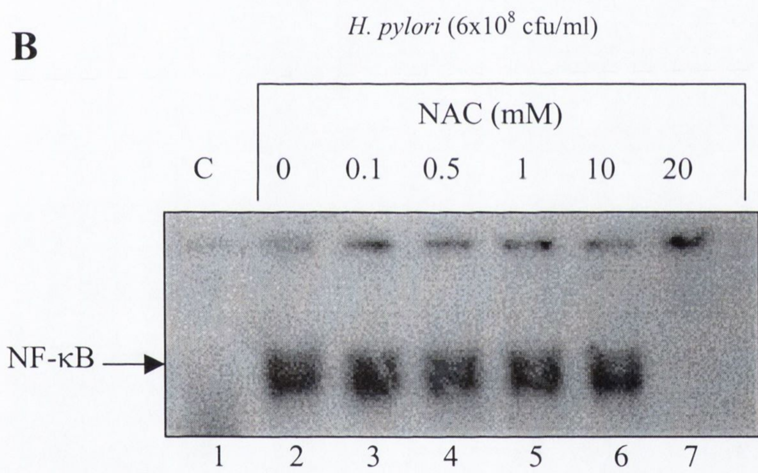


Fig. 4.3. Effects of ascorbic acid **(A)** and NAC **(B)** on NF-κB DNA-binding activity by *H. pylori*. AGS cells were pretreated with ascorbic acid (0.1-60 mM) or NAC (0.1-20 mM) prior to stimulation with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for 2 h. Nuclear extracts were prepared and analysed by EMSA. Each experiment was repeated three times and representative gels are shown.

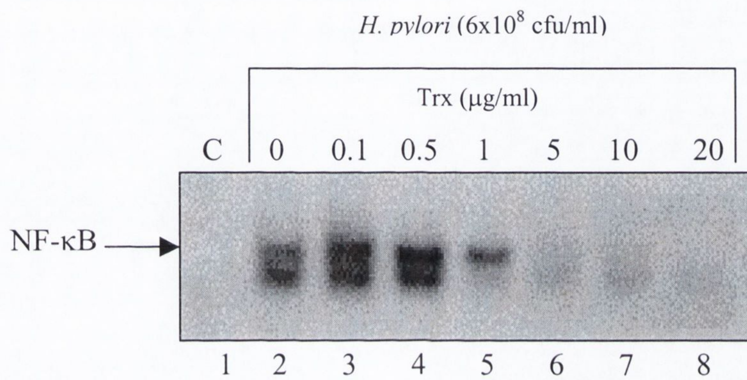
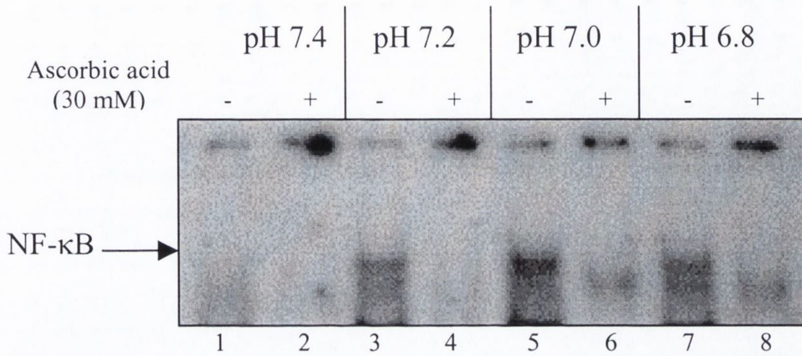


Fig. 4.4. Effects of Trx on *H.pylori*-induced NF-κB DNA-binding activity. AGS cells were pretreated with Trx (0.1-20 μg/ml) prior to stimulation with *H. pylori* strain 11638 (6x10^8 cfu/ml) for 2 h. Unstimulated AGS cells and *H. pylori*-induced NF-κB in AGS cells are indicated in lanes 1 and 2, respectively. Nuclear extracts were prepared and analysed by EMSA. A representative result of three different experiments is shown.

A



B

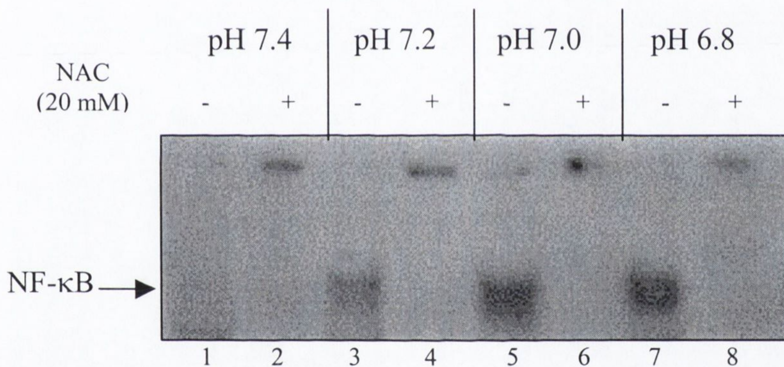
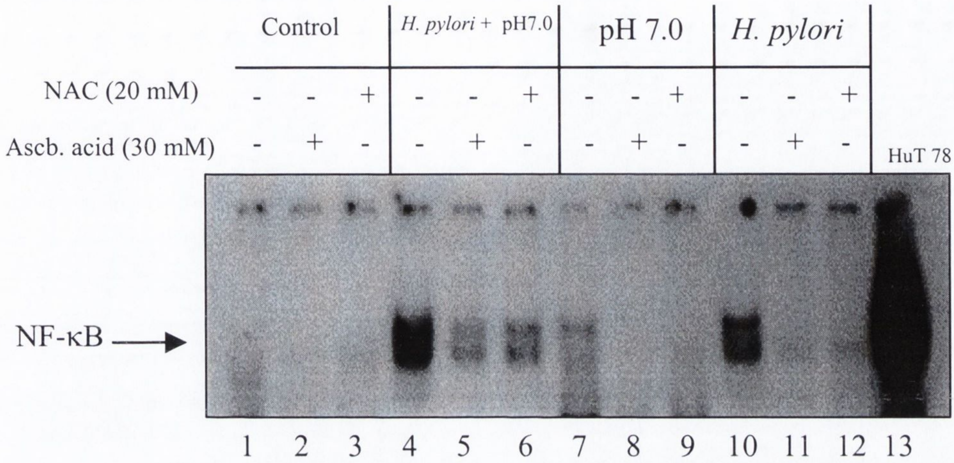


Fig. 4.5. Effects of ascorbic acid and NAC on NF-κB DNA-binding activity in AGS cells exposed to different values of pH. AGS cells were preincubated in the presence or absence of ascorbic acid (30 mM) **(A)** or NAC (20 mM) **(B)** for 1 h prior to incubation in media of different pH values (7.4 to pH 6.8). Nuclear extracts were prepared and NF-κB was assayed using EMSA. Each experiment was performed at least three times and one result is shown.

A



B

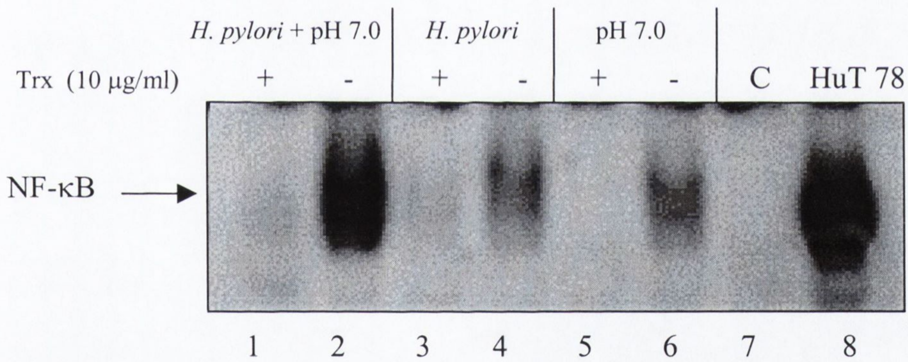


Fig. 4.6. Effects of ascorbic acid, NAC and Trx on NF-κB DNA-binding activity by low pH or low pH combined with *H. pylori*. AGS cells were treated with ascorbic acid (30 mM), NAC (20 mM) **(A)** or Trx (10 μg/ml) **(B)** for 2 h and incubated at pH 7.0 or with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) or *H. pylori* at pH 7.0. Nuclear extracts were analyzed in EMSA for NF-κB DNA-binding activity. HuT 78 cells were used as a positive control. Representative gels of two independent experiments with similar results are shown.

treatment with IL-1β (10 ng/ml), TNF-α (20 ng/ml) or PMA (10 ng/ml) for 2 h. These pro-inflammatory agents induce NF-κB in AGS cells. Here, the results demonstrate that the antioxidant pretreatment blocked NF-κB activation by the proinflammatory cytokines IL-1β and TNF-α and the mitogen PMA (Fig. 4.7A and B).

4.3.5 Effect of Trx on NF-κB activation by cytokine rich supernatants

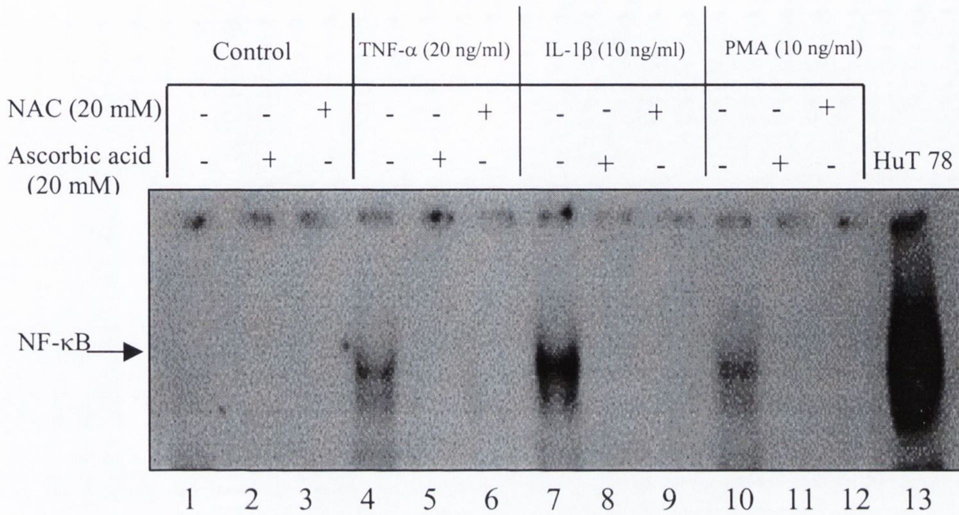
The effect of Trx on NF-κB activation in AGS cells stimulated with a cytokine rich supernatant obtained from PBMCs that had been activated with *H. pylori* strain 11638, TNF-α or PMA/PHA was investigated. Pretreatment of AGS cells with Trx (10 μg/ml) also prevented NF-κB activation by cytokine rich supernatants obtained from *H. pylori*- and cytokine- and mitogen-activated PBMCs (Fig. 4.8).

4.3.6 Effect of hydrogen peroxide on NF-κB DNA-binding activity

To examine the effect of H_2O_2 on NF-κB activation, AGS cells were exposed to a range of doses of H_2O_2 (0.01-10 mM) or 2 h. Treatment of AGS cells with H_2O_2 had no effect on NF-κB DNA-binding activity in the concentration range tested (Fig. 4.9).

4.3.7 Effect of antioxidants on IκB-α protein level

AGS cells were preincubated with either vitamin C (30 mM) or NAC (20 mM) for 1 h, the medium was refreshed, and then cells were incubated with *H. pylori*, IL-1β, TNF-α, PMA or acidic conditions for 2 h. Total cell extracts were prepared and analyzed by Western blot analysis using antibody against IκB-α. Vitamin C and NAC (Fig. 4.10 A and B) inhibited IκB-α degradation in response to treatment with *H. pylori*, IL-1β, TNF-α, PMA and pH 7.0. Pretreatment of AGS cells with vitamin C (30 mM) or NAC (20 mM) up-regulated IκB-α level in AGS cells (lane 2, Fig. 4.10A and B) compared to resting AGS cells. The degradation of IκB-α is coincident with the activation of NF-κB. Trx also inhibited IκB-α degradation in response to *H. pylori* and TNF-α (Fig. 4.10C).



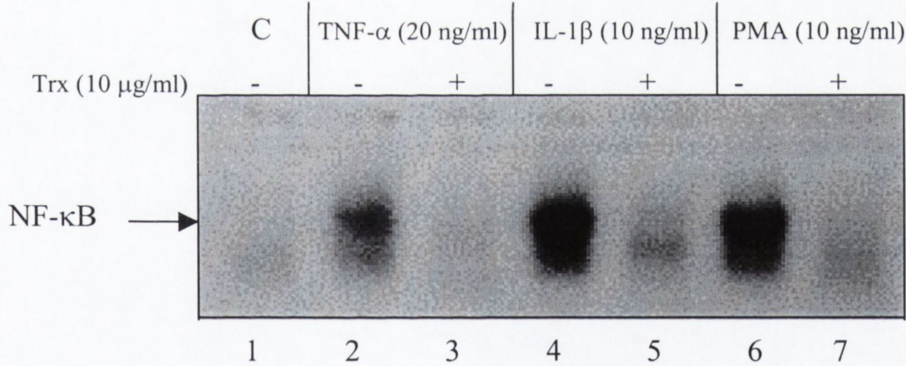


Fig. 4.7. Effects of ascorbic acid, NAC and Trx on NF-κB DNA-binding in response to cytokines and mitogen. AGS cells were preincubated with ascorbic acid (30 mM), NAC (20 mM) **(A)** or Trx (10 μg/ml) **(B)** for 1 h and then the cells stimulated with IL-1β (10 ng/ml), TNF-α (20 ng/ml) or PMA (10 ng/ml) for 2 h. NF-κB DNA-binding activity was assayed by EMSA on nuclear extracts. Each experiment was repeated at least three times with similar results and one result is shown.

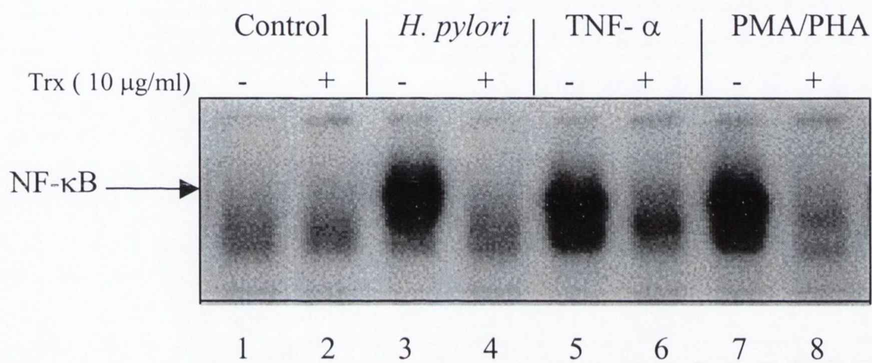


Fig. 4.8. Effects of Trx on NF-κB DNA-binding activity by cytokine-rich supernatants prepared from PBMC. AGS cells were pretreated with Trx (10 μg/ml) for 1 h and then the cells were treated with PBMC supernatants activated with *H. pylori* strain 11638 ($6x10^8$ cfu/ml), TNF-α (20 ng/ml) or PMA (10 ng/ml)/PHA (10 μg/ml)) for 2 h. Nuclear cell extracts were prepared and analyzed in EMSA for NF-κB DNA-binding activity. A representative blot is shown.

treatment with IL-1β (10 ng/ml), TNF-α (20 ng/ml) or PMA (10 ng/ml) for 2 h. These pro-inflammatory agents induce NF-κB in AGS cells. Here, the results demonstrate that the antioxidant pretreatment blocked NF-κB activation by the proinflammatory cytokines IL-1β and TNF-α and the mitogen PMA (Fig. 4.7A and B).

4.3.5 Effect of Trx on NF-κB activation by cytokine rich supernatants

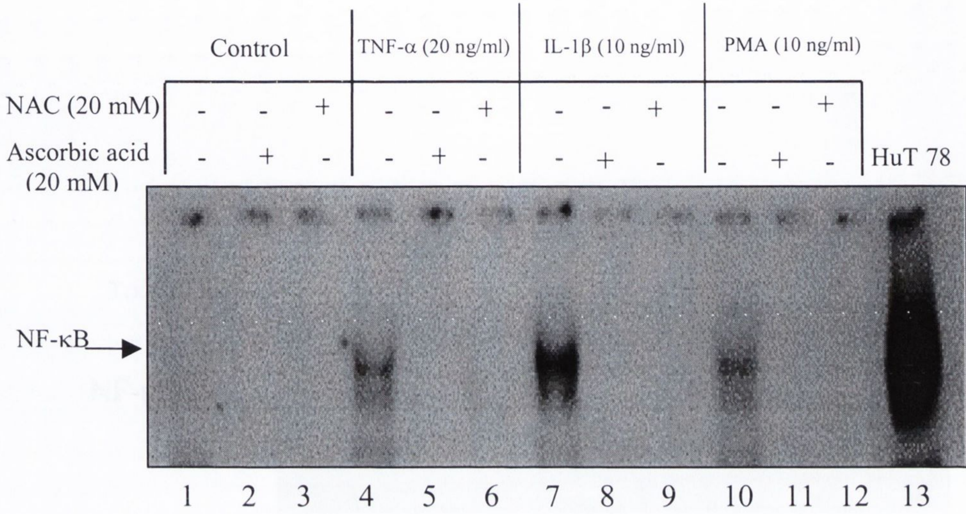
The effect of Trx on NF-κB activation in AGS cells stimulated with a cytokine rich supernatant obtained from PBMCs that had been activated with *H. pylori* strain 11638, TNF-α or PMA/PHA was investigated. Pretreatment of AGS cells with Trx (10 μg/ml) also prevented NF-κB activation by cytokine rich supernatants obtained from *H. pylori*- and cytokine- and mitogen-activated PBMCs (Fig. 4.8).

4.3.6 Effect of hydrogen peroxide on NF-κB DNA-binding activity

To examine the effect of H_2O_2 on NF-κB activation, AGS cells were exposed to a range of doses of H_2O_2 (0.01-10 mM) or 2 h. Treatment of AGS cells with H_2O_2 had no effect on NF-κB DNA-binding activity in the concentration range tested (Fig. 4.9).

4.3.7 Effect of antioxidants on IκB-α protein level

AGS cells were preincubated with either vitamin C (30 mM) or NAC (20 mM) for 1 h, the medium was refreshed, and then cells were incubated with *H. pylori*, IL-1β, TNF-α, PMA or acidic conditions for 2 h. Total cell extracts were prepared and analyzed by Western blot analysis using antibody against IκB-α. Vitamin C and NAC (Fig. 4.10 A and B) inhibited IκB-α degradation in response to treatment with *H. pylori*, IL-1β, TNF-α, PMA and pH 7.0. Pretreatment of AGS cells with vitamin C (30 mM) or NAC (20 mM) up-regulated IκB-α level in AGS cells (lane 2, Fig. 4.10A and B) compared to resting AGS cells. The degradation of IκB-α is coincident with the activation of NF-κB. Trx also inhibited IκB-α degradation in response to *H. pylori* and TNF-α (Fig. 4.10C).



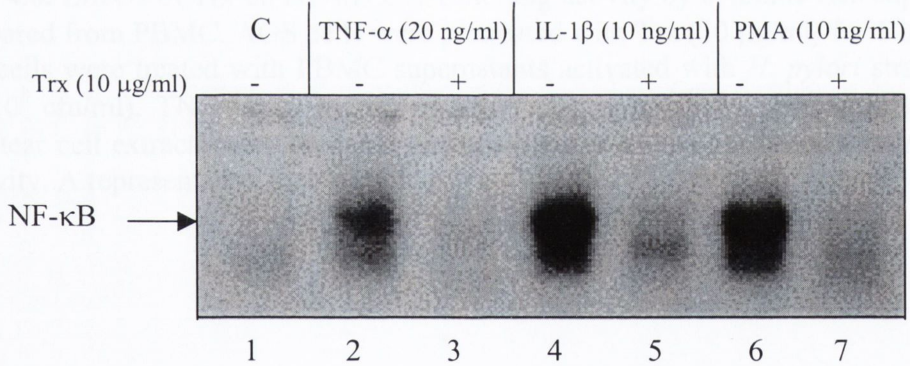


Fig. 4.7. Effects of ascorbic acid, NAC and Trx on NF-κB DNA-binding in response to cytokines and mitogen. AGS cells were preincubated with ascorbic acid (30 mM), NAC (20 mM) **(A)** or Trx (10 μg/ml) **(B)** for 1 h and then the cells stimulated with IL-1β (10 ng/ml), TNF-α (20 ng/ml) or PMA (10 ng/ml) for 2 h. NF-κB DNA-binding activity was assayed by EMSA on nuclear extracts. Each experiment was repeated at least three times with similar results and one result is shown.

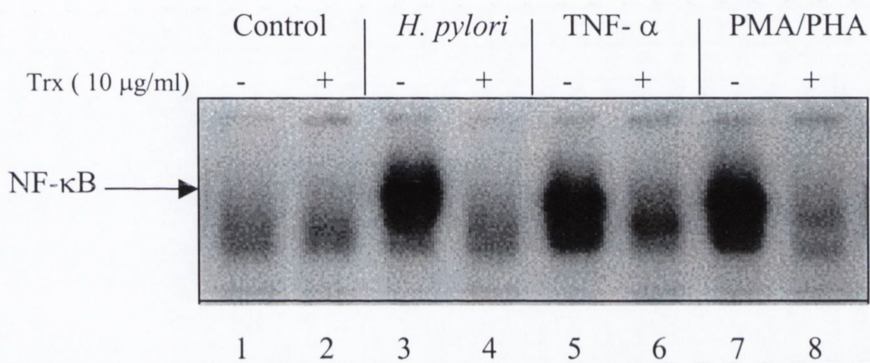


Fig. 4.8. Effects of Trx on NF-κB DNA-binding activity by cytokine-rich supernatants prepared from PBMC. AGS cells were pretreated with Trx (10 μg/ml) for 1 h and then the cells were treated with PBMC supernatants activated with *H. pylori* strain 11638 ($6x10^8$ cfu/ml), TNF-α (20 ng/ml) or PMA (10 ng/ml)/PHA (10 μg/ml)) for 2 h. Nuclear cell extracts were prepared and analyzed in EMSA for NF-κB DNA-binding activity. A representative blot is shown.

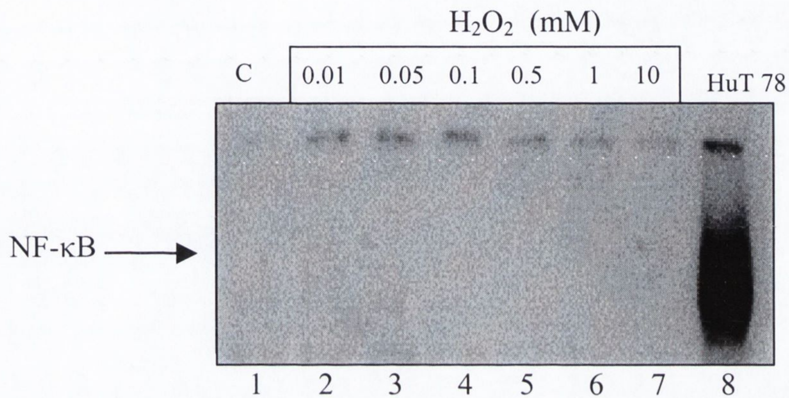
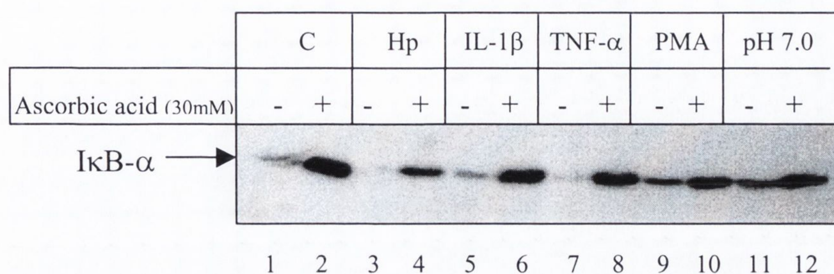
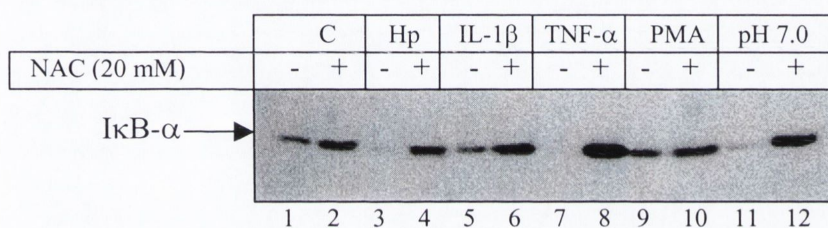


Fig. 4.9. Effect of hydrogen peroxide on NF-κB DNA-binding activity in AGS cells. AGS cells were incubated with (0.01-10 mM) H_2O_2 (lanes 2-7) for 2 h and nuclear extracts were prepared and assayed by EMSA for NF-κB DNA-binding activity. HuT 78 cells were used as a positive control (lane 8). This experiment was performed at least three times and one representative result is shown.





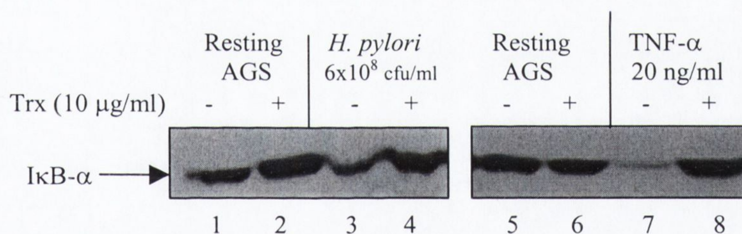


Fig. 4.10. The effect of antioxidants on IκB-α protein level in AGS cells. AGS cells were pretreated with either ascorbic acid (30 mM) **(A)** or NAC (20 mM) **(B)** for 1 h. The cells were incubated with *H. pylori* strain 11638 ($6x10^8$ cfu/ml, lanes 3 and 4), IL-1β (10 ng/ml, lanes 5 and 6), TNF-α (20 ng/ml, lanes 7 and 8), PMA (10 ng/ml, lanes 9 and 10) or at pH 7.0 (lanes 11 and 12), respectively, for 2 h. **(C)** Effect of Trx pretreatment on IκB-α in AGS cells stimulated with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) or TNF-α (20 ng/ml) for 2 h. Following stimulation, total cell extracts from AGS cells were prepared and analyzed by Western blotting using antiserum against IκB-α. A representative blot of three independent experiments with similar results is shown.

4.3.8 Effect of antioxidants on NF-κB p65 translocation

Pretreatment of AGS cells with vitamin C (30 mM), NAC (20 mM) or Trx (10 μg/ml) for 1 h blocked p65 activation by *H. pylori* as determined by Western blot analyses (Fig. 4.11A). Similarly, Trx pretreatment blocked *H. pylori*-induced p65 protein (Fig. 4.11B).

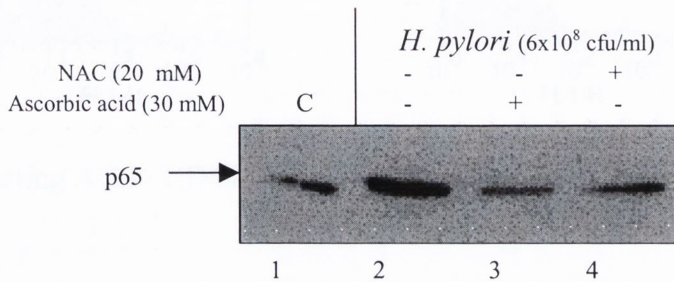
4.3.9 Effect of Trx on the expression of cell adhesion molecules

Pretreatment of AGS cells with Trx (10 μg/ml) for 24 h down-regulated CD44 expression on AGS cells (Fig. 4.12B) compared to untreated AGS cells (Fig. 4.12A). Trx was also effective in preventing the up-regulation of CD44 on AGS cells in response to stimulation with *H. pylori* strain 11638 for another 24 h (Fig. 4.12C and D). Pretreatment with Trx (10 μg/ml) for 24 h also down-regulated of ICAM-1 expression on AGS cells (Fig. 4.13B) compared to untreated cells (Fig. 4.13A). Likewise, pretreatment of AGS cells with Trx diminshed the *H. pylori*-induced up-regulation of ICAM-1 expression on AGS cells (Fig. 4.13C and D).

4.3.10 Effect of Trx on cell proliferation

The effect of Trx on AGS cell proliferation was investigated. The amounts of Trx (1-20 μg/ml) which resulted in inhibition of NF-κB DNA-binding activity had no effect on AGS cell proliferation compared to untreated cells (Fig. 4.14A). Cell viability was compromised only by elevated levels of thioredoxin (≥50 μg/ml). Incubation of AGS cells with *H. pylori* thioredoxin (10 μg/ml) for different periods of time up to 72 h showed no effect on cell viability (Fig. 4.14B). Moreover, incubation of AGS cells with thioredoxin at 10 μg/ml in the presence or absence of serum had no effect on cell proliferation. Incubation of AGS, PBMCs and HuT78 cells with *H. pylori* Trx (10 μg/ml) for 24 h had no effect on cell proliferation (Fig. 4.15A). Interestingly, pretreatment of AGS cells with thioredoxin (10 μg/ml) inhibited *H. pylori*, TNF-α- and PMA-induced cell proliferation (Fig. 4.15B).

A



B

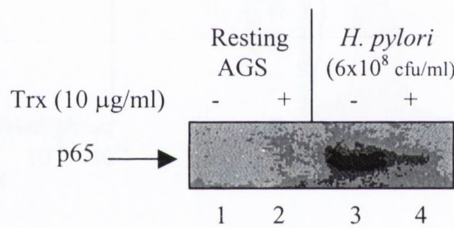


Fig. 4.11. Effect of Trx on NF-κB p65 translocation. AGS cells were pretreated with either ascorbic acid (30 mM), NAC (20 mM) **(A)** or Trx (10 µg/ml) **(B)** for 1 h. The cells were then incubated with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for 2 h. Following stimulation, nuclear cell extracts from AGS cells were prepared and analyzed by Western blotting using antiserum against NF-κB p65.

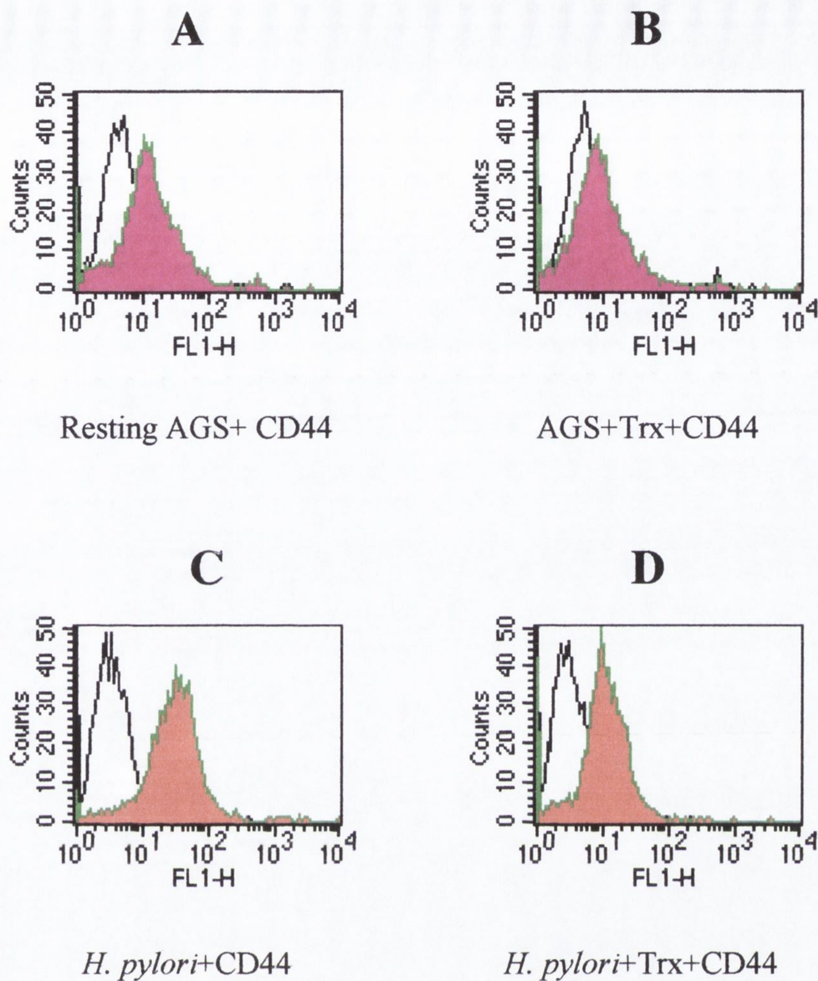


Fig. 4.12. FACScan profiles of CD44 expression on gastric epithelial cells. AGS cells ($5x10^5$ cells/ml) were incubated with medium alone **(A)** or Trx (10 μg/ml) **(B)** for 24 h. Cells were stained with FITC-labelled anti-CD44 (L3D.1) (pink peaks) or FITC-labelled isotype control (anti-IE, unshaded peaks). **(C)** Effect of *H. pylori* strain 11638 ($6x10^8$ cfu/ml) on CD44 expression in AGS cells (orange peaks). Unshaded peaks represent FITC-labelled isotype control (anti-IE). **(D)** AGS cells pretreated with Trx at 10μg/ml for 24 h and then stimulated with *H. pylori* for 24 h.

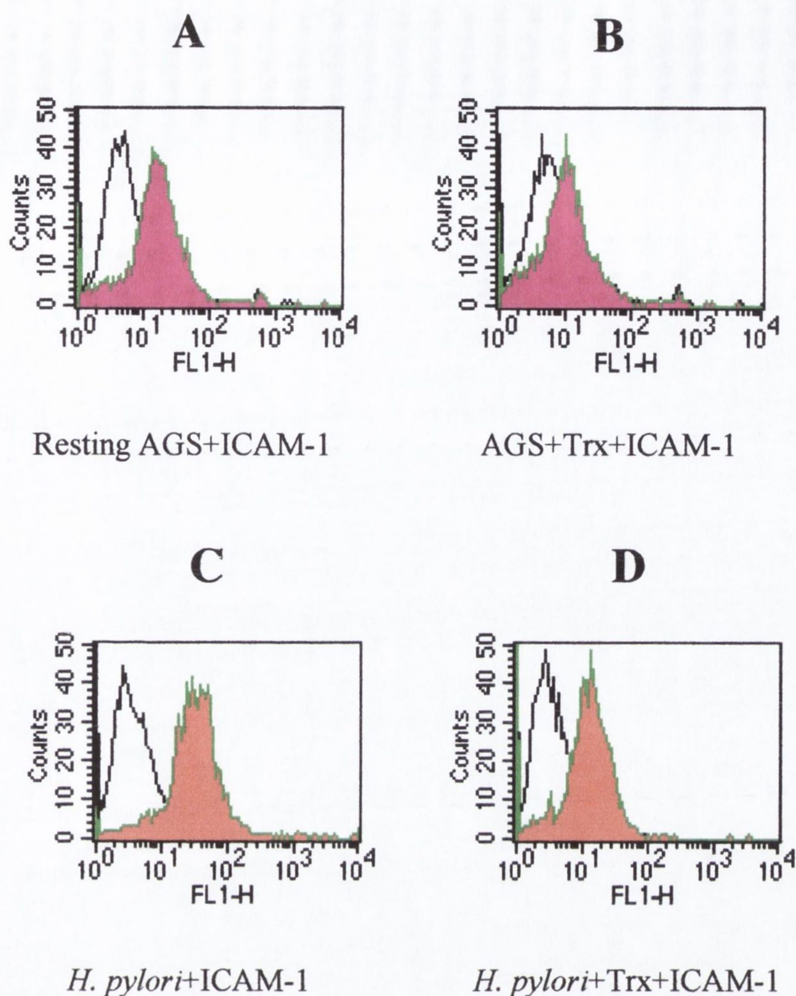
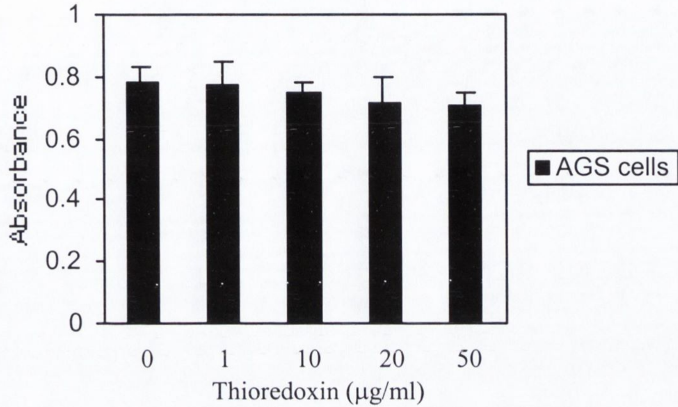


Fig. 4.13. FACScan profiles of ICAM-1 expression on gastric epithelial cells. AGS cells ($5x10^5$ cells/ml) were incubated with medium alone **(A)** or Trx (10 μg/ml) **(B)** for 24 h. Cells were stained with FITC-labelled anti-ICAM-1 (pink shaded peaks). **(C)** Effect of *H. pylori* strain 11638 ($6x10^8$ cfu/ml) on ICAM-1 expression. Cells were stained with FITC-labelled with anti-ICAM-1 (orange peaks) or FITC-labelled isotype control (anti-IE, unshaded peaks). **(D)** The effect of preincubation with Trx (10 μg/ml) on ICAM-1 expression in *H. pylori*-treated AGS cells.

A



B

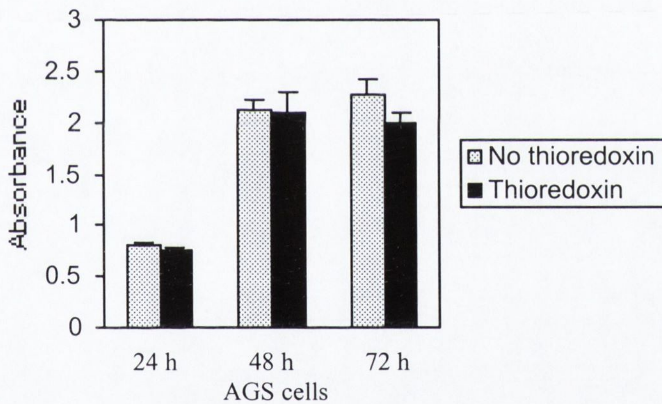
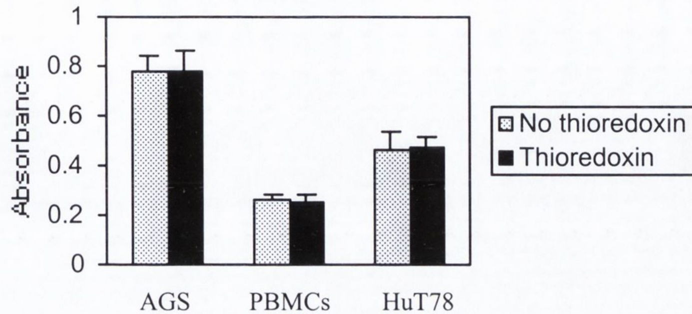


Fig. 4.14. Effect of Trx on AGS cell proliferation. **(A)** Dose-response effect of Trx. AGS ($1x10^5$ cells/ml) were incubated with various amounts of Trx (1-50 μg/ml) for 24 h. **(B)** Time-course of thioredoxin incubation with AGS cells, cells were incubated with Trx (10 μg/ml) for 24 to 72 h. To the cultured cells, 20 μl of freshly prepared PMS/MTS solution was added and the plates were incubated for 4 h at 37^0C. The absorbance was read at 490 nm using an ELISA plate reader. Each experiment was repeated three times and results are shown as the mean ±SD (n=3).

A



B

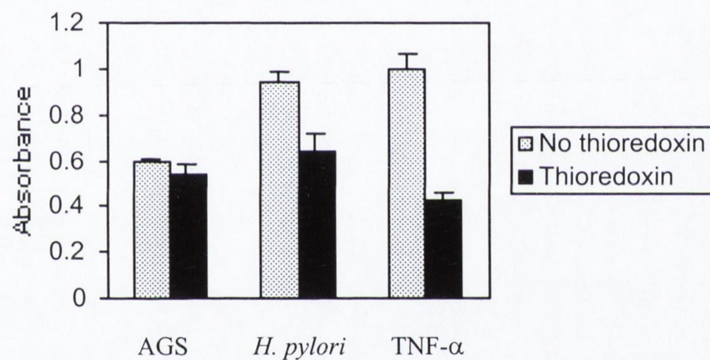


Fig. 4.15. (A) Effect of Trx on the proliferation of different cell lines. AGS, PBMC or HuT78 cells seeded at concentration of 1×10^5 cells/ml were incubated with Trx (10 μg/ml) for 24 h. **(B)** Effect of Trx on *H. pylori*- and TNF-α-induced cell proliferation. AGS cells (1×10^5 cells/ml) were pretreated with Trx for 24 h prior to incubation with *H. pylori* strain 11638 (6×10^8 cfu/ml) or TNF-α (20 ng/ml) for a further 24 h. To the cultured cells, 20 μl of freshly prepared PMS/MTS solution was added to each well and the plates were incubated for 4 h at 37^0C. The absorbance was read at 490 nm using ELISA plate reader. Each experiment was performed three times and results are shown as the mean ±SD (n=3).

4.4 DISCUSSION

The activity of the transcription factor NF-κB is modulated by the redox state of the cell. Many antioxidants such as vitamin C, NAC and PDTC have been shown to block the activation of NF-κB in response to a variety of stimuli (Bowie and O'Neill, 2000; Schreck *et al.*, 1992b; Meyer *et al.*, 1993; Harakeh *et al.*, 1990; Dröge *et al.*, 1992). This leads to the prediction that thiol compounds are potent inhibitors of NF-κB activation in response to various stimuli. The findings presented here demonstrate that vitamin C, NAC and *H. pylori* Trx inhibited *H. pylori*-induced NF-κB DNA-binding activity. Inhibition of NF-κB activation by vitamin C and NAC required relatively high concentrations in the millimolar range, 10-30 mM and 20 mM, respectively, whereas inhibition by *H. pylori* Trx was obtained in the micromolar range. No apparent effect on cell viability was noted at this range of concentrations of vitamin C, NAC or Trx as measured by EB/AO staining.

Vitamin C is an acidic molecule with strong reducing activity and is essential component of most living tisssues. Although vitamin C is present at significantly higher levels in gastric juice and gastric mucosal cells (50-300 μM/l) than in plasma or serum (Zhang *et al.*, 1998; Sobala *et al.*, 1991; Reed, 1999), its effect on gastric epithelial cells remains unclear. In addition to vitamin C thioredoxin has also been reported to be an efficient electron donor to human plasma glutathione peroxidase (Bjornstedt *et al.*, 1994). Trx is present in plasma at concentrations up to 6 nM and has been suggested to have an antioxidant role in plasma (Nakamura *et al.*, 1997). However, plasma contains considerably higher levels of reduced glutathione, around 1 μM (Hogarth *et al.*, 1996).

Vitamin C plays an important role in the overall antioxidant defense system of the body. Vitamin C scavenges oxygen free radicals and reacts with nitrite and converts it to nitrous oxide, thus preventing the formation of such carcinogens (Mirvish, 1986; Mirvish, 1994). Elevated gastric pH and the damage to the gastric surface epithelium were inversely associated with the vitamin C concentration in gastric juice. Several studies have shown that *H. pylori*-associated gastritis is correlated with decreased

concentrations of gastric juice ascorbic acid (Sobala *et al.*, 1993; Banerjee *et al.*, 1994). Hence understanding the role of physiological levels of vitamin C in the stomach is important in elucidating the protective properties of vitamin C against *H. pylori* infection and gastric carcinogenesis. Here the results demonstrate that vitamin C inhibited NF-κB activation in response to *H. pylori* and other agents, as demonstrated by mobility shift assays and Western blot analyses. Vitamin C also prevented *H.pylroi*-induced IκB-α degradation which appears to be the initial step in NF-κB activation. O'Toole *et al.* (1997) reported that vitamin C blocked NF-κB induction in response to *H. pylori*-activated PBMCs.

The exact mechanism wherby vitamin C and NAC inhibit NF-κB activation is not known. In this study, pretreatment of gastric epithelial cells with vitamin C up-regulated IκB-α level in comparison with untreated AGS cells. Consistent with this, Bowie and O'Neill (2000) have demonstrated that treatment of the endothelial cells ECV304 with vitamin C blocked IL-1- and TNF-mediated degradation and phosphorylation of IκB-α, due to inhibition of IKK activation. The inhibition of TNF-induced IKK activation was mediated by p38 MAPK, as treatment of cells with vitamin C led to a rapid and sustained activation of p38 MAPK. Other studies have shown that high vitamin C doses prevented *H. pylori* infection (Jarosz *et al.*, 1998) and also protected against gastric carcinoma (Drake *et al.*, 1996; Zhang *et al.*, 1997). Wang *et al.* (2000) have shown that treatment of *H. pylori* infected mice with algal cell extract containing the antioxidant astaxanthin and vitamin C reduce gastric inflammation and inhibit bacterial infection.

Doses of of vitamin C and NAC in millimolar concentrations were required to inhibit NF-κB activation in our experiment. Normal gastric juice vitamin C levels are in the micromolar concentration range and whether at these lower concentrations, an inhibitory effect on NF-κB is seen *in vivo* is unclear. Vitamin C is acetively secreted into the gastric juice and higher vitamin concentrations may be found close to the gastric epithelial cells. Levels of ascorbic acid in gastric tissue (from antral and body regions) are at least ten-fold greater than either plasma or gastric juice ascorbic acid

concentrations (Waring *et al.*, 1996). Clearly, in both normal and diseased human stomach, factors other than plasma and gastric mucosal levels of ascorbic acid are important in determining the concentration of gastric juice ascorbic acid such as reduced secretion, increased ascorbate oxidation with subsequent breakdown or back-diffusion of dehydroascorbic acid through unhealthy mucosa or increased utilisation by *H. pylori* that colonise the stomach (Marshall, 1986).

In this study, the results also demonstrate that Trx inhibits the activation of NF-κB in AGS cells in response to *H. pylori*. Dose-response experiments showed that the inhibitory effect of Trx on NF-κB activation was detected at a dose of 1 μg/ml with maximal inhibition at doses of 10 μg/ml. Trx also inhibited NF-κB activation by IL-1β and TNF-α and PMA. Moreover, Trx blocks the induction of NF-κB by acidic conditions and *H. pylori*- and cytokine-activated PBMC supernatants.

Pretreatment of AGS cells with Trx inhibited *H. pylori*-and cytokine-induced cell proliferation. Incubation of AGS cells with various amounts of Trx, over the same range of Trx concentrations which inhibited NF-κB activation, had no effect on the number of the cells.

NAC also inhibited *H. pylori*-induced NF-κB activation by *H. pylori*. Previous reports have shown that NAC suppresses NF-κB induction by IL-1, TNF, PMA or LPS treatment of various cell types (Schreck *et al.*, 1991; Staal *et al.*, 1990; Meyer *et al.*, 1993; Brennan *et al.*, 1995). In addition, the activation of NF-κB by cytokines and acidic conditions was blocked by NAC. Hydrogen peroxide did not activate NF-κB after the exposure of AGS cells to different concentrations of H_2O_2. None of the concentrations of H_2O_2 tested were toxic to AGS cells. The involvement of H_2O_2 in activating of NF-κB appears to be restricted to certain cell types. H_2O_2 has been shown to activate NF-κB in HeLa and Jurkat T cells (Brennan and O'Neill 1995; Meyer *et al.*, 1995; Schreck *et al.*, 1991). A number of other cell types have proved to be insensitive to H_2O_2, including monocytic cells, astrocytoma, standard Jurkats, EL4.NOB-1 T cells, KB epidermal cells and human umbilical vein endothelial cells (Schreck and Baeuerle,

1994; Moynagh *et al.*, 1994; Anderson *et al.*, 1994; Brennan and O'Neill, 1995; Bowie *et al.*, 1997). Previous studies suggest that cells can release glutathione peroxidase, a H_2O_2 metabolizing enzyme extracellulary (Avissar *et al.*, 1994). Although elevated levels of oxidised glutathione inhibit NF-κB, it has been suggested that a certain amount of oxidised glutathione is required for optimal activation (Galter *et al.*, 1994). Sen *et al.* (1996) have also shown that differences in the kinetics of intracellular Ca^{2+} increases in response to H_2O_2 may be the basis for the difference in the sensitivity of NF-κB to H_2O_2 in Jurkat subclones.

The results of flow cytometry demonstrated that Trx down-regulates the expression of *H. pylori*-induced up-regulation of CD44 and ICAM-1. Parallel to these results, it has been shown that preincubation of murine B-lymphocytes with NAC diminished the CD40-mediated JNK response (Lee and Koretzky, 1998). Similarly, PDTC and NAC were able to inhibit the expression of VCAM-1 and tissue factor genes induced by pro-inflammatory stimuli in endothelial cells (Marui *et al.*, 1993; Weber *et al.*, 1994). PDTC also inhibited the production of IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor by endothelial cells in response to inflammatory mediators (Münoz *et al.*, 1996), most likely through inhibition of NF-κB. Moreover, pharmacological inhibitors of IκB-α phosphorylation or proteasome activity inhibit NF-κB activation and subsequent transcription of adhesion molecule genes (Read *et al.*, 1995). These findings suggest that Trx and other related molecules play a critical role in the redox regulation of transcription processes.

The activity of NF-κB is modulated by the redox state of the cell and is activated by a prooxidant state and is therefore inhibited by antioxidants (Bowie and O'Neill, 2000; Schreck *et al.*, 1992a and 1992b; Schmidt *et al.*, 1995). Inhibition of NF-κB activation by antioxidants can occur by scavenging of free radicals. Schenk *et al.* (1994) suggested that Trx interferes with the release of IκB as the major regulatory step of NF-κB activation. Hayashi *et al.* (1993) reported that the redox regulation of NF-κB by Trx might be exerted at a step after dissociation of the inhibitory molecule IkappaB. On the other hand, NAC can act directly as free radical scavenger and also increase

intracellular levels of glutathione (Aruoma *et al.*, 1989). It has been shown that NAC blocks NF-κB activation by preventing a decrease in intracellular thiol levels (Staal *et al.*, 1990) and suppressing the formation of reactive oxygen species (Schreck *et al.*, 1991). Drake *et al.* (1996) have suggested that vitamin C acts through scavenging of free radicals generated in human gastric mucosa. Mirvish (1986) found that vitamin C is capable of eliminating oxygen radicals, which may damage the gastric epithelium. Therefore, the combination of reduced vitamin C levels in association with *H. pylori* infection and altered acid secretion may result in an exaggerated inflammatory response through induction of NF-κB, Egr-1 and other related genes.

The main finding of this study is that vitamin C, NAC and Trx inhibited NF-κB activation in AGS cells and other cells in response to *H. pylori*. The use of antioxidants could protect against the mucosal inflammation associated with *H. pylori* infection and gastric carcinoma. The inhibition of NF-κB by novel compounds such as *H. pylori* Trx could represent a new approach for therapeutic intervention in treating inflammatory disease states.

CHAPTER V

H. PYLORI INDUCES THE EXPRESSION OF THE EARLY GROWTH RESPONSE (Egr)-1 TRANSCRIPTION FACTOR IN GASTRIC EPITHELIAL CELLS

CHAPTER V

5.1 INTRODUCTION

The early growth response-1 (Egr-1) transcription factor is the prototype of a family of zinc finger transcription factors including Egr-2, Egr-3, Egr-4 and NGFI-B that are involved in regulating of cell proliferation and differentiation and cell death. Egr-1 is expressed in the nucleus of cells following cell activation and is capable of altering the transcription of several genes during the inflammatory process.

Although no studies have been yet described the activation of transcription factor Egr-1 in *H. pylori* infected patients, accumulating evidence suggests that this factor regulates diverse biological processes, particularly those of inflammatory and immune responses (McMahon and Monroe, 1996). Following *H. pylori* infection, a series of cellular changes takes place in the gastric epithelium that can result in the development of peptic ulcer disease. These events involve the inducible expression of a wide variety of genes involved in the inflammatory response. It appears that the induction of the transcription factor Egr-1 may play a critical role in these initial changes. As Egr-1 can activate many pathophysiologically relevant target genes such as cell surface adhesion molecules, pro-inflammatory cytokines and proteins that can alter cell survival, all of which are expressed during *H. pylori* infection. Therefore, identification of the role of Egr-1 in *H. pylori* infection could lead to understanding of the mechanisms of *H. pylori*-induced inflammation. In this chapter, the role of *H. pylori* in the induction of Egr-1 in gastric epithelial cells is explored.

5.2 OBJECTIVES

The aims of this chapter were to examine:

1. The effect of *H. pylori* on Egr-1 expression in gastric epithelial cells.
2. The signalling pathways of Egr-1 activation by *H. pylori*.
3. The effect of low pH, the cytokines IL-1β and TNF-α and the mitogen PMA on Egr-1 expression.
4. Signalling pathways of low pH-, cytokine- and mitogen-induced Egr-1 activation.

5. Effect of oxidants and antioxidants on Egr-1 activation.
6. The expression of Egr-1 *in vivo* in antral gastric biopsies from patients infected with *H. pylori*.

5.3 RESULTS

5.3.1 *H. pylori* induces the expression of Egr-1

To examine whether Egr-1 expression could be induced by *H. pylori*, AGS cells were cocultured with a freshly harvested suspension of *H. pylori* strain 11638 for different periods of time. Whole cell extracts were prepared and subjected to Western blot analysis. Fig. 5.1A shows that *H. pylori* induces the expression of the 84-kDa Egr-1 protein in AGS cells cocultured with *H. pylori* strain 11638 (6×10^8 cfu/ml) for 2 h at a multiplicity of infection (MOI) 100:1. A transient increase in Egr-1 protein levels was observed in AGS cells cocultured with *H. pylori* compared to control cells with a maximum induction of Egr-1 expression seen at 2-4 h (Fig. 5.1B) and this induction was maintained up to 8 h. The expression of Egr-1 exhibited a dose-dependent response to *H. pylori* (Fig. 5.1C).

5.3.2 Effect of *H. pylori* clinical isolates on Egr-1 expression

Coculture of AGS cells with $CagA^+$ and $VacA^+$ *H. pylori* strains, NCTC 11637 and NCTC 11638 (6×10^8 cfu/ml) for 2 h, induced Egr-1 expression in AGS cells. *H. pylori* clinical isolates deficient in *cagA* gene and do not express CagA protein [92-701 and 93-1000 (both $CagA^-$ and $VacA^-$) and 92-1099 ($CagA^-$ and $VacA^+$) cocultured at the same concentration for the same period of time were unable to induce Egr-1 expression (Fig. 5.2A).

5.3.3 Effect of other bacteria on Egr-1 expression

The induction of Egr-1 expression in the gastric epithelial cells AGS appears to be specific to *H. pylori* as no induction of Egr-1 was observed when AGS cells were cocultured with either *E. coli* or *C. jejuni* (6×10^8 cfu/ml) for 2 h (Fig. 5.2B). Egr-1 induction was only seen with live *H. pylori*. Heat killed *H. pylori* and *H. pylori* LPS did not induce Egr-1.

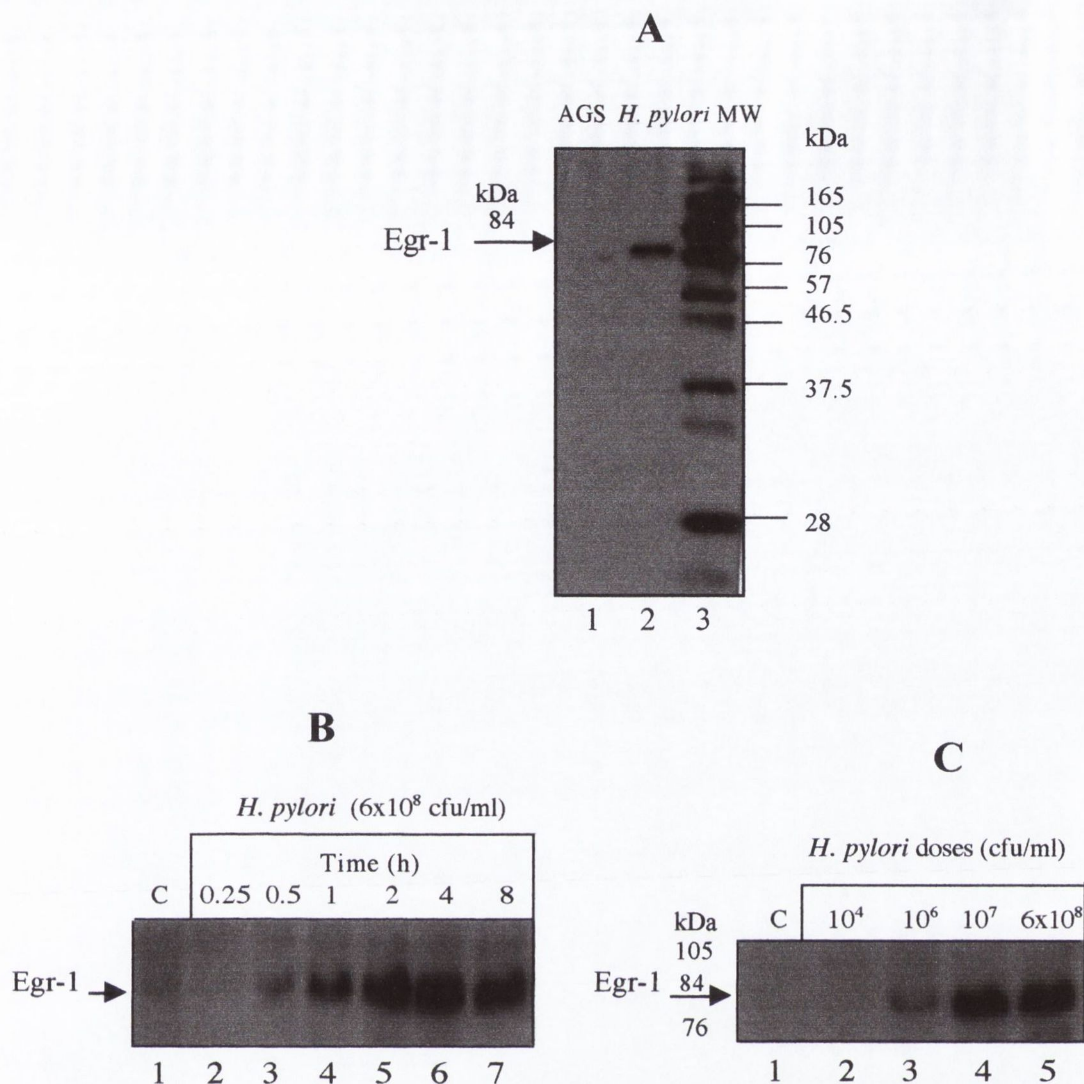
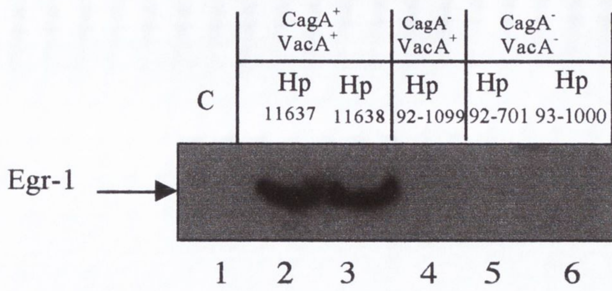


Fig. 5.1. *H. pylori*-induced Egr-1 expression in AGS cells. (A) The induction of 84-kDa Egr-1 protein in AGS cells cocultured with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) at a multiplicity of infection of 100:1 for 2 h, and MW; molecular weight marker. **(B)** Time-course of Egr-1 expression in *H. pylori*-treated AGS cells. AGS cells were treated with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for various periods of time, as indicated above each lane. **(C)** Effect of *H. pylori* dose on Egr-1 expression in AGS cells. Cells were cocultured with different amounts of *H. pylori* strain 11638 between $1x10^4$ and $6x10^8$ cfu/ml for 2 h. Following incubation AGS cells were washed three times with PBS and total cell extracts from stimulated and unstimulated AGS cells were prepared. Proteins in cell extracts (50 μg protein/lane) were separated by 10% polyacrylamide gel and blotted onto PVDF membrane. Western blot analysis for the 84-kDa Egr-1 protein was performed using anti-Egr-1 antiserum. Experiments were performed at least three times with similar results and a representative experiment is shown.

A



B

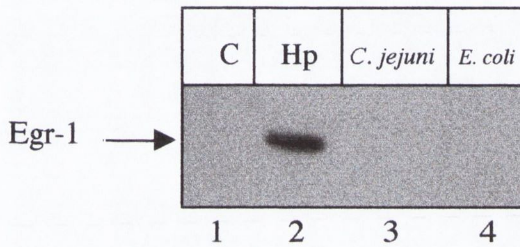


Fig. 5.2. (A) Effect of different *H. pylori* strains, NCTC 11637, 11638 (both $CagA^+$ and $VacA^+$, lanes 1 and 2, respectively), strain 92-1099 ($CagA^-$ and $VacA^+$, lane 3) and strains 92-701 and 93-1000 (both $CagA^-$ and $VacA^-$, lanes 4 and 5, respectively) on Egr-1 expression in AGS cells. **(B)** Effects of *H. pylori* (lane 2), *Campylobacter jujeni* (lane 3), *E. coli* (lane 4); at $6x10^8$ cfu/ml on Egr-1 expression. Total cellular extracts from stimulated and unstimulated AGS cells were prepared and subjected to analysis by Western blotting for Egr-1 protein expression. A representative gel of three different experiments with similar results is shown.

5.3.4 *H. pylori* induces Egr-1 expression in other gastric and non-gastric cells

To examine the induction of Egr-1 expression in other gastric epithelial cells, KATO-3 cells were incubated with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for 2 h and Egr-1 protein was detected with anti-Egr-1 antibody using Western blotting. Fig. 5.3A shows that *H. pylori* also activates Egr-1 in KATO-3 (lane 2) compared to untreated cells (lane 1).

The induction of Egr-1 by *H. pylori* was further examined in two colonic cell lines (T84 and HT29 cells). T84 and HT29 cells were cocultured with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for 2 h and, following stimulation, the total cell extracts were prepared and assayed for Egr-1 expression. As shown in Fig. 5.3 B and C, *H. pylori* strongly induced Egr-1 expression in these cells.

5.3.5 Effect of *H. pylori* on Egr-1 DNA-binding activity

Incubation of AGS cells with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) resulted in a significant gel retardation of the DNA-protein complex. This expression was detected as early as 30 min and reached maximal induction at 2 h, followed by a decrease to the basal level by 24 h (Fig. 5.4A). These findings support the observations from Western blot analyses. *E. coli* had no effect on Egr-1 expression as determined by EMSA. The addition of anti-Egr-1 antibody resulted in a decrease of the intensity of this DNA-protein complex (Fig. 5.4B), lane 2, confirming that Egr-1 was bound to this site. The specificity of this assay was confirmed in competition assays using a 100-fold molar excess of double-stranded oligonucleotide containing the Egr-1 consensus sequence, which totally blocked the formation of this complex (Fig. 5.4B), lane 3.

5.3.6 Signalling pathways involved in Egr-1 induction by *H. pylori*

In order to determine the signalling pathways involved in Egr-1 induction by *H. pylori*, a panel of PKC inhibitors Gö6976 (5 μM), rottlerin (10 μM) and calphostein C (100 nM); PI3-kinase inhibitor worthmannin (100 nM); tyrosine kinase inhibitor herbimycin A (10 μM); p38 MAP kinase inhibitor SB203580 (10 μM) and MEK MAP

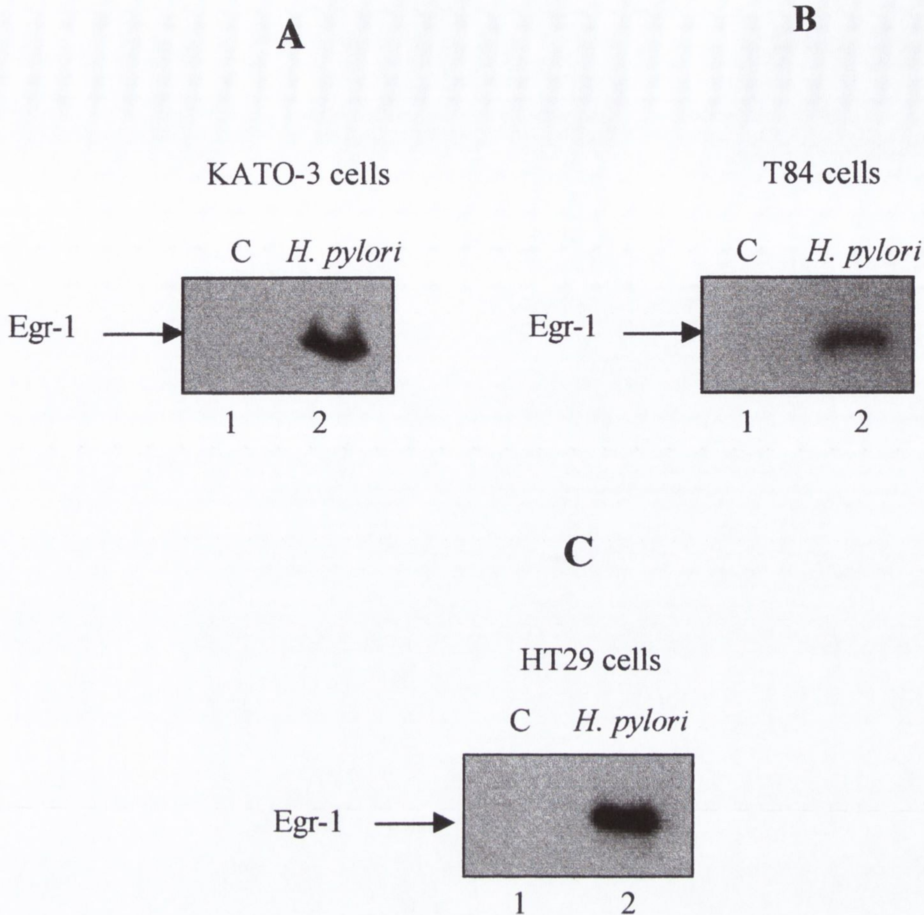
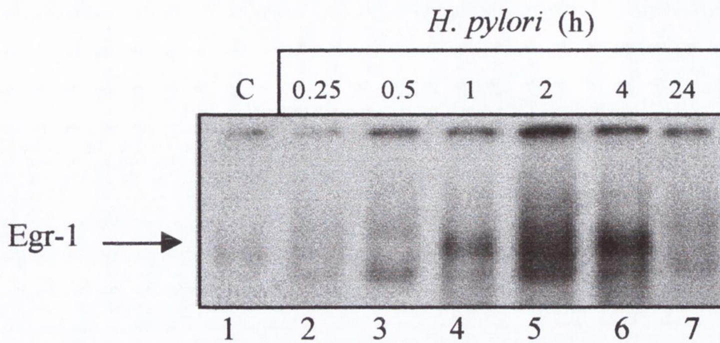


Fig. 5.3. Induction of Egr-1 by *H. pylori* in different cell lines. (A) *H. pylori* induces Egr-1 in KATO-3 cells. Cells were cocultured with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for 2 h (lane 2) or left untreated (lane 1). Following stimulation, total cell extracts were prepared and analyzed for Egr-1 expression by Western blotting using anti-Egr-1 antiserum. **(B)** *H. pylori* induces Egr-1 in T84 cells. T84 cells were cocultured with *H. pylori* (lane 2) or left untreated (lane 1), and Egr-1 expression was assayed as described in A. **(C)** *H. pylori* induces Egr-1 in HT29 cells. HT29 cells were left untreated (lane 1) or cocultured with *H. pylori* (lane 2), and detection of Egr-1 was performed as described in A.

A



B

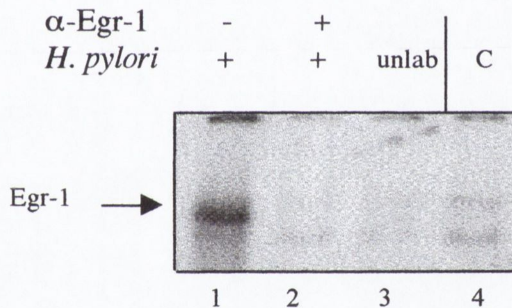


Fig. 5.4. Electrophoretic mobility shift assay (EMSA) analysis of Egr-1 expression in *H. pylori*-induced AGS cells. (A) Time course of Egr-1 induction by *H. pylori* in AGS cells. AGS cells were incubated with a freshly harvested suspension of *H. pylori* strain 11638 ($6x10^8$ cfu/ ml) for the indicated periods of time, as indicated above each lane. **(B)** Supershift assay was performed using 2 μl of antibody against Egr-1 in *H. pylori* treated AGS (lane 2). Competition assay was also performed for Egr-1 in AGS cells treated with *H. pylori* in which excess (2 μl) of unlabeled probe was added (lane 3). Nuclear extracts were prepared and analysed in EMSA as described in the Methods section. Results represent of three different experiments.

kinase inhibitor, PD98059 (20 μM). Confluent AGS cells were preincubated with these inhibitors for 1 h followed by coculture of AGS cells with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for 2 h. Treatment of AGS cells with any of these inhibitors or vehicle alone (0.1% v/v DMSO) had no effect on Egr-1 expression. Western blot analysis of Egr-1 expression showed that the PKC inhibitor rottlerin (10 μM), Gö6976 (5 μM) and calphostein C (100 nM) or the phosphatidylinositol 3-kinase inhibitor worthmannin (100 nM), had no effect on *H. pylori*-induced Egr-1 expression (Fig. 5.5A). Neither the tyrosine kinase inhibitor herbimycin A (10 μM) nor the p38 MAP kinase inhibitor SB203580 (10 μM), had any effect on *H. pylori*-induced Egr-1 expression, while the MEK MAP kinase inhibitor, PD98059 (20 μM), partially inhibited Egr-1 induction by *H. pylori* (Fig. 5.5B).

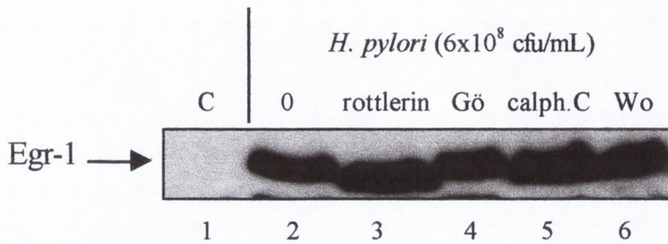
5.3.7 Effect of pH reduction on Egr-1 expression

The effect of low pH on Egr-1 expression was examined in AGS cells. The pH of the culture medium was decreased from pH 7.4, the pH of the untreated medium, to 6.4 by the addition of 0.1 M HCl. Cells counts and pH value were routinely checked before and after each experiment. No change in cell numbers or pH value was noticed over the time course studied. As the pH decreased from 7.4 to 6.4, induction of Egr-1 expression was observed (Fig. 5.6A). However, incubation at pH values greater than pH 7.4 or less than pH 6.4 had no effect or no further effect, respectively, on Egr-1 expression in AGS cells. At pH values less than 6, there was no effect on Egr-1 expression as the cell viability was greatly compromised as assessed microscopically. The time course of Egr-1 induction was determined by incubating AGS cells at pH 7.0 for various periods of time. As shown in Fig. 5.6B, incubation of the cells at pH 7.0 resulted in a rapid and transient induction of Egr-1 expression peaking at 2 h.

5.3.8 Effect of low pH combined with *H. pylori* on Egr-1 expression

To further investigate the combined effect of low pH and *H. pylori* on the expression of Egr-1, AGS cells were incubated with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) in media of different pH values ranging from pH 7.4 to 6.4 for 2 h. Incubation of AGS cells at low pH in the presence of *H. pylori* resulted in stronger induction of Egr-1

A



B

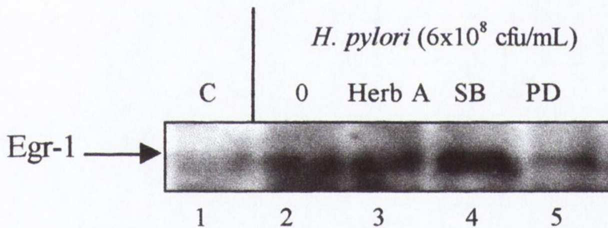
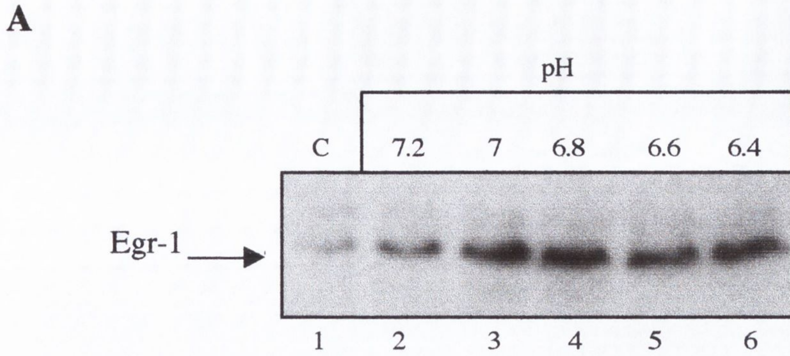
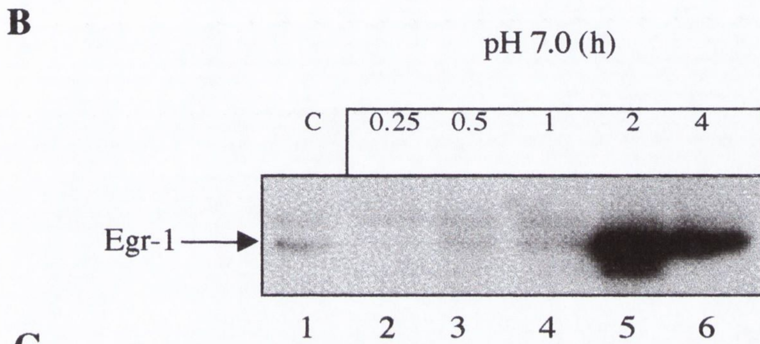


Fig. 5.5. Effect of different inhibitors on *H. pylori*-induced Egr-1 expression in AGS cells. AGS cells were preincubated for 1 h with the following inhibitors: PKC inhibitors rottlerin (10 μM), Gö6976 (5 μM) and calphostein C (100 nM); the PI3-kinase inhibitor worthmannin (100 nM); the tyrosine kinase inhibitor herbimycin A (10 μM) and the MAP kinase inhibitor SB203580 (10 μM) and PD98059 (20 μM). AGS cells were then treated with *H. pylori* strain 11638 (6x10^8 cfu/ml) for 2 h **(A and B)**. Total cellular extracts were prepared and analyzed by Western blotting using anti-Egr-1 antibody. Results are representative of three independent experiments.





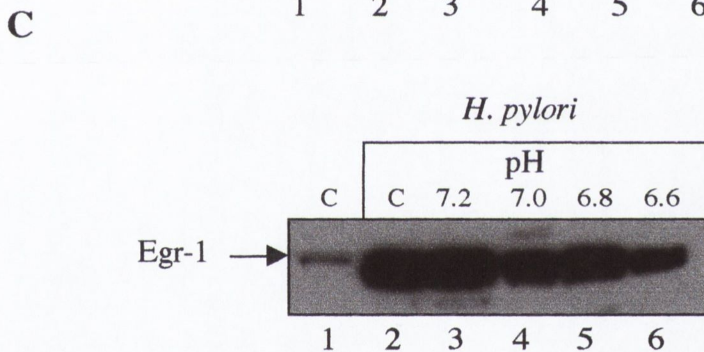


Fig. 5.6. Effect of low pH on Egr-1 expression in AGS cells. (A) Effect of media of different pH values on Egr-1 expression. AGS cells were incubated for 2 h in RPMI 1640 medium titrated to different values of pH. **(B)** Time course of Egr-1 expression in AGS cells incubated at pH 7.0. The cells were incubated at pH 7.0 for various periods of time, as indicated above each lane. The induction of Egr-1 protein expression was analyzed by Western blotting on total cellular extracts using anti-Egr-1 antiserum. **(C)** Effects of the combined treatment of low pH and *H. pylori* or low pH alone on the expression of Egr-1 in AGS cells. AGS cells were incubated in media of various pH values in the absence or presence of *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for 2 h and total cellular extracts were prepared and analyzed for Egr-1 expression by Western blot analysis. Experiments were performed three times with similar results and a representative experiment is shown.

expression than that seen upon incubation of the cells at low pH alone (Fig. 5.6C). However, this is likely to be due to *H. pylori* alone as the combined effect of low pH and *H. pylori* did not increase the induction of Egr-1 expression above the levels seen in the control cells cocultured with *H. pylori* at pH 7.4, rather than resulted in a decrease in Egr-1 levels.

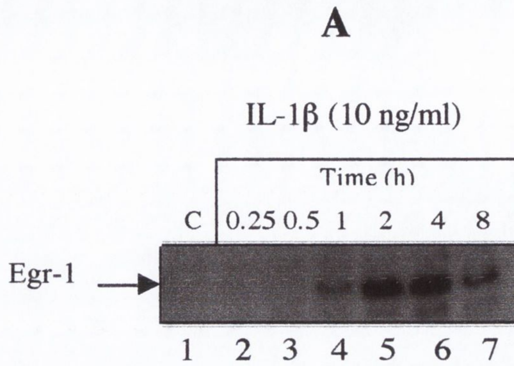
5.3.9 Effect of the cytokines IL-1β and TNF-α on Egr-1 expression

To further investigate whether Egr-1 expression was also induced by cytokine treatment, the gastric epithelial cells were treated with IL-1β (10 ng/ml) or TNF-α (20 ng/ml) for various periods of time and total cell extracts were prepared and subjected to analysis by Western blotting. The time course of Egr-1 expression in AGS cells after treatment with IL-1β or TNF-α showed a transient induction of Egr-1. As shown in Fig. 5.7A and B, a significant induction of Egr-1 protein was observed at 2 h and remained above the basal level after treatment of the cells up to 8 h.

The induction of Egr-1 expression by IL-1β or TNF-α occurred in a dose-dependent manner (Fig. 5.8A and B). The minimum amount of IL-1β and TNF-α required to induce Egr-1 expression was 100 pg/ml and 500 pg/ml, respectively. Maximum induction was observed using 10 ng/ml of both IL-1β and TNF-α after 2 h stimulation. No further increase in Egr-1 expression was observed with higher amounts of IL-1β and TNF-α.

5.3.10 Effect of the phorbol ester PMA on Egr-1 expression

The effect of PMA on Egr-1 expression in AGS cells was examined and preliminary experiments showed that PMA induced Egr-1 in a time- (Fig. 5.9A) and dose-dependent (Fig. 5.9B) manner. Maximal induction was observed with PMA (10 ng/ml) after 2 h incubation with no further increase in Egr-1 induction apparent when higher amounts of PMA were used.



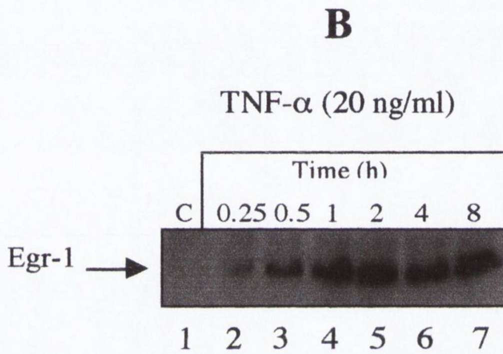
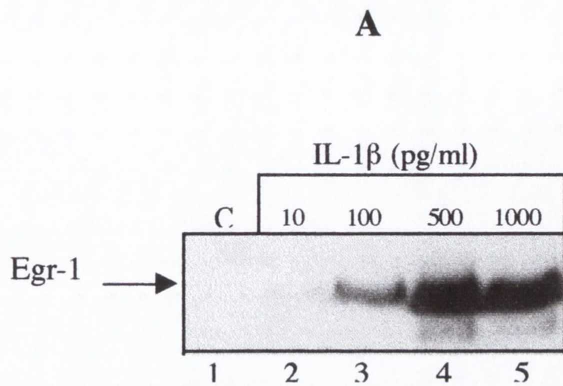


Fig. 5.7. Effects of cytokines IL-1β and TNF-α on Egr-1 expression. Time-course of Egr-1 expression induced by IL-1β **(A)** and TNF-α **(B)** in AGS cells. Total cellular extracts from AGS cells stimulated with either IL-1β (10 ng/ml) or TNF-α (20 ng/ml) for the indicated times were prepared and subjected to analysis by Western blotting for Egr-1 protein expression. Results represent three different experiments.

A



B

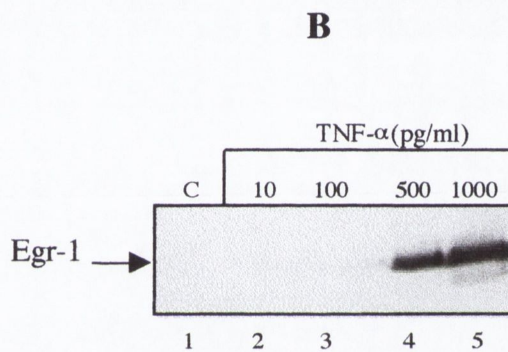
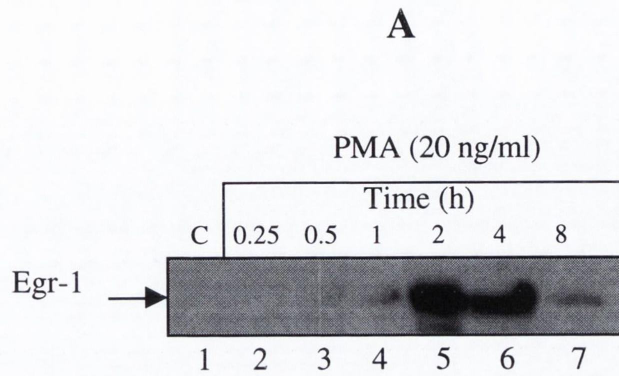


Fig. 5.8. Dose response of cytokines IL-1β- and TNF-α-induced Egr-1 expression in AGS cells. Total cellular extracts were prepared from AGS cells treated with various amounts of either IL-1β **(A)** or TNF-α **(B)** as indicated above each lane for 2 h and analysed for Egr-1 protein expression by Western blot analysis. Results are representative of three independent experiments.



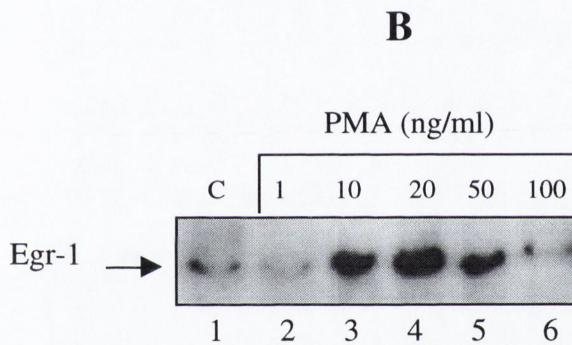


Fig. 5.9. (A) Time-course of Egr-1 expression in PMA-treated AGS cells. Egr-1 expression in AGS cells treated with PMA (20 ng/ml) for the indicated times. **(B)** Effect of PMA doses on Egr-1 induction in AGS cells exposed to PMA for 2 h. Egr-1 expression was determined by Western blotting on the total cellular extracts. Each experiment was repeated three times and a representative blot is shown.

5.3.11 Signalling pathways involved in cytokine-induced Egr-1 expression

Next, the effect of inhibitors on Egr-1 induction by the cytokines IL-1 β and TNF- α was examined. Confluent AGS cells were preincubated for 1 h with the following inhibitors: the PKC inhibitors Gö6976 (5 μM), rottlerin (10 μM) and calphostein C (100 nM); the PI3-kinase inhibitor worthmannin (100 nM); the tyrosine kinase inhibitor herbimycin A (10 μM); the p38 MAP kinase inhibitor SB203580 (10 μM) and the MEK MAP kinase inhibitor, PD98059 (20 μM), followed by treatment with IL-1β (10 ng/ml) and TNF-α (20 ng/ml) for2 h. Treatment of AGS cells with any of these inhibitors or vehicle alone had no effect on Egr-1 expression. The PKC inhibitor Gö6976 (5 μM) partially blocked IL-1β- and TNF-α-induced Egr-1 activation, but rottlerin (10 μM) and calphostein C (100 nM) had no effect on this induction (Fig. 5.10A and B), respectively. The PI3-kinase inhibitor worthmannin (100 nM) partially blocked Egr-1 activation by these cytokines. The tyrosine kinase inhibitor herbimycin A (10 μM) had no effect on this induction. On the other hand, the p38 MAP kinase inhibitor SB203580 (10 μM) had no effect on cytokine-induced Egr-1 expression, while the MEK MAP kinase inhibitor, PD98059 (20 μM), completely inhibited Egr-1 induction. These observations suggest that different signalling pathways are involved in Egr-1 induction as PKC, PI3-kinase and MAP kinase kinase (MEK) pathways were responsible for cytokine-induced Egr-1 expression.

5.3.12 Signalling pathways involved in PMA- and pH-induced Egr-1 activation

Western blot analyses of Egr-1 expression also showed that the PKC inhibitor Gö6976 (5 μM) inhibited PMA- and pH-induced Egr-1 activation, but rottlerin (10 μM) and calphostein C (100 nM) were ineffective (Fig. 5.10C and D). The tyrosine kinase inhibitor herbimycin A (10 μM) inhibited pH-induced Egr-1 activation, but it did not affect PMA-induced Egr-1 expression. Moreover, pretreatment of AGS cells with the MAP kinase inhibitor SB203580 (10 μM) or PD98059 (20 μM) followed by exposure of AGS cells to PMA or acidic conditions demonstrated that SB203580 had no effect on Egr-1 activation, while PD98059 partially blocked this induction (Fig. 5.10C and D).

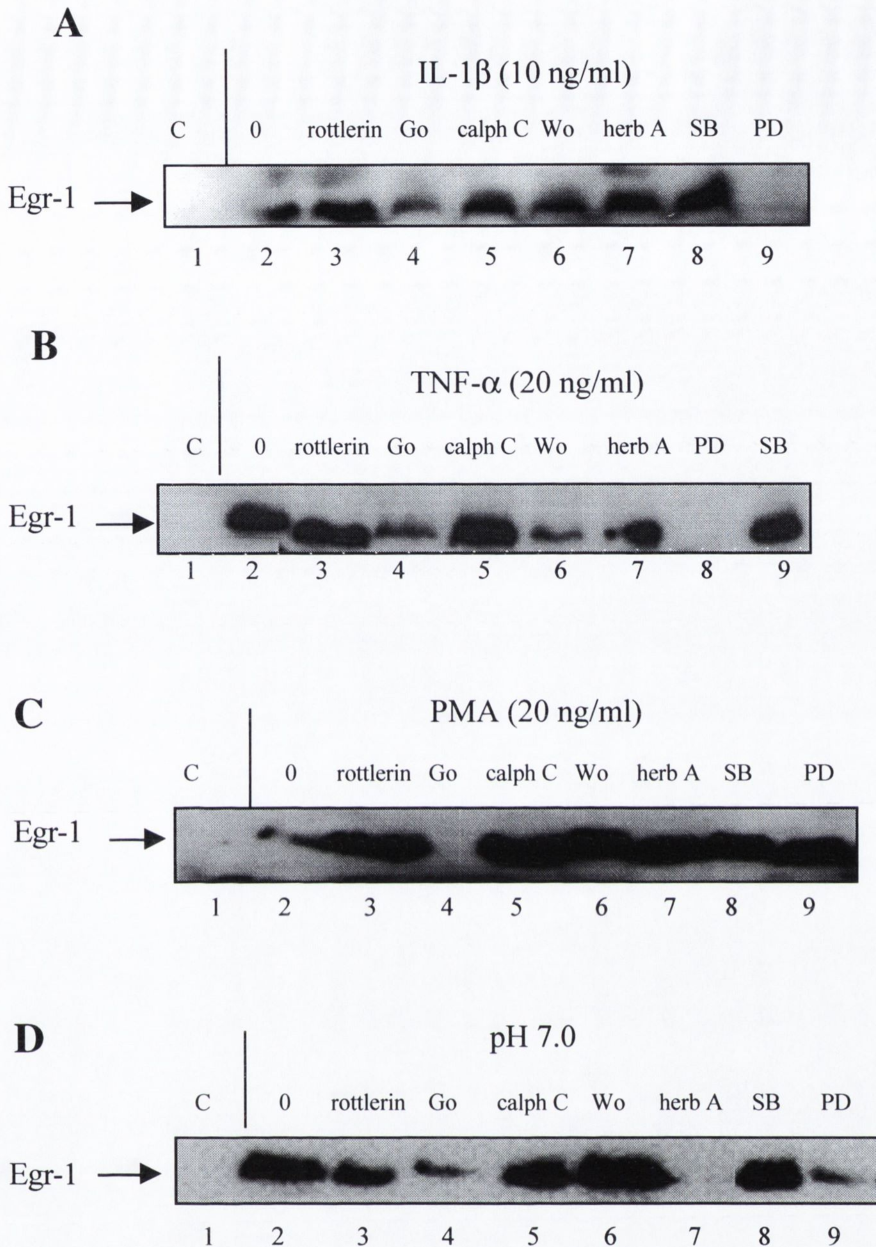


Fig. 5.10. Effect of different inhibitors on induction of Egr-1 expression in AGS cells by various stimuli. AGS cells were preincubated for 1 h with the following inhibitors: PKC inhibitors rottlerin (10 μM), Gö6976 (5 μM) and calphostein C (100 nM); the PI3-kinase inhibitor worthmannin (100 nM); the tyrosine kinase inhibitor herbimycin A (10 μM) and the MAP kinase inhibitor SB203580 (10 μM) and PD98059 (20 μM). AGS cells were then treated with the cytokines **(A)** IL-1β (10 ng/ml) and **(B)** TNF-α (20 ng/ml), **(C)** PMA (20 ng/ml) or **(D)** low pH (pH 7.0) 2 h. Total cellular extracts were prepared and analyzed by Western blotting using anti-Egr-1 antibody. Results are representative of three independent experiments.

5.3.13 Effect of oxidants and antioxidants on Egr-1 expression

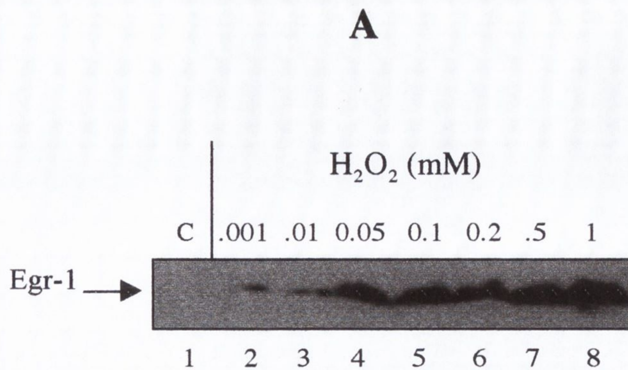
As redox activity has been shown to regulate the expression of Egr-1 (Ohba *et al.*, 1994; Nose *et al.*, 1996), the effect of H_2O_2 on Egr-1 activation in AGS cells was examined. Exposure of AGS cells to H_2O_2 induced Egr-1 expression in a dose dependent-manner. Low concentrations of H_2O_2 (10 μM-1 mM) caused increased Egr-1 levels in AGS cells (Fig. 5.11A). Concentrations of H_2O_2 greater than 1mM had no effect on Egr-1 expression and these doses were toxic to the cells. The induction of Egr-1 by 200 μM H_2O_2 was inhibited by pretreatment of AGS cells with either ascorbic acid (30 mM) or NAC (20 mM) for 1 h prior to H_2O_2 treatment (Fig. 5.11B). Treatment of AGS cells with ascorbic acid or NAC had no effect on expression of Egr-1 at the doses tested.

To further test the effect of antioxidants on *H. pylori*-induced Egr-1 expression. AGS cells were preincubated with ascorbic acid (30 mM) or NAC (20 mM) for 1 h, the cell culture medium was refreshed, and then cells were cocultured with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) or exposed to other agents fro 2 h. Preincubation of AGS cells with ascrobic acid or NAC inhibited the activation of Egr-1 in AGS cells by *H. pylori* strain 11638 ($6x10^8$ cfu/ml), pH 7.0, IL-1β (10 ng/ml), TNF-α (20 ng/ml) and PMA (20 ng/ml) (Fig. 5.12A and B). This suggests that an oxidizing environment is involved in the activation of Egr-1 by these stimuli. Taken together, it appears that reactive oxygen species play an important role in the signalling pathways involved in Egr-1 expression.

5.3.14 Expression of Egr-1 in patients infected with *H. pylori*

To assess whether Egr-1 is expressed in the gastric tissue of *H. pylori*-infected patients, Egr-1 expression in gastric biopsies from normal, chronic gastritis (*H. pylori*-negative) and *H. pylori*-positive patients was studied by immunohistochemistry. Egr-1 expression was significantly increased in all biopsies obtained from *H. pylori*-infected patients compared to non-infected patients with chronic gastritis and normal gastric mucosa. Egr-1 expression was clearly evident in *H. pylori*-positive patients (Fig. 5.13A and B). Specimens from *H. pylori*-negative patients with chronic gastritis

A



B

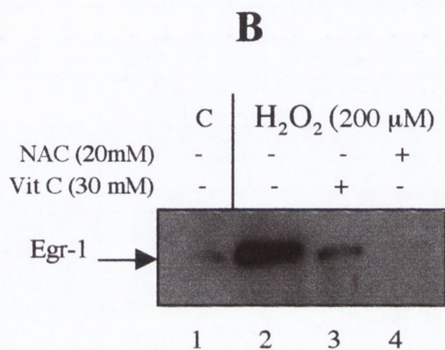
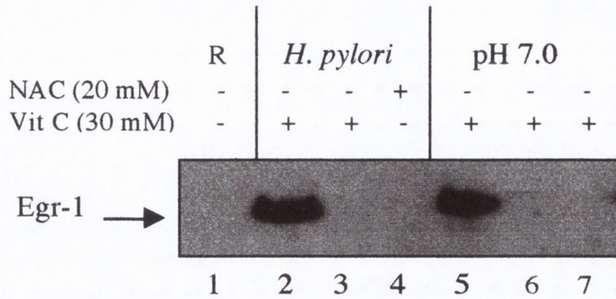


Fig. 5.11. Effects of H_2O_2 on Egr-1 expression in AGS cells. **(A)** AGS cells were incubated with different doses of H_2O_2 for 2 h and the induction of Egr-1 protein expression was determined by Western blot analysis on total cellular extracts. In some experiments, **(B)** AGS cells were preincubated with either ascorbic acid (30 mM) or NAC (20 mM) for 1 h and the cells were then stimulated with H_2O_2 (200 μM) for 2 h and total cellular extracts were prepared and analyzed for Egr-1 expression. Each experiment is repeated three times and a representative gel is shown.

A



B

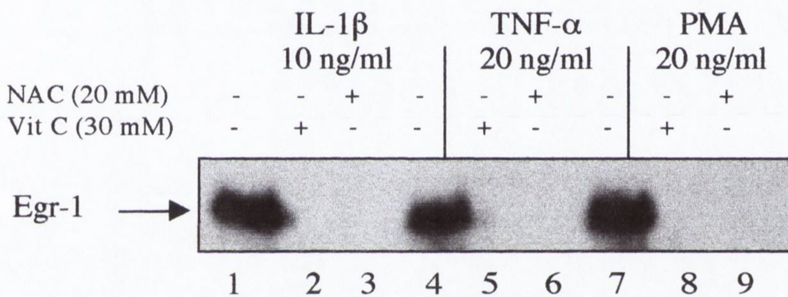
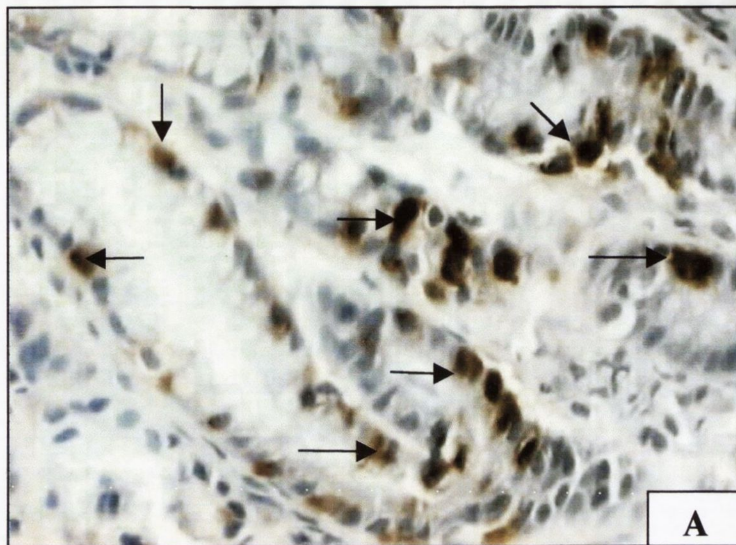
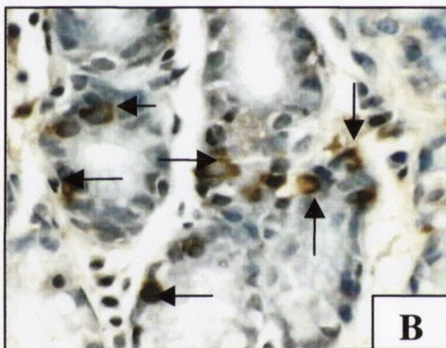


Fig. 5.12. Effects of antioxidants on Egr-1 induction in AGS cells in response to various stimuli. AGS cells were preincubated with either ascorbic acid (30 mM) or N-acetyl cysteine (20 mM) for 1 h and then the cells were stimulated with either *H. pylori* strain 11638 ($6x10^8$ cfu/ml) or low pH for 2 h as shown in **(A)**. AGS Cells were treated with IL-1β (10 ng/ml), TNF-α (20 ng/ml) or PMA (20 ng/ml) for 2 h **(B)**. Total cellular extracts were prepared and analyzed for Egr-1 protein expression by Western blotting. Results are representative of three independent experiments.





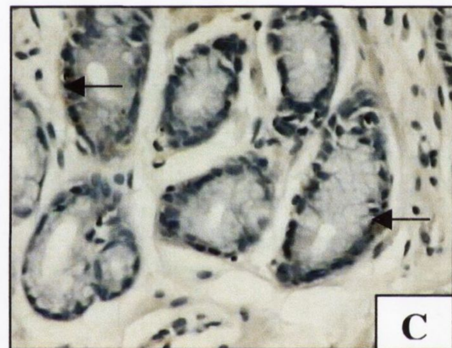


Fig. 5.13. Immunohistochemical staining of Egr-1 expression in antral gastric biopsies of *H. pylori* infected patients. Immunohistochemical staining was performed using Egr-1 polyclonal antibody (1:50). **(A)** *H. pylori*-positive antral gastric biopsy showing increased Egr-1 expression, as indicated by arrows (brown staining). **(B)** Egr-1 expression is present in both the nucleus and the cytoplasm of epithelial gastric cells of patients infected with *H. pylori*. **(C)** Antral gastric samples from patients with chronic gastritis show a slight immunostaining for Egr-1 expression in un-infected patients. (Magnifications, A and B; X 600 and C; X400).

showed slight Egr-1 expression (Fig. 5.13C). In contrast, no or only slight expression of Egr-1 was observed in antral samples of normal biopsies (Fig. 5.14A and B). In control experiments, antral samples obtained from *H. pylori*-positive patients were stained with appropriate controls such as the omission of the primary antibody, (Fig. 5.14D, or using a rabbit polyclonal Ig antibody (10 μg/ml, Z 147, DAKO) (Fig. 5.14E).

5.4 DISCUSSION

The early growth response-1 (Egr-1) is inducibly expressed in a variety of cell types by diverse stimuli and is functionally implicated in the regulation of a wide range of cellular effects including the inflammatory and immune responses (Gashler and Sukhatme, 1995; Sukhatme, 1990; McMahon and Monroe, 1996). The results presented here demonstrate that Egr-1 is induced in a dose- and time-dependent manner in gastric epithelial cells when the cells are exposed to the gastric pathogen *H. pylori*. Importantly, Egr-1 expression is elevated in gastric biopsies obtained from *H. pylori*-positive individuals. Coculture of gastric epithelial cells with $CagA^+$ *H. pylori* strains resulted in greater activation of Egr-1 expression compared with $CagA^-$ isolates. These differences among *H. pylori* strains in their ability to induce Egr-1 and other proteins may play a critical role in the outcome of *H. pylori* infection.

Agents known to be capable of inducing Egr-1 expression include viruses (Fu *et al.*, 1993; Hallahan *et al.*, 1991), bacterial LPS (Coleman *et al.*, 1992), peptidoglycan (Xu *et al.*, 2001), and enteropathogenic *E. coli* (de Grado *et al.*, 2001). However, purified LPS (15 μg/ml) of *H. pylori* had no effect on Egr-1 expression in gastric epithelial cells. Others have demonstrated too that intestinal epithelial cells are unresponsive to LPS exposure (Jung *et al.*, 1995; de Grado *et al.*, 2001), presumably to prevent intestinal inflammation occurring in the host in response to non-pathogenic commensal gut flora. Although the mechanism whereby *H. pylori* induces Egr-1 expression is not clear at present preliminary data indicate the potential involvement of CagA or other gene products of the *cag* PAI in this process. However, these data must be interpreted with caution as the CagA deficient clinical isolates (by PCR and

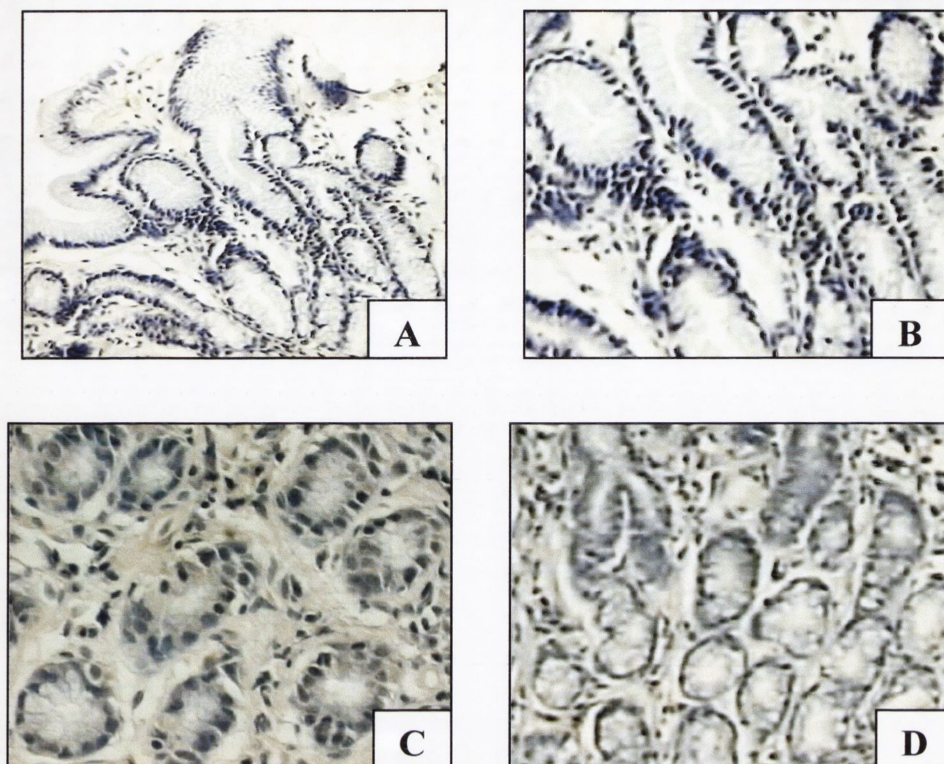


Fig. 5.14. Immunohistochemical staining of Egr-1 expression in antral gastric biopsies of normal individuals and *H. pylori*-positive patients. Normal antral gastric biopsy showed little or no immunostaining for Egr-1 expression **(A and B)**. Antral gastric biopsy from the same *H. pylori* positive Egr-1 stained subjects (as in Fig. 5.13A) stained without adding the polyclonal Egr-1 antibody, showing no Egr-1 staining of cells **(C)** or use of an appropriate Ig control, the rabbit polyclonal antibody **(D)** were used as controls. (Magnifications A, X 200; B, C and D, X 400).

immunoblotting) used are not isogenic mutants. The appropriate isogenic mutants were not available during this study. These observations indicate an association between Egr-1 expression and *H. pylori*-induced gastric pathology. It is not known however whether Egr-1 expression levels are sustained throughout the course of chronic *H. pylori* infection. Prolonged exposure of gastric tissue to *H. pylori* provokes a host inflammatory response and consequent gastritis. These findings may have particular mechanistic importance with respect to the regulation of the inflammatory and immune responses to this organism.

Exposure of AGS cells to low pH also resulted in a significant increase in the level of Egr-1 protein in a time-dependent fashion. The data indicate that a small change in extracellular pH effects the expression of Egr-1 protein. Moreover, the combined treatment of low pH and *H. pylori* did not augment the induction of Egr-1 over that observed with *H. pylori* alone, suggesting that this effect is not additive. Furthermore, treatment of gastric cells with IL-1β, TNF-α or PMA resulted in a marked but transient increase in the level of Egr-1 protein. These results are consistent with a number of reports demonstrating that Egr-1 is induced during cytokine and mitogenic stimulation of different cell types (Fitzgerald and O'Neill, 1999; Gashler and Sukhatme, 1995; Chaudhary *et al.*, 1996). It has been demonstrated that both IL-1β and TNF-α are present in increased amounts in gastric biopsies of patients with *H. pylori* infection (Fan *et al.*, 1993; Noach *et al.*, 1994). These findings suggest that Egr-1 may be induced by *H. pylori* directly to a level comparable to that seen with proinflammatory cytokines. Moreover, it is likely that a combination of factors could contribute to the observed Egr-1 expression in *H. pylori* infection.

It is clear that the activation of Egr-1 by various unrelated stimuli arises due to activation of different signalling pathways. PKC, tyrosine kinase and phosphatidylinositol 3-kinase inhibitors had no effect on the induction of Egr-1 by *H. pylori.* According to these data, it appears that PKC and PI3-kinase pathways play a role in regulating Egr-1 induction by the cytokines IL-1 β and TNF-α, PMA and low pH but not by *H. pylori*. Earlier studies demonstrated that inhibition of protein kinase

pathways in various cell lines prevents the expression of Egr-1 (Chaudhary *et al.*, 1996; Huang and Adamson, 1995). The MAP kinase kinase (MEK) PD98059 inhibitor partially inhibited the induction of Egr-1 in response to *H. pylori*. PD98059 is a specific inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK1/2), which binds inactive MEK and prevents phosphorylation by Raf without affecting other known serine/threonine or tyrosine kinases. Guha *et al.* (2001) have demonstrated that the MEK-ERK1/2 inhibitor PD980059 reduced LPS induction of tissue factor (TF) and TNF-α by inhibiting Egr-1 expression. In addition, mutation of the Egr-1 sites in the TF and TNF-α promoters reduced expression of these proinflammatory genes. However, the MAP kinase p38 inhibitor, SB350580 is not involved in Egr-1 induction by *H. pylori* or the cytokines IL-1 β and TNF-α. The tyrosine kinase inhibitor herbimycin A had no effect on *H. pylori*- and cytokine-induced Egr-1 acivation but caused inhibition of Egr-1 induction by low pH.

The documented ability of *H. pylori* (Wessler *et al.*, 2000; Meyer-ter-Vehn *et al.*, 2000) and other bacteria (Hobbie *et al.*, 1997; Tang *et al.*, 1998; Warny *et al.*, 2000) to activate MAP kinase cascades in host cells provides a potential mechanism whereby Egr-1 induction may be facilitated. Consistent with this, there is a requirement for the MAP kinase kinase (MEK) pathway in *H. pylori*-induced Egr-1 activation whereas MEK, PKC and PI3-kinase pathways appear to be involved in regulating cytokine-induced Egr-1 expression. Inhibition of some participants in these signalling cascades resulted in partial or complete abrogation of bacterial- and/or cytokine-induced Egr-1 activation. A requirement for protein kinase pathways in Egr-1 activation has been documented in various cell lines in response to a variety of stimuli (Huang and Adamson, 1995; Chaudhary *et al.*, 1996; Cantley *et al.*, 1991). The activation of Egr-1 by acid and growth factors has been reported to be mediated via tyrosine kinase pathways (Yamaji *et al.*, 1994).

Activation of Egr-1 was sensitive to the cellular redox environment. The results showed that incubation of gastric epithelial cells with hydrogen peroxide at low concentrations resulted in the activation of Egr-1, and that this induction was blocked

by pre-treatment with ascorbic acid and NAC. It has been shown that mild oxidative stress induces Egr-1 gene transcription in mouse osteoblastic cells (Nose and Ohba, 1996; Nose *et al.*, 1991). However, it appears that this may be cell type dependent as several studies have reported that the activity of Egr-1 protein was impaired by treatment of the cells with oxidants (Huang *et al.*, 1999; Esposito *et al.*, 1994). Moreover, the induction of Egr-1 by *H. pylori*, IL-1β, TNF-α, PMA or low pH was inhibited by the treatment with antioxidants. Taken together, these findings indicate that redox reactions are involved in regulating Egr-1 expression in gastric epithelial cells in response to various agents. This may be of particular note as reduced vitamin C levels have been demonstrated in gastric juices of patients infected with *H. pylori* (O'Connor *et al.*, 1989; Sobala *et al.*, 1991).

In conclusion, the induction of Egr-1 by *H. pylori* may play a critical role in regulating the expression of several genes during the inflammatory and immune responses. The results might provide a clue to understanding the mechanism by which *H. pylori* causes chronic inflammation and damage to gastric epithelial cells.

CHAPTER VI

FUNCTIONAL ROLE OF Egr-1 EXPRESSION IN THE REGULATION OF GENES INVOLVED IN *H. PYLORI*-INDUCED ADHESION MOLECULE EXPRESSION AND APOPTOSIS

CHAPTER VI

6.1 INTRODUCTION

Evidence is accumulating which suggests that Egr-1 is involved in the regulation of multiple genes involved in cell adhesion and apoptosis in various cell types. Egr-1 and its role in transcription regulation may have potential relevance to the pathogenesis of a variety of inflammatory diseases. Khachigian *et al.* (1996) demonstrated that elevated Egr-1 levels can be found at sites of inflammation and it was suggested that inducible Egr-1 expression may contribute to the expression of many genes during vascular injury.

Infection with *H. pylori* leads to gastritis and damage of gastric epithelium. The gastric epithelium appears to play an active role in mucosal inflammation as part of the host response to *H. pylori* infection. *H. pylori* elaborates several substances that can attract and activate neutrophils and other inflammatory cells causing tissue injury (Wallace, 1991; Blaser, 1992). Neutrophil adhesion to endothelial cells is enhanced by various adhesion complexes that initiate ligand-receptor interactions in response to inflammatory stimuli (Harlan and Liu, 1992; Carlos and Harlan, 1990). The CD44 and ICAM-1 adhesion molecules regulate lymphocyte function by controlling migration patterns and intracellular contacts. Fan *et al.* (1996b) demonstrated that *H. pylori* infection either directly or indirectly through a local inflammatory response up-regulates the expression of CD44 and its variant CD44v9 on gastric epithelial cells. *H. pylori* also up-regulates ICAM-1 expression on gastric epithelial cells (Fan *et al.*, 1995a). The expression of ICAM-1 and CD44 by *H. pylori* on gastric epithelial cells is not fully understood.

Several studies have shown that the *H. pylori* colonized stomach contains more apoptotic cells than normal stomach (Moss *et al.*, 1996; Jones *et al.*, 1997). Moss et al. (1996) indicated that the increased numbers of apoptotic epithelial cells decrease to normal following eradication of *H. pylori*. Consistent with this, *in vitro* co-culture of gastric cancer cell lines with *H. pylori* can induce apoptosis in these cells (Wagner *et*

al., 1997; Rudi *et al.*, 1998). It has been postulated that *H. pylori*-induced apoptosis occurs through the up-regulation of the Fas (APO-1/CD95) ligand receptor on gastric epithelial cells (Rudi *et al.*, 1998; Koyma *et al.*, 2000). In addition to CD95/Fas ligand expression, *H. pylori* can also induce up-regulation of CD95 expression (von Herbay and Rudi, 2000).

The induction of Egr-1 is critical for up-regulation of CD44 (Maltzman *et al.*, 1996a) and ICAM-1 (Maltzman *et al.*, 1996b) adhesion molecules. Following stimulation, Egr-1 binding sites in the regulatory regions of both the ICAM-1 and CD44 genes were found to be occupied by Egr-1. Furthermore, Li-Weber *et al.* (1999) have shown that the three Egr-1 binding sites (-120, -180 and -680) are required for the full function of the CD95L promoter. These findings indicate that Egr-1-mediated gene transcription plays a key role in controlling the inflammatory response. This chapter investigates the role of Egr-1 on the expression of CD44, ICAM-1 and CD95L in response to *H. pylori*.

6.2 OBJECTIVES

The aims of this chapter were to examine:

1 The regulation of expression of the cell adhesion molecules CD44 and ICAM-1 in gastric epithelial cells in response to *H. pylori* infection using transient transfection with CAT-reporter constructs for CD44 and ICAM-1.

2 The functional role of Egr-1 in regulating the expression of the cell adhesion molecules CD44 and ICAM-1 in gastric epithelial cells in response to *H. pylori* infection using reporter genes for CD44 and ICAM-1 containing Egr-1 sites.

3. The effect of Egr-1 in regulating the expression of CD95L in gastric epithelial cells by *H. pylori* using wild-type and mutant CD95L luciferase constructs.

2. The effect of antisense Egr-1 oligonucleotide on *H. pylori*-induced Egr-1 and CD44 expression.

6.3 RESULTS

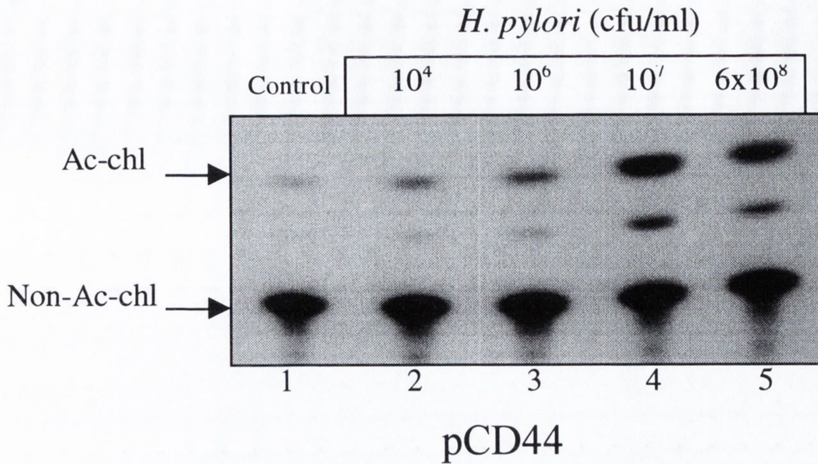
6.3.1 *H. pylori* up-regulates the CAT activity of the CD44 promoter construct

The effect of *H. pylori* on the induction of the CD44 expression in AGS cells transfected with a plasmid carrying a CAT-reporter gene under the control of 1.7 kb of the CD44 promoter was examined. Incubation of AGS cells with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) caused an elevation of the CAT activity driven by the CD44 promoter (Fig. 6.1A and B). *H. pylori* induced this effect in a dose-dependent manner and this response correlated with the dose-response shown for Egr-1 expression in AGS cells (Fig. 5.1C).

6.3.2 Involvement of Egr-1 in the regulation of *H. pylori*-induced CD44 expression

The transcriptional regulation of CD44 expression was investigated by studying the effect of *H. pylori* on a CAT-reporter gene under the control of the CD44 promoter containing an Egr-1 binding site. Several studies have shown that the induction of CD44 is dependent on the activation of the transcription factor Egr-1 (Matlzman *et al.*, 1996a; Fitzgerald and O'Neill, 1999). To test whether the transcriptional activity of the CD44 promoter is dependent on the Egr-1 binding site, CAT-reporter plasmids containing a 550 bp region of the CD44 promoter spanning -701 to -151 with (pBLCD44) or without (pBLmCD44) the Egr-1 binding site at position -301 were used (Fig. 6.2). The mutation within the pBLCD44 at -301-bp site consists of a 3-bp mutation in the -301-bp site that abolishes the ability of Egr-1 to bind to the -301-bp site but, importantly, retains Sp1-binding activity.

Following stimulation of the transfected cells with *H. pylori* strain 11638 ($6x10^8$ cfu/ml), the cell lysates were examined for CAT activity. As shown in Fig. 6.3A and B, coculture of AGS cells with *H. pylori* resulted in inducing of CD44 reporter gene transcription, and this effect was enhanced as the dose of *H. pylori* was increased. The presence of the Egr-1 binding site was important for CD44 promoter activity, as the absence of the -301 Egr-1 binding site significantly abrogated CD44 induction. This reduced effect suggests that binding to the Egr-1 site is essential for CD44 promoter activity.



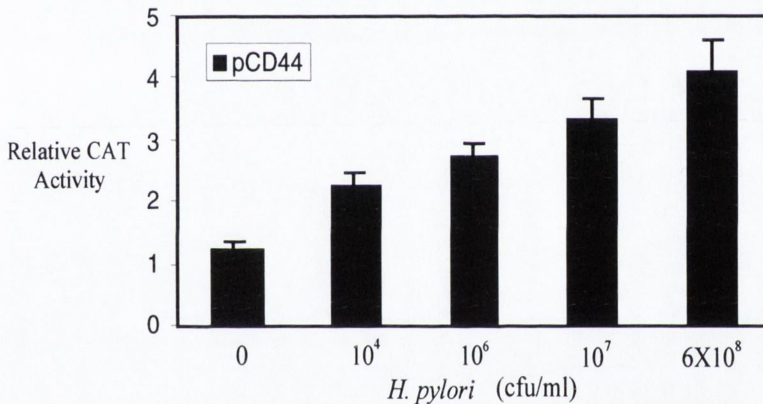


Fig. 6.1. *H. pylori* activates CD44 promoter activity in AGS cells. AGS cells were transfected with the reporter plasmid CD44-CAT. The transfected cells were allowed to recover overnight. Following stimulation with different amounts of *H. pylori* strain 11638 (10^4, 10^6, 10^7 and $6x10^8$ cfu/ ml) for 24 h, cells were harvested and analyzed for CAT activities as described in Methods and Materials. Acetylated (Ac-chl) and non-acetylated (non-Ac-chl) forms of chloramphenicol were separated by thin layer chromatography, followed by autoradiography **(A)** and quantitation using scintillation counting and the results were expressed as relative CAT activity **(B)**. A β-Galactosidase expression plasmid was transfected into the cells to monitor transfection efficiency. All transfection assays were performed in triplicate with two different plasmid preparations. A representative gel is shown and the results of relative CAT activity are shown as means ± SD.

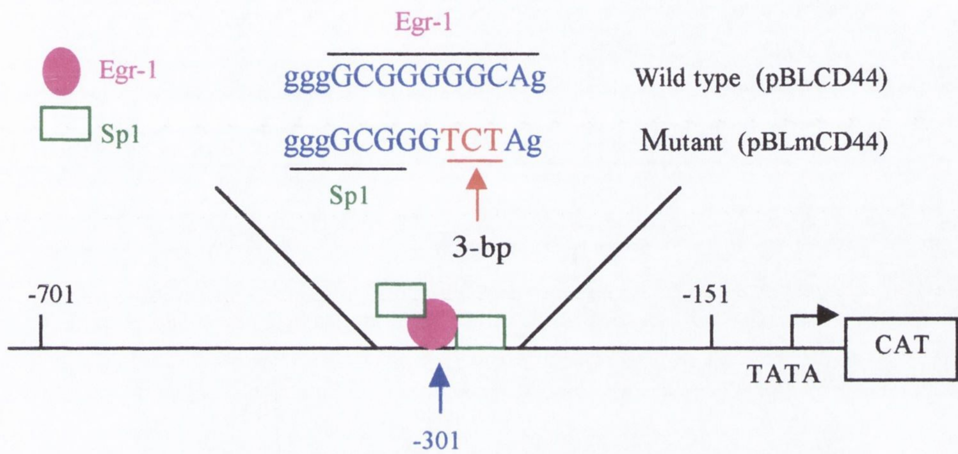
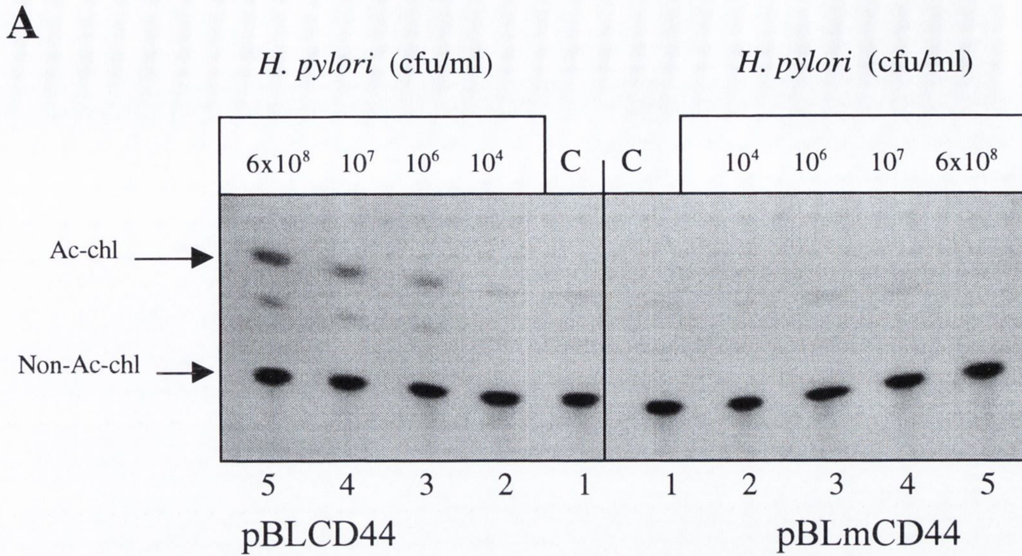


Fig. 6.2. Schematic representation of the CD44 promoter gene constructs. The wild-type CAT-reporter construct pBLCD44 and the mutant pBLmCD44 differ by the indicated 3-bp mutation (red colour) that abolishes Egr-1 binding at a position -301-bp of the CD44 promoter.

A



B

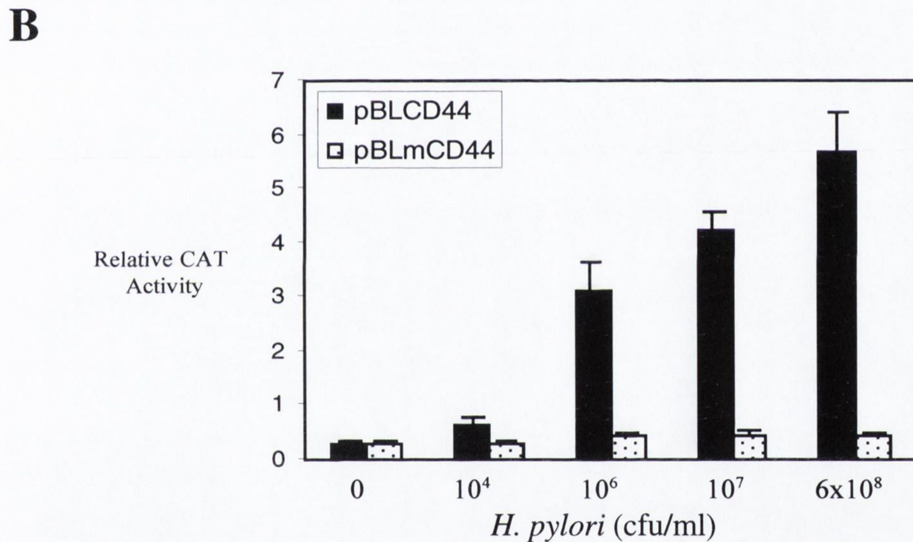


Fig. 6.3. Regulation of transcription of CD44 promoter activity by Egr-1 in *H. pylori*-stimulated AGS cells. AGS cells were transfected with pCAT-CD44 constructs, pBLCD44 and pBLmCD44 and the transfected cells were allowed to recover overnight. Following stimulation with different amounts of *H. pylori* strain 11638 (10^4, 10^6, 10^7 and $6\text{x}10^8$ cfu/ ml) for 24 h, cell lysates were prepared and analyzed for CAT activities as described under Methods and Materials. Acetylated (Ac-chl) and non-acetylated (non-Ac-chl) forms of chloramphenicol were separated by thin layer chromatography, followed by autoradiography **(A)** and quantitation using scintillation counting and the results are expressed as relative CAT activity **(B)**. A representative gel is shown (from three independent experiments) and the results of relative CAT activity are shown as means ± SD.

6.3.3 Effect of *H. pylori* strains on the CD44 promoter activity

Next, the effect of CagA$^+$ and CagA$^-$ *H. pylori* strains, 11638 and 92-701; respectively, at the same concentration (6x10^8 cfu/ml) on the CD44 promoter activity was examined by transfecting AGS cells with plasmids containing the CD44 upstream regulatory region linked to a reporter gene, CAT. The results showed that CagA$^+$ *H. pylori* strain 11638 enhances the CAT activity of the CD44 promoter compared to CagA$^-$ strain 92-701 (Fig. 6.4A and B). The presence of the Egr-1 binding site within the CD44 promoter is critical for the induction of CD44 transcriptional activity, as mutation of this Egr-1 site at position -301 in the CD44 promoter construct spanning -151 to -701 abrogated its activity in response to *H. pylori*. These results are in agreement with Western blotting data shown in chapter 5 which demonstrated that CagA$^+$ *H. pylori* strain strongly induces Egr-1 expression, while CagA$^-$ strain does not.

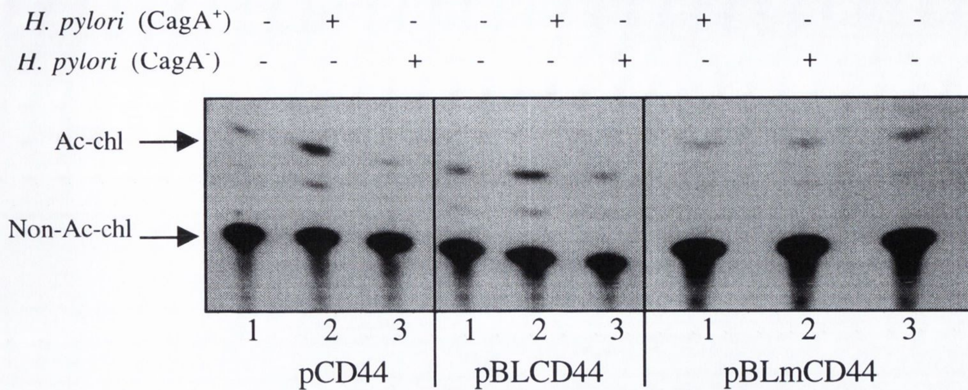
6.3.4 *H. pylori* up-regulates the CAT activity of the ICAM-1 promoter construct

When AGS cells were transfected with a plasmid carrying the CAT reporter gene under the control of the murine ICAM-1 promoter (1.1 kb), the CAT activity was enhanced in *H. pylori*-stimulated cells compared to unstimulated cells (Fig. 6.5 A and B).

6.3.5 Regulation of ICAM-1 activation by Egr-1

The relationship between Egr-1 and ICAM-1 induction was investigated using plasmids containing either 1.1 kb of the murine ICAM-1 promoter (-1091 – +34 bp) upstream of the CAT reporter gene (pBLICAM-1) or the promoter mutated at the -701 bp Egr-1 binding site (pBLmICAM-1) for transient transfection experiments. The mutation within the pBLmICAM1 construct consists of a 5-bp substitution that abolishes the ability of Egr-1 to bind to the -701-bp site but, importantly, retains Sp1-binding activity (Fig. 6.6). AGS cells were transfected with either pBLICAM-1 or pBLmICAM-1 constructs and then the transfected cells were stimulated for 24 h with *H. pylori* strain 11638 (6x10^8 cfu/ml) or left untreated. The CAT activity was enhanced in *H. pylori*-treated cells compared to unstimulated cells. A significant increase in CAT activity was observed in AGS cells transfected with the wild type

A



B

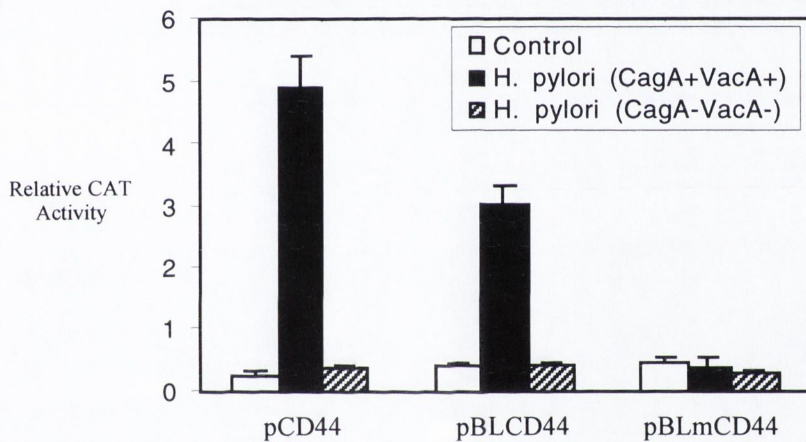
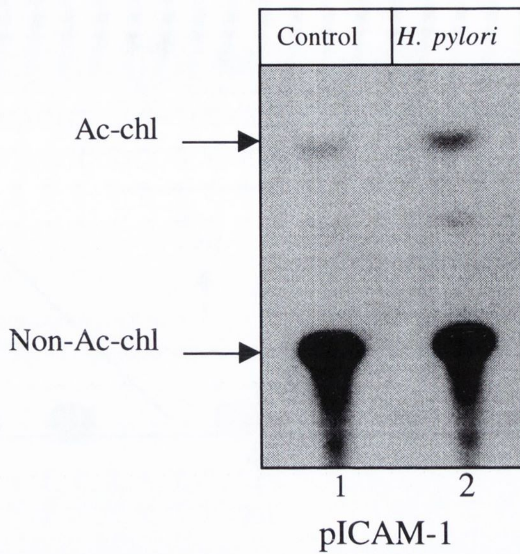


Fig. 6.4. Effect of *H. pylori* isolates on the CAT gene expression under the control of CD44 reporter gene. AGS cells were transfected with reporter plasmid CD44-CAT and CD44 constructs (pBLCD44 and pBLmCD44). The transfected cells were allowed to recover overnight and then cocultured with either *H. pylori* strain 11638 ($CagA^+$ and $VacA^+$) or *H. pylori* strain 92-701 ($CagA^-$ and $VacA^-$) at the same concentration of $6x10^8$ cfu/ ml for 24 h. Cell lysates were prepared and analyzed for CAT activities as described under Methods and Materials. Acetylated (Ac-chl) and non-acetylated (non-Ac-chl) forms of chloramphenicol were separated by thin layer chromatography, followed by autoradiography **(A)** and quantitation using scintillation counting and the results are expressed as relative CAT activity **(B)**. A representative gel is shown (from three independent experiments) and the results of relative CAT activity are shown as means ± SD.

A



B

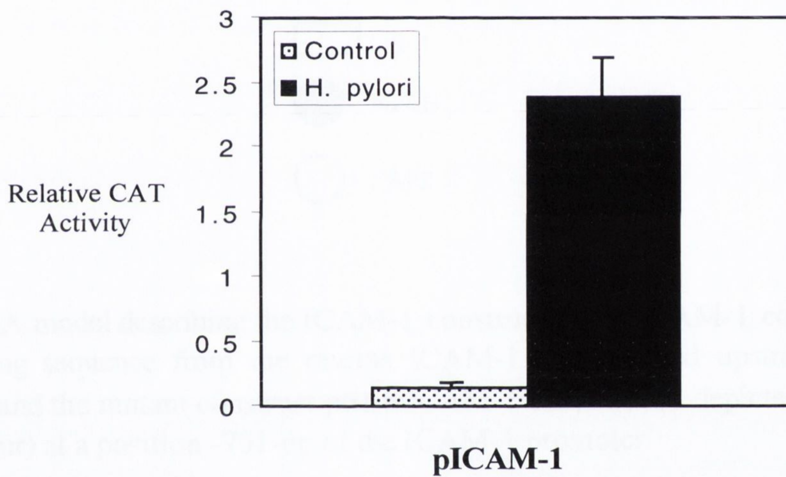
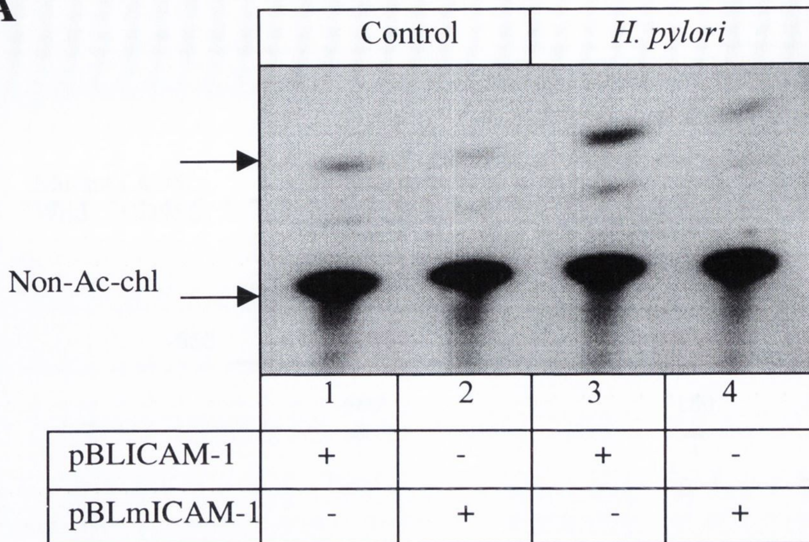


Fig. 6.5. Effect of *H. pylori* on CAT activity under the control of ICAM-1 reporter gene. AGS cells were transfected with reporter plasmid ICAM-1-CAT. The transfected cells were allowed to recover overnight and then cocultured with *H. pylori* strain 11638 ($6x10^8$ cfu/ ml) for 24 h. Cell lysates were prepared and analyzed for CAT activities as described under Methods and Materials. Acetylated (Ac-chl) and non-acetylated (non-Ac-chl) forms of chloramphenicol were separated by thin layer chromatography, followed by autoradiography **(A)** and quantitation using scintillation counting and the results are expressed as relative CAT activity **(B)**. A representative gel is shown (from three independent experiments) and the results of relative CAT activity are shown as means ± SD.



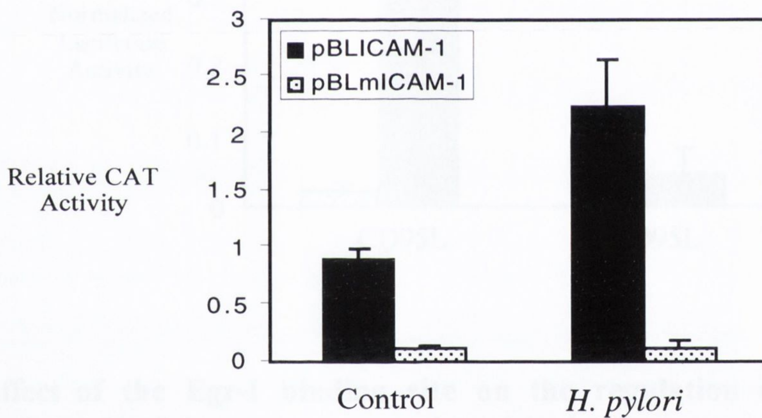


Fig. 6.7. Effect of Egr-1 expression on regulation of ICAM-1 promoter activity. AGS cells were transfected with pCAT-ICAM-1 constructs of pBLICAM-1 and pBLmICAM-1 and following stimulation with *H. pylori* strain 11638 ($6x10^8$ cfu/ ml) for 24 h, cell lysates were prepared and assayed for CAT activities. Acetylated (Ac-chl) and non-acetylated (non-Ac-chl) forms of chloramphenicol were separated by thin layer chromatography, followed by autoradiography **(A)** and quantitation using scintillation counting and the results are expressed as relative CAT activity **(B)**. The experiments were repeated three times with similar results and a representative result is shown.

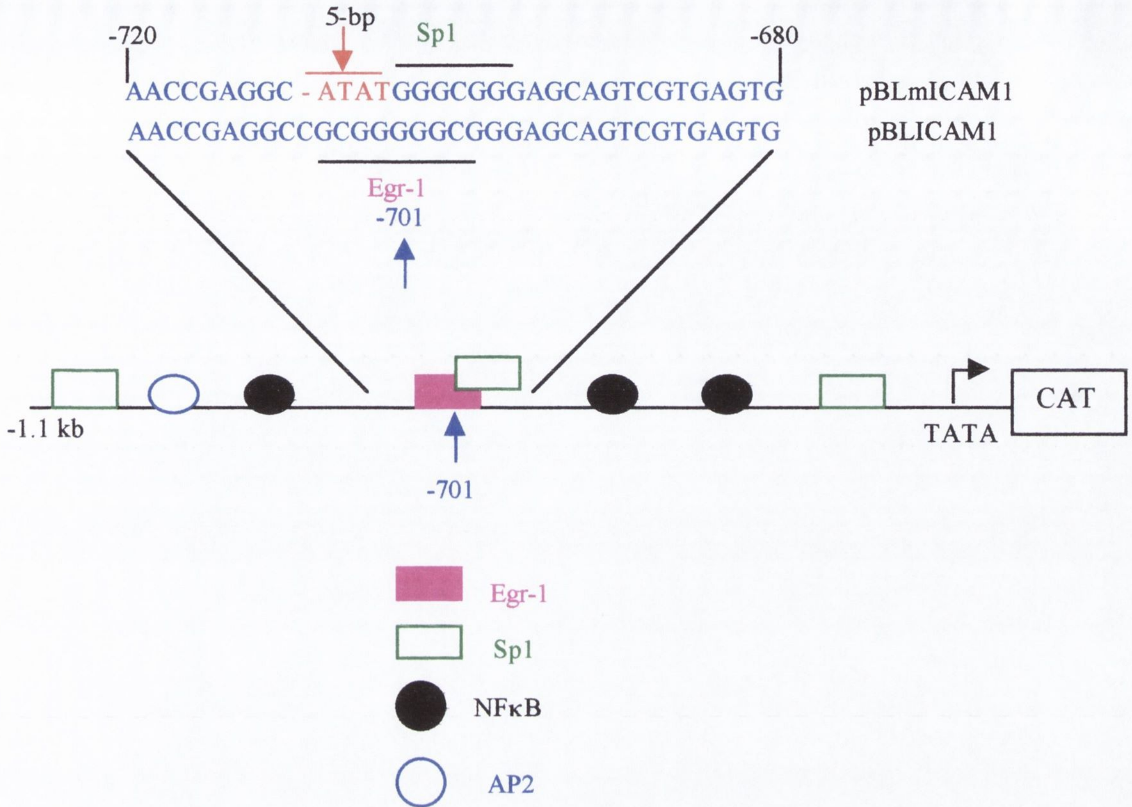
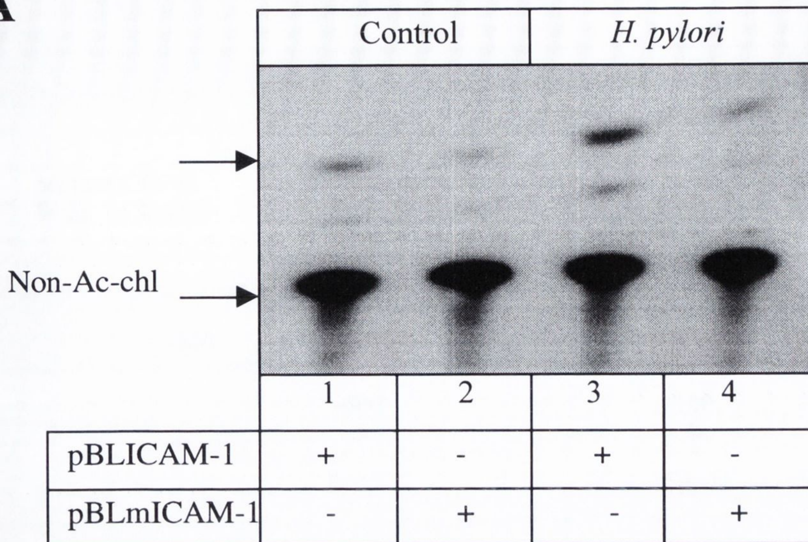


Fig. 6.6. A model describing the ICAM-1 constructs. pBLICAM-1 contains 1.1 kb of 5′ flanking sequence from the murine ICAM-1 gene cloned upstream of a CAT reporter and the mutant construct pBLmICAM-1 contains the depicted 5-bp mutation (red colour) at a position -701-bp of the ICAM-1 promoter.



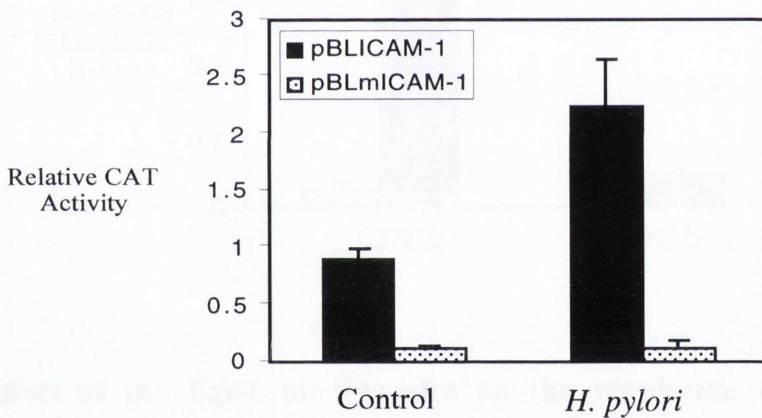


Fig. 6.7. Effect of Egr-1 expression on regulation of ICAM-1 promoter activity. AGS cells were transfected with pCAT-ICAM-1 constructs of pBLICAM-1 and pBLmICAM-1 and following stimulation with *H. pylori* strain 11638 ($6x10^8$ cfu/ ml) for 24 h, cell lysates were prepared and assayed for CAT activities. Acetylated (Ac-chl) and non-acetylated (non-Ac-chl) forms of chloramphenicol were separated by thin layer chromatography, followed by autoradiography **(A)** and quantitation using scintillation counting and the results are expressed as relative CAT activity **(B)**. The experiments were repeated three times with similar results and a representative result is shown.

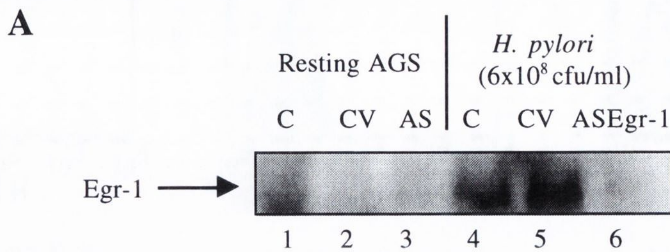
pBLICAM-1 construct over the mutated promoter after stimulation with *H. pylori* (Fig. 6.7A and B). Reduction of the transcriptional activity of the ICAM-1 promoter as a result of the mutation at the -701 Egr-1 binding site provides additional support for the involvement of the Egr-1 binding site in the activation of the ICAM-1 promoter.

6.3.6 Involvement of Egr-1 binding sites in the regulation of CD95L activation

The effect of *H. pylori* strain 11638 on AGS cells transfected with the luciferase reporter plasmid carrying the wild type CD95L promoter spanning -860 to +100 with or without mutations at the -680, -180 and -120 Egr-1 binding sites (Fig. 6.8A) was investigated. *H. pylori* strain 11638 ($6x10^8$ cfu/ml) caused an enhancement of the luciferase activity of the wild type CD95L promoter. This activation is attributed to the induction of Egr-1, as mutation of the consensus Egr-1 binding sites markedly abolished the CD95L promoter activity (Fig. 6.8B). Thus, Egr-1 expression in gastric epithelial cells may participate in the regulation of several cellular events including cell differentiation and apoptosis in response to *H. pylori* infection.

6.3.7 Effect of the antisense Egr-1 oligonucleotide on *H. pylori*-induced Egr-1 expression

To examine whether an antisense Egr-1 oligonucleotide directed against Egr-1 suppresses Egr-1 protein expression after stimulation with *H. pylori*, AGS cells were transfected with human Egr-1 antisense (Egr-1/PcDNA3.1+/Zeo) or the respective control empty vector (PcDNA3.1+/Zeo) at doses 1 μg or 5 μg. The antisense oligonucleotide was designed and prepared based on the DNA sequence (GenBank™ accession number M62829). The sequence of the antisense oligonucleotide was from 131 to 2131, as confirmed by sequencing the clone (Du *et al.*, 2000). For control experiments, the sense oligonucleotide with a similar sequence but in the opposite orientation relative to the promoter was used. Fourty-eight hours after transfection, the cells were incubated for 8 h with *H. pylori* strain 11638 ($6x10^8$ cfu/ml). Following treatment, total cell lysates were prepared and analyzed for Egr-1 expression by Western blotting. Antisense Egr-1 oligonucleotide blocked *H. pylori*-induced Egr-1 expression, whereas the respective control vector had no effect on Egr-1 protein levels



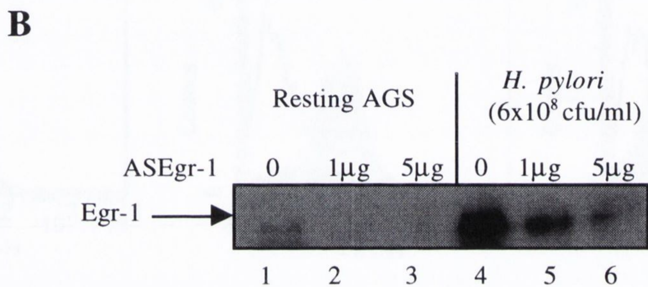


Fig. 6.9. Effect of antisene Egr-1 oligonucleotide on *H. pylori*-induced Egr-1 expression. (A) Antisense Egr-1 blocks *H. pylori*-induced Egr-1 expression. AGS cells were transfected with 5 µg antisense Egr-1 oligonucleotide (Egr-1/PcDNA3.1+/Zeo), the control vector (PcDNA3.1+/Zeo) or left alone for 48 h, as described under Material and Methods. Following incubation of AGS cells with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for 8 h, total cell lysates were prepared and analyzed for Egr-1 expression by Western blotting. Egr-1 protein expression was detected using chmeiluminescense. Lanes 1 and 4; untransfected AGS cells, Lanes 2 and 5; AGS cells transfected with control vector; Lanes 3 and 6 AGS cells transfected with antisense Egr-1 oligonucleotide. **(B)** Dose-dependent inhibition of Egr-1 protein expression by *H. pylori* in AGS cells by 1 µg and 5 µg of antisense Egr-1 oligonucleotide. One representative result is shown of three independent experiments with similar results.

(Fig. 6.9A). The inhibition of Egr-1 protein by antisense Egr-1 is dose-dependent (Fig. 6.9B).

6.3.8 Effect of the antisense Egr-1 on *H. pylori*-induced CD44 expression

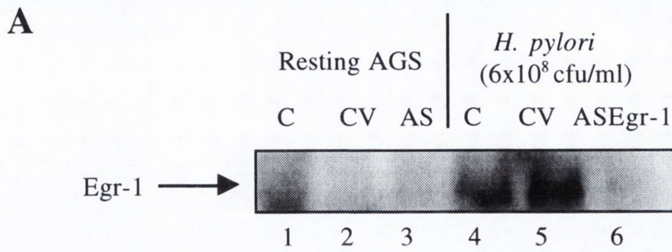
Having shown that *H. pylori*-induced Egr-1 expression in AGS cells, the effect of antisense Egr-1 oligonucleotide on *H. pylori*-induced CD44 expression was examined. Transfection of AGS cells with antisense Egr-1 (5 μg) resulted in down-regulation of CD44 expression by *H. pylori* strain 11638, while the corresponding vector had no significant effect on *H. pylori*-induced CD44 expression (Fig. 6.10).

6.4 DISCUSSION

Egr-1 is known to be capable of initiating the transcription of many genes. Of these gene products, TNF-α (Kramer *et al.*, 1994), IL-2 (Decker *et al.*, 1998), CD44 (Fitzgerald and O'Neill, 1999; Maltzman *et al.*, 1996a), ICAM-1 (Maltzman *et al.*, 1996b) and p53 (Nair *et al.*, 1997) are known to be elevated in *H. pylori* positive subjects. As the promoter regions of the corresponding genes contain one or more Egr-1 consensus binding domains, it is tempting to hypothesise that the induction of Egr-1 could regulate the transcription of several genes during *H. pylori* infection *in vivo*.

It has previously been shown that *H. pylori* up-regulates the expression of the cell adhesion molecules CD44 and CD44 v9 in gastric epithelial cells from patients infected with *H. pylori* (Fan *et al.*, 1996b). CD44 v9 has also been associated with metastatic gastric carcinoma (O'Connor, 1992). Moreover, it has been shown that Egr-1 is involved in the regulation of CD44 expression in endothelial cells (Fitzgerald and O'Neill, 1999). *H. pylori* also up-regulates ICAM-1 expression on gastric epithelial cells (Fan *et al.*, 1995a; Crowe *et al.*, 1995). The mechanisms of CD44 and ICAM-1 expression by *H. pylori* on gastric epithelial cells are poorly understood. To answer the question of whether CD44 and ICAM-1 activation by *H. pylori* is dependent on the presence of an Egr-1 binding site, AGS cells were transfected with pCAT-reporter plasmids carrying CD44 or ICAM-1 promoters containing the Egr-1 binding site. *H. pylori* increased the CAT activity driven by the CD44 and ICAM-1 promoters in an

A



B

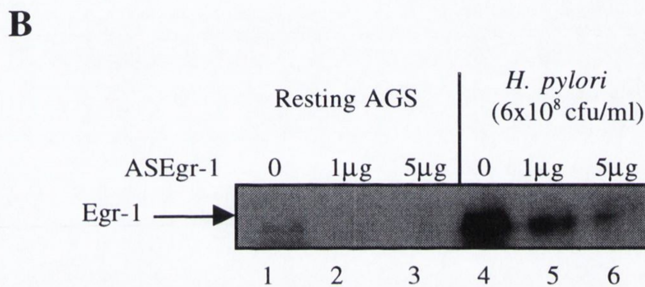


Fig. 6.9. Effect of antisene Egr-1 oligonucleotide on *H. pylori*-induced Egr-1 expression. (A) Antisense Egr-1 blocks *H. pylori*-induced Egr-1 expression. AGS cells were transfected with 5 μg antisense Egr-1 oligonucleotide (Egr-1/PcDNA3.1+/Zeo), the control vector (PcDNA3.1+/Zeo) or left alone for 48 h, as described under Material and Methods. Following incubation of AGS cells with *H. pylori* strain 11638 ($6x10^8$ cfu/ml) for 8 h, total cell lysates were prepared and analyzed for Egr-1 expression by Western blotting. Egr-1 protein expression was detected using chmeiluminescense. Lanes 1 and 4; untransfected AGS cells, Lanes 2 and 5; AGS cells transfected with control vector; Lanes 3 and 6 AGS cells transfected with antisense Egr-1 oligonucleotide. **(B)** Dose-dependent inhibition of Egr-1 protein expression by *H. pylori* in AGS cells by 1 μg and 5 μg of antisense Egr-1 oligonucleotide. One representative result is shown of three independent experiments with similar results.

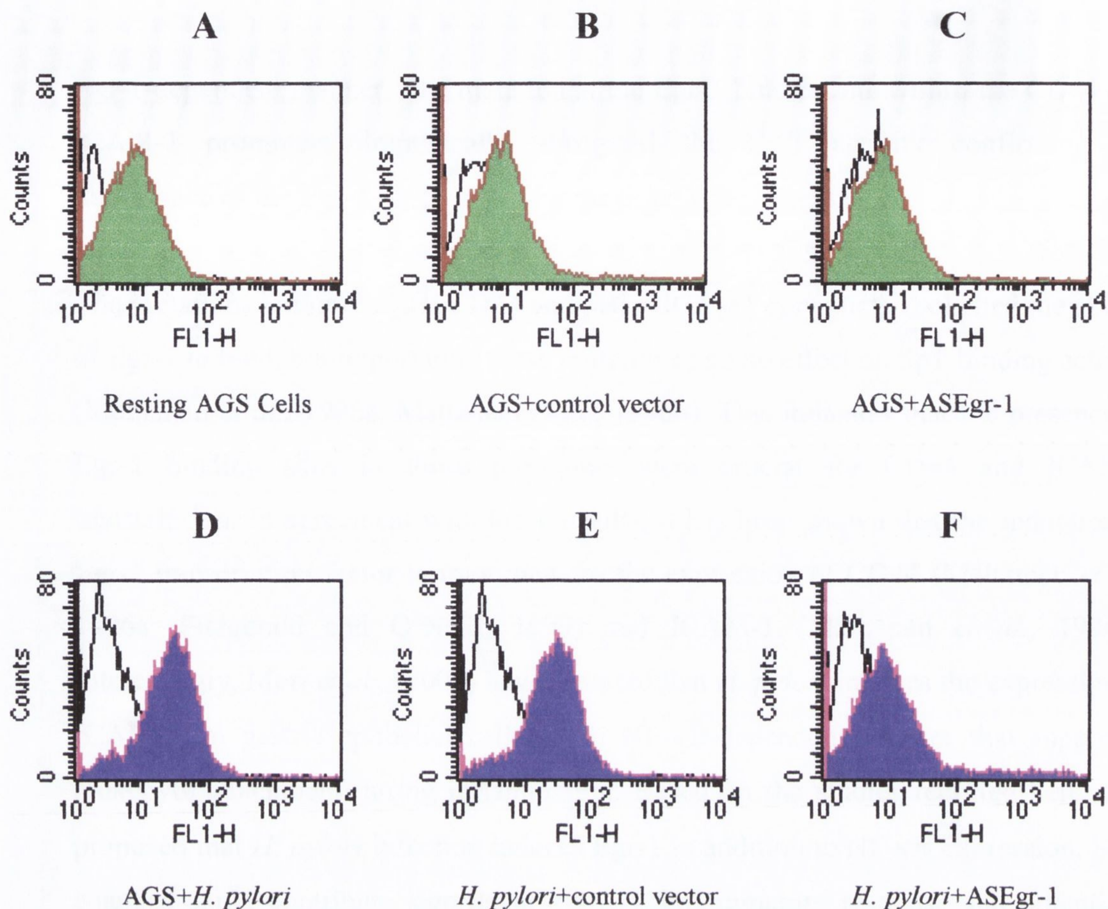


Fig. 6.10. Effect of antisense Egr-1 oligonucleotide on FACSan profiles of CD44 expression by *H. pylori* on AGS cells. AGS cells ($5x10^5$ cells/ml) were transfected with 5μg antisense Egr-1, 5μg control vector or left untransfected. Following incubation for 8 h with *H. pylori* strain 11638 ($6x10^8$ cfu/ml), cells were stained with FITC-labelled with anti-CD44 (L3D.1; shaded peakes) or FITC-labelled isotype control (anti-IE; unshaded peaks). **Panel A** shows resting untransfected AGS cells, **panel B** shows AGS cells transfected with control vector and **panel C** shows AGS cells transfected with antisense Egr-1 oligonucleotide, stained with FITC-labelled with anti-CD44. **Panel D** shows resting untransfected AGS cells and **panel E** shows *H. pylori*-induced CD44 expression on AGS cells transfected with control vector. **Panel F** shows down-regulation of *H. pylori*-induced CD44 expression on AGS cells by antisense Egr-1 oligonucleotide. One representative result of three independent experiments with similar results is shown.

Egr-1-dependent manner. The mutation at the Egr-1 binding site within the CD44 and ICAM-1 promoters dramatically abrogated the CAT activity confirming the importance of the Egr-1 binding site for maximal gene expression.

The mutations within the pBLCD44 and pBLmICAM1 constructs abolished the ability of Egr-1 to bind, but importantly these mutations had no effect on Sp1-binding activity (Maltzman *et al.*, 1996a; Maltzman *et al.*, 1996b). This indicates that the presence of Egr-1 binding sites in those promoters were crucial for CD44 and ICAM-1 transcription. In agreement with these results, it has been shown that the induction of Egr-1 transcription factor is important for the expression of CD44 (Maltzman *et al.*, 1996a; Fitzgerald and O'Neill, 1999) and ICAM-1 (Maltzman *et al.*, 1996b). Interestingly, Mori *et al.*, (2000) have reported that *H. pylori* induces the expression of ICAM-1 on gastric epithelial cells in an NF-κB-dependent manner that supported leukocyte attachment during inflammation. Based on the studies reported here, it is proposed that *H. pylori* infection induces Egr-1 in addition to NF-κB expression. Such changes could contribute significantly to the inflammatory response and potentially alter cell proliferation status.

Egr-1 also plays a role in the regulation of the cell cycle. In some cell types, high expression is associated with resistance to apoptosis (Huang *et al.*, 1998; de Belle *et al.*, 1999). However, Egr-1 may also have anti-proliferative effects (Nair et al., 1997). Earlier studies have demonstrated that CD95L (APO-1/Fas) ligand, a type II transmembrane protein of the TNF family (Suda *et al.*, 1993), is highly expressed in activated cells and plays an essential role in apoptosis (Alderson *et al.*, 1995). Induction of apoptosis by *H. pylori* was found to be linked to overexpression of Fas receptor and its ligand in *H. pylori* infected patients (Rudi *et al.*, 1998). In order to demonstrate whether the induction of Egr-1 is involved in the regulation of the human CD95L promoter activity, AGS cells were transfected with a luciferase-reporter carrying CD95L promoter activity with or without an Egr-1 binding site. Coculture of gastric epithelial cells with *H. pylori* resulted in an increase in luciferase activity in AGS cells transfected a plasmid carrying a luciferase reporter gene under control of

the wild type CD95L promoter which has intact Egr-1 binding sites. The mutations at the Egr-1 sites abolished this activation, suggesting a crucial role for Egr-1 in regulation of CD95L promoter activity. These findings are in agreement with a recent study, which has shown that the activation of Egr-1 is involved in regulation of CD95L promoter activity (Li-Weber *et al.*, 1999). It is thus tempting to speculate that increased Egr-1 protein levels may play a role in the regulation of cell proliferation and differentiation in gastric epithelial cells exposed to *H. pylori* infection.

Human Egr-1 antisense oligonulceotide inhibited *H. pylori*-induced Egr-1 expression, whereas the respective control empty vector had no significant effects on Egr-1 protein levels. Also, induction of Egr-1 is required for up-regulation of CD44 expression by *H. pylori* and Egr-1 antisense oligonucleotide down-regulated the expression of CD44 in response to *H. pylori*. These data indicate that Egr-1 is an important regulatory component of CD44 expression in AGS cells.

To conclude, the data presented in this chapter indicate the involvement of the transcription factor Egr-1 in the regulation of multiple genes such as those genes coding for adhesion molecules and apoptosis which play a role in the host inflammatory response to *H. pylori* infection. Importantly, *H. pylori*-mediated transcriptional activation of Egr-1 could be one of the possible mechanisms that correlate gastric inflammation and cell damage due to specific gene transcription.

CHAPTER VII

GENERAL DISCUSSION

CHAPTER VII

General Discussion

7.1 *Helicobacter pylori*: A prominent factor in the pathogenesis of peptic ulceration

The discovery of *H. pylori* on the gastric mucosa stimulated a growth of interest in gastric immunity. It is now clear that *H. pylori* is a dominant pathogenic factor in both peptic ulcer disease and gastric adenocacrinoma (Blaser, 1992; Blaser and Parsonnet, 1994; Nomura *et al.*, 1991). *H. pylori* has evolved a sophisticated mechanism to live in the acidic environment of the stomach. Colonization of the gastric epithelium by *H. pylori* occurs through the production of a number of virulence factors that permit *H. pylori* to evade the bactericidal effect of the gastric acid. Direct bacterial damage to the gastric epithelium may result from the bacterial production of urease, phospholipase and the vacuolating cytotoxin.

The host cells at the site of the infection respond to the bacterial pathogen by producing cytokines and chemokines. At the site of colonization, the rate of cell proliferation and apoptosis of the epithelial cells are also affected. Infection with *H. pylori* is also associated with elevated levels of proinflammatory cytokines, IL-1β, IL-2, IL-6, TNF-α and IL-8 compared with specimens from uninfected individuals (Crabtree *et al.*, 1991b and 1994; Yamoka *et al.*, 1997). The variation in the clinical manifestations of *H. pylori* infection among individuals is potentially due to the difference in the virulence of different *H. pylori* strains. *H. pylori* strains that possess the cag PAI cause more peptic ulceration and gastric carcinoma than do non cytotoxic strains (Crabtree *et al.*, 1994; Blaser *et al.*, 1995). Molecular studies have shown over 30 genes are coexpressed with CagA these form a pathogenicity island that is present in approximately 60% of *H. pylori* strains (Censini *et al.*, 1996; Covacci *et al.*, 1998).

Strains of *H. pylori* expressing the cytotoxin associated gene (*cagA*) exert direct cytotoxic effects on the mucosal epithelium and potentiate tissue damage by stimulating an inflammatory response in the gastric mucosa and the submucosa. In this study, *in vitro* infection of AGS cells with $CagA^+$ and $VacA^+$ *H. pylori* strains resulted

in the induction of transcription factors NF-κB and Egr-1 compared to $CagA^-$ and $VacA^-$ strains. Importantly, *ex vivo* work has shown that $CagA^+$ strains induce significantly elevated levels of IL-1α, IL-1β, IL-6, TNF-α and IL-8 compared to $CagA^-$ strains (Crabtree *et al.*, 1994; Yamaoka *et al.*, 1997). Previous studies have reported that attachment of *H. pylori* to cultured gastric epithelial cells such as AGS cells can induce tyrosine phosphorylation of a 145 kDa protein (CagA) and also the subsequent activation of NF-κB (Asahi *et al.*, 2000; Aihara *et al.*, 1997; Sharma *et al.*, 1998). Recently, it has been shown that CagA interacts directly with a tyrosine phosphatase (Higashi *et al.*, 2002).

7.2 *Helicobacter pylori* and Transcriptional Regulation

Transcriptional control plays a vital role in regulating several cellular events such as proliferation, differentiation and cell death. Changes in gene expression are affected by altering the activity of transcription factors that bind to their target genes. Evidence from the literature suggests that genetic variation in transcription factors and/or their binding sites may contribute to common multifactorial disorders including peptic ulceration. Thus, the focus of this study was the role of transcription factors in the pathogenesis of *H. pylori* infection. A growing body of evidence indicates that *H. pylori* infection is associated with expression and activation of the transcription factor NF-κB (Keates *et al.*, 1997; Münzenmaier *et al.*, 1997, Maeda *et al.*, 2001), which is one of the transcription modulators in the host response to bacterial invasion.

Activation of NF-κB is critical for the inducible expression of genes coding for various cytokines, adhesion molecules and cell surface receptors (Baeuerle and Henkel, 1994). As demonstrated by gel mobility shift assays and immunoblotting, *H. pylori* activates this transcription factor in gastric epithelial cells. Further confirmation of the expression of this factor was provided by immunohistochemical staining of NF-κB in antral gastric biopsies obtained from patients infected with *H. pylori*. **Fig. 7.1** illustrates the induction of cellular events including the activation of NF-κB after infection with *H. pylori*. Infection with *H. pylori* leads to changes in many factors including the vitamin C content of gastric juice, reactive oxygen metabolites, epithelial

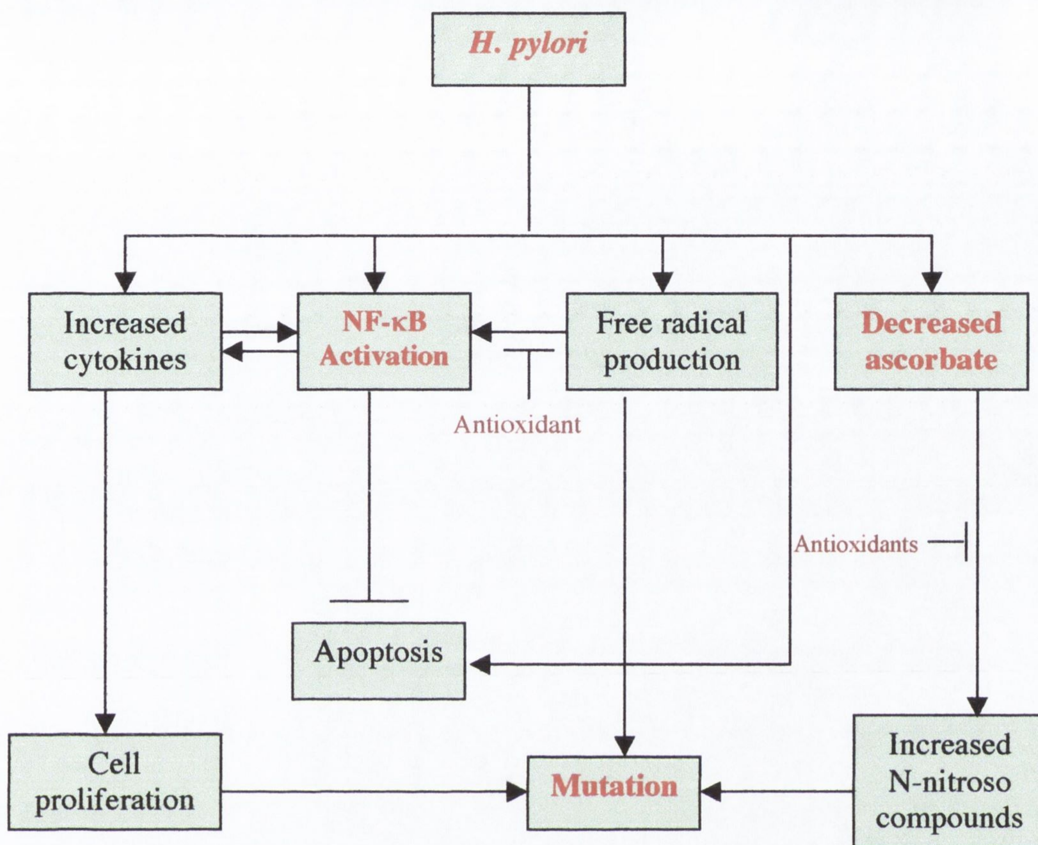


Fig. 7.1. Schematic model shows consequences of *H. pylori* infection in the development of peptic ulcer disease and gastric carcinoma with the possible sites for antioxidant action.

cell proliferation and cytokine levels (Banerjee *et al.*, 1994; Davies and Rampton, 1994; Brenes *et al.*, 1993), and eradication of *H. pylori* infection reverses these changes.

To my knowledge, the data presented in this thesis is the first reported demonstration that *H. pylori* induces Egr-1 in gastric epithelial cells. Egr-1 expression is associated with the up-regulation of genes encoding cytokines and cell adhesion molecules, which play crucial roles in regulating cell proliferation, differentiation, cell adhesion and apoptosis. The activation of Egr-1 signalling pathways, Egr-1 transcription and cellular events regulated by Egr-1 is shown in **Fig. 7.2**. Importantly, *H. pylori*-positive patients exhibit increased Egr-1 expression compared to normal individuals, as demonstrated by immunohistochemical staining. Thus, Egr-1 expression represents a potential mechanism, in addition to NF-κB activation, whereby the inducible expression of multiple genes following *H. pylori* infection is regulated.

The activation of Egr-1 by various unrelated stimuli arises due to activation of different signalling pathways. In this study, the MAP kinase kinase (MEK) PD98059 inhibitor, which binds inactive MEK and prevents phosphorylation by Raf without affecting other known serine/threonine or tyrosine kinases, partially inhibited the induction of Egr-1 in response to *H. pylori*. Similar studies by Guha *et al.* (2001) demonstrated that the MEK-ERK1/2 inhibitor PD980059 reduced LPS induction of tissue factor (TF) and TNF-α by inhibiting Egr-1 expression. Inhibitors for PKC, tyrosine kinase and phosphatidylinositol 3-kinase inhibitors had no effect on the induction of Egr-1 by *H. pylori.* According to these data, it appears that PKC and PI3-kinase pathways play a role in regulating Egr-1 induction by the cytokines IL-1 β and TNF-α, PMA and low pH but not by *H. pylori*. It has been demonstrated that inhibition of protein kinase pathways in various cell lines prevents the expression of Egr-1 (Chaudhary *et al.*, 1996; Huang and Adamson, 1995). Moreover, the induction of Egr-1 by *H. pylori* and other antigens was inhibited by the pretreatment with vitamin C and NAC in the millimolar range. Taken together, these findings indicate

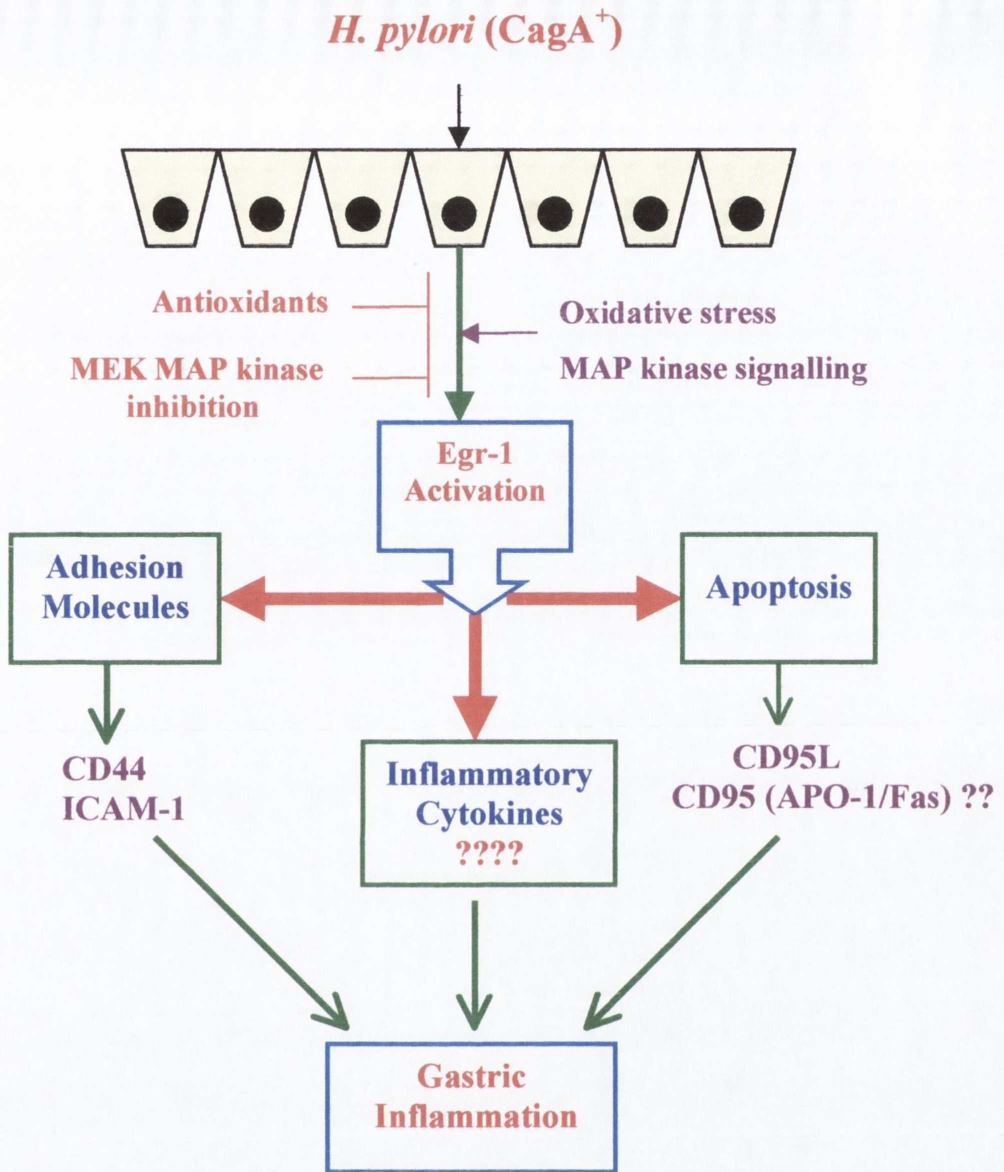


Fig. 7.2. Schematic model of Egr-1 activation by *H. pylori* in gastric epithelial cells and the multiple pathways through which *H. pylori* may contribute to gastric inflammation and cell damage.

that redox reactions are involved in regulating Egr-1 expression in gastric epithelial cells in response to *H. pylori* infection.

Using reporter genes under the control of the CD44 and ICAM-1 promoter, this study has shown that the induction of CD44 and ICAM-1 expression is dependent on the presence of Egr-1 within the CD44 and ICAM-1 promoters since mutation of the Egr-1 binding site abrogated the activity. These findings indicate the critical involvement of Egr-1 in the regulation of these adhesion molecules in response to *H. pylori* infection. Furthermore, induction of apoptosis by *H. pylori* has been reported in gastritis and duodenal ulcer patients (Moss *et al.*, 1996). The mechanisms involved in *H. pylori*-induced apoptosis are poorly understood. One of the proposed mechanisms is that interaction between *H. pylori* and gastric epithelial cells occurs through the upregulation of Fas (CD95) and its ligand receptor (von Herbay and Rudi, 2000; Rudi *et al.*, 1998). Using luciferase reporter constructs under the control of the CD95L promotor, it is clear that induction of the Egr-1 transcription factor is important for *H. pylori*-induced apoptosis. This effect required the presence of the three Egr-1 binding sites (-120, -180 and -680), as mutations in these sites abolished CD95L promoter activity. Li-Weber *et al.* (1999) have also demonstrated that the three Egr-1 binding sites are required for CD95L promoter activity. These results suggest that *H. pylori* directly induces apoptosis in gastric epithelial cells via increased expression of the CD95L receptor which is regulated by Egr-1.

The functional significance of these findings is provided by the observation that antisense oligonucleotides directed against Egr-1 inhibited *H. pylori*-induced Egr-1 and CD44 expression, whereas the respective control empty vector had no significant effects on Egr-1 protein levels. These data suggest that induction of Egr-1 is required for the up-regulation of CD44 expression by *H. pylori* as Egr-1 antisense oligonucleotide down-regulated this induction. As Egr-1 binds to the proximal promoters of many genes involved in various cellular events, identification of additional pathophysiologically relevant target genes of this transcription factor may reveal other potential therapeutic targets. Of these gene products, TNF-α (Kramer *et*

al., 1994), IL-2 (Decker *et al.*, 1998), CD44 (Fitzgerald and O'Neill, 1999; Maltzman *et al.*, 1996a), ICAM-1 (Maltzman *et al.*, 1996b) and p53 (Nair *et al.*, 1997) are known to be elevated in *H. pylori* positive subjects.

7.3 Effect of *H. pylori* and Low pH on Transcriptional Regulation

The production of gastric acid constitutes the first defense line against enteropathogenic bacteria (Sarker and Gyr, 1992). The majority of bacterial species can not tolerate low pH which is considered the most powerful barrier to colonization posed by the stomach. However, *H. pylori* can survive in acidic pH by producing urease enzyme which protects it by local neutralization of the acid (Marshall *et al.*, 1990). The results presented in this thesis demonstrate that exposure of gastric epithelial cells to conditions of low pH resulted in increased NF-κB DNA-binding activity. This effect could be directly related to the acidic environment of the stomach. Interestingly, when gastric epithelial cells are exposed to low pH and *H. pylori*, pronounced NF-κB activation was observed compared to the effects of either pH or *H. pylori* alone. Also exposure of gastric epithelial cells to media of low pH enhanced Egr-1 expression. This induction was not increased further in the presence of *H. pylori*. However, it is possible that low pH and *H. pylori* might be synergistic under certain conditions. The association between low pH and *H. pylori* infection may play an integral role in the pathogenesis of peptic ulcer disease. This suggests that in addition to *H. pylori*, conditions of low pH are also contributing factors to inflammation.

H. pylori infection exerts diverse effects on gastric acid secretion. In one group of subjects with *H. pylori*-associated chronic gastritis, hyposecretion of gastric acid is observed (Chandrakumaran *et al.*, 1994), while in duodenal ulcer patients, hypersecretion of gastric acid occurs (Blaser, 1992; Moss and Calam, 1992). The alteration in acid secretion in *H. pylori* infected patients appears to be dependent on the extent and severity of *H. pylori*-induced gastritis.

7.4 *Helicobacter pylori* and Vitamin C Levels

Gastric juice vitamin C levels are altered in the presence of *H. pylori* infection. Reduced levels of vitamin C may contribute to the potenial harmful effects of circulating oxidized substances. Vitamin C is secreted into the stomach leading to gastric juice concentrations (50-300 μM/l) greater than those in plasma (Sobala *et al.*, 1989). Sobala *et al.* (1991) have demonstrated that the acute inflammatory response to *H. pylori* is accompanied by a failure of vitamin C secretion into the gastric juice. Subsequent studies have been reported that *H. pylori*-associated gastritis was correlated with decreased concentrations of gastric juice ascorbic acid (Sobala *et al.*, 1993; Banerjee *et al.*, 1994). Therefore, altered levels of vitamin C may also contribute to the potent induction of NF-κB and Egr-1 in *H. pylori*-asscoicated gastritis and as a consequence to an immune response to the bacteria. The ability of antioxidants such as vitamin C, NAC and *H. pylori* Trx to attenuate *H. pylori*-induced NF-κB and Egr-1 induction in gastric epithelial cells supports the fact that the induction of NF-κB and Egr-1 through these mechanisms may involve redox-signalling pathway. The exact protective mechanisms of vitamin C on *H. pylori* infection and the development of gastric cancer are unclear. In this study, vitamin C in the millimolar range potently inhibited NF-κB and Egr-1 activation secondary to *H. pylori* and other antigens. Vitamin C also up-regulated IκB-α levels, which is the natural inhibitor of NF-κB, which could be one of the mechanisms through which vitamin c protects against *H. pylori* infection. Bowie and O'Neill (2000) have also demonstrated that millimolar doses of vitamin C inhibit multiple pathways to NF-κB, including IL-1 and TNF, in endothelial cells. This inhibition was mediated vitamin C-induced activation of the stress-activated protein kinase p38 MAPK.

Prolonged exposure *to H. pylori* with altered levels of vitamin C and gastric acid may play a dominant role in the series of events leading to pathological abnormalaties of the gastroduodenal epithelium. Several studies have been shown that high vitamin C doses prevented *H. pylori* infection (Jarosz *et al.*, 1998) and also protected against gastric carcinoma (Drake *et al.*, 1996; Zhang *et al.*, 1997). Recently, Everett *et al.* (2002) have demnostrated that vitamin supplements (vitamin C and E) were not

effective either alone or in combination with *H. pylori* eradication. Supplements of vitamin C and E do not significantly reduce mucosal reactive oxygen species damage in *H. pylori* gastritis in the short term. However, the combination of reduced vitamin C levels in association with *H. pylori* infection and altered acid secretion may result in an exaggerated inflammatory response through induction of NF-κB, Egr-1 and other related genes.

7.5 Future Prospects

The data presented in this thesis indicate a role for NF-κB and Egr-1 in *H. pylori*-induced inflammation and provide insights into the mechanisms controlling the expression of several genes during the inflammatory response and the subsequent outcome of peptic ulcer disease. Further work still remains to be done to investigate directly whether other transcription factor(s) may be involved in the pathogenesis of *H. pylori* infection, and if these factor(s) and NF-κB and Egr-1 work in concert to regulate the inflammatory response. Antioxidants such as vitamin C and NAC efficiently blocked NF-κB and Egr-1 activation. These findings suggest the possibility that dietary antioxidants may protect against the mucosal inflammation associated with *H. pylori* infection. Modulation of expression of NF-κB and Egr-1 represent potential targets for therapeutic intervention, as these proteins regulate the transcription of pro-inflammatory cytokines and cell adhesion molecules involved in the initiation of the inflammatory process in response to *H. pylori*. Early administration of such inhibitors might prevent these acute reactions. As redox-regulated transcription factors become an attractive drug target, inhibition of DNA binding of NF-κB and Egr-1 has emerged as an important mechanism to control gene expression.

Elucidation of the biochemical and molecular mechanisms whereby *H. pylori* induces activation of the NF-κB and Egr-1 genes will be a fruitful exercise as inhibition of activation would clearly represent novel therapeutic strategies in the treatment and prevention of *H. pylori*-associated disease. Moreover, identification of the mechanisms of NF-κB and Egr-1 activation will likely elucidate important pathogenic mechanisms of bacterial-host interaction.

REFERENCES

Abate C, Patel L, Rauscher FJ 3rd, Curran T (1990). Redox regulation of fos and jun DNA-binding activity in vitro. Science **249**: 1157-1161.

Abbadie C, Kabrun N, Bouali F, Smardova J, Stephelin D, Bandenbunder B, Enrietto PJ (1993). High levels of c-rel expression are associated with programmed cell death in the developing avian embryo and in bone marrow cells *in vitro*. Cell **75**: 899-912.

Ahmed MM, Venkatasubbarao K, Fruitwala SM, Muthukkumar S, Wood DP, Jr, Sells SF, Mohiuddin M, Rangnekar VM (1996). EGR-1 induction is required for maximal radiosensitivity in A375-C6 melanoma cells. J Biol Chem **271**: 29231-29237.

Aihara M, Tsuchimoto D, Takizawa H, Azuma A, Kilkuchi M, Mukaida N, Matsushima K (1997). Mechanisms involved in *Helicobacter pylori*-induced interleukin-8 producrion by a gastric cancer cell line, MKN 45. Infect Immun **65**: 3218-3224.

Akira S, Kishimoto T (1992). IL-6 and NF-IL6 in acute-phase response and viral infection. Immunol Rev **127**: 25-50.

Alderson MR, Tough TW, Davis-Smith T, Braddy S, Falk B, Schooley KA, Goodwin RG, Smith CA, Ramsdell F, Lynch D (1995). Fas ligand mediates activation-induced cell death in human T lymphocytes. J Exp Med **181**: 71-77.

Alpert D, Vilćek J (2000). Inhibition of IκB kinase activity by sodium salicylate *in vitro* does not reflect its inhibitory mechanism in intact cells. J Biol Chem **275**: 10925-10929.

Andersen LP, Gaarslev K (1992). IgG subclass antibodies against *Helicobacter pylori* heat-stable antigens in normal persons and in dyspeptic patients. APMIS **100**: 747-751.

Anderson D, Yu TW, Phillips BJ, Schmezer P (1994). The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the COMET assay. Mutat Res **307**: 261-271.

Aruoma OI, Halliwell B, Hoey BM, Butler J (1989). The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med **6**: 593-597.

Asahi M, Axuma T, Ito S, Ito Y, Suto H, Nagai Y, Tsubokawa M, Tohyama Y, Maeda S, Omata M, Suzuki T, Sasakawa C (2000). *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. J Exp Med **191**: 593-602.

Atherton JC, Cover TL, Peek PM, Blaser MJ (1995). Subtyping of *Helicobacter pylori* strains into two groups by polymerase chain reaction amplification of the *vacA* gene and correlation of these groups with CagA status. Am J Gastroenterol **89**: 1291 (Abstract).

Auphan N, Didonato JA, Rosette C, Helmberg A, Karin M (1995). Immunosuppression by glucocortiicoid: inhibition of NF-κB activity through induction of IκB synthesis. Science **270**: 286-290.

Avissar N, Kerl EA, Baker SS, Cohen HJ (1994). Extracellular glutathione peroxidase mRNA and protein in human cell lines. Arch Biochem Biophys **309**: 239-246.

Backert S, Ziska E, Brinkmann V, Zimny-Arndt U, Fauconnier A, Jungblut PR, Naumann M, Meyer TF (2000). Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secertion appartus. Cellular Microbiol **2**: 155-164.

Baeuerle PA (1991). The inducible transcription activator NF-kappa B: regulation by distinct protein subunits. Biochim Biophys Acta **1072**: 63-80.

Baeuerle PA, Baichwal V (1997). NF-kappa B as a frequent target for immunosuppressive and inflammatory molecules. Adv Immunol **65**: 111-137.

Baeuerle PA, Baltimore D (1989). A 65-kDa subunit of active NF-kappaB is required for inhibition of NF-kappaB by I kappaB. Genes Dev **3**: 1689-1698.

Baeuerle PA, Baltimore D (1988a). Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53**: 211-217.

Baeuerle PA, Baltimore D (1988b). IκB: a specific inhibitor of the NF-κB transcription factror. Science **242**: 540-546.

Baeuerle PA, Baltimore D (1996). NF-κB: Ten years after. Cell **87**: 13-20.

Baeuerle PA, Henkel T (1994). Function and activation of NF-κB in the immune system. Annu Rev Immunol **12**: 141-179.

Baggiolini M, Wymann MP (1990). Turining on the respiratory burst. Trens Biochem Sci **15**: 65-72.

Baldwin AS (1996). The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol **14**: 649-681.

Baldwin AJ, Azizkhan JC, Jensen DE., Beg AA, Coadly LR (1991). Induction of NF-κB DNA-binding activity during the G0-to-G1 transition in mouse fibroblsats. Mol Cell Biol **11**: 4943-4951.

Bamford KB, Fan X, Crowe SE, Leary JF, Gourley WK, Luthra GK Brooks EG, Graham DY, Reyes VE, Ernst PB (1988). Lymphocytes in the human gastric mucosa during *Helicobacter pylori* have a T helper cell 1 phenotype. Gastroenterology **114**: 482-492.

Banerjee S, Hawksby C, Miller S, Dahill S, Beattle AD, McColl KE (1994). Effect of *Helicobacter pylori* and its eradication in gastric juice ascorbic acid. Gut **35**: 317-322.

Barnes PJ, Adcock T (1993). Anti-inflammatory actions of steroids: molecular mechanisms. Trends Pharmacol Sci **14**: 436-441.

Beales IL, Calam J (1998). Interleukin 1 beta and tumour necrosis factor alpha inhibit acid secretion in cultured rabbit parietal cells by multiple pathways. Gut **42**: 227-234.

Beales I, Calam J, Post L, Srinivasan S, Yamada T, Del Valle J (1997). Effect of tumor necrosis factor- and IL-8 on somatostatin release from canine fundic G cells. Gastroenterology **112**: 136-143.

Beg AA, Baldwin AS, Jr (1993). The Ikappa B proteins: multifunctional regulators of Rel/NF-kappa B transcription factors. Genes Dev **7**: 2064-2072.

Beg AA, Baltimore D (1996). An essential role for NF-kappa B in preventing TNF-alpha-induced cell death. Science **274**: 782-784.

Beg AA, Finco TS, Nantermet PV, Baldwin AS, Jr (1993). Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκB-α: a mechanism for NF-κB activation. Mol Cell Biol **13**: 3301-3310.

Beg AA, Sha WC, Branson RT, Ghosh S, Baltimore D (1995). Embryonic lethality and liver degeneration in mice lacing the RelA component of NF-κB. Nature **376**: 167-170.

Beil W, Birkholz C, Wagner S, Sewing KF (1994). Interaction of *Helicobacter pylori* and its fatty acids with parietal cells and gastric H+/K(+)-ATPase. Gut **35**: 1176-1180.

Bellocq A, Suberville S, Philippe C, Bertrand F, Perez J, Fouqueray B, Cherqui G, Baud L (1998). Low environmental pH is responsible for the induction of nitric-oxide synthase in macrophages. Evidence for involvement of nuclear factor-kappaB activation. J Biol Chem **273**: 5086-5092.

Betts JC, Nabel GJ (1996). Differential regulation of NF-κB2 (p100) processing and control by amino-terminal sequences. Mol Cell Biol **16**: 6363-6369.

Bielinska A, Shivdosami RA, Zhang IQ, Nabel GJ (1990). Regulation of gene expression with double-stranded phosphorothioate oligonucleotides. Science **250**: 997-1000.

Birkholz S, Schneider T, Knipp U, Stallmach A, Zeitz M (1998). Decreased *Helicobacter pylori*-specific gastric secretory IgA antibodies in infected patients. Digestion **59**: 638-645.

Bizzozero G (1893). Über die Schlauchformigen drüsen des Magendarmkanals und die Beziehungen ihres epithels Zu dem Obserflächenepithel der Schleimahut. Arch F Mikr Anat **23**: 82-94.

Bjornstedt M, Xue J, Huang W, Akesson B, Holmgren A (1994). The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. J Biol Chem **269**: 29382-29384.

Blackwell FR, Burke F (1989). The cytokine network. Immunol Today **10**: 299-304.

Blair AJ, Feldman M, Barnett C, Walsh JH, Richardson CT (1987). Detailed comparison of basal and food-stimulated gastric acid secretion rates and serum gastrin release in duodenal ulcer patients. Gut1 **33**: 601-603.

Blank V, Kourilsky P, Israel A (1992). NF-κB and related proteins: Rel, dorsal homologies meet ankyrin-like repeats. Trends Biochem Sci **17**: 135-140.

Blaser MJ (1992). Hypotheses on the pathogenesis and natural history of *Helicobacter pylori*-induced inflammation. Gastroenterology **102**: 720-727.

Blaser MJ (1994). *Helicobacter pylori* phenotypes associated with peptic ulceration. Scand J Gastroenterol **29** (Supp 205): 1-5.

Blaser MJ, Parsonnet J (1994). Parasitism by the "slow" bacterium *Helicobacter pylori* leads to altered gastric homeostasis and neoplasia. J Clin Invest **94**: 4-8.

Blaser MJ, Perez-Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH, Stermmermann GN, Nomura A (1995). Infection with *Helicobacter pylori* strains possessing CagA associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res **55**: 2111-2116.

Bonizzi G, Piette J, Schoonbroodt S, Greimers R, Havard L, Merville MP, Bours V (1999). Reactive oxygen intermediate-dependent NF-kappa activation by interleukin-1 beta requires 5-lipoxygenase or NADPH oxidase activity. Mol Cell Biol **19**: 1950-1960.

Bontkes HJ, Veenendaal RA, Pena AS, Goedhard JG, van Duijn W, Kuiper J, Meijer JL, Lamers CB (1992). IgG subclass response to *Helicobacter pylori* in patients with chronic active gastritis and duodenal ulcer. Scand J Gastroenterol **27**: 129-133

Boren T, Falk P, Roth KA, Larson G, Normark S (1993). Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. Science **262**: 1892-1895.

Bottcher G (1874). Dorpater Med Z **5**: 148.

Bours V, Dejardin E, Goujon-Letawe F, Merville MP, Castronovo V (1994). The NF-kappa B transcription factor and cancer: high expression of NF-kappa B- and I kappa B-related proteins in tumor cell lines. Biochem Pharmacol **47**: 145-149.

Bours V, Franzoso G, Azarenko V, Park S, Kanna T, Brown K, Siebenlist U (1993). The oncoprotein Bcl-3 directly transactivates through κB motifs via association with DNA-binding p50 homodimers. Cell **72**: 729-739.

Bowie AG, Moynagh PN, O'Neill LAJ (1997). Lipid peroxidation of NF-kappa B by tumor necrosis factor but not interleukin-1 in the human endothelial cell line ECV304. Lack of involvement of H_2O_2 in NF-kappa B activation by either cytokine in both primary and transformed endothelial cells. J Biol Chem **272**: 25941-25950.

Bowie AG, O'Neill LAJ (2000). Oxidative stress and nuclear factor-κB activation: a reassessment of the evidence in the light of recent discoveries. Biochem Pharmacol **59**: 13-23.

Bowie AG, O'Neill LAJ (2000). Vitamin C inhibits NF-κB activation by TNF via the activation of p38 mitogen-activated protein kinase. J Immunol **165**: 7180-7188.

Boyum A (1968). Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest **97** (Supp 1): 77-89.

Busam K, Gieringer C, Freudenberg M, Hohmann HP (1992). *Staphylococcus aureus* and derived exotoxins induce nuclear factor kappa B-like activity in murine bone marrow macrophages. Infect Immun **60**: 2008-2015.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram levels of protein utilising the pricible of protein-dye binding. Anal Biochem **72**: 248-254.

Brenes F, Correa P, Hunter F, Rhamakrishnan T, Fantha E, Shi TY (1993). *Helicobacter pylori* causes hyperproliferation of the gastric epithelium: pre- and post-eradication indices of proliferating cell nuclear antigen. Am J Gastroenterol **88**: 1870-1875.

Brennan P, O'Neill LA (1995). Effects of oxidants and antioxidants on nuclear factor kappa activation in three cell lines: evidence against a universal hypothesis involving oxygen radicals. Biochim Biophys Acta **1260**: 167-175.

Brown K, Gertberger S, Carlson L, Franzoso G, Siebenlist U (1995). Control of Ikappa-alpha proteolysis by site-specfic signal-induced phosphorylation. Science **267**: 1485-1488.

Cahill RJ, Xia H, Kilgallen C, Beatie S, Hamilton H, O'Morian C (1995). Effect of eradication of *Helicobacter pylori* infection on gastric epithelial cell proliferation. Dig Dis Sci **40**: 1627-1631.

Calam J (1995). The somatostatin-gastrin link of *Helicobacter pylori* infection. Ann Med **27**: 569-573.

Calam J, Beales ILP, Gibbons A, Ghatei M, Del Valle J (1996). Effects of abnormalites of gastrin and somatostatin in *Helicobacter pylori* infection on acid secretion. In. *Helicobacter pylori*: Basic mechanisms to clinical cure. Hunt, R. H., and Tytgat, G. N. J. (eds). Dordrecht: Kluwer Academic. pp 108-121.

Calam J, Gibbon A, Healey ZV, Bliss P, Arebi N (1997). How does *Helicobacter pylori* cause mucosal damage? Its effect on acid and gastric physiology. Gastroenterology **113**: S43-49.

Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, Soltoff S (1991). Oncogenes and signal transduction. Cell **64**: 281-302

Cao X, Mahendran R, Guy GR, Tan YH (1993). Detection and characterization of cellular EGR-1 binding to its recognition site. J Biol chem. **268**: 16949-16957.

Carlos TM, Harlan JM (1990). Membrane proteins involved in phagocyte adherence to endothelium. Immunol Rev **114**: 5-28.

Cave DR, Vargas M (1989). Effect of a *Campylobacter pylori* protein on acid secretion by parietal cells. Lancet **2**: 187-189.

Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodousky M, Rappuoli R, Covacci A (1996). *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type 1-specific and disease-associated virulence factors. Proc Natl Acad Sci USA **93**: 14648-14653.

Chance B, Sies H, Boveris A (1979). Hydroperoxide metabolism in mammalian organs. Physiol Rev **59**: 527-605.

Chandrakumaran K, Vaira D, Hobsley M (1994). Duodenal ulcer, *Helicobacter pylori*, and gastric secretion. Gut **35**: 1033-1036.

Chaudhary LR, Cheng SL, Avioli LV (1996). Induction of early growth response-1 gene by interleukin-1 beta and tumor necrosis factor-alpha in normal human bone marrow stromal osteoblastic cells: regulation by a protein kinase C inhibitor. Mol Cell Biochem **159**: 69-77.

Chen Z, Hagler J, Palombella VJ, Melandri F, Scherer D, Ballard D, Maniatis T (1995). Signal-induced site-specific phosphorylation targets I kappa alpha to the ubiquitin-proteasome pathway. Genes Dev **9**: 1586-1597.

Chinnaiyan, A. M., Tepper CG, Seldin MF, O'Rourke K, Kischkel FC, Hellbardt S, Krammer PH, Peter ME, Dixit VM (1996). FADD/MORT1 is a common mediator of CD95 (Fas/Apo-1) and tumor necrosis factor receptor-induced apoptosis. J Biol Chem **271**: 4961-4969.

Chiverton SG, Hunt RH (1989). Initial therapy and relapse of duodenal ulcer: possible acid secretory mechanism. Gastroenterology **96**: 632-639.

Christy BA, Lau LF, Nathans D (1988). A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc Natl Acad Sci USA **85**: 7857-7861.

Christy BA, Nathans D (1989). DNA binding site of the growth factor-inducible protein Zif68. Proc Natl Acad Sci USA **86**: 8737-8741.

Cogswell PC, Mayo MW, Baldwin AS, Jr (1997). Involvement of Egr-1/Rela synergy in distinguishing T cell activation from tumor necrosis factor-α-induced NF-kappa B1 transcription. J Exp Med **185**: 491-497.

Coleman DL, Bartiss AH, Sukhatme VP, Liu J, Rupprecht HD (1992). Lipopolysacchride induces Egr-1 mRNA and protein in murine peritoneal macrophages. J Immunol **149**: 3045-3051.

Correa P (1991). Is gastric carcinoma an infectious disease? N Engl J Med **325**: 1170-1171.

Corthésy-Theulas I, Porta N, Pringault E, Racine L, Bugdanova A, Kraehenbuhl JP, Blum AL, Michetti P (1996). Adhesion of *Helicobacter pylori* to polarized T84 human intestinal cell monolayers in pH dependent. Infect. Immun **64**: 3827-3832.

Courey AJ, Tjian R (1992). Mechanisms of transcriptional control as revealed by studies of human transcription factor Sp1. In: Transcriptional regulation. Cold spring, Harbor, NY. Vol 28. pp 743-769.

Covacci A, Censini S, Bugnoli M, Petracca R, Burroni D, Macchia G, Massone A, Papini E, Xiang Z, Figura N, et al (1993). Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc Natl Acad Sci USA **90**: 5791-5795.

Covacci A, Falkow S, Berg DE, Rappuoli R (1997). Did the inheritance of a pathogenicity island modify the virulence of *Helicobacter pylori*? Trends Microbiol **5**: 205-208.

Cover TL, Blaser MJ (1992). Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. J Biol Chem **267**: 10570-10575.

Crabtree JE (1996). Immune and inflammatory response to *Helicobacter pylori* infection. Scand J Gastroenterol **31** (Supp 215): 3-10.

Crabtree JE, Covacci A, Farmery SM, Ziang Z, Tompkins DS, Perry S, Farmery SM, Xiang Z, Tompkins DS, Perry S, Lindley IJ, Rappuoli R (1995). *Helicobacter pylori* induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype. J Clin Pathol **48**: 41-45.

Crabtree JE, Farmery SM, Lindley IJ, Figura N, Peichl P, Tompkins DS (1994). CagA/cytotoxic strains of *Helicobacter pylori* and interleukin-8 in gastric epithelial cell lines. J Clin Pathol **47**: 945-950.

Crabtree JE, Taylor JD, Wyatt JI, Heatley RV, Shallcross TM, Tompkins DS, Rathbone BJ (1991a). Mucosal IgA recognition of *Helicobacter pylori* 120 kDa protein, peptic ulceration, and gastric pathology. Lancet **338**: 332-335.

Crabtree JE, Shallcross TM, Heatley RV, Wyatt JI (1991b). Mucosal tumor necrosis factor-alpha and interleukin-6 in patients with *Helicobacter pylori* associated gastritis. Gut **32**: 1473-1477.

Crabtree JE, Wyatt JI, Sobala GM, Miller G, Tompkins DS, Primrose JN, Morgan AG (1993). Systemic and mucosal humoral responses to *Helicobacter pylori* in gastric cancer. Gut **34**: 1339-1343.

Crowe SE, Alvarez L, Dytoc M, Hunt RH, Muller M, Sherman P, Patel J, Jin X, Ernst PB (1995). Expression of interleukin 8 and CD54 by human gastric epithelium after *Helicobacter pylori* infection *in vitro*. Gastroenterology **108**: 65-74.

Davies N, Ghosh S, Simmons DL, Tempst P, Liou HC, Baltimore D, Boso HJ (1991). Rel-associated pp40: an inhibitor of the rel family of transcription factors. Science **253**: 1268-1271.

Davies GR, Rampton DS (1994). *Helicobacter pylori*, free radicals and gastroduodenal disease. Eur J Gastroenterol Hepatol **6**: 1-10.

Davies GR, Simmonds NJ, Stevens TR, Grandison A, Blake DR, Rampton DS (1992). Mucosal reactive oxygen metabolites production in duodenal ulser disease. Gut **33**: 1467-1472.

Davies GR, Simmonds NJ, Stevens TR, Sheaff MT, Banatvala N, Laurenson IF, Blake DR, Rampton DS (1994). *Helicobacter pylori* stimulates antral mucosal reactive oxygen metabolite production in vivo. Gut **35**: 179-185.

Day ML, Wu S, Blaser JW (1993). Prostatic nerve growth factor inducible A gene binds a novel element in the retinoblastoma gene promoter. Cancer Res **53**: 5597-5599.

de Belle I, Huang RP, Fan Y, Liu C, Mercola D, Adamson ED (1999). p53 and Egr-1 additively suppress transformed growth in HT1080 cells but Egr-1 counteracts p53-dependent apoptosis. Oncogene **18**: 3633-3642.

de Grado M, Rosenberger CM, Gauthier A, Vallance BA, Finlay BB (2001). Enteropathogenic Escherichia coli infection induces expression of the early growth response factor by activating mitogen-activated protein kinase cascades in epithelial cells. Infect Immun **69**: 6217-24

de Grendele CH, Estess P, Siegelman MH (1996). Requirement for CD44 in activated T cell extravasation into an inflammatory site. Science **278**: 672-275.

de Martin R, Vanhove B, Cheng Q, Hofer E, Csizmadia V, Winkler H, Bach FH (1993). Cytokine-inducible expression in endothelial cells of an IκB-α-like gene is regulated by NF-κB. EMBO J **12**: 2773-2779.

Decker E, Sherka C, Zipfel P (1998). The early growth response protein (Egr-1) regulates interleukin-2 transcription by synergistic interactions with the nuclear factor of activated T cells. J Biol Chem **273**: 26923-26930.

D'Elios MM, Manghetti M, Almerigogna F, Amedei A, Costa F, Burroni D, Baldari CT, Romagnani S, Telford JL, Del Prete G (1997). Different cytokine profile and antigen-specificity repertoire in *Helicobacter pylori*-specific T cell clones from the antrum of chronic gastritis patients with or without peptic ulcer. Eur J Immunol **27**: 1751-1755.

Delhase M, Hayakawa M, Chen Y, Karin M (1999). Positive and negative regulation of IκB kinase activity through IKKβ subunit phosphorylation. Science **284**: 309-313.

Del Prete G (1998). The concept of type 1 and type II helper T cells and their cytokines in humans. Intern Rev Immunol **16**: 427-455.

Deschner EE, Winawer SJ, Lipkin M (1972). Patterns of nucleic acid and protein synthesis in normal gastric mucosa and atrophic gastritis. J Natl Cancer Inst **48**: 1567-1574.

Dick LR, Cruikshank AA, Grenier L, Melandri FD, Nunes SL, Stein RL (1996). Mechanistic studies on the inactivation of the proteasome by lactacystein. J Biol Chem **271**: 7273-7276.

Dinkel A, Aicher WK, Haas C, Zipfel PF, Peter H-H, Eibel H (1997). Transcription factor Egr-1 activity down-regulates Fas and CD23 expression in B cells. J Immunol **159**: 2678-2684.

Di Tommaso A, Xiang Z, Bugnoli M, Pileri P, Figura N, Bayeli PF, Rappuoli R, Abrignani S, De Magistris MT (1995). *Helicobacter pylori*-specific CD4+ T-cell clones from peripheral blood and gastric biopsies. Infect Immun **63**: 1102-1106.

Dixon MF (1991). *Helicobacter pylori* and peptic ulceration: histopathological aspects. J Gastroenterol Hepatol **6**: 125-130.

Doenges JL (1938). Spirochates in the gastric glands of Macacus rhesus and humans without definite history of related disease. Proc Soc Exp Med Biol **38**: 536-538.

Dooley CP (1991). *Helicbacter pylori*: Review of research findings. Aliment Pharmacol Ther **5**: 129-143.

Dooley CP, Cohen H, Fitzgibbons PL, Bauer M, Appleman MD, Perez-Perez GI, Blaser MJ (1989). Prevalence of *Helicobacter pylori* infection and histological gastritis in asymptomatic persons. N Engl J Med **321**: 1562-1566.

Dougherty GJ, Murdoch S, Hogg N (1988). The function of human intercellular adhesion molecule-1 (ICAM-1) in the regulation of an immune response. Eur J Immunol **18**: 35-39.

Drake IM, Davies MJ, Mapstone NP, Dixon MF, Schorah CJ, White KL, Chalmers DM, Axon AT (1996). Ascorbic acid may protect against human gastric cancer by scavenging mucosal oxygen radicals. Carcinogenesis **17**: 559-562.

Drake IM, Mapstone NP, Schorah CJ, White KL, Chalmers DM, Dixon MF, Axon AT (1998). Reactive oxygen species activity and lipid peroxidation in *Helicobacter pylori* asssociated gastritis: relation to gastric mucosal ascorbic acid concentration and effect of *H. pylori* eradication. Gut **42**: 768-771.

Dröge W, Eck HP, Mihm S (1992). HIV-induced cysteine deficiency and T-cell dysfunction-a rationale for treatment with N-acetylcysteine. Immunol Today **13**: 211-214.

Drumm B, Perez-Perez GI, Blaser MJ, Sherman PM (1990). Intrafamilial clustering of *Helicobacter pylori* infection. N Engl J Med **322**: 359-363.

Du B, Fu C, Kent KC, Bush H Jr., Schulick AH, Kreiger K, Collins T, McCaffrey TA (2000). Elevated Egr-1 in human atherosclerotic cells transcriptionally represses the transforming growth factor-β type II receptor. J Biol Chem **275**: 39039-39047.

Dunn BE (1993). Pathogenic mechanisms of *Helicobacter pylori*. Gastroenterol Clin N America **22**: 43-57.

Duyao MP, Buckler AJ, Sonenshem GE (1990). Interaction of an NF-κB-like factor with a site upstream of the C-myc promoter. Proc Natl Acad Sci USA **87**: 4727-4732.

Eaton KA, Morgan DR, Brooks C, Krakowka S (1989). Essential role of urease in the pathogensis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infec Immun **59**: 2470-2475.

El-Kaissouni J, Bene MC, Faure GC (1998). Activation of epithelial cells in gastritis. Digestion **59**: 53-59.

El-Omar EM (2001). The importance of interleukin 1 beta in *Helicobacter pylori* associated disease. Gut **48**: 743-747.

El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr, Rabkin CS (2000). Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature **404**: 398-402.

El-Omar EM, Penman I, Dorrian CA, Ardill JE, McColl KE (1993). Eradicating *Helicobacter pylori* infection lowers gastrin-mediated acid secretion by two-thirds in duodenal ulcer patients Gut **34**: 1060-1065.

Engstrand L, Gustavsson S, Jorgensen A, Schwan A, Scheynius A (1990). Inoculation of barrier-born pigs with *Helicobacter pylori*: a useful animal model for gastritis type B. Infect Immun **58**: 1763-1768.

Ericson ML, Horling J, Wendel-Hansen V, Holmgren A, Rosen A (1992). Secretion of thioredoxin after in vitro activation of human B cells. Lymphokine Cytokine Res **11**: 201-207.

Esposito F, Agosti V, Morrane G, Morra F, Cuomo C, Russo T, Venuta S, Cimino F (1994). Inhibition of the differentiation of human myeloid cell lines by redox changes induced through glutathione depletion. Biochem J **301**: 649-653.

Esteves MI, Schrenzel MD, Marini RP, Taylor NS, Xu S, Hagen S, Feng Y, Shen Z, Fox JG (2000). *Helicobacter pylori* gastritis in cats with long-term natural infection as a model of human disease. Am J Pathol **156**: 709-721.

Evans DG, Evans DJ, Moulds JJ, Graham DY (1988). N-acetylneuraminyllactose-binding fibrillar hemagglutinin of *Campylobacter pylori*: a putative colonization factor antigen. Infect Immun **56**: 2898-2906.

Everett SM, Drake IM, White KL, Mapstone NP, Chalmers DM, Schorah CJ, Axon AT (2002). Antioxidant vitamin supplements do not reduce reactive oxygen species actiivty in *Helicobacter pylori* gastritis in the short term. Br J Nutr **87**: 3-11.

Fan XJ, Chua A, O'Connell MA, Kelleher D, Keeling PW (1993). Interferon-gamma and tumor necrosis factor production in patients with *Helicobacter pylori* infection. Ir J Med Sci **162**: 408-411.

Fan XG, Fan XJ, Xia HX, Keeling PWN, Kelleher D (1995a). Upregulation of CD44 and ICAM-1 expression on gastric epithelial cells by *H. pylori*. APMIS **103**: 744-748.

Fan XG, Kelleher D, Fan XJ, Xia HX, Keeling PWN (1996a). *Helicobacter pylori* increases proliferation of gastric epithelial cells. Gut **38**: 19-22.

Fan XJ, Long A, Fan XG, Keeling PWN, Kelleher D (1995b). Adhesion molecules expression on gastric intraepithelial lymphocytes of patients with *Helicobacter pylori* infection. Eur. J. Gastroenterol Hepatol **7**: 541-546.

Fan X, Long A, Goggini M, Fan X, Kelling PW, Kelleher D (1996b). Expression of CD44 and its variants on gastric epithelial cells of patients with *Helicobacter pylori* colonization. Gut **38**: 507-512.

Feliciello I, Chinali G (1993). A modified alkaline lysis method for the preparation of highly purified plasmid DNA from *Escherchia coli*. Anal Biochem **212**: 394-401.

Figura, N, Guglielmetti, P., Rossolini A, Barberi A, Cusi G, Musmanno RA, Russi M, Quaranta S (1989). Cytotoxin production by *Campylobacter pylori* strains isolated from patients with peptic ulcers and from patients with chronic gastritis only. J Clin Microbiol **27**: 225-226.

Figura N, Vindigni C, Covacci A, Presenti L, Burroni D, Vernillo R, Banducci T, Roviello F, Marrelli D, Biscontri M, Kristodhullu S, Gennari C, Vaira D (1998). CagA positive and negative *Helicobacter pylori* strains are simultaneously present in the stomach of most patients with non-ulcer dyspepsia: relevance to histological damage. Gut **42**: 772-778.

Finco TS, Baldwin AS (1995). Mechanistic aspects of NF-kappa B regulation: the emerging role of phosphorylation and proteolysis. Immunity **3**: 263-272.

Finco TS, Beg AA, Baldwin AS, Jr (1994). Inducible phosphorylation of I kappa B alpha is not sufficient for its dissociation from NF-kappa B and is inhibited by protease inhibitors. Proc Natl Acad Sci USA **91**: 11884-11888.

Fitzgerald KA, O'Neill LA (1999). Characterization of CD44 induction by IL-1: a critical role for Egr-1. J Immunol **162**: 4920-4927.

Fox SB, Fawcett J, Jackson DG, Collins I, Gatter KC, Harris AL, Gearing A, Simmons DL (1994). Normal human tissues, in addition to some tumors, express multiple different CD44 isoforms. Cancer Res **54**: 4539-4546.

Franzoso G, Bours V, Park S, Tomita YM, Kelly K, Siebenlist U (1992). The candidate oncoprotein Bcl-3 is an antagonist of p50 NF-κB-mediated inhibition. Nature **359**: 339-342.

Fu ZF, Weihe E, Zheng YM, Schafer MK, Sheng H, Corisdeo S, Rauscher FJ 3rd, Koprowshi H, Dietzschold B (1993). Differential effects of Rabies and borna disease viruses on immediate-early- and late-response gene expression in brain tissues. J Virol **67**: 6674-6681.

Furuta T, Baba S, Takashima M, Futami H, Arai H, Kajimura M, Hanai H and Kaneko E (1998). Effect of *Helicobacter pylori* infection on gastric juice pH. Scand J Gastroenterol **33**: 357-363.

Galter D, Mihm S, Droge W (1994). Distinct effects of glutathione disulphide on the nuclear transcription factor kappa B and the activator protein-1. Eur J Biochem **221**: 639-648

Ganchi PA, Sun SC, Greene WC, Ballard DW (1992). I kappa B/MAD-3 masks the nuclear localization signal of NF-kappa B p65 and requires the transactivation domain to inhibit NF-kappa B p65 DNA binding. Mol Biol Cell **3**: 1339-1352.

Gashler A, Sukhatme VP (1995). Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors. Prog Nucleic Acid Res Mol **50**: 191-224.

Gashler AL, Swaminathan S, Sukhatme VP (1993). A novel repression module, an extensive activation domain, and a bipartite nuclear localization signal defined in the immediate-early transcription factor Egr-1. Mol Cell Biol **13**: 4556-4571.

Ghosh S, May MJ, Kopp EB (1998). NF-kappa B and Rel protein: evoluationarily conserved mediators and immune responses. Annu Rev Immunol **16**: 225-260.

Gillen D, El-Omar EM, Wirz AA, Ardill JE, McColl KE (1998). The acid response to gastrin distinguishes duodenal ulcer patients from *Helicobacter pylori*-infected healthy subjects. Gastroenterology **114**: 50-57.

Gilvarry J, Buckley MJ, Beattie S, Hamilton H, O'Morain CA (1997). Eradication of *Helicobacter pylori* affects symptoms in non-ulcer dyspepsia. Scand J Gastroenterol **32**: 535-540.

Ginn-Pease ME, Whisler RL (1998). Redox signals and NF-κB activation in T cells. Free Rad Biol Med **25**: 346-361.

Gleason FK, Holmgren A (1988). Thioredoxin and related proteins in procaryotes. FEMS Microbiol Rev **4**: 271-97.

Glocker E, Lange C, Covacci A, Bereswill S, Kist M, Pahl HL (1998). Proteins encoded by the cag pathogenicity island of *Helicobacter pylori* are required for NF-κB activation. Infect Immun **66**: 2346-2348.

Goodwin CS, Armstrong JA, Chilvers T, Peters M, Collins DM, Sly LI, McDonnell W, Harper WES (1989). Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to Helicobacter gen. nov. as *Helicobacter pylori comb. nov.* and *Helicobacter mustelae comb. nov.*, respectively. Int J Syst Bacteriol **39**: 397-405.

Gottlicher M, Heck S, Herrlich P (1998). Transcriptional cross-talk, the second mode of steroid hormone receptor actions. J Mol Med **76**: 480-489.

Graham DY (1989). *Campylobacter pylori* and peptic ulcer disease. Gastroenterology **96**: 615-625.

Graham DY, Alpert LC, Smith JL, Yoshimura HH (1988). Iatrogenic *Campylobacter pylori* infection is a cause of epidemic achlohydria. Am J Gastroenterol **83**: 974-980.

Graham GY, Lidsky MD, Cox AM, Evans DJ, Jr, Evans DG, Alpert L, Klein PD, Sessoms SL, Michaletz PA, Saeed ZA (1991). Long-term non steroidal-anti-inflammatory drug use and *Helicobacter pylori* infection. Gastroenterology **100**: 1653-1657.

Graham DY, Go MF, Lew GM, Genta RM, Rehfeld JF (1993). *Helicobacter pylori* infection and exaggerated gastrin release. Effects of inflammation and progastrin processing. Scand J Gastroenterol **28**: 690-694

Graham DY, Lew GM, Klein PD, Evans DG, Evans DJ, Jr, Saeed ZA, Malaty HM (1992). Effect of treatment of *Helicobacter pylori* infection on the long-term recurrence of gastric or duodenal ulcer. A randomized, controlled study. Ann Inter Med **116**: 705-708.

Graham DY, Opekun A, Lew GM, Evans DJ Jr, Klein PD, Evans DG (1990). Ablation of exaggerated meal-stimulated gastrin release in duodenal ulcer patients after clearance of *Helicobacter (Campylobacter) pylori* infection. Am J Gastroenterol **85**: 394-398.

Griffiths B (1992). Scaling-up of animal cell cutures. In: Animal cell culture- a practical approach (2nd ed). Freshney RI. (ed). Oxford, University Press, New york, USA.

Grilli M, Jason J-S, Lenardo MJ (1993). NF-κB and Rel: participatants in a multiform transcriptional regulatory system. Int Rev Cytol **143**: 1-62.

Grippo JF, Tienrungroj W, Dahmer MK, Housley PR, Pratt WB (1983). Evidence that the endogenous heat-stable glucocorticoid receptor-activating factor is thioredoxin. J Biol Chem **258**: 13658-13664.

Guha M, O'Connell MA, Pawlinski R, Hollis A, Patricia M, Yan S-F, Stein D, Mackman N (2001). Lipopolysaccharide activation of the MEK-ERK1/2 pathway in human monocytic cells mediates tissue factor and tumor necrosis factor α expression by inducing Elk-1 phosphorylation and Egr-1 expression. Blood **98**: 1429-1439.

Hallahan DE, Sukhatme VP, Sherman ML, Virudachalam S, Kufe D, Weichselbaum RR (1991). Protein kinase C mediates x-ray inducibility of nuclear signal transducers Egr-1 and Jun. Proc Natl Acad Sci USA **88**: 2156-2160.

Hansson LE, Nyren O, Bergstrom R, Wolk A, Lindgren A, Baron J, Adami HO (1994). Nutrients and gastric cancer risk. A population-based case-control study in Sweden. Int J Cancer **57**: 638-644.

Harakeh S, Jariwalla RJ, Pauling L (1990). Suppression of human immunodeficiency virus replication by ascorbate in chronically and acutely infected cells. Proc Natl Acad Sci USA **87**: 7245-7249.

Harlan JM, Liu DY (1992). In: Adhesions, its role in inflammatory disease. New York: Freeman WH and company.

Harlow E, Lane D (1988). Antibodies-a laboratory manual. Cold Spring Harbour Laboratory, New York, USA.

Haskill S, Beg AA, Tompkins SM, Morris JS, Yurachka AD, Sampson JA, Modal K, Ralph P, Baldwin AJ (1991). Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell **65**: 1281-1289.

Hatz RA, Brooks WP, Kramling H-J, Enders G (1992). stomach immunology and *Helicobacter pylori* infection. Curr Opin Gastroenterol **8**: 993-1001.

Hatz RA, Rieder G, Stalte M, Bayerdorffer E, Meimarakis G, Schildberg FW, Enders G (1997). Pattern of adhesion molecule expression on vascular endothelium in Helicobacter pylori-associated antral gastritis. Gastroenterology **112**: 1908-1918.

Hay RJ (1992). Cell line characterization and preservation. In: Animal cell culture-a practical approach, (2nd ed). Freshney RI (ed). Oxford University Press, New York, USA.

Hayashi T, Ueno Y, Okamoto T (1993). Oxidoreductive regulation of nuclear factor kappa B. Involvement of a cellular reducing catalyst thioredoxin. J Biol Chem **268**: 11380-11388.

Haynes BF, Hale LP, Patton KL, Martin ME, McCallum RM (1991). Measurement of an adhesion molecule as an indicator of inflammatory disease activity: up-regulation of the receptor for hyalurnate (CD44) in rheumatoid arthritis. Arthritis Rheum **34**: 1434-1443.

Haynes BF, Telen MJ, Hale LP, Denning SM (1989). CD44: a molecule involved in leukocyte adherence and T cell activation. Immunol Today **10**: 423-428.

Hazell SL, Evans DJ Jr, Graham DY (1991). *Helicobacter pylori* catalase. J Gen Microbiol **137**: 57-61

Hazell SL, Lee A, Brady L, Hennessy W (1986). *Campylobacter pyloridis* and gastritis; association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. J Infect Dis **153**: 658-663.

Henkel T, Machleidt T, Alkalay F, Kronke M, Ben-Neriah Y, Baeuerle PA (1993). Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. Nature **365**: 182-185.

Hessey SJ, Spencer J, Wyatt JI, Sobala G, Rathbone BJ, Axon AT, Dixon MF (1990). Bacterial adhesion and disease activity in *Helicobacter* associated chronic gastritis. Gut **31**: 134-138.

Higashi H, Tsutsumi R, Muto S, Sugiyama T, Azuma T, Asaka M, Hatakeyama M (2002). SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. Science **295**: 683-686.

Higuchi K, Arakawa T, Uchida T, Nakagawa K, Nakamura S, Matsumoto T, Fukudo T, Kabayashi S, Kuroki T (1997). In situ expression of cell adhesion molecules in chronic gastritis with *Helicobacter pylori* infection. J Clin Gastroenterol **25**: 5215-5221.

Hinz M, Krappmann D, Eihten A, Heder A, Scheidereit C, Strauss M (1999). NF-kappa B function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. Mol Cell Biol **19**: 2690-2698.

Hobbie S, Chen LM, Davis RJ, Galan JE (1997). Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella typhimurium* in cultured intestinal epithelial cells. J Immunol **159**: 5550-5559.

Hogarth L, English M, Price L, Wyllie R, Pearson ADJ, Hall AG (1996). The effect of treatment with high dose melphalan, cisplatin or carboplatin on levels of glutathione in plasma, erythrocytes, mononuclear cells and urine. Cancer Chemother Pharmacol **37**: 479-485.

Hollstein M, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sorlie T, Hovig E, Smith-Sorensen B, Montesano R, Harris CC (1994). Database of *p53* gene somatic mutations in human tumors and cell lines. Nucleic Acids Res **22**: 3551-3555.

Holmgren A (1981). Regulation of ribonucleotide reductase. Curr Top Cell Regul **19**: 47-76.

Holmgren A (1985). Thioredoxin. Annu Rev Biochem **54**: 237-271.

Holmgren A (1988). Thioredoxin and glutaredoxin: small multi-functional redox proteins with active-site disulphide bonds. Biochem Soc Trans **16**: 95-106.

Hopkins RJ, Vial PA, Ferreccio C, Ovalle J, Prado P, Sotomayor V, Russell RG Wasserman SS, Morris JG, Jr (1993). Seroprevalence of *Helicobacter pylori* in Chile: Vegetables may serve as one route of transmission. J Infect Dis **168**: 222-226.

Houghton J, Korah RM, Condon MR, Kim KH (1999). Apoptotic in *Helicobacter pylori*-asssociated gastric and duodenal ulcer disease is mediated via the Fas antigen pathway. Dig Dis Sci **44**: 465-478.

Hoyos B, Ballard DW, Böhnlein E, Siekevitz M, Greene WC (1989). Kappa B-specific DNA binding proteins: role in the regulation of human interleukin-2 gene expression. Science **244**: 457-460.

Hsu SM, Raine L, Fanger H (1981). Use of Avidin-Biotin-Peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem **29**: 577-580.

Hsu H, Shu HB, Pan MG, Goeddel DV (1996). TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell **84**: 513-521.

Hu Y, Baud V, Delhase M, Zhang P, Deerinck T, Ellisman M, Johnson R, Karin M (1999). Abnormal morphogenesis but intact IKK activation in mice lacking the IKK subunit of IκB kinase. Science **284**: 316-320.

Hu RM, Levin ER (1994). Astrocyte growth is regulated by neuropeptides through Tis 8 and basic fibroblast growth factor. J Clin Invest **93**: 1820-1827.

Huang RP, Adamson ED (1993). Characterization of the DNA-binding properties of the early growth response-1 (egr-1) transcription factor: evidence for modulation by a redox mechanism. DNA Cell Biol **12**: 265-273.

Huang RP, Adamson ED (1995). A biological role for Egr-1 in cell survival following ultra-violet irradiation. Oncogene **10**: 467-475.

Huang RP, Darland T, Okamura D, Mercola D, Adamson ED (1994). Suppression of v-sis-dependent transformation by the transcription factor, Egr-1. Oncogene **9**: 1367-1377.

Huang RP, Fan Y, deBelle I, Ni Z, Matheny W, Adamson ED (1998). Egr-1 inhibits apoptosis during the UV response: correlation of cell survival with Egr-1 phosphorylation. Cell Death Differ **5**: 96-106.

Huang RP, Fan Y, Ni Z, Mercola D, Adamson E (1997). Reciprocal modulation between SP1 and Egr1. J Cell Biochem **66**: 489-499.

Huang J, Keeling PW, Smyth CJ (1992). Identification of erythrocyte-binding antigens in *Helicobacter pylori*. J Gen Microbiol **138**: 1503-1513.

Huang RP, Liu C, Fan Y, Mercola D, Adamson ED (1995). Egr-1 negatively regulates human tumor cell growth via the DNA-binding domain. Cancer Res **55**: 5054-5062.

Huang RP, Peng A, Hossain MZ, Fan Y, Jagdale A, Boynton AL (1999). Tumor promotion by hydrogen peroxide in rat liver epithelial cells. Carcinogenesis **20**: 485-492.

Hudson L, Hay FC (1976). Cell counts with a haemocytometer. In: Practical Immunology. Hudson L and Hay FC (eds). Blackwell Scientific Publications, Oxford, UK. pp 32-33.

Huesca M, Borgia S, Hoffman P, Lingwood CA (1996). Acidic pH changes receptor binding specificity of *Helicobacter pylori*: a binary adhesion model in which surface heat shock (stress) proteins mediate sulfatide recognition of host cell proteins. Infect Immun **64**: 2643-2648.

Hunt RH (1992). Spontaneous hypochlorhydria. In: *Campylobacter pylori* and gastroduodenal disease. Rathbone BJ and Heatley RV (eds) (2nd ed). Oxford, Blackwell Scientific. pp 176-183.

Inoue J, Kerr LD, Kakizuka A, Verma IM (1992). IκB gamma, a 70 kDa protein identical to the C-terminal half of p110NF-κB: a new member of the IκB family. Cell **68**: 1109-1121.

International Research Agency on Cancer (1994). Schistosomes, liver flukes and *Helicobacter pylori*. IARC monographs on the evaluation of carcinogenesis risks in humans. Lyon. IARC 61.

Jablonowski H, Hengels KJ, Kraemer N, Geis G, Opferkuch W, Strohmeyer G (1994). Effects of *Helicobacter pylori* on histamine and carbachol stimulated acid secretion by human parietal cells. Gut **35**: 755-757.

Jankowski J, Bridges AB, Scott N (1991). Circulation free-radical markers and peptic ulcer disease. Eur J Gastroenterol Hepatol **3**: 823-828.

Janssen YMW, Van Houten B, Borm PJA, Mossman BT (1993). Cell and tissue responses to oxidative damage. Lab Invest **69**: 261-274.

Jarosz M, Dzieniszewski J, Dabrowska-Ufniarz E, Wartanowicz M, Ziemlanski S, Reed PI (1998). Effects of high dose vitamin C treatment on *Helicobacter pylori* infection and total vitamin C concentration in gastric juice. Eur J Cancer Prev **7**: 449-454.

Jobin C, Sartor B (2000). The IκB/NF-κB system: a key determinant of mucosal inflammation and protection. Am J Physiol Cell Physiol **278**: C451-C462.

Jones DM, Curry A (1992). The ultrastructure of *Helicobacter pylori*. In: *Helicobacter pylori* and gastroduodenal disease (2nd ed). Rathbone BJ, Heatley RV (eds). Blackwell Scientific Publication, Oxford, UK. pp 29-41.

Jones NL, Shannon PT, Cutz E, Yeger H, Sherman PM (1997). Increase in proliferation and apoptosis of gastric epithelial cells early in the natural history of *Helicobacter pylori* infection. Am J Pathol **151**: 1695-1703.

Jung M, Zhang Y, Lee S, Dritschilo A (1995). Correction of radiation sensitivity in a taxia telengiectasia by a trancated IκB-α. Science **268**: 1619-1621.

Kaiser S, Curthoys NP (1991). Effect of pH and bicarbonate on phosphoenolpyruvate carboxykinase and glutaminase mRNA levels in cultured renal epithelial cells. J Biol Chem **266**: 9397-9402.

Kalebic T, Kinter A, Poli G, Anderson ME, Meister A, Fauci AS (1991). Suppression of human immunodeficiency virus expression in chronically infected monocytic cells by glutathione, glutathione ester, and N-acetylcysteine. Proc Natl Acad Sci USA **88**: 986-990.

Karin M (1999). The beginning of the end: IκB kinase (IKK) and NF-κB activation. J Biol Chem **274**: 27339-27342.

Karita M, Morshed MG, Ouchi K, Okita K (1994). Bismuth-free triple therapy for eradicating *Helicobacter pylori* and reducing the gastric ulcer recurrence rate. Am J Gastroenterol **89**: 1032-1035.

Karttunen R, Karttunen T, Ekre HPT, MacDonald TT (1995). Interferon gamma and interleukin-4 secreting cells in the gastric antrum in *Helicobacter pylori* positive and negative gastritis. Gut **36**: 341-345.

Kaufmann SH, Schoel B, van Embden JD, Koga T, Wand-Wurttenberger A, Munk ME, Steinhoff U (1991). Heat-shock protein 60: Implications for pathogenesis of and protection against bacterial infection. Immunol Rev **121**: 67-90.

Kawano S, Tsujii M, Fusamoto H, Sato N, Kamada T (1991). Chronoc effect of intragastric ammonia on gastric mucosal structurs in rats. Dig Dis Sci **36**: 33-38.

Keates S, Hitti YS, Upton M, Kelly CP (1997). *Helicobacter pylori* infection activates NF-kappa B in gastric epithelial cells. Gastroenterology **113**: 1099-1109.

Kelleher D, Long A (1992). Development and characterization of a protein kinase C β-isozyme-deficient T-cell line. FEBS Letts **301**: 310-314.

Kelleher D, Windle H, Fan X (1997). Human immune response to *Helicobacter pylori* infection. In. Pathogenesis and host response in *Helicobacter pylori* infections. Moran AP and O'Morian CA (eds.). Normed Verlag, Hamburg, Germany. pp 148-158.

Kelly CP, Michetti P (1998). Pathogenesis of *Helicobacter pylori* infection. Curr Opin Gastroenterol **14**: 57-63.

Khachigian L, Linder L, Williams A, Collins T (1996). Egr-1-induced endothelial gene expression: a common theme in vascular injury. Science **271**: 1427-1431.

Kieran M, Blank V, Logeat F, Vandekerckhove J, Lottspeich F, Le Bail O, Urban MB, Kourilsky P, Baeuerle PA, Israel A (1990). The DNA binding subunit of NF-kappa B is identical to factor KBF1 and homologous to the rel oncogene product. Cell **62**: 1007-1018.

Kim S-J, Jeang K-T, Glick AB, Sporn MB, Roberts AB (1989). Promoter sequences of the human transforming growth factor-β1 gene responsive to transforming growth factor-β1 autoinduction. J Biol Chem **264**: 7041-7045.

Kirkland T, Viriyakosol S, Perez-Perez GI, Blazer MJ (1997). *Helicobacter pylori* lipopolysacchride can activate 70Z/3 cells via CD14. Infect Immun **65**: 604-608.

Klein PD, Graham DY, Gaillaur A, Opekun AR, Smith EO (1991). Water source as risk factor for Helicobacter pylori infection in Peruvian children. Lancet **337**: 1503-1506.

Knipp U, Birkholz S, Koup W, Opferkuch W (1993). Immune suppressive effects of *Helicobacter pylori* on human peripheral blood mononuclear cells. Med Microbiol Immunol **182**: 63-76.

Kobayashi H, Kamiya S, Suzuki T, Kohda K, Muramatsu S, Kurumada T, Ohta U, Miyazawa M, Kimura N, Mutoh N, Shirai T, Takagi A, Harasawa S, Tani N, Miwa T (1996). The effect of *Helicobacter pylori* on gastric acid secretion by isolated parietal cells from a guinea pig. Association with production of vacuolating toxin by *H. pylori*. Scand J Gastroenterol **31**: 428-433.

Kodama M, Fujioka T, Kodama R, Takahashi K, Kubota T, Murakami K, Nasu M (1998). p53 expression in gastric mucosa with *Helicobacter pylori* infection. J Gastroenterol Hepatol **13**: 215-219.

Köngten F, Grumont RJ, Strasser A, Metcalf D, Li R, Tarlinton D, Gerondakis S (1995). Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. Genes Dev **9**: 1965-1977.

Kopp E, Ghosh S (1994). Inhibition of NF-kappa B by sodium salicylate and aspirin. Science **265**: 956-959.

Kopp EB, Ghosh S (1995). NF-kappa B and rel proteins in innate immunity. Adv Immunol **58**: 1-27.

Koyama S (2000). Apoptotic depletion of infiltration mucosal lymphocytes associated with fas ligand expression by *Helicobacter pylori*-infected gastric mucosal epithelium: human glandular stomach as a site of immune privilege. Dig Dis Sci **45**: 773-780.

Kramer B, Meichle A, Hensel G, Charany P, Kranke M (1994). Characterization of an Krox-24/Egr-1-responsive element in the human tumor necrosis factor promoter. Biochim Biophys Acta **1219**: 413-421.

Krienitz W (1906). Ueber das Auftreten von Spirochaetne verschie-gener form im mogen-inhalt bei Carcinoma ventriculi. Dtsch Med Wochenscher **32**: 872.

Kumar S, Rabson AB, Gelinas C (1992). The RxxRxRxxC motif conserved in all Rel/kappa B proteins is essential for the DNA-binding activity and redox regulation of the v-Rel oncoprotein. Mol Cell Biol **12**: 3094-3106.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **277**: 680-685.

Lambert I, Clyne M, Drumm B (1993). *H. pylori* in dental plaques. Lancet **341**: 957.

Lambert JR (1993). The role of *Helicobacter pylori* in non-ulcer dyspepsia: a debate for. Gastroenterol Clin N Am **22**: 141-151.

Lambert JR, Lin SK, Arenda-Michel J (1995). *Helicobacter pylori.* Scand J Gastroenterol **208** (Supp 30): 33-46.

Lambert JR, Lin SK, Nicholson L, Sievert W, Sharma V, Kaldor J, Goulepis AG, Gust I (1990). High prevalence of *Helicobacter pylori* antibodies in institutionalized adults. Gastroenterology **98**: A74.

Lane DP (1992). Cancer. p53, guardian of the genome. Nature **358**: 15-16.

Langenberg M, Tytgat GN, Schipper MEI, Rietra PJGM, Zanen HC (1984). Campylobacter-like organisms in the stomach of patients and healthy individuals (Letter). Lancet **1**: 1348-1349.

Langton SR, Cesareo SD (1992). *Helicobacter pylori* associated phospholipase A_2 activity: a factor in peptic ulcer production? J Clin Pathol **45**: 221-224.

Lee A (1996). The nature of *Helicobacter pylori.* Scand J Gastroenterol **214** (Suppl 31): 5-8.

Lee JR, Koretzky GA (1998). Production of reactive oxygen intermediates following CD40 ligation correlates with c-Jun N-terminal kinase activation and IL-6 secretion in murine B lymphocytes. Eur J Immunol **28**: 4188-4197.

Lee SK, Singh J, Taylor RB (1975). Subclasses of T cells with different sensitivities to cytotoxic antibody in the presence of anaesthetics. Eur J Immunol **5**: 259-262.

Lemaire P, Revelant O, Bravo R, Charnay P (1988). Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. Proc Natl Acad Sci USA **85**: 4691-4695.

Lenardo MJ, Fan CM, Maniatis T, Baltimore D (1989). The involvement of NF-κB in β-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. Cell **57**: 287-294.

Levi S, Beardshall K, Haddad G, Playford R, Ghosh P, Calam J (1989). *Campylobacter pylori* and duodenal ulcers: the gastrin link. Lancet **1**: 1167-8.

Li Q, Lu Q, Hwang JY, Büsher D, Lee K-F, Izpisua-elmonte JC, Verma IM (1999). IKK1-deficient mice exhibit abnormal development of skin and skeleton. Genes&Dev **13**: 1322-1328.

Li N, Karin M (1999). Is NF-kappa B the sensor of oxidative stress. FASEB J **13**: 1137-1143.

Lim CP, Jain N, Cao Y (1998). Stress-induced immediate-early gene, egr-1 involves activation of p38/JNK1. Oncogene **16**: 2915-2926.

Lim RW, Varnum BC, Herschman HR (1987). Cloning of the tetradecanoyl phorbol ester-induced "primary response" sequenses and their expression in density-arrested swiss 3T3 cells and a TPA non-proliferative variant. Oncogene **1**: 263-270.

Ling L, Cao Z, Goeddel DV (1998). NF-κB-inducing kinase activates IKK-alpha by phosphorylation of Ser-176. Proc Natl Acad Sci USA **95**: 2791-2797.

Lingwood CA, Wasfy G, Han H, Huesca M (1993). Receptor affinity purification of a lipid-binding adhesin from *Helicobacter pylori*. Infect Immun **61**: 2474-2478.

Link E, Kerr LD, Schreck R, Zabel U, Verma I, Baeuerle PA (1992). Purified IκB-β is inactivated upon dephosphorylation. J Biol Chem **267**: 239-246.

Li-Weber M, Laur O, Krammer PH (1999). Novel Egr/NF-AT composite sites mediate activation of the CD95 (APO-1/Fas) ligand promoter in response to t cell stimulation. Eur J Immunol **29**: 3917-3027.

Logan RP, Walker MM, Misiewicz JJ, Gummett PA, Karim QN, Baron JH (1995). Changes in the intragastric distribution of *Helicobacter pylori* during treatment with omeprazole. Gut **36**: 12-16.

Luck JM, Seth TN (1924). Gastric urease. Biochem J **18**: 1227-1231.

Macchia G, Massone A, Burroni D, Covacci A, Censini S, Rappuoli R (1993). The Hsp 60 protein of *Helicobacter pylori*: structure and immune response in patients with gastroduodenal diseases. Mol Microbiol **9**: 645-652.

Mackay CR, Terpe HJ, Stauder R, Marston WL, Stark JH, Gunthert U (1994). Expression and modulation of CD44 variant isoforms in humans. J Cell Biol **124**: 71-82.

Maeda S, Yoshida H, Ogura K, Mitsuno Y, Hirata Y, Yamaji Y, Akanuma M, Shiratori Y, Omata M (2000). *H. pylori* activates NF-kappaB through a signaling pathway involving IkappaB kinases, NF-kappaB-inducing kinase, TRAF2, and TRAF6 in gastric cancer cells. Gastroenterology **119**: 97-108.

Mai UEH, Perez-Perez GI, Wahl LM, Wahl SM, Blaser MJ, Smith PD (1991). Soluble surface proteins from *Helicobacter pylori* activate monocytes/macrophages by lipopolysaccharide-independent mechanism. J Clin Invest **87**: 894-900.

Mai UEH, Perez-Perez GI, Allen JB, Wahl SM, Blaser MJ, Smith PD (1992). Surface proteins from *Helicobacter pylori* exhibit chemotactic activity for human leukocytes and are present in gastric mucosa. J Exp Med **175**: 517-525.

Maltzman JS, Carman JA, Monroe JG (1996a). Role of EGR1 in regulation of stimulus-dependent CD44 transcription in B lymphocytes. Mol Cell Biol **16**: 2283-2294.

Maltzman JS, Carmen JA, Monroe JG (1996b). Transcriptional regulation of the *Icam-1* gene in antigen receptor- and phorbol-stimulated B lymphocytes: role for transcription factor Egr-1. J Exp Med **183**: 1747-1759.

Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB (1998). Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappa B and activated protein-1. J Biol Chem **273**: 13245-13254.

Marshall B (1983). Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet **1**: 1273-1275.

Marshall BJ (1996). Campylobacter pyloridis and gastritis. J Infect Dis **153**: 650-657.

Marshall BJ, Armstrong JA, McGechie DB, Glancy R (1985). Attempt to fufill Kock's postulates for *pyloric Campylobacter*. Med J Aust **142**: 436-439.

Marshall BJ, Barrett LJ, Prakash C, McCallum RW, Guerrant RL (1990). Urea protects *Helicobacter (Campylobacter) pylori* from the bactericidal effects of acid. Gastroenterology **99**: 697-702.

Marshall BJ, Warren JR (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet **1**: 1311-1315.

Marui N, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM (1993). Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. J Clin Invest **92**: 1866-1874.

Matthews JR, Wakasugi N, Virelizier JL, Yodoi J, Hay RT (1992). Thioredoxin regulates the DNA binding activity of NF-kappa B by reduction of a disulphide bond involving cysteine 62. Nucleic Acids Res **20**: 3821-3830.

Mauch F, Bode G, Ditschuneit H, Malfertheiner P (1993). Demonstration of a phospholipid-rich zone in the human gastric epithelium damaged by *Helicobacter pylori*. Gastroenterology **105**: 1698-1704.

McCord JM, Fridovich I (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem **244**: 6049-6055.

McMahan SB, Monroe JC (1996). The role of early growth response gene 1 (Egr-1) in regulation of the immune response. J Leukoc Biol **60**: 159-166.

McMahan SB, Monroe JG (1995). A ternary complex factor-dependent mechanism mediates induction of egr-1, through selective serum response elements following antigen receptor cross-linking in B-lymphocytes. Mol Cell Biol **15**: 1086-1093.

Mégraud F (1996). Diagnosis of *Helicobacter pylori* infection. Scand J Gastroenterol **214** (Suppl 31): 44-46.

Mégraud F, Bonnet F, Garnier M, Lamouliatte H (1987). Characterization of "*Campylobacter pyloridis*" by culture, enzymatic profile, and protein content. J Clin Microbial **25**: 597-599.

Mégraud F, Lamouliatte H (1992). *Helicobacter pylori* and duodenal ulcer evidence suggesting causation. Dig dis Sci **37**: 769-770.

Mendall MA, Goggin PM, Malineaux N, Levy J, Tossy T, Strachan D, Northfield TC (1992). Childhood living conditions and *Helicobacter pylori* seropositivity in adult life. Lancet **329**: 896-897.

Mercurio F, DiDonato JA, Rosette C, Karin M (1993). p105 and p98 presursor proteins play an active role in NF-kappa B-mediated signal transduction. Genes Dev **7**: 705-718.

Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Yaung DB, Barbosa M, Mann M, Manning A, Rao A (1997). IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. Science **278**: 860-866.

Meyer M, Schreck R, Baeuerle PA (1993). H_2O_2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1 in intact cells. EMBO J **12**: 2005-2015.

Meyer-ter-Vehn T, Covacci A, Kist M, Pahl HL (2000) .*Helicobacter pylori* activates mitogen-activated protein kinase cascades and induces expression of the proto-oncogenes c-fos and c-jun. J Biol Chem **275**: 16064-16072.

Mihm S, Ennen J, Pessara U, Kurth R, Drage W (1991). Inhibition of HIV-1 replication and NF-kappa B activity by cysteine and cysteine derivatives. AIDS **5**: 497-503.

Milbrandt J (1987). A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science **238**: 797-799.

Mirochnitchenko O, Inouye M (1996). Effect of overexpression of human Cu, Zn superoxide dismutase in transgenic mice on macrophage functions. J Immunol **156**: 1578-1586.

Mirvish SS (1994). Experimental evidence for inhibition of N-nitroso compound formation as a factor in the negative correlation between vitamin C consumption and the incidence of certain cancers. Cancer Res **54** (Suppl 7): 1948s-1951s.

Mirvish SS (1986). Effects of vitamin C and E on N-nitroso compound formation, carcinogenesis, and cancer. Cancer **58** (Suppl 8): 1842-1850.

Mitchell HM, Lee A, Carrick J (1989). Increased incidence of *Campylobacter pylori* infection in gastroenterologists: Further evidence of support person-to-person transmission of *C. pylori*. Scan J Gastroenterol **24**: 396-400.

Mobley HLT, Hu L-T, Foxall PA (1991). *Helicobacter pylori* urease: properties and role in pathogenesis. Scand J Gastroenterol **187** (Suppl 26): 39-46.

Mohamed AH, Hunt RH (1994). Inflammation and disease associated with *H. pylori*. Mucosal Immunology Update (Fall) **2**: 1

Mooney C, Keenan J, Munster D, Wilson I, Allardyce R, Bagshaw P, Chapman B, Chadwick V (1991). Neutrophil activation by *Helicobacter pylori*. Gut **32**: 853-857.

Moran AP, Linder B, Walsh EJ (1997). Structural characterization of the lipid A component of *Helicobacter pylori* rough-and smooth-form lipopolysacchrides. J Bacteriol **179**: 6453-6463.

Mori N, Wada A, Hirayama T, Parks TP, Stratowa C, Yamamoto N (2000). Activation of intercellular adhesion molecule 1 expression by *Helicobacter pylori* is regulated by NF-kappaB in gastric epithelial cancer cells. Infect Immun **68**: 1806-1814.

Mori M, Suzuki H, Suzuki M, Kai A, Miura S, Ishii H (1997). Catalase and superoxide dismutase secreted from *Heliocbacter pylori*. Helicobacter **2**: 100-105.

Moss SF, Alam S, Pou R, Wang S, Krajewski S, Reed JC, Holt PR (1997). Increased expression of the pro-apoptotic Bcl-2 homologue, BAK, in *H. pylori* infected gastric mucosa. Gastroenterology **112**: A225 (Abstract).

Moss S, Calam J (1992). *Helicobacter pylori* and peptic ulcers: the present position. Gut **33**: 289-292.

Moss SF, Calam J (1993). Acid secretion and sensitivity to gastrin in duodenal ulcer patients: effect of eradication of *H. pylori*. Gut **34**: 888-892.

Moss SF, Calam J, Wang S, Holt PG (1996). Induction of gastric epithelial apoptosis by *Helicobacter pylori*. Gut **38**: 498-501.

Moss SF, Legon S, Davies J, Calam J (1994). Cytokine gene expression in *Helicobacter pylori* associated antral gastritis. Gut **35**: 1567-1570.

Moynagh PN, Williams DC, O'Neill LA (1994). Activation of NF-kappa B and induction of vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 expression in human glial cells by IL-1. Modulation by antioxidants. J Immunol **153**: 2681-2690.

Münoz C, Pascual-Salcedo D, Castellanos MC, Alfranca A, Aragones J, Vara A, Redondo MJ, de Landazuri MO (1996). Pyrrolidine dithiocarbamate inhibits the production of interleukin-6, interleukin-8, and granulocyte-macrophage colony-stimulating factor by human endothelial cells in response to inflammatory mediators: modulation of NF-kappa B and AP-1 transcription factors activity. Blood **88**: 3482-3490.

Münzenmaier A, Lange C, Glocker E, Covacci A, Moran A, Bereswill S, Baeuerle AP, Kist M, Pahl HL (1997). a secreted/shed product of *Helicobacter pylori* activates transcription factor nuclear factor-kappa B. J Immunol **159**: 6140-6147.

Muthukkumar S, Nair P, Sells SF, Maddiwar NG, Jacob RJ, Rangnekar VM (1995). Role of EGR-1 in thapsigargin-inducible apoptosis in the melanoma cell line A375-C6. Mol Cell Biol **15**: 6262-6272.

Nabel GJ, Verma IM (1993). Proposed NF-kappa B/Ikappa B family nomenclature (Letter). Genes Dev **7**: 2063.

Nagarajan L, Lange B, Cannizzaro L, Finan J, Nowell PC, Huebner K (1990). Molecular anatomy of a 5q interstitial deletion. Blood **75**: 82-87.

Nair P, Muthukkumar S, Sells SF, Han SS, Sukhatme VP, Rangnekar VM (1997). Early growth response-1 dependent apoptosis is mediated by p53. J Biol Chem **272**: 20131-20138.

Nakamura H, Nakamura K, Yodoi J (1997). Redox regulation of cellular activation. Ann Rev Immunol **15**: 351-369.

Naumann M, Scheidereit C (1994). Activation of NF-kappa B *in vivo* is regulated by multiple phosphorylations. EMBO J **13**: 4597-4607.

Negrini R, Lisato L, Zanella I, Cavazzini L, Gullini S, Villanacci V, Poiesi C, Albertini A, Ghielmi S (1991). *Helicobacter pylori* infection induces antibodies cross-reacting with human gastric. Gastroenterology **101**: 437-445.

Neguyen H, Hoffman-Liebermann B, Liebermann D (1993). The zinc finger transcription factor Egr-1 is essential for and restricts differentiation along the macrophage lineage. Cell **72**: 197-209.

Neurath MF, Becker C, Barbulescu K (1998). Role of NF-κB in immune and inflammatory responses in the gut. Gut **43**: 856-860.

Neurath MF, Pettersson S, zum Buschenfelde K-HM, Straber W (1996). Local administration of antisense phosphorothioate oligonucleotide to the p65 subunit of NFκB abrogates established experimental colitis in mice. Nature Med **2**: 998-1004.

Nielsen H, Andersen LP (1992). Activation of human phagocyte oxidative metabolism by *Helicobacter pylori*. Gastroenterology **103**: 1747-1753.

Noach LA, Bosma NB, Jansen J, Hoek FJ, van Deventer SJ, Tytgat GN (1994). Mucosal tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 production in patients with *Helicobacter pylori* infection. Scand J Gastroenterol **29**: 425-429.

Nolan GP, Fujita T, Bhatia K, Huppi K, Liou H-C, Scott ML, Baltimore D (1993). The bcl-3 pro-oncogene encodes a nuclear IκB-like molecule that preferentially interacts with NF-κB p50 in a phosphorylation-dependent manner. Mol Cell Biol **13**: 3557-3566.

Nomura A, Stemmermann GN, Chyou PH, Kato I, Perez-Perez GI, Blaser MJ (1991). *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. N Engl J Med **325**: 1132-1136.

Nose K, Ohba M (1996). Functional activation of the egr-1 (early growth response-1) gene by hydrogen peroxide. Biochem J **316**: 381-383.

Nose K, Shibanuma M, Kikuchi K, Kageyama H, Sakiyama S, Kuroki T (1991). Transcriptional activation of early-response genes by hydrogen peroxide in mouse osteoblastic cell line. Eur J Biochem **201**: 99-106.

O'Connell MA, Cleere R, Long A, O'Neill LA, Kelleher D (1995). Cellular proliferation and activation of NF-kappa B are induced by autocrine production of tumor necrosis factor alpha in the human T lymphoma cell line Hut78. J Biol Chem **270**: 7399-7404.

O'Connor HJ (1992). *Helicobacter pylori* and gastric cancer. Eur J Gastroenterol Hepatol **4**: 103-109.

O'Connor HJ, Schorah CJ, Habibzedah N, Axon AT, Cockel R (1989). Vitamin C in the human stomach: relation to gastric pH, gastroduodenal disease, and possible sources. Gut **30**: 436-442.

Oehm A, Behrmann I, Falk W, Pawlita M, Maier G, Klas C, Li-Weber M, Richards S, Dhein J, Trauth BC, Panstingl H, Krammer PH (1992). Purification and cloning of the Apo-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. J Biol Chem **267**: 10709-10715.

Ohba M, Shibanuma M, Kuroki T, Nose K (1994). Production of hydrogen peroxide by transforming growth factor-beta1 and its involvement in induction of egr-1 in mouse osteoblastic cells. J Cell Biol **126**: 1079-1088.

Okamoto T, Ogiwara H, Hayashi T, Mitsui A, Kawabe T, Yodoi J (1992). Human thioredoxin/adult T cell leukemia-derived factor activates the enhancer binding protein of human immunodeficiency virus type 1 by thiol redox control mechanism. Int Immunol **4**: 811-819.

O'Morain C, Dettmer A, Rambow A, von Fritsch E, Fraser AG (1996). Double-blind, multicenter, placebo-controlled evaluation of clarithromycin and omeprazole for Helicobacter pylori-associated duodenal ulcer. Helicobacter **1**: 130-137.

O'Morain C, Gilvarry J (1993). Eradication of *Helicobacter pylori* in patients with non-ulcer dyspepsia. J Scand J Gastroenterol **196** (Supp 1): 30-33.

O'Toole D, Long A, Murphy AM, Bowie A, O'Neill LA, Weir DG, Kelleher D (1997). *Helicobacter pylori* induces expression of nuclear factor kappa B in gastric epithelial cells and N-acetylcysteine attenuates this induction. Gastroenterology **112** (Supp 1): A956.

Ormand JE, Talley NJ, Shorter RG, Conley CR, Carpenter HA, Fich A, Wilson WR, Phillips SF (1991). Prevalence of *Helicobacter pylori* in specific forms of gastritis. Further evidence supporting a pathogenic role for *H. pylori* in chronic non specific gastritis. Dig Dis Sci **36**: 142-145.

Orian A, Whiteside S, Israel A, Stancovski I, Schwartz AL, Ciechanover A (1995). Ubiquitin-mediated processsing of NF-κB transcriptional activator precursor p105. J Biol Chem **270:** 21707-21714.

Osborn L, Kunkel S, Nabel GJ (1989). Tumor necrosis factor alpha and interleukin1 stimulate the human immunodeficience virus enhancer by activation of the nuclear factor kappa B. Proc Natl Acad Sci USA **86**: 2336-2340.

Owen RJ (1995). Bacteriology of *Helicobacter pylori.* In: Bailliere's Clinical Gastrornterology: *Helicobacter pylori*. Calam J (ed) Vol. 9. pp 415-446.

Palombella VJ, Rando OJ, Goldberg AL, Maniatis T (1994). The ubiquitin proteasome pathway is required for theprocessing of NF-kappa B precursor and the activation of NF-kappa B. Cell **78**: 773-785.

Parkin DM, Laora E, Muir CS (1988). Estimates of the worldwide frequency of sixteen major cancers in 1980. Int J Cancer **41**: 184-197.

Parkin DM, Pisani P, Ferlay J (1993). Estimates of the worldwide incidence of eighteen major cancers in 1985. Int J Cancer **45**: 594-606.

Parsonnet J, Friedman GD, Orentreich N, Vogelman H (1997). Risk for gastric cancer in people with CagA positive or CagA negative *Helicobacter pylori* infection. Gut **40**: 297-301.

Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK (1991). *Helicobacter pylori* infection and the risk of gastric carcinoma. N Engl J Med **325**: 1127-1131.

Patchett S, Beattie S, Leen E, Keane C, O'Morain C (1992). *Helicobacter pylori* and duodenal ulcer recurrence. Am J Gastroenterol **87**: 24-27.

Perkins ND (2000). The Rel/NF-κB family: friend and foe. Trends Biochem Sci **25**: 434-440.

Piotrowski J, Piotrawski E, Skrodzka D, Slomiany A, Slomiany BL (1997). Induction of acute gastritis and epithelial apoptosis by *Helicobacter pylori* lipopolysacchride. Scand J Gastroenterol **32**: 203-211.

Pisani P, Parkin DM, Munoz N, Ferlay J (1997). Cancer and infection: estimates of the attributable fraction in 1900. Cancer Epidemiology, Biomarkers and Prevention **6**: 389-400.

Podolsky I, Lee E, Cohen R, Peterson WL (1989). Prevalence of *C. pylori* (CP) in healthy subjects and patients with peptic diseases. Gastroenterology **96**: A394.

Rachal MJ, Yoo H, Becker FF, Lapeyre JN (1989). In vitro DNA cytosine methylation of cis-regulatory elements modulates c-Ha-ras promoter activity *in vivo* Nucleic Acids Res **17**: 5135-5147.

Rademaker JW, Hunt RH (1991). *Helicobacter pylori* and gastric acid secretion: the ulcer link? Scand J Gastroenterol **26** (Supp 187): 71-77.

Rathbone BJ, Wyatt JI, Worsley BW, Shires SE, Trejdosiewicz LK, Heatley RV, Losowsky MS (1986). Systemic and local antibody responses to gastric *Campylobacter pyloridis* in non-ulcer dyspepsia. Gut **27**: 642-647.

Rauws EA, Langenberg W, Houthoff HJ, Zanen HC, Tytgat GN (1988). *Campylobacter pyloridis*-associated chronic active antral gastritis: A prospective study of its prevalence and the effects of antibacterial and antiulcer treatment. Gastroenterology **94**: 33-40.

Rauws EA, Langenberg W, Oudbier JH, Mulder CJJ, Tytgat GNJ (1989). Familial clustering of peptic ulcer disease colonized with *C. pylori* of the same DNA composition. Gastroenterology **96**: A409.

Rauws EA, Tytgat GN (1990). Cure of duodenal ulcer associated with eradication of *Helicobacter pylori*. Lancet **335**: 1233-1235.

Ray A, Prefantaine KE (1994). Physical association and functional antagonism between the p65 subunit of transcription factor NF-κB and the glucocorticoid receptor. Proc Natl Acad Sci USA **91**: 752-756.

Read MA, Neish AS, Luscinskas FW, Palombella VJ, Maniatis T, Collins T (1995). The proteasome pathway is required for cytokine-induced endothelial-leukocyte adhesion molecule expression. Immunity **2**: 493-506.

Reed PI (1999). Vitamin C, *Helicobacter pylori* infection and gastric carcinogenesis. Int J Vitam Nutr Res **69**: 220-227.

Régnier CH, Song HX, Gao X, Goeddel DV, Cao Z, Rothe M (1997). Identification and characterization of an IκB kinase. Cell **90**: 373-383.

Reichard P (1993). The anaerobic ribonucleotide reductase from *Escherichia coli*. J Biol Chem **268**: 8383-8386.

Rock Kl, Gramm C, Rothstein L, Clark K, Stein R, Dick L, Hwang D, Goldberg, AL (1994). Inhibitors of the proteasome block the degradation of peptides presented on MHC class I molecules. Cell **78**: 761-771.

Rogler G, Brand K, Vogl D, Page S, Hofmeister R, Andus T, Knuechel R, Baeuerle PA, Scholmerich J, Gross V (1998). Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterology **115**: 357-69.

Rollason TP, Stone J, Rhodes JM (1984). Spiral organisms in endoscopic biopsies of the human stomach. J Clin Pathol **37**: 23-26.

Rothwarf DM, Zandi E, Natali G, Karin M (1998). IKK-gamma is an essential regulatory subunit of the IKKB kinase complex. Nature **395**: 297-300.

Rudi J, Kolb C, Maiwald M, Kuch D, Zuna I, Vanherbay A, Galle PR, Stremmel W (1997). Serum antibodies against *Helicobacter pylori* proteins VacA and CagA are associated with increased risk for gastric carcinoma. Dig Dis Sci **42**: 1652-1659.

Rudi J, Strand S, Van Herbay A, Mariani SM, Krammer PH, Galle PR, Stremmel W (1998). Involvement of the CD95 (Apo-1/Fas) receptor and ligand system in *Helicobacter pylori*-induced gastric epithelial apoptosis. J Clin Invest **102**: 1506-1514.

Russo MW, Matheny C, Milbrandt J (1993). Transcriptional activity of the zinc finger protein NGFI-A is influenced by its interaction with a cellular factor. Mol Cell Biol **13**: 6858-6865.

Russo MW, Sevetson B, Milbrandt J (1995). Identification of NAB1, a repressor of NGFI-A and Krox 20 mediated transcription. Proc Natl Acad Sci USA **92**: 6873-6877.

Sambrook J, Gething MJ (1989). Protein structure. Chaperones, paperones. Nature **342**: 224-225.

Sarker SA, Gyr K (1992). Non-immunological defence mechanisms of the gut. Gut **33**: 987-993.

Sarosiek J, Peura DA, Guerrant RL, Marshall BJ, Laszewicz W, Gabryelewicz A, McCallum RW (1991). Mucolytic effects of *Helicobacter pylori*. Scand J Gastroenterol **26** (Supp 187): 47-55.

Scandalios JG (1997). Oxidative stress and the molecular biology of antioxidant defences. Cold Spring, Harbor Labortory, Cold Spring Harbr, NY.

Schenk H, Klein M, Erdbrugger W, Drage W, Schulze-Osthoff K (1994). Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-kappa B and AP-1. Proc Natl Acad Sci **91**: 1672-1676.

Schmid RM, Alder G (2000). NF-κB/Rel/IκB: Implications in gastrointestinal disease. Gastroenterology **118**: 1208-1228.

Schmidt A, Hennighauson L, Siebenlist U (1990). Inducible nuclear factor binding to the I kappa B elements of the human immunodeficiency virus enhancer in T cells can be blocked by cyclosporin A in a signal-dependent manner. J Virol **64**: 4037-4041.

Schmidt KN, Amstad P, Cerutti P, Baeuerle PA (1995). The roles of hydrogen peroxides and superoxide as messengers in the activation of transcription factor NF-kappa B. Chem Biol **2**: 13-22.

Schouton GJ, Vertegoal AC, Whiteside ST, Israel A, Toebes M, Dorsman JC, van-der-EB AJ, Zantema A (1997). IkappaB is a target for the mitogen-activated 90 kDa ribosomal S6 kinase. EMBO J **16**: 3133-3144.

Schreck R, Albermann K, Baeuerle PA (1992a). Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells. Free Rad. Res Comms **17**: 221-237.

Schreck R, Baeuerle PA (1994). Assessing oxygen radicals as mediators in activation of inducible eukaryotic transcription factor NF-kappa B. Methods Enzymol **234**: 151-163.

Schreck R, Meier B, Mannel DN, Drage W, Baeuerle PA (1992b). Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. J Exp Med **175**: 1181-1194.

Schreck R, Rieber P, Baeuerle PA (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. EMBO J **10**: 2247-2258.

Schreiber S, Nikolaus S, Hampe J (1998). Activation of nuclear factor kappa B in inflammatory bowel disease. Gut **42**: 477-84.

Schulze-Osthoff K, Bakker AC, Los M, Baeuerle PA (1995). Redox signalling by transcription factors NF-kappa B and AP-1 in lymphocytes. Biochem Pharmacol 50: 735-741.

Scott D, Weeks D, Melchers K, Sach G (1998). The life and death of *Helicobacter pylori*. Gut **43** (Supp 1): S56-S60.

Searle PF, Davison BL, Stuart GW, Wilkie TM, Norstedt G, Palmiter RD (1984). Regulation, linkage, and sequence of mouse metallothionein I and II genes. Mol Cell Biol **4**: 1221-1230.

Secreaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI (1992). Genimoic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc Natl Acad Sci. USA **89**: 12160-12164.

Segal ED, Falkow S, Tompkins LS (1996). *Helicobacter pylori* attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins. Proc Natl acad Sci USA **93**: 1259-1264.

Semeraro, N, Montemurro P, Piccoli C, Muolo V, Colucci M, Giuliani G, Fumarola D, Pece S, Moran AP (1996). Effect of *Helicobacter pylori* lipopolysacchride (LPS) and LPS derivatives on the production of tissue factor and plasminogen activator type 2 by human blood mononuclear cells. J Infec Dis **174**: 1255-1260.

Sen R, Baltimore D (1986). Inducibility of kappa immunoglobulin enhancer-binding protein NF-kappa B by a posttranslantional mechanism. Cell **47**: 921-928.

Seyfert VL, McMahan S, Glenn W, Cao X, Sukhatme VP, Monroe JG (1990). Egr-1 expression in surface Ig-mediated B cell activation; kinetics and asssociation with protein kinase C activation. J Immunol **145**: 3647-3653.

Seyfert VL, Sukhatme VP, Monroe JG (1989). Differential expression of a zinc finger-encoding gene in response to positive versus signalling through receptor immunoglobulin inmurine B-lymphocytes. Mol Cell Biol **9**: 2083-2088.

Sharma SA, Miller GG, Peek RA, Perez-Perez GI, Blaser BJ (1997). T-cell antibody, and cytokine response to homologs of the 60-kilodalton heat shock protein in *Helicobacter pylori* infection. Clin Diag Lab Immunol **4**: 440-446.

Sharma SA, Tummuru MKR, Blaser MJ, Kerr LD (1998). Activation of IL-8 gene expression by *Helicobacter pylori* is regulated by transcription factor nuclear factor-κB in gastric epithelial cells. J Immunol **160**: 2401-2407.

Sharma SA, Tummuru MKR, Miller GG, Blaser MJ (1995). Interleukin-8 response of gastric epithelium cell lines to *Helicobacter pylori* stimulation in vitro. Infect Immun **63**: 1681-1687.

Shimada T, Terano A (1998). Chemokine expression in *Helicobacter pylori*-infected gastric mucosa. J Gastroenterol **33**: 613-617.

Shimoyama T, Crabtree JE (1998). Bacterial factors and immune pathogenesis in *Helicobacter pylori* infection. Gut **43** (Supp 1): S2-S5.

Siebenlist U, Franzoso G, Brown K (1994). Structure, regulation and function of NF-kappa B. Annu Rev Cell Biol **10**: 405-455.

Silverman ES, Collins T (1999). Pathways of Egr-1 mediated gene transcription in vascular biology. Am J Pathol **154**: 665-670.

Silverman ES, Du J, de Sanctis GT, Radmark O, Samuelsson B, Drazen JM, Collins T (1998b). Egr-1 and SP1 interact functionally with the 5-Lipoxygenase promoter and its naturally occuring mutants. Am J Cell Mol Biol **19**: 316-323.

Silverman ES, Du J, Williams A, Wadgaonkar R, Drazen J, Collins T (1998a). cAMP-response-element-binding-protein-binding protein (CBP), and p300 are transcriptional co-activators of Egr-1. Biochem J **336**: 183-189.

Silverman ES, Khachigian LM, Linder V, Williams AJ, Collins T (1997). Inducible PDGFIA-chain transcription in smooth muscle cells is mediated by Egr-1 displacement of Spi and SP3. Am J Physiol **273**: H1415-H1426.

Skerka C, Decker EL, Zibfel PF (1995). A regulatory element in the human interleukin-2 gene promoter is a binding site fot the zinc finger protein Sp1 and Egr-1. J Biol Chem **270**: 22500-22506.

Slomiany BL, Bilski J, Sarasiek J, Murty VLN, Jdworkin B, Van Horn K, Zielenski J, Slomiany A (1987). *Campylobacter pyloridis* degrades mucin and undermines gastric mucosal integritey. Biochem Biophys Res Commun **144**: 307-314.

Smoot DT, Bobley HLT, Chippendale GR, Lewison JF, Resau JH (1990). *Helicobacter pylori* urease activity is toxic in human gastric epithelial cells. Infect Immun **58**: 1992-1994.

Sobala GM, Axon AT, Dixon MF (1992). Morphology of chronic antral gastritis: relationship to age, *Helicobacter pylori* status, and peptic ulceration. Eur J Gastroenterol Hepatol **4**: 825-829.

Sobala GM, Crabtree JE, Dixon MK, Schorah CJ, Taylor JD, Rathbone BJ, Heatley RV, Axon AT (1991). Acute *Helicobacter pylori* infection: clinical features, local and systemic immune response, gastric mucosal histology and gastric juice ascorbic acid concentrations. Gut **32**: 1415-1418.

Sobala GM, Schorah CJ, Shires S, Lynch DA, Gallacher B, Dixon MF, Axon AT (1993). Effect of eradication of *H. pylori* on gastric ascorbic acid concentrations. Gut **34**: 1038-1041.

Sobala GM, Schorah CJ, Sanderson M, Dixon MF, Tompkins DS, Godwin P, Axon AT (1989). Ascorbic acid in the human stomach. Gastroenterology **97**: 357-363.

Soll AH, Weinstein WM, Kurata J, McCarthy D (1991). Nonsteroidal anti-inflammatory drugs and peptic ulcer disease. Ann Intern Med **114**: 307-319.

Sozzi M, Valentini M, Figura N, De Paoli P, Tedeschi RM, Gloghini A, Serraino D, Poletti M, Carbone A (1998). Atrophic gastritis and intestinal metaplasia in *Helicobacter pylori* infection: the role of CagA status. Am J Gastroenterol **93**: 375-379.

Staal FJ, Roederer M, Herzenberg LA, Herzenberg LA (1990). Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. Proc Natl Acad Sci USA **87**: 9943-9947.

Steer HW (1975). Ultrastructure of cell migration through the gastric epithelium and its relationship to bacteria. J Clin Pathol **28**: 639-646.

Steer HW (1992). Ultrastructure of *Helicobacter pylori* in vivo. In: *Helicobacter pylori* and gastroduodenal disease (2nd ed). Rathbone BJ and Heatley RV (eds). Blackwell Scientific Publication, Oxford, UK. pp 42-50.

Stein M, Rappuoli R, Covacci A (2000). Tyrosine phosphorylation of the *Helicobacter pylori* CagA antigen after *cag*-driven host cell translocation. PNAS **97**: 1263-1268.

Strickland RG, Mackkay IR (1973). A reappraisal of the nature and significance of chronic atophic gastritis. Am J Dig Dis **18**: 426-440.

Sukhatme VP (1990). Early transcriptional events in cell growth: the Egr family. J Am Soc Nephrol **1**: 859-866.

Sukhatme VP, Cao X, Chang LC, Tsai-Morris CH, Stamenkovich D, Ferreira PC, Cohen DR, Edwards SA, Shows TB, Curran T (1988). A zinc finger-encoding gene coregulated with c-Fos during growth and differentiation, and after cellular depolarization. Cell **53**: 37-43.

Sukhatme VP, Kartha S, Toback FG, Taub R, Hoover RG, Tsai-Morris CHA (1987). novel early growth response gene rapidly induced by fibroblast, epithelial cell and lymphocyte mitogens. Oncogene Res **1**: 343-355.

Suzuki YJ, Mizuno M, Packer L (1994). Signal transduction for nuclear factor-κB activation. Proposed location of antioxidant inhibitable step. J Immunol **153**: 5008-5015.

Svaren J, Sevetson B, Apel ED, Zimonjic D, Popescu N, Milbrandt J (1996). NAB2, a corepressor of NGFI-A (Egr-1), and Krox 20, is induced by proliferative, and differentiative stimuli. Mol Cell Biol **16**: 3545-3553.

Swirnoff AH, Apel EP, Svaren J, Sevetson BR, Zimonjic DB, Popescu NC, Milbrandt J (1998). NAB1, a corepressor of NGFI-A (Egr-1), contains an active transcriptional repression domain. Mol Cell Biol **18**: 512-524.

Swirnoff AH, Mibrandt J (1995). NGFI-A and related zinc-finger transcription factors. Mol Cell Biol **15**: 2275-2287.

Szabo S, Khomenko T, Gambos Z, Deng XM, Jadus MR, Yoshida M (2000). Transcription factors and growth factors inulcer healing. Aliment Pharmacol Ther **14** (Supp 1): 33-43.

Takahashi S, Igarashi H, Nakamura K, Masubuchi N, Saito S, Aoyagi T, Itoh T, Hirata I (1993). *Helicobacter pylori* and urease activity--comparative study between urease positive and negative mutant strains. Nippon Rinsho **51**: 3149-3153.

Talley NJ (1990). Is *Helicobacter pylori* a cause of non-ulcer dyspepsia? In: Malfertheiner P and Ditschuneit H (eds.). *Helicobacter pylori*, Gastritis and peptic ulcer. Berlin: Springer-Verlag. pp 361-369.

Tang P, Sutherland CL, Gold MR, Finlay BB (1998). Listeria monocytogenes invasion of epithelial cells requires the MEK-1/ERK-2 mitogen-activated protein kinase pathway. Infect Immun **66**: 1106-1112.

Taniuchi T, Mortensen ER, Ferguson A, Greenson J, Merchant JL (1997). Overexpression of ZBP-89, a zinc finger DNA binding protein in gastric cancer. Biochem Biophys Res Commun **233**: 154-160.

Taylor DN, Blaser MJ (1991). The epidemiology of *Helicobacter pylori* infection. Epidemiol Rev **13**: 42-59.

Telford JL, Ghiara P, Dell'Orca M, Comanducci M, Burroni D, Bugnoli M, Tecce MF, Censini S, Covacci A, Xiang Z, et al (1994). Gene structure of *Helicobacter* cytotoxin and evidence of its key role in gastritis disease. J Exp Med **179**: 1653-1658.

Tewari M, Dobrzanski P, Mohn KL, Cressmann DE, Hsu J-C, Bravo R, Taub R (1992). Rapid induction in regenerating liver of RL/IF-1 (an IκB that inhibits NF-κB, RelB-p50, and c-Rel-p50) and PHF, a novel κB site binding complex. Mol Cell Biol **12**: 2898-2908.

The EUROGAST Study Group (1993). Epidemiology of, and risk factors for, *Helicobacter pylori* infection among 3194 asymptomatic subjects in 17 populations. Gut **343**: 1672-1676.

Thomas JE, Gibson GR, Darboe MK, Dale A, Weaver LT (1992). Isolation of *Helicobacter pylori* from human faeces. Lancet **340**: 1194-1195.

Tojima Y, Fujimoto A, Delhase M, Chen Y, hatakeyama S, Nakayama K, Kaneko Y, Nimura Y, Motoyama N, Ikeda K, Karin M, Nakanishi m (2000). NAK is an IkappaB kinase-activting kinase. Nature **404**: 778-782.

Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, Mckenney K, Fitzegerald LM, Lee N, Adams MD, Venter JC (1997). The complete genome sequence of the gastric pathogen *Helicobacter*. Nature **388**: 539-547.

Towbin HT, Staehelin T, Gordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76**: 4350-4354.

Traenckner EB, Pahl HL, Henkel T, Schmidt KN, Wilks S, Baeuerle PA (1995). Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. EMBO J **14**: 2876-2883.

Treisman R (1994). Ternary complex factors: growth factors regulated transcriptional activators. Curr Opin Gen Dev **4**: 96-101.

Tsai-Morris CH, Cao XM, Sukhatme VP (1988). 5′ flanking sequence and genomic structure of Egr-1, a murine mitogen inducible zinc finger encoding gene. Nucleic Acids Res **16**: 8835-8846.

Tummuru MK, Cover TL, Blaser MJ (1993). Cloning and expression of a high molecular-mass antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. Infect Immun **61**: 1799-1809.

Tytgat GN, Rauws EA (1990). *Campylobacter pylori* and its role in peptic ulcer disease. Gastroenterol Clin North Am **1**: 183-96.

Van Antwerp D, Martin SJ, Kafri T, Green DR, Verma I (1996). Suppression of TNFα-induced apoptosis by NF-κB. Science **274**: 787-789.

van den Brink GR, ten Kate FJ, Ponsioen CY, Rive MM, Tytgat GN, van Deventor SJH, Peppelenbosch MP (2000). Expression and activation of NF-κB in the antrum of the human stomach. J Immunol **164**: 3353-3359.

Verma IM, Stevenson JK, Schwarz EM, Antwerp DV, Miyamato S (1995). Rel/NF-κB/IκB family: Intimate tales of association and dissociation. Genes Dev **9**: 2723-2735.

von Herbay A, Rudi J (2000). Role of apoptosis in gastric epithelial turnover. Microsc Res Tech **48**: 303-311.

Wagner S, Beil W, Westermann J, Logan RP, Bock CT, Trautwein C, Bleck JS, Manns MP (1997). Regulation of gastric epithelial cell growth by *Helicobacter pylori*: evidence for a major role of apoptosis. Gastroenterology **113**: 1836-1847.

Wahl C, Liptay S, Adler G, Schmid RM (1998). Sulphasalazine: a potent and specific inhibitor of nuclear factor κB. J Clin Invest **101**: 1163-1174.

Wallace JL (1991). Possible mechanisms and mediators of gastritis-associated with *Helicobacter pylori* infection. Scand J Gastroentrol **26** (Supp 187): 65-70.

Wang X, Willen R, Wadstrom T (2000). Astaxanthin-rich algal meal and vitamin C inhibit *Helicobacter pylori* infection in BALB/cA mice. Antimicrob Agents Chemother **44**: 2452-2457.

Wang C-Y, Cusack JC, Jr, Liu R, Baldwin AS, Jr (1999). Control of inducible chemoresistance: increased apoptosis by inhibition of NF-κB. Nature Med **5**: 412-417.

Wang C-Y, Mayo MW, Baldwin AS (1996). TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science **274**: 784-787.

Wang C-Y, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS (1998). NF-κB antiapoptosis: induction of TRAF2, IAP1 and c-IAP2 to suppress caspase-8 activation. Science **281**: 1680-1683.

Waring AJ, Drake IM, Schorah CJ, White KL, Axon AT, Dixon MF (1996). Ascorbic acid and total vitamin C concentrations in plasma, gastric juice and gastrointestinal mucosa: effects of gastritis and oral supplementation. Gut **38**: 171-176.

Warny M, Keates AC, Keates S, Castagliuolo I, Zacks JK, Aboudola S, Qamar A, Pothoulakis C, LaMont JT, Kelly CP (2000). p38 MAP kinase activation by *Clostridium difficile* toxin A mediates monocyte necrosis, IL-8 production, and enteritis. J Clin Invest **105**: 1147-1156.

Weber C, Erl W, Pietsch A, Strobel M, Ziegler-Heitbrock HW, Weber PC (1994). Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-kappa B mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. Arterioscler Thromb **14**: 1665-1673.

Weichsel A, Gasdaska JR, Powis G, Montfort WR (1996). Crystal structures of reduced, oxidized, and mutated human thioredoxins: evidence for a regulatory homodimer. Structure **4**: 735-751.

Weigert N, Schaffer K, Schusdziarra V, Classen M, Schepp W (1996). Gastrin secretion from primary cultures of rabbit antral G cells: stimulation by inflammatory cytokines. Gastroenterology **110**:147-54.

Weiss SJ (1989). Tissue destruction by neutophils. N Engl J Med **320**: 365-376.

Wessler S, Hocker M, Fischer W, Wang TC, Rosewicz S, Haas R, Wiedenmann B, Meyer TF, Naumann M (2000). *Helicobacter pylori* activates the histidine decarboxylase promoter through a mitogen-activated protein kinase pathway independent of pathogenicity island-encoded virulence factors. J Biol Chem **275**: 3629-3636.

Whelan J, Ghersa P, Hooft VHR, Gray J, Chandra G, Talabat F, DeLamarter JF (1991). An NF-κB-like factor is essential but not sufficient for cytokine induction in endothelial leukocyte adhesion molecule1 (ELAM-1) gene transcriptio. Nucl Acids Res **19**: 2645-2653.

Wilson KT, Ramanujam KS, Mobley HLT, Musselman RF, James SP, Meltzer SJ (1996). *Helicobacter pylori* stimulates inducible nitric oxide synthase expression and activity in a murine macrophage cell line. Gastroenterology **111**: 1524-1533.

Windle HJ, Fox A, Ni Eidhin D, Kelleher D (2000). The thioredoxin system of *Helicobacter pylori*. J Biol Chem **275**: 5081-5089.

Windle HJP, Kelleher D (1997). Identification and characterization of a metalloprotease activity from *Helicobacter pylori*. Infect Immun **65**: 3132-3137.

Wright JJ, Gunter KC, Mitsuya H, Irving SG, Kelly K, Siebenlist U (1990). Expression of a zinc finger gene in HTLV-I and HTLV-II transformed cells. Science **248**: 588-591.

Wright J, Schwartz JH, Olson R, kosowsky JM, Tauber AI (1986). Proton secretion by the sodium/hydrogen ion antiporter in the human neutrophil. J Clin Invest **77**: 782-788.

Wyatt JI, Rathbone BJ, Dixon MF, Heatley RV (1987). *Campylobacter pyloridis* and acid induced gastric metaplasia in the pathogenesis of duodenitis. J Clin Pathol **40**: 841-848.

Xia HX, English L, Keane CT, O'Morain C (1993). A. Enhanced cultivation of *Helicobacter pylori* in liquid media. J Clin Pathol **46**: 750-753.

Xiang Z, Bugnoli M, Rappuoli R, Covacci A, Ponzetto A, Crabtree JE (1993). *Helicobacter pylori*: host responses in peptic ulceration. Lancet **341**: 900-901.

Xiang Z, Censini S, Bayeli PF, Telford JL, Figura N, Rappuloi R, Covacci A (1995). Analysis of expression of CagA and VacA virulence factors in 43 strains of *Helicobacter pylori* reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. Infect Immun **63**: 94-98.

Xu JK, Goodwin CS, Cooper M, Robinson J (1990). Intracellular vasculization caused by the urease of *Helicobacter pylori*. J Infect Dis **161**: 1302-1304.

Xu Z, Dziarski R, Wang Q, Swartz K, Sakamoto KM, Gupta D (2001). Bacterial peptidoglycan-induced TNF-alpha transcription is mediated through the transcription factors Egr-1, Elk-1, and NF-kappaB. J Immunol **167**: 6975-6982.

Yahiro K, Niidome T, Hatakeyama T, Aoyagi H, Kurazona H, Padilla PI, Wada A, Hirayama T (1997). *Helicobacter pylori* vacuolating cytotoxin binds to the 140-kDa protein in human gastric cancer cell lines, AZ-521 and AGS. Biochem Biophys Res Commun **238**: 629-632.

Yamaji Y, Moe OW, Miller RT, Alpern RJ (1994). Acid activation of immediate early genes in renal epithelial cells. J Clin Invest **94**: 1297-1303.

Yamaoka Y, Kita M, Kodama T, Sawi N, Kashima K, Imanshi J (1997). Induction of various cytokines and development of severe mucosal inflammation by cagA gene positive *Helicobacter pylori* strains. Gut **41**: 442-451.

Yan YX, Nakagawa H, Lee MH, Rustgi AK (1997). Transforming growth factor-alpha enhances cyclin D1 transcription through the binding of early growth response protein to a cis-regulatory element in the cyclin D1 promoter. J Biol Chem **272**: 33181-33190.

Yan SF, Zou YS, Gao Y, Zhai C, Mackman N, Lee SL, Milbrandt J, Pinsky D, Kisiel W, Stern D (1998). Tissue factor transcription driven by Egr-1 is a critical mechanism of murine pulmonary fibrin deposition in hypoxia. Proc Natl Acad Sci USA **95**: 8298-8303.

Yin MJ, Yamamoto Y, Gaynor RB (1998). The anti-inflammatory agents aspirin and salicylate inhibit the activity of I (kappa) B kinase-beta. Nature **396**: 77-80.

Yoshido N, Granger DN, Evans DJ, Jr, Evans DG, Graham DY, Anderson DC, Wolf RF, Kvietys PR (1993). Mechanisms involved in *Helicobacter pylori*-induced inflammation. Gastroenterology **105**: 1431-1440.

Zabel U, Henkel T, Silva MS, Baeuerle PA (1993). Nuclear uptake control of NF-κB by MAD-3, an IκB protein present in the nucleus. EMBO J **12**: 201-211.

Zabel U, Schreck R, Baeuerle PA (1991). DNA binding of purified transcription factor NF-kappa B. Affinity, specificity, Zn^{2+}dependence, and differential half-site recognition. J Biol chem **266**: 252-260.

Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M (1997). The Ikappa kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for I kappa B phosphorylation and NF-kappa B activation. Cell **91**: 243-252.

Zarrilli R, Ricci V, Romano M (1999). Molecular response of gastric epithelial cells to *Helicobacter pylori*-induced cell damage. Cellular Microbiol **1**: 93-99.

Zhang ZW, Patchett SE, Perett D, Katelaris PH, Domizio P, Farthing MJG (1998). The relation between vitamin C concentrations, mucosal histology, and CagA seropositivity in the human stomach. Gut *43*: 322-326.

Zhang HM, Wakisaka N, Maeda O, Yamamoto T (1997). Vitamin C inhibits the growth of a bacterial risk factor for gastric carcinoma: *Helicobacter pylori*. Cancer **80**: 1897-1903.

Zheng YM, Schafer MK, Weihe E, Sheng H, Corisdeo S, Fu ZF, Koprowski H, Dietzschold B (1993). Severity of neurological signs and degree of inflammatory lesions in the brain of rats with Borna disease correlated with the induction of nitric oxide synthase. J Virol **67**: 5786-5791.

Zimmermann AG, Spychala J, Mitchell BS (1995). Characterization of the human inosine-5′-monophosphate dehydrogenase type II gene. J Biol Chem **270**: 6808-6814.

APPENDICES

Appendix A

Reagents and Buffers

1- Cell Culture Reagent

Culture cocktail

Penicillin (100 U/ml) /Streptomycin (10 µg/ml)	100 ml
L-glutamine (20 mM)	100 ml

This mixture was stored at 20°C in aliquots of 10 ml.
One aliquot was used for each medium bottle.

Complete RPMI 1640 medium

RPMI 1640	500 ml
FCS	50 ml
Culture cocktail	10 ml

Dulbecco's MEM-nutrient mix F-12 complete medium

Dulbecco's MEM-nutrient mix F-12 medium	500 ml
Foetal calf serum	50 ml
Culture cocktail	10 ml

Hanks balanced salts washing solution (HBSS)

HBSS	500 ml
HEPES buffer (1 M)	10 ml

Cryopreservative solution

Foetal calf serum	9.0 ml
Dimethylsulphoxide	1.0 ml

Ethidium bromide (EB) stock

EB	100 mg
PBS	20 ml

Acridine orange (AO) stock

AO	20 mg
PBS	20 ml

EB/AO working solution

EB solution	4 ml
AO solution	4 ml
PBS	100 ml

2- <u>Cell Protein Preparation</u>

10X Phosphate Buffered Saline (PBS)

$Na_2HPO_4.2H_2O$ (8 mM)	14.24 g
KH_2PO_4 (1.5 mM)	2.04 g
NaCl (137 mM)	80.0 g
KCl (2.7 mM)	2.0 g

Adjust to pH 7.4 and made up to 1 litre

1% Nonidet P40

PBS	100 ml
EGTA	0.038 g
Nonidet P40	946 µl

Protease inhibitors

Leupeptin	2 mg/ml
PMSF	1 M

BSA buffer solution

Bovine serum albumin	50 mg

This solution was made up to 50 ml with a 1:1 mixture of PBS and 1% NP40

Bradford reagent

Coomassie Blue G	100 mg
95% ethanol	50 ml
0.85% orthophosphoric acid	100 ml

This solution was made up to 1 litre with distilled water

3- <u>Polyacrylamide Gel Electrophoresis</u>

5x Sample buffer

Glycerol	5 ml
2-mercaptoethanol	6.25 ml
20% SDS	5 ml
Stacking gel buffer	1.25 ml
0.2% Bromophenol blue	0.3 ml

Make up to 25 ml with distilled water

Resolving gel buffer

Tris base	36.3 g
Distilled water	100 ml

Adjust to pH 8.8 with conc HCl

Stacking gel buffer

Tris base	6.0 g
Distilled water	100 ml

Adjust to pH 6.8 with conc HCl

1% Ammonium persulphate (APS)

APS	0.1 g
Distilled water	10 ml

Make up fresh before use.

10% Sodium dodecyl sulphate (SDS)

SDS	1.0 g
Distilled water	10 ml

Acrylamide/Bisacrylamide Mix

Acrylamide	30 g
Bisacrylamide	0.8 g
Distilled water	100 ml

Water-saturated butanol

Butanol	10 ml
Distilled water	10 ml

This was mixed well, allowed to settle and pipetted from top layer

10X Electrode running buffer

Tris base	30 g
Glycine	114 g
SDS	5.0 g
Distilled water	1000 ml

Dilute 1/10 in distilled water before use

4- Semi-Dry Transfer

Transfer buffer

Tris base	2.9 g
Glycine	1.45 g
SDS	0.185 g
Methanol	100 ml

Make up to 500 ml with distilled water

Coomassie blue gel stain

Coomassie Brilliant Blue R	0.5 g
Methanol	200 ml
Glacial acetic acid	35 ml
Distilled water	265 ml

Gel destain solution

Methanol	400 ml
Glacial acetic acid	70 ml
Distilled water	530 ml

5- Immunoblotting

Blotto-Tween blocking solution

Skimmed dried milk (Marvel)	5 g
PBS	100 ml
Tween 20	46 µl

Make up fresh before use

0.05% PBS-Tween washing solution

PBS	1000 ml
Tween 20	0.5 ml

Primary antibody solution

Primary antibody	10 µl
Blotto-Tween	10 ml

Secondary antibody solution

Swine anti-rabbit peroxidase-conjugated Ig	10 µl
Blotto-Tween	20 ml

6- Enhanced Chemiluminescence

Developing solution

Luminol	14 mg
Iodophenol	4 mg
DMSO	500 µl
H_2O_2	18 µl
0.1 M Tris-HCl (pH 8.8)	50 ml

7- Flow Cytometry Analysis

FACS washing buffer

PBS	500 ml
30% BSA	17 ml
10% sodium azide	500 µl

5% Para-formaldehyde solution

Para-formaldehyde	5 g
PBS	100 ml

The solution was heated up to 65°C to be dissolved, cooled, filtered and stored at 4°C

8- Nuclear Extract Preparation

Extraction buffers

All stock solutions should be autoclaved

Buffer A	**Stock Solution**	**Volume**
Hepes, pH 7.9 (10 mM)	1 M	100 µl
Magensium chloride (1.5 mM)	1 M	15 µl
Potassium chloride (10 mM)	1 M	100 µl
PMSF	1 M	5 µl
DTT	1 M	5 µl

Make up to 10 ml with sterile water

Buffer C		
Hepes, pH 7.9 (20 mM)	1 M	200 µl
Magensium chloride (1.5 mM)	1 M	15 µl
Sodium chloride (420 mM)	5 M	840 µl
EDTA (0.2 mM)	0.5 M	4 µl
Glycerol 25%	--	2.5 ml
PMSF (0.5 mM)	100 m M	50 µl

Make up to 10 ml with Sterile water

BufferD		
Hepes, pH 7.9 (10 mM)	1 M	100µl
Potassium chloride (50 mM)	1 M	400µl
EDTA (0.2 mM)	0.5 M	1.6µl
Gylcerol 20%	--	800µl
PMSF (0.5 mM)	100 mM	20µl
DTT (0.2 mM)	1 M	2µl

Make up to 10 ml with sterile water

9- Electrophoretic Mobility Shift Assay

10X Tris borate buffer (TBE)	
Tris base	108 g
Boric acid	55 g
0.5 M EDTA	40 ml

Make up to 1 litre with distilled water

TE buffer (1X)	
Tris-HCl (pH 8.0)	10 mM
EDTA	1 mM

Binding reaction buffer (10X)	**Stock**	**Volume**
Tris pH 7.5 (100 mM)	1 M	50 µl
NaCl (1 M)	5 M	100 µl
Glycerol (40%)	---	200 µl
EDTA (10 mM)	0.5 M	10 µl
DTT (50 mM)	1 M	25 µl
Nuclease free BSA (1 mg/ml)	10 mg/ml	50 µl

Make up to 500 µl with sterile water

10- Plasmid Preparation

All stock solutions should be autoclaved

L-broth medium (LB)

Tryptamine	10 g
Yeast extract	5 g
Sodium chloride	5 g
Agar	15 g

Make up to 1 litre with distilled water

Antibiotic Selection

Ampicillin	100 mg/ml

STE solution

NaCl (0.1 M)	0.292 g
TE	50 ml

Solution I

Glucose (50 mM)	0.90 g
TE	50 ml

Solution II

NaOH (1 M)	1 ml
SDS (10%)	500 µl
Sterile water	3.5 ml

This solution should be prepared freshly from stocks

Acetic acid (57%)

Acetic acid	5.7 ml
Distilled water	4.3 ml

Potassium acetate solution (5 M)

Potassium acetate	4.907 g
Distilled water	10 ml

This solution should be filter sterilised

First precipitation solution

Potassium acetate (filter sterilise) (5 M)	4 ml
57%v/v Acetic acid (10 M)	1ml

Final precipitation solution

Potassium acetate (5 M)	1 ml
Sterile water	2 ml
Isopropanol	22 ml

TE/RNase solution

TE	250 µl
RNase (DNase free)	10 µg/ml

STE, solution I and first precipitation solution should be stored on ice before use

11- Agarose Gel Electrophoresis

TAE buffer (10X)

Tris base	24.2 g
Glacial acetic acid	5.71 ml
EDTA, pH 8.0 (0.5 M)	20 ml

Agarose gel (0.8%)

Agarose	0.32 g
TAE (1X)	40 ml

This solution was heated up until dissolved

Electrode running buffer

TAE	50 ml
Distilled water	450 ml

Gel loading dye

Bromophenol blue (0.25%)	0.125 g
Xylene cyanol (0.25%)	0.125 g
Glycerol (30%)	15 g

Make up to 50 ml with distilled water

12- Immunohistochemistry

Citrate buffer

Citric acid monohydrate	2.1 g

Make up to 1 litre with distilled water

Tris buffered saline

Tris-HCl (pH 8.0)	10 mM
NaCl	150 mM

Appendix B

Publications

Helicobacter pylori Induces the Early Growth Response-1 Transcription Factor in Gastric Epithelial Cells.
Mohamed M. M. Abdel-Latif, Henry J. Windle, Katherine A. Fitzgerald, Yeng S. Ang, Dèirdre Nì Eidhin, Min Li-Weber, Kamal Sabra, Dermot Kelleher.
Manuscript submitted to Gastroenterology.

Low pH and *Helicobacter pylori* Increase Nuclear Factor Kappa B Binding in Gastric Epithelial Cells: A Common Pathway for Epithelial Cell Injury.
Dermot O'Toole, **Mohamed M. M. Abdel-Latif**, Aideen Long, Ann M. Murphy, Henry Windle, Andrew Bowie, Luke A. J. O'Neill, Donald G. Weir, Dermot Kelleher.
Manuscript submitted to Gut.

Modulation of Cytokine-, Mitogen- and *H. pylori*-induced Nuclear Factor-kappa in Gastric Epithelial Cells by *H. pylori* Thioredoxin.
Mohamed M. M. Abdel-Latif, Henry J. Windle, Dermot O'Toole, Dermot Kelleher.
Manuscript submitted to J. Immunology.

Effects of Ascorbic Acid and N-acetylcysteine on Nuclear Factor Kappa B Activation by *Helicobacter pylori* in Gastric Epithelial Cells.
Mohamed M. M. Abdel-Latif, Dermot O'Toole, Aideen Long, Henry J. Windle, Donald G. Weir, Dermot Kelleher.
Manuscript in preparation.

Presentations

Helicobacter pylori Induces the Early Growth Response-1 Gene in Gastric Epithelial Cells.
M. M. Abdel-Latif, H. J. Windle, D. Nì Eidhin, K. Sabra, D. Kelleher. Ir. J. M. Sci., 1999; 168 (Suppl 5): p-15. (Won a prize at Irish Society for Immunology (ISI), 1999).

Induction of the Early Growth Response-1 Transcription Factor in Gastric Epithelial Cells by *Helicobacter pylori*.
Mohamed M. Abdel-Latif, Henry J. Windle, Yeng S. Ang, Deirdre Nì Eidhin, Kamal Sabra, Dermot Kelleher.
Digestive Disease Week (DDW) 2000, San Diego, California: p-678.

Helicobacter pylori Induces the Early Growth Response-1 Gene in Gastric Epithelial Cells.
M. M. Abdel-Latif, H. J. Windle, D. Nì Eidhin, K. Sabra, D. Kelleher.
American Society of Health-system Pharmacista (ASHP), 1999, Orlando, Florida.

Helicobacter pylori Induces the Early Growth Response-1 Gene in Gastric Epithelial Cells.
M. M. Abdel-Latif, H. J. Windle, D. Nì Eidhin, K. Sabra, D. Kelleher.
Assiut University Second Pharmaceutical Sciences Conference 2000, Assiut, Egypt.

Effects of Antioxidants on NF-kappa B Activation by *Helicobacter pylori* in Gastric Epithelial Cells.
M. M. Abdel-Latif, H. J. Windle, D. Nì Eidhin, K. Sabra, D. Kelleher.
Ir. J. M. Sci., 1999; 168 (Suppl 9): p-27.

Effects of Antioxidants on NF-kappa B Activation by *Helicobacter pylori* in Gastric Epithelial Cells.
M. M. Abdel-Latif, H. J. Windle, D. Nì Eidhin, K. Sabra, D. Kelleher.
Assiut University Second Pharmaceutical Sciences Conference 2000, Assiut, Egypt.